Manual of Quality Analyses for Soybean Products in the Feed Industry

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1. Introduction

The use of soybean products in the feed and food industry has increased steadily over the past decennia. Fifty years ago world soybean production was estimated to be 17 million tons with China being the major producer (UNEP, 1999). A little more than 50 years later and the production for 2003 is expected to reach more than 190 million tons with the major centers of production being the USA, Brazil and Argentina (USDA, 2003). The USA remains the largest producer of soybeans and soybean meals but its production is leveling off while Brazilian production and crushing of beans is increasing rapidly.

Of the total world production of soybeans, less than 10 % is directly used for human consumption. The overwhelming majority is used in animal feed in the form of various types of soybean meals or specialty soy products. The current world production of soybean meal is estimated to be in excess of 130 million mt (USDA, 2003). With global animal feed production estimates approximating 1.100 mt (Speedy, 2002), and compound feed production well above the 600 million mt (Gill, 2003), soybean meals represent the dominant source of protein in animal diets. However, total use and importance of soybeans or soybean products is likely to be higher than indicated by major statistics as a plethora of different soybean products are entering the feed and food chain.

This dominant position of soybeans and their products is no doubt associated with their high quality especially with respect to protein and amino acid profile. Following proper treatment or extraction, digestibility of the protein fraction is high and the amino acid profile provides a close match with cereals to meet animal requirements. Nevertheless, in their untreated form, soybeans contain a number of factors that have the potential to seriously diminish their nutritive value - to the point of decreasing animal performance and health (Liener, 2000). A treatment of soybeans to eliminate these anti nutritional factors (ANF) is thus necessary especially in the case of monogastric diets. These treatments, combined with varietal differences in the production process of soybean meals or other products lead to potentially large variations in quality.

While basic standard specifications for soybean meals have been established (NOPA, 1997) no official specifications exist for other soy products that are routinely used in the feed industry. Furthermore the NOPA specifications only refer to four chemical characteristics. Current evaluations of soy products are based on a much larger array of tests allowing a more accurate evaluation of the

nutritive value of the different products. However, under practical conditions of feed production the choice of tests differ greatly among producers and feed compounders and not all tests are applied on a regular basis (West, 2002). It is most likely that in the future more analyses of greater complexity will be needed. Developments in the technological modification of soybean products, along with a better understanding of the effects on performance and health of relatively unknown compounds, such as isoflavones, will add value to soy products. Accurate analysis to measure the effects of new treatments and the relatively unexplored compounds will be of great importance.

In order for results of quality tests to have real value and to be comparable between producers it is important that tests are standardized in method as well as equipment. This standardization is becoming increasingly important as trade in soybean products grows more global and competition amongst suppliers increases. Identity preservation and traceability associated with detailed quality characterization are issues of major importance in the (future) trade of soy products. Accurate and consistent quality procedures and analyses along with precise descriptions of the product are necessary. These tests must be reproducible at different levels of the supply chain. Furthermore, the increasing demands of the implemented quality systems (HACCP, ISO or GMP) will dictate the establishment of more detailed quality procedures and a larger analytical capacity. For the information that is generated at the various production stages to be consistent and comparable it is important that a single reference is available.

This quality manual intends to provide clear directives and explanations for the quality analysis needed at all stages of the soy protein supply chain in the feed industry. The objective is to supply information that is applicable at all levels of operation, from the crusherto the compounder and from the quality operator in the plant to the nutritionist. Applications of the methods and analyses presented will enhance the value of soy products through improved knowledge and application resulting in improved performance and health.

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2. Soybeans, Soybean Products and Production Processes

A large number of soybean varieties exist, producing soybeans that vary greatly in shape and color. For the complete range of soybeans shapes vary from flat to spherical and colors range from yellow to green, brown and black. Modern varieties, mainly grown for their oil content, are generally spherical in shape with a yellow or green as the accepted seed coats. These characteristics logically will affect many of the soybean products obtained from these beans. Official limits have been set on the minimal size requirements for the beans (see below) but generally soybeans grown for industrial purposes will weigh between 18 – 20 g per 100 beans.

The soybean consists of two cotyledons which represent approximately 90 % of the weight, a seed coat or hull (8% of weight), and two much smaller and lighter structures the hypoctyl and the plumule. The cotyledons contain the proteins and lipids (oils) that constitute the main nutritional components of the soybean products obtained from soybeans. They are also the main storage area for the carbohydrates and various other components of importance, most notably the enzymes (lipoxygenase, urease) and the ANF. The various soybean products are obtained through the separation or extraction of the different component of the soybean.

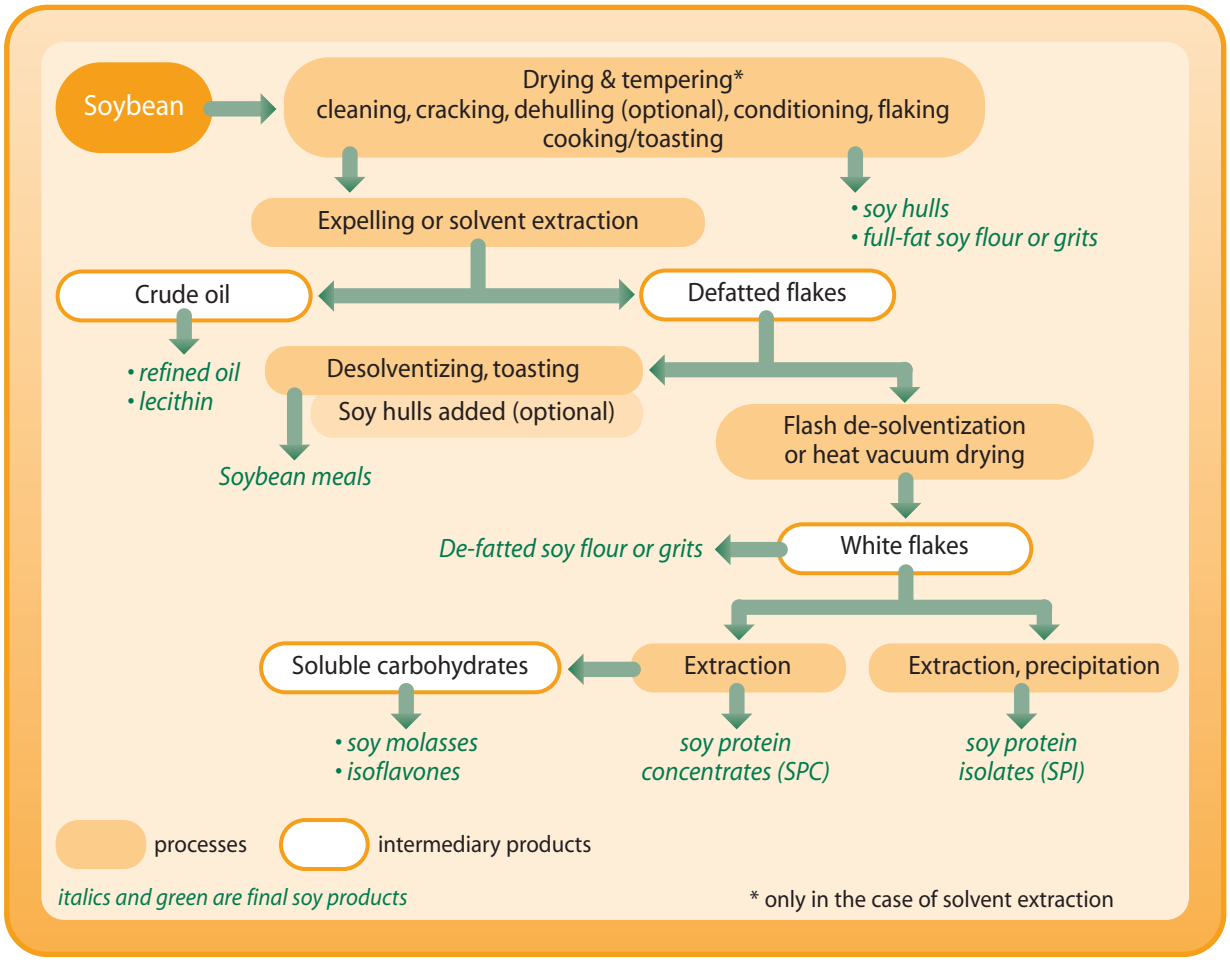
A large array of different manufacturing processes is applied to obtain the many soy products used in animal and human nutrition (Berk, 1992). Figure 1 provides a schematic representation of the transformation from soybean into the various products. In the "crushing" process of soybeans, which includes a series of preparatory operations, crude oil is obtained as a major product. The crude oil is refined and separated into lecithin and refined oil used in human as well as animal nutrition; especially in young animal diets.

The soybean meals, which on a volume basis are the most important products obtained from soybeans, have the defatted flakes as an intermediary product that requires further treatment. Two main processes are used to extract the oil and obtain the defatted flakes: the expeller process (mechanical extraction of the oil by a screw press) or solvent extraction where non-polar solvents (commonly hexane and hexane isomers) are used to extract the oil. Solvent extraction is the most efficient and widely used process at present. In the case of solvent extraction the flakes are desolventized. All flakes are toasted in order to eliminate the heat-labile anti nutritional factors. Sometimes the hulls obtained in the preparatory steps are added

back to the toasted flakes. This is done in variable degrees resulting in soybean meals with variable levels of fiber and crude protein. When no hulls are added the high protein meals are obtained. These are the meals used predominantly in poultry diets. Flash desolventization or heat vacuum drying of the defatted flakes produces the white flakes that are higher in protein quality (solubility) and do not have the undesirable darker color. Through a series of different extraction and precipitation process soy protein isolates (SPI) or soy protein concentrates (SPC) are produced. Whereas SPI production is fairly standardized, different methods of extraction are used to obtain the SPC resulting in slightly different compositional characteristics. SPC but also the white flakes can be further elaborated (grinding, texturizing; separation on basis of molecular weight) to obtain a large array of products used in human nutrition. SPI and SPC are used in animal nutrition but are limited to specialty diets due to the relatively high cost. The use of these ingredients in animal diets is mainly as a replacement of high quality protein sources such as animal or milk proteins or as a replacement of fishmeal in aquaculture diets.

Figure 1

Schematic representation of the manufacturing of soybean products



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3. Definition and Application of Soybeans and Soy Products

The number of soy products currently being used in the feed industry is large, and an exhaustive review is hardly possible. Recent years have seen a dramatic expansion of specialty products based on soybeans. Classical, commodity products such as raw soybeans and soybean meals are relatively well defined with thorough descriptions and specifications. This is not necessarily the case for some of the recent modifications or adaptations of these products (i.e. Rumen-protected soybean meal) or further elaborated products (i.e. soy concentrates). These evolved, value-added products may differ significantly among producers with each producer applying proprietary knowledge and specialized treatments. Typically, value–added products must be evaluated on the basis of the entity that produces them taking into account the guarantees provided by the manufacturer or distributor. Consistent analysis of these producer-specific products allows classification and the building of a database along with confidence about the product. This increased level of knowledge will allow an analysis schedule of decreased intensity and increase inclusion rates in diets.

Commodities as well as the value-added products can be classified in a specific class or group of products for which a sufficiently specific description can be developed. For an efficient and correct use - as well as a meaningful interpretation of analytical results - a precise and generally agreed upon definition of the product is needed. Trading, purchasing, formulation, and the entire operation of feed manufacturing depend on the precise referencing of a raw material and the consistent use of the correct name and description. Also the quality control mechanisms that have been introduced in the feed industry require a precise description and classification for all ingredients.

Although many databases and ingredient tables have their own classification system, the most widely recognized system is probably the IFN system (International Feed Name and number) (INFIC, 1980). In this system, ingredients have been divided into eight fairly arbitrary feed classes on the basis of their composition and use (NRC, 1982). The system is widely used in the UK, the US and in Canadian feed composition tables but less so in other countries.

In the IFN system, ingredients are assigned a six digit code with the first digits denoting the International Feed Class number. With the exception of soybean hay, soybean hulls (class 1), lecithin, soybean mill run and soybean mill feed (class 4), soy products listed in table 2 (page 16) fall in the class of protein supplements (5) defined as products that contain more than 20 % crude protein on a dry matter basis. The five

digits following the class number is the link between the INF and chemical and biological data in the USA databank (NRC, 1982). The number appears generally on official US ingredient specifications and, although the system may not be used by all feed producers or manufacturers, it provides an easy and systematic reference for quality systems and formulation purposes.

A brief and general description is available for many soy products. This description has the advantage of providing information that is not generally captured in compositional tables. It also provides for a general appreciation of the origin and quality and thus the potential applications or uses in a feed. Although these definitions might differ slightly between different sources, they are in general sufficiently similar to use them interchangeably. AAFCO publishes at regular intervals reference specifications for soybean products (AAFCO, 2001). These definitions have been used as a basis for the specifications listed in Table 1.

Table 1

Description and classification of soybean products *

1. **Condensed Soybean Solubles** is the product obtained by washing soy flour or soybean flakes with water and acid at a pH of 4.2-4.6. The wash water is then concentrated to a solid content of not less than 60%. IFN 5-09-344.
2. **Dried Soybean Solubles** is the product resulting from the washing of soy flour or soybean flakes with water and acid; water, alkali and acid; or water and alcohol. The wash water is then dried. IFN 5-16-733.
3. **Ground Extruded Whole Soybeans** is the meal product resulting from extrusion by friction heat and/or steam of whole soybeans without removing any of the component parts. It must be sold according to its crude protein, fat and fiber content. IFN 5-14-005.
4. **Ground Soybean Hay** is the ground soybean plants including the leaves and beans. It must be reasonably free of other crop plants and weeds and must contain no more 33% crude fiber. IFN 1-04-559.
5. **Ground Soybeans** are obtained by grinding whole soybeans without cooking or removing any of the oil. IFN 5-04 -596.
6. **Heat Processed Soybeans** (Dry Roasted Soybeans) is the product resulting from heating whole soybeans without removing any of the component parts. It may be ground, pelleted, flaked or powdered. It must be sold according to its crude protein content. Maybe required to be labeled with guarantees for maximum crude fat, maximum crude fiber and maximum moisture (CFIA 2003). IFN 5-04-597.
7. **Kibbled Soybean Meal** is the product obtained by cooking ground solvent extracted soybean meal, under pressure and extruding from an expeller or other mechanical pressure device. It must be designated and sold according to its protein content and shall contain not more than 7% crude fiber. IFN 5-09-343.

** In alphabetical order; adapted from the AAFCO Official Publication 2001 and the CFIA. 2003.*

8. **Protein Modified Soybean** is a product that has been processed to primarily modify the natural protein structure by utilizing acids, alkalies or other chemicals and without removing significant amounts of any nutrient constituent. The defined name under section 84 of the applicable soybean product so modified shall be declared in the product name. IFN 5-26-010.
9. **Soy Flour** is the finely powdered material resulting from the screened and graded product after removal of most of the oil from selected sound cleaned and dehulled soybeans by a mechanical or solvent extraction process. It must contain not more than 4.0% crude fiber. Some organisms also require labeling guarantees for minimum crude protein and maximum crude fat and moisture. IFN 5-12-177.
10. **Soy Grits** is the granular material resulting from the screened and graded product after removal of most of the oil from selected, sound, clean and dehulled soybeans by a mechanical or solvent extraction process. It must contain not more than 4% crude fiber. Soybean grits mechanical extracted: IFN 5-12-176. Soybean grits solvent extracted: IFN 5-04-592.
11. **Soy Lecithin** or **Soy Phosphate** is the mixed phosphatide product obtained from soybean oil by a degumming process. It contains lecithin, cephalin and inositol phosphatides, together with glycerides of soybean oil and traces of tocopherols, glucosides and pigments. It must be designated and sold according to conventional descriptive grades with respect to consistence and bleaching. IFN 4-04-562.
12. **Soy Protein Concentrate** is prepared from high quality, sound, dehulled soybean seeds by removing most of the oil and water soluble non-protein constituents from selected, sound, cleaned, dehulled soybeans (CFIA 2003) and must contain not less than 65% protein on a moisture-free basis. It shall be labeled with guarantees for minimum crude protein, maximum crude fat, maximum crude fiber, maximum ash and maximum moisture.
    IFN Number: 5-08-038.
13. **Soy Protein Isolate** is the major proteinaceous fraction of soybeans prepared from dehulled soybeans by removing the majority of non-protein components, and contains not less than 90% protein on a moisture-free basis. The CFIA (2003) adds that the original material must consist of selected, sound, cleaned, dehulled soybeans and that it shall be labeled with guarantees for minimum crude protein (90%), maximum ash and maximum moisture. IFN Number 5-08-038 (CFIA lists this product with the IFN Number 5-24-811.
14. **Soybean Feed, Solvent Extracted** is the product remaining after the partial removal of protein and nitrogen free extract from dehulled solvent extracted soybean flakes. IFN 5-04-613.
15. **Soybean Flour Solvent Extracted** (or **Soy flour**) is the finely powdered material resulting from the screened and graded product after removal of most of the oil from dehulled soybeans by a solvent extraction process. It shall contain less than 4 percent crude fiber. It shall be labeled with guarantees for minimum

crude protein, maximum crude fat, maximum crude fiber and maximum moisture. IFN 5-04-593.

16. **Soybean Hulls** consist primarily of the outer covering of the soybean. IFN-1-04-560.
17. **Soybean Meal, Mechanically Extracted** is the product obtained by grinding the cake or chips which remain after removal of most of the oil from soybeans by a mechanical extraction process. It must contain not more than 7% crude fiber. It may contain an inert, non-toxic conditioning agent either nutritive or non-nutritive or any combination thereof, to reduce caking and improve flowability in an amount not to exceed that necessary to accomplish its intended effect and in no-case exceed 0.5%. The name of the conditioning agent must be shown as an added ingredient. IFN 5-04-600.
18. **Soybean Meal, Dehulled, Solvent-Extracted** is obtained by grinding the flakes remaining after removal of most of the oil from dehulled soybeans by a solvent extraction process. It must contain not more than 3.3% crude fiber. It may contain an inert non-toxic conditioning agent either nutritive or non-nutritive or any combination thereof, to reduce caking and improve flowability in an amount not to exceed that necessary to accomplish its intended effect and in no-case to exceed 0.5%. The name of the conditioning agent must be shown as an added ingredient. IFN 5-04-612. It may also be required to be labeled with guarantees for minimum crude protein, maximum crude fat and maximum moisture(CFIA 2003).
19. **Soybean Meal, Solvent-Extracted**, is the product obtained by grinding the flakes which remain after removal of most of the oil from soybeans by a solvent extraction process. It must contain not more than 7% crude fiber. It may contain an inert, non-toxic conditioning agent either nutritive or non-nutritive and any combination thereof, to reduce caking and improve flowability in an amount not to exceed that necessary to accomplish its intended effect and in no-case exceed 0.5%. It shall contain less than 7 percent crude fiber. The CFIA (2003) specifies that it shall be labeled with guarantees for minimum crude protein, maximum crude fat and maximum moisture. IFN 5-04-604.
20. **Soybean Mill Feed** is composed of soybean hulls and the offal from the tail of the mill which results from the manufacture of soy grits or flour. It must contain not less than 13% crude protein and not more than 32% crude fiber. IFN 4-04-594.
21. **Soybean Mill Run** is composed of soybean hulls and such bean meats that adhere to the hulls which results from normal milling operations in the production of dehulled soybean meal. It must contain not less than 11% crude protein and not more than 35% crude fiber. IFN 4-04-595.
22. **Soybean Oil** consists of the oil from soybean seeds that are commonly processed for edible purposes. It consists predominantly of glyceride esters of fatty acids. If an antioxidant(s) is used, the common name or names shall be indicated on the label. It shall be labeled with guarantees for maximum

moisture, maximum insoluble matter, maximum unsaponifiable matter and maximum free fatty acids. IFN 4-07-983.

23. **Soyflour Chemically and Physically modified** is the product resulting from treating soy flour by chemical and physical (heat and pressure) means. It shall be labeled with guarantees for minimum crude protein, maximum crude fat, maximum crude fiber and maximum moisture. IFN 5-19-651.

The list in Table 1 gives an overview of the large diversity of soy products and different methods of producing them. It provides a brief description of how the product is obtained and for some products, compositional reference points. The common name and IFN is provided which allows for a consistent and non-equivocal use of ingredients, important in quality systems. The description gives an adequate back ground of the products for trading and classification purposes, references in quality systems and production purposes. It is sufficiently precise to provide clear reference points for product definition and contract agreements but general enough to cover a substantial variation in composition and production processes. For proper use of an ingredient additional analytical data should complement the information provided in the description. However, for analytical purposes the descriptions provide general back ground information as to what can be expected and how analysis should be carried out or what results may be expected. For formulation objectives the description only serves as a classification aide and more precise compositional data will be necessary.

The products listed in Table 1 only represent the major soy products produced. At present, a large number of additional specialty products are marketed and the list does not adequately reflect the acceleration seen in the development of new soy products; mostly branded products. Many new, more elaborated products have come on the market over the past 10 - 20 years. The most important examples of these are the different types of soy protein concentrates and soy isolates. These products, characterized by strongly reduced anti-nutritional factors, can effectively be used in diets for young animals, pets and aquaculture, replacing other protein sources such as milk or animal proteins (fish meal). Additional new soy products have often been developed with applications in human or pet food nutrition in mind. In this area special importance is attached to the functional properties of soybean proteins which include the ability of the proteins to increase viscosity, emulsify, form gels, foam, produce films and absorb water and/or fat. Specific applications allow the production of texturized structures, a much sought after property in certain human and pet food products. The functional properties of soy proteins are related to the amino acid composition and sequence (primary structure) as well as the spatial configuration of the protein molecule and the inter-molecular forces (secondary and

tertiary structures). Soybean protein products with unique functional properties may constitute important tools in the formulation of the so-called specialty diets used in animal nutrition. However, these techniques and products remain insufficiently explored in the production of specialty diets for domestic livestock, with economic considerations probably being the major limiting factor at present.

The most important products in terms of volume of use are soybean meals (SBM) solvent extracted or dehulled (18 and 19) resulting from the original use of soybeans i.e. the removal of oil. This is also the case for the mechanically extracted SBM (17) although this type of SBM is much less common. Fullfat soybeans in ground, extruded or heated form are defined and their use is increasing due to their high energy content, especially in formulations where previously animal products (meat and bone meals and fats) were of interest. Two fiber-rich products are included in the list: ground soybean hay (4) and soybean hulls (16). While soybean hay has little application in the compound feed industry, the interest in soybean hulls is important and increasing. Soy flour and soy grits are primarily products destined for human consumption although minor amounts may find an application in specialty diets. Technological modifications of these products have produced different types of flour and grits. They are further classified and commercialized according to their application objectives with the main differences being the level of fat content or heat treatment.

The remaining products are mainly modifications of different types of soybean meal with the objective of rendering the product more digestible; either through the modification of the protein structure or a removal of the ANF. The specifications do not make reference to these factors leaving the decision as to how the product compares in this respect to the interpretation of the nutritionist or guarantees provided by the producer. Quality analysis must provide a more precise indication of the product in terms of these characteristics in order to assure that the diet meets proper nutritional and animal performance objectives.

With the increased complexity of production processes aimed at removing ANF and improving protein digestibility, a clear understanding of the products and the production process becomes more important and adapted quality procedures/ analysis more critical. Quality differences between producers/suppliers for these products can be substantial, especially for the more evolved products. These differences need to be verified and understood at the feed manufacturer's level. Nevertheless, it remains the responsibility of the user to carry out the needed quality analysis and classify suppliers and products accordingly. Reliable manufacturer's information is, of course, important but verification remains the basis of this tool and

of the overall quality assurance program. The quality of the information provided by the manufacturer must be an integral part of the "supplier classification process".

The quality of ingredients play a determining role in the level at which these ingredients are used in animal diets. Quality criteria used to determine the inclusion level for an ingredient go beyond the standard nutrient levels, and have often more to do with residual ANF, storage and contaminations (see Chapter 5) and the physiological characteristics of the animal. The inherent variation in quality and chemical characteristics associated with these ingredients make repeated quality analyses necessary which in turn will determine more precisely the inclusion levels employed. The nutritionist's experience and interpretation of the quality analyses play a major role in defining the final inclusion level used in particular diets. Table 2 gives thus only general estimates of maximum inclusion levels of each product under practical conditions of diet formulation. The inclusion levels suggested are for inclusion in complete diets and are thus necessarily general. They will also need to be adjusted to the specific diet (inclusion of other ingredients) and feeding objectives. Also, the precise nutrient and ANF concentrations and the diet requirements (the ability of the animal to use nutrients or deal with anti nutritional factors) will need to be taken into consideration. Fine tuning of inclusion levels for each product is very much a company-specific decision reflecting depth of understanding of the formulation complexities and confidence in proprietary data relative to the ingredients. The suggestions listed in Table 2 must therefore be regarded as general recommendations that need to be further defined for each feed manufacturer, the manufacturing process and the feed being formulated.

Some of the maximums suggested are not defined by any inability of the animal to use the nutrients in a given product, but rather by the effects of specific nutrients on carcass or product quality. Such is for instance the case for whole heat treated soybeans or soybean oil. Other maximums are controlled by economic considerations. While higher inclusions in diets may be possible, those levels will inevitably lead to additional costs with no or limited gain in performance.

Some soy products listed in Table 1 are not included in the recommendations for use in animal diets. This is the case of "protein modified" soybean meal, soy flour or grits. Although these ingredients could be used in animal diets (and they actually may be when quality is not sufficient to include in human diets) they are primarily produced for utilization in human foods. Included in small amounts, they may convey major nutritional or technological advantages to certain food items (Liu, 1997). Evaluation of these products in pet foods or certain specialty diets merit consideration.

Table 2

Application of soybean products[1]

Product	Species[2] Po	Sw	R	A	Pe	Level (%)[3]
1. Condensed Soybean Solubles			√			10
2. Dried Soybean Solubles			√			15
3. Ground Extr. Whole Soybeans	√	√	√			35
				√	√	5[4]
4. Ground Soybean Hay			√			20
5. Ground Soybeans			√			15
6. Heat Processed Soybeans	√	√	√			15
7. Kibbled Soybean Meal	√	√				10(Y)
				√	√	7
8. Soy Lecithin or Soy Phosphate	√	√	√	√	√	3
9. Soy Protein Concentrate	√	√	√			7(Y)
				√	√	5[4]
10. Soy Protein Isolate	√	√	√			10(Y)
				√	√	15[4]
11. Soybean Feed, Solvent Extracted	√	√	√			5(Y)
				√	√	3
12. Soybean Flour Solvent Extracted	√	√	√			40
13. Soybean Hulls		√	√			25
14. SBM Mechanically Extracted	√	√	√			30
15. SBM Dehulled Solvent Extracted	√	√	√			35
16. SBM Solvent Extracted	√	√	√			35
17. Soybean Mill Feed	√	√	√			10
18. Soybean Mill Run	√	√	√			10
19. Soybean oil	√	√	√[5]		√	8

(1) Suggested upper-use levels in diets of different domestic species; this will vary with age of animal, quality, composition and analysis of product; does not include young animal diets unless specifically indicated. Detailed and extensive analyses will allow discretionary changes in usage level.

(2) Species: Production diets (growing/finishing) for Poultry (Po), Swine (Sw), Ruminants (R), Aqua (salmonids) (A); Pets (dogs) (Pe).

(3) On a diet dry matter basis. "Y" indicates primarily in young animal diets.

(4) Higher levels may be used in salmon and trout grower, finisher diets.

(5) Maximum inclusion of oil in Ruminant diets should not exceed 2%.

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4. Chemical and Nutritional Composition of Soybean Products

The compositional data provided in the Tables 3 (p.19) and 4 (p.20) (with additional details in Appendix Tables 1, 2) are better descriptors of the nutritional characteristics of soybean products. They require however, a more in-depth understanding of the chemical, analytical and nutritional aspects of the products. The composition data also provide an indication of the specific processes that have been used to obtain the product. This is especially true for the data in Table 4. Along with the general description provided above, these data give thus a rather complete picture of the various properties and potential applications for each product. The total number and types of soybean products commercialized is clearly much larger that the ones listed in the tables.
The tables only provide values for the main products. A large variety of different soy products are produced by different companies and for a large number of specific applications. Soy protein concentrates or heat or formaldehyde treated products for ruminant diets are an excellent example of this. The nutritional concentration as analyzed may not differ significantly from an ingredient listed but the nutritional value (due to a change in digestibility or degradability) may vary greatly. Since the tables only report composition that can be directly analyzed, such differences do not show up and are therefore not included.

The nutrient concentration of the different soy products in Tables 3 and 4 are compiled from a wide range of official sources and publications (NRC 1982, 1998, 2001; INRA-AFZ 2002; CVB 2000; FEDNA, 1994 and others). Besides completing the descriptive information provided in Table 2 the major purpose of the composition tables is to provide reference values that can be used to either evaluate the analytical data that are obtained in the laboratory or to further classify a specific ingredient. Since the data are obtained from a wide range of publications, the user may want to refer to the original publications if the sample corresponds more closely to one of the sources in his region. This is especially true in the case of soybean meals or soy by-products where crushing and further handling of the ingredient determine to a large extent the nutrient quality of the products.

The table values provide means based on a large number of samples covering many years and a wide range in origin. They cannot be used as standard values but only as reference points around which analysis of individual samples should be situated if they are to be identified by the specific ingredient name. Most individual samples will be within an acceptable statistical range of

these means (see Chapter 10). This level of precision is adequate for classification, storage and trading agreements, as those are generally based on a small set of analyses (proximate analysis or just humidity, protein and fat). More detailed analyses concerning the more difficult to determine nutrients may show larger variations from the means and possibly inconsistencies with some values above and others below the table values. This is often the case for amino acids or micro minerals. As such they may point to consistent differences in the production process of a given supplier or, alternatively, reflect problems in the analytical procedure. The experience and know-how of a lab technician in interpreting the result is here of great value. Cross-checking of values known to be affected in a similar fashion by a production process or a laboratory procedure may provide an explanation of a discrepancy and confirm the true value and classification for the ingredient.

For most users of soy products the detailed nutrient concentrations serve as a basis to formulate diets and to calculate total nutrient supply to animals. Since animal performance is determined by nutrient concentration and the relationship between nutrients, knowing the precise nutritional composition of the ingredients that make up the diet allows the prediction of animal performance and thus a detailed estimation of the value of each ingredient. Clear compositional descriptions of soy products are thus not only necessary for quality control reasons, but also for the evaluation in a diet or feeding operation. For precise formulations the analytical data on the ingredient in the plant should be used. The use of the table values, especially because of the large contribution that soy products make to the protein and amino acid supply, may lead to significant variations in nutrients between the formulated value and the real diets.

The compositional data in Table 4 includes nutrients that can be directly analyzed in a large and well equipped laboratory. Routine analyses, as carried out in standard quality control procedures or smaller laboratories, mainly concern the proximate analysis, the van Soest fiber components (with the exception of lignin) and the minerals calcium and phosphorus. These analyses (especially the proximate) are most often used to derive other nutrient values such as amino acids or energy. In advanced formulation systems they are generally combined with estimates of digestibility for each individual nutrient. No digestibility data are included here as this information is not necessarily the result of direct observations but rather of literature compilations and research conducted by feed compounders. Thus digestibility data used in formulationsystems can differ considerably among users and are generally considered proprietary information. In the Appendix tables (1, 2) specific energy values have been included however, because of their importance as descriptive parameters for individual soy products and because of their importance in classifying and referencing ingredients.

Table 3

Composition of some soy protein ingredients used in animal feeds

	Unit	Heat processed FF soybean seeds	SBM mechanically extracted	SBM solvent extracted 44	SBM solvent extracted 48	SBM solvent extracted 50	Soybean hulls	Soy protein concentrate	Soy protein isolate
Dry matter	%	89.44	89.80	88.08	87.58	88.20	89.76	91.83	93.38
Crude protein	%	37.08	43.92	44.02	46.45	48.79	12.04	68.60	85.88
Crude fiber	%	5.12	5.50	6.26	5.40	3.42	34.15	1.65	1.32
Ether extracts	%	18.38	5.74	1.79	2.1	1.30	2.16	2.00	0.62
Ash	%	4.86	5.74	6.34	6.02	5.78	4.53	5.15	3.41
NDF	%	12.98	21.35	13.05	11.79	9.95	56.91	13.50	-
ADF	%	7.22	10.20	8.76	7.05	5.00	42.05	5.38	-
ADL	%	4.30	1.17	0.75	0.90	0.40	2.05	0.40	-
Starch	%	4.66	7.00	5.51	5.46	3.28	5.95	-	-
Total sugars	%	-	-	9.06	9.17	9.29	1.40	-	-
Gross energy	kcal/kg	5013	-	4165	4130	4120	3890	4280	5370
Lysine	%	2.34	3.50	2.85	2.89	3.00	0.73	4.59	5.26
Threonine	%	1.53	2.21	1.80	1.84	1.90	0.73	2.82	3.17
Methionine	%	0.52	0.80	0.62	0.63	0.67	0.14	0.87	1.01
Cystine	%	0.55	0.77	0.68	0.73	0.73	0.16	0.89	1.19
Tryptophane	%	0.49	0.74	0.56	0.63	0.65	0.12	0.81	1.08
Calcium	g/kg	2.62	2.96	3.12	3.07	2.68	4.96	2.37	1.50
Phosphorus	g/kg	5.70	6.64	6.37	6.37	6.36	1.59	7.63	6.50
Magnesium	g/kg	2.80	2.84	2.72	3.03	2.88	2.23	1.85	0.80
Potasium	g/kg	15.93	20.28	19.85	22.00	20.84	12.15	12.35	2.75
Sodium	g/kg	0.29	0.33	0.18	0.18	0.88	0.10	0.55	2.85
Linoleic acid C18:2	%	9.70	2.87	0.64	0.80	0.56	1.21	-	-

FF Soybean = Full Fat Soybean; SBM = Soybean meal. For more detailed compositional data on soybean products see Appendix table 1, 2.
Source: compilation of NRC, INRA-AFZ, CVB, FEDN and selected suppliers
NDF = Neutral detergent Fiber; ADF= Acid Detergent Fiber; ADL = Acid Detergent Lignin (Klason Lignin)

Protein quality analyses (Urease Index, KOH soluble N, or PDI) are also not included as these do not generally differ among soy protein products. A number of these analyses do exist and they are important in evaluating soy protein quality especially in terms of digestibility of amino acids. Methods and optimal values for these tests are detailed further in Chapter 8. In many respects they refer to the residual values for the ANF listed in Table 5 (p. 22) but only the heat labile ones such as trypsin inhibitors, lectins and goitrogens (Liener, 2000). There is no proven

relationship between heat stable ANF and protein quality indexes. For many diets, especially in the case of diets for young animals, aquatic species and pets, the application and use of soy products depends to a much larger extent on the residual ANF than on the nutrient concentration. In such diets the more elaborated soy products such as SPC or SPI are more frequently used. Accurate analyses for most of these ANF are difficult to carry out and under most practical conditions the suppliers' guarantees are accepted. As Table 5 indicates, the range in some of these ANF is considerable and a thorough supplier classification is thus important. In many cases, if an analysis for a specific ANF is indicated, the choice to use external laboratories may be advised. External, specialized, laboratories will provide reliable results and generally are in a position to give advice as to the quality and level of an ANF relative to other samples of a similar product. If preference is given to install an analysis for ANF (generally trypsin inhibitor) in a laboratory the adherence to a ring test or systematic comparisons of results with a well established laboratory is necessary.

Table 4

Analytical characteristics of common types of soy protein products

Product type	Unit	Soybean seeds	SBM	Enzyme treated SPC	Alcohol extracted SPC	SPI
Humidity	%	10 - 12	10 - 12	6 - 7	6 - 7	6 - 7
Crude protein	%	33 - 17	42 - 50	55 - 60	63 - 67	>85
Fat	%	17 - 20	0.9 - 3.5	2.5	0.5 - 3.0	0.1 - 1.5
Ash	%	4.5 -5.5	4.5 - 6.5	6.2 - 6.8	4.8 - 6.0	2 - 3.5
Oligosacharides	%	14	15	<1	<3.5	<0.4
Stachyose	%	4 - 4.5	4.5 - 5	<0.3	1 - 3	<0.2
Raffinose	%	0.8 - 1	1 - 1.2	<0.2	<0.2	<0.1
Trypsin inhibitor TIA	mg/g CP	45 - 60	4 - 8	1 - 2	2 - 3	<1
Glycinin	mg/g	150 - 200	40 - 70	<0.1	<0.1	<0.01
ß-conglycin	mg/g	50 - 100	10 - 40	<0.1	<0.1	<0.005
Lectins	ppm	50 - 200	50 - 200	<1	<1	<1
Saponins	%	0.5	0.6	0	0	0
Phytic acid bound P	%	0.6	0.6	0.6	0.6	-

SBM = defatted soybean meal; SPC = soy protein concentrate; SPI = soy protein isolate.
Adapted from: Hansen (2003) and Peisker (2001)

Anti-nutritional factors decrease in concentration as the elaboration increases and the soy product becomes richer in protein. The increased concentration of protein associated with a lower level of ANF increases the value of soy products in a proportionally greater fashion than the increase in cost of production. They are therefore much sought after products in specialty diets. However, they remain uneconomical in diets of older livestock animals as those animals are less sensitive to the ANF and their protein requirements can be met with lower concentrations and/or quality of proteins.

5. Official Standards of Some Soybean Products

While a large number of compositional tables and publications for soybean products exist, those data cannot be considered as standard values, especially not for trading purposes. For trading and contractual purposes they are too detailed and thus unpractical. Furthermore, they do not provide the required borderline minimum or maximum values for limited number readily identifiable parameters.

A limited number of official standards have been published to start with the basic material: whole, untreated soybeans or seeds (IFN 5-04-610). As is the case for all other grains and seeds the USDA publishes official standards for soybean grains as defined under the United States Grain Standards Act. These standards do not generally change much over time and under the act soybeans are defined as grains that consists of 50 percent or more of whole or broken soybeans (Glycine max (L) Merr.) that will not pass through an 8/64"round hole sieve (3183 microns) and does not contain more than 10.0 percent of other grains for which standards have been established under the United States Grain Standards Act (USDA, 1999).

For trading purposes – especially in view of specific applications and export requirements – additional specifications are provided by dividing soybeans into classes and grades. Only two classes of soybeans have been defined (yellow soybeans and mixed soybeans) but 5 grades are specified. The grades and grade requirements for the major export countries (USA, Brazil and Argentina) are similar. However, while Brazil and Argentina have a special export grade, the United States does not define a specific export grade as soybeans are exported from the US at any pre-defined specification or grade. The USDA (1999) description of grades is provided in 5.

Next to whole soybeans only three soybean products (two soybean meals and soybean oil) have standard values. Used as official references standards they have been developed by the National Oil Processors Association (NOPA, 1997) and are also published by the American Soybean Association (ASA, 1998) in the Soy Importers Handbook. These standards are now widely accepted and provide minimums or maximums on only a few, easily identifiable, key parameters. In the case of soybean meals their main purpose is the classification of soybean meals into two main categories: solvent extracted SBM and dehulled, hipro SBM.

Table 5

US grades and grade requirements for soybeans

Grade	Minimum test weight		Maximum limits of:				
			Damaged kernels				
	per bushel (lbs)	per hl (kg)	Heat damaged %	Total %	Foreign material %	Splits %	Soybeans of other colors %
U.S. No.1	56	72	0.2	2.0	1.0	10.0	1.0
U.S. No.2	54	69	0.5	3.0	2.0	20.0	2.0
U.S. No.3 (1)	52	67	1.0	5.0	3.0	30.0	5.0
U.S. No.4 (2)	49	63	3.0	8.0	5.0	40.0	10.0
U.S. Sample grade (3)							

(1) Soybeans that are purple mottled or stained are graded not higher than U.S. No 4.

(2) Soybeans that are materially weathered are graded not higher than U.S. No 4.

(3) Soybeans that do not meet the requirements for U.S. Nos. 1,2,3 or 4, or

i) Contain 8 or more stones which have an average weight in excess of 0.2% of the sample weight, 2 or more pieces of glass, 3 or more Crotalaria seeds, 2 or more castor beans, 4 or more particles of an unknown substance(s), 10 or more rodent pellets, bird droppings or equivalent quantity of other abnormal filth per 1,000 grams of soybeans; or

ii) Have a musty, sour or commercially objectionable foreign odor (except garlic odor);

iii) Are heating or otherwise of distinctly low quality.

See also: USDA, 2001: http://www.usda.gov/gipsa/reference-library/brochures/soyinspection.pdf

For soybean oil the NOPA standards refer to crude degummed soybean oil mainly with food application purposes in mind. These standards serve as a general guide for transactions, thus assuring a minimal degree of quality and consistency in at least the three main types of soy products being traded. However, the standards and trading guidelines proposed by NOPA are not binding. Organizations, companies or individuals participating in a transaction involving soybean meals are free to adopt, modify or disregard the NOPA rules. They principally serve the trading and marketing of US soybean products within the USA but due to their wide acceptance, their impact goes well beyond US meals (and oils) as they are generally applied to compare and benchmark soybean products from other origins.

Solvent extracted soybean meal can be the result of blending back soybean hulls in the dehulled meal. The blending of different types of soybean meals or soybean components at the point of shipping is allowed under NOPA regulations and standards for minimum blending procedures are provided. As a matter of fact, this can be the source of a significant variation in quality and chemical composition. However, blending of soybeans is not permitted. For soybean meals only soy hulls, soybean mill run and soybean mill feed are permitted to be blended with soybean meals before the point of sampling. The blending must lead to a meal of uniform quality representative of the contract terms.

Table 6

Specifications for solvent extracted and dehulled soybean meals (%)

	Min/Max	Solvent extracted SBM	Dehulled SBM
Moisture	max.	12	12
Protein	min.	44	47.5 - 49
Fat	min.	0.5	0.5
Crude fiber	max.	7	3.3 - 3.5
Anti-cacking agent	max.	0.5	0.5

NOPA, 1997

For SBM, the NOPA standards clearly aim at providing a minimum number of primary quality characteristics and as such are only a basis for contract specifications (Table 6). The only characteristics defined are moisture, crude protein, fat and crude fiber with a maximum tolerance for an anti-caking agent. Beyond purchasing and possibly storage allocations these specifications have little impact on normal feed milling operations; neither from a specific quality point of view nor from a formulation perspective. They do not provide a sufficiently detailed overview of the nutritional characteristics required for proper quality management or further use. Meals purchased under NOPA contract specifications will therefore still need additional analysis. In order to provide greater quality assurances and meet the nutritional requirements of the feed compounder or nutritionist additional recommendations have been added by NOPA (Table 7- next page).

These are, again, only recommendations that apply in a non-binding manner to all soybean meals. Rather than guidelines they should be regarded as further suggestions to both producers of soybean meal and buyers, provided in an effort to improve the quality of US soybean meals. Under practical conditions there remains a large variation around these recommendations and from a feed compounder's point of view, information on quality requirements for SBM needs to be still more detailed. Also, new parameters have been added and more recently evaluations have changed slightly. For instance there is a definite tendency for KOH values to shift to the high end of the established range (close to the 85 % value).

Table 7

Recommended additional specifications for soybean meal

Lysine	2.85 % (basis 88 % dry matter)
Ash	< 7.5 %
Acid insoluble ash (silica)	< 1 %
Protein solubility in 0.2 % KOH	73 - 85 %
Urease activity	0.01 - 0.35 pH unit rise
Bulk density	57 - 64 g/100 cc
Screen analysis (mesh)	95 % thru # 10, 45 % thru #20, 6 % thru # 80
Texture	Uniform, free flowing, no lumps, cakes, dust
Color	Light tan to light brown
Odor	Fresh - not musty, sour, ammonia, burned
Contaminants	No urea, ammonia, pesticides, grains, seeds, molds

NOPA, 1997

The Protein Dispersibility Index (PDI), an additional measure of protein quality, has been added as a routine quality evaluation. This follows the general application of this method in evaluating protein quality in products for human consumption (AACC, 1976). The results of this method are considered to be superior to the KOH solubility especially where it concerns cases of inadequate heat treatments (Batal et al., 2000). The KOH solubility index is considered better to estimate overheating of SBMs. Nevertheless, consistent application of the recommendations in Table 7 would go a long way in meeting product quality and nutritional requirements.

An additional degree of detail is necessary for the regular and detailed formulation changes that are required to meet the performance guarantees of

animal diets and the constant cost-reduction objectives. The generation of this information is, at present, considered to be the responsibility of the in-house quality control and analytical services organization of the feed compounder. As a matter of fact, this is often regarded as part of the proprietary know-how by feed manufacturers. It does, however, offer the crusher an opportunity to provide a more consistent and better quality product and therefore a means to add value to a commodity. As identity preservation (IP) and traceability tools improve, a greater detail and guarantee on nutritional characteristics will be possible.

The NOPA standards for soybean oil have the same objectives as those for SBM i.e. providing a framework for trading and contract negotiations. However the emphasis is on oil for human consumption as the designated types are for edible oil (officially referred to as crude degummed soy oil). As a matter of fact, no standards for oil used in animal feed is available and most feed companies or users of oil in animal feed have developed in-house standards for oils and fats or mixtures of both. These proprietary standards for animal feed will generally be slightly more relaxed (see Table 8) but information for additional parameters such as iodine and peroxide numbers are often required. On the other hand, information on P levels and flash point are not considered. This difference in standards allows for the use of soy oils which are rejected for human consumption to be used in animal feed provided they

Table 8

Standards for edible crude degummed soybean oil and vegetable oils in animal feed

Analytical parameter	Unit	NOPA[1] Max	Feed[2] Max
Unsaponifiable matter	%	1.5	1.5
Free fatty acids (as Oleic acid)	%	0.75	1
MIU (Moisture, Isolubles, Volatile matter)	%	0.3	1
Flash point	°F	250	–
Phosphorous	%	0.02	–
Iodine value	g/100g EE	–	130 - 136[3]
Peroxide value	Meq/kg	–	2

[1,2] NOPA, 1999; Feed refers to common values for vegetable oil.
[3] Range for soybean oil.

meet the still stringent formulation and feed quality guarantees. In general, soy oil usage in animal feed is reserved for specialty feeds often for those diets where highly digestible energy sources are needed. This is typically the case in young animal diets.

Besides the basic products (soybeans, soybean oil, solvent extracted SBM and dehulled SBM) there are no published requirements or recommendations for the large array of other soy products that are marketed in various forms and conditions. This leaves it up to the user to set internal quality control measures. Those may include most of the criteria considered for the 3 main (basic) products but they will need to go beyond this and include a measure of anti-quality components (anti-nutritional factors – ANF), expanded amino acid profiles, in vitro digestibility and measures of microbial contamination. It is interesting to notice that no specific requirements have been published on the degree of microbial presence in soybeans or SBM. The end user will therefore have to apply industry norms as established by local governments or organizations.

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6. Sampling Soy Products

The quality of any analysis carried out on feed or the feed ingredients stands or falls with the sampling tools and procedures. It seems evident - but is not necessarily recognized under routine operating conditions - that in order for any subsequent analytical work and interpretation to make sense, the collection of a correct, representative sample is fundamental.

The objective of any sampling procedure, no matter what the subject to be evaluated may be, is collection of a truly representative sampling; a sample that represents to the greatest possible degree the composition and characteristics or the material to be analyzed or studied. This always leads to a compromise between cost of sampling and analysis and the degree or precision or confidence that is acceptable. Statistical tools have been developed to asses the minimal number of samples needed to achieve a given level of confidence regarding the composition of the ingredients (see Chapter 10). As the number of samples that have been collected and analyzed increases and variation for a particular nutrient and ingredient is better understood, a more precise number of samples and sampling frequency can be established. In the animal feed business, separate estimates of the number of samples per supplier are not only recommended but are routine procedures for many feed producers.

The sampling techniques and procedures vary with the ingredient, the form or particle size of the ingredient, the conditioning and size of the consignment, methods of loading or unloading and storage conditions. The soy products that are used in animal feeds cover the entire range of physical forms from seeds to flakes and powder and sampling methods will therefore need to be adapted to the specific ingredient that enters a feed plant. Details to this extent need to be included in quality control (QC) procedures and do now appear routinely on QC documents. These techniques are fairly standard throughout the world and a detailed description of sampling techniques for grains and seeds have been provided by Herrman (2001). They apply to the majority of the soy products, in bag or bulk. Also NOPA has published basic rules for the sampling of soybean meal at vessel loading facilities using an automatic sampling device (see Appendix 3 - 6). These procedures are practical and can be implemented under almost any condition or operating procedure. A small degree of local adaptation may be necessary and may even be advisable to assure the collection of a truly representative sample. The experience

and training of the samplers and persons in charge of the quality program will determine to a large extent the efficacy of the sampling program and thus the precise way to sample.

Prior to sampling soybean products a sampling scheme or frequency has to be established. For a given ingredient this will depend to a large extent on the supplier and the information received prior to delivery. Additional considerations are laboratory capacity and availability, analytical cost, size of the consignment and the use of the soy product (in which feed it will used as an ingredient and at which percentage). In general, random sampling of different consignments (corrected for experience or prior knowledge about the supplier and ingredient) is combined with systematic sampling of the vessel, truck or container. To this purpose a pre-determined sampling grid is established. Details on the sampling of open containers with soybean products are taken from Herrman (2001) and GIPSA (1995) and are summarized in Appendix 3- 6. A first, rapid evaluation of the material to be sampled and of the sample is considered part of the sampling procedure. The total load (bags, container or carrier) is evaluated for homogeneity and possible local damage during loading or transport. In the case of a homogeneous delivery a pre-established sampling grid is applied and samples are collected accordingly (Appendix 3A). Separate sampling schemes have been developed to allow sampling of sound versus damaged areas (Appendix 3B).

The tools that are used to sample depend on the material and form in which the ingredient has been transported. While automatic sampling of trucks or containers is increasingly implemented, hand-sampling remains a dominant means of obtaining sample of soy products. In the case of hand- sampling, slotted grain probes can be used to correctly sample soy beans and meals from a bag or a container (Appendix 4 - Figure 1A). Tapered bag triers (Appendix 4 - Figure 1B) are used to sample powder and granular material, such as SPC and SPI from bags. For the sampling of soybeans or soybean meals from a conveyer belt or a discharging truck, a Pelican Probe sampler can be used (Appendix 4 - Figure 1D). The sampler is pulled through a stream of falling grain or meal, collecting a sample into a leather bag. NOPA has established special procedures for sampling soybean meals at vessel loading facilities (Appendix 6).

The sampling of oil follows principles of sampling of other liquid feed ingredients. A bomb or zone sampler (Appendix 4 - Figure 1 C) is used to collect liquids such as soy oil from bulk containers. This sampler consists of a closed cylinder (30 to 40 cm long by 4.5 to 7.5 cm in diameter) which is lowered at pre-defined

places in an oil tanker and filled with a 100 to 1000 ml sample of oil. Drums are sampled using a glass or stainless steel tube 1 – 1.5 cm in diameter and 50 – 100 cm long (Herrmann, 2001). A minimum of 500 ml sample of liquid must be obtained for storage and sub-sampling.

The size of the sample depends on the homogeneity of the load (or lack thereof), and - again - previous experience is of importance. A larger sample should be collected than that what is ultimately retained for further analysis and storage (for the minimal legally required period). A minimum sample size of 2 kg is recommended. In order to reduce the sample to the minimal required size, the sample is passed through a gated riffle sample splitter (25 mm riffles) or a Boerner divider (Appendix 6 - Figure 2 A and B respectively). This is done repeatedly until the sample is homogeneous. A sub-sample (minimum 500 g) is obtained for further analysis and storage.

The sample obtained prior to reduction as well as the final sample is rapidly evaluated for test or specific weight and a number of physical and organoleptic characteristics. The reduced sample is divided in two portions of roughly equal size (250 g). Both are stored in airtight containers. One container is dispatched to the laboratory for further analysis; the second container is stored in a dry storage area, reducing to a minimum any type of chemical changes due to deterioration as the sample may be used for subsequent analysis in the case of claims.

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7. Physical Evaluation and Equipment

Following the sampling, three types of evaluations are carried out on soybean products. These are: Physical, Chemical and Microbiological. The Physical examination of the material aims at establishing the general soundness of the product, its origin and a rapid, general approximation of nutritive quality. This is a series of tests the merchandise has to pass in order to be accepted by the buyer. The chemical analysis will establish the nutritive value of the product. The specific analysis carried out may differ according to future use (animal species). Results of these analyses aim at providing the basis for a detailed nutritional profile possibly resulting in adaptations in the formulation matrix. As such they establish the maximum and minimum level of use in a feed as well as a precise price: quality relationship for the ingredient and the individual nutrient supplied by the ingredient. The micro-biological evaluation intends to reveal any specific microbial, fungal or yeast contamination. It mainly refers to levels of salmonella and specific mycotoxins (mainly zearalenone and ochratoxins). Exceeding pre-set (often legal) limits will lead to a rejection of the material for further use or modifications in the inclusion level and/or the production technology. All measures - physical, chemical and biological – when found to be outside the contractual or legal limits may lead to claims and or changes in the contractual agreement.

Soybean products are evaluated for a number of physical and organoleptic criteria. A first evaluation of this type is carried out prior to sampling, but is repeated on the original sample. In general a vessel, container, truck or bag is inspected before unloading and a sample is taken. Only when the merchandise is considered acceptable - upon general evaluation and a rapid analysis of the sample - will unloading proceed. This inspection approaches the more detailed physical evaluation of the sample and requires a certain level of expertise of the quality control person. Inspection criteria should be part of a pre-established quality system. Most important are those referring to the physical characteristics provided in Tables 5 and 7. More stringent in-house standards or requirements may apply. At this stage the important criteria for whole soybeans and soybean meals are: contamination or foreign materials, bulk density, texture, particle size or screen analysis, color and odor.

The latter, color and odor are rapidly evaluated on the entire load by a trained person. They are the first evaluation but are of crucial importance. Deviations from the standard colors indicate excessive contamination with foreign material or excessive or inadequate heat treatment. Deviations from the characteristic odor may

confirm the visual observations but will also provide a first idea of the past storage conditions, contamination with other substances (especially liquids) and the excessive presence of molds.

All further physical evaluations should be carried out at a plant laboratory or special QC area. A first appreciation of the degree of contamination with foreign material is obtained visually. A detailed count is obtained from the sample by physically (hand-) separating a sub sample and weighing the various fractions. It is recommended at this stage to take a sample for light microscopic analysis. Evaluation of a sub-sample under a microscope permits a more detailed analysis of the material and the contaminants. In general a wide field stereoscopic microscope with a magnification of 20 to 40 times is adequate. Additional equipment required for microscopic evaluation is a microscope-illuminator, forceps or probe and in the case of large clumps a mortar and pestle. Precise analysis of contamination is possible with a microscope but requires an experienced operator and may require additional techniques specific to light microscopy in feed analysis.

Bulk density is measured by taking a liter of material (in an official container – kettle) and weighing the content. Bulk density (expressed in lbs/bu, g/100 cc or kg/hl) is a first appreciation of various attributes of the received ingredient namely: the moisture content, texture and level of damage or contamination. The range of required bulk density (test weight) for soybeans increases with the grade from 63 kg/hl for grade 4 to 72 kg per hl for grade 1 (Table 5). For soybean meals a single range of 57 to 64 kg/hl is recommended (Table 7). The importance of this measure has come under some criticism, especially from foreign operators. While it is widely used in North America, only a minor number of processors or compounders outside the USA use test weight on a regular basis. The equipment required for these measures is relatively simple. Besides the kettle used to measure bulk density, a balance with a minimum accuracy of + 0.1 grams is required (ASAE, 1993).

Texture may be considered as primarily a visual observation (verifying the absence of lumps, cakes or coarse particles). A first rapid evaluation can be carried out by hand-sieving a sample in a 0.525 Tyler (0.530US standard equivalent; 13.5 mm) sieve. For a more precise and objective estimation of particle size (especially the presence of small or dust particles) an analysis with an official particle separator needs to be conducted. Special equipment for particle size separation exists. Generally, a RoTap Sieve Shaker is used for this purpose. This allows separation of particles to a size down to 150 micron (0.0059 inch) covering adequately requirements for standards advised for soybean meal (see Table 7).

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8. Chemical Analyses

The nutritional quality of a feed ingredient, and thus soybean products, is dependent on the content of several chemical elements and compounds which carry a nutritional function. These elements and compounds are referred to as feed nutrients. When feeding animals, nutritionists select a combination of ingredients that supply the right amounts of a series of feed nutrients. Therefore, when preparing rations, ingredients are treated as carriers of feed nutrients. Thus, the quality and value of a given ingredient will largely depend on the concentration of its nutrients. Because determining the content of all feed nutrients is extraordinarily time consuming and almost impossible, nutritionists use different systems for estimating or approximating the nutritional value of a feed. The most common system is the so-called Weende system (developed in Germany more than 100 years ago). The system measures water or humidity, crude protein, crude fat, crude fiber, ash and nitrogen-free extract. This method has been proven to be useful for assessing the value of ingredients, however, as with any system, it has a number of shortcomings. The most important one refers to the crude fiber fraction (and consequently the nitrogen-free extract which is not directly determined but calculated by difference). Nowadays, as will be discussed later in this chapter, there are improved methods to determine nutrients within the fibrous fraction of soybean products.

Soybean meal is one of the most consistent (least variable) and highest quality protein source for animal nutrition. However, some variation does occur in both the nutrient concentration (chemical determination) and quality (digestibility or bioavailability) among different samples and sources of soybean meal. These variations can be attributed to the different varieties of soybeans, growing conditions, storage conditions and length, and processing methods. Because soybean products, especially soybean meals, are such an important fraction of feeds (in poultry they can account for 35% of the total formula) it is crucial to monitor the quality of soybean products. Small changes in quality might translate into important changes in animal performance due to their high inclusion rate in the ration.

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8.1 Moisture

Moisture content is one of the simplest nutrients to determine, but at the same time is one of the most important. The moisture content of soybean products is important for three main reasons:

1. To establish the appropriate acquisition price based on the concentration of the nutrients on a dry matter basis and thus not paying more than necessary for water.
2. A wrong determination of moisture will affect the rest of the nutrients when expressed on a dry matter basis, potentially leading to erroneous concentrations of nutrients in formulated diets.
3. To assure that mold growth cannot occur.

In general, samples with moisture content above 12.5% present a high risk of molding, and should be accepted with caution and correspondent penalties for quality. However, moisture is not evenly distributed across the sample particles. A sample batch containing an average of 15.5 percent moisture may, for example, contain some particles with 10 percent moisture and others with 20 percent moisture. The particles with the highest moisture content are the ones most susceptible to mold growth. Consequently, at the early stages of development mold growth is often concentrated in specific areas of a batch of soy products underlining the importance of good sampling methods. To determine moisture content it is necessary to have a forced-air drying oven, capable of maintaining 130°C (± 2°C), porcelain crucibles or aluminum dishes and an analytical balance with a precision of 0.01 mg.

The official method (AOAC, 1990) to determine the moisture content of soybean products consists of:

- Hot weighing porcelain crucibles and registering their tare.
- Placing 2 ± 0.01 g of ground sample in a porcelain crucible and drying at 95-100°C to a constant weight (usually about 5 hours is sufficient).
- Hot weighing crucible and sample.
- Calculating the moisture content as a percentage of original weight:

$$\text{Moisture, \%} = \frac{\text{Original weight} - \text{Final weight}}{\text{Original weight}} \times 100$$

and

$$\text{Dry matter, \%} = 100 - \text{moisture, \%}$$

An alternative, but less accurate method that has the advantage of being fast and simple is the determination of moisture with a microwave. In this method a sample of 100 g is placed in a microwave oven for about 5 minutes. It is important not to run the microwave for more than 5 minutes to avoid burning the sample. Reweigh and record the weight, and place the sample in the microwave for 2 more minutes. Repeat the process until the change in weight is less than 0.5 g than the previous one. This weight would be considered the dry or final weight. The calculations are performed as indicated above.

In feed plants, for routine QC procedures, moisture is often determined by the Brabender test. Like the microwave method, this test is rapid, simple and considered less accurate than the oven dried reference method. This test requires a small, semi-automatic Brabender moisture tester, a scale and aluminum dishes. For most soy products the thermo-regulator of the Brabender moisture tester is set to 140°C with the blower on. Allow the unit to stabilize (± 0.5°C). Tare an aluminum dish on the analytical balance. Weigh ~10 g of sample in the dish and record exact weight. Place the dish (or dishes, up to 10) in the oven, close door. Start timing when temperature returns to 140°C and then dry for one hour. Re-weigh the sample hot after the specified drying time. Calculate moisture with equation above.
Moisture can also be determined by near infrared spectroscopy (see Chapter 9).

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8.2 Ash

Ash determination requires a muffle furnace, porcelain crucibles, and an analytical balance (precision of 0.01 mg).

The ash content of soybean products is determined by weighing 2 ± 0.1 g of sample in a tared porcelain crucible and placing it in a furnace at 600°C for 2 hours. The oven is turned off, allowed to return to room temperature and the crucible plus ash weighed. To obtain the ash content of the sample, the final weight should be divided by the initial weight and then multiplied by 100 to express it in a percentage basis. The ash content is thus calculated as:

$$\text{Ash, \%} = \frac{\text{Final weight}}{\text{Original weight}} \times 100$$

Monitoring ash content is not only a way to assess the nutritional quality of soybean products but also to detect possible contaminations, especially soil.
For example, the ash content of soybean meal should not exceed 7%.

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8.3 Protein

Protein is no doubt the most important and frequently analyzed nutrient in soy products. The protein content of soybean products is estimated as total nitrogen in the sample multiplied by 6.25. This assumes that protein in soybean products has 16% nitrogen; however, the actual amount of nitrogen in soybean protein is 17.5%. Nevertheless, like for most other ingredients used in feed formulation, the standard value of 6.25 is used. Determining crude protein from nitrogen content has the

drawback that part of the nitrogen present in soybean products is considered to be part of proteins (or amino acids), which is not the case as there is nitrogen in the form of ammonia, vitamins and other non-protein compounds. However, the nitrogen fraction that is not in the form of amino acids or protein in soybean products is very small and corrections for the difference in N content in soybean products relative to other ingredients are carried out at the amino acid level.

The most accurate method for determining the nitrogen content of soybean products is the Kjeldahl method. This method consists of digesting the sample in sulfuric acid (H_2SO_4) and a copper and titanium catalyst to convert all nitrogen into ammonia (NH_3). Then, the NH_3 is distilled and titrated with acid. The amount of nitrogen in the sample is proportional to the amount of acid needed to titrate the NH_3. The Kjeldahl method requires:

- A digestion unit that permits digestion temperatures in the range of 360 – 380°C for periods up to 3 hours.
- Special Kjeldahl flasks (500 – 800 ml).
- A distillation unit that guarantees air-tight distillation from the flask with the digested sample into 500 ml Erlenmeyer flasks (distillation receiving flask).
- A buret to measure exactly the acid that needs to be titrated in the receiving flask to neutralize the collected ammonia hydroxide.
- All Kjeldahl installations require acid-vapor removing devices. This may be by a fume removal manifold or exhaust-fan system, water re-circulation or a fume cupboard.

The chemical needs for the procedure are as follows:

- Kjeldahl catalyst: contains 10 g of K_2SO_4 plus .30 g of $CuSO_4$.
- Reagent grade, concentrated H_2SO_4
- Mixed indicator solution: 3125g methyl red and .2062 g methylene blue in 250 ml of 95% ethanol (stirred for 24 hours).
- Boric Acid Solution: 522 g U.S.P. boric acid in 18 l of deionized water. Add 50 ml of mixed indicator solution and allow stirring overnight.
- Zinc: powdered or granular, 10 mesh.
- Sodium hydroxide: 50% wt/vol. aqueous (saturated).
- Standardized .1 N HCl solution.

The procedure is as follows:

- Weigh a 1 g sample and transfer into an ash free filter paper, and fold it to prevent loss of sample.
- Introduce one catalyst in the Kjeldahl flask.
- Add 25 ml of reagent grade, concentrated H_2SO_4 to each Kjeldahl flask.
- Start the digestion by pre-heating the digester block to 370°C, and then place the Kjeldahl flaks on it for 3 hours.
- After removing flasks from the digester, and once they are cool, add 400 ml of deionized water.

- Prepare the receiving flask for steam distillation by adding 75 ml of prepared boric acid solution to a clean 500 ml Erlenmeyer flask and place on distillation rack shelf. Place delivery tube from condenser into the flask.
- Turn the water on the distillation system and all the burners on.
- Prepare the sample for distillation by adding approximately .5 g of powdered zinc to flask, mix thoroughly and allow to settle.
- After digest has settled, measure 100 ml of saturated, aqueous NaOH (50% wt/vol) into a graduated cylinder. Slant Kjeldahl flask containing prepared digest solution about 45° from vertical position. Pour NaOH slowly into flask so that a layer forms at the bottom. All these operations need to be performed wearing gloves and a face mask.
- Attach flask to distillation-condenser assembly. Do not mix flask contents until firmly attached. Holding flask firmly, making sure cork is snugly in place, swirl contents to mix completely. Immediately set flask on heater. Withdraw receiving flask from distillation-condenser delivery tube momentarily to allow pressure to equalize and prevent back suction.
- Continue distillation until approximately 250 ml of distillate has been collected in receiving flask.
- Turn heater off. Remove receiving flask partially and rinse delivery tube with deionized water, collecting the rinse water into receiving flask.
- Replace receiving flask with a beaker containing 400 ml of deionized water. This water will be sucked back into the Kjeldahl flask as it cools, washing out the condenser tube.
- Titrate green distillate back to original purple using 0.1 N HCl and record volume of acid used in titration.
- It is recommended to use a couple of blanks and controls or standards on every run. Blanks - Kjeldahl reagents generally contain small amounts of nitrogen, which must be measured and corrected for in calculations. Prepare blanks for dry samples by folding one ash free filter paper and placing it into the Kjeldahl flask. Treat blanks exactly like samples to be analyzed.

Standards: weigh two 0.1 g samples of urea, transfer into an ash free filter paper and treat exactly like the rest of samples. Calculate percent recovery of nitrogen from urea and make sure the obtained result is the one expected.

The calculation is:

$$\text{Crude protein, \%} = \frac{(\text{ml of acid} - \text{ml of blank}) \times \text{normality} \times .014 \times 6.25 \times 100}{\text{Original weight}} \times 100$$

A more recent and alternatively way to determine nitrogen content is by the Dumas method. The method requires very little sample but the sample size will differ with the type of ingredient to be analyzed. Sample size depends largely on the expected level of crude protein in the material. In the case of soybean products a sample size of 50 – 150 mg is recommended (AOAC, 2000). The sample is placed in a

tin foil cup for subsequent burning at 850 - 900°C to determine the amount of N_2 by nitrometer. This method has the advantage over the Kjeldahl that is faster, better suited for automation and creates little residues. However, the Kjeldahl method continues to be the reference method. Total Dumas nitrogen can be slightly higher than values obtained with the classical Kjeldahl method. However, for most purposes, especially in the case of soy products, the difference is extremely small.

Crude protein can also be predicted by NIR, with an acceptable relative standard deviation of about 0.42% (see Chapter 9).

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8.4 Protein quality

Protein quality is a function of the amino acid profile and the proportion of each amino acid that is available to the animal. When soybean meals are intended for monogastric feeding it is well known that proper heat processing has a dramatic positive effect on amino acid digestibility, consequence of the destruction of anti-nutritional factors (Table 1). However, over-heating can result in a decrease in both concentration (Table 9) and digestibility of several amino acids, especially lysine. The reduction in digestibility is due to the Maillard reaction which binds free amino acids to free carbonyl groups (i.e., from carbohydrates). The Maillard reaction-end products are not bio-available for all livestock species.

Table 9

Effect of heat processing on amino acid digestibility of raw soybeans in poultry (adapted from Anderson-Haferman et al., 1992)

Autoclaving (minutes)	Lysine	Methionine	Threonine
0	73	65	64
9	78	70	68
18	87	86	82

Table 10

Effect of heat-processing soybean meals on amino acid concentration (adapted from Parsons et al., 1992)

Autoclaving (minutes)	Lysine %	Methionine %	Cystine %	Threonine %
0	3.27	0.70	0.71	1.89
20	2.95	0.66	0.71	1.92
40	2.76	0.63	0.71	1.87

There are several methods (Table 12-page 41) to determine protein quality of soybean products for monogastric species.

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8.4.1. Urease Index

The urease index (AOCS, 1980) is the most common test used to evaluate the quality of the soybean processing treatment. The method requires a pH meter, volumetric flasks (250 ml), a small water bath that allows maintenance of temperature at 30°C for at least 30 minutes, test tubes and a pipette.

The method determines the residual urease activity of soybean products as an indirect indicator to assess whether the anti-nutritional factors, such as trypsin inhibitors, present in soybeans have been destroyed by heat processing.
Both enzymes, urease and trypsin inhibitor, are deactivated during heating.
The laboratory method for urease involves mixing soybean meal with urea and water for one minute.

Procedure:

- Place 0.2 g of soybean sample in a test tube.
- Add 10 ml of a urea solution (30 g of urea into 1 l of a buffer solution, composed of 4.45 g of Na_2HPO_4 and 3.4 g of KH_2PO_4).
- Place the test tube in a water bath at 30°C for 30 minutes.
- Determine pH and compare it with the original pH of the urea solution.

The test measures the increase in pH consequence of the release of ammonia, which is alkaline, into the media arising from the breakdown of urea by the urease present in soybean products (urea is broken down into ammonia and carbon dioxide). Depending on the protocol used, the endpoint is determined differently. In the American Oil Chemists Society (AOCS, 1980) method, the endpoint is determined by measuring the increase in pH of the sample media. In the EEC method, the endpoint reflects the amount of acid required to maintain a constant static pH. Results of these two methods differ slightly from one another.

The optimum pH increase is considered to be between 0.05 (McNaughton et al., 1980) and 0.20 (Waldroup et al., 1985). Usually, all overheated samples yield urease indexes below 0.05, but that does not imply that all samples with urease tests below 0.05 have been overheated. It is recommended that, when using soybean products for swine or poultry the increase in pH is not greater than 0.35 (Waldroup et al., 1985). Animal performance is severely impaired with urease indexes above 1.75 pH units.

The urease test is useful to determine whether the soybean has been sufficiently heated to deactivate anti-nutritional factors, but it is not a good indicator to assess whether the soybean product has received an excessive heat treatment.

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8.4.2. KOH Protein Solubility

This method consists of determining the percentage of protein that is solubilized in a potassium hydroxide (KOH) solution (Araba and Dale, 1990). The method requires volumetric flasks (250 ml), a small magnetic stirrer, filtering funnels or a centrifuge, and the Kjeldahl equipment to measure nitrogen.

Procedure:

- Determine nitrogen content of soybean sample using official methods.
- Place 1.5 g of soybean sample in 75 ml of a 0.2% KOH solution (.036 N, pH 12.5) and stir at 8,500 rpm for 20 minutes at a temperature of 22°C.
- Then, about 50 ml is taken and immediately centrifuged at 2500 x g for 15 minutes.
- Take aliquot of about 10 ml to determine nitrogen content in the liquid fraction by Kjeldahl method.
- The results are expressed as a percentage of the original nitrogen content of the sample.

The KOH protein solubility is not sensitive enough to gauge the level of heat processing that a soybean product has undergone, but it is effective in differentiating overheated products from correctly processed ones.

Table 11

Effect of autoclaving soybean meal on chick performance (1-18 days), KOH protein solubility and urease activity (adapted from Araba and Dale, 1990)

Autoclaving (120°C) minutes	Weight gain g	Feed : gain ratio	KOH protein solubility %	Urease Index (pH units change)
0	450[a]	1.79[c]	86.0	0.03
5	445[a]	1.87[bc]	76.3	0.02
10	424[a]	1.83[bc]	74.0	0.00
20	393[b]	1.89[b]	65.4	0.00
40	316[c]	2.04[b]	48.1	0.00
80	219[d]	2.55[a]	40.8	0.00

a, b, c, d Means within a column with common superscripts are not significantly different ($P < 0.05$).

The solubility values have been correlated with growth rates in poultry and swine (Lee and Garlich, 1992; Araba and Dale, 1990), with a clear decline in performance with solubility values below 72%. Raw soybeans and well heat-processed soybean products should have a protein solubility around 90% (that is 90% of the protein present in the product is solubilized in a KOH solution).

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8.4.3. Protein Dispersibility Index (PDI)

Among the available tests for determining protein quality in soybean products, the PDI is the simplest, most consistent, and most sensitive method. This test measures the solubility of soybean proteins in water and is probably the best adapted to all soy products. The PDI method measures the amount of soy protein dispersed in water after blending a sample with water in a high-speed blender. The water solubility of soybean protein can also be measured with a technique called Nitrogen Solubility Index (NSI). Thee two methods differ in the speed and vigor at which the water containing the soybean product is stirred. In animal nutrition the PDI method is used.

Both methods require a blender (8,500 ppm), filtering funnels or a centrifuge, and the routine Kjeldahl equipment for N analysis.

Procedure:

- Determine nitrogen content of soy sample using official methods.
- Place a 20 g sample of a soybean product in a blender.
- Add 300 ml of deionized water at 30°C.
- Stir at 8,500 rpm for 10 minutes (AOCS, 1993a).
- Filter and centrifuge for 10 minutes at 1000g.
- Analyze nitrogen content of the supernatant.
- The results are expressed as a percentage of the original nitrogen content of the sample.

The NSI method uses a 5 g soybean sample into 200 ml of water at 30°C stirred at 120 rpm for 120 minutes (AOCS, 1989). With either method, the final step consists of determining the nitrogen content of the liquid fraction and the results are expressed as a percentage of the original nitrogen content of the sample.

Nowadays, most soybean producers and users of soy products advocate the PDI method as the best for assessing protein quality in soybean meals. Because this test is more recent it is often used as a complement to the urease and KOH solubility measurements. As a matter of fact, the PDI method has proven to be especially useful in determining the degree of under heating soybean meals to remove ANF. Furthermore, Batal et al. (2000) described a greater consistency in the results of heating of soy flakes obtained with the PDI procedure than those from urease or protein solubility. Since the work of Batal et al. (2000) which recommended PDI values below 45 % recommendations have shifted slightly under the influence of practical experience. Consequently, current recommendations are for soybean meals with PDI values between 15 and 30 %, KOH solubilities between 70 and 85 % and a urease index of 0.3 pH unit change or below. These meals are considered adequately heat processed, without under- nor over-processing.

Table 12

A brief description of available methods to determine protein quality of soybean meal

Urease Index

1. Mix 0.2 g of soybean meal with 10 ml of urea solution (3% of urea)
2. Place in 30°C water bath for 30 minutes
3. Determine pH
4. Calculate pH increase (final pH - initial pH)

KOH Protein Solubility

1. Mix 1.5 g soybean meal with 75 ml of 0.2% KOH solution and stir for 20 minutes
2. Centrifuge at 2,500 x g for 20 minutes
3. Measure soluble nitrogen in the liquid fraction

Protein Dispersibility Index (PDI)

1. Mix 20 g of soybean meal with 300 ml of deionized distilled water
2. Blend at 8,500 RPM for 20 minutes at a temperature of 22°C.
3. Centrifuge (1000 x g for 10 minutes) or filter and measure nitrogen content of the liquid fraction

Nitrogen Solubility Index (NSI)

1. Mix 5 g of soybean meal with 200 ml of water
2. Stir at 120 RPM for 120 minutes at 30°C
3. Centrifuge at 1,500 RPM and measure soluble nitrogen in the liquid fraction

Absorbance at 420 nm

1. The supernatant (if centrifuged) or the liquid fraction (if filtered) from the PDI technique is diluted 80 times.
2. Filter through .2 µm pore size filter.
3. Read the absorbance of the clear filtrate at 420 nm with a spectrophotometer.

(Adapted from Dudley-Cash, W.A, 1999)

All these assays will give slightly different results depending on the particle size of the sample used, temperature of the solutions and centrifugation speeds and times. For example, protein solubility indexes will yield greater values as mean particle size decreases (Parsons et al., 1991; Whitle and Araba, 1992). Therefore, it is recommended to grind the sample at a consistent mesh size (1 mm), and to maintain (at least within the same laboratory and company) rigorously the same duration for treating the samples in the respective solutions and for centrifugation.

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8.4.4. Protein quality in ruminants

For ruminants, protein quality of soybean meals will depend on its rumen degradation and its intestinal digestion. The trypsin inhibitor factors present in soybeans are irrelevant in ruminants, as they are mostly inactivated in the rumen (Caine et al., 1998).

Amino acids are supplied to the duodenum of ruminants by microbial protein synthesized in the rumen, undegraded dietary protein, and endogenous protein. Microbial protein usually accounts for a substantial portion of the total amino acids entering the small intestine. Ruminal degradation of protein from dietary feed ingredients is one of the most important factors influencing intestinal amino acid supply to ruminants. Soybean meal is extensively degraded in the rumen, providing an excellent source of degradable intake protein for the ruminal microbes, but not enough undegradable protein to meet the demands of high producing ruminants. Because soybeans contain a high quality protein with a good amino acid profile and they are highly digestible in the small intestine, various processing methods and treatments have been used to increase its undegradable protein value. The most common methods for protecting soybean proteins from ruminal degradation are heat application, incorporating chemicals such as formaldehyde or a combination of heat and chemicals such as lignosulfonate combined with xylose.

To assess the extent of protein degradation of a soybean product several techniques are available.

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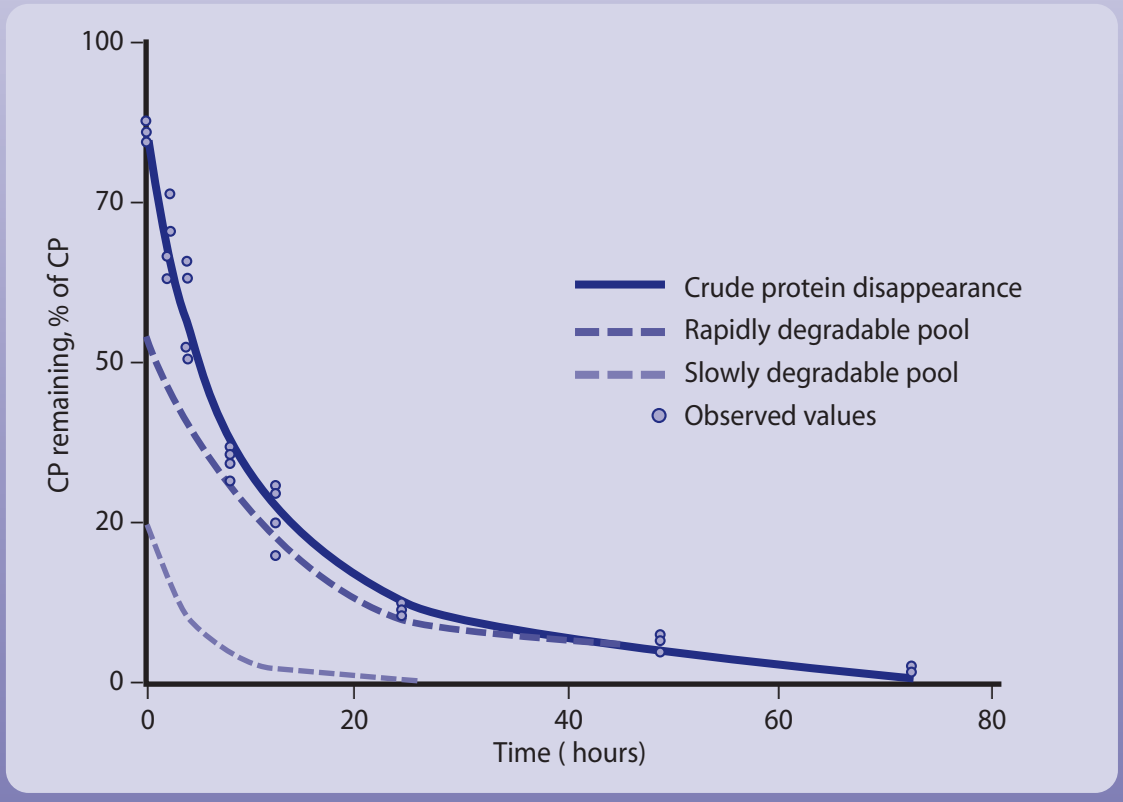
8.4.4.1. In situ technique

Although this technique is relatively expensive, labor intensive, and requires access to rumen cannulated animals, it is very useful to determine the rate of degradation of proteins from soybeans. This technique requires consecutive times of ruminal incubation of the samples under study so that the rate of protein degradation can be determined. The in situ technique determines degradation of the insoluble fraction only. The soluble fraction is considered to be totally and instantaneously degraded. To accurately predict rate of protein degradation, sufficient time points must be included in early as well as later stages of degradation (Figure 2).

Figure 2

Protein disappearance from soybean meal and curve peeling processa



Adapted from Bach et al. (1998).

After ruminal incubation, the data are fitted to different models to determine the rate of protein degradation in the rumen. Bach et al. (1998) studied the effects of different mathematical approaches (curve peeling, linear and nonlinear regression) to estimate the rate of protein degradation in soybean samples and concluded that using curve peeling (Shipley and Clark, 1972) allowed for the best separation of the different protein pools in soybean proteins.

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8.4.4.2. In vitro technique

There are several *in vitro* methods that require the use of rumen fluid, such as the Tilley and Terry (1963) technique, or the in vitro inhibitor technique (Broderick, 1987). Like the *in situ* technique, these two methods present the disadvantage that they require access to cannulated animals. The in vitro technique consists of incubating a small feed or ingredient sample with strained rumen fluid and a buffer under anaerobic conditions in a test tube or container. The test tube or container is located in a water bath that is maintained at 37 – 38°C throughout the incubation.

At regular, pre-determined intervals a sample is removed from the incubator, centrifuged and analyzed for dry matter and nitrogen disappearance (using the Kjeldahl method). Data are analyzed as described for the in situ technique.

There are a number of enzymatic techniques which have the important advantage that they are completely independent of the animal, and should result in less variation, making this technique relatively simple to standardize.

The most common enzymatic techniques are the Ficin technique (Poos-Floyd et al., 1985) and the *Streptomyces griseus* technique (Nocek et al., 1983). The biological value of the results from these techniques may be limited due to incomplete enzymatic activity compared with the ruminal environment. Mahadevan et al. (1987) found large differences when comparing digestion of different protein sources using protease from Streptomyces griseus with an extract of ruminal microbial enzymes. Chamberlain and Thomas (1979) reported that, although rate constants can be calculated using these proteases, these results do not always rank proteins in the same order as degradabilities estimated in vivo. When using enzymatic techniques to predict microbial fermentation in the rumen, it is crucial that the enzyme concentration is sufficient to saturate the substrate.
Some researchers have attempted to use near infrared reflectance spectroscopy (NIR) to estimate protein degradation of feedstuffs in the rumen. Tremblay et al. (1996) evaluated NIR as a technique for estimating ruminal CP degradability of roasted soybeans and found a coefficient of determination between NIR and undegraded protein estimated by the inhibitor in vitro technique of .70. However, the use of NIR for this purpose would require continuous access to cannulated animals to maintain the prediction equations.

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8.5. Amino Acids

Determining the amino acid composition of proteins is essential to characterize their biological value. The greater the proportions of essential amino acids the greater the biological value of a protein.

The amino acid analysis requires the use of high performance liquid chromatography (HPLC) or the combination of commercial kits and gas chromatography (GC). The analysis involves four steps:

- Hydrolysis (using HCl or barium hydroxide); this breaks the peptide bonds and releases the free amino acids.
- Separation; column chromatography separates amino acids on the basis of their functional groups.

- Derivatization; a chromogenic reagent enhances the separation and spectral properties of the amino acids and is required for sensitive detection.
- Detection; a data processing system compares the resulting chromatogram, based on peak area or peak height, to previously known and calibrated standard.

HPLC analysis for amino acids is a highly specialized laboratory procedure requiring skilled personnel and sophisticated equipment. For amino acid analysis the sample preparation is critical and differs with the type of ingredient and the amino acid of major interest. Most amino acids can be hydrolyzed by a 23 or 24 h hydrolysis in HCl (6 mol/l). For sulfur amino acids hydrolysis should be preceded by performic oxidation and for tryptophane a hydrolysis with barium hydroxide (1.5 mol/l) for 20 h is required. In general it is recommended to use a specialized laboratory to conduct the amino acid analysis.

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8.6. Crude Fiber

The original method was intended to quantify the materials in the feed that form part of the cell wall and provide relatively low energy as their digestibility is usually low. Thus, the technique was meant to quantify cellulose, certain hemicelluloses and lignin. However, later it was shown that crude fiber also included pectines, and that not all the lignin was recovered in the crude fiber fraction. The major disadvantage of this technique is that hemi-cellulose, lignin and pectines are inconsistently accounted for.

The method requires the following reagents:

- Sulfuric acid solution, .255N, 1.25 g of H_2SO_4/100 ml.
- Sodium hydroxide solution, .313N, 1.25 g of NaOH/100 ml, free of Na_2CO_3.
- Alcohol - Methanol, isopropyl alcohol, 95% ethanol, reagent ethanol.
- Antifoam agent (n-octanol).

Equipment:

- Digestion apparatus.
- Ashing dishes.
- Desiccator.
- Filtering device (Buchner filter).
- Suction filter: To accommodate filtering devices. Attach suction flask to trap in line with vacuum source.
- Vacuum source with valve to break or control vacuum.

The procedure described by the AOAC (1980) can be summarized as follows:

- Weigh 2 g of sample *(A)*. Remove moisture and fat using ether (removing fat is not necessary if the sample has less than 1% ether extract).
- Transfer to a 600 ml beaker, avoiding fiber contamination from paper or brush. Add approximately 1 g of prepared asbestos, 200 ml of boiling 1.25% H_2SO_4, and 1 drop of diluted antifoam. Avoid using excessive antifoam, as it may overestimate fiber content.
- Place beaker on digestion apparatus with pre-adjusted hot plate and boil for 30 minutes, rotating beaker periodically to prevent solids from adhering to sides.
- Remove beaker and filter as follows:
  - Filter through Buchner filter and rinse beaker with 50 to 75 ml of boiling water.
  - Repeat with three 50 ml portions of water and apply vacuum until the sample is dried. Remove mat and residue by snapping bottom of Buchner against top, while covering stem with the thumb and replace in beaker.
  - Add 200 ml of boiling 1.25% NaOH, and boil 30 more minutes.
- Remove beaker and filter as described above. Wash with 25 ml of boiling 1.25% H_2SO_4, three 50 ml portions of H_2O, and 25 ml of alcohol.
- Dry mat and residue for 2 h at 130°C.
- Remove, place in desiccator, cool, weigh and record *(B)*.
- Remove mat and residue, and transfer to an ashing dish.
- Ignite for 30 minutes at 600°C. Cool in desiccator and reweigh *(C)*.
- Calculate crude fiber content on dry matter basis as:

$$\text{Crude fiber, \%} = \frac{\text{weight after acid and base extraction } (B) - \text{weight after ashing} (C)}{\text{Original weight } (A) \times \text{\% dry matter}} \times 100$$

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8.7. Neutral Detergent Fiber (NDF)

Neutral detergent fiber (NDF) accounts for the cellulose, hemicellulose and lignin content of soybean products. These fractions represent, most of the fiber or cell wall fractions of soybean products, with the exemption that pectines are not included in the NDF fraction.

The neutral detergent fiber (NDF) was first described by Goering and Van Soest (1970) and later modified by Van Soest et al. (1991). The NDF determination requires a refluxing apparatus 600 ml and Berzelius beakers.

The technique is as follows.

Reagents:

- NDF solution: dilute 30 g of sodium lauryl sulfate, 18.61 g of disodium dihydrogen ethylene diamine tetra acetic dihydrate, 6.81 g of sodium borate

decahydrate, 4.56 g of disodium hydrogen phosphate, 10 m of triethylene glycol 65 in 1 l of deionized water.
- Acetone.

The Goering and Van Soest (1970) procedure for NDF determination is as follows:
- Weigh 0.5 to 1.0 g sample (to precision of ± 0.0001 g) in a 600-ml Berzelius beaker *(A)*.
- Add 100 ml of neutral detergent fiber solution.
- Heat to boiling (5 to 10 min). Decrease heat as boiling begins. Boil for 60 minutes.
- After 60 minutes, filter contents onto a pre-weighted, ash-free filter paper *(B)* under vacuum. Use low vacuum at first, and increase it as more force is needed.
- Rinse contents with hot water, filter, and repeat twice.
- Wash twice with acetone.
- Dry at 100°C in forced air oven for 24 h.
- Cool filter paper and sample residue in desiccator; weigh and record *(C)*.
- Fold filter paper and place in a pre-weighted aluminum pan.
- Ash in muffle at 500°C for 4 h.
- Cool in desiccator. Weigh and record *(D)*.

The NDF content on a dry matter basis is then calculated as:

$$\text{NDF, \%} = \frac{\left[(\text{Weight of NDF residue, } C - \text{Weight of filter paper, } B) - \text{Weight after ashing, } D\right]}{\text{Original weight of sample, } A \times \text{\% Dry matter}} \times 100$$

For the Ankom system the following procedure applies:
- Number filter bags.
- Weigh 0.5 g sample in filter bag, record exact weight (± 0.0001 g) *(A)* and one blank bag (included in extraction to determine blank bag correction).
- Seal bags within 0.5 cm from the open edge.
- Spread sample uniformly inside the filter bag by shaking and lightly flicking the bag to eliminate clumping.
- Pre-extract soybean products containing more than10% fat with acetone
- Place bags containing samples in a 500 ml bottle with a screw cap. Fill the bottle with acetone into bottle to cover bags (at least 15 ml/bag) and secure top. Swirl gently after 3 and 6 min has elapsed and allow bags to soak for a total of 10 min. Repeat with fresh acetone.
- Pour out acetone, press bags gently between two layers of absorbent paper, and place bags in a hood to air dry for at least 15 min.
- Place 24 bags in the suspender, putting 3 bags per basket.
- Stack baskets on center post with each basket rotated 120°C.
- Include one standard and one blank.
- Place duplicate samples in separate batches and in reverse order of top to bottom

- Bring center post with bags in the vessel and agitate lightly to remove air.
- Close the vessel and boil at 100°C for 60 minutes.
- Drain liquid from vessel.
- Add 2 liter of boiling water to vessel along with 4 ml thermamyl and continue to boil for 5 minutes. Drain and repeat this part of the procedure with 2 ml of thermamyl.
- Drain, remove bags and squeeze excess water carefully.
- Clean bags with acetone and again squeezing bags carefully.
- Leave bags to air dry for 30 minutes.
- Dry bags for 8 hours at 103°C and cool afterwards in desiccator. Weigh *(B)*.
- Weigh blank bag *(C)*.
- Ash bags on pre-registered and weighed aluminum pan *(D)*; D_b for blank) for 6 hours at 550°C in muffle furnace, cool, place in desiccator and weigh blank *(E)* and pans with samples *(F)*.

The NDF content (dry matter basis) is then calculated as:

$$\text{NDF, \%} = \frac{(B - C) - (F - D) - (E - D_b)}{A \times \text{\% Dry matter}} \times 100$$

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8.8. Acid Detergent Fiber (ADF)

It is recommended that ADF is determined sequentially, that is using the residue left from NDF determination. If not done sequentially, some fractions of pectines and hemicellulose could contaminate and overestimate the ADF fraction. For doing sequential analysis, the Ankom procedure is recommended. Like for the NDF procedure the ADF analysis requires 600 ml Berzelius beakers, a fiber digestion apparatus and a filtering flask. Also sintered glass crucibles of 40 to 50 ml with coarse porosity are required.

Reagents needed are:

- Acid Detergent Solution. For this add 27.84 ml of H_2SO_4 to a volumetric flask and bring to 1 l volume with deionized water (it is recommended that before adding the acid, some water is placed in the volumetric flask). Then add 20 g of $CH_3(CH_2)15N(CH_3)3Br$ to this solution.
- Acetone.
- 72% H_2SO_4 standardized to specific gravity of 1.634 at 20°C.

Extraction of sample

- Transfer 1 (± 0.0001) g air-dried sample to Berzelius beaker *(A)*.
- Add 100 ml acid detergent solution.
- Heat to boil (5 to 10 minutes), and then boil for exactly 60 minutes.
- Filter with light suction into previously tared crucibles.

- Wash with deionized hot water 2 to 3 times.
- Wash thoroughly with acetone until no further color is removed. Suction dry.
- Dry in oven at 100°C for 24 h.
- Cool in desiccator. Weigh and record weight *(B)*.
- Ash in muffle at 500°C for 4 h.
- Cool in desiccator. Weigh and record *(C)*.

The ADF content on a dry matter basis is then calculated using the following equation:

$$\text{ADF, \%} = \frac{\text{Weight of ADF residue and crucible, } B - \text{Weight after ashing, } C}{\text{Original weight , } A \text{ x \% Dry matter}} \text{ x100}$$

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8.9. Lignin

Lignin is a polymer of hydroxycinnamyl alcohols that can be linked to phenolic acids, and also non-phenolic compounds. Lignin acts like a shield that prevents the action of enzymes and bacteria, by physical means. Lignin, not only is totally indigestible, but also limits digestion of some nutrients (especially fiber fractions) of soybean products. The determination of lignin is thus, important to estimate the digestibility and energy value of certain, fiber-rich, soybean products.
There are two methods to determine lignin, the Klason lignin and the permanganate lignin. The method of choice is the Klason lignin.

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8.9.1 Klason lignin

Klason lignin requires 72% sulfuric acid and sintered glass crucibles.
The technique consists of adding 25 ml of sulfuric acid to the residue of an ADF determination (without ashing), filtering and adding distilled water three times.
Procedure:

- Place ADF crucible in a 50 ml beaker on a tray. For the original weight use same as for ADF analysis *(A)*.
- Cover contents of crucible with 72% H_2SO_4. (Fill approximately half way with acid).
- Stir contents with a glass rod to a smooth paste.
- Leave rod in crucible, refill hourly for 3 h, each time stirring the contents of the crucible.
- After 3 h, filter contents of crucible using low vacuum at first, increasing progressively as more force is needed.
- Wash contents with hot deionized water until free of acid (minimum of five times).

- Rinse rod and remove.
- Dry crucible in oven at 100°C for 24 h.
- Cool in desiccator. Weigh and record weight *(B)*.
- Ash in muffle at 500°C for 4 h.
- Cool in desiccator. Weigh and record *(C)*.

Calculate Klason lignin (on dry matter basis) as:

$$\text{Lignin, \%} = \frac{\text{Weight of lignin residue and crucible, } B - \text{Weight after ashing, } C}{\text{Original weight, } A \times \text{\% Dry matter}} \times 100$$

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8.9.2. Permanganate lignin

The permanganate lignin requires 80% ethanol, a permanganate buffer solution, acetone, fiber crucibles and a Fibertec apparatus or a vacuum system. The permanganate buffer solution consists of 2 parts of potassium permanganate and one part of lignin buffer solution. The lignin buffer solution in turn is made up of : 300 ml of distilled water, 18 g of ferric nitrate, .45 g of silver nitrate, 1.5 l of glacial acetic acid, 15 g of potassium acetate and 1.2 l of tertiary butyl alcohol.

- Determine ADF following the above-described procedure using crucibles (not Ankom) *(B)*. For the original weight, use same as for ADF analysis *(A)*.
- Place crucibles with ADF digested samples (not ashed) on an enamel pan.
- Fill the pan with distilled water to the bottom of the filter plate of the crucible.
- Place a stirring rod in each crucible and gently break the matt residue with a little of distilled water.
- Fill the crucibles about half way, with the permanganate-buffer solution. Stir, and keep filling crucibles as solution drains out.
- Leave the permanganate solution on for 90 minutes, stirring occasionally.
- Filter the permanganate using the vacuum system of the Fibertec.
- Place crucibles on another enamel pan.
- Fill crucibles with distilled water (avoiding overflow) and refill as necessary.
- Add demineralizing solution to the samples and leave until they turn white.
- Place on cold extractor and filter the demineralized solution using vacuum.
- Wash with 80% ethanol 2 to 3 times.
- Rinse with acetone. Air dry.
- Place in a 105°C oven overnight.
- Place in desiccator, cool, weigh and record weights *(C)*.

Calculate Permanganate lignin (on dry matter basis) as:

$$\text{Lignin, \%} = \frac{\text{Weight of ADF residue and crucible, } B - \text{Weight after oxidation, } C}{\text{Original weight, } A \times \text{\% dry matter}} \times 100$$

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8.10. Starch

Starch occupies only a small part of most soy products but the nitrogen free extract (NFE) fraction- with which it is often identified – may represent a large part of the product. Chemically speaking, starch is defined as a polymer of linear alpha-1,4 linked glucose units (amylose) or alpha-1,5 branched chains of alpha-1,4 linked glucose units (amylopectine).

The starch content of soybean products can be determined with a large number of methods of which the most common methods are the polarimetric method and the enzymatic. The polarimateric method, also referred to as the Ewers method, will recuperate free sugars, pectins and a selection of non-starch polysaccharides.
It is generally recommended not to use this method for samples high in the above mentioned substances or rich in optically active substances that do not dissolve in ethanol (40%) (v/v). The most common alternative method of starch determination is the enzymatic method. This method is based on the selective enzymatic digestion of amyloses and amylopectins by an amylo-glucosidase.

The polarimatric method and the various enzymatic methods do not generally provide the same numeric starch value for an ingredient, feed or digesta sample. The Ewers value being generally higher. However, the enzymatic method(s) are more accurate and are better in discriminating between true starch and related molecules. A comparison of starch analysis in the CVB (2000) tables shows that the two methods give close to identical results for ingredients high in starch. For raw materials with low to intermediate starch levels and ingredients rich in NSPs or cell wall components, starch determination is higher with the Ewers method compared to the enzymatic method. Consequently, for soy products high in (soluble) sugar content (see appendix Tables 1, 2) the polarimatric method will result in higher values than the enzymatic method and the enzymatic method should be preferred.

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8.10.1 Polarimatric starch determination

The Polarimetric method requires: Erlenmeyers volumetric flasks, pipettes, filter paper, a water bath, and a polarimeter or saccharo-meter plus the following reagents:

- 2.5% HCl.
- 1.128% HCl (this solution must be verified by titration with a 0.1 N NaOH solution in presence of 0.1% (w/v) methyl red in 94% (v/v) ethanol.
- Carrez solution I: made by dissolving 21.9 g of zinc acetate and 3 g of glacial acetic acid into 100 ml of water.

- Carrez solution II: dissolve 10.6 g of potassium ferro-cyanide in 100 ml of deionized water.
- 40% (v/v) ethanol.

The polarimetric procedure has two parts, the total optical rotation and the determination of the optical rotation of the dissolved substances in 40% ethanol:

Total optical rotation determination:

- Weigh 2.5 g of soybean sample previously ground through a 5-mm mesh into a 100 ml volumetric flask.
- Add 25 ml of HCl and stir to obtain a homogenized solution and add 25 additional milliliters of HCl.
- Immerse and continuously shake the volumetric flask in a boiling water bath for 15 minutes.
- Remove the flasks from the water bath, add 30 ml of cold water and immediately cool to 20°C.
- Add 5 ml of Carrez solution I and stir for 1 minute.
- Add 5 ml of Carrez solution II and stir, again, for 1 additional minute.
- Add water to the 100 ml level.
- Measure the optical rotation of the solution in a 200 mm tube with the polarimeter or saccharo-meter.

Optical rotation determination of dissolved substances in 40% ethanol:

- Weigh 2.5 g of soybean sample previously ground through a 5-mm mesh into a 100 ml volumetric flask.
- Add 80 ml of 40% ethanol and let react for 1 hour at room temperature, stirring every 10 minutes.
- Complete to volume (100 ml) with ethanol, stir and filter.
- Pipette 50 ml of the filtrate into a 250 ml Erlenmeyer.
- Add 2.1 ml of HCl and shake vigorously.
- Place Erlenmeyer (with cooling device) in a boiling water bath for exactly 15 minutes.
- Transfer the sample into a 100 ml volumetric flask.
- Cool and maintain at room temperature.
- Clarify the sample with Carrez solution I and II and fill to the 100-ml level with water.
- Filter and measure optical rotation in a 200 mm tube with a polarimeter or saccharo-meter.
- The starch content of the sample is then calculated using the following equation:

$$\text{Starch, \%} = \frac{2000 \times (\text{total rotation} - \text{dissolved rotation})}{\text{Specific optical rotation of pure starch}}$$

The specific optical rotation of pure starch will depend on the type of starch used. Table 13 depicts the generally accepted values for some common starch-rich ingredients.

Table 13.

Optical rotation of various pure starch sources

Starch source	Optical rotation
Rice starch	185.9º
Potato starch	185.4º
Corn starch	184.6º
Wheat starch	182.7º
Barley starch	181.5º
Oat starch	181.3º

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8.10.2. Enzymatic or colorimetric starch determination

The enzymatic method is much longer than the polarimetric one.

Reagents needed are:

- Acetate buffer solution, .2 M at pH 4.5.
- Amyloglucosidase enzyme.
- Glucose reagent kit containing: NAD, ATP, hexokinase, glucose-6-phosphate, magnesium ions, buffer and non reactive stabilizers and filters.
- Glucose standards. Prepare three solutions of 100 ml each with 100, 300, and 800 mg/dl of glucose, and 10, 30 and 300 mg/dl of urea nitrogen.

The total procedure takes three days.

Day on:

- Weigh 125 Erlenmeyer flaks are record their weight to the nearest tenth of gram.
- Add 25 ml of distilled water.
- Add .1 g of soybean product and swirl gently.
- Place Erlenmeyers with samples on autoclave at 124°C and 7 kg of pressure, once these conditions are reached, leave the samples in the autoclave for 90 minutes.
- Turn autoclave to liquid cool and leave sample in the autoclave overnight.

Day two:

- Remove from autoclave and cool to room temperature.
- Add 25 ml of acetate buffer and swirl gently.
- Add .2 g of amylo-glucosidase enzyme and swirl.
- Cover tight with aluminum foil caps and put in drying oven at 60°C for 24 hours.

Day three:

- Remove flasks from oven and let to cool at room temperature.
- Remove foil caps and weigh to the nearest tenth of gram and record weight.
- Pour contents into 50 ml centrifuge tubes and centrifuge at 1000 x g for 10 minutes.
- Save supernatant in a plastic scintillation vial.
- Prepare a standard curve using the standard solutions:

Table 14.

Solutions to prepare standard curve for colorimetric starch determination

Working standards	*Combined standards*
50	1:1 dilution of 100 mg/dl standard and water
100	Use 100 mg/dl standard
200	1:3 dilution of 800 mg/dl standard and water.
300	Use 300 mg/dl standard
400	1:1 dilution of 800 mg/dl standard and water
800	Use 800 mg/dl

- Set up a series of test tubes for the color determination step. Include tubes for standards and a blank (i.e. glucose reagent only).
- Prepare glucose reagent kit according to the instructions provided by the supplier of the kit.
- Add 1.5 ml of glucose reagent agent into test tubes.
- Read and record absorbance at 340 nm vs water as a reference. This will be INITIAL A (the blank) in the calculations.
- Add 10 µl of sample to the test tube. Mix gently.
- Incubate tubes for 5 minutes at 37°C.
- Read and record the absorbance at 340 nm vs water as a reference. This will be FINAL A in the calculations.
- Subtract INITIAL A from FINAL A to obtain change in absorbance (Δ A in the calculations).
- Calculate glucose concentration using the following equation:

$$\text{Glucose, mg/dl} = \text{standard concentration} \times \frac{\text{FINAL A (sample)} - \text{INITIAL A (sample)}}{\text{FINAL A (standard)} - \text{INITIAL A (standard)}}$$

- Calculate the content of alpha linked glucose polymers:

  Alpha-linked glucose polymer, mg/g = Glucose concentration in standard x (V/100) x (1/sample weight)

  where, V is the flask volume difference (sample + flask weight - flask weight)

- Calculate starch content as:

$$\text{Starch, \%} = \frac{\text{Alpha linked glucose polymer, mg/g}}{1.111}$$

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8.11. Non starch polysaccharides (NSP) and monosaccharides

A large part of the NFE fraction of soy products may belong to the group of non-starch polysaccharides. This group is composed of fairly simple, soluble and insoluble sugars, most notably raffinose, stachyose, β-mannans and xylans. A major proportion of these sugars are not readily digested, especially by young animals and they are thus often considered part of the ANF. Consequently, a correct estimation of these sugars or the mono-saccharides that make-up these NSP is important when formulating special diets.

The precise analysis for simple sugars requires HPLC equipment. The first part of the procedure requires the elimination of starch from the sample. This is accomplished with the following procedure:

- Weigh 2.5 g of sample in Hungate tubes.
- Add 2.5 ml of acetate buffer (70 ml 0.1 M sodium acetate and 30 ml of 0.1 M acetic acid).
- Add 2.5 µm of α-amylase.
- Place in boiling water bath for 1 hour, shaking every 10 minutes.
- Cool to 40°C.
- Add 50 µl of glucosidase.
- Place in water bath at 60°C for 6 hours and shake every 30 minutes.
- Cool to room temperature.
- Add 10.5 ml of pure ethanol.
- Place in refrigerator for 1 hour.
- Centrifuge at 1000 x g for 5 minutes.
- Discard the supernatant, rinsing the pellet twice with distilled water.
- Dry overnight at 40°C.

The total NSP fraction can be estimated as follows:

Total NSP, % = 100 – (humidity, % + ash, % + protein,% + lipids,% + NDF,% + starch,%)

Once starch has been removed it is necessary to conduct the hydrolysis of sugars.

- Detach the sample from the tube walls.
- Add 1.5 ml of sulfuric acid (75 ml of 96% sulfuric acid and 25 ml of water).
- Vortex.
- Place in water bath 30°C for 1 hour.
- Transfer sample into a 100-ml Erlenmeyer and add 40 ml of distilled water.
- Add 5 ml of myo-inositol (2mg/l) as an internal standard.
- Cover Erlenmeyer with aluminum foil and autoclave (125°C) for 1 hour.
- Filter sample.
- Re-suspend the filtrate into 50 ml of distilled water.

After hydrolysis, the derivatization needs to be performed:

- Place 1 ml of filtrate into a 5-ml plastic test tube.
- Neutralize with 200 µl of 12 M ammonium hydroxide.
- Vortex.
- Add 100 µl of 3 M ammonium hydroxide containing 150 mg/ml of KBH_4 (Borate is very toxic; all following steps must be conducted under a hood).
- Place in a 40°C water bath for 1 hour.
- Add 100 µl of glacial acetic acid and vortex.
- Transfer 500 µl into a 30 ml glass tube.
- Add 500 µl of 1-metilimidazol.
- 5 ml acetic acid, vortex and wait 10 minutes.
- Add 1 ml of ethanol, vortex and wait 10 minutes.
- Add 5 ml of distilled water.
- Add 5 ml of 7.5 M KOH, vortex, and wait 3 minutes.
- Add, again, 5 ml of 7.5 M KOH, vortex, and wait 3 minutes.
- Cover tubes.
- Take a 1-ml aliquot and transfer into a 5-ml test tube.
- Add 50 mg of anhydrous sodium sulfate.
- Decant supernatant into a GLC vial.
- Dry at 40°C for 8-10 hours
- Add 0.5 ml of chloroform.

Chromatography:

- Run samples against stand and blank through a gas chromatograph following equipment-specific procedures.

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8.12. Ether Extract

The ether extract (EE) method measures the proportion of a feed that is soluble in ether. It is equivalent to the total amount of lipids present in a feed and it represents mostly true fats and oils. However, it also includes some ether-soluble material that are not true fats, such as fat-soluble vitamins, carotenes, chlorophylls, sterols, phospholipids, waxes and cutins.

Fatty acids will readily form insoluble complexes with free cations, most notably calcium. These reactions may occur in soy products that have a relatively high concentration of positively charged minerals. To assure that all the fat components are extracted from a mineral rich sample it is recommended to perform an acid hydrolysis in hot HCl prior to the ether extraction.

The EE technique requires a Soxhlet extraction system, funnels, filter paper, HCl (3 *N*), and anhydrous diethyl ether.

The procedure is as follows:

- Weight approximately 2 g of sample ground trough 1 mm-mesh into an Erlenmeyer.
- Add 100 ml of 3 N HCl and boil for 1 h.
- Cool at room temperature.
- Filter through a filter paper and rinse with distilled water to remove all HCl.
- Remove the moisture of the sample by drying it in an oven at 105°C for 24 hours. (If the sample were not dried the ether would have difficulties penetrating all the areas of the ingredient).
- Place sample with anhydrous diethyl ether in a Soxhlet extractor. Turn the heater coil high enough to evaporate 2-3 drops of ether per second in the condenser. Extract for 24 hours. After that time, the ether should be removed, and replaced with clean ether, leaving the samples in the Soxhlet for 8 more hours.
- Remove from Soxhlet, air-dry for about 2 hours and oven dry at 105°C for 12 hours.

The calculation of crude fat is as follows:

$$\text{Crude fat, \%} = \frac{\text{Final weight after extraction, g}}{\text{Original weight, g}} \times 100$$

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8.13. Lipid quality

Fat or oil quality depends on the fatty acid profile, specific physical characteristics and the oxidation level. While fatty acid characteristics and composition determine the physical and nutritional quality of the true lipid fraction,

the physical characteristics and oxidation level are the aspects that are of greatest importance in the routine QC procedures that are applied when oils or fats enter the feed production process. Consequently, the two most common physical tests to assess quality of oils are the insoluble impurities and the unsaponifiable matter. Along with moisture in the oil or fat sample, these characteristics are collectively referred to as the MUI (Moisture, Unsaponifiables, Insolubles) value.

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8.13.1. Moisture

Through the crushing and various treatments of soy oil water may settle in oil samples especially if these samples have undergone significant temperature changes. Generally the moisture content is small but it may have a large effect on the oil quality. The procedure is simple but calls for a forced air drying oven capable of maintaining 130°C ± 2°C, aluminium sample pans with tight fitting covers and a desiccator. Attention, high temperatures may cause the fat sample to ignite.

The procedure is following:

- Accurately weigh 5.0 ± 01 g of sample into a tared moisture dish that has been previously dried and cooled in a desiccator.
- Place the dish in a forced air oven and dry it for 30 min at 130°C + 1°C. Remove from the oven, cool to room temperature in a desiccator and weigh.

Repeat until the loss in weight does not exceed 0.05% per 30 min drying period.

$$\text{Moisture content, \%} = \frac{\text{Loss in weight, g}}{\text{Weight of sample, g}} \times 100$$

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8.13.2. Insoluble impurities

This is a measure of the content of non-lipid compounds in oil. It should be less than 1 %.

The method is as follows:

- Place 15 ml of sample into a graduate cylinder (if sample is not liquid it should be liquefied applying a mild increase in temperature using a hot plate). Maintain in liquid state for the duration of the test. The lower values of the tube should be clearly identified to ensure easy reading following the procedure.
- Let the sample settle in the graduate cylinder for 24 hours.
- Observe the amount of insolubles that have settled out of the sample and collected at both at the top and bottom of the tube.

- The insoluble impurities are then calculated as:

$$\text{Insoluble impurities, \%} = \frac{\text{Reading of settled insolubles, ml}}{\text{Total sample volume, ml (15)}} \times 100$$

- If no insoluble matter is seen in the tube, report the insoluble matter as < 0.2%.

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8.13.3. Unsaponifiable matter

The method measures those substances which cannot be saponified by a caustic alkali treatment. It includes compounds such as aliphatic alcohols, sterols, pigments and hydrocarbons. They do not have a recognized energy value, and thus are of little nutritional interest.

The technique (AOCS, 1993b) requires Erlenmeyer or Soxhlet flasks, beakers, separator funnels, a balance(accuracy of ± .001g), pipettes, a water bath, a reflux condenser, an explosion-proof hot plate, a 50ml burette with its stand, a Soxhlet fat cup and Soxhlet HT2 system, and a desiccator.

The reagents for this method are:

- 85% Ethanol.
- Petroleum Ether.
- NaOH, ACS grade.
- Phenolphthalein indicator solution.
- 0.2 M HCL standard.
- Deionized water.

The procedure is as follows:

- Accurately weigh 5 ± 0.0001 g of well mixed sample into an extraction flask. If the sample is fluid at room temperature, shake to mix well before weighing out sample, and if the sample is solid at room temperature, melt the sample in a water bath, set at 60°C, until the sample is liquefied. Remove and shake to mix well.
- Add 30 ml of 85% ethanol to the sample.
- Add 5 ml of 45% aqueous potassium hydroxide.
- Assemble the extractor by turning on the hot plates and the water taps. Reflux the solution gently but steadily for 1 hour or until completely saponified.
- Quantitatively transfer the solution to a 500 ml separator funnel and rinse the flask into the funnel with approximately 10 ml of 85% ethanol.
- Wash the flask into the separator funnel with approximately 5ml of warm water and pour it into the separator funnel.
- Add approximately 5ml of cool distilled water, swirl and pour it into the separator funnel.

- Complete the transfer from the flask by rinsing with approximately 5ml of petroleum ether.
- Allow the solution to cool to room temperature.
- Add approximately 50 ml of petroleum ether.
- Insert the stopper and shake vigorously by repetitions of inverting for at least one minute. After every few seconds, release the accumulated pressure in the funnel by inverting and opening the stopcock.
- Allow to settle until the solution has separated into two layers.
- Transfer the bottom fat layer back into the original flask and transfer the petroleum ether layer into a clean 250ml Erlenmeyer flask.
- Repeat the former 4 steps until the petroleum ether layer is clear and colorless (about 6 times).
- Once the washes are completed, discard the fat portion of the sample in a waste container and transfer all of the petroleum ether to the 500ml separator funnel.
- Add 30ml of 10% ethanol to the petroleum ether.
- Insert the stopper and shake vigorously by repetitions of inverting for at least one minute. Release any pressure in the funnel by inverting the funnel and opening the stopcock.
- Allow the mixture to settle until the solution has separated into two layers.
- Draw off the alcohol, or bottom layer, and discard, being careful not to remove any of the ether layer.
- Continue the alcohol washes until the petroleum ether layer is clear, approximately 5 or 6 times or until the bottom layer no longer turns into a pink color after addition of 1 drop of phenolphthalein indicator solution.
- Transfer 60 ml of the ether layer (top layer) to a previously tared Soxhlet fat cup.
- Evaporate the petroleum ether layer.
- Repeat the ether evaporation on the Soxhlet system from the same fat cup until all petroleum ether has been completely evaporated from the separator funnel.
- Place the cup in the oven, set at 100°C, for approximately 20 minutes.
- Allow to cool to room temperature in a desiccator and weigh.
- After weighing, dissolve the residue in 50 ml of the phenolphthalein indicator solution. Heat on a hot plate to the point where the alcohol is just starting to boil, then transfer to a 250 ml Erlenmeyer flask.
- Titrate with standardized 0.02 N sodium hydroxide to a faint pink of the same intensity as the original indicator solution. No titration is needed if the sample is already pink when poured into the flask. The amount of ml added times 0.0056 will yield the weight of fatty acids in the sample.
- The unsaponifiable matter is calculated as follows:

$$\text{Unsaponifiable matter, \%} = \frac{(\text{Weight of fat cup plus residue} - \text{Weight of fat cup}) - \text{Weight of fatty acids}}{\text{Weight of sample}}$$

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8.13.4. Iodine value

The iodine value is an estimate of the proportion of unsaturated fatty acids present in a sample. Iodine will bind to unsaturated or double bonds in fatty acids. The greater the amount of iodine bound to the sample the greater the proportion of unsaturated fatty acids. The procedure requires the following reagents:

- Glacial acetic acid.
- Carbon tetrachloride.
- Iodine trichloride.
- Iodine.
- Potassium iodide (100 g/l aqueous solution).
- Sodium thiosulfate, 0.1 N (19.76 g of sodium thiosulfate into 230.24 ml of water).
- Potassium iodate, 0.4 N.
- starch solution: 10g/l aqueous dispersion recently prepared from natural soluble starch.
- Wijs solution: Add 9 g of trichloride into a brown glass bottle (1500 ml capacity). Dissolve in 1 l of a mixture composed of 700 ml of acetic acid and 300 ml of carbon tetrachloride.

The procedure is as follows:

- Determine the halogen content of the Wijs solution by taking 5 ml of the solution and adding 5 ml of the potassium iodide and 30 ml of water. Then add 10 ml of pure iodine and dissolve by shaking. Determine again the halogen content as previously described. The titer should now be equal one and half times that of the first determination. If this were not the case, add a small amount of iodine until the content slightly exceeds the limit of one and half times. Let the solution stand, then decant the clear liquid into a brown glass bottle.
- Place about 100 g of sample in a flask with 15 ml carbon tetrachloride and 25 ml of Wijs reagent. Insert a stopper and shake gently.
- Let sample sit in a dark location for 60 min for fats with expected iodine numbers below 150, and for 120 min for fats with expected iodine values above 150.
- Remove the flask from the dark and add 20 ml of the aqueous potassium iodide solution and 150 ml of distilled water.
- Titrate the solution with 0.1 N sodium thio-sulfate until the yellow color has mostly disappeared.
- Add 1 to 2 ml of starch indicator solution and continue the titration until the blue color has just disappeared after vigorous shaking.

Determine the iodine value using the following equation:

$$\text{Iodine Value} = \frac{12.69 \times 0.1 \times (\text{ml titration of blank} - \text{ml titration of sample})}{\text{Weight of original sample, g}}$$

The iodine test can also be useful as an indicator of lipid oxidation by comparing the initial iodine value and monitoring it across time. The oxidation process destroys the double bonds or reduction of di-enoic acids (see later in this chapter), and thus if the iodine value decreases with time it is an indication of lipid oxidation in the sample under study.

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8.13.5. Acid value

The acid value is a measurement of the proportion of free fatty acids in a given sample. It is determined by measuring the milligrams of potassium hydroxide required to neutralize 1 g of fat. Oxidation is not involved directly in free fatty acid formation, but in advanced states of oxidation, secondary products such a butyric acid may contribute to FFA formation (Shermer et al, 1985).

The technique requires the following reagents: Solvent mixture (95% ethanol/diethyl ether, 1/1, v/v), 0.1 M KOH in ethanol accurately standardized with 0.1 M HCl (pure ethanol may be also used if aqueous samples are analyzed), 1% phenolphthalein in 95% ethanol.

The procedure is as follows:

- Weigh 0.1 to 10 g of oil (according to the expected acid value) in glass vial and dissolve in at least 50 ml of the solvent mixture (if necessary by gentle heating).
- Titrate, while shaking, with the KOH solution (in a 25 ml burette, graduated in 0.1 ml) to the end point of the indicator (5 drops of indicator), the pink color persisting for at least 10 seconds.
- The acid value is calculated by the formula:

$$\text{Acid value} = 56.1 \times \text{KOH} \times \frac{\text{ml of KOH}}{\text{Weight of original sample, g}}$$

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8.13.6. Lipid Oxidation

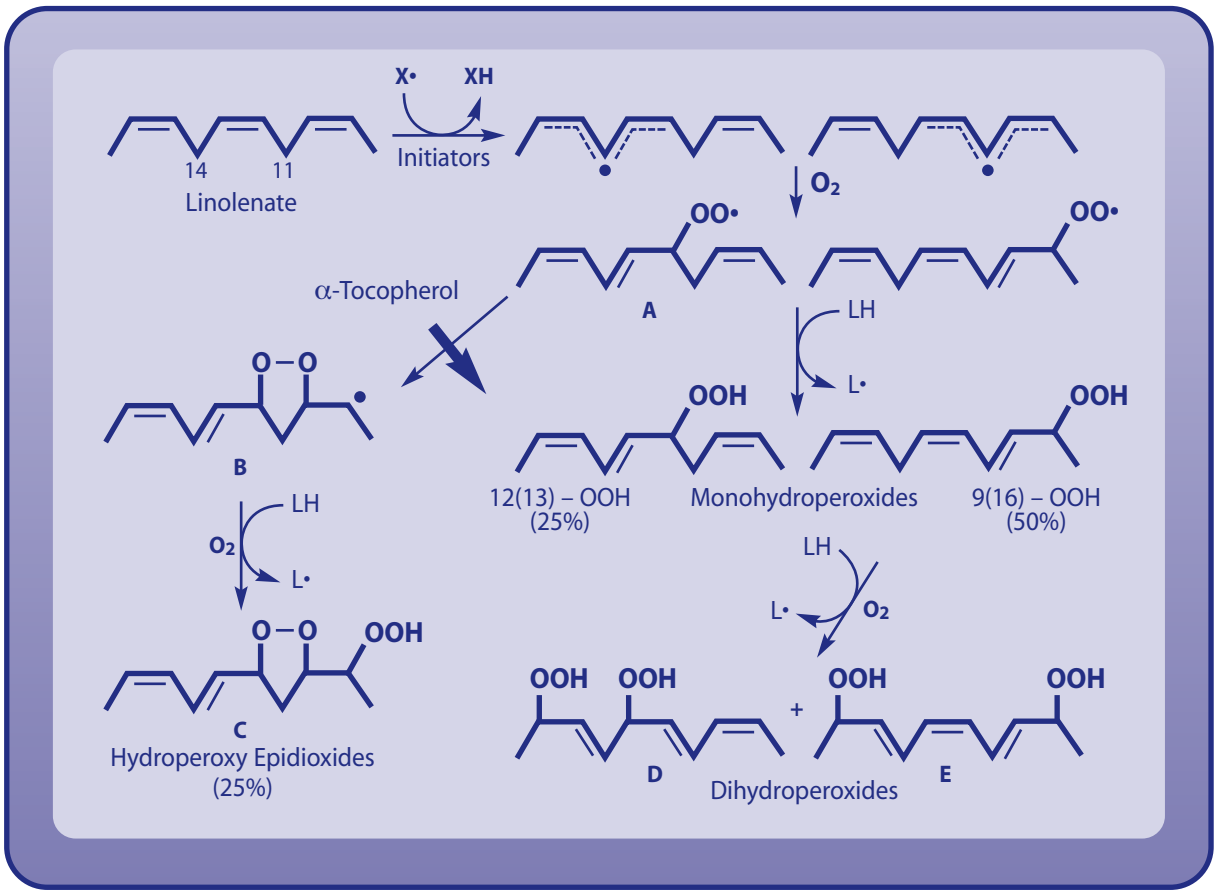
Lipids, especially oils, can undergo oxidation, leading to deterioration. In feeds, these reactions can lead to rancidity, loss of nutritional value, destruction of vitamins (A, D, and E) and essential fatty acids, and the possible formation of toxic compounds and changes in color of the product.

The important lipids involved in oxidation are the unsaturated fatty acid moieties, oleic, linoleic, and linolenic. The rate of oxidation of these fatty acids increases with the degree of unsaturation. The overall mechanism of lipid

oxidation consists of three phases: (1) initiation, the formation of free radicals; (2) propagation, the free-radical chain reactions; and (3) termination, the formation of non-radical products. Chain branching consists in the degradation of hydro-peroxides and the formation of new hydroxyl radicals which will then induce a new oxidation. During the process, there are secondary products being formed from the decomposition of lipid hydro-peroxides producing a number of compounds that may have biological effects and cause flavor deterioration in feed. These compounds include aldehydes, ketones, alcohols, hydrocarbons, esters, furans and lactones (Figure 3).

Figure 3

Auto-oxidation of linolenic acid



Soybean products are relatively sensitive to oxidation because they are rich in unsaturated FA especially linoleic acid. If soybeans are cracked or ground they become more susceptible to oxidation, as fat becomes exposed to oxygen and light. The finer the soybeans are ground, the greater the exposure and thus, the greater the risk of oxidation. Evidently, soybean oil in its pure form (no additives) is very susceptible to oxidation.

There are several techniques to determine the oxidation state of a soybean product or soybean oil. These tests can be classified according to the type of oxidation compound quantified:

- Determination of primary products of oxidation: peroxide value.
- Determination of secondary products of oxidation:
  – Colorimetric methods: TBA and anisidine value.
  – Volatile compounds determination: Chromatography.
- Stability tests: AOM and OSI.

CONTENTS

8.13.6.1. Peroxide value

The peroxide value is an indicator of the products of primary oxidation (peroxides). They can be measured by techniques based on their ability to liberate iodine from potassium iodide, or to oxidize ferrous to ferric ions.

The peroxide value is determined by the amount of iodine liberated from a saturated potassium iodine solution at room temperature, by fat or oil dissolved in a mixture of glacial acetic acid and chloroform (2:1). The liberated iodine is titrated with standard sodium thiosulfate, and the peroxide value is expressed in milli-equivalents of peroxide oxygen per kilogram of fat.

Procedure:

- Place 5 g of sample in a 250 ml Erlenmeyer flask and add 30 ml of the acetic acid-dodecane solution.
- Swirl until the sample is dissolved and add 0.5 ml of a saturated potassium iodide solution (150 g potassium iodide to 100 ml).
- Allow the solution to stand with occasional shaking for exactly one minute, and then add 30 ml of distilled water.
- Titrate with 0.01N sodium thiosulfate adding it gradually and with constant and vigorous shaking. Continue the titration until the yellow color has almost disappeared, and add 1 ml of a starch indicator solution. Continue the titration until the solution acquires a blue color.

The calculations are as follows:

Peroxide value, milliequivalents/1000 = Titration (ml used) x Acid normality x 1000

Although the peroxide value is applicable to peroxide formation at the early stages of oxidation, it is, nevertheless, highly empirical. During the course of oxidation, peroxide values reach a peak and then decline. Consequently the accuracy of this test is sometimes questionable as the results vary with the duration of the procedure used. Thus, a single peroxide value cannot be indicative or the real oxidation state of a product. Also, this test is extremely sensitive to temperature changes potentially leading to poor repeatability of this test.

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8.13.6.2. Thiobarbituric acid (TBA)

TBA is the most widely used test for measuring the extent of lipid peroxidation in foods due to its simplicity and because its results are highly correlated with sensory evaluation scores. The thio-barbituric acid has a high affinity to carbonyl substances (aldehydes and ketones) and its reaction with aldehydes (especially with malon-aldehyde, secondary oxidation product of fatty acids with three or more double bonds) forms a colorimetric complex with maximum absorbance at 530 nm.

The basic principle of the method is the reaction of one molecule of malon-aldehyde and two molecules of TBA to form a red malon-aldehyde-TBA complex , which can be quantified with a spectrophotometer (530nm). However, this method has been criticized as being nonspecific and insensitive for the detection of low levels of malon-aldehyde. Other TBA-reactive substances including sugars and other aldehydes could interfere with the malon-aldehyde-TBA reaction.

The procedure was first described by Witte et al. (1970). The technique requires a spectrophotometer, a water bath, pipettes, test tubes and the following reagents:

- TBA solution: 0.02 M (1.44 g/500 ml of distilled water) 4, 6-dihydroxypyrimidine-2-thiol.
- m-phosphoric acid solution 1.6% (v/v).
- Standard solution: 1,1,3,3-tetraethoxipropyl (TEP) 10.2 M (0.2223 g/100 ml of TCA solution).
- Construct calibration curve using several dilutions.

The procedure is as follows:

- Place 5 g of sample in a beaker and add 50-ml of a 20% tri-chloro-acetic acid and 1.6% of m-phosphoric acid solution for about 30 minutes.
- Filter the slurry.
- Dilute the residue with 5 ml of freshly prepared 0.02 M (1.44 g in 500 ml of distilled water) 4, 6-dihydroxypyrimidine-2-thiol and mixed.
- Tubes are then stored in the dark for 15 hours to develop the color.
- The color is measured by a spectrophotometer at a wavelength of 530 nm.

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8.13.6.3. Anisidine value

The principle of this technique is the preparation of a test solution in 2,2,4-trimethylpentane (iso-octane). Reaction with an acetic acid solution of *p*-anisidine and measurement of the increase in absorbance at 350 nm.

The anisidine value is mainly a measure of 2-alkenals. In the presence of acetic acid, p-anisidine reacts with aldehydes producing a yellowish color and an absorbance increase if the aldehyde contains a double bond.

CONTENTS

8.13.6.4. Lipid Stability tests

Lipid stability tests are either predictive or indicative tests. They measure the stability of lipids under conditions that favor oxidative rancidity. The predictive tests use accelerated conditions to measure the stability of an oil or fat. Indicator tests are intended to quantify the rancidity of an oil or fat. The most important tests to determine lipid stability are the Active Oxygen Method (AOM) and Oxygen Stability Index (OSI).

CONTENTS

8.13.6.4.1. AOM (active oxygen method)

This method predicts the stability of a lipid by bubbling air through a solution of oil using specific conditions of flow rate, temperature and concentration. It measures the time required (in hours) for a sample to attain a predetermined peroxide value (in general 100 mEq/kg oil) under the specific and controlled conditions of the test. The length of this period of time is assumed to be an index of resistance to rancidity. Peroxide value is determines as under 8.13.6.1.

The more stable the lipid (oil) the longer it will take to reach the predetermined value (100 mEq/kg). For products other than oils such as full fat soybeans, the oil must first be gently extracted. The method is very time-consuming since stable oil or fat may take 48 hours or more before reaching the required peroxide concentration. While still being used today, the AOM method is being replaced by faster, automated techniques.

CONTENTS

8.13.6.4.2. OSI (oil stability index)

The OSI method is similar in principle to the AOM method, but it is faster and more automated. Air is passed through a sample held at constant temperature. After the air passes through the sample, it is bubbled through a reservoir of deionized water. Volatile acids produced by the lipid oxidation are dissolved in the water. These organic acids are the stable secondary reaction products when oils are oxidized by bubbling steam. They are responsible for an increase in conductivity of the water. This conductivity is monitored continuously and the OSI value is defined as the hours required for the rate of conductivity change to reach to pre-determined value.
A major advantage of this method is that multiple samples can be tested simultaneously.

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8.13.7. Fatty acid profile

The fatty acid (FA) profile is, from a nutritional point of view, the most important characteristic of oils. The FA composition of the oil is often a fingerprint for the origin, treatment and storage of the oil and it determines largely the quantity that can be used in specific animal diets. On average, palmitic, stearic, oleic, linoleic and linolenic acid proportion of total fatty acids in soybeans is about 10, 4, 25, 51.5 and 7.5% respectively. However, there seems to be a recent trend for oil from soybeans to be richer in palmitic, stearic and oleic acids, and poorer in linoleic and linolenic acids. Part of this decrease has been attributed to global warming, as high temperatures induce a reduction in poli-unsaturated acids in soybeans. However, this assumption will need further substantiation.

The fatty acid profile can be determined by gas or liquid chromatography. The most common is the gas liquid chromatography procedure (GLC). For this analysis a pure sample of oil is used after removal of moisture, insoluble impurities and unsaponifiable substances. Sample preparation requires the following reagents:

- Metanolic-HCl (5% v/v): Add 10 ml of acetyl chloride into 100 ml of anhydrous methanol.
- 6% K_2CO_3: 15 g of K_2CO_3 into 250 ml of distilled water.

Procedure to prepare samples for GLC (adapted from Sukhija and Palmquist, 1988):

- Weight 0.15 g of sample into 10 ml test tubes.
- Add 0.5 ml of an internal standard (i.e. 2mg of C19 per 1 ml of toluene).
- Add 0.5 ml of toluene.
- Add 1.5 ml of metanolic-HCl.
- Close tubes to avoid sample loses.
- Vortex for 1 min.
- Place in water bath at 70°C for 2 hours.
- Cool at room temperature.
- Add 2.5 ml of the K_2CO_3 solution.
- Add 1 ml of toluene.
- Vortex 30 for seconds.
- Centrifuge at 3000 rpm for 5 minutes.
- Keep the supernatant and add 0.5 g of anhydrous Na_2SO_4.
- Vortex for 30 seconds.
- Centrifuge at 4000 rpm for 10 minutes.
- Collect the supernatant and place in gas chromatography (GC) vial for subsequent C analysis.

For operation of the GC equipment and analyses of fatty acids it is recommended to follow the specific procedure provided by the manufacturer of the chromatographic equipment. The chromatography methods are based on the separation and quantitative measurement of specific fractions, such as volatile, polar, or polymeric compounds or individual components such as pentane or hexane.

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8.14. Minerals

Mineral composition of soy products can vary considerably among and within products. The concentration of minerals depends greatly on a number of factors most notably the origin and crop-growing conditions of the soybean, the variety and the different types of extraction processes that are applied to obtain the soy product. Although a measure of the concentration of these minerals is important for most feed applications, under routine feed production conditions table values are used to formulate. Generally, in feed production, formulators count on the contribution of the minerals in the premix to cover mineral requirements of animals. This is especially the case for the micro-elements. Regular analyses are generally only considered necessary for the macro minerals calcium and phosphorus. For these elements, rather than table values analytical values are used to formulate.

In certain regions, especially in areas of intensive animal production, the regulatory limits on phosphorus use and excretion by animals make a precise estimate of this element in the feed necessary. Phosphorus concentrations in soy products are high and with the exception of soybean hulls and soybean mill feed, P levels in these products are a multiple of Ca levels. This makes analyses for P, both from a regulatory and nutritional point of view important. In addition to Ca and P, salt (NaCl) analysis may be carried out on a routine basis for QC purposes.

Routinely, under more sophisticated laboratory conditions, most minerals are analyzed by atomic absorption or flame emission. However, this requires a considerable amount of investment and expertise. For normal QC objectives, classical wet chemistry can be used to estimate the content of the most important minerals.

CONTENTS

8.14.1. Calcium

The determination of calcium by wet chemistry requires a set of porcelain dishes, volumetric flasks (250 ml), beakers (250 ml), filter paper and funnels, and a burette.

The following reagents are needed:

- Hydrochloric acid (1 to 3 v/v).
- Nitric acid (70%).
- Ammonium hydroxide (1 to 1 v/v).
- Methyl red indicator (Dissolve 1 g in 200 ml alcohol).
- Ammonium oxalate (4.2% solution).
- Sulphuric acid (98%).
- Standard potassium permanganate solution (0.05N).

Ca is determined as follows: weigh 2.5 g finely ground material into a porcelain dish and ash (see section 8.2; alternatively use residue from ash determination). Add 40 ml hydrochloric acid and a few drops of nitric acid to the residue, boil, cool and transfer to a 250 ml volumetric flask. Dilute to volume and mix.

Pipette a suitable aliquot of the solution (100 ml for cereal feeds; 25 ml for mineral feeds) into a beaker, dilute to 100 ml and add 2 drops of methyl red. Add ammonium hydroxide drop-wise until a brownish orange color is obtained, then add two drops of hydrochloric acid to give a pink color. Dilute with 50 ml water, boil and add - while stirring - 10 ml of hot 4.2% ammonium oxalate solution. Adjust pH with acid to bring back pink color if necessary. Allow precipitate to settle out, and filter, washing precipitate with ammonium hydroxide solution (1 to 50 v/v). Place the filter paper with precipitate back in beaker and add a mixture of 125 ml water and 5 ml sulphuric acid. Heat to 70°C and titrate against the standard permanganate solution.

Calculation:

$$\text{Calcium (\%)} = \frac{\text{ml, permanganate solution}}{\text{wt. of sample, g}} \times \frac{\text{Aliquot used (ml)}}{250} \times 0.1$$

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8.14.2. Phosphorus

The method for phosphorus analysis requires a spectrophotometer able to read at 400 nm, volumetric flasks (100 ml) and the following reagents:

- Molybdo-vanadate reagent. To obtain this dissolve 40 g ammonium molybdate 4H0 in 400 ml hot water and cool. Dissolve 2 g ammonium meta-vanadate in 250 ml hot water, cool and add 450 ml 70% perchloric acid. Gradually add the molybdate to the vanadate solution with stirring and dilute to 2 liters.
- Phosphorous standards. Prepare stock solution by dissolving 8.788 g potassium di-hydrogen ortho-phosphate in water and making up to 1 liter. Prepare the

working solution by diluting the stock 1 in 20 (working concentration = 0.1 mg P/ml).

To determine phosphorus: pipette an aliquot of the sample solution prepared as for the calcium determination into a 100 ml flask and add 20 ml of the molybdo-vanadate reagent. Make up to volume, mix and allow to stand for 10 minutes. Transfer aliquots of the working standard containing 0.5, 0.8, 1.0 and 1.5 mg phosphorus to 100 ml flasks and treat as above. Read sample at 400 nm setting the 0.5 mg standard at 100% transmission. Determine mg phosphorus in each sample aliquot from a standard curve.

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8.14.3. Sodium chloride

The reagents used for the determination of salt in feed samples or feed ingredients are:

- Standard 0.1N silver nitrate solution.
- Standard 0.1N ammonium thio-cyanate solution.
- Ferric indicator - saturated aqueous solution of ferric aluminum.
- Potassium permanganate solution - 6% w/v.
- Urea solution - 5% w/v.
- Acetone (A.R. grade).

The method consists of: weighing a 2 g sample into a 250 ml conical flask. Moisten the sample with 20 ml water and then pipette, 15 ml 0.1N silver nitrate solution - mix well. Add 20 ml concentrated nitric acid and 10 ml potassium permanganate solution and mix. Heat mixture continuously until liquid clears and nitrous fumes are evolved. Cool. Add 10 ml urea solution and allow to stand for 10 minutes. Add 10 ml acetone and 5 ml ferric indicator and back titrate the excess silver nitrate with the 0.1N thio-cyanate solution to the red brown end point.

Calculation:

$$\text{NaCl}(\%) = \frac{15 - \text{ml } 0.1\text{ N NH4CNS} \times 0.585}{\text{wt. of sample, g}}$$

For rapid, routine QC procedures, Quantabs, a bench-top test kit is used.

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8.15. Isoflavones

In many diets, human as well as animals, soybean products are the main dietary source of isoflavones. These secondary metabolic compounds may play an important role in preventing cancers and reducing risk of cardiovascular diseases. There is also an increasing interest in the role and use of isoflavones in animal production as these compounds have been implicated in enhancing immunity and improving growth performance and carcass traits (Cook, 1998; Payne et al., 2002; Kerley and Allee, 2003).

Two forms of isoflavones can be determined: the bound glucoside form (genistin, daidzin, glycitin) and the free aglycone form (genistein, daidzein, glycitein). Lee et al. (2003) reported that the total isoflavone contents in soybean cultivars grown in Korea ranged from 110 to 330 mg 100 g^{-1}. The USDA and Iowa State University (2002) have developed a database on isoflavones from scientific articles. The analysis of isoflavones was carried out according to the method of Wang and Murphy (1994) using high-performance liquid chromatography (HPLC).
For the analysis of isoflavones the following reagents are needed:

- Acetonitrile.
- HCl (0.1 N) or phosphoric acid.
- Isoflavone standards (commercial source).

Besides normal laboratory equipment the essay requires an HPLC instrument with a YMC-pack ODS-AM-323 column (10 µm, 25 cm x 10 mm i.d.).

The procedure consists of an Isoflavone extraction and an HPLC quantification step. For the extraction two grams of ground soybean products are mixed with 2 ml of HCl and 10 ml of acetonitrile in a 125 ml flask, stirred for 2 hours and filtered. The filtrate is dried under vacuum at a temperature below - 30°C and then re-dissolved in 10 ml of 80 % HPLC grade methanol in distilled water. The sample is then filtered through a 0.45 mm filter unit and then transferred to 1 ml vials.

The HPLC quantification of isoflavones requires a column temperature of 25°C and a mobile phase employing a linear HPLC gradient using 0.1 % glacial acetic acid in distilled water (solvent A) and 0.1 % glacial acetic acid in acetonitrile (solvent B). Following the injection of 20 µL of the sample, solvent B is increased from 15 to 35 % over 50 min and then held at 35 % for 10 min. The recommended flow rate is 1 ml min^{-1} and the detection wavelength: 200 - 350 nm.
The content of each isoflavone is expressed on a $w.w^{-1}$ basis.

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8.16. Antinutritional factors (ANF)

One of the most important restrictions on the use of soybeans and their products in animal diets is the presence of a relatively large number of antinutritional factors. The presence of these factors is also the main reason why different technological treatments are applied to soybeans or their products. The ANF in soybeans include compounds classified as protease inhibitors, phyto-hemaglutins (lectins), urease, lipoxygenases and antivitamin factors which can relatively easily be destroyed by heat application or fermentation (Liener, 2000). The methods referred to under section 8.4 provide a relative estimate of the effectiveness with which they have been destroyed. The effect of heat treatment on ANF is a direct function of the degree and duration of the heat application along with particle size and moisture level. ANFs that are not destroyed by heat are the poorly digested carbohydrates, Saponins, Estrogens, Cyanogens and Phytate (Liener, 2000). In the case of soybean products, the most important and best known ANF is the trypsin inhibitors. The quality of technological treatment to destroy ANF is mainly related to this specific factor.

To analyze for any ANF a large number of different methods and procedures are available ranging from instrumental (HPLC, GC, CE) to thin-layer chromatography (TLC) and immuno-assays. The reliability and accuracy of results obtained with these methods varies and no preferred method has been defined for all ANF. When possible, and for practical routine QC purposes, the use of ELISA (enzyme-linked immuno-sorbent assay) tests are recommended.

The ELISA tests rest on the principle that the compound called the antigen (in this case an ANF obtained by extraction from the feed or ingredient) will bind with enzyme-linked antibodies. Upon this reaction, the enzyme-linked antibodies will be released from the surface to which they were attached (this maybe a stick, plate or tube). The enzyme-linked antibodies are then washed away and an enzyme substrate is added to allow a reaction with the remaining enzyme-linked antibodies. This procedure results in a color change which is inversely related to the antigen concentration. Thus, the deeper the color, the smaller the antigen (ANF) concentration since less antibody-antigen complexes have been formed and washed away leaving more enzyme-linked antibodies to react with the color causing enzyme substrate.

CONTENTS

8.16.1. Trypsin inhibitors

The residual trypsin inhibitor in soy products combines with the trypsin in the small intestine and forms an inactive complex thus reducing digestibility of

protein. In addition to the negative effect on protein digestibility, the trypsin inhibitor induces pancreatic hypertrophy and leads therefore to an increase in secretion of trypsin (endogenous nitrogen). The combined effect on the animal is a reduction in nitrogen retention, growth and feed conversion.

The procedure described to determine trypsin inhibitors activity is based on the ability of the inhibitors to form a complex with the enzyme and thus to reduce the enzyme activity. Uninhibited trypsin catalyzes the hydrolysis of a synthetic substrate BAPNA, forming a yellow-colored product and thus producing a change in absorbance. The reference procedures proposed by the American Oil Chemists' Society (AOCS) and the French Association for Normalization (AFNOR) are based upon the work of Kakade et al. (1969, 1974). Here, the AOCS (1997) procedure is summarized but the only difference with the AFNOR (1997) procedure is the composition of the extraction buffer, which is alkaline whereas it is acid in the other case. Still, these procedures are not very well adapted for routine QC use, and a well equipped lab with skilled technician is necessary.

For practical reasons, the method described measures total trypsin inhibitors. It reflects thus the concentration and effects of two distinctively different types of inhibitors namely the KTI (Kunitz trypsin inhibitor) and the BBI (Bowman-Birk inhibitor).

Reagents needed are:

- Hexane or petroleum ether.
- Sodium hydroxyde solution (0.01 N).
- Tris buffer: dissolve 6.05 g tris (hydroxyl-metyl)-amino-methan and 2.94 g calcium chloride in 900 ml of water, adjust to pH 8.2 and dilute to 1 L. Bring to 37°C before using.
- Trypsin solution: dissolve 4 mg, accurately weighed, twice-crystallized, salt-free trypsin in 200 ml hydrochloric acid (0.001 N).
- BAPNA solution: In a water bath, dissolve 40 mg N α-benzoyl DL-arginine p-nitroanilide (BAPNA) in 1 ml dimetyl sulfoxide. Dilute to 100 ml with tris buffer (at 37°C). Prepare new solution daily. Maintain at 37°C for use.
- Acetic acid solution (30 %): mix 30 ml glacial acetic acid and 70 ml water (caution).

Equipment required: a grinding mill, with screen size 0.15 mm or smaller and a Spectrophotometer capable to read at 410 nm.

The procedure is as follows:

- Samples should be finely ground without excessive heating. Samples with more than 5 % fat should be defatted with hexane or petroleum ether and desolventized before grinding.

- One gram of ground sample is subsequently weighed into a beaker containing a magnetic stirring bar. 50 ml sodium hydroxide solution is added and the suspension is agitated slowly. After 3 hr, the pH is measured; pH should range between 8.4 and 10.0.
- An aliquot of suspension should be taken with a serological pipette and diluted with distilled water so that soybean trypsin inhibitor concentration is sufficient for 40 - 60 % trypsin inhibition. When it is not possible to estimate the expected trypsin inhibitor units, more than one dilution should be made.
- With serological pipettes, 0, 0.6, 1.0, 1.4 and 1.8 ml of the diluted suspension is added to duplicate sets of test tubes. Water is then added to bring the volume to 2 ml in each tube.
- With a regular time interval for the different tubes, 2 ml trypsin solution is added to each tube and quickly mixed on the Vortex stirrer and placed in the 37°C water bath. 5 ml BAPNA is added to each tube, mixed on Vortex stirrer. The samples are incubated for 10 min at 37°C. After exactly 10 min, the reaction is stopped by addition of 1 ml acetic acid solution followed by mixing on the Vortex stirrer.
- Prepare a blank sample as above, except that trypsin is added after acetic acid.
- The contents of each tube are filtered and absorbance is measured at 410 nm.

Calculation of trypsin inhibitors activity. One trypsin unit is arbitrarily defined as the amount of enzyme, which will increase absorbance at 410 nm by 0.01 unit after 10 minutes of reaction for each 10 ml of reaction volume. Trypsin inhibitor activity is defined as the number of trypsin units inhibited (TIU).

$$\text{TIU (/ml)} = \frac{\text{Absorbance blank} - \text{absorbance sample}}{0.01 \times \text{volume of diluted sample solution, ml}}$$

TIU is plotted against the volume of the diluted sample solution.
The extrapolated value of the inhibitor volume to 0 ml gives the final TIU /ml.
This value is used to calculate the TIU per g sample:

$$\text{TIU(/g)} = \text{TIU (/ml)} \times d \times 50$$

where d = dilution factor (final volume divided by the amount of aliquot taken).
The results of this analytical method should not exceed 10 % of the average value for repeated samples.

CONTENTS

8.16.2. Soy antigens

Immunoassay techniques are used to determine concentrations of soy antigens (glycinin and ß-conglycinin) in soy products. The ELISA tests require little training and can be used in small laboratories. Various types of ELISA tests with

specific polyclonal antisera (Pabs) or monoclonal antibodies (Mabs) can be used to assess soy antigens contents (Table 15).

To apply the different ELISA tests, the protein fraction of the soy product is first extracted in borate buffer (100 mM $NaBO_3$, 0.15 M NaCl, pH 8) for 1.5 hr (Tukur et al., 1993). The level of glycinin and ß-conglycinin can be measured by a specific competitive inhibition ELISA using anti-soy globulin Pabs (Heppell et al., 1987). Serial, four-fold dilutions of the sample are incubated with a standard dilution of rabbit antiserum to test protein and the residual unbound antibodies are quantified.

Table 15

ELISA formats used for analysis of soy globulins

	Antibody	*ELISA format*	*Specificity*
Glycinin	Pab LJR J4	inhibition	intact glycinin
	Mab IFRN 0025	inhibition	binds proteolytic intermediates and thermally denatured glycinin; epitope lies within acidic polypeptides
	Mab IFRN 0025 & Pab $R103b_3$	two-site	recognize proteolytic intermediates and thermally denatured glycinin
ß-conglycinin	Pab LJR J2	inhibition	intact ß-conglycinin
	Mab IFRN 0089	inhibition	recognizes epitopes in acidic regions of a and α' subunits of ß-conglycinin
	Mab IFRN 0089 & Pab $R195b_3$	two-site	recognition of thermally denatured ß-conglycinin is 3-fold greater than native

(from Tukur et al., 1996)

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8.16.3. Lectins

Lectin is a protein with a specific binding affinity for sugar residues. The lectin-sugar interaction is important at the level of the membrane receptors in the gut where it is thought to be responsible for agglutination and mitosis. As for most leguminous plants or seeds of these plants, lectins have been shown to be an important ANF in raw soy products (Pusztai, 1991).

Table 16

Anti nutritional factor contents in various soy products

Product	PDI (%)	Trypsin inhibitor activity (mg/g)	Lectins (mg/g)	Antigens (mg/g)
Untoasted soy flour	90	23.9	7.3	610
Slightly toasted soy flour	70	19.8	4.5	570
Toasted soy flour	20	3.1	0.05	125
Ethanol/water-extracted soy concentrate	6	2.5	<0.0001	<0.02

(adapted from Huisman and Tolman, 1992)

Lectins are heat sensitive and are therefore only present at residual levels in soybean products. Heat treatment to inactivate antinutritional factors in soy products is less efficient for antigens than for trypsin inhibitors or lectins (Table 16).

The level of soy lectins can be estimated by measuring the hemaglutination activity. More recently, ELISA (total lectins) and FLIA (functional lectins) tests have been developed and these methods are more sensitive and selective (Delort-Laval, 1991). Lectins can vary considerably (chemical structure, molecular weight a.o.), therefore a specific essay is required for each legume seed tested (de Lange et al., 2000).

The procedure as presented by Schulze et al. (1995) can be summarized as follows:

One gram of sample is mixed with 20 ml tris-HCl buffer (50 mM, pH 8.2) and stirred for 1 hr. Extracts are centrifuged at 7500 x g for 15 min and the supernatant is used for serial dilutions. Lectins are is determined in the supernatant.

Polyclonal antibodies against soy-lectins (ELISA) are coated to micro-titer plates overnight at 4°C. The plates were then blocked with 0.5 % BSA (bovine serum albumin) and 0.2 % Tween-20 in TBS for 1 hr at 37°C. Subsequently, the plates are washed and samples are diluted at appropriate concentrations. A reference soy-lectin sample is run in parallel. All samples are transferred to micro-titer wells and incubated for 2 hr at 37°C. The plates are washed and peroxidase-conjugated anti-lectin antibodies are applied and incubated for 2 hr at 37°C. Finally, the plates are washed again and bound conjugated antibodies are developed for peroxidase activity using 1,2-phenylendiamine. Absorbance is read at 492 nm. Data can be evaluated by the parallel line assay using a computer software package connected to the ELISA reader system. Lectin concentrations are expressed in $w.w^{-1}$ on a dry matter basis.

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8.17. Mycotoxins; rapid tests

Mycotoxins are a major quality concern for the feed industry. Although soy products do not generally show the same level or range of mycotoxin contamination as cereal grains, they do occur occasionally and routine QC methods should be in place to control their presence. This is especially the case now that regulatory restrictions on mycotoxin levels are becoming increasingly more stringent. The most common mycotoxins occurring in feed ingredients are aflatoxins, deoxynivalenol (DON), zearalenone, ochratoxin and fumonisins. All these mycotoxins can potentially be found in soy products but the most important mycotoxins in the case of soy products are ochratoxin (produced by the molds *Aspergillus ochraceous* or *Penicillium verrucosum* under poor storage conditions) and zearalenone (produced by the fungus *Fusarium graminearum*).

As in the case of ANFs, the analyses for mycotoxins and their metabolites can be carried out by a range of methods. No preferred method has been defined for all mycotoxins. For practical QC purposes, however, the use of the TLC and ELISA tests are recommended. In the case of mycotoxins, these tests can be separated in screening and quantitative analysis with the former detecting a simple presence of the mycotoxin and the later providing rather precise estimates of mycotoxins levels present in a sample. Qualitative analysis will require additional equipment such as long-wave microwell strip readers, UV lights or fluorometers.

The precision of these quantitative measures varies with the type and manufacturer of the test and some prior evaluation and training as to which test most suitable for a particular laboratory setting is recommended. Minimum detection levels may vary among producers and types of test kits. However, the significant improvements in the quantitative ELISA tests over the last 10 to 20 years have made these tests perfectly suited for routine quality procedures and several have been validated by the AOAC and received approval (AOAC International, 1995; Trucksess et al., 1989). Nevertheless, due to the many factors that may affect the results of the ELISA test kits, the variation between laboratories and analysts may be considerable. In some instances, limits of detection are also inadequate to meet the increasingly stringent demands for measurement at low levels. False positive or negative readings are known to occur and for purposes other than routine quality procedures, classical instrumental analysis as referred to above will be needed. Also, test kits have been developed that will qualitatively detect several mycotoxins in a single test.

General procedure:

Before performing the rapid test, the mycotoxins need to be extracted from the sample. Most of mycotoxins can be extracted by grinding the sample to 0.6 mm-

mesh, then blending 25 g of that sample with 125 ml of a 70% methanol solution (7 parts of methanol and 3 parts of de-ionized water). Stir vigorously in a high-speed blender for 2-3 minutes. The ELISA test should be performed as indicated by the manufacturer of the test kits.

When choosing the ELISA test for mycotoxin analyses it is necessary to make sure that the kit has been validated for use with soybean products.

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8.17.1. Ochratoxin

This mycotoxin is often considered the most common mycotoxin in soybean products. It is thought to be principally produced during storage under humid and warm (>20°C) conditions. Damage to grains by insects or through mechanical means will provide an entry for the fungi and enhance initial contamination. Ochratoxin is a mycotoxin produced by several species of the mold genera *Aspergillus* and *Penicillium*. Usually, the rapid tests for ochratoxins have a lower limit of detection of 0.01 ppm in the case of screening methods while quantitative tests have a lower detection limit at 0.005 ppm. It seems that at levels of 0.2 ppm clinical signs associated with ochratoxins will appear in monogastric species.

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8.17.2. Zearalenone

Zearalenone is primarily produced by *Fusarium graminearum*. By itself, zearalenone is not toxic, but once metabolized, its end-products have estrogenic activity, which may cause some reproductive alterations in animals. Sensitivity to zearalenone differs considerably among livestock species with swine considered most sensitive. Levels above 1 ppm result in noticeable effects on reproduction in swine. Usually, the rapid screening tests for zearalenone have a lower limit of detection of 0.1 ppm with quantitative tests having a lower detection limit of 0.2 ppm.

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8.17.3. Fumonisins

Fumonisins includes a group of mycotoxins produced by *Fusarium moniliforme* and *Fusarium proliferatum*. Horses are especially sensitive to fumonisins. Usually, the rapid tests for fumonisins have a lower limit of detection of .2 ppm, a limit of quantification of 1 ppm up to 6 ppm.

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8.17.4. Aflatoxins

Aflatoxin is often considered the most common mycotoxin in feeds and grains. However, the occurrence of this toxin in soy products is relatively rare. Aflatoxin is a mycotoxin produced by *Aspergillus flavus* and *Aspergillus parasiticus*. Not all strains of these fungi are capable of aflatoxin production. Drought conditions associated with warm temperatures and physical damage to the grain strongly increase the probability of aflatoxin occurrence. There are several types of aflatoxins, with the most common in order of prevalence being B1, B2, G1 and G2. To date, aflatoxins are the only mycotoxins for which official maximum levels have been defined. The FDA as well as the EEC has established a maximum level of total aflatoxins of 20 ppb in ingredients for the feed industry.

Usually, the quantitative rapid tests for aflatoxins have a lower limit of detection of 1 ppb and a limit of quantification of 5 ppb up to 50 ppb.

In addition to the method described above, aflatoxins can be extracted, by weighing 10 ml of soybean products into a wide mouthed bottle and thoroughly mixing it in 10 ml of water. Add 100 ml of chloroform, stopper with a chloroform resistant bung and shake for 30 minutes. Filter the extract through diatomaceous earth.

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8.17.5. Deoxynivalenol

Deoxynivalenol (DON), commonly referred to as vomitoxin, is a trichothecene primarily produced by *Fusarium graminearum*. Fusarium growth requires a minimum moisture level of 19 % thus DON levels are not known to develop or increase during normal storage conditions. The FDA has established advisory levels for DON. Maximum levels for ingredients other than wheat and wheat by-products have been set at 5 ppm for swine and 10 ppm for ruminants (with a 20 % limit at the inclusion rates of these contaminated ingredients in the case of swine diets).

The extraction of DON from soybean should not be performed with ethanol. It should be conducted with about 10 g of sample ground to 0.6 mm. Shake vigorously in 50 ml of de-ionized water for 3 minutes. Then the sample is filtered and the liquid fraction is kept for subsequent ELISA analyses.

Usually, the rapid tests for DON have a lower limit of detection of 2.0 ppm for the screening tests and 0.5 ppm for the quantitative tests.

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8.18. Genetically modified organisms (GMO)

Some soybeans have been genetically modified. As market demands for traceability are growing and market demands for non-GMO products are decreasing, it is important to be able to distinguish between genetically modified and traditional products. Certain official maximum limits on the presence of GMO material in non-GMO products exist. In the EEC these levels are now fixed at a maximum of 0.9 %. Japanese legislation allows food products containing less than 5% of approved biotech crops, like corn and soybeans, to be labeled as non-GMO. In the presence of the extensive use of GMO soybean varieties, the risk of commingling and analytical variability, these minimum levels reflect in part the inability to guarantee complete absence of GMO material in products labeled as GMO-free.

The GMO varieties are characterized by the insertion of a new, functional gene (or cluster of genes) into their genomes. The expression of these genes provides the soybeans with some advantages, such as resistance to insects and herbicides. Several commonly used GMO testing protocols, including biological tests, as well as ELISA and PCR (polymerase chain reaction) tests, exist. The ELISA methods are based on the same principle as described above for the detection of mycotoxins. A popular version of the ELISA test, used for screening purposes only, uses lateral flow strips that deliver results in a couple of minutes. This makes this test especially suited for QC purposes at feed mills. Quantitative ELISA tests also exist. They are normally presented as plate tests with the degree of color change being indicative of the level of GMO material present in the sample. An important limitation of the ELISA tests is that they have limited accuracy when applied to heat-processed ingredients; especially in the case of high temperature application (extrusion). The limitation applies to all products in which the application of high temperatures leads to substantial denaturation of the soy proteins, thereby making detection of proteins difficult.

The PCR tests (more sensitive than the ELISA methods) are based on the detection of DNA sequences in the genome of the soybean product. The PCR is an extremely sensitive technique and is able to identify different types of GMOs at very low levels. It is also the only method that can effectively detect GMOs in heat treated ingredients and feeds which makes this method the preferred procedure in the case of most soy products. However, due to the requirements for equipment, the delay in obtaining results (2 to 3 days) and the level of expertise required, the test is not suited for routine QC analyses at the feed plant level. This test should be carried out in a proper laboratory setting. An additional disadvantage of this procedure is its tendency to give false positives which may require replicate testing.

Biological tests are mainly limited to the herbicide resistant soybean varieties and can only be applied to the untreated bean. The advantage of these tests is that they are relatively inexpensive and produce clear-cut results. In these tests seeds are placed in a germination media. The seeds are then moistened with a diluted solution containing the herbicide against which the seed is thought to be resistant or the germinated seeds are sprayed with the herbicide in question. Herbicide tolerant GMO seeds will germinate and/or grow normally while the non-GMO seeds will fail to germinate or grow. A minimum one week period is needed to carry out this test.

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9. NIR Analyses

Near infra-red reflectance (NIR) spectroscopy has been used for more than 35 years to rapidly analyze grains, animal feeds and forages. The first application of NIR spectroscopy was developed by Norris and associates in the early sixties to measure water content in grains and seeds (Givens et al., 1997). Since these early developments NIR spectroscopy has matured to a well established and broadly accepted method to measure a wide array of chemical compounds in feed and food ingredients or diets.

Concerning soybean products, the largest and most evident application is in the rapid determination of proximate components previously carried out by the time-consuming and laborious conventional wet chemistry. The potential of NIR to carry out more evolved analysis such as protein quality and ANF is a real possibility since the technique has been used to measure characteristics of similar complexity such as digestibility of individual amino acids (van Kempen and Bodin, 1998). However, despite the rapid answers and the major time savings made possible by NIR, the development of the calibrations required for protein solubility and ANF have as yet received little attention.

NIR spectroscopy is based on the principle that infra-red radiation of a sample results in the reflection or transmittance of the radiation that is not absorbed by the sample. The characteristics of the reflected or transmitted radiation can be used to describe certain chemical characteristics of the sample. Since this relationship is not mathematical, the relationship between the reflected radiation and the chemical compound of interest must be based on a calibration. In this calibration the amount of light reflected (or absorbed) at one or more wavelengths are related to a specific chemical compound or compounds. More precisely, it is the chemical bonds and functional groups of the compound that are related to the reflectance at a specific wavelength. Consequently, molecules characterized by a repetitive bond and structure are often more suited for detection by NIR. The choice of a wavelength or a combination of wavelengths to detect a chemical compound is not necessarily constant. The optimum choice of wavelengths to correlate with a specific compound differs not only among ingredients but also among laboratories, equipment and even years. Also, the scattered reflectance from other compounds leads to interference.

Consequently, the wavelength best related to the compound of interest is the one at which absorption by the compound is maximized and interference by reflectance of other sample constituents is minimized.

A number of items interfere with the near infra-red reflectance spectra. The reflectance obtained from a sample is characterized by scatter due to instrument type and function, sample preparation (grinding and thus particle size), temperature, water content and interference of reflectance from other compounds. Variations in water content of the sample are important because water absorbs radiation strongly. In order to increase the precision of NIR analyses the factors interfering with the NIR spectra need to be standardized when analyzing an ingredient or they need to be eliminated through the application of mathematical corrections on the spectrum or calibrations. Since standardization of sample preparations is not always practical and since it reduces the major benefit of NIR analyses (time savings) preference is given to mathematical corrections. A series of mathematical tools have been developed to correct the spectral data and improve the predictive capacity of the calibrations. The choice and application of these corrections differ considerably among the constituents to be analyzed. The range of mathematical tools that is available to treat spectral data is increasing rapidly thus improving the quality of the analysis and the requirements for sample preparation.

Before routine analyses can be carried out equations need to be developed for each individual constituent and often the individual ingredients. Sometimes, a common equation can be developed for ingredients and/or their by-products. In the case of soy products a single equation can by used for a number of products if they are sufficiently alike in composition and preparation. This is for instance the case for all soybean meals. However as a general rule of thumb it may be said that the larger the physical and chemical differences among ingredients, the greater the need to develop separate equations.

NIR calibrations are equations developed from a dataset composed of the component of interest analyzed by a standard reference method (i.e. crude protein) and the infra-red reflectance spectra. Least square multiple linear regression analysis are used to develop the prediction equation (calibration) i.e. chose the equation that provides the best fit between the analytical component and reflectance or absorption at one or more wavelengths. The calibration data set should include samples that represent the total chemical, physical and spectral variation normally

found in the population of samples that will be analyzed with the calibration. For instance in the case of a calibration to measure crude protein in all soybean meals the calibration dataset should include samples of SBM ranging from 42 to 50 % crude protein. Calibration sets should have the widest possible range in composition but above all they should be representative of all samples to be routinely analyzed with the equation. It is generally not recommended to include samples with extreme values (Shenk and Westerhaus, 1991). Extrapolation beyond the range of values covered in the calibrations is not acceptable. Thus for most soybean products separate equations will need to be developed for groups of products with similar characteristics and values (i.e. Full fat soybeans, SBMs, SPCs, oils etc.).

The quality of a calibration depends greatly on the number of samples and the choice of the samples. The number of samples required to develop a reliable equation remains a subject of discussion. No definite numbers can be provided as the size of the calibration dataset is related to the variability within a set and the range of values that needs to be covered. Under most conditions applicable to soybean products, the number of samples will be no less than 40. The larger the set of well prepared and selected samples the stronger the calibration will be. Once the calibration established, validation of the calibration will be necessary. Samples for validation are subject to the same criteria for representation and number as those used for samples to establish the equation. Generally a smaller number are allowed when samples are representative of the population. Routine procedures to verify the validity and quality of the equation need to be established. The calibration can and should be strengthened through a continuous updating and expansion of the calibration set by adding critically selected samples.

A number of statistical measures are used to describe the quality of a calibration or evaluate its predictive capacity. Most of these refer directly to the least square multiple linear regression techniques used to develop the equations. Most common measures are the regression coefficient (R^2), the standard error of prediction or estimate (SEP) and bias (D). The R^2 is a measure of the variability in the reference data accounted for by the regression equation; the SEP is the variability between predicted values and reference values when the equation is applied to the data other than the calibration set, and D is the average difference between the predicted and reference values. Ideally R^2 should be as close as possible to 1.0 while SEP and D should be as small as possible.

Analyses obtained by NIR are potentially subject to a large number of errors related to the equipment, the calibration and validation process or sample preparation (Williams, 1987). Not all errors are of equal importance and their occurrence and impact is being reduced by the development and installation of more sophisticated NIR techniques and equipment. Users have learned to manage the equipment better and increased their understanding of the special requirements needed for NIR analysis. While the routine use of the equipment is quite simple, the maintenance and development of calibrations require a high level of expertise. For proper operation and in order to reduce errors clear protocols should be drawn up and implemented at all levels of NIR operations. It is important that these protocols assure continuity between the use of NIR for routine analytical functions and the development of new calibrations. When used for routine quality assurance analyses, it is important to provide a separate dust-free environment. This is often difficult to realize in operations dealing with commodities and feed production.

An important number of the errors that can occur in NIR are related to the equipment. There is a relatively large variation between NIR equipments. Consequently, in the case of monochromatic equipment for instance calibrations cannot be transferred directly from one NIR to another without adjustments or corrections followed by a series of validations. Universal calibrations have been developed to solve the problem of transferability of calibrations. These equations are based on a larger dataset than normal covering often different regions and years. Results of these calibrations are often less accurate that those of equipment-specific calibrations. More recently the concept of cloning or networking NIRs has been developed. In these networks and through a series of mathematical corrections the NIRs are calibrated to provide identical spectral results. This of course facilitates enormously the transfer of calibrations and the verification of the different NIRs in the network.

While in principle all organic compounds of a feed or feed ingredient can be analyzed by NIR, for most ingredients and especially for soybean products, best results in terms of accuracy and precision are obtained for humidity, crude protein and lipids. NIR results for fiber components and non-fiber carbohydrates (starch, sugars) normally give larger SEPs and biases and lower R^2 values. NIR cannot be used for the analyses of minerals although a rough estimate for ash and minerals may be obtained by relating the reflectance at specific wavelengths to the organic matter or components of the organic matter (Givens et al., 1997). NIR can be used to analyze

other organic compounds such as amino acids (van Kempen and Bodin, 1998) ANF or fatty acids in soy products, however, the number of publications on this subject is limited and more work is needed.

Equipment required for NIR analysis of soy products:

- Drying equipment (force draught oven).
- Wet chemistry laboratory (to conduct analyses for reference values used in calibration development – see previous sections).
- Grinder (preferably Retch grinder but this is optional; calibrations can be developed for un-ground, homogeneous material).
- NIR equipment.

Procedure (calibration development).

- Dry sample to constant weight (see Section 8.1).
- Grind (optional).
- Split sample in 2 sub-samples, one for reading on NIR equipment and one for analysis by the reference method(s) (wet chemistry).
- Fill sample holder (as described in manual).
- Insert sample holder in NIR and read reflectance or analyte concentration.
- Obtain analytical results for analyte of interest by reference method (see Chapter 8).
- Using a statistical software perform multiple linear regression analysis between wavelength spectra (independent variable) and results of chemical analysis (dependent variable).
- Establish regression equation (high R^2, low SE); beware of over-parameterization (use of too many wave lengths).
- Validate equation with samples not used to establish equation.
- Re-evaluate calibration regularly.

Procedure (application):

- Dry sample to constant weight (see Section 8.1).
- Grind (optional).
- Fill sample holder (as described in manual).
- Insert sample holder in NIR and read reflectance or analyte concentration. (Modern apparatus have integrated computers that will give a direct reading of the component concentration).

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10. Data Management

10.1 Sample statistics

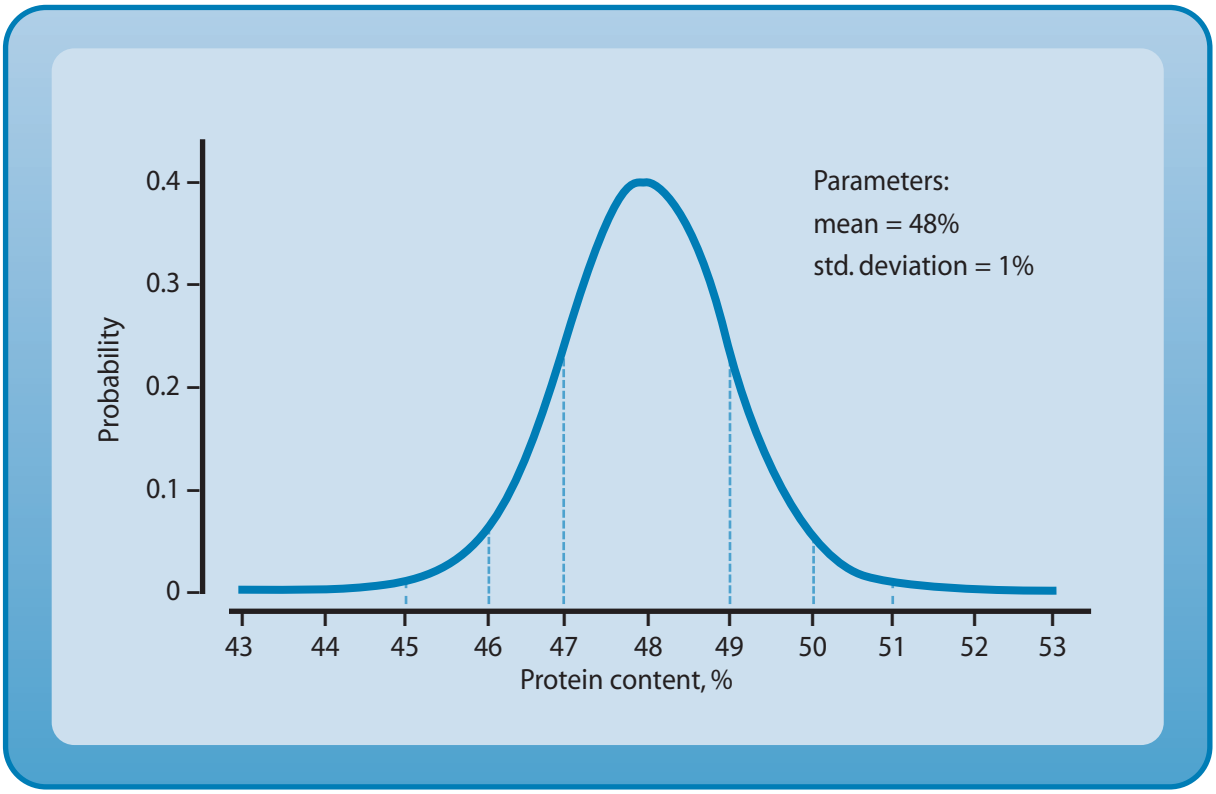
The physical, chemical and microbiological analyses that are performed on feed or soybean products provide information on the nutritional or health value of a selected lot (statistically speaking: the population). The analysis of the whole population is generally not possible. Therefore, statistical procedures are required to obtain information from samples to describe the population accurately.

a. Basic assumptions:

The distribution of a measured parameter (X) in the population of size N is assumed to be normal. In statistical terms, this is expressed as:
$X_i \sim N(\mu,\sigma^2)$. Where μ is the population mean and σ^2 the population variance).

Figure 4

Example of a density curve describing a normal distribution



| μ−3σ|μ−2σ| μ±1σ |μ+2σ | μ+3σ|

Note: The area under the curve gives the proportion of observations that falls in a particular range of values.

Properties of the normal distribution:

68 % of the observations fall within $\pm 1\sigma$ of the mean μ.

95 % of the observations fall within $\pm 2\sigma$ of the mean μ.

99.7 % of the observations fall within $\pm 3\sigma$ of the mean μ.

The population can be characterized by its mean μ and variance σ^2 (unknown). The normal distribution is the most common random distribution about the mean value. An example of this could be the distribution of crude protein content (CP) in a load of soybean meal (SBM) guaranteed to contain 48 % of CP (Figure 4).

b. Parameter estimates:

Sample statistics are used to estimate the population parameters from a sample of smaller size (n). In our SBM example, this would be the estimation of the crude protein of all SBM in the load on the basis of a set of samples of SBM from that load. Main parameter estimates (Table 17) can be calculated simply from the measured results on the samples.

Table 17

Common notation of parameters and parameter estimates

	Parameters (population)	Parameter estimates (sample)
Mean	μ	$\bar{X}$
Variance	σ^2	S^2
Standard deviation	σ	S

Mean

The mean $\bar{x}$ represents the average value of the analyzed component and is calculated by taking the sum of the measurements and dividing by the number of samples.

- Mean ($\bar{x}$): $\bar{x} = \frac{\Sigma x_i}{n}$

  Where x_i: individual sample measurement, n: number of samples

Variability

More important than the mean of a parameter maybe the variability in the observations on the samples as it provides information about the spread in values within the population. For our example: how many samples have crude protein values above or below the mean and how much do they differ from the mean value? Different parameters can be used as indicators of the variability present in a set of measurements:

- Range (w): $w = x_{max} - x_{min}$
- Relative percent difference (RPD) used for duplicates:

  $$RPD = \frac{W}{\bar{x}} \times 100\%$$
- Variance (S^2) obtained from at least three replicates:

  $$S^2 = \frac{\Sigma(x_i - \bar{x})^2}{n-1} \quad \text{or } S^2 = \frac{\Sigma x_i^2 - (\Sigma x_i)^2/n}{n-1}$$
- Standard deviation (s): square root of the variance. The standard deviation is often preferably calculated because it is expressed in the same physical unit as the original data.
- Coefficient of variation (CV):

  $$CV = \frac{S}{\bar{x}} \times 100\%$$

CV is mainly used when the size of the standard deviation changes with the magnitude of the mean.

c. Presentation of analytical results (example):

A cargo of SBM was sampled and 14 samples were collected (n = 14 replicates) to determine protein content of the SBM. The sampling was conducted to be representative on the entire load. The results of the analyses are presented in Table 18.

Table 18.

Protein content of soybean meal: calculation steps to determine the mean and variance

n° sample	measurement: x_i	$x_i - \bar{x}$	$(x_i - \bar{x})^2$
1	50.2	1.79	3.19
2	54.0	5.59	31.20
3	48.7	0.29	0.08
4	44.2	-4.21	17.76
5	45.4	-3.01	9.09
6	46.8	-1.61	2.61
7	51.3	2.89	8.33
8	49.7	1.29	1.65
9	47.7	-0.71	0.51
10	47.6	-0.81	0.66
11	42.9	-5.51	30.41
12	48.0	-0.41	0.17
13	52.1	3.69	13.58
14	49.2	0.79	0.62
Sum Σ	677.8	0	119.86
Σ / n	$\bar{x} = 48.41$	-	-
Σ / (n-1)	-	-	$S^2 = 9.22$

In this example, the mean protein content in the sample was of 48.41 % of DM and the standard deviation of 3.04 % of DM.

The construction of histograms is helpful to visualize the data (average value and range) and to determine if they follow a normal distribution. Histograms are an important tool in quality control (QC) because they help to identify the cause of problems by the shape (i.e., uni- or bimodal) and the width of the distribution.

d. How to construct a histogram?

This procedure was developed from the above example.

- Calculate the range of the values: w = 54.0 – 42.9 = 11.1 % of DM
- Choose a number of intervals (ex. 7).

  The size of the interval is equal to: w / 7 (= 1.6)

For practical considerations, it is better to round the interval size (ex. 2 % of DM).

- Calculate the frequency of occurrences for each interval:

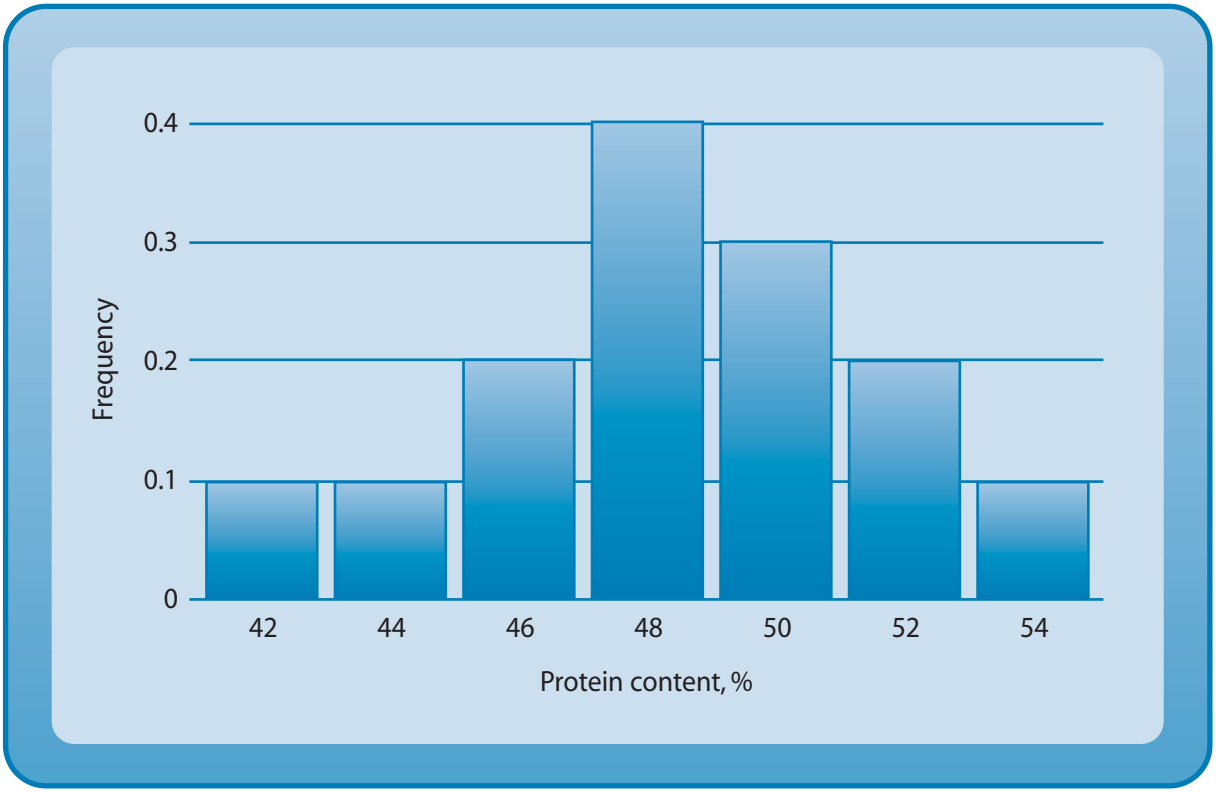
  Ex. Interval: 41 - 43 > occurrence: 1

  Interval: 45 - 47 > occurrence: 2

- Draw the corresponding figure (Figure 5).

Figure 5

Histogram of the data based on seven intervals



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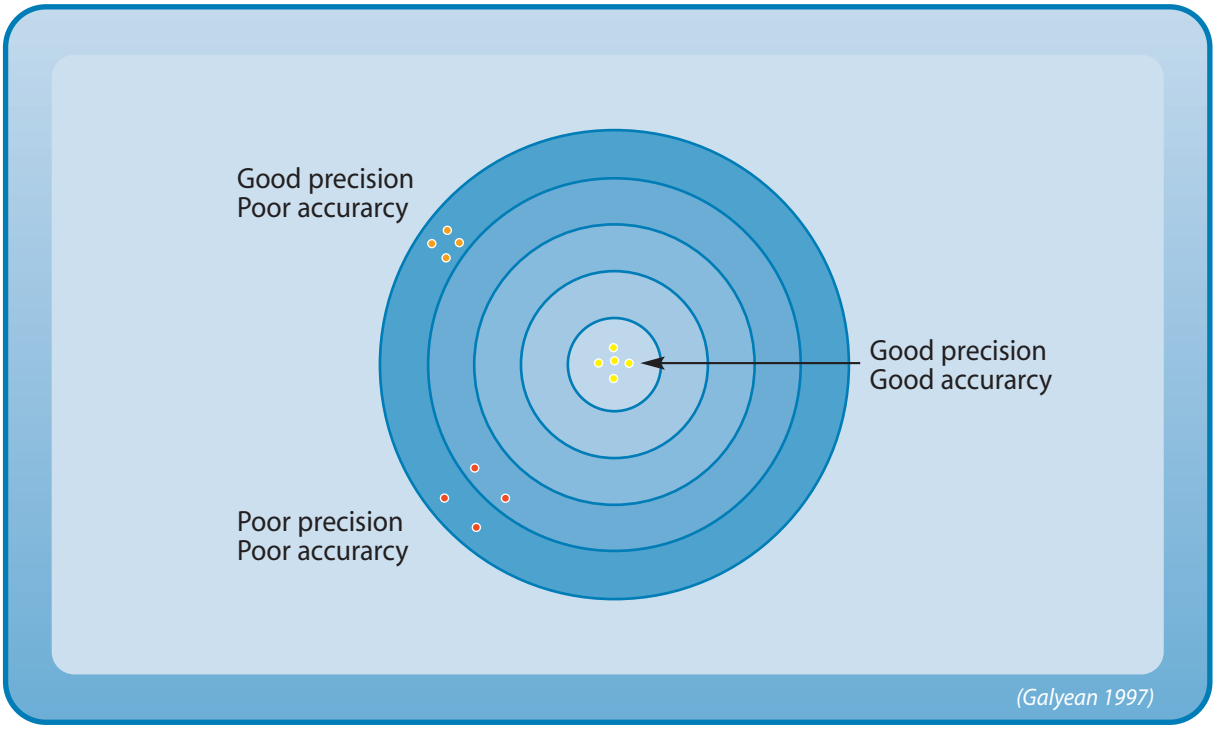
10.2. Quality indicators

The reliability of analytical results and thus the quality of our estimations concerning the population (SBM) depend on critical parameters. First of all, the analytical method should be specific for the compound to be measured (ex. crude protein). The method should also be sensitive to variations in the amount of the compound under study. A small change in CP content should result in a relatively equivalent change in the instrumental response. Finally, accuracy and precision of the method are required (Figure 6) and quality indicators can help to evaluate these two measures.

Figure 6

Definition of accuracy versus precision



In the above example, the method for crude protein analysis in SBM could present a poor accuracy (mean value of 46 % of DM when 48 % of DM should be measured) but a good precision (small range: 45.5 - 46.5 % of DM). On the contrary, the method could present a good accuracy (48 % of DM) and a poor precision (large range: 45 - 51 % of DM).

a. Accuracy

Accuracy is a measure of the bias between the analytical results (X_i) and the true value (X_t). The accuracy can be tested on a sample, when the composition is known.

Accuracy can be determined by: the absolute error (X_i - X_t) or the relative error: 100 x (X_i - X_t) / X_t.
For example, if the CP value of SBM is 48 % of DM and the analytical result yields 50, the method is not accurate: the absolute error for this result is 2 % of DM and the relative error is 4.17 %.

How to check the accuracy of a method?

- Certified reference materials (CRM).
  When available, CRM are materials issued and certified by an external organization and whose properties are validated and reliable. The use of CRM is a powerful tool to assess the good performance in the analytical method.
- Laboratory reference materials (LRM)
  Because of the high cost of CRM, in-house reference standards are generally preferred. The standard recovery is a good indicator of the accuracy of the method.
- "Spiked" sample.
  Accuracy can also be estimated by the ability to measure an amount of substance in a "spiked" sample. A sample is "spiked" when it contains a precisely measured amount of substance. This amount is adjusted to a desired and known level (S).
  The percent recovery is then calculated as follows:
  $$\% \text{ Recovery} = \frac{Q_S - Q_N}{S} \times 100$$
  where QS is the measured quantity in spiked sample, QN: the measured quantity in unspiked sample and S: the quantity of substance in spiked sample.
- Blank.
  A blank is a QC sample designed to check for contamination into the sampling and analytical procedure. A method blank should be free of the molecule to be measured.
- Inter-laboratory comparisons.
  Inter-laboratory comparisons programs should be conducted to compare accuracy of analytical results.

b. Precision

Precision is a measure of the ability to reproduce analytical results.

How to check the precision of a method?

The precision can be estimated with laboratory duplicate samples. These samples should be collected at the same time and location and analyzed in the same conditions. Laboratory duplicates intended to be identical to the original sample. Precision can be determined by calculating the relative percent difference of the

duplicates. It can also be calculated by the standard deviation or coefficient of variation when three or more replicates are used. High variability (RPD, s, CV) among duplicates reflects low precision. Table 19 depicts typical and acceptable coefficients of variation for common analyses.

Table 19

Typical ranges and acceptable coefficients of variation for proximate analysis in feedstuffs (Galyean, 1997)

Analysis	Typical range, %	Acceptable CV, %
DM	80 - 100	0.5
Ash	0 - 20	2.0
CP	5 - 50	2.0
ADF	5 - 70	3.0
NDF	10 - 80	3.0
ADL	0 - 20	4.0
EE	1 - 20	4.0

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10.3. Significance of parameter estimates

a. Hypothesis tests

These tests can be performed to address the uncertainty of the sample estimates and to take decisions about the validity of the data (Feinberg, 1996). For example, it can help to determine if an observed value of a statistic differs from a hypothesized value of a parameter. For our example on SBM the question is: "Is the crude protein analyzed in the sample really different from the population of all SBM in the load?" To answer this, generally two hypotheses can be tested:

H_0: "null hypothesis". The population mean is equal to a reference value ($\mu - \mu_0 = 0$).

The mean value of crude protein in all SBM is equal to 48 % of DM.

H_1: "alternative hypothesis". The population mean is different to the reference value ($\mu - \mu_0 \neq 0$). The mean value of crude protein in all SBMs differs from 48 % of DM.

Select a level of significance (α):

The level of significance represents the probability to reject the hypothesis Ho. By convention, α is set at 5 % - sometimes 10 % is accepted but this increases the probability of being wrong (10 % vs. 5 %).

Calculate the test statistics, in other words test the hypothesis from the sample data:

The test procedure measures the compatibility between the null hypothesis and the data. Several statistical tests exist. The choice of the statistical test will depend on the sample (size), the knowledge on population parameters (ex. variance), the accepted/assumed probability and the hypotheses under question.

For example: can it be concluded from the sampling procedures that the mean value of CP in SBM is 48 % of DM? The Student t-test of the population mean is the test of choice for this case (n small, σ unknown); the following formula can be used for one-sample testing:

$$t = \frac{\bar{x} - \mu_o}{\frac{s}{\sqrt{n}}}, \text{ therefore } t = \frac{48.41 - 48}{\frac{3.04}{\sqrt{14}}} = 0.51$$

Determine the P-value:

The probability value (*P*-value) of a statistical hypothesis test is the probability to obtain results equal to or more "extreme" in future experiments (given that Ho is true). This probability (*P*) can be determined using statistical tables to compare the value of the test statistic (ex. 0.51) with values from the probability distribution (ex. Student distribution). The Student t-test and the Normal z tables are presented in Appendix 7 and 8.

In the above example, the lower and upper bounds for a Student-t test statistic with n-1 = 13 degrees of freedom: (t_p13) can be determined with the tables in appendix 7: $t_{0.4}$ (13) < 0.51 < $t_{0.25}$ (13), therefore P ranges from 0.25 to 0.4.
The *P*-value for a two-sided test is twice the *P*-value of a one-sided test; consequently, in the above example P is between 0.50 and 0.80. The computed actual *P*-value is equal to 0.62.

Set up decision rules:

***P*-value ≤ α**

The difference is said to be "statistically significant" when *P*, the probability that Ho is true given the sample data, is less or equal to the level of significance. In that case, it can be concluded that results are not due to chance and the hypothesis Ho can be rejected.

***P*-value > α**

The difference is attributed to chance or to an error of measurement. In that case, the null hypothesis cannot be rejected; alternatively, Ho is accepted. In the above example, *P*-value is 0.62 (p > 0.05) therefore it is concluded that the crude protein content of SBM is not statistically different from 48 % of DM.

Two types of errors may occur (Table 20-next page). Ho is rejected when it is true (type I error). Ho is accepted when H_1 is true (type II error). The probability α represents the "producer's risk" whereas β represents the "consumer's risk". For example, α is the risk of rejecting a "good" lot and β, the risk of accepting a "bad" lot.

Table 20

Error types in hypothesis testing

		Actual situation	
		H_0	H_1
Decision	Reject Ho	Type I error ($P : \alpha$)	Correct ($P : 1 - \beta$)
	Retain Ho	Correct ($P : 1 - \alpha$)	Type II error ($P : \beta$)

The results of the tests should always be applied with caution. It is particularly important to choose an appropriate sample size to answer the question and detect differences. The ability of the test to detect differences ($P = 1 - \beta$), called power of the test, depends on the size of the difference, the sample size and the level of significance. The test's power increases as sample size increases but decreases as the level of significance increases. Typical power probabilities are set at 0.80, the sample size needed to reach this value can then be estimated.

b. Confidence interval

The sample mean and the population mean are rarely exactly the same but sometimes we like to be able to say that we are pretty sure that the population is within a given amount of our sample mean. Statistically it is possible to calculate an interval around the sample mean with a given level of confidence (probability). Interval estimates are dependent on the heterogeneity or variance associated with the measured variable (s^2), the number of samples (n) and the probability of being wrong (α).

The confidence interval for the mean μ of the population (σ unknown) can be determined with the z or t values in statistical tables (Appendix 7, 8):

- in the case of a small sample from a normal population:

$$\bar{x} \pm t_{\alpha/2\,(n-1)} \frac{s}{\sqrt{n}}$$

- in the case of a large sample from a normal population (n > 30):

$$\bar{x} \pm z_{\alpha/2} \frac{s}{\sqrt{n}}$$

For our example:

$$\bar{x} \pm t_{\alpha/2\,(n-1)} \frac{s}{\sqrt{n}} = 48.41 \pm t_{0.025\,(13)} \frac{3.04}{\sqrt{14}}$$

$$= 48.41 \pm 2.16 \times \frac{3.04}{\sqrt{14}}$$

$$= [46.66 - 50.16]$$

Thus we are 95 % confident that the average crude protein content in SBM is between 46.7 and 50.2 % of DM.

c. Sample size determination

Sampling is costly and time-consuming, therefore it is important to know what sample size should be selected to obtain a desired precision. The sample size can be determined if we know the confidence required (*P*-value; ex. $\alpha = 0.05$), the variability in the population and the precision required. The precision is expressed as H, representing half the width of the confidence interval. The answer should be rounded up to next following whole number.

- unknown population variance: $n = \left(\frac{t_{(\alpha/2)}s}{H}\right)^2$

- known population variance (σ): $n = \left(\frac{z_{(\alpha/2)}\sigma}{H}\right)^2$

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10.4. Control charts

Control charts are efficient devices to control an analytical method and to check its stability over time (Daudin and Tapiero, 1996). They are used to indicate the range of variability of a process and to decide whether the process is under statistical control. In certification schemes (HCAPP, ISO, GMP) and solid quality control programs, they have become fundamental tools. For routine QC procedures, different types of charts are developed depending on the controlled parameter (average or range) and the number of replicates per sample.

- Measurements in group (X or range chart)
- Individual measurements (individual X or moving range chart)

Historical data and experience are generally used to establish the specific charts.

Basic Principles

A control chart is composed of:

- A centerline:
  This value is calculated as the average value of a large number of samples plotted (n > 30).
- Horizontal lines:
  These lines represent the upper control limits (UCL) and the lower control limits (LCL). Typically, these limits are calculated based on the mean and standard deviation:
  - warning control limits: mean ± 2s
  - action control limits: mean ± 3s

The data is plotted over time.

The results of the analytical measurements are plotted in chronological order on the control chart. If the process is in control, the sample points will fall between the control limits. However, points that plot outside of the control limits are interpreted as evidence that the process is out of control. Exceeding a warning control limit generally means that the process is not operating properly. The analyst can try to assess the source of errors, however, no action is needed, providing that next results fall within the warning limits. Exceeding an action control limit leads to the necessary identification and elimination of the causes of errors.

How to develop an individual control chart?

When samples are individual measurements, control charts can be drawn up very simply. In this case, the moving range $|x_i - x_{i-1}|$ can be calculated for each pair of data (see Table 21).

The lines are then defined as follows:

Centerline: $\bar{x} = 48.41$

The standard deviation of the process is estimated from the average moving range (MR) divided by 1.128 (conversion factor d_2 for n = 2).

Action control limits:

$$\bar{x} \pm 3 \frac{\overline{MR}}{1.128}$$

(LCL = 39.98 and UCL = 56.84).

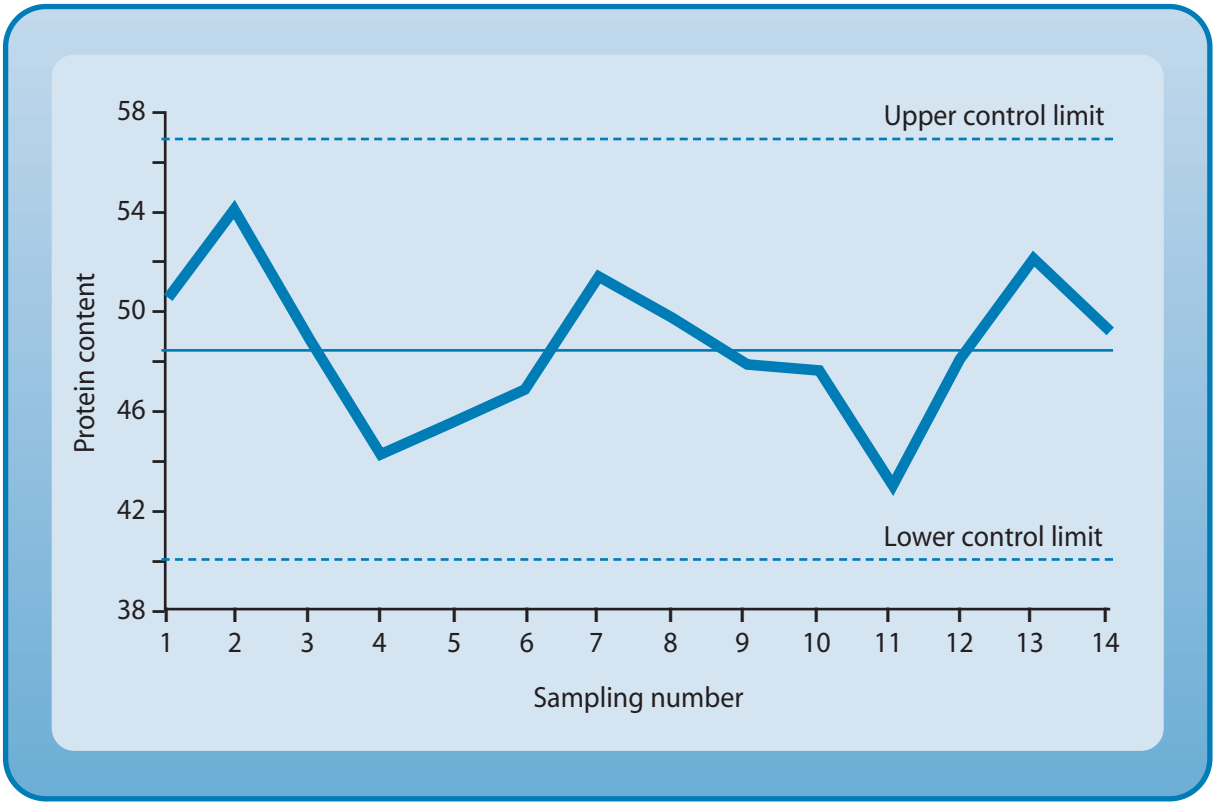
Table 21

Protein content (% of DM) in soybean meal samples

n° sample	measurement: x_i	moving range
1	50.2	-
2	54	3.8
3	48.7	5.3
4	44.2	4.5
5	45.4	1.2
6	46.8	1.4
7	51.3	4.5
8	49.7	1.6
9	47.7	2
10	47.6	0.1
11	42.9	4.7
12	48	5.1
13	52.1	4.1
14	49.2	2.9
Average	$\bar{x} = 48.41$	$\overline{MR} = 3.17$

Figure 7

Control chart for protein content (% of DM) analyses in soybean meal samples



The process can be said to be "in control" since none of the points fall outside the control limits (Figure 7).

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10.5. Follow-up and application of analytical results

Analyses of any type are always associated with uncertainty. Indeed, both systematic and random errors can occur. Therefore, it is important to evaluate the size of the errors and to have an estimation of the reliability of the analytical results. This procedure should be part of a standard quality control procedure and needs to be developed through a joint effort between analysts and nutritionists. Each has a specific responsibility/task, which can be summarized as follows:

a. Analyst:

- Perform the sampling and analysis correctly.
- Use proper QC measures to validate the data and to keep systematic and random errors under control: calibration standards, controls, duplicate field samples and blanks to estimate sampling errors, laboratory duplicates to estimate analytical errors.
- Establish quality objectives (precision, accuracy) or quality acceptance limits. The acceptable level of variation between duplicates varies by test and by concentration of nutrient (Table 3).
- Propose corrective actions (re-sampling, re-calibration...) if needed.

b. Nutritionist:

- Define the parameters that need to be analyzed.
- Include ingredient quality specifications in the purchasing agreement and provide this information to the analyst.
- Adjust formulation.
- Find alternative ingredient if quality specifications are not met.

The objectives of the analyst and the nutritionist may be to reduce variation (increase quality of the results) but also to maximize the value of a raw material. There is a subjective judgment associated with quality control. The risk of type I or type II errors exists. It is possible to reduce these risks (higher significance level, higher power of the tests, and larger number of samples) but this is generally associated with a higher cost.

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CONTENTS

12. Annex

Appendix 1

Average nutrient composition of soy protein products used in livestock diets

Nutrient	Unit	Soybean seeds extruded	Ground FF soybean seeds	Heat processed FF soybean seeds	Soy protein concentrate	Soy protein isolate	Soybean hulls
Dry matter	%	88.10	89.72	89.44	91.83	93.38	89.76
Crude protein	%	34.80	37.50	37.08	68.60	85.88	12.04
Crude fiber	%	5.20	5.03	5.12	1.65	1.32	34.15
Ether extracts	%	17.90	18.04	18.38	2.00	0.62	2.16
Ash	%	5.20	4.77	4.86	5.15	3.41	4.53
NDF	%	11.00	12.50	12.98	13.50	–	56.91
ADF	%	6.40	8.80	7.22	5.38	–	42.05
ADL	%	1.00	4.10	4.30	0.40	–	2.05
Starch	%	0.00	4.59	4.66	–	–	5.95
Total sugars	%	7.70	–	–	–	–	1.40
Gross energy	kcal/kg	4870	5027	5013	–	5370	3890
DE-swine	kcal/kg	3800	4157	4088	4517	4545	1944
ME energy-swine	kcal/kg	3560	3739	3714	3661	3955	1687
NE-swine	kcal/kg	2560	2920	2803	2000	2000	1074
App. ME-broiler	kcal/kg	3350	3475	3332	–	4060	–
App. ME-adults	kcal/kg	3450	3621	3564	2472	3945	334
ME-ruminants	kcal/kg	3400	3373	3400	2690	–	1241
NE-dairy	kcal/kg	2159	1986	2097	1600	–	1544
NE-beef	kcal/kg	2311	2080	2230	1610	–	1618
Amino acids							
Lysine	%	2.16	2.39	2.34	4.59	5.26	0.73
Threonine	%	1.40	1.54	1.53	2.82	3.17	0.73
Methionine	%	0.53	0.51	0.52	0.87	1.01	0.14
Cystine	%	0.56	0.55	0.55	0.89	1.19	0.16
Tryptophane	%	0.44	0.49	0.49	0.81	1.08	0.12
Isoleucine	%	1.61	1.78	1.79	3.68	4.25	0.41
Valine	%	1.66	1.88	1.85	3.69	4.21	0.49
Leucine	%	2.59	2.81	2.76	5.41	6.65	0.75
Phenylalanine	%	1.74	1.84	1.87	3.60	4.35	0.47
Tyrosine	%	1.23	1.18	1.22	1.55	3.14	0.43
Histidine	%	0.93	0.95	0.96	2.41	2.25	0.29
Arginine	%	2.57	2.73	2.71	7.34	6.87	0.62
Alanine	%	1.41	1.54	1.52	–	3.33	0.51
Aspartic acid	%	3.88	4.09	4.06	–	8.29	1.14
Glutamine	%	6.17	6.37	6.35	–	12.0	1.49
Glycine	%	1.48	1.52	1.59	3.32	3.38	0.85
Serine	%	1.78	1.94	1.89	5.19	4.81	0.67
Proline	%	1.83	1.89	1.86	–	4.45	0.55

Nutrient	Unit	Soybean seeds extruded	Ground FF soybean seeds	Heat processed FF soybean seeds	Soy protein concentrate	Soy protein isolate	Soybean hulls
Minerals							
Calcium	g/kg	3.10	2.58	2.62	2.37	1.50	4.96
Phosphorus	g/kg	5.50	5.83	5.70	7.63	6.50	1.59
Magnesium	g/kg	2.30	2.98	2.80	1.85	0.80	2.23
Potasium	g/kg	18.50	14.60	15.93	12.35	2.75	12.15
Sodium	g/kg	0.80	0.20	0.29	0.55	2.85	0.10
Chloride	g/kg	0.40	0.25	0.33	0.20	0.20	0.25
Sulfur	g/kg	2.80	2.20	2.43	0.70	7.00	0.95
Manganese	mg/kg	23.00	36.00	31.79	27.50	5.00	10.67
Zinc	mg/kg	40.00	57.00	47.80	47.00	34.00	37.75
Copper	mg/kg	34.00	6.90	15.17	17.00	14.00	10.68
Iron	mg/kg	146.00	84.00	128.01	137.00	137.00	437.50
Selenium	mg/kg	0.28	0.11	0.17	0.14	0.14	0.16
Cobalt	mg/kg	–	–	0	–	–	0.06
Molybdenum	mg/kg	4.00	–	2.00	–	–	0.60
Fatty acids							
Myristic acid-C14:0	%	0.01	0.03	0.03	–	–	0
Palmitic acid-C16:0	%	1.05	1.47	1.95	–	–	0.24
Palmitoleic acid-C16:1	%	0.02	0.03	0.04	–	–	0
Stearic acid-C18:0	%	0.38	0.53	0.71	–	–	0.09
Oleic acid-C18:1	%	2.17	2.97	3.96	–	–	0.50
Linoleic acid-C18:2	%	5.31	7.28	9.70	–	–	1.21
Linolenic acid-C18:3	%	0.74	1.06	1.40	–	–	0.17

FF Soybean is Full Fat Soybean; SBM = Soybean meal.
Source: compilation on NRC, INRA-AFZ, CVB, SRTNA and selected suppliers.

Appendix 2

Average nutrient composition of soy protein products used in livestock diets

Nutrient	Unit	SBM mechanically extracted	SBM solvent extracted 44	SBM solvent extracted 48	SBM solvent extracted 50	Soybean mill feed	Soybean oil
Dry matter	%	89.80	88.08	87.58	88.20	89.70	99.25
Crude protein	%	43.92	44.02	46.45	48.79	12.93	1.40
Crude fiber	%	5.50	6.26	5.40	3.42	33.47	–
Ether extracts	%	5.74	1.79	2.13	1.30	1.70	97.20
Ash	%	5.74	6.34	6.02	5.78	4.73	0.40
NDF	%	21.35	13.05	11.79	9.95	–	–
ADF	%	10.20	8.76	7.05	5.00	41.40	–
ADL	%	1.17	0.75	0.90	0.40	–	–
Starch	%	7.00	5.51	5.46	3.28	–	–
Total sugars	%	–	9.06	9.17	9.29	–	–
Gross energy	kcal/kg	–	4165	4130	4120	–	–
DE-swine	kcal/kg	–	3394	3446	3776	1167	8915
ME energy-swine	kcal/kg	–	2986	3210	3299	925	8400
NE-swine	kcal/kg	–	1903	1955	1992	–	6760
App. ME-broiler	kcal/kg	–	1929	1973	2147	–	8600
App. ME-adult	kcal/kg	–	2171	2208	2464	774	8805
ME-ruminants	kcal/kg	–	2831	2840	3010	1630	8180
NE-dairy	kcal/kg	–	1706	1748	1826	1001	4520
NE-beef	kcal/kg	–	1838	1847	1993	965	5022
Amino acids							
Lysine	%	3.50	2.85	2.89	3.00	0.65	–
Threonine	%	2.21	1.80	1.84	1.90	0.30	–
Methionine	%	0.80	0.62	0.63	0.67	0.13	–
Cystine	%	0.77	0.68	0.73	0.73	0.14	–
Tryptophane	%	0.74	0.56	0.63	0.65	0.13	–
Isoleucine	%	2.88	2.26	2.17	2.30	041	–
Valine	%	2.73	2.19	2.30	2.38	0.38	–
Leucine	%	4.29	3.42	3.60	3.60	0.58	–
Phenylalanine	%	2.79	2.16	2.37	2.37	0.38	–
Tyrosine	%	1.52	1.61	1.68	1.64	0.23	–
Histidine	%	1.44	1.64	1.21	1.21	0.18	–
Arginine	%	3.98	2.99	3.48	3.53	0.75	–
Alanine	%	1.85	2.53	2.05	2.04	–	–
Aspartic acid	%	5.16	4.03	5.49	5.55	–	–
Glutamine	%	8.18	6.29	8.62	8.52	–	–
Glycine	%	2.29	3.46	1.97	2.09	0.48	–
Serine	%	2.20	2.13	2.38	2.49	0.30	–
Proline	%	2.35	2.17	2.37	2.43	–	–

Nutrient	Unit	SBM mechanically extracted	SBM solvent extracted 44	SBM solvent extracted 48	SBM solvent extracted 50	Soybean mill feed	Soybean oil
Minerals							
Calcium	g/kg	2.96	3.12	3.07	2.68	4.05	–
Phosphorus	g/kg	6.64	6.37	6.37	6.36	1.75	–
Magnesium	g/kg	2.84	2.72	3.03	2.88	3.20	–
Potasium	g/kg	20.28	19.85	22.00	20.84	15.20	–
Sodium	g/kg	0.33	0.18	0.18	0.88	2.50	–
Chloride	g/kg	0.72	0.42	0.35	0.53	–	–
Sulfur	g/kg	3.37	3.51	2.76	4.30	0.55	–
Manganese	mg/kg	40.86	37.85	43.11	33.92	290.00	–
Zinc	mg/kg	58.98	53.56	50.12	53.70	–	–
Copper	mg/kg	21.90	20.03	18.04	17.10	–	–
Iron	mg/kg	218.45	304.84	319.43	190.75	–	–
Selenium	mg/kg	0.10	0.31	0.30	0.30	–	–
Cobalt	mg/kg	0.18	0.14	0.17	0.09	–	–
Molybdenum	mg/kg	3.80	–	2.45	3.56	–	–
Fatty acids							
Myristic acid-C14:0	%	–	0.00	0.00	0.01	–	0.10
Palmitic acid-C16:0	%	–	0.77	0.14	1.05	–	10.50
Palmitoleic acid-C16:1	%	–	0.00	0.00	0.02	–	0.15
Stearic acid-C18:0	%	–	0.28	0.05	0.38	–	4.20
Oleic acid-C18:1	%	–	0.28	0.27	0.21	–	23.30
Linoleic acid-C18:2	%	2.87	0.64	0.80	0.56	–	52.00
Linolenic acid-C18:3	%	0.42	0.55	0.12	0.08	–	6.90

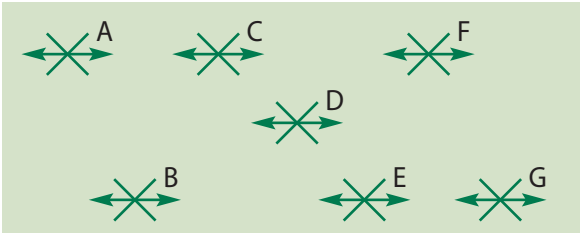
FF Soybean is Full Fat Soybean; SBM = Soybean meal.
Source: compilation on NRC, INRA-AFZ, CVB, SRTNA and selected suppliers.

Appendix 3

Sampling patterns for bulk carriers

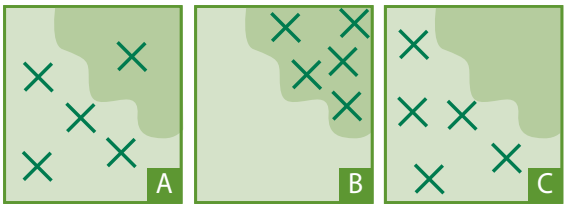
(From: Herrman, 2001)

A. Sampling pattern for bulk carriers containing a homogeneous load



Sampling pattern as recommended by GIPSA (1995) for the sampling of bulk truck or rail shipments of soybean seeds or soybean meals using a hand-held sampling devise or an automatic sampler. Site A: Probe the grain approx. 0.6 m. from the front and side. Site B: Probe approx. half-way between the front and center; Site C: Probe approx. three-quarter of the way between the front and center; Site D: Probe grain in the center of the carrier. Site E,F,G: follow a similar pattern described above for the back part of the carrier.

B. Sampling pattern for bulk carriers containing areas with damaged material



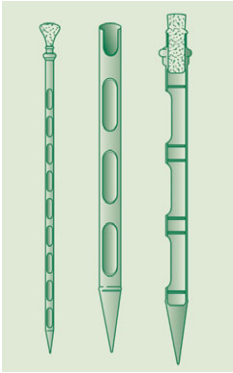
Recommended stratified sampling patterns for carriers containing inferior or damaged portions of soybean seeds or soybean meals. In this case a three step procedure is recommended. A: Probe the carrier as a whole (inferior and sound portions) as if the load was homogeneous. B: Probe the portion or portions containing the inferior grain thoroughly so as a representative cross section is obtained of the damaged or inferior material. C: Probe the portion or portions with the sound material to collect a representative sample. The sample of each step should be a minimum of 2 kg. Samples should be analyzed individually and proportions of sound to inferior material noted.

Appendix 4

Sampling devices for soy bean products

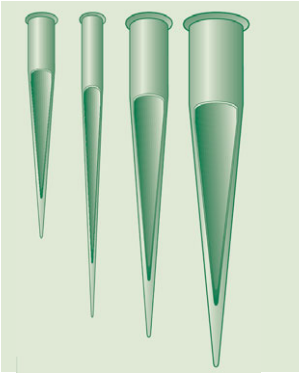
(From: Hermann, 2001)

Figure 1A



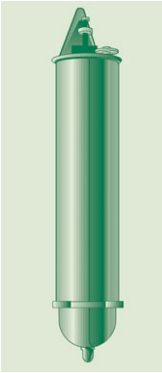
Grain probes

Figure 1B



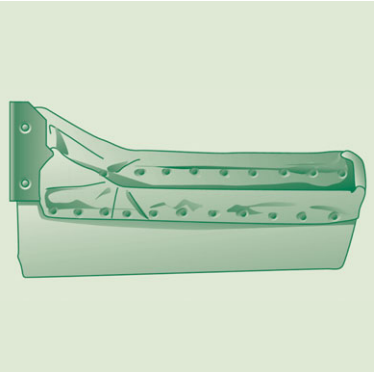
Tapered bag triers

Figure 1C



Bomb sampler

Figure 1D



Pelican grain probe sampler

Appendix 5

Sampling guidelines for bagged material

Sampling of 1 bag: Stand bag up and insert sampling probe in top corner of the bag. Lower the probe diagonally through the bag to reach the opposite corner and withdraw sample.

For lots up to 10 bags, each bag should be samples.

Sampling of more than 10 bags: sample 10 bags selected at random.

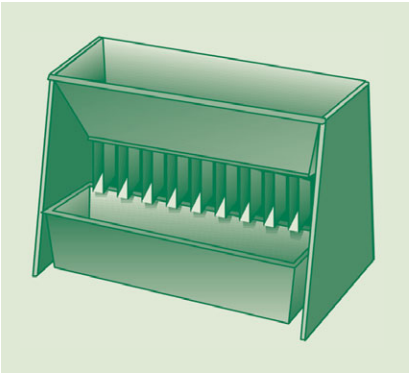
Enough material should be collected to perform the necessary assays and retain a sample. Generally a 0.5 kg sample is adequate.

Appendix 6

Devices for splitting of samples

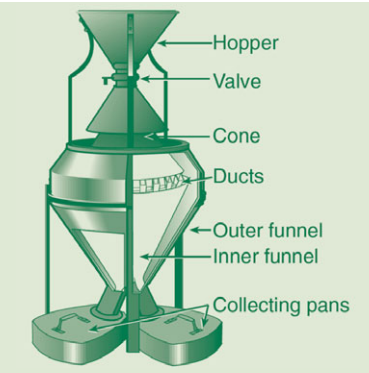
(From: Hermann, 2001)

Figure 2A



Riffle sample splitter

Figure 2B



Boerner divider

Appendix 7

Student's *t* test: $t_p(df)$

Degrees of freedom	Probability, p							
	0.40	0.25	0.10	0.05	0.025	0.01	0.005	0.0005
1	0.324920	1.000000	3.077684	6.313752	12.70620	31.82052	63.65674	636.6192
2	0.288675	0.816497	1.885618	2.919986	4.30265	6.96456	9.92484	31.5991
3	0.276671	0.764892	1.637744	2.353363	3.18245	4.54070	5.84091	12.9240
4	0.270722	0.740697	1.533206	2.131847	2.77645	3.74695	4.60409	8.6103
5	0.267181	0.726687	1.475884	2.015048	2.57058	3.36493	4.03214	6.8688
6	0.264835	0.717558	1.439756	1.943180	2.44691	3.14267	3.70743	5.9588
7	0.263167	0.711142	1.414924	1.894579	2.36462	2.99795	3.49948	5.4079
8	0.261921	0.706387	1.396815	1.859548	2.30600	2.89646	3.35539	5.0413
9	0.260955	0.702722	1.383029	1.833113	2.26216	2.82144	3.24984	4.7809
10	0.260185	0.699812	1.372184	1.812461	2.22814	2.76377	3.16927	4.5869
11	0.259556	0.697445	1.363430	1.795885	2.20099	2.71808	3.10581	4.4370
12	0.259033	0.695483	1.356217	1.782288	2.17881	2.68100	3.05454	4.3178
13	0.258591	0.693829	1.350171	1.770933	2.16037	2.65031	3.01228	4.2208
14	0.258213	0.692417	1.345030	1.761310	2.14479	2.62449	2.97684	4.1405
15	0.257885	0.691197	1.340606	1.753050	2.13145	2.60248	2.94671	4.0728
16	0.257599	0.690132	1.336757	1.745884	2.11991	2.58349	2.92078	4.0150
17	0.257347	0.689195	1.333379	1.739607	2.10982	2.56693	2.89823	3.9651
18	0.257123	0.688364	1.330391	1.734064	2.10092	2.55238	2.87844	3.9216
19	0.256923	0.687621	1.327728	1.729133	2.09302	2.53948	2.86093	3.8834
20	0.256743	0.686954	1.325341	1.724718	2.08596	2.52798	2.84534	3.8495
21	0.256580	0.686352	1.323188	1.720743	2.07961	2.51765	2.83136	3.8193
22	0.256432	0.685805	1.321237	1.717144	2.07387	2.50832	2.81876	3.7921
23	0.256297	0.685306	1.319460	1.713872	2.06866	2.49987	2.80734	3.7676
24	0.256173	0.684850	1.317836	1.710882	2.06390	2.49216	2.79694	3.7454
25	0.256060	0.684430	1.316345	1.708141	2.05954	2.48511	2.78744	3.7251
26	0.255955	0.684043	1.314972	1.705618	2.05553	2.47863	2.77871	3.7066
27	0.255858	0.683685	1.313703	1.703288	2.05183	2.47266	2.77068	3.6896
28	0.255768	0.683353	1.312527	1.701131	2.04841	2.46714	2.76326	3.6739
29	0.255684	0.683044	1.311434	1.699127	2.04523	2.46202	2.75639	3.6594
30	0.255605	0.682756	1.310415	1.697261	2.04227	2.45726	2.75000	3.6460
∞	0.253347	0.674490	1.281552	1.644854	1.95996	2.32635	2.57583	3.2905

Appendix 8

Standard normal Z table

z	0.00	0.01	0.02	0.03	0.04	0.05	0.06	0.07	0.08	0.09
0.0	0.0000	0.0040	0.0080	0.0120	0.0160	0.0199	0.0239	0.0279	0.0319	0.0359
0.1	0.0398	0.0438	0.0478	0.0517	0.0557	0.0596	0.0636	0.0675	0.0714	0.0753
0.2	0.0793	0.0832	0.0871	0.0910	0.0948	0.0987	0.1026	0.1064	0.1103	0.1141
0.3	0.1179	0.1217	0.1255	0.1293	0.1331	0.1368	0.1406	0.1443	0.1480	0.1517
0.4	0.1554	0.1591	0.1628	0.1664	0.1700	0.1736	0.1772	0.1808	0.1844	0.1879
0.5	0.1915	0.1950	0.1985	0.2019	0.2054	0.2088	0.2123	0.2157	0.2190	0.2224
0.6	0.2257	0.2291	0.2324	0.2357	0.2389	0.2422	0.2454	0.2486	0.2517	0.2549
0.7	0.2580	0.2611	0.2642	0.2673	0.2704	0.2734	0.2764	0.2794	0.2823	0.2852
0.8	0.2881	0.2910	0.2939	0.2967	0.2995	0.3023	0.3051	0.3078	0.3106	0.3133
0.9	0.3159	0.3186	0.3212	0.3238	0.3264	0.3289	0.3315	0.3340	0.3365	0.3389
1.0	0.3413	0.3438	0.3461	0.3485	0.3508	0.3531	0.3554	0.3577	0.3599	0.3621
1.1	0.3643	0.3665	0.3686	0.3708	0.3729	0.3749	0.3770	0.3790	0.3810	0.3830
1.2	0.3849	0.3869	0.3888	0.3907	0.3925	0.3944	0.3962	0.3980	0.3997	0.4015
1.3	0.4032	0.4049	0.4066	0.4082	0.4099	0.4115	0.4131	0.4147	0.4162	0.4177
1.4	0.4192	0.4207	0.4222	0.4236	0.4251	0.4265	0.4279	0.4292	0.4306	0.4319
1.5	0.4332	0.4345	0.4357	0.4370	0.4382	0.4394	0.4406	0.4418	0.4429	0.4441
1.6	0.4452	0.4463	0.4474	0.4484	0.4495	0.4505	0.4515	0.4525	0.4535	0.4545
1.7	0.4554	0.4564	0.4573	0.4582	0.4591	0.4599	0.4608	0.4616	0.4625	0.4633
1.8	0.4641	0.4649	0.4656	0.4664	0.4671	0.4678	0.4686	0.4693	0.4699	0.4706
1.9	0.4713	0.4719	0.4726	0.4732	0.4738	0.4744	0.4750	0.4756	0.4761	0.4767
2.0	0.4772	0.4778	0.4783	0.4788	0.4793	0.4798	0.4803	0.4808	0.4812	0.4817
2.1	0.4821	0.4826	0.4830	0.4834	0.4838	0.4842	0.4846	0.4850	0.4854	0.4857
2.2	0.4861	0.4864	0.4868	0.4871	0.4875	0.4878	0.4881	0.4884	0.4887	0.4890
2.3	0.4893	0.4896	0.4898	0.4901	0.4904	0.4906	0.4909	0.4911	0.4913	0.4916
2.4	0.4918	0.4920	0.4922	0.4925	0.4927	0.4929	0.4931	0.4932	0.4934	0.4936
2.5	0.4938	0.4940	0.4941	0.4943	0.4945	0.4946	0.4948	0.4949	0.4951	0.4952
2.6	0.4953	0.4955	0.4956	0.4957	0.4959	0.4960	0.4961	0.4962	0.4963	0.4964
2.7	0.4965	0.4966	0.4967	0.4968	0.4969	0.4970	0.4971	0.4972	0.4973	0.4974
2.8	0.4974	0.4975	0.4976	0.4977	0.4977	0.4978	0.4979	0.4979	0.4980	0.4981
2.9	0.4981	0.4982	0.4982	0.4983	0.4984	0.4984	0.4985	0.4985	0.4986	0.4986
3.0	0.4987	0.4987	0.4987	0.4988	0.4988	0.4989	0.4989	0.4989	0.4990	0.4990

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