QUALITY MANUAL AND ANALYSIS FOR SOYBEAN PRODUCTS IN THE FEED INDUSTRY

3rd Edition

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1. INTRODUCTION

Soybeans are one of the most valuable crops in the world, contributing increasing amounts of high-quality oil and protein to human and animal diets. Worldwide soybean production has increased steadily over the past decades. Since 1960, world soybean production has increased more than tenfold to exceed 366 million metric tons in the 2020-2021 season. With slightly more than 31% of total world production, or 114 million metric tons in 2020-2021, of which 61 million metric tons was exported, the United States continues to be an important producer and supplier of soybeans and soy products. However, its share of worldwide soybean production has decreased steadily since 1970, when the United States produced more than 70% of the world's soybeans. The main production region is now South America. Two countries, Brazil and Argentina, produce the lion's share, while surrounding countries, including Uruguay, Paraguay and Bolivia, produce smaller, but growing quantities. Soy production is also increasing significantly in other parts of the world, such as Asia, Oceania and Europe, reflecting its critical importance and value in the food chain.

Of the total soybean volume, the majority is crushed to yield soy oil and soybean meal (SBM). Soybean meal is predominantly used in animal feed and consists of the portions of the soybean constituting protein, carbohydrates, and minerals. A relatively small fraction of the soybean is used for direct human consumption in a wide variety of soy products. Approximately 19% of the soybean's composition is oil, 36% is protein, 19% is insoluable carbohydrate (fiber), 9% is soluable carbohydrate, and the remainder is moisture and minerals. Efficient processors can extract most of the oil, which is used in human and animal nutrition, and in industrial applications. Moisture is removed through drying. The remaining components typically compose soy meal.

Most soy used in animal feed is in the form of various types of SBM. Smaller quantities are fed as full-fat soybeans (FFSB) or value-added specialty soy products. The current world production of SBM is estimated to be more than 235 million MT, which amounts to approximately 69% of major protein meals (USDA, 2018). With global compound feed estimates at well over 1 billion MT (IFIF, 2017), and with SBM accounting for 70% of world protein meals in 2020/2021, SBM represents the dominant source of protein meal in animal diets. However, total use of soybeans and soy products is likely to be higher than major statistics indicate, as a plethora of specialty soy products have been developed for 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 (AA) profile. Following proper treatment or extraction, digestibility of the protein fraction is high, and the AA profile provides an ideal complement to the AA profile of many cereal grains to meet animal requirements.

Nevertheless, in their untreated form, soybeans contain a number of factors with potential to diminish their nutritive value — to the point of decreasing animal performance and health (Liener, 2000). Soybeans need a treatment to eliminate these anti-nutritional factors (ANF), especially in the case of monogastric diets. These treatments, combined with varietal and environmental or geographical differences, potentially lead to large variations in the nutritional quality of SBM and other soy products.

While basic standard specifications for SBM have been established by the National Oilseed Processors Association (NOPA), no official specifications exist for other soy products routinely used in the feed industry. Furthermore, the NOPA specifications (2017) only refer to four chemical characteristics.

Industry nutritionists and formulators currently base evaluations of soy products on a much larger array of tests, allowing more accurate assessment of the nutritive value of different products. However, under practical conditions of feed production, test choices differ greatly among producers and feed compounders, and not all tests are applied on a regular basis. As nutritional requirements become more precisely defined, especially for sensitive species or age groups, more analyses of greater complexity likely will be needed. Developments in the technological modification of soybean products, along with a better understanding of performance and health effects of relatively unknown compounds such as isoflavones, will add to their value. Accurate analysis to measure the effects of new treatments and relatively unexplored compounds will be of great importance.

For results of quality tests to have real value and to be comparable between producers, tests should be standardized in method and equipment. This standardization is becoming increasingly important as global soybean product trade grows. Identity preservation and traceability associated with detailed quality characterization are major issues for soy products. Accurate and consistent quality procedures and analyses, along with precise product descriptions, are necessary. These tests must be reproducible at different levels of the supply chain. Furthermore, increasing demands of implemented quality systems, like Hazard Analysis Critical Control Point (HACCP), International Organization for Standardization (ISO), Feed Additives and Premixture Quality System (FAMI-QS), or Good Manufacturing Practice (GMP), will dictate the establishment of more detailed quality procedures and larger analytical capacities. For the information generated at various production stages to be consistent and comparable, a single reference needs to be available.

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

Acronyms and Abbreviations:

AA — Amino acids
AACC — American Association for Cereal Chemists
AAFCO — Association of American Feed Control Officials
AAS — Atomic absorption spectrometry
ACS — American Chemical Society
ADF — Acid detergent fiber
ADFCP — Acid detergent fiber crude protein
ADIN — Acid detergent insoluble nitrogen
ADL — Acid detergent lignin (Klason lignin)
AFNOR — French Association for Normalization
AFZ — French Association for Animal Production
AID — Apparent ileal digestibility
ANF — Anti-nutritional factors
AMEn — Apparent metabolizable energy, N-corrected
AOAC — Association of Official Analytical Collaboration International
AOCS — American Oil Chemists' Society
AOM — Active oxygen method
aP — Available phosphorus
ASA — American Soybean Association
ASAE — American Society of Agricultural Engineers
BBI — Bowman-Birk inhibitor
CE — Capillary electrophoresis
CF — Crude fiber
CFIA — Canadian Food Inspection Agency
CIRAD — French Agricultural Research Centre for International Development
CP — Crude protein
CRM — Certified reference materials
CVB — Centraal Veevoederbureau, Dutch Central Animal Feed Agency
DAA — Digestible amino acids
DAS — Diacetoxyscirpenol
DE — Digestible energy
DM — Dry matter
DNP-lysine — dinitrophenyl lysine
DON — Deoxynivalenol
EAA — Endogenous amino Acids
EE — Ether extract (oil and fats)
EEC — European Economic Community
ELISA — Enzyme-linked immuno-sorbent assay
ESBM — Enzymatically treated soybean meal
EU — European Union
FA — Fatty acids
FAAS — Flame atomic absorption spectrometry
FAMI-QS — Feed Additives and Premixture Quality System
FAO — Food and Agriculture Organization of the United Nations
FDA — United States Food and Drug Administration

FEDNA — Spanish Foundation for the Development of Animal Nutrition
FFA — Free fatty acids
FFSB — Full-fat soybeans
FGIS — Federal Grain Inspection Service
FLIA — Functional lectins
FSBM — Fermented soybean meal
GC — Gas chromatography
GE — Genetically engineered
GIPSA — Grain Inspection, Packers and Stockyards Administration
GLC — Gas liquid chromatography
GM — Genetically modified
GMP — Good Manufacturing Practice
GMO — Genetically modified organism
HACCP — Hazard Analysis Critical Control Point
Hi-pro — High protein
HPLC — High-performance liquid chromatography
IEAA — Ileal endogenous amino acids
IFIF — International Feed Industry Federation
IFN — International Feed Name and International Feed Number
INFIC — International Network of Feed Information Centres
INRA — French National Institute of Agricultural Research
IP — Identity preservation
ISAAA — International Service for the Acquisition of Agri-biotech Applications
ISO — International Organization for Standardization
IV — Iodine value
KOHPS — Potassium hydroxide protein solubility
KTI — Kunitz trypsin inhibitor
LRM — Laboratory reference materials
LC-MS/MS — Liquid chromatography-tandem mass spectrometry
Mabs — Monoclonal antibodies
MAFF — Japanese Ministry of Agriculture, Forestry and Fisheries
Max. — Maximum
ME — Metabolizable energy
Min. — Minimum
MIU — Moisture, insolubles, unsaponifiables
NCP — Non-cellulosic polysaccharides
ND — Neutral detergent fiber solution
NDF — Neutral detergent fiber
NE — Net energy
NFE — Nitrogen-free extract
NIR — Near-infrared reflectance
NIRS — Near-infrared reflectance spectroscopy
NOPA — National Oilseed Processors Association
nPP — Non-phytate (phytin) phosphorus
NRC — National Research Council
NSI — Nitrogen solubility index

NSP — Non-starch polysaccharides
OSI — Oil stability index
Pabs — Polyclonal antisera
PCR — Polymerase chain reaction (test)
PDI — Protein dispersibility index
PFR — Precision-fed cecectomized rooster assay
PP — Phytic or phytin phosphorus
QC — Quality control
RFP — Rapid feed passage
SBM — Soybean meal
SIAAD — Standardized ileal amino acid digestibility assay — poultry
SID — Standardized ileal digestibility — swine
SPC — Soy protein concentrates
SPI — Soy protein isolates
TDF — Total dietary fiber
TI — Trypsin inhibitors
TIA — Trypsin inhibitor activity
TID — True ileal digestibility
TId — Trypsin inhibited
TLC — Thin–layer chromatography
TU— Trypsin unit
UA — Urease activity
UI — Urease index
UNEP — United Nations Environment Programme
U.S. — United States
USDA — United States Department of Agriculture
USP — United States Pharmacopeia
USSEC — United States Soybean Export Council

Compounds and elements

$AgNO_3$ — Silver nitrate
ATP — Adenosine triphosphate
BAEE — Nα-benzoyl-L-arginine ethyl ester
$Ba(OH)_2$ — barium hydroxide
BAPNA — α-Benzoyl-DL-arginine-p-nitroanilide
$BH_{12}N_3O_3$ — ammonium borate
BSA — Bovine serum albumin
C_2H_3N — Acetonitrile
$C_2H_3NaO_2$ — Sodium acetate
$C_4H_6N_2$ — 1-metilimidazole
$C_5H_{12}O$ — 3-methylbutan-1-ol
$C_6FeK_4N_6$ — Potassium ferrocyanide
$C_6H_8N_2$ — 1,2-phenylendiamine
$C_6H_8O_7$ — Citric acid
$C_6H_{12}O_6$ — Myo-inositol
$C_6H_{13}O_9P$ — Glucose-6-phosphate
$C_6H_{14}O_4$ — Triethylene glycol

$C_{10}H_9NO_4S$ — 1-amino-2-naphthol-4-sulfonic acid
$C_{10}H_{14}N_2Na_2O_8 \cdot 2H_2O$ — disodium dihydrogen ethylene diamine tetra-acetic dihydrate
Ca — Calcium
$CaCl_2$ — Calcium chloride
CCl4 — Carbon tetrachloride
Cd — Cadmium
$(CH_3)_2SO$ — Dimethyl sulfoxide
$(CH_3)_3COH$ — Tertiary butyl alcohol
CH_3COCl — Acetyl chloride
CH_3CO_2K — Potassium acetate
Cl — Chloride
Co — Cobalt
CO_2 — Carbon dioxide
Cr_2O_3 — Chromic oxide
Cu — Copper
$CuSO_4$ — Copper sulfate
DMSO — Dimethylsulfoxide
EDTA — Ethylenediaminetetraacetic acid
FDNB — 1-fluoro-1,4-dinitro benzene
Fe — Iron
$Fe(NO_3)_3$ — Ferric nitrate
H_2SO_4 — Sulfuric acid
H_3BO_3 — Boric acid
H_3COOH — Acetic acid
H_3PO_4 — Phosphoric acid
HCl — Hydrochloric acid
$HClO_4$ — Perchloric acid
HgI_2 — Mercuric iodide
HNO_3 — Nitric acid
I — Iodine
I_2Cl_6 — Iodine trichloride
K — Potassium
K_2CO_3 — Potassium carbonate
K_2SO_4 — Potassium sulfate
KBH_4 — Potassium borohydride
KH_2PO_4 — Potassium dihydrogen orthophosphate or potassium acid phosphate
KI — Potassium iodide
KIO_3 — Potassium iodate
$KMnO_4$ — Potassium permanganate
KOH — Potassium hydroxide
L-BAPNA — N α-benzoyl L-arginine p-nitroanilide
Mg — Magnesium
Mn — Manganese
Mo — Molybdenum
N — Nitrogen
Na — Sodium
Na_2CO_3 — Sodium carbonate
Na_2HPO_4 — Disodium phosphate

$Na_2S_2O_3$ — Sodium thiosulfate
Na_2SO_3 — Sodium sulfite
Na_2SO_4 — Sodium sulfate
$NaBO_3$ — Sodium perborate
NaCl — Sodium chloride
NAD — Nicotinamide adenine dinucleotide
$NaHSO_3$ — Sodium bisulfite
NaOH — Sodium hydroxide
NH_3 — Ammonia
$(NH_4)_2C_2O_4$ — Ammonium oxalate
$(NH_4)_2MoO_4$ — Ammonium molybdate
NH_4OH — Ammonium hydroxide
NH_4SCN — Ammonium thiocyanate
NH_4SO_4 — Ammonium sulfate
NH_4VO_3 — Ammonium metavanadate
O — Oxygen
OMIU — O-methylisourea
P — Phosphorous
Pb — Lead
S — Sulfur
Se — Selenium
SLS — Sodium lauryl sulfate
TBA — Thiobarbituric acid
TCA — Trichloroacetic acid
TEP — 1,1,3,3-tetraethoxipropyl
Ti — Titanium
Tris — (Hydroxyl-methyl)-amino-methane
Zn — Zinc
$ZnC_4H_6O_4$ — Zinc acetate

Measurements

bu — bushel
C — Celsius
cc — cubic centimeter
cm — centimeter
dL — deciliter
g — gram
g — relative centrifugal force
hL — hectoliter
kcal — kilocalorie
kg — kilogram
L — liter
lb — pound
mEq — milliequivalent
mg — milligram
mL — milliliter
mm — millimeter

mmol — millimole
mol — mole
MT — metric ton
N — normality
nm — nanometers
rpm — revolutions per minute
TIU — trypsin inhibitor units
TUI — trypsin units inhibited
U/S — unsaturated:saturated ratio
v/v — volume per volume
w/v — weight per volume
w/w — weight per weight
x g — times gravity
µL— microliter
µm — micrometer

Statistics

CV — Coefficient of variation
D — Bias
H_1 — Alternative hypothesis
Ho — Null hypothesis
LCL — Lower control limits
m — Mean
MLR — Multiple linear regression
MR — Moving range
MSE — Mean squared error
N — Population size
n — Number of samples
r — Correlation coefficient
R^2 — Regression coefficient
RPD — Relative percent difference
P — Probability
P-value — Probability value
PCA — Principle component analysis
PLS — Partial least squares
s — Standard deviation
s_2 — Variance
SE — Standard error
SEP — Standard error of prediction
UCL — Upper control limits
w — Range
$\bar{x}$ — Mean
Xi — Analytical result
Xt — True value

2. SOYBEANS, SOYBEAN PRODUCTS AND PRODUCTION PROCESSES

Soybeans originated in China, where cultivation goes back more than 4,000 years. The first soybeans were introduced in the United States as animal feed in 1765, with the first plantings in 1922 (Liu et al., 2017). In the 1940s, breeding programs released the first cultivars using original plant introductions to yield varieties adapted to North American conditions. Subsequent breeding efforts focused on yield increases and pest resistance (Carter et al., 2004; Rincker et al., 2014). In this effort, genetically modified (GM) or genetically engineered (GE) soy - first introduced in 1996 - played a major role. At present, much of the worlwide production is GM soy.

As a result of its long history of cultivation and more recent breeding efforts, a large number of soybean varieties exist producing soybeans that vary greatly in shape and color. Carter et al. (2004) estimated that approximately 45,000 unique soybean accessions were maintained in germplasm collections worldwide. However, only about 1,000 accessions have been used in applied breeding programs.

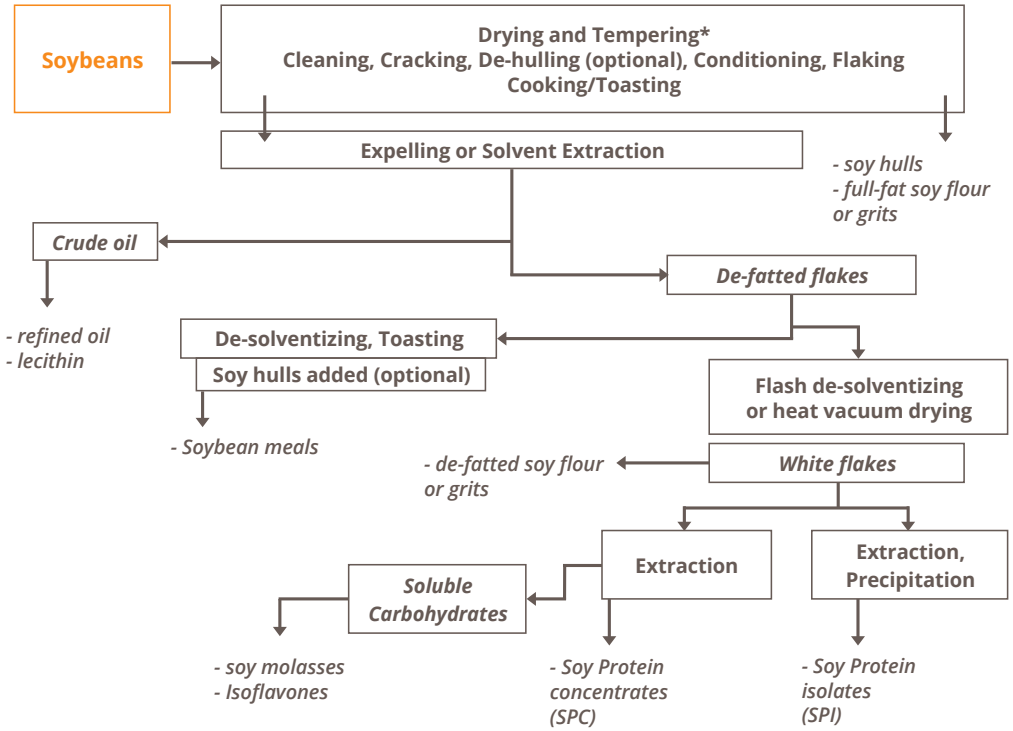
Concerning soybeans' physical aspects, shapes vary from flat to spherical and colors range from yellow to green, brown and black. Modern varieties, mainly grown for their oil content, are generally spherical in shape with yellow or green as the accepted seed coats. The color of the seedcoat, along with the soil characteristics where the soybeans are grown, will affect many of the products obtained from soybeans. Official limits have been set on the minimal size requirements for the beans (see Table 5), but generally soybeans grown for industrial purposes will weigh 18-20 g per 100 beans.

Morphologically speaking, the soybean consists of two cotyledons that comprise approximately 90% of the weight, a seed coat or hull, which is 8% of the weight, and two much smaller and lighter structures, the hypocotyl and the plumule. The cotyledons contain the proteins and lipids, or oils, that constitute the main components of nutritional interest of the soybeans and its products. They are also the main storage area for the carbohydrates and various other components of importance, most notably the enzymes like lipoxygenase and urease) and the anti-nutritional factors (ANF).

The various soybean products are obtained through separation or extraction of different components of the soybean. Different manufacturing processes are 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 soybean crushing process, 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 and animal nutrition, especially in young animal diets. The soybean meal (SBM), which on a volume basis is the most important product obtained from soybeans, has defatted flakes as an intermediary product that requires further treatment. Two main processes are used to extract oil and obtain defatted flakes: the expeller process, mechanical extraction of oil by a screw press, or solvent extraction, using non-polar solvents, commonly hexane and hexane isomers, to extract oil. At present, solvent extraction is the most widely used process. All flakes are toasted to eliminate the heat-labile ANF. Sometimes the hulls obtained in the preparatory steps are added back to the toasted flakes. This is practiced in varying degrees, resulting in SBM with variable levels of fiber and crude protein (CP). When no hulls are added, a high-protein (Hi-Pro) SBM is obtained. These are the SBM predominantly used in poultry diets. SBM can be treated further with enzymes or fermentation to produce enzymatically treated soybean meal (ESBM) or fermented soybean meal (FSBM). A combination of the enzymatical treatment and fermentation is also possible. These additional treatments result in products of higher nutritive value due to elimination of oligosaccharides, which may cause diarrhea in young animals (Liying et al., 2003). Enzymatical treatment may also reduce the concentration of glycinin and beta-conglycinin, which are antigens naturally present in soybeans (Cervantes-Pahm and Stein, 2010).

Figure 1. Schematic representation of soybean product manufacturing



* Only in the case of solvent extraction.

Where:
Boxes with regular script = Processes.
Boxes with italics = Intermediary products.
Italics outside boxes = Final soy products.

Source: Author's reference.

Flash de-solventizing or heat-vacuum-drying of the de-fatted 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 processes, 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. Three extraction methods for SPC are commonly used: 1) aqueous alcohol extraction, 2) extraction with diluted acid (pH of ±4.5), and 3) a method based on heat denaturation followed by a water washing process. The resulting SPC but also the white flakes can be further processed (grinding, texturizing, separation on the basis of molecular weight) to obtain a large array of products used in human nutrition. SPC and sometimes SPI 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.

3. DEFINITION AND APPLICATION OF SOYBEANS AND SOY PRODUCTS

The feed industry currently uses a large number of soy products, 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 meal (SBM) are relatively well-defined with thorough descriptions and specifications. This is not necessarily the case for some recent modifications or adaptations of these products like rumen-protected SBM or further processed products such as soy protein concentrates (SPC). These evolved, value-added products differ among producers because they use proprietary knowledge and specialized treatments during production. Typically, value-added products must be evaluated based on 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 an accurate database, along with improved confidence about the product. This increased level of knowledge will allow an analysis schedule of decreased intensity and drive increased inclusion rates in diets.

Commodities and value-added products can be classified as specific descriptions are developed. For efficient and correct use — as well as meaningful interpretation of analytical results — products need a precise and generally agreed upon definition. Trading, purchasing, formulation and the entire operation of feed manufacturing depend on the precise referencing of raw materials and the consistent use of correct names and descriptions. Also, quality control (QC) 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 International Feed Name and Number (IFN) system (INFIC, 1980). In this system, ingredients have been divided into eight arbitrary feed classes on the basis of composition and use (NRC, 1982). The system is widely used in U.K., U.S. and 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 IFN class number. With the exception of soybean hulls in class 1 and soybean oil, ground soybean hay, lecithin, soybean mill run and soybean mill feed in class 4, the soy products listed in Tables 1A and 1B fall in class 5, protein supplements, defined as products containing more than 20% crude protein (CP) on a dry-matter (DM) basis. The five digits following the class number link the IFN with chemical and biological data in the U.S. databank (NRC, 1982). The number appears generally on official U.S. ingredient specifications. Although the system may not be used by all feed producers or manufacturers, it provides an easy, systematic reference for quality systems and formulation purposes. The Association of American Feed Control Officials (AAFCO) continues utilizing the IFN system (AAFCO, 2018).

A brief, general description is available for many soy products. This description provides information that is not generally captured in compositional tables. It also provides a general appreciation of origin and quality, and thus the potential applications or uses in a feed. Although these definitions might differ slightly between sources, they are sufficiently similar to be used interchangeably. AAFCO regularly publishes reference specifications for soybean products. These definitions have been used as the basis for the specifications listed in Tables 1A and 1B.

Table 1A. Description and classification of most important soybean products[1]

1. Raw soybeans (AAFCO: Ground soybeans) are obtained by grinding whole soybeans without cooking or removing any of the oil. IFN 5-04-596.
2. Full-fat soybeans (FFSB), extruded, (AAFCO: 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 (CP), fat and fiber content. IFN 5-14-005.
3. FFSB, roasted, (AAFCO: Heat-processed 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 CP content. May be required to be labeled with guarantees for maximum crude fat, maximum crude fiber (CF) and maximum moisture (CFIA, 2003). IFN 5-04-597.
4. Soy protein concentrate (SPC) is prepared from high-quality, sound, cleaned, dehulled soybeans by removing most of the oil and water-soluble non-protein constituents (CFIA, 2003) and must contain not less than 65% protein on a moisture- free basis. It shall be labeled with guarantees for minimum CP, maximum crude fat, maximum CF, maximum ash and maximum moisture. IFN 5-32-183.
5. Soy protein isolate (SPI) 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 CP at 90%, maximum ash and maximum moisture. IFN 5-08-038. CFIA lists this product with IFN 5-24-811.
6. Soybean hulls consist primarily of the outer covering of the soybean. IFN 1-04-560.
7. Soybean meal (SBM), mechanical-extracted, also known as expelled soybean, is the product obtained by grinding the cake or chips that remain after removal of most of the oil from soybeans by a mechanical extraction process. It must contain no more than 7% CF. It may contain an inert, non-toxic conditioning agent, either nutritive, 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 exceeding 0.5%. The name of the conditioning agent must be shown as an added ingredient. IFN 5-04-600.
8. SBM, 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 no more than 3.3% CF. It may contain an inert non-toxic conditioning agent, either nutritive, 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 exceeding 0.5%. The name of the conditioning agent must be shown as an added ingredient. It may also be required to be labeled with guarantees for minimum CP, maximum crude fat and maximum moisture (CFIA, 2003). IFN 5-04-612.
9. SBM, 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 no more than 7% CF. It may contain an inert, non-toxic conditioning agent, either nutritive, 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 exceeding 0.5%. It shall contain less than 7% CF. The CFIA (2003) specifies that it shall be labeled with guarantees for minimum CP, maximum crude fat and maximum moisture. IFN 5-04-604.

10. Soybean oil consists of the oil from soybeans that are commonly processed for edible purposes. It consists predominantly of glyceride esters of fatty acids (FA). If any antioxidants are 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 (FFA). IFN 4-07-983.
11. Enzymatically treated soybean meal (ESBM) is the product resulting from the treatment of SBM with a single enzyme or a mixture of enzymes; primarily proteases or carbohydrases resulting in a reduction of anti-nutritional factors (ANF), a higher concentration of CP and amino acids (AA) and a higher digestibility. IFN not applicable.
12. Fermented soybean meal (FSBM) is the product resulting from an incubation of SBM with specific micro-organisms (beneficial bacteria or fungi) using predominantly a solid-state fermentation process. The resulting product has a higher CP and AA level, a lower level of low molecular weight carbohydrates including oligosaccharide, and improved digestibility of CP. IFN not applicable.

Table 1B. Description and classification of additional soybean products[1]

13. Condensed soybean soluble 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.
14. Dried soybean soluble 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.
15. Ground soybean hay is 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 (CF). IFN 1-04-559.
16. Kibbled soybean meal (SBM), or soybean expellers, is the product obtained by cooking ground solvent-extracted SBM 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 no more than 7% CF. IFN 5-09-343.
17. Protein-modified soybean is a product that has been processed to primarily modify the natural protein structure by utilizing acids, alkalis 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.
18. 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 no more than 4% CF. Some organizations also require labeling guarantees for minimum crude protein (CP) and maximum crude fat and moisture. IFN 5-12-177.
19. Soy grits are 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 no more than 4% CF. Soybean grits, mechanical-extracted: IFN 5-12-176. Soybean grits, solvent-extracted: IFN 5-04-592.
20. 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.
21. Soybean feed, solvent-extracted is the product remaining after the partial removal of protein and nitrogen (N)-free extract from dehulled, solvent-extracted soybean flakes. IFN 5-04-613.

22. 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% CF. It shall be labeled with guarantees for minimum CP, maximum crude fat, maximum CF and maximum moisture. IFN 5-04-593.
23. 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% CP and not more than 32% CF. IFN 4-04-594.
24. 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 SBM. It must contain not less than 11% CP and not more than 35% CF. IFN 4-04-595.
25. Soy flour, chemically and physically modified, is the product resulting from treating soy flour by chemical and physical means, including heat and pressure. It shall be labeled with guarantees for minimum CP, maximum crude fat, maximum CF and maximum moisture. IFN 5-19-651.

1 Source: Adapted from the Association of American Feed Control Officials (AAFCO) Official Publication 2018 and the Canadian Food Inspection Agency (CFIA), 2003.

The list in Tables 1A and 1B gives an impression of the large diversity of soy products and production methods. Although they only represent major soy products, these tables provide brief descriptions of how products are obtained and, for some, a compositional reference point. The common name and IFN are provided, allowing consistent and non-equivocal use of ingredients, which is important in quality systems. The descriptions give adequate product background for trading and classification , references in quality systems and production purposes. They are sufficiently precise to provide clear reference points for product definition and contract agreements, but general enough to cover substantial variation in composition and production processes. For proper use of an ingredient, additional analytical data should complement the description information, such as digestible amino acid (DAA) and anti-nutritional factor (ANF) values. However, for analytical purposes, descriptions provide general background information, how analysis should be carried out and expected results. When considering formulation objectives, the descriptions only serve as classification aides, and more precise compositional data will be necessary.

The products listed in Tables 1A and 1B only represent major soy products. At present, a large number of additional specialty products are marketed. The list does not adequately reflect the acceleration in development of new soy ingredients, mostly branded products. Many new further-processed products such as SPC and SPI have become available over the past 10-20 years. These products, characterized by considerably reduced ANF, can effectively be used in diets for young animals, pets and aquaculture, replacing other protein sources such as milk or animal proteins, including fishmeal. Additional new soy products have been developed for applications in human or pet food nutrition. In this area, special importance is attached to the functional properties of soybean proteins, including abilities to increase viscosity, emulsify, form gels, foam, produce films, absorb water and absorb 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 relate to the AA composition and sequence, or primary structure, as well as the spatial configuration of the protein molecule and the inter-molecular forces, or secondary and tertiary structures. Soybean protein products with unique functional properties may constitute important tools in the formulation of specialty diets used in animal nutrition. However, these techniques and products remain insufficiently explored in the production of specialty diets for domesticated livestock, with economic considerations probably the major limiting factor at present.

The most important products in terms of volume are solvent-extracted or dehulled SBM (see items 8 and 9, Table 1A), resulting from the original use of soybeans, i.e., the removal of oil. This is also the case for the mechanical-extracted SBM or expelled soybean (see item 7, Table 1A), although this type of SBM is much less common. Extruded and roasted full-fat soybeans (FFSB; see items 2 and 3, Table 1A) are increasing due to their high energy content, especially in formulations where previously animal products like meat, bone meals and fats were of interest (see Chapter 10). The list includes two fiber-rich products: ground soybean hay (see item 15, Table 1B) and soybean hulls (see item 6, Table 1A). 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 animal 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 SBM with the objective of rendering the product more digestible, either through modifying protein structures or removing ANF. The specifications do not reference these factors, leaving the decision as to how the product compares to the interpretation of nutritionists or guarantees provided by the producer. Quality analysis must provide a more precise indication of the product in terms of these characteristics to assure that the diet meets proper nutritional and animal performance objectives (see Chapter 8).

With the increased complexity of production processes aimed at removing ANF and improving protein digestibility, a clear understanding of the products and production process becomes more important. Likewise, adapted quality procedures and analyses become increasingly critical. Quality differences between producers or suppliers can be substantial, especially for more evolved products. These differences need to be verified and understood at the feed manufacturer level. Nevertheless, it remains the responsibility of the user to carry out the needed quality analysis and classify suppliers and products accordingly. Reliable manufacturer information is important, but verification remains the basis of this tool and 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 plays a determining role in the level they 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 analysis necessary, which in turn will determine more precisely the inclusion levels employed. Nutritionist experience and quality analysis interpretation play a major role in defining the final inclusion level used in particular diets. Table 2 gives only general estimates of maximum inclusion levels of each product under practical conditions of diet formulation. The levels suggested are for inclusion in complete diets and are necessarily general. They will 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, or the ability of the animal to use nutrients or deal with ANF, will need to be taken into consideration. The increasing relevance of FFSB and the simultaneous inclusion of SBM in the same feed may create the necessity of a maximum for the total of these two products, SBM + FFSB, in specific formulas. In this case, least-cost formulation and ANF levels of each soy product will determine the optimal composition.

Fine-tuning inclusion levels for each product is a company-specific decision, reflecting depth of understanding of formulation complexities and confidence in proprietary data relative to the ingredients. The suggestions listed in Table 2 must 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 maxima 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. This is the case for extruded or roasted FFSB or soybean oil in swine and ruminants. Other maxima are controlled by economic considerations. While higher inclusion 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 1B are not included in the recommendations for use in animal diets. This is the case of protein-modified SBM, soy flour and grits. Although these ingredients actually are used in animal diets when quality is insufficient 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 merits consideration.

Table 2. Application of soybean products[1]

	Product	Species[2]					Level (%)[3]
		Poultry	Swine	Ruminant	Aqua	Pet	
1.	Condensed soybean solubles			√			10
2.	Dried soybean solubles			√			15
3.	Full-fat soybeans (FFSB)	√					30
			√	√			15
					√	√	5[4]
4.	Ground soybean hay			√			20
5.	Kibbled soybean meal (SBM)	√	√				10 (Y)
					√	√	7
6.	Soy lecithin or soy phosphate	√	√	√	√	√	3
7.	Soy protein concentrate (SPC)	√	√	√			7 (Y)
					√	√	5[4]
8.	Soy protein isolate (SPI)	√	√	√			10 (Y)
					√	√	15[4]
9.	Soybean feed, solvent-Extracted	√	√	√			5 (Y)
					√	√	3
10.	Soybean flour, solvent-extracted	√	√	√			40
11.	Soybean hulls		√	√			25
26.	SBM, mechanical-extracted	√[5]	√	√			35
27.	SBM, dehulled, solvent-extracted	√[5]	√	√			35
28.	SBM, solvent-extracted	√[5]	√	√			35
15.	Soybean mill feed	√	√	√			10
16.	Soybean mill run	√	√	√			10
17.	Soybean oil	√	√	√[6]		√	8
18.	Fermented soybean meal (FSBM)	√	√	√	√	√	10
19.	Enzyme-treated soybean meal (ESBM)	√	√	√	√	√	10

Source: Authors' recommendation.

[1] Suggested upper-use levels in diets of different domestic species. This will vary with animal age, as well as product quality, composition and analysis. The table 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, swine, ruminants, aqua (salmonids) and pets (dogs).

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

[4] Higher levels may be used in salmon and trout grower and finisher diets.

[5] Turkey poults starters may contain 50% SBM.

[6] Maximum inclusion of soy oil in ruminant diets should not exceed 2%.

4. CHEMICAL AND NUTRITIONAL COMPOSITION OF SOYBEAN PRODUCTS

The compositional data provided in Tables 3 and 4, with additional details in Annexes 1 and 2, describe the nutritional characteristics of soybean products. Proper use of these descriptors requires a more in-depth understanding of the chemical, analytical and nutritional aspects of the products. The compositional data also provide an indication of the specific processes used to obtain the products. This is especially true for the data in Table 4. Along with the general descriptions provided above (see Chapter 3), these data provide a detailed picture of the various properties and potential applications for each product. More commercialized soybean products exist than are listed in the tables. The tables only provide values for standard products. A large range of soy products are produced by different companies for many specific applications. Soy protein concentrates (SPC) and heat- or formaldehyde-treated products for ruminant diets are excellent examples. Production processes and equipment differ among producers, adding more variation. Although the nutritional concentration as analyzed or declared may not differ from an ingredient listed, the nutritional value may vary greatly due to changes in digestibility or degradability. Since the tables only report composition that can be directly analyzed, quality parameters not analyzed are not included in the tables. As an example, standard analysis for lysine provides its total quantity in an ingredient. But heat damage may occur in some ingredients, which results in some lysine being present in a form that precludes animal utilization (Moughan et al., 1996). However, the standard amino acid (AA) analysis does not account for the degree to which lysine may have been heat damaged.

The nutrient concentration of the different soy products in Tables 3 and 4 are compiled from a wide range of official sources and public databases, including the National Research Council (NRC), National Institute of Agricultural Research (INRA), French Association for Animal Production (AFZ), Dutch Central Animal Feed Agency (CVB), Spanish Foundation for the development of Animal Nutrition (FEDNA), Evonik Industries and others. Besides completing the descriptive information provided in Tables 1A and 1B, the composition tables provide reference values that can be used to evaluate laboratory-obtained analytical data to further classify a specific ingredient. Since the data are obtained from a wide range of publications, users may want to refer to the original publications if the sample corresponds more closely to one of the regional sources. This is especially true of soybean meal (SBM) or soy by-products where crushing and further handling of the ingredient largely determines 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 of origins. They cannot be used as standard values, but only as reference points around which analysis of individual samples should be situated if they are identified by the specific ingredient name. Most individual samples will be within an acceptable statistical range of these means. This level of precision is adequate for classification, storage and trading agreements, which are generally based on a small set of analyses — proximate analysis or just moisture, protein and fat. More detailed analyses concerning the more difficult-to-determine nutrients and anti-nutritional factors (ANF) may show larger variations from the mean and possible inconsistencies with some values above, and others below, the table values. This is often the case for AA or micro minerals. As such, they may point to consistent differences in the production process of a given supplier or reflect problems in the analytical procedure. The experience and know-how of a lab technician in interpreting the result is of great value. Cross-checking values known to be affected by a production process or laboratory procedure may explain a discrepancy and confirm the true value and classification for the ingredient.

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

The compositional data in Table 4 includes nutrients and ANF that can be directly analyzed in large, well-equipped laboratories. Routine analyses, as carried out in standard QC procedures or smaller laboratories, mainly concern the proximate analysis, the Van Soest fiber components except lignin, and the minerals calcium (Ca) and phosphorus (P). These analyses, especially the proximate, are most often used to derive other nutrient values, such as AA 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 the information available is very limited. In fact, the digestibility data used in formulation systems can differ considerably among users and are generally considered proprietary information. In Annexes 1 and 2, specific energy values have been included because of their importance as descriptive parameters for individual soy products and in classifying and referencing ingredients.

Protein quality analyses like reactive lysine, urease index (UI), potassium hydroxide protein solubility (KOHPS), or protein dispersibility index (PDI) are also not included. These largely reflect a heat treatment effect and may not be applicable indiscriminately among soy protein products. These analyses do apply to SBM, and they are important in evaluating SBM quality, especially in terms of digestible amino acids (DAA) and ANF. Methods and optimal values for these tests are detailed further in Chapter 8. In many respects they refer to the residual values for the heat-labile ANF listed in Table 5, such as trypsin inhibitors (TI), lectin and goitrogens (Liener, 2000). There is no relationship between heat-stable ANF and protein quality indexes. For many diets, especially for young animals, aquatic species and pets, the application and use of soy products depends more on the entire range of residual ANF than on nutrient concentrations. In such diets, the more elaborated soy products such as enzymatically treated soybean meal (ESBM), fermented soybean meal (FSBM), SPC or soy protein isolate (SPI) are used more frequently. 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 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 generally provide reliable results and may be able 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 standardize an analysis for an ANF in a laboratory, generally TI, the adherence to a ring test or systematic comparisons of results with a well-established laboratory is necessary.

Table 3. Key nutrient composition of some soy protein ingredients used in animal feeds[1,2]

	Unit	Full-fat soybeans (FFSB)	Soybean meal (SBM); mechanical extracted	SBM; solvent extracted 44	SBM; solvent extracted 48	SBM; solvent extracted 50	Soybean hulls	SBM fermented	Soy protein concentrate (SPC)	Soy protein isolate (SPI)
Dry matter (DM)	%	89.4	89.8	88.1	87.6	88.2	89.8	90.1	91.8	93.4
Crude protein (CP)	%	37.1	43.9	44.0	46.4	48.8	12.0	52.1	68.6	85.9
Crude fiber (CF)	%	5.1	5.5	6.3	5.4	3.4	34.1	3.6	1.7	1.3
Ether extract (EE)	%	18.4	5.7	1.8	2.1	1.3	2.2	2.7	2.0	0.6
Ash	%	4.9	5.7	6.3	6.0	5.8	4.5	6.6	5.2	3.4
Neutral detergent fiber (NDF)	%	13.0	21.4	13.0	11.8	10.0	56.9	9.0	13.5	-
Acid detergent fiber (ADF)	%	7.2	10.2	8.8	7.0	5.0	42.1	5.6	5.4	-
Acid detergent lignin (ADL)[3]	%	4.3	1.2	0.7	0.9	0.4	2.1	0.3	0.4	-
Starch[4]	%	<0.5	<1.0	<1.0	1.1	0.8	0.7	<0.5	0.0	0.0
Non-starch polysaccharides (NSP)[5]	%	24.3	32.2	34.2	31.1	30.9	68.5	27.9	15.2	3.0
Gross energy[6]	kcal/kg	5013	4420	4165	4130	4120	3890	4700	4665	5370
Lysine	%	2.26	2.61	2.73	2.90	3.00	0.73	3.02	4.42	5.31
Threonine	%	1.44	1.66	1.73	1.82	1.90	0.43	2.01	2.73	3.23
Methionine	%	0.52	0.60	0.62	0.64	0.67	0.14	0.65	0.96	1.09
Cystine	%	0.53	0.64	0.66	0.70	0.73	0.19	1.03	0.95	1.02
Arginine	%	2.66	3.05	3.23	3.46	3.56	0.62	3.52	5.15	6.42
Isoleucine	%	1.68	1.95	2.01	2.15	2.21	0.44	2.24	3.17	4.12
Valine	%	1.75	2.07	2.11	2.26	2.30	0.52	2.48	3.29	4.21
Glycine	%	1.58	1.81	1.89	2.01	2.11	0.91	2.15	2.80	3.59
Serine	%	1.86	2.08	2.24	2.40	2.50	0.65	2.35	3.45	4.37
Tryptophane	%	0.49	0.58	0.60	0.63	0.65	0.16	0.66	0.84	1.14
Calcium (Ca)	g/kg	2.62	2.96	3.12	3.07	2.68	4.96	2.90	2.37	1.50
Phosphorus (P)	g/kg	5.70	6.64	6.37	6.37	6.36	1.59	5.57	7.63	6.50
Magnesium (Mg)	g/kg	2.80	2.84	2.72	3.03	2.88	2.23	1.00	1.85	0.80
Potassium (K)	g/kg	15.93	20.28	19.85	22.00	20.84	12.15	16.96	12.35	2.75
Sodium (Na)	g/kg	0.29	0.33	0.18	0.18	0.30	0.10	0.79	0.55	2.85
Chloride (Cl)	g/kg	0.3	0.4	0.30	0.30	0.30	-	-	-	-
Linoleic acid — C18:2	%	9.7	2.9	0.6	0.8	0.6	1.2	0.5	-	-

[1]As-fed basis. FFSB as obtained by a range of different processes.
For more detailed compositional data on soybean products, see Annexes 1 and 2.
SBM, mechanical-extracted also called soybean expeller.

[2]Sources: Compilation of National Research Council (NRC), 2001, 2012, 2016; National Institute of Agricultural Research – French Association for Animal Production (INRA-AFZ) 2004; INRA 2018; Dutch Central Animal Feed Agency (CVB) 2018; Feedipedia; Spanish Foundation for the Development of Animal Nutrition (FEDNA) 2017; and Evonik Industries, 2010, 2016.

[3] ADL — Klason Lignin.

[4]As determined by the enzymatic (amyloglucosidase) method.

[5]Estimated; basis: 100 – (% humidity + % ash + % protein + % lipids + % lignin + % starch).

[6]Based on Equation INRA-AFZ Tables; Sauvant et al., 2004.

Concentrations of ANF in soy products decrease as processing conditions (extraction, heat treatment and fermentation or enzymatic treatment) increase. Consequently, the protein content of the final product increases. The increased concentration of crude protein and higher digestibility 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 i.e., diets for young animals, which are most sensitive to the ANF. However, they remain uneconomical in diets for older 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.

Table 4. Analytical characteristics of common types of soy protein products[1,2]

Item	Units	Raw soybean seeds	Full-fat soybeans (FFSB)	Soybean meal (SBM)	Fermented soyben meal (FSBM)	Enzyme - treated soy protein concentrate (SPC)	Alcohol-extracted SPC	Soy protein isolate (SPI)
Dry matter (DM)	%	88 - 90	87- 90	88 - 90	89 - 91	92 - 94	92 - 94	92 - 94
Crude protein (CP)	%	33 - 37	33 -37	42 - 50	49 - 54	55 - 60	63 - 67	> 85
Fat	%	17 - 20	17 - 20	0.9 - 3.5	1.0 - 2.5	2.5	.5 - 3.0	.1 - 1.5
Ash	%	4.5 - 5.5	4.5 - 5.5	4.5 - 6.5	4.5 - 6.5	6.2 - 6.8	4.8 - 6.0	2 - 3.5
Oligosacharides	%	14	14	15	< 3.0	< 1.0	< 3.5	< 0.4
Stachyose	%	4 - 4.5	4 - 4.5	4.5 - 5	0.04 - 0.08	< 0.3	1 - 3	< 0.2
Raffinose	%	0.8 - 1	0.8 - 1	1 - 1.5	0.01 - 0.04	< 0.2	< 0.2	< 0.1
Verbascose	%	--	--	0.3 - 0.4	--	--	--	--
Trypsin inhibitors (TI)[3]	mg/g	25 - 50	1.0 - 14.0	1.6 - 5.0	< 4.5	1 - 2	2 - 3	< 1
Glycinin	mg/g	150 - 200	--	20 - 70	<3.0	<0.01	< 0.1	< 0.01
β-conglycinin	mg/g	50 - 100	--	3 - 40	<1.0	<0.01	<0.01	<0.005
Lectins[3]	ppm	2100 - 3500	< 1 - 20	20 - 600	< 50	< 1.0	< 1.0	< 1.0
Saponins	%	0.5	0.5	0.6	0.2	0	0	0
Phytic acid-bound Phosphorus (P)	%	0.35	0.35	0.40 - 0.45	< 0.3	0.6	0.6	--

[1] Sources: Adapted from Hansen (2003), Peisker (2001), Fasina et al. (2004), Maenz et al. (1999), Dutch Central Animal Feed Agency (CVB, 2018), AV Vista (2018) and personal data.

[2] All values as-fed basis.

[3] For TI and lectins in FFSB and SBM, these are typical commercial ranges, not recommended ranges. For recommended ranges, see Table 7.

5. OFFICIAL STANDARDS OF SOME SOYBEAN PRODUCTS

Many publications and compositional tables describe and specify the nutritional quality of soybeans and soy products. The data provided in these publications reflect different sources and analyses selected along variable criteria. Thus, they cannot be considered standard values, especially for trading purposes. For trading and contractual objectives, such data are too detailed and unpractical. Nor do they provide the required borderline minimum or maximum values for a limited number of readily identifiable parameters.

A limited number of official standards have been published, starting with the basic material: whole, untreated soybeans or seeds (IFN 5-04-610). As for all other grains and seeds, the U. S. Department of Agriculture (USDA) publishes official standards for soybean grains as defined under the U.S. Grain Standards Act. These standards do not change much over time and under this specific act, soybeans are defined as, "Grain that consists of 50% or more of whole or broken soybeans that will not pass through an 8/64-inch, round- hole sieve (3,183 microns) and do not contain more than 10.0% of other grains for which standards have been established under the U.S. Standards, Subpart J-United States Standards for Soybeans" (USDA, 2007). Associated with this statement, whole soybeans are defined as soybeans with three-fourths or more of the soybean present (USDA, 2007).

For trading and inspection purposes — considering specific applications or export requirements — additional specifications are provided by dividing batches of soybeans into classes and grades. Only two classes of soybeans have been defined: yellow soybeans and mixed soybeans. Yellow soybeans are defined as soybeans that have a yellow or green seed coat, which upon section, are yellow or have a yellow tinge. Batches of soybeans in this class may contain up to 10% soybeans of other colors. All other soybeans fall in the class of mixed soybeans.

Within each class, five grades are specified: U.S. #1-4 and a fifth grade referred to as sample grade. The grades and grade requirements for the major export countries, the United States, 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. Soybeans are exported from the United States at any predefined specification or grade. The USDA (2007) description of grades is provided in Table 5. The USDA publication further stipulates that determination of class, heat-damaged kernels, damaged kernels, splits and soybeans of other colors must be made on the basis of the grain when free from foreign material. All other determinations are to be made based on the whole grain.

Table 5. U.S. grades and grade requirements for soybeans

Soybean grade and grade requirements					
Grading factors	Grades: U.S. numbers				Sample grade*
	1	2	3	4	
Minimum test weight; lbs/bu	56	54	52	49	See below for specifications
Minimum test weigh; g/L	721	695	669	630	
Maximum % limits of					
Damaged kernels:					
Heat (part of total)	0.2	0.5	1.0	3.0	
Total	2.0	3.0	5.0	8.0	
Foreign material	1.0	2.0	3.0	5.0	
Splits	10.0	20.0	30.0	40.0	
Soybeans of other color[1]	1.0	2.0	5.0	10.0	
Maximum count limits of					Undefined
Other materials:					
Animal filth	9	9	9	9	
Castor beans	1	1	1	1	
Crotalaria seeds	2	2	2	2	
Glass	0	0	0	0	
Stones[2]	3	3	3	3	
Unknown foreign substance	3	3	3	3	
Total[3]	10	10	10	10	

Source: U.S. Department of Agriculture (USDA) 2007.
*U.S. Sample Grade is soybeans that fall into one of these categories.

(a) Do not meet the requirements for grades U.S. No.1, 2, 3, or 4.

(b) Contains 4 or more stones which have an aggregate weight in excess of 0.1% of the sample weight, 1 or more pieces of glass, 3 or more crotalaria seeds (Crotalaria spp.), 2 or more castor beans (Ricinus communis L.), 4 or more particles of an unknown foreign substance(s) or a commonly recognized harmful or toxic substance(s), 10 or more rodent pellets, bird droppings or an equivalent quantity of other animal filth in 1,000 grams of soybeans.

(c) Contain 11 or more animal filth, castor beans, crotalaria seeds, glass, stones or unknown foreign substance(s) in any combination.

(d) Have a musty, sour or commercially objectionable foreign odor (except garlic odor).

(e) Are heating or otherwise of distinctly low quality.

[1]Disregard for mixed soybeans.

[2]In addition to the maximum count limit, stones must exceed 0.1% of the sample weight.

[3]Includes any combination of animal filth. castor beans, crotalaria seeds, glass, stones and unknown foreign substances.

The weight of stones is not applicable for total other material.

The grading factors in Table 5 have been further specified. As such, it is understood that "damaged kernels" are considered soybeans and pieces of soybeans that are badly ground-, weather-, disease-, insect-, germ-, mold-, frost-, heat- or sprout-damaged or damaged in any other way. Heat-damaged kernels are further specified as soybeans and pieces of soybeans that are materially discolored and damaged by heat.

"Foreign material" is defined as all matter that passes through an 8/64 inch (0.125 inch; 3.175 mm) round-hole sieve and all matter other than soybeans remaining in the sieved sample after sieving, according to procedures prescribed in the Federal Grain Inspection Service (FGIS) instructions.

"Splits" are defined as soybeans or parts of soybeans of which more than one-fourth of the bean is removed and that are not otherwise damaged.

"Soybeans of other color" are defined as soybeans that have a green, black, brown or bicolored seed coat. The hilum of a soybean is not considered a part of the seed coat for this determination. Also included in the reference to color are purple-mottled or stained soybeans that are discolored due to the growth of a fungus or by dirt, provided this is not a toxic substance (USDA, 2007).

Additional specifications beyond those defined above are possible. These are optional and can be requested or provided to further specify quality or value. These specifications include odor, insect infestation or simple compositional characteristics such as moisture or other components. Modern technology such as near-infrared reflectance spectroscopy (NIRS) analyses (see Chapter 9) can greatly facilitate this more detailed information.

Besides whole soybeans, only three soybean products, two soybean meals (SBM) and soybean oil, have standard values. Used as official reference standards, they have been developed by the National Oil Processors Association (NOPA, 2017) and are also published by the U.S. Soybean Export Council (USSEC) in the U.S. Soy: International Buyers' Guide (2006). These standards are widely accepted and provide minimums or maximums on only a few, easily identifiable, key parameters. In the case of SBM, the main purpose of these standards is the classification of SBM products into two main categories: solvent-extracted SBM and dehulled, high-protein (Hi-Pro) SBM. NOPA (2017) defines SBM as ground soybean cake, ground soybean chips or ground soybean flakes, sold according to protein content. Table 6 provides the NOPA specifications for both meals. In the table footnote, the methods of analyses for all required constituents are provided. NOPA (2017) specifies further that in terms of analyses, moisture content shall be performed in duplicate on the sample after grinding. It is this moisture content that should be used to convert the average constituent values to the average moisture content of the unground sample, and to a 12% moisture basis.

Organizations, companies or individuals participating in a transaction involving SBM are free to adopt, modify or disregard the NOPA rules. They principally serve the trading and marketing of U.S. soybean products within the United States. But due to their wide acceptance, their impact goes well beyond U.S. SBM and oils, as they are generally applied to compare and benchmark soybean products from other origins. Solvent-extracted SBM can be the result of blending back soybean hulls in the dehulled meal. The blending of different types of SBM or soybean components at the point of shipping is allowed under NOPA regulations and standards for minimum blending procedures are provided. This can be the source of a significant variation in quality and chemical composition. However, blending of soybeans is not permitted. For SBM, only soy hulls, soybean mill run and soybean mill feed are permitted to be blended 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 meal (SBM) by %

Item[1]	Minimun (min.)/ maximum (max.)	Solvent-extracted soybean meal (SBM)	Dehulled SBM
Moisture	Max.	12	12
Protein	Min.	44	47.5-49.0
Fat	Min.	0.5	0.5
Crude Fiber	Max.	7	3.3-3.5
Anti-caking agent[2]	Max.	0.5	0.5

[1]Testing methods to be used follow.
Moisture = American Oil Chemists' Society (AOCS) Method Ba 2a-38, 2011a.
Protein = AOCS Method Ba 4f-00, 2011d.
crude fiber (CF) = AOCS Method Ba 6-84, 2017.
Fat/oil = AOCS Method Ba 3-38, 2017.
[2]A non-nutritive inert, non-toxic conditioning agent to reduce caking and improve flowability.

Source: Adapted from National Oilseed Processors Association (NOPA), 2017.

For SBM, the NOPA (2017) standards clearly aim at providing a minimum number of primary quality characteristics only as a basis for contract specifications (see Table 6). The only characteristics defined are moisture, crude protein (CP), fat and crude fiber (CF) 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. SMB purchased under NOPA contract specifications will, therefore, still need additional analysis. To provide greater quality assurances and meet the nutritional requirements of the feed compounder or nutritionist, additional recommendations have been added (see Table 7).

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

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 KOH protein solubility (KOHPS), especially where it concerns inadequate heat treatments (Batal et al., 2000). The KOHPS index is considered better to estimate overheating of SBM. Nevertheless, consistent application of the recommendations in Table 7 would go a long way in meeting product quality and nutritional requirements.

Table 7. Recommended additional specifications for soybean meal (SBM)

Total lysine	> 2.85%, basis 88% dry matter (DM)
Digestible lysine	Equal or > 88% of total lysine
Ash	< 7.5%
Acid-insoluble ash (silica)	< 1%
Protein solubility in 0.2% potassium hydroxide (KOH)	78 – 85%
Urease activity	0.000 – 0.075 pH unit rise
Trypsin inhibitors (TI)	1.60 – 2.50 mg/g (~3 – 4 TIU/mg)
Bulk density	57 – 64 g/100 cc
Screen analysis (mesh)	95% through #10, 45% through #20, 6% through #80
Texture	Uniform, free flowing, no lumps, cakes or dust
Color	Light tan to light brown
Odor	Fresh – not musty, sour, ammonia, burned
Contaminants	No urea, ammonia, pesticides, grains, seeds, molds or raw soybeans

Source: Authors' value.

An additional degree of detail is necessary for the regular and detailed formulation changes required to meet performance guarantees of animal diets and constant cost- reduction objectives. The generation of this information is, at present, considered to be the responsibility of the in-house quality control (QC) and analytical services organization of the feed compounder. Feed manufacturers often regarded this as part of the proprietary know-how. However, these recommendations do offer crushers an opportunity to provide a more consistent and better-quality product, and a means to add value to a commodity. As identity preservation (IP) and traceability tools improve, guarantees on the nutritional characteristics of SBM will increase.

The NOPA standards for soybean oil have the same objectives as those for SBM, 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, which is officially referred to as crude de-gummed soy oil. No standards for oil used in animal feed are 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 than those used for oils destined for human consumption (see Table 8), but information for additional parameters such as iodine value (IV) and peroxide numbers are often required. On the other hand, information on phosphorus (P) levels and flash point are not considered.

This difference in standards between oils for human consumption and oils for animal feeds allows for soy oils that are rejected for human consumption to be used in animal feed, provided they meet 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, including full-fat soybeans (FFSB) that are marketed in various forms and conditions. This leaves it up to the user to set internal QC measures. Those may include most of the criteria considered for the three basic products, but they will need to go beyond to include a measure of anti-quality components or anti-nutritional factors (ANF), expanded amino acid (AA) profiles, in vitro-digestibility and measures of microbial contamination. No specific requirements have been published on the degree of microbial presence in soybeans or SBM. The end user will have to apply industry norms as established by local governments or organizations.

Table 8. Standards for edible crude de-gummed soybean oil and vegetable oils in animal feed

Analytical parameter	Unit	NOPA[1] maximum	Feed[2] maximum
Unsaponifiable matter	%	1.5	1.5
Free fatty acids (FFAs) likeas oleic acid	%	0.75	1
Moisture, volatile matter, impurities	%	0.3	1
Flash point	°F	250	-
Phosphorous (P)	%	0.02	-
Iodine value (IV)	g/100g EE*	-	124-136[3]
Peroxide value	mEq/kg	-	2

[1] National Oilseed Processors Association (NOPA) 2017.
[2] Feed refers to common values for vegetable oil.
[3] Range for soybean oil, Noller (1965).
* Ether extract (EE)

6. SAMPLING SOY PRODUCTS

Sampling is a necessary and critical step in the inspection and determination of the quality of any soy product. Consequently, it has a decisive effect on the evaluation of the soy product and the ensuing feed product. The quality of any analysis carried out on feed or feed ingredients stands or falls with the sampling tools and procedures. It seems evident, but routine operating conditions do not necessarily recognize that 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 the subject to be evaluated, is the collection of a truly representative sample. A sample or set of samples should represent to the greatest possible degree the composition and characteristics of the material to be analyzed or studied. This generally leads to a compromise between cost of sampling and analysis and the acceptable degree of precision or confidence. Statistical tools have been developed to assess the minimum number of samples needed to achieve a given level of confidence regarding ingredient composition (see Section 11.3.3). As the number of samples collected and analyzed increases and nutrient and ingredient variation 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 ingredient, its form or particle size, the consignment conditioning and size, loading or unloading methods and storage conditions. The soy products used in animal feeds cover the entire range of physical forms, from seeds to flakes and powder. The sampling methods need to be adapted to the specific ingredient traded or entering 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 considered standard throughout the world, and a detailed description of sampling techniques for grains and seeds have been provided by Herrman (2001). They apply to most soy products in bag or bulk, but they focus primarily on sampling techniques used for feed production (see Annexes 3-6). The U.S. Department of Agriculture (USDA) and National Oilseed Processors Association (NOPA) have also published basic rules (USDA, 2014; NOPA, 2017) for the sampling of soybeans and soybean meal (SBM) from vessels, containers and trucks using manual or automatic sampling devices. Those procedures focus primarily on sampling lots for trading purposes, and they emphasize the official and legal aspects associated with sampling. Since these sampling procedures often concern official inspection purposes, the type and status of the sampling equipment are subject to strict rules and regulations. Policies and procedures regarding equipment, installation and testing of manual and mechanical sampling systems have been published (USDA, 2017). The same or slightly modified procedures and techniques can be applied to container or truck loads as they are delivered to feed mills or large farms. The procedures are practical and can be implemented under almost any condition or operating procedure. A degree of local adaptation may be necessary and even advisable to assure the collection of a truly representative sample. The experience and training of samplers and persons in charge of the quality program will largely determine the efficacy of the sampling program and the precise way to sample.

Prior to sampling soybean products, a sampling scheme or frequency must be established. When a soy product is used as a feed ingredient, the sampling scheme and frequency will depend to a large extent on the supplier and the information received prior to delivery. Additional considerations are assumed or recorded, including variability, laboratory capacity and availability, analytical cost, consignment size and its percentage use as an ingredient in specific feed. 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 or trucks with soybean products are summarized in Annexes 3-6, taken from

Herrman (2001) and USDA (2014). USDA (2014) also provides primarily information for seeds shipped on vessels, containers and trucks, and information on the minimum number of containers and samples that should be collected (see Annex 3.F).

In all sampling schemes, a first, rapid evaluation of the material and of the required sample number and size 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 (see Annex 3A, C-G). Separate sampling schemes have been developed to allow sampling of sound versus damaged areas (see Annex 3B).

The tools used to collect samples depend on the ingredient material and transport form. Whereas automatic sampling of trucks or containers is increasingly implemented, hand-sampling remains an important means of obtaining samples of soy products. In the case of hand-sampling, slotted grain probes can be used to correctly sample soybeans and SBM from a bag or a container (see Annex 4, Figure 1A). Tapered bag triers (see Annex 4, Figure 1B) are used to sample powder and granular material, such as soy protein concentrate (SPC) and soy protein isolate (SPI), from bags. For the sampling of soybeans or SBM from a conveyer belt or a discharging truck, a Pelican Probe sampler can be used (see Annex 4, Figure 1D). The sampler is pulled through a stream of falling grain or meal, collecting a sample into a leather bag. Increasingly, sampling is mechanical and automatized using a pre-programmed auger system adjusted for the size and conditions of the carrier (see Annex 4, Figure 1E). The sampling grid for auger samplers is the same as for manual sampling.

NOPA (2017) has established procedures for mechanical sampling of SBM for trading purposes at vessel loading facilities. This includes brief guidelines for sample handling and certification of the equipment (see Annex 5B).

The sampling of oil follows principles of sampling of other liquid feed ingredients. A sampler (see Annex 4, Figure 1C) is used to collect liquids such as soy oil from bulk containers. This sampler consists of a closed cylinder, 30-40 cm long by 4.5-7.5 cm in diameter, that is lowered at predefined places in an oil tanker and filled with a 100-1,000 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 (Herrman, 2001). A minimum 500 mL sample of liquid must be obtained for storage and sub-sampling.

The sample size and number from a larger volume depend on the load's homogeneity — or lack thereof — and previous experience is important. A larger sample should be collected than needed to be retained for further analysis and storage for the minimum legally required period. A minimum sample size of 2 kg is recommended. To reduce the sample to the minimum required size, the sample is passed through a gated riffle sample splitter with 25 mm riffles or a Boerner divider (see Annex 6, Figures 2A and 2B, respectively). This is done repeatedly until the sample is homogeneous. A minimum 500 g sub-sample 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 each. Both are stored in airtight containers. One container is dispatched to the laboratory for analysis; the second container is stored in a dry storage area, minimizing any type of chemical changes due to deterioration as the sample may be used for subsequent analysis in the case of claims.

7. PHYSICAL EVALUATION AND EQUIPMENT

Following sampling, three types of evaluations are carried out on soybean products: physical, chemical and microbiological.

Physical examination of the material aims at establishing the general soundness of the product, its origin and a rapid, general approximation of nutritive quality. The merchandise must pass a series of tests to be accepted by the buyer. The first step in the physical evaluation of a soy product is a visual appraisal of the entire lot delivered at a plant or shipping station, followed by an evaluation of the collected sample. In both cases, the product is evaluated to confirm that it corresponds correctly to the description provided in the contract or agreement. Subsequently, the product is evaluated for color, smell, homogeneity and presence of foreign material, including insects. The physical parameters provided in Chapter 5 in the official standards for soybeans and soybean meal (SBM) can be used as part of the physical evaluation.

Chemical analyses provide information about the nutritive value of the product. These analyses may differ according to future use, including animal species. Results of these analyses aim at providing the basis for knowledge about the detailed nutritional profile, and this information is used in the formulation matrix. As such, they establish the maximum and minimum use level in a feed, as well as a precise price:quality relationship for the ingredient and the individual nutrients it supplies.

Microbiological evaluation reveals specific microbial, fungal or yeast contamination. The evaluation mainly refers to levels of Escherichia coli, salmonella, most notably S. enteritidis and S. typhimurium, 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 or production technology. All physical, chemical and biological measures, when outside the contractual or legal limits, may lead to claims or changes in the contractual agreement.

Soybean products are evaluated for several 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 — should unloading proceed. This inspection approaches the more detailed physical evaluation of the sample and requires a certain level of expertise of the quality control (QC) 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 SBM are contamination or foreign materials, bulk density, texture, particle size or screen analysis, color and odor.

Color and odor are rapidly evaluated on the entire load by a trained person. They are the first evaluation, but of crucial importance because they are also the first evaluation of product nutritional quality. Deviations from the predefined, standard colors are indicative of excessive contamination with foreign material or excessive or inadequate heat treatment. Contamination with foreign material dilutes the nutritional content of soy products, altering bulk density, or in the worst of cases, may originate from contamination that reduces the nutritive value. Excessive heat treatment during production or drying of soy products may result in amino acids (AA), especially lysine, undergoing Maillard reactions with reducing sugars, rendering them undigestible by the animal. Deviations from the characteristic odor may confirm the visual observations, but will also provide a first idea of past storage conditions, contamination with other substances, especially liquids, and the excessive presence of molds, and thus possible mycotoxins.

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 separating by hand a sub-sample and weighing the various fractions. It is recommended at this stage to collect a sample for light microscopic analysis. Evaluation of a sub-sample under a microscope permits a more detailed analysis of the material and contaminants. In general, a wide field stereoscopic microscope with a magnification of 20-40 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 or test weight is measured by taking 1 L of material in an official container or kettle and weighing the content. 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% of the charge mass can be used, according to the American Society of Agricultural Engineers (ASAE, 1997). The official procedure states that the soybeans or SBM is placed in a hopper above the kettle. When the hopper gate is opened, the soybeans or SBM drops into the kettle until it flows over on all sides. The operator strikes off the top of the kettle with a leveling stick and weighs the kettle and contents. The weight of the soybeans or SBM in the kettle is measured in grams or pounds. This delivers the bulk weight in g/L or, when multiplied by 35.239, the bulk weight in units of lbs/bu.

Bulk density, expressed in g/L, kg/hL or lbs/bu, is a first evaluation of the attributes of the received ingredient, namely: moisture content, texture and level of damage or contamination. The range of required bulk density or test weight for soybeans increases with the grade from 630 g/L or 63 kg/hL for grade 4 to 720 g/L or 72 kg per hL for grade 1 or 49-56 lbs/bu respectively (see Table 5). For SBM, a single range of 57-64 kg/hL is recommended (see 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 United States use test weight on a regular basis.

Texture may be considered as primarily a visual observation, verifying the absence of lumps, cakes or coarse particles. A first evaluation can be carried out by hand-sieving a sample in a 0.525 Tyler or 0.530 U.S. standard equivalent, 13.5 mm sieve. For a more precise and objective estimation of particle size, especially the presence of small or dust particles, conduct an analysis with an official particle separator. 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, or 0.0059 inch, adequately covering requirements for standards advised for SBM (see Table 7).

8. CHEMICAL ANALYSES

The nutritional quality of a feed ingredient, including soybean products, depends on the content of several chemical elements and compounds that carry nutritional functions. 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 feed nutrient carriers. The quality and value of a given ingredient will largely depend on the concentration of its nutrients.

Accurate chemical analysis is critical to the formulation of a balanced diet. Nutritionists use nutritional analysis data from several laboratory methods to estimate or approximate the nutritional value of feed ingredients. Older analytical systems are still being used such, as the Weende system developed by Henneberg and Stohmann, Germany, in 1867. The system measures water or humidity, crude protein (CP), crude fat, crude fiber (CF), ash and nitrogen-free extract (NFE). This method has been useful for assessing the value of ingredients; though as with any system, it has shortcomings. The Weende analytical system is the base of our approximate definition of ingredients, hence the name “proximate analysis,” as the system is commonly referred to. Today, numerous regression equations for animal nutrition are based partially or completely on the proximate analysis of ingredients. Over time, each component of the proximate analysis has evolved into a more defined and sophisticated analysis. The shortcomings of analysis for -CF and NFE, which is not directly determined but calculated by difference, have resulted in development of improved methods to determine nutrients, especially in the fibrous fraction of soybean products.

Soybean meal (SBM) is one of the most consistent and least variable protein sources for animal nutrition. However, some variation does occur in both the nutrient concentration by chemical determination and quality, in terms of digestibility or bioavailability, among different SBM samples and sources. These variations can be attributed to different soybean varieties, growing conditions, storage conditions and length, and processing methods. Differences in processing are considered to have a major effect on quality and this may lead to important differences among crushers and crushing plants. In addition to processing, one source of quality variation that has been studied is the geographic origin of soybeans and soybean products (Coca-Sinova et al., 2008; Wang et al., 2011).

These variations result in differences in nutrient content and the digestibility or utilization of nutrients by the animal. In the case of soybeans, this is especially important for the level of amino acids (AA), energy and phosphorus (P). Energy is not directly determined, but estimated based on nutrient composition and digestibility. Variation in energy content combines variability in nutrient content and utilization, as well as differences in laboratory analyses of the component nutrients.

The level of digestible amino acids (DAA), digestible energy (DE), metabolizable energy (ME) and net energy (NE) are the dominant nutritive components determining the quality and value of any ingredient. Under most formulation conditions, the largest cost of an ingredient is associated with its energy value followed by DAA values. For many ingredients, P or more precisely, digestible P, the P that is absorbed from the diet by the animal, follows DAA and ME. This is also the case for protein ingredients, including full-fat soybeans (FFSB) or SBM. However, for these types of ingredients, the value of DAA is increased relative to energy or P. Consequently, in any comparative evaluation of soy products, an accurate determination of DAA, ME and P digestibility is important.

Direct and routine analyses of AA and energy are difficult. Therefore, nutritionists have developed equations to estimate parameters like DAA, DE, ME and NE on the basis of components that are more readily and accurately determined, i.e., proximate analyses. The quality and accuracy of these more routine analyses

associated with the robustness of the equations developed to predict DAA and DE will determine the accuracy of the estimated value for each ingredient. In the case of P in soy products, regular analyses for total P are generally carried out and table values are used to determine digestible P. Digestibility of P is corrected if microbial phytase is used to reduce the level of phytate-bound P and increase P digestibility.

For nutritional and diet-formulation purposes, the true value of FFSB, SBM or other soybean product can only be determined by an accurate estimate of its constituent nutrients as they relate to the cost or market value of the soy product. This price to quality relationship determines the real value of soybean products to the feed formulator. A comparison among ingredients based on their respective price to quality relationships defines their true value in feed formulation, to the feed compounder and to the final user. Commercial feed formulation requires an assessment of the key nutrients of different ingredients. Only on this basis can a meaningful comparison be made among ingredients and — by corollary — the different types of soy or protein ingredients. This also applies in selecting similar ingredients of different origins, such as SBM from the U.S. versus SBM from South America or Asia.

Because soybean products, especially SBM and FFSB, are often the primary sources of DAA in poultry and swine diets, it is crucial to monitor accurately the quality and value of soybean products. Small changes in quality will translate into important changes in animal performance and value to the producer.

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 concentration of all other 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. Mold growth will result in a significant increase in the internal temperature of soybeans, full-fat soybeans and SBM in storage over time. This increase of the internal temperature may result in auto-combustion at the center of the pile with subsequent irreversible damage to the quality of the product.

SBM with moisture content above 12.5% presents a high risk of molding and should be accepted with caution and corresponding quality penalties. Trading rules establish a maximum moisture content for SBM of 12.0% (± 0.5%), according to the National Oilseed Processors Association (NOPA, 2017). Therefore, the absolute maximum is 12.5%. However, moisture is not evenly distributed across the lot. A sample batch containing an average of 15.5% moisture may, for example, contain some particles with 10% moisture and others with 20% moisture. The particles with the highest moisture content are the ones most susceptible to mold growth. At the early stages of development, mold growth is often concentrated in specific areas of a batch of soy products, underlining the importance of applying correct sampling methods.

To determine moisture content, it is necessary to have the following equipment:

- Air forced-air drying oven.
- Porcelain crucibles or aluminum dishes.
- An analytical balance with a precision of 0.01 mg.

The official method (AOAC, 2005) to determine the moisture content of soybean products consists of the following procedure:

- Dry porcelain crucibles or aluminum dishes in oven at 100 °C for 15-30 minutes.
- Cool porcelain crucibles or aluminum dishes in desiccator, weigh and record weight.
- Place 2 g (+ 0.01 g) of ground sample in the porcelain crucible or aluminum dish and dry at 95-100°C to a constant weight. Usually about five hours is sufficient.
- Cool the containers and samples in a desiccator, weigh back and record weight.
- Calculate the moisture content as a percentage of original weight.

$$Moisture, \% = \frac{(Original\ weight - Final\ weight)}{Original\ weight}\ x\ 100$$

and

$$Dry\ matter, \% = (100 - moisture, \%)$$

This procedure applies to all soy products and feeds prepared with soy ingredients. In the case of fermented soy products or feeds, in their original (non-dried) state, freeze drying should be used.

For dry ingredients or feeds, 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 five 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 two 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 quality control (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 reaches 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 reflectance spectroscopy (NIRS) (see Chapter 9).

8.2 Ash and organic matter

Ash refers to the inorganic residue remaining after ignition and/or complete oxidation of the organic matter in an ingredient or feed sample. Consequently, when expressed on a dry matter basis, the organic matter content of a sample is the difference between the sample dry matter weight and the ash content.

The analysis for ash content is an integral part of the proximate analysis, and "ash" enters as a variable in many equations for estimation of energy content, emphasizing its importance and nutritional significance.

Ash determination requires the following equipment:

- A muffle furnace.
- Porcelain crucibles or aluminum pans.
- An analytical balance with a precision of 0.01 mg.

The procedure follows:

- The crucible or aluminum pan is dried in an oven at 100 °C for 15-30 minutes, cooled in a desiccator and weighed. The ash content of soybean products is determined by weighing 2 g (+ 0.1 g) of dried sample in the tared, dried porcelain crucible or aluminum pan and placing it in a furnace at 600 °C for 16 hours.
- Then, the oven is turned off and allowed to return to room temperature before opening.
- The crucible plus sample is placed in a desiccator 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 calculated as shown.

$$Ash,\% = \left(\frac{Final\ weight}{Original\ weight} \right) x100$$

The percentage of organic matter in an ingredient or feed sample is the difference between the dry matter and the ash content.

$$Organic\ Matter,\ \% = 100 - Ash\ (\%)$$

The ash content of SBM should not exceed 7% and consequently, the organic content should not be less than 93%.

Monitoring ash content is both a way to assess the nutritional quality of soybean products and to detect possible contamination, especially soil contamination. The procedure outlined above is also a preparatory step for analysis of specific minerals by spectrophotometric or atomic absorption (see Section 8.14.4). The additional preparation of the ash for mineral analysis involves several steps:

- Completely transfer the ash residue, obtained as outlined above, to a 250 mL beaker.
- Add 50 mL hydrochloric acid (HCl) (1part HCl + 3 parts water) and add several drops of nitric acid (HNO_3).
- Bring to a boil under a hood; boil for 15 minutes.
- Cool and filter through an ash-free filter paper into a 50 or 100 mL volumetric flask that has been rinsed with dilute acid.
- Dilute to volume with deionized water.

Certain minerals like selenium (Se), lead (Pb) and cadmium (Cd) may be volatilized by high-temperature ashing. For the analyses of these minerals, a special wet-ashing procedure is used. Wet-ashing involves digestion of a sample with nitric and perchloric acids. This procedure requires special caution because of the use of perchloric acid (HClO4), and the use of a special a perchloric acid fume hood is required.

8.3 Protein

The protein content of soybean products is estimated as total nitrogen (N) in the sample multiplied by 6.25. This assumes that protein in soybean products has 16% N; however, the actual amount of N in soybean protein is 17.5%. Nevertheless, as for most other ingredients used in feed formulation, the standard value is used. The reason for using the standard value of 6.25 is generally referred to as figuring crude protein (CP). Determining CP from N content has the drawback that all the N in soybean products is considered to be part of proteins or amino acids (AA), which is not the case as there is N in the form of ammonia, vitamins and other non-protein compounds. However, the N fraction not in AA or protein is very small, and corrections for the difference in N content in soybean products relative to other ingredients are carried out at the AA level.

In the 1800s, two methods for determining N in organic matter were developed: the Dumas method based on N combustion and the Kjeldahl method based on the reaction of sulfuric acid ($H2SO_4$) with organic matter to convert all N into ammonia (NH_3). For over a century, the Kjeldahl method was the predominant method because the procedure lends itself to routine analysis of large numbers of samples. The basic procedure for the Kjeldahl method involves three major steps, as follows:

1. Digestion: The sample is digested in sulfuric acid and copper (Cu) and titanium (Ti) catalysts are added to convert all N into ammonium sulfate (NH_4SO_4).
2. Distillation: The ammonium sulfate reacts with sodium hydroxide (NaOH) to release ammonia, which is distilled and received on a boric acid (H_3BO_3) or a sulfuric or hydrochloric acid (HCl) volumetric solution.
3. Titration: When using boric acid as the absorbing solution, ammonia is converted into ammonium borate ($BH_{12}N_3O_3$). An acid-base titration is performed using standard solutions of sulfuric acid or hydrochloric acid and a mixture of indicators to quantify the amount of ammonia. Alternatively, the endpoint can be determined potentiometrically with a pH-electrode, direct titration. When using sulfuric acid standard solution as absorbing solution, the residual sulfuric acid is titrated with NaOH and by difference the amount of ammonia is calculated, back titration.

The Kjeldahl method requires all the following equipment:

- A digestion unit that permits digestion temperatures in the range of 360 °C-380 °C for periods up to three 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 ammonium hydroxide (NH_4OH).
- 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 10 g of potassium sulfate (K_2SO_4) plus 0.30 g of copper sulfate ($CuSO_4$).
- Reagent-grade, concentrated sulfuric acid.
- Mixed indicator solution: 3,125 g methyl red and 0.2062 g methylene blue in 250 mL of 95% ethanol, stirred for 24 hours.
- Boric acid solution: 522 g USP boric acid in 18 L of deionized water. Add 50 mL of mixed indicator solution and allow stirring overnight.

- Zinc (Zn): powdered or granular, 10 mesh.
- NaOH: 50% w/v aqueous, or saturated. Standardized 0.1 N HCl solution.
- Standardized 0.1 N hydrochloric acid 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 sulfuric acid to each Kjeldahl flask.
- Start the digestion by preheating the digester block to 370 °C, and then place the Kjeldahl flaks on it for three hours.
- After removing flasks from the digester, and once they are cool, add 400 mL of de ionized 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 0.5 g of powdered Zn to flask, mix thoroughly and allow to settle.
- After digest has settled, measure 100 mL of saturated, aqueous NaOH (50% w/v) 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 de ionized 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 hydrochloric acid and record volume of acid used in titration.
- It is recommended to use at least two blanks and controls or standards on every run.
    - Blanks: Kjeldahl reagents generally contain small amounts of N that 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 N from urea and make sure the obtained result is the one expected.
- Calculate as follows.

$$Crude\ Protein,\% = \left(\frac{(ml\ of\ acid - ml\ of\ blank)\ x\ Normality\ x0.014\ x\ 6.25\ x\ 100}{Original\ weight} \right) x100$$

The Dumas 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 CP 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 in the presence of oxygen. This leads to the release of carbon dioxide (CO2), water and N. The gases are then passed over special columns, such as an aqueous solution of potassium hydroxide (KOH), that absorb the CO_2 and water. A column containing a thermal conductivity detector at the end is then used to separate the N from any residual CO_2 and water and the remaining N2 content is measured. This method has the advantage over the Kjeldahl that is faster, better suited for automation and creates little residues. The reason the Dumas method was not a method of choice for the industry before the 1990s is automation. Now that automation has been accomplished and is commercially available, the Dumas method is an officially recognized method (AOAC, 2005; AOCS, 2011d).

A comparison of the Kjeldahl and Dumas methods for protein analysis in soybean products was conducted by Jung et al. (2003). They used nine soybean products having protein contents ranging from 0.5-90%. The Kjeldahl method gave slightly, but significantly, lower values than the Dumas method. Both methods had equivalent variabilities, i.e., similar standard deviations.

CP can also be predicted by near-infrared reflectance spectroscopy (NIRS), with an acceptable relative standard deviation (see Chapter 9).

8.4 Protein quality

Protein quality is a function of the amino acid (AA) profile and the proportion of each AA available to the animal. When soybean meal (SBM) is intended for monogastric feeding, proper heat processing has a positive effect on digestible amino acid (DAA) because heating or toasting destroys anti-nutritional factors (ANF). However, over-heating can result in a decrease in both concentration and digestibility of several AA, especially lysine (see Tables 9 and 10). The reduction in digestibility is due to the Maillard reaction, which binds free AAs to free carbonyl groups from carbohydrates. The Maillard reaction end-products are not bioavailable for all livestock species.

Table 9. Effect of heat processing on digestible amino acid (DAA) of raw soybeans in poultry

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

Source: Adapted from Anderson-Hafermann et al., 1992.

TABLE 10. Effect of heat processing soybean meal (SBM) on amino acid (AA) concentration

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

Source: Adapted from Parsons et al., 1992.

Several methods to determine protein quality of soybean products for monogastric species have been developed (see Table 12).

8.4.1. Urease index

The urease index (UI), also called urease activity (UA) test, was developed by Caskey and Knapp (1944) to differentiate properly cooked SBM from those that didn't undergo adequate heat treatment. The initial concern was ruminants being fed inadequately processed SBM in the presence of urea supplementation. To avoid ammonia toxicity, a quantitative test was developed to measure UA expressed as delta pH. The same method was validated for poultry (Bird et al., 1947) and later it was reported that UA was highly correlated with trypsin inhibitor activity (TIA) (McNaughton et al., 1980). A reduction of UA resulted in a simultaneous reduction of TIA as heat treatment was applied.

The method requires the following:

- A pH meter
- 250 mL volumetric flasks
- A small water bath that allows maintenance of temperature at 30°C for at least 30 minutes
- Test tubes
- A pipette

The method determines the residual UA of soybean products as an indirect indicator to assess whether the ANF, such as trypsin inhibitors (TI), present in soybeans has been destroyed by heat processing. Both enzymes, urease and TI, are deactivated during heating. The laboratory method for urease involves mixing SBM with urea and water for one minute.

The procedure follows:

- 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 ºC for 30 minutes.
- Determine pH and compare it with the original pH of the urea solution.

The test measures the increase in pH due to release of ammonia (NH_3), which is alkaline, into the media arising from the breakdown of urea by the urease present in soybean products. Urea is broken down into NH_3 and carbon dioxide (CO_2). Depending on the protocol used, the endpoint is determined differently. In the American Oil Chemists' Society (AOCS, 2011a) method, the endpoint is determined by measuring the increase in pH of the sample media. In the European Union (EU) method, the endpoint reflects the amount of acid required to maintain a constant static pH. Results of these two methods differ slightly.

The urease test is an indirect measurement, and the procedure must, therefore, be correlated with the concentration of TI. These represent the most critical ANF in soybeans, and TI are associated with a decrease in poultry and swine well-being and performance when fed at excessive levels. It has also been suggested that TI in SBM are associated with the occurrence of incomplete digestion, wet litter, rapid feed passage (RPF) and enteritis in broilers and broiler breeders (Ruiz et al., 2005). The excreta of broilers exposed to excess TI in the diet routinely display undigested feed and sloughed off intestinal tissue. The maximum level of TI in soy products for broilers is a moving target, and current levels may need reconsideration in light of modern production conditions. Using the work of Havenstein et al. (2003), who compared broiler genetics of 1957 to the genetics of 2001, it is possible to estimate that, at the same SBM inclusion level at 42 days of age, modern broilers consume over three times the amount of TI relative to their 1957 predecessors.

Schulze (1994) studied the effects of TI and lectins in growing pigs fed a semi-purified diet. Separate experiments studied the effect of adding crystalline TI and crystalline lectins to diets. Inclusion of 2.4 mg of TI/g of diet increased the daily flow of total nitrogen (N) at the terminal ileum. This N was determined to be of both endogenous and exogenous origin, thus explaining the increased amount of undigested feed at the terminal ileum.

Consequently, TIA values below 2.35 mg/g of soy may be considered as necessary to avoid the wet litter or RFP syndrome in broilers. Based on field observations and expressed in terms of TI intake, at an average inclusion of 23% SBM in broiler diets, a modern broiler may consume 1,600-2,350mg of TIA for SBM containing 1.60-2.35 mg/g by day 42. It is of interest to notice that this corresponds to a UA in SBM below 0.05 pH units. This is considerably below the previously suggested range of 0.05-0.3 of pH units (Ewing, 1963; Hayward, 1975). Thus, for broiler diets, a lower UA should be recommended. Clarke and Wiseman (2007) reached a similar conclusion while measuring DAA in vivo in broilers fed full-fat soybeans (FFSB) with different TIA contents. They measured DAA for methionine, cysteine, glutamine, lysine and valine at 14.8, 9.6. 4.5 and 1.9 mg of TIA/g of FFSB (see Table 23). The highest digestibility was observed at 1.9 mg/g. Also, Huisman (1991) indicated that the maximum tolerance level for broilers is close to 0.5 mg of TIA/g of complete diet. This is in the same order of magnitude as the recommended maximum of 0.54-0.58 mg of TIA/g of commercial broiler feed (Ruiz, 2012).

In the case of FFSB, the target value for TI is similar as in SBM: at or below 2 mg/g, or 3-4 TIU/mg) of FFSB for pigs and poultry. Specific quality tests used for SBM may not directly apply to FFSB. This seems to be the case for the UI and the potassium hydroxide protein solubility (KOHPS) test (see Section 8.4.2.). Like in SBM, the UI measures a degree of heat treatment in FFSB, but the relationship between TI and UA seems to differ between the two soy products. While UI and TI are strongly correlated in SBM (McNaughton et al., 1980), in FFSB the correlation is weak. Given the fact that TIs are the principal ANF, it makes sense to use TI analysis (see Section 8.16.1) as the quality analysis test of choice for FFSB (Perilla et al., 1997). Varga-Visi et al. (2009) also reported inconsistencies in the use of UA as a quality predictor for FFSB, and Azcona et al. (2012), working with experimental FFSB, reached a similar conclusion.

8.4.2. Potassium hydroxide protein solubility

The solubility of the protein of vegetable origin in solutions of potassium hydroxide (KOH) was initially demonstrated by Evans and St. John in 1945 when searching for a method to assess the nutritional effects of heating on protein quality. Rinehart and co-workers at the Ralston Purina Company developed the technique as it is known today. The objective of the test is to differentiate overheated lots of SBM from those correctly processed.

This method consists of determining the percentage of protein that is solubilized in a KOH solution (Araba and Dale, 1990). The method requires the following equipment:

- 250 mL volumetric flasks
- A small magnetic stirrer
- Filtering funnels or a centrifuge
- The Kjeldahl equipment to measure N

The procedure includes the following steps:

- Determine N 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 ºC.
- Then, about 50 mL is taken and immediately centrifuged at 2500 x g for 15 minutes.
- Take aliquot of about 10 mL to determine N in the liquid fraction by the Kjeldahl method.
- The results are expressed as a percentage of the original N content of the sample.

A well-documented *in vitro/in vivo* correlation exists between KOHPS values and DAA for SBM in broilers (see Table 11; Araba and Dale, 1990; Parsons et al., 1992), turkeys (Lee et al., 1992) and swine (Parsons et al., 1991). Well-preserved raw soybeans should have a solubility in 0.2 KOH solution of equal to or more than 90%, while correctly heat-processed SBM should have a protein solubility around 78-85% (see Table 7).

The correlation between DAA and KOHPS is not the same for autoclaved SBM as for industrially produced commercial SBM.

In contrast to other *in vitro* techniques used to assess the quality of soy products, the KOHPS test has not reached the level of standardization of an AOCS or an AOAC method. In other words, there is no official method for KOHPS, but rather a brief procedure (Araba and Dale, 1990) that is used worldwide. This is probably the reason for considerable inter-laboratory variation, which becomes evident when claims about over-processing of SBM arise between traders and end-users. However, the KOHPS technique - when correctly standardized intra-laboratory - can be used quite effectively to differentiate between or among commercial lots of SBM at a given company. This is especially true when working with an internal central laboratory or a commercial analytical laboratory where the technique has been standardized. Sample particle size (Whittle et al. 1992) and intensity of the agitation (Ruiz, 1996) are two of the main factors that affect repeatability of the results.

The KOHPS procedure applies to SBM, but not necessarily to other soy products. When commercially available FFSB was used, no significant correlation between KOHPS and the in vivo digestible lysine could be demonstrated (r=0.24; P=0.11) (Ruiz et al., 2004). A further confirmation that protein solubility and DAA may not be correlated in FFSB was demonstrated in an experiment with 10 lots of commercial FFSB, processed at a single extruder over a period of 1.5 years. Samples were selected based on their KOHPS values which ranged on average between 60.0-81.3%. In vivo DAA coefficients were determined for each of the 10 samples. No correlation was observed (P>0.05) between the protein solubility values and the digestibility coefficient for the AA (Ruiz et al., 2015).

Table 11. Effect of autoclaving soybean meal (SBM) on chick performance (1-18 days), potassium hydroxide protein solubility (KOHPS) and urease activity (UA)

Autoclaving (120 ºC), 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

Source: Adapted from Araba and Dale, 1990.
[a, b, c, d] Means within a column with common superscripts are not significantly different (P<0.05).

8.4.3. Protein dispersibility index

Among the available tests for determining protein quality in soybean products, the protein dispersibility index (PDI) is the simplest, most consistent method. This test measures the solubility of soybean proteins in water and may be the test best adapted to all soy products. The PDI method measures the amount of soy protein dispersed after blending a sample in water in a high-speed blender. The water solubility of soybean protein can also be measured with a technique called Nitrogen Solubility Index (NSI). These 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 this equipment:

- An 8,500 ppm blender
- Filtering funnels or a centrifuge
- The routine Kjeldahl equipment for N analysis

Procedure:

- Determine N 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, 2011e).
- Filter and centrifuge for 10 minutes at 1,000 g.
- Analyze N content of the supernatant.
- The results are expressed as a percentage of the original N 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, 2011c). With either method, the final step consists of determining the N content of the liquid fraction and the results are expressed as a percentage of the original N content of the sample.

Some soybean producers and users of soy products advocate the PDI method as the best for assessing protein quality in SBM. Because this test is more recent, it is often used as a complement to UA and KOHPS measurements, and the PDI method has proven to be especially useful in determining the degree of possible under heating SBM. Batal et al. (2000) described a greater consistency in the results of heating of soy flakes obtained with the PDI procedure than those from UA or KOHPS. Since the work of Batal et al. (2000), which recommended PDI values below 45%, recommendations under laboratory experimental conditions have shifted slightly due to the influence of practical experience with commercial SBM.

As in the case of SBM (Batal et al., 2000), Qin et al. (1996) reported a linear relationship between increasing time of heat treatment and PDI at two different processing temperatures of FFSB. Time of extrusion at 102 °C and at 120 °C highly correlated with descending PDI ($r=0.99$; $P < 0.001$). Furthermore, the correlation between TIA and PDI was also high ($r=0.96$; $P < 0.01$), indicating that PDI as a quality analysis may be utilized to estimate the TI content of FFSB on a routine basis. Hsu and Satter (1995) demonstrated that PDI is also a useful quality analysis to predict the adequacy of processing FFSB for dairy cows.

Despite the many positive aspects of the PDI test, more recent work has demonstrated that the PDI values of SBM are not stable and will change over time (Swick, personal communication; Serrano et al., 2013). The decrease in PDI values with storage is such that only recent analyses are valid to assess protein quality. Repeat PDI analyses may be necessary every 30 days.

Current recommendations are for SBM with PDI values between 15 – 30%, KOHPS between 78 – 85% and UI of less than 0.05 pH unit change. Such SBM is considered adequately heat processed, without under- or over-processing. The suggested quality parameters are consistent with TIA in the range of 1.60 – 2.35 mg/g.

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; Whittle et al., 1992). PDI in FFSB is similar (Hsu et al., 1995). Therefore, it is recommended to grind samples at a consistent mesh size, less than 500 microns, and to rigorously maintain the same standard procedures for stirring times, duration for treating the samples in the respective solutions and centrifugation within the same laboratory and company. It is equally important to use the same equipment, particularly stirrers and centrifuges, time after time when conducting these determinations. This is primarily to reduce intra-laboratory variability (Parsons et al., 1991.; Ruiz, 1996).

A summary of routine quality parameters for SBM is provided below.

Table 12. Available methods to determine protein quality of soybean meal (SBM)

Urease Index (UI)

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

Potassium hydroxide protein solubility (KOHPS)

1. Mix 1.5 g SBM with 75 mL 0.2% KOH solution and stir for 20 minutes.
2. Centrifuge at 2,500 x g for 20 minutes.
3. Measure soluble nitrogen (N) in the liquid fraction.

Protein Dispersibility Index (PDI)

1. Mix 20 g SBM with 300 mL deionized distilled water.
2. Blend at 8,500 RPM for 20 minutes at a temperature of 22 ºC.
3. Centrifuge at 1,000 x g for 10 minutes, and filter and measure N content of the liquid fraction.

Nitrogen Solubility Index (NSI)

1. Mix 5 g SBM with 200 mL distilled water.
2. Stir at 120 rpm for 120 minutes at 30 °C.
3. Centrifuge at 1,500 rpm and measure soluble N 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.

Source: Adapted from Dudley-Cash, 1999.

8.4.4. Protein quality in ruminants

For ruminants, protein quality of soybean meals will depend on its rumen degradation and its intestinal digestion. As for other protein ingredients used in ruminant nutrition, the protein fraction of soy products is divided in three distinct sub-fractions based on their availability in the rumen: rumen-soluble (a), rumen-degradable (b) and rumen-undegradable protein (c). The contribution of the soy product to the amino acid supply of the animal will greatly depend on the relative size of these fractions. Soy products that have not been specially treated to resist rumen degradation, are generally high in the rumen-soluble and degradable fraction (a and b) and low in rumen undegradable fraction (c). The anti-nutritional factors present in heat treated soybeans and soy products are considered of lesser importance in ruminants, as their residual contents are largely destroyed or des-activated in the rumen (Caine et al., 1998; Nobar et al., 2009).

Amino acids are supplied to the duodenum of ruminants in the form of 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, like most soy products, are extensively degraded in the rumen, providing an excellent source of degradable intake protein for the ruminal microbes. However, this may lead at the same time, to a situation where not enough undegradable protein is supplied to meet the demands of high producing ruminants. Because soybeans contain a high-quality protein with a good amino acid profile and are highly digestible in the small intestine, various processing methods and treatments have been developed to increase its undegradable protein value. The most common methods for protecting soybean proteins from ruminal degradation is the application of heat, incorporating chemicals such as formaldehyde or a combination of heat and chemicals, such as lingo-sulfonate combined with xylose.

Assessment of protein quality of soy-products for ruminants is realized through the determination of the relative proportion of the three protein sub-fractions and the rate of rumen protein degradation. Several techniques are available for this analysis.

8.4.4.1. In situ technique

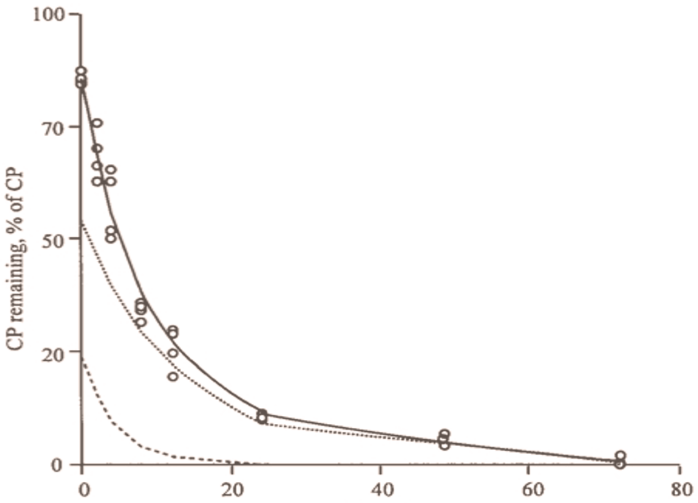
Although this technique is relatively expensive, labor intensive and requires access to rumen cannulated animals, it is very useful to determine the rate and extent of degradation of soybean proteins. This technique requires consecutive times of ruminal incubation of feed or ingredient samples under study. To this purpose, a sample is placed in a nylon or dacron bag of specific pore size. Generally, the sample size is approximately 6 g ground to pass a 2 mm screen; pore size is 45-µm; and the bag size is 10 cm×20 cm; (Nocek, 1988). The sample bags are subsequently suspended in the rumen of the cannulated animal. Normally several bags of the same feed or ingredient are suspended at the same time (T0), such that duplicate or triplicate bags can be removed at fixed time intervals after the onset of incubation or fermentation. This allows for determination of the rate and extent of protein degradation at any given time after the onset of fermentation.

The *in-situ* technique determines the rate of degradation and size of the insoluble protein fraction. The soluble protein fraction is assumed to be totally and instantaneously degraded. The size of this fraction is sometimes determined through washing of the sample in distilled water. The rumen undegradable fraction is equal to 100 minus the soluble and the rumen degradable fraction.

To predict the rate of protein degradation, sufficient time points must be included over the entire incubation period, however, a greater density of time points (shorter intervals) should be allocated to the early stages of degradation (Figure 2).

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 et al., 1972) allowed for the best separation of the different protein pools in soybean proteins.

Figure 2. Protein disappearance from soybean meal (SBM) and curve peeling process



— Crude protein (CP) disappearance
--- Rapidly degradable pool
... Slowly degradable pool
O Observed values

Source: [1]Adapted from Bach et al. (1998).

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 require access to cannulated animals as a source of rumen fluid. The in vitro technique consists of incubating a small sample of feed or an ingredient, normally 0.5 g, with strained rumen fluid and a buffer, under anaerobic conditions in a test tube or container. The test tube or container is placed in a water bath that is maintained at 37-38°C throughout the incubation. At predetermined intervals, a sample is removed from the incubator, centrifuged and analyzed for dry matter (DM) and N disappearance using the Kjeldahl method. Data are analyzed as described for the in-situ technique (see above).

There are several enzymatic techniques that have the important advantage of being completely independent of the animal. Consequently, they should result in lower variation, making these techniques easier to standardize. However, results depend on the selection of the enzyme or enzyme mix, which clearly do not reflect the complete effect of rumen microbial fermentation. 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 to the ruminal environment. Mahadevan et al. (1987) observed 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 of degradability as 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 (NIRS) to estimate protein degradation of feedstuffs in the rumen. Most of this is related to analyzing the protein fractions in forages. However, Tremblay et al. (1996) evaluated NIRS as a technique for estimating ruminal CP degradability of roasted soybeans and found a coefficient of determination between NIRS and undegraded protein estimated by the inhibitor in vitro technique of 0.70. More recent work by Foskolos et al. (2015), using an in-situ technique and a wide range of ingredients including soy products, concluded that NIRS equations to predict the effective degradability and fractions a and b of CP could be adequate for screening purposes ($R^2 > 0.77$). They could also be potentially suitable for quantitative purposes by separating feeds into more precisely defined groups, possibly with soy products as a select group. However, to reduce variability, this will require a larger number of soy ingredients with determined degradation characteristics. The development and maintenance of reliable NIRS calibration equations for this purpose seems possible, but will remain laborious, time-consuming and costly.

8.5. Amino acids

Determining the amino acid (AA) composition of proteins is essential to characterize their biological value. The greater the proportion of some of the essential AA, the greater the biological value of a protein. For all soybean products, an important part of their value depends on the concentration of the most limiting indispensable AA and the degree to which they are available to the animal. In the absence of direct analyses, an accurate estimate of the essential AA is necessary. Several systems and equations to estimate AA concentrations have been developed and are available (e.g., Evonik, 2016; Novus, 1996) and many feed companies have developed proprietary equations. In these systems, individual AA are normally estimated on the basis of total crude protein (CP) and possibly a second nutritional parameter.

For pigs and poultry, digestible amino acids (DAA) are estimated applying digestibility coefficients to total AA values that have been determined in vivo for most commercial ingredients. DAA values are available in tabulated form (CVB, 2018; FEDNA, 2019; INRA-AFZ, 2002; Rostagno, 2017). These digestibility coefficients can be adjusted by secondary parameters that — in the case of soy products — are generally one of the protein quality indexes (see Section 8.4). Under practical conditions, DAA estimates depend greatly on the accuracy of analyses for other nutritional components. Near-infrared reflectance spectroscopy (NIRS; see Chapter 9) might offer potential for more direct and quicker analyses, but reliable calibrations and proper equipment are needed.

The classical AA 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 tryptophan, a hydrolysis with barium hydroxide sodium hydroxide (1.5 mol/l) for 20 h is required.

8.5.1. Amino acid digestibility methods

Different methodologies exist to determine in vivo DAA of feed ingredients for monogastrics. In poultry and pigs, digestion of dietary protein is largely complete by the end of the ileum, and any uptake of AA from the hindgut is considered of little or no importance. Microbial fermentation in the hindgut modifies the amount and composition of AA in the digesta. Consequently, the contribution of AA from specific feeds or ingredients to the overall AA supply should be evaluated at the terminal-ileum level. The most accurate and widely used digestibility coefficients for AA refer now to ileal digestibility.

For poultry, two methods exist:

1. The precision-fed cecectomized rooster assay (PFR) (Parsons, 1985) is based on the Sibbald method (1979).
2. The apparent ileal digestibility (AID) and standardized ileal amino acid digestibility (SIAAD) assays are from Ravindran et al. (1998).

The first method utilizes adult cockerels whose ceca have been surgically removed to reduce the effect of hindgut microbes on AA fermentation:

- Birds are placed in individual cages, which facilitates the total collection of excreta.
- Following a 24-hour fast, 5 birds are given 30 g of the ingredient to test for DAA via crop intubation. An additional 5 cockerels are fasted throughout the experimental period to measure endogenous excretion of AA.
- A plastic tray is placed under each cage, and excreta are collected for 48 hours.
- Excreta samples are lyophilized, weighed and ground to pass through a 60-mesh screen.
- AA concentration of the ingredient and excreta samples are analyzed by HPLC. Each sample is divided into three test sub-samples to analyze for:
    - Tryptophan by alkaline hydrolysis and subsequent HPLC.
    - Sulfur AA by performic oxidation before 24-hour acid hydrolysis.
    - The remaining AA by direct hydrolysis for 24 hours at 110 °C with 6 N hydrochloric acid, followed by HPLC.
- Calculations for standardized PFR follow.

Standardized AA digestibility,%=

$$\left\{\frac{[AA\ intake\ (mg) - \ AA\ excreted\ (mg) + \ EAA\ (mg)]}{AAintake\ (mg)}\right\} x\ 100$$

Where: EAA =Endogenous AA excretion from fasted cockerels.

The second method, AID and SIAAD assays, use day-old chicks:

- Chicks are fed a starter diet until day 16.
- After an overnight period of fasting, birds are weighed individually and randomized to the dietary treatments with 5 birds per battery cage and 4 replicates, or cages, per dietary treatment. If only one ingredient is to be analyzed for DAA, then there is only one treatment.
- For determination of SIAAD, a special treatment consisting of a nitrogen (N)-free diet is included. The difference between the AID assay and the SIAAD one is the feeding of the N-free diet to correct for endogenous losses.
- Except for the N-free diet, the dietary treatments contain approximately 20% protein. In the case of most soy products, all the protein will be provided by the soy product to be evaluated. An inert marker is added at a fixed level to all diets as an indigestible marker, for instance, chromic oxide (Cr_2O_3) at 0.30%. All diets are fed in mash form.
- Experimental diets are fed for a 5-day period. On day 21, birds are euthanized by carbon dioxide (CO_2) asphyxiation and ileal digesta is collected immediately from the ileum from Meckel´s diverticulum to approximately 1cm proximal to the ileo-cecal junction.
- The ileal digesta from birds within cages or groups are pooled, frozen and stored at -20 °C until they are processed.
- Calculations follow.

$$Apparent\ ileal\ AA\ digestibility\ (AID) = [1\text{- }(marker\ in\ diet/marker\ in\ digesta)\ \text{x}\ (AA\ in\ digesta/AA\ in\ diet)]\ \text{x}\ 100$$

$$Standardized\ \ ileal\ AA\ digestibility\ (SIAAD), \% = AID + \left[\frac{IEAA\ flow, \frac{g}{kg}\ of\ DM\ intake}{AA\ in\ the\ diet\ or\ feed\ ingredient, \frac{g}{kg}\ of\ DM\ intake}\right] x\ 100$$

Where: IEAA = Ileal endogenous AA flow values from the 21-day-old broilers fed an N-free diet.

Several comparisons have demonstrated that PFR and the SIAAD assay are basically equivalent methods providing similar results across different ingredients, including soybean meal (SBM) (Kim et al., 2012a; Kim et al., 2012b). Significant differences have been observed in DAA between methods, but such differences were not consistent between methods or AA. One important difference between the above discussed methods is the higher cost and time consumed with the SIAAD method. In fact, most of the DAA coefficients available in the public domain for poultry were obtained with the PFR method.

For swine, the equivalent of both systems as used in poultry exist for the determination of in vivo DAA of ingredients and diets. However, the predominant system in use today is the ileal AA digestibility with digesta being collected from the terminal ileum through a canula. Generally, ileal digestibility is determined in growing pigs, and with the use of the canula, pigs can be used for repeated measures. Normally 6-10 pigs will be used per treatment. Treatment of samples for storage and AA analysis are identical to those described above for poultry.

Ileal digestibility values may be expressed as AID, standardized ileal digestibility (SID) or true ileal digestibility (TID) (Stein et al., 2007). These terms are used to specify how ileal endogenous amino acid (IEAA) losses are accounted for in digestibility values. IEAA losses may be separated into basal losses, which are not influenced by feed ingredient composition, and specific losses, which are induced by feed ingredient characteristics such as levels and types of fiber and anti-nutritional factors (ANF). Values for AID are established when total ileal outflow of AA, or the sum of endogenous losses and nondigested dietary AA, is related to dietary AA intake. A concern with the use of AID values is that they are not additive in feed ingredient mixtures (Nyachoti et al., 1997). This concern may be overcome by correcting AID values for defined basal endogenous losses of AA, which yields SID values.

If AID values are corrected for basal and specific endogenous losses, then values for TID can be calculated. However, reliable procedures to routinely measure specific endogenous losses are not yet available. It is recommended that basal IEAA losses are measured in digestibility experiments using a defined protein-free diet, and that these losses are reported with observed AID and SID values. It is suggested that SID values are used for feed formulation, at least until more information on TID values becomes available (Stein et al., 2007).

The calculation for the SID of AA follows.

$$Standardized\ \ ileal\ AA\ digestibility\ (SID), \% =$$

$$AID + \left[\frac{Endogenous\ \ AA\ flow\ , \frac{g}{kg}\ of\ DM\ intake}{AA\ in\ the\ diet\ or\ feed\ ingredient, \frac{g}{kg} of\ DM\ intake}\right] x\ 100$$

In ruminants, determination of intestinal digestibility of AA is more complex than in monogastric animals, and several techniques have been used. These include the use of multi-cannulated animals, allowing for duodenal perfusion and ileal collection, the use of small mobile dacron bags, in vitro procedures and digestibility in monogastric animals. All of these techniques are based on the collection of a feed or ingredient sample at the end of an in situ or in vitro rumen incubation (See Section 8.4.4.). The determined DAA is considered an estimate of true DAA of the rumen undegradable fraction of the feed or ingredient. Combined with the AA concentration of the microbial protein produced in the rumen, it is used in mathematical models to estimate supply and requirements of the essential AA.

8.5.2 Reactive lysine methods

Reactive lysine methods have been proposed — and used — as procedures to estimate the effect of heat treatment on the availability of lysine and other AA. This has been especially true for soy products. Lysine is considered the most susceptible AA to react with other compounds in the same ingredient or in the food or feed during thermal processing. The reactive site in the lysine moiety is the ε-amino group. The more ε-amino groups in a protein molecule react, the less digestible is the lysine in that protein. Maillard (1912) was the first to report the non-enzymatic reaction between an AA and a sugar in the presence of heat. Such a reaction involves the amino group of an AA and the carbonyl group of a reducing sugar. The Maillard reaction is also called a non-enzymatic browning reaction.

The *in vivo* determination of DAA as discussed in the previous section aims to quantify the total AA — including lysine — available for digestion and absorption by monogastric animals. An alternative method to estimate the amount of lysine available for protein synthesis is a chemical determination of the actual amount of intact ε-amino groups in the protein of a given ingredient. This quality analysis is called the reactive lysine method. Since soy products are important protein sources and lysine is the most abundant

essential AA in soy protein, reactive lysine methods are relevant in evaluating soy protein quality.

There are at least two reactive lysine methods. Both methods intend to measure the free ε-amino group of lysine and the amount of lysine potentially available for digestion and absorption.

- The 1-fluoro-1,4-dinitro benzene (FDNB) method also is called the Carpenter method. FDNB reacts with the ε-amino group of a protein-bound lysine to yield ε-dinitrophenyl lysine (DNP-lysine) upon acid hydrolysis. Ether-soluble interfering compounds are removed by extraction and the absorbance of the residual aqueous layer is measured (Carpenter, 1960).
- In the guanidination method (Moughan et al., 1996), O-methylisourea (OMIU) reacts with the ε-amino group of a protein-bound lysine to yield homoarginine upon acid hydrolysis, which in turn is measured using conventional AA analysis.

For reactive lysine methods to be reliable procedures to quantify the amount of available lysine for digestion and absorption in soy products, three key criteria must be met:

1. The derivatizing reagent, FDNB or OMIU, must be specific for the ε-amino group of lysine to allow for the determination of both free and bound reactive lysine.
2. The derivatized lysine compound, DNP-lysine or homoarginine, must be acid-stable, since they are formed while lysine is still bound to protein. A normal acid hydrolysis follows to quantify either DPN-lysine or homoarginine.
3. Derivatization must be quantitative.

The guanidination method was developed by Moughan and Rutherfurd (1996) to replace the FDNB method because of the limitations of the latter, per some of the above-mentioned criteria. However, the guanidination method has also been found limiting on some of the very same criteria (Hulshof et al., 2017). More precisely, either method fails to be specific and exclusive for the ε-amino group of lysine. In other words, both reagents also react with the ε-amino groups wherever they are available for reaction. Homoarginine appears to be more acid-stable than DPN-lysine, but the reaction of OMIU with the α-amino group is not quantitative.

One important objective of having a quantitative assessment of digestible lysine in soy products — as well as all major macro-ingredients — is to use data to formulate poultry and swine feeds. None of the current techniques to assess the digestibility or availability of lysine, in vivo DAA, FDNB and OMIU, are rapid methods. Consequently, nutritionists and formulators look for significant correlations that allow the development of reliable regression to improve the feed formulation based on the best analytical estimation of the ingredients in the bins. Frikha et al. (2012) measured SIAAD of lysine for poultry and reactive lysine by the guanidination method on 22 commercial samples of SBM of different origins, and results indicated only a weak correlation between in vivo digestible lysine and reactive lysine. For routine use, a stronger correlation is required. A considerable number of in vivo DAA coefficients have been determined for soy products in multiple laboratories worldwide, and these have been published, particularly for SBM.

Even though there are valid shortcomings in the in vivo methodology, in vivo DAA coefficients remain the most consistent estimators of DAA. Until a more reliable reactive lysine procedure is developed that meets the three key criteria discussed above, a practical use of the concept of reactive lysine as a quality analysis and a formulation tool seems unwarranted.

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 (CF) also included pectin, and that not all the lignin was recovered in the CF fraction. The major disadvantage of this technique is that hemi-cellulose, lignin and pectins are inconsistently accounted for.

The method requires the following reagents:

- Sulfuric acid solution, 0.255 N, 1.25 g of H_2SO_4/100 mL
- Sodium hydroxide solution, 0.313 N, 1.25 g of NaOH/100 mL, free of sodium carbonate (Na_2CO_3)
- Alcohol: methanol, isopropyl alcohol, 95% ethanol or reagent ethanol
- Antifoam agent (n-octanol)

Equipment is as follows:

- 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 with valve to break or control vacuum

The procedure described by the AOAC (2005) 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 (EE).
- 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% sulfuric acid, 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 50-75 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% sulfuric acid, three 50 mL portions of water, and 25 mL of alcohol.
- Dry mat and residue for 2 hours 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 CF content on dry matter (DM) basis.

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

8.7. Neutral detergent fiber

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 pectin is not included in the NDF fraction.

The NDF method was first described by Goering and Van Soest (1970) and later modified by Van Soest et al. (1991) and Mertens (1992). The NDF determination requires the following equipment:

- A refluxing apparatus
- A number of 600 mL Berzelius beakers
- Pre-weighted, ash-free filter paper
- Optional: 50 mL fritted glass (Gooch) crucibles with coarse porosity
- An analytical electronic balance, accurate to 0.1 mg

The technique is as follows:

Reagents needed:

- NDF solution: Dilute 30 g of sodium lauryl sulfate (SLS), 18.61 g of disodium dihydrogen ethylene diamine tetra-acetic dihydrate ($C_{10}H_{14}N_2Na_2O_8 \cdot 2H_2O$), 6.81 g of sodium borate decahydrate ($Na_2B_4O_7 \cdot 10H_2O$), 4.56 g of disodium phosphate (Na_2HPO_4), and 10 mL of triethylene glycol ($C_6H_{14}O_4$) 65 in 1 L of de ionized water
- Acetone

The Goering and Van Soest (1970) procedure for NDF determination is as follows:

- Weigh 0.5-1.0 g sample (+0.0001 g) in a 600 mL Berzelius beaker *(A)*.
- Add 100 mL of NDF solution.
- Heat to boiling, 5-10 minutes. 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.
    - Note: Gooch crucibles can be used for the filtration. In this case, use crucible that have been dried at 100 °C and hot-weighed *(B)*, recording weight to nearest 0.1 mg.
- Remove sample from heating unit and allow to settle for 30-60 seconds before filtering.
- Rinse contents with hot water, filter and repeat twice.
- Wash twice with acetone.
- Dry at 100 °C in forced-air oven for 24 hours.
- Cool filter paper or crucibles and sample residue in desiccator; weigh and record *(C)*.
- Fold filter paper and place in a pre-weighted aluminum pan.
- Ash filter paper or crucibles in muffle furnace at 500 °C for 8 hours or overnight.
- Cool in desiccator. Weigh and record *(D)*.
- Calculate the NDF content on a dry matter (DM) basis.

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

A double treatment with reagent-grade, heat-stable alpha-amylase solution is recommended at present, and it is especially applicable to starch-rich ingredients. To this purpose, the above NDF procedure is slightly modified. The sample size is limited to 0.45–0.55 g and crucibles should be used. After the solution is brought to boiling for a duration of 5 minutes in the third step, 2 mL of the standardized amylase solution is added.

Subsequently, return the beaker to the reflux unit after assuring that all feed particles are suspended in the NDF solution and allow to come to a boil.

Prior to the sixth step, reheat the Gooch crucible for filtering by adding 40 mL of boiling water. Remove water from crucible with vacuum. Reduce vacuum and empty the content of the beaker in the crucible, assuring that all particles are transferred. Immediately fill crucible half full of hot water and add 2 mL of standardized amylase solution. Allow to react for approximately 45–60 seconds. Subsequently, continue with above procedure.[1]

For Ankom[1] systems the following procedure applies:

- Use any number of filter bags, constructed from chemically inert and heat-resistant filter media, capable of being heat-sealed closed and able to retain 25 micron particles while permitting solution penetration.
- Weigh 0.45-0.50g sample in filter bag, record exact weight (+ 0.0001g) (A) and one blank bag, included in extraction to determine blank bag correction.
- Completely seal each bag within 0.4 cm from the open edge using a heat sealer.
- Spread sample uniformly inside the filter bag by shaking and lightly flicking the bag to eliminate clumping.
- Pre-extract soybean products containing more than 5% fat with acetone extract samples by placing bags with samples into a container with a top. Pour enough acetone into the container to cover the bags and secure the top. Shake the container 10 times and allow bags to soak for 10 minutes. Repeat with fresh acetone. Pour out acetone and place bags on a wire screen to air-dry.
- Place bags containing samples in a 500 mL bottle with a screw cap. Fill the bottle with acetone to cover bags, at least 15 mL/bag, and secure top. Swirl gently after three and six minutes have elapsed and allow bags to soak for a total of 10 minutes. Repeat with fresh acetone. Pour out acetone and place bags on a wire screen to air-dry.
    - **Exception:** roasted soybean. Due to the processing of roasted soy, a modification to the extraction is required. Place roasted soy samples into 500 mL bottle with a screw cap. Pour enough acetone into the container to cover the bags and secure the top. Shake the container 10 times and pour off the acetone. Add fresh acetone and allow samples to soak for 12 hours. After the soaking time, pour out the acetone and place the bags on a wire screen to dry.
- Spread the sample uniformly inside the filter bags by shaking and flicking the bags to eliminate clumping.
- Place up to three bags on each of eight bag suspender trays, for a maximum of 24 bags.
- Stack suspenders on center post with each basket rotated 120°.
- Include one standard and one blank.
- Place duplicate samples in separate batches and in reverse order of top to bottom. All 9 trays must be used, regardless of the number of bags being processed.
- Open the Ankom vessel and insert the suspender with bags into the vessel and place the bag suspender weight on top of the empty ninth tray to keep the bag suspender submerged.
- When processing 24 sample bags, add 1,900-2,000mL of ambient temperature NDF solution to the fiber analyzer vessel. If processing less than 20 bags, add 100 mL/ bag of NDF solution. Use a minimum of 1,500 mL to ensure suspender is covered. Add 20 g of sodium sulfite (Na_2SO_3), or 0.5 g per 50 mL of NDF solution, and 4 mL of alpha-amylase to the solution in the vessel.
- Turn agitate and heat on and confirm agitation. Set the timer for 75 minutes and close the lid.
- When the NDF extraction is complete, turn agitate and heat off. Open the drain valve and exhaust the hot solution before opening the vessel lid.

- Note: The solution in the vessel is under pressure. The exhaust valve needs to be opened to release the pressure and solution prior to opening the vessel lid.

- Close the exhaust valve and open the vessel lid. Add 1,900-2,000 mL of 70-90 °C rinse water and 4 mL of alpha-amylase to the first and second rinses. Turn agitate on and rinse for five minutes. Repeat five minute hot-water rinse one more time for a total of three rinses.
- When the NDF extraction and rinsing procedures are complete, open the vessel lid and remove the filter bags. Gently press out excess water from the bags. Place bags in a 250 mL beaker and add enough acetone to cover bags and soak for three to five minutes.
- Remove the filter bags from the acetone and place them on a wire screen to air- dry. Completely dry in an oven at 102 °C (± 2 °C). In most ovens the filter bags will be completely dry within two to four hours. Do not place bags in the oven until the acetone in the bags has completely evaporated. Weigh *(B)*.
- Weigh blank bag *(C)*.
- Ash bags on pre-registered and weighed aluminum pan (D; Db for blank) for six hours at 550 °C in muffle furnace, cool, place in desiccator and weigh blank *(E)* and pans with samples *(F)*.
- Calculate the NDF content on a DM basis.

[1] For A200 and A200I

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

- Calculate the ash-free NDF content on a DM basis.

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

8.8. Acid detergent fiber

It is recommended that acid detergent fiber (ADF) is determined sequentially, that is using the residue left from neutral detergent fiber (NDF) determination. If not done sequentially, some fractions of pectin 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 similar equipment:

- 600 mL Berzelius beakers
- A fiber digestion apparatus
- A filtering flask
- Sintered glass crucibles of 40 – 50mL with coarse porosity

Reagents needed:

- Acid Detergent Solution. For this, add 27.84 mL of H2SO4 to a volumetric flask and bring to 1 L volume with de-ionized water (it is recommended that before adding the acid, some water is placed in the volumetric flask). Then add 20 g of quaternaryammonium salt solution, CH3(CH2)15N(CH3)3Br, to this solution.
- Acetone.
- 72% sulfuric acid standardized to specific gravity of 1.634 at 20 °C. Extraction of sample.

Extract sample:

- Prepare samples: oven-dried at 55 °C to ≥85% dry matter (DM) and ground to pass a 1 mm screen.
- Dry 50 mL fritted glass crucibles overnight at 100 °C and record weight to nearest 0.1 mg.

- Transfer air-dried sample of 0.9-1.1g to Berzelius beaker. Record weight of sample accurate to 0.1 mg (A).
- Add 100 mL acid detergent solution.
- Heat to boil, 5-10 minutes), and then boil for exactly 60 minutes, adjusting boiling to slow, even level.
- After 30 minutes, wash down sides of beaker with minimal amount of acid detergent solution.
- After 60 minutes, remove beaker, swirl and filter through previously tared, fritted glass crucible, using minimal vacuum. Rinse the Berzelius beaker with boiling water while inverted over the crucible to insure quantitative transfer of all fiber particles into the crucible.
- Filter with light suction into crucible.
- Wash sample in crucible with deionized hot water 2-3 times, breaking up mat.
- Wash with acetone until no further color is removed. Suction dry.
- Dry in forced-air oven at 100 °C for three hours or overnight.
- Cool in desiccator. Weigh and record weight *(B)*.
- Ash in muffle at 500 °C for four hours.
- Cool in desiccator. Weigh and record (C).
- The ash-free ADF content on a DM basis is then calculated using the following equation.

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

8.8.1. Acid detergent insoluble nitrogen

Acid detergent insoluble nitrogen is the nitrogen (protein) bound to the acid detergent fiber residue. While some occurs naturally in all plant material, in the case of soy products, it reflects excess heat treatment during crushing, extrusion, drying or storage. ADIN is usually unavailable to animals especially to monogastric animals.

ADIN is determined by measuring the N content in the ADF residue. It is generally impossible to collect all of the ADF residue from the fritted glass crucible, therefore, only a subsample of the total ADF residue is analysed for nitrogen. Consequently, the nitrogen content of the ADF residue must be determined by dividing the nitrogen in the ADF sample by the ADF sample weight. When expressed on a DM basis, ADIN is calculated by multiplying the nitrogen content of ADF by the ADF content in the dry matter.

Caution should be exercised when sampling ADF residues from fritted glass crucibles, to avoid scraping glass particles into the partial ADF residue.

Acid detergent fiber crude protein (ADFCP) is ADIN expressed as crude protein on a dry matter basis. For equipment and reagents, see Section 8.3 on protein and Section 8.8 on ADF.

To analyze for ADIN, it is recommended to use filter paper in the ADF procedure:

- Using procedure with filter paper, dry filter paper three hours in a forced-air oven at 100 °C and weigh hot, recording weight to nearest 0.1mg *(B)*.
- Following the ADF procedure, after vacuum drying the paper and ADF sample residue, fold the filter paper to retain the sample and dry three hours in a forced-air oven at 100 °C.
- Weigh hot, recording weight *(A)* to nearest 0.1mg.
- Subsequently, insert filter paper and sample into a Kjeldahl flask, add 5 mL additional acid to digest the filter paper, and determine Kjeldahl N (see Section 8.3).
- Calculate ADIN content on a DM basis using the following equation.

$$ADF, \% = \frac{(mgN \text{ in } ADF + \text{filter paper, } A - mgN \text{ in blank filter peper, } B)}{(\text{Original sample weight, } x \ \% \text{ Dry matter})} \ x\ 100$$

8.9. Lignin

Lignin is a polymer of hydroxycinnamyl alcohols that can be linked to phenolic acids and non-phenolic compounds. Lignin acts like a shield that physically prevents the action of enzymes and bacteria. Lignin is totally indigestible and also limits digestion of some nutrients, especially fiber fractions, of soybean products.

Determination of lignin is, therefore, 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.

8.9.1 Klason lignin

The Klason lignin requires the following materials:

- 72% sulfuric acid (H2SO4)
- Sintered glass crucibles

The technique consists of adding 25 mL of sulfuric acid to the residue of an acid detergent fiber (ADF) determination without ashing, filtering and adding distilled water three times.

The procedure follows:

- Place crucible with ADF residue 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% sulfuric acid. Fill approximately halfway with acid.
- Stir contents with a glass rod to a smooth paste.
- At room temperature, leave rod in crucible, refill hourly for three hours, each time stirring the contents of the crucible.
- After three hours, 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, a minimum of five times.
- Rinse rod and remove.
- Dry crucible in oven at 100 °C for 24 hours.
- Cool in desiccator. Weigh and record weight *(B)*.
- Ash in muffle at 500 °C for four hours.
- Cool in desiccator. Weigh and record *(C)*.
- Calculate Klason lignin on a dry matter (DM) basis as shown.

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

8.9.2. Permanganate lignin

The permanganate lignin requires the following materials:

- 80% ethanol
- Permanganate buffer solution, which consists of two parts potassium permanganate ($KMnO_4$) and one part lignin buffer solution
  - Lignin buffer solution is made up of:
    - 300 mL of distilled water
    - 18 g ferric nitrate ($Fe(NO_3)_3$)
    - 0.45 g silver nitrate ($AgNO_3$)
    - 1.5 L glacial acetic acid (H_3COOH)
    - 15 g of potassium acetate (CH_3CO_2K)
    - 1.2 L of tertiary butyl alcohol ($(CH_3)_3COH$)
- Acetone.
- Fiber crucibles.
- A Fibertec apparatus or a vacuum system.

The procedure follows:

- 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 mat residue with a little distilled water.
- Fill the crucibles about halfway with the permanganate buffer solution. Stir, and keep filling crucibles as solution drains out.
- Leave the permanganate buffer solution on for 90 minutes, stirring occasionally.
- Filter the permanganate using the vacuum system of the Fibertech.
- 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 two to three 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 a DM basis.

$$Lignin, \% = \frac{(Weight\ of\ ADF\ residue\ and\ crucible, B - Weight\ after\ oxidation, C)}{(Original\ weight, A\ x\%\ Dry\ matter)}\ x\ 100$$

8.10. Starch

Data of official tables and databases indicate that soy products, including soybean meal (SBM), contain 0-7% starch (Choct, 1997; CVB, 2018; Sauvant et al., 2004; NRC, 2012). Higher starch concentrations, 10.4-11.7%, have been reported in immature soybeans (Stevenson et al., 2006), indicating the rapid rearrangement or conversion of starch to protein and other carbohydrate fractions shortly before maturity. In addition to harvest time, two reasons have been advanced to explain the large variation in starch values. First and foremost, the starch values presented in tables and individual publications are the result of different analytical methods. These methods vary in the degree they inadvertently include variable fractions of free sugars or carbohydrates in the analyzed starch fraction. Secondly, in leguminous seeds especially, starch is encapsulated to variable degrees by the dominant protein fraction. This close association, or physical entrapment, also referred to as resistant starch (Englyst et al., 1994), along with the hydrophobic properties of these proteins, may interfere with the release and identification of starch molecules during analyses (Jezierny et al., 2017). In practice, the real starch content of commercial SBM, based on the enzymic method, is close to 1%.

The total carbohydrate fraction, which includes starch, has historically been equated to the nitrogen free extract (NFE) fraction. However, this calculated fraction represents a much larger part of soy products, ranging between 20-40% (CVB 2018; FEDNA, 2019). Consequently, other carbohydrates represent a more important part of most soy products. Two important sub-categories of carbohydrates are distinguished: the non-starch polysaccharides (NSP) and reducing sugars as determined by the Luff-Schoorl method. Contrary to many of the NSP constituents, pure starch and the reducing sugars are generally highly digestible. However, the difference between NSP and reducing sugars is largely represented by poorly digestible carbohydrates. These include cellulose, pectic and non-pectic NSP, and especially the oligosaccharides stachyose, raffinose and verbascose. In fact, the latter are considered anti-nutritional factors (ANF) for young animals.

Quantification of starch content, reducing sugars and NSP in soy products allows for a better identification and utilization of soy products in diets for different types of livestock and age groups.

Chemically speaking, starch is defined as a mixture of two polymers: linear alpha-1,4 linked glucose units, or simply amylose, and alpha-1,5 branched chains of alpha-1,4 linked glucose units, called amylopectin. In soybeans, the ratio between amylose and amylopectin is close to 20:80 (Stevenson et al., 2006).

Several analytical methods are used to estimate the starch content of soybean products, but the two most common methods are the polarimetric method and the enzymatic method. The polarimetric method — also referred to as the Ewers method — recuperates free sugars, pectin and a selection of NSP. Although it is the official method used for starch analysis in the European Union (Official Journal European Union, 2009), the method cannot be recommended for samples high in the above-mentioned substances or rich in optically active substances that do not dissolve in 40% ethanol (v/v). For soy products, this applies notably to SBM and full-fat soybeans (FFSB). 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 amyloglucosidase. The polarimetric method and the enzymatic method generally do not provide the same numerical starch value for an ingredient, feed or digesta sample. The Ewers value, or polarimetric method, is normally higher. The enzymatic method is more accurate and better in discriminating between true starch and related molecules. A comparison of starch analysis in the CVB (2018) 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 NSP 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 Annexes 1 and 2), the polarimetric method will result in higher values than the enzymatic method, and the enzymatic method should be preferred.

8.10.1 Polarimetric starch determination

The polarimetric method requires the following equipment and reagents:

- 250 mL Erlenmeyer flasks with standard ground-glass joint and with reflux condenser
- A polarimeter or saccharimeter
- Pipettes
- Filter paper
- A water bath plus
- 25% (w/w) hydrochloric acid (HCl), d: 1,126 g/mL
- 1.128% hydrochloric acid. This solution must be verified by titration with a 0.1 N sodium hydroxide (NaOH) solution in the presence of 0.1% (w/v) methyl red in 94% (v/v) ethanol
- Carrez solution I: Dissolve 21.9 g zinc acetate ($ZnC_4H_6O_4$) and 3 g glacial acetic acid (H_3COOH) into 100 mL water
- Carrez solution II: Dissolve 10.6 g potassium ferrocyanide ($C_4FeK_6N_4$) in 100 mL of de ionized water
- 40% (v/v) ethanol, d: 0.948 g/mL at 20 °C
- 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 0.5 mm mesh into a 100 mL volumetric flask.
- Add 25 mL hydrochloric acid and stir to obtain a homogenized solution and add another 25 mL hydrochloric acid.
- 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 one minute.
- Add 5 mL of Carrez solution II and stir, again, for one minute.
- Add water to the 100 mL level.
- Measure the optical rotation of the solution in a 200 mm tube with the polarimeter or saccharimeter.
- Optical rotation determination of dissolved substances in 40% ethanol
- Weigh 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 one 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 hydrochloric acid 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 to 20 °C 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 mL tube with a polarimeter or saccharimeter.
- The starch content of the sample is then calculated using the following equation. See measurement by polarimeter.

$$Starch,\ \% = \frac{2000\ x\ (Total\ rotation\ -\ dissolved\ rotation)}{specific\ optical\ rotation\ of\ pure\ starch}$$

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
Other types of starch or starch mixtures in compound feeds	184.0º

Measurement by saccharimeter.

$$\text{Starch content, \%} = \frac{2000}{[\alpha]_D^{20^\circ}} \times \frac{(2N \times 0.665) \times (S\text{-}S')}{100} - \frac{26.6N \times (S\text{-}S')}{[\alpha]_D^{20^\circ}}$$

Where:

S = total optical rotation in saccharimeter degrees.

S' = optical rotation in saccharimeter degrees of the substances soluble in 40% (V/V) ethanol.

N = weight (g) of saccharose in 100 ml of water yielding an optical rotation of 100 saccharimeter degrees when measured using a 200 mm tube (20,00 g for mixed saccharimeters).

$[\alpha]_D^{20^\circ}$ = specific optical rotation of pure starch.

8.10.2. Enzymatic or colorimetric starch determination

Several commercial kits are available for the determination of starch by this method. In this case, a strict adherence to the procedure provided with each kit is necessary.

The enzymatic method is much longer than the polarimetric one.

Reagents needed are:

- Acetate buffer solution, 0.2 mol at pH 4.5.
- Amyloglucosidase enzyme.
- Glucose reagent kit containing: Nicotinamide adenine dinucleotide (NAD), adenosine triphosphate (ATP), hexokinase, glucose-6-phosphate ($C_6H_{13}O_9P$), magnesium (Mg) ions, buffer and non-reactive stabilizers and filters.
- Glucose standards. Prepare three solutions of 100 mL each with 100, 300 and 800 mg/dL glucose, and 10, 30 and 300 mg/dL of urea nitrogen (N).

The total procedure takes three days.

Day one

- With each set of determinations, reagent blanks and glucose controls (100 µg, quadruplicate) should be included as well as a standard flour or starch sample.
- Grind soy product to be analyzed to pass a 0.5 mm screen.
- Weigh 125 mL Erlenmeyer flaks and record their weight to the nearest tenth of gram. Number of flasks to correspond to the number of samples (in duplicate), blank, glucose control and standard starch sample.
- To each Erlenmeyer add 25 mL of distilled water.
- Add 0.1 g of soybean product and swirl gently
- Place Erlenmeyers with samples on autoclave at 124 ºC 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 0.2 g of amyloglucosidase enzyme and swirl.

Day three

- Remove flasks from oven and let to cool at room temperature.
- Remove foil caps, weigh to the nearest 0.1 g and record weight.
- Pour contents into 50 mL centrifuge tubes and centrifuge at 1,000 x g for 10 minutes.
- Save supernatant in a plastic scintillation vial.
- Prepare a standard curve using the standard solutions as shown in Table 14.
- 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.
- Add 1.5 mL of glucose reagent agent into test tubes.

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

- Read and record absorbance at 340 nm vs. water as a reference. This will be INITIAL A, or the blank, in the calculations.
- Add 10µL of sample to the test tube. Mix gently.
- Incubate tubes for five 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, ΔA in the calculations.
- Calculate glucose concentration using the following equation.

$$glucose,\ mg/dl\ =\ standard\ concentration\ x\ \frac{FINAL\ A\ (sample)\ -\ INITIAL\ A\ (sample)}{FINAL\ A\ (standard)\ -\ INITIAL\ A\ (standard)}$$

- Calculate the content of alpha-linked glucose polymers as shown.

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

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

- Calculate starch content.

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

8.11. Reducing sugars and non-starch Polysaccharides

8.11.1 Determination of reducing sugars (Luff-Schoorl)

Reducing sugars are defined as any mono-, di-, oligo- or poly-saccharide capable of acting as a reducing agent. The Luff-Schoorl method allows for the determination of glucose, reducing sugars expressed as glucose and total sugars expressed as sucrose in feedstuffs. The method quantifies the amount of readily digestible and fermentable sugars in soy products, but it does not recuperate the non-reducing and undigestible sugars raffinose, stachyose and verbascose.

Reagents needed follow:

- Ethanol solution: 40% (v/v) neutralized to phenolphthalein.
- Carrez solution I: Dissolve 21.9 g zinc acetate dihydrate ($ZnC_4H_6O_4 \cdot 2H_2O$) and 3 g glacial acetic acid (H_3COOH) in water. Dilute to 100 mL with water.
- Carrez solution II: Dissolve 10.6 g potassium ferrocyanide ($C_6FeK_4N_6$) in water. Dilute to 100 mL.
- Methyl orange solution, 0.1% (w/v).
- Hydrochloric acid (HCl) solution, 4 mol/L.
- Hydrochloric acid solution, 0.1 mol/L.
- Sodium hydroxide (NaOH) solution, 0.1 mol/L.
- Luff-Schoorl reagent.
  - Copper sulfate solution: Dissolve 25 g copper sulfate ($CuSO_4$) pentahydrate, free from iron (Fe), in 100 mL water.
  - Citric acid solution: Dissolve 50 g citric acid ($C_6H_8O_7$) monohydrate in 50 mL water.
  - Sodium carbonate solution: Dissolve 143.8 g anhydrous sodium carbonate (Na_2CO_3) in approximately 300 mL of warm water. Leave to cool. Stir carefully.
  - Pour the citric acid solution into the sodium carbonate solution. Add the copper sulfate solution and make up to 1L with water. Leave to settle overnight and filter. Check the concentration of the reagent thus obtained: Cu 0.05 mol/L; Na_2CO_3 2N 1 mol/L. The solution's pH should be approximately 9.4.
- Sodium thiosulfate ($Na_2S_2O_3$) solution, 0.1 mol/L.
- Starch solution: Dissolve 5 g soluble starch in 30 mL water and add this to 1 L boiling water. Boil for three minutes, allow to cool and add 10 mg mercuric iodide (HgI_2) as preservative.
- Sulfuric acid (H_2SO_4) solution, 3 mol/L.
- Potassium iodide (KI) solution, 30% (w/v).
- Granulated pumice stone boiled in hydrochloric acid, washed in water and dried.
- 3-methylbutan-1-ol ($C_5H_{12}O$).
- Ethanol solution, 80% (v/v).

Apparatus used:

- Rotary shaker, 35-40 rpm.

Procedure follows:

- Sample extraction.
  - Weigh 2.5 g of the prepared sample to the nearest mg and transfer to a 250 mL volumetric flask.
  - Add 200 mL 40% ethanol and mix on the rotary shaker for one hour.
  - Add 5 mL Carrez solution I and stir for approximately 30 seconds.

- Add 5 mL of Carrez solution II and stir for one minute.
- Make up to volume with 40% ethanol, mix and filter.
- Remove 200 mL of the filtrate and evaporate to approximately half-volume in order to eliminate most of the ethanol.
- Transfer the residue quantitatively to a 200 mL volumetric flask using warm water, cool, make up to volume with water, mix and filter if necessary. This solution is used to determine the amount of reducing sugars and, after inversion, of total sugars.

- Determination of reducing sugars.
  - Using a pipette, remove not more than 25 mL of the solution containing less than 60 mg reducing sugars expressed as glucose.
  - If necessary, dilute the solution to 25 mL with distilled water and determine the content of reducing sugars by the Luff-Schoorl method as below.

- Determination of total sugars after inversion.
  - Using a pipette, take 50 mL of the solution and transfer to a 100 mL graduated flask.
  - Add a few drops of methyl orange solution.
  - Carefully and stirring continuously, add hydrochloric acid until the liquid turns a definite red.
  - Add 15 mL hydrochloric acid and immerse the flask in a boiling water bath for 30 minutes.
  - Cool rapidly to approximately 20 °C and add 15 mL NaOH solution.
  - Make up to 100 mL with water and mix.
  - Remove not more than 25 mL of the solution containing less than 60mg reducing sugars expressed as glucose.
  - If necessary, dilute the solution to 25 mL with distilled water and determine the content of reducing sugars by the Luff-Schoorl method.
- Titration by the Luff-Schoorl method.
  - Using a pipette, take 25 mL of Luff-Schoorl reagent and transfer to a 300 mL Erlenmeyer flask and add 25 mL of the clarified sugar solution.
  - Add 2 granules of pumice stone and 1 mL 3-methylbutan-1-ol, heat, while swirling by hand, over a free flame of medium height, so as to bring the liquid to a boil in approximately two minutes.
  - Place the flask immediately on an asbestos-coated wire gauze with a hole approximately 60 mm in diameter, under which a flame has been lit. The flame shall be regulated in such a way that only the base of the flask is heated.
  - Fit a reflux condenser to the Erlenmeyer flask and boil for exactly 10 minutes.
  - Cool immediately in cold water and after approximately five minutes, titrate as below.
  - Add 10 mL of KI solution and immediately add 25 mL of sulfuric acid added carefully in small increments to prevent excessive foaming.
  - Titrate with $Na_2S_2O_3$ solution until a dull yellow color appears; add the starch indicator and complete the titration.

- Blank titration.
  - Carry out the same f, without boiling, on a mixture of 25 mL of Luff-Schoorl reagent and 25 mL of water, after adding 10 mL of KI and 25 mL of sulfuric acid.
- Calculation.
  - Calculate the difference between the sample titration and the blank titration expressed in mg $Na_2S_2O_3$ solution 0.1 mol/L.
  - Using Table 15, establish the amount of glucose in mg which corresponds to the difference between the values of the two titrations.
  - Express the result as a percentage of the sample.

Table 15. Values for 25 mL of Luff-Schoorl Reagent

$Na_2S_2O_3$	Glucose, Fructose invert. Sugars		$Na_2S_2O_3$	Glucose, Fructose invert. Sugars	
0.1 mole/liter	$C_6H_{12}O_6$		0.1 mole/liter	$C_6H_{12}O_6$	
ml	mg	difference	ml	mg	difference
1	2.4	2.4	13	33	2.7
2	4.8	2.4	14	35.7	2.8
3	7.2	2.5	15	38.5	2.8
4	9.7	2.5	16	41.3	2.9
5	12.2	2.5	17	44.2	2.9
6	14.7	2.5	18	47.1	2.9
7	17.2	2.6	19	50	3
8	19.8	2.6	20	53	3
9	22.4	2.6	21	56	3.1
10	25	2.6	22	59.1	3.1
11	27.6	2.7	23	62.2	

Values show mL of sodium thiosulfate ($NA_2S_2O_3$) 0.1 mol/L after two minutes heating and 10 minutes boiling.

8.11.2. Non-starch polysaccharides and monosaccharides

A large part of many soy products belongs to the group of compounds classified as non-starch polysaccharides (NSP). As the name indicates, this group represents the totality of carbohydrates in the soy products minus the starch content. The NSP are an extremely heterogenous group of carbohydrate compounds. They are routinely split into three main groups, namely cellulose, non-cellulosic polymers and pectic polysaccharides, all of them mainly present in the primary or secondary cell wall of plants. On a dry matter (DM) basis, the total NSP fraction of soybean meal (SBM), approximately 40%, is composed of cellulose, + 8%, a mixture of pectic polysaccharides representing + 20%, and 10-14% in the form of free sugars, mainly sucrose, stachyose and raffinose, with trace quantities of verbascose (Choct et al., 2010). Removal of the cellulose fraction from NSP results in a sub-fraction commonly referred to as non-cellulosic polysaccharides (NCP) (Bach Knudsen, 2014).

In monogastric animals, the cellulose fraction is enzymatically undigestible and considered inert, except for its bulk-enhancing characteristic. This leaves the NCP fraction that can be divided in soluble and insoluble carbohydrates. The water-insoluble fraction represents + 65%, with the remainder being soluble NCP. However, estimates vary greatly depending on variety, origin, technological treatment and storage conditions (Choct et al., 2010). The soluble fraction, + 35%, is of considerable nutritional and physiological importance, since it increases the viscosity of the digesta and reduces digestibility and absorption of nutrients. This is of

special importance in young animals and the reason this fraction is often considered part of the ANF.

Consequently, a correct estimation of these carbohydrates, or the monosaccharides that make-up this fraction of the NSP, is important when formulating diets.

The total dietary fiber (TDF) fraction can be measured by a gravimetric method, the traditional method for collective analyses of NSP. However, it is not an accurate estimation of total NSP since the recovery of cellulose, hemicelluloses and lignin is low. The method involves chemical or enzymatic solubilization of dietary protein, starch and fat, followed by weighing of the insoluble residue and subtraction of the separately determined lignin fraction.

A method based on a colorimetric analysis or gas chromatography (GC) was proposed by Englyst et al. (1994). The method calls for complete enzymatical removal of starch and the determination of NSP as the sum of the constituent sugars released by acid hydrolysis. The individual sugars can subsequently be measured by GC.

For analysis of the constituent simple sugars, high-performance liquid chromatography (HPLC) is preferred, requiring 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, ground to pass a 0.5 mm screen, in Hungate tubes.
- Add 2.5 mL of acetate buffer, 70 mL 0.1 mol sodium acetate ($C_2H_3NaO_2$) and 30mL of 0.1 mol acetic acid (H_3COOH).
- 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 six hours and shake every 30 minutes.
- Cool to room temperature.
- Add 10.5mL of pure ethanol.
- Place in refrigerator for one hour.
- Centrifuge at 1,000 x g for five 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.

$$Total\ NSP,\ \% = 100 - (humidity,\ \% + ash,\ \% + protein,\ \% + lipids,\% + lignin,\ \% + starch,\%)$$

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, from 7 5mL of 96% sulfuric acid and 25 mL of water.
- Vortex.
- Place in water bath 30 °C for one hour.
- Transfer sample into a 100 mL Erlenmeyer and add 40mL of distilled water.
- Add 5 mL of myo-inositol ($C_6H1_2O_6$), (2mg/L, as an internal standard.
- Cover Erlenmeyer with aluminum foil and autoclave at 125 °C for one hour.
- Filter sample.
- Resuspend 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 mol ammonium hydroxide (NH_4OH).
- Vortex.
- Add 100 µL of 3 mol NH_4OH containing 150 mg/mL of potassium borohydride (KBH_4). Borate is very toxic; all following steps must be conducted under a hood.
- Place in a 40 °C water bath for one hour.
- Add 100 µL of glacial acetic acid and vortex.
- Transfer 500 µL into a 30 mL glass tube.
- Add 500 µL of 1-metilimidazole ($C_4H_6N_2$).
- Add 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 mol potassium hydroxide (KOH), vortex and wait three minutes.
- Add, again, 5 mL of 7.5 mol KOH, vortex and wait three minutes.
- Cover tubes.
- Take a 1 mL aliquot and transfer into a 5mL test tube.
- Add 50 mg of anhydrous sodium sulfate (Na_2SO_4).
- Decant supernatant into a gas liquid chromatography (GLC) vial.
- Dry at 40 °C for 8-10 hours.
- Add 0.5 mL of chloroform.

Chromatography is the final step.

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

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 (Ca). 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 hydrochloric acid (HCl) prior to the ether extraction.

The EE technique requires the following materials:

- A Soxhlet extraction system
- Funnels
- Filter paper
- 3 N hydrochloric acid
- Anhydrous diethyl ether

The procedure is as follows:

- Weigh approximately 2 g of sample, ground through 1 mm mesh into an Erlenmeyer.
- Add 100 mL of 3 N hydrochloric acid and boil for one hour.
- Cool at room temperature.
- Filter through a filter paper and rinse with distilled water to remove all hydrochloric acid.
- Remove the moisture of the sample by drying it in an oven at 105 °C for 24 hours. If the sample was 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 eight more hours.
- Remove from Soxhlet, air-dry for about two hours and oven dry at 105 °C for 12 hours.
- Calculate crude fat as follows.

$$Crude\ Fat,\% = \left(\frac{Final\ weight\ after\ extraction, g}{Original\ weight, g} \right) x100$$

8.13. Lipid quality

Fat or oil quality depends on the fatty acid (FA) profile, specific physical characteristics and oxidation level. While FA characteristics and composition determine physical and nutritional quality of the true lipid fraction, the physical characteristics and oxidation level are most important in the routine quality control (QC) procedures 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 Moisture, Insolubles, Unsaponifiables (MIU) value.

8.13.1. Moisture

Through the crushing and various treatments of soy oil, water may settle in oil samples, especially if they have undergone significant temperature changes. Generally the moisture content is small, but it may have a large effect on oil quality. The procedure is simple and calls for the following equipment:

- A forced air-drying oven capable of maintaining 130 °C (± 2 °C)
  - **Attention:** High temperatures may cause the fat sample to ignite
- Aluminum sample pans with tight-fitting covers
- A desiccator

The procedure follows.

- Accurately weigh 5.0 g (± 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 for 30 minutes 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-minute drying period.
- Calculate.

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

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.

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

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

8.13.3. Unsaponifiable matter

The method measures those substances that 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, 1993) requires the following materials:

- Erlenmeyer or Soxhlet flasks
- Beakers
- Separator funnels
- A balance with accuracy of ± 0.001g
- 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
- A desiccator

The reagents for this method follow:

- 85% Ethanol
- Petroleum ether
- 0.02 N sodium hydroxide (NaOH)
- 45% aqueous potassium hydroxide (KOH)
- Phenolphthalein indicator solution
- 0.2 mol hydrochloric acid (HCL) standard
- Deionized water

The procedure is as follows:

- Accurately weigh 5 g (+ 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. If the sample is solid at room temperature, melt it 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 KOH.
- Assemble the extractor by turning on the hot plates and the water taps. Reflux the solution gently but steadily for one 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 5 mL of warm water and pour it into the separator funnel.

- Add approximately 5 mL of cool distilled water, swirl and pour it into the separator funnel.
- Complete the transfer from the flask by rinsing with approximately 5 mL 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 250 mL Erlenmeyer flask.
- Repeat the previous four steps until the petroleum ether layer is clear and colorless, about six times.
- Once the washes are completed, discard the fat portion of the sample in a waste container and transfer all the petroleum ether to the 500 mL separator funnel.
- Add 30 mL 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 five or six times, or until the bottom layer no longer turns into a pink color after addition of one drop of phenolphthalein indicator solution.
- Transfer 60 mL of the ether layer, or 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 NaOH 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 FA in the sample.
- The unsaponifiable matter is calculated as follows.

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

8.13.4. Iodine value

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

- Glacial acetic acid (H_3COOH)
- Carbon tetrachloride (CCl_4)
- Iodine trichloride (I_2Cl_6)
- Iodine (I)
- Potassium iodide (KI), 100 g/L aqueous solution
- Sodium thiosulfate ($Na_2S_2O_3$), 0.1 N, or 19.76 g $Na_2S_2O_3$ into 230.24 mL water
- Potassium iodate (KIO_3), 0.4 N
- Starch solution: 10 g/L aqueous dispersion recently prepared from natural soluble starch
- Wijs solution: Add 9 g of trichloride into a brown glass bottle with 1,500 mL capacity. Dissolve in 1 L of a mixture composed of 700 mL of acetic acid and 300 mL of CCl_4

The procedure is as follows:

- Determine the halogen content of the Wijs solution by taking 5 mL of the solution and adding 5 mL KI and 30 mL water. Then add 10 mL of pure iodine and dissolve by shaking. Determine again the halogen content as previously described. The titer should now equal 1.5 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 1.5 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 CCl_4 and 25 mL of Wijs reagent. Insert a stopper and shake gently.
- Let sample sit in a dark location for 60 minutes for fats with expected IV below 150, and for 120 minutes for fats with expected IV above 150.
- Remove the flask from the dark and add 20 mL aqueous KI solution and 150 mL distilled water.
- Titrate the solution with 0.1 N $Na_2S_2O_3$ until the yellow color has mostly disappeared.
- Add 1-2mL 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.

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

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 and leads to 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.

8.13.5. Acid value

The acid value is a measurement of the proportion of free fatty acid (FFA) in a given sample. It is determined by measuring the mg of KOH required to neutralize 1 g of fat. Oxidation is not involved directly in FFA 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 of 95% ethanol/diethyl ether, 1/1, (v/v).
- 0.1 mol KOH in ethanol accurately standardized with 0.1 mol hydrochloric acid. Pure ethanol may be also used if aqueous samples are analyzed.
- 1% phenolphthalein in 95% ethanol.

The procedure is as follows:

- Weigh 0.1-10 g of oil according to the expected acid value in glass vial and dissolve in at least 50 mL of the solvent mixture 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 (five drops of indicator), the pink color persisting for at least 10 seconds.
- The acid value is calculated by the following formula.

$$Acid\ value = 56.1\ x\ KOH\ Normality\ x\ \frac{ml\ of\ KOH}{weight\ of\ orginal\ sample, g}$$

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 FA, and the possible formation of toxic compounds and changes in color of the product.

The important lipids involved in oxidation are the unsaturated FA moieties, oleic, linoleic and linolenic. The oxidation rate of these FA 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.

(3) Termination, the formation of non-radical products.

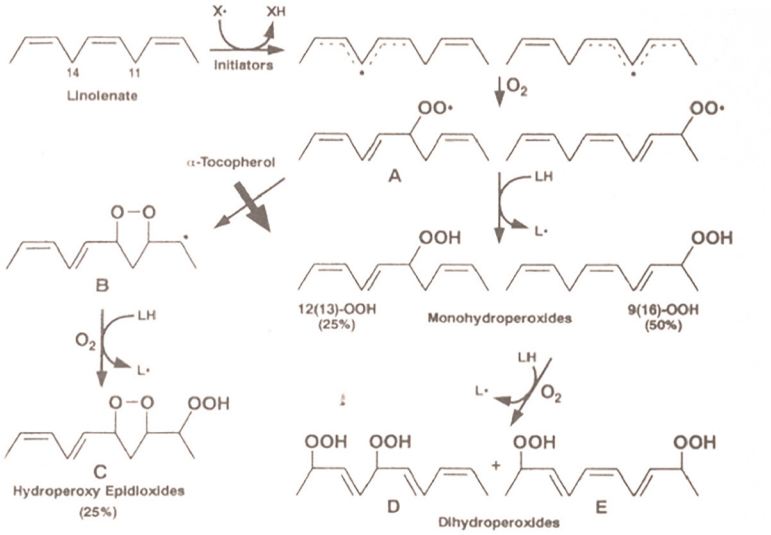
Chain branching consists of the degradation of hydroperoxides and the formation of new hydroxyl radicals, which will then induce a new oxidation. During the process, secondary products are formed from the decomposition of lipid hydroperoxides, producing a number of compounds that may have biological effects and cause flavor deterioration in feed. These compounds include aldehydes, ketones, alcohols, hydrocarbons, esters, furans and lactones (see Figure 3).

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 soybeans are ground, the greater the exposure and thus, the greater the risk of oxidation. Evidently, soybean oil in its pure form, with no additives, is very susceptible to oxidation.

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

- Determination of primary products of oxidation provides peroxide value.
- Determination of secondary products of oxidation can be done with:
  - Colorimetric methods of thiobarbituric acid (TBA) and anisidine value.
  - Volatile compounds determination through chromatography.
- Stability tests are the active oxygen method (AOM) and oil stability index (OSI).

Figure 3. Auto-oxidation of linolenic acid



Source: Frankel, 1984.

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 KI solution, 150 g KI 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 0.01 N $Na_2S_2O_3$, 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 follow.

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

Although the peroxide value is applicable to peroxide formation at the early stages of oxidation, it is highly empirical. During 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. A single peroxide value cannot be indicative of a product's real oxidation state. Also, this test is extremely sensitive to temperature changes, potentially leading to poor repeatability.

8.13.6.2. Thiobarbituric acid

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. TBA has a high affinity to carbonyl substances, aldehydes and ketones. Its reaction with aldehydes, especially with malonaldehyde, a secondary oxidation product of FA 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 malonaldehyde and two molecules of TBA to form a red malonaldehyde-TBA complex, which can be quantified with a spectrophotometer at 530 nm. However, this method has been criticized as being nonspecific and insensitive for the detection of low levels of malonaldehyde. Other TBA- reactive substances, including sugars and other aldehydes, could interfere with the malonaldehyde-TBA reaction.

The procedure was first described by Witte et al. (1970). The technique requires and the following reagents and equipment:

- A spectrophotometer
- A water bath
- Pipettes
- Test tubes
- TBA solution: 0.02 mol 4, 6-dihydroxypyrimidine-2- thiol, i.e., 1.44 g/500 mL distilled water
- M-phosphoric acid (H_3PO_4) solution 1.6% (v/v)
- Standard solution: 10.2 mol 1,1,3,3-tetraethoxipropyl (TEP), 0.2223 g/100 mL
- Trichloroacetic acid (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% TCA 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 mol 4, 6-dihydroxypyrimidine-2-thiol and mix
- 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

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). This reacts with an acetic acid solution of p-anisidine, measuring 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.

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 AOM and OSI.

8.13.6.4.1. 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, generally 100 mEq/kg oil, under the specific and controlled conditions of the test. The length of this time period is assumed to be an index of resistance to rancidity. Peroxide value is determined as under Section 8.13.6.1.

The more stable the lipid or oil, the longer it will take to reach the predetermined value. For products other than oils, such as full-fat soybeans (FFSB), 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.

8.13.6.5.2. 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 the predetermined value. A major advantage of this method is that multiple samples can be tested simultaneously.

8.13.7. Fatty acid profile

The FA profile is, from a nutritional point of view, the most important characteristic of oils for at least two reasons. First, 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 FAs 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 is attributed to global warming, as high temperatures induce a reduction in polyunsaturated acids in soybeans. Second, the FA composition, which can be expressed as the unsaturated:saturated (U/S) ratio, is exponentially related to the usable energy in poultry and swine (Wiseman et al., 1991; Wiseman et al., 1998).

Therefore, by determining the FA composition, it can be verified if a given oil is soybean oil. Using the U/S ratio, energy estimations can be accomplished for loads of soybean oil of different qualities using Wiseman's equations. Besides the U/S ratio, Wiseman's equations require the FFA content, the acid value as in Section 8.13.5 above, of the oil. By correcting the right-hand side of the equations with the MIU value, a reasonable and practical assessment of the energy content of a commercial load of soybean oil is obtained.

The FA profile can be determined by gas or liquid chromatography. The most common procedure is the gas liquid chromatography (GLC) procedure. For this analysis, a pure sample of oil is used after removal of MIU. Sample preparation requires the following reagents:

- Metanolic-HCl (5% v/v): Add 10 mL acetyl chloride (CH_3COCl) into 100 mL anhydrous methanol
- 6% potassium carbonate (K_2CO_3): 15 g K_2CO_3 into 250 mL distilled water

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

- Weigh 0.15 g of sample into 10 mL test tubes.
- Add 0.5 mL of an internal standard, i.e., 2 mg of C19 per 1 mL of toluene.
- Add 0.5 mL of toluene.
- Add 1.5 mL of metanolic-hydrochloric acid.
- Close tubes to avoid sample loses.
- Vortex for one minute.
- Place in water bath at 70 °C for two hours.
- Cool at room temperature.
- Add 2.5 mL of the K_2CO_3 solution.
- Add 1 mL of toluene.
- Vortex for 30 seconds.
- Centrifuge at 3,000 rpm for 5 minutes.
- Keep the supernatant and add 0.5 g of anhydrous sodium sulfate (Na_2SO_4).
- Vortex for 30 seconds.
- Centrifuge at 4,000 rpm for 10 minutes.
- Collect the supernatant and place in gas chromatography (GC) vial for subsequent GC analysis.

For operation of the GC equipment and analyses of FA, 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.

8.14. Minerals

Mineral composition of soy products can vary considerably among and within products. The concentration of minerals depends on soybean origin and crop growing conditions, variety and the different types of extraction processes applied to obtain the soy product. Although a measure of the concentration of these minerals is important for most feed applications, table values are used to formulate under routine feed production conditions. 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 (Ca) and phosphorus (P). 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 P use and excretion by animals make a precise estimate of this element in the feed necessary. P concentrations in soy products are high, and except for soybean hulls and soybean mill feed, P levels in these products are a multiple of Ca levels unless limestone has been added as a flow agent. This makes analysis for P, both from a regulatory and nutritional point of view, important. In addition to Ca and P, sodium (Na) and chlorine (Cl) analyses should be carried out on a routine basis for quality control (QC) purposes

Atomic absorption spectrophotometry

Under more sophisticated laboratory conditions, a wider spectrum of minerals, including micro-minerals, can be analyzed by a range of spectrophotometric methods, including atomic absorption spectrophotometry (AAS) or flame-atomic absorption spectrometry (FAAS). This requires a considerable amount of investment in equipment and expertise. However, following proper installation and training, the method is very suitable for precise, routine analysis of most minerals.

AAS requires a relatively simple, but critical, sample preparation. Like in many other analyses, sample preparation in mineral analysis is responsible for most of the analytical errors.

Liquid samples can be analyzed directly after filtration or purification. Solid feed or food samples — thus soy products — involve digestion, extraction and preparation of the analytes before the analysis. The principal objectives of AAS sample preparation are dissolution of the analytes in a suitable solvent and concentration if necessary, as in case of extreme diluted mineral levels. Dissolution can be accomplished by digestion of the ash residue obtained after dry-ashing in a mineral acid (see Section 8.2). Alternatively, the following wet-ashing procedure can be applied (Uddin et al., 2016):

- Accurately weigh 0.5 g (±0.001 g) sample and place in a 100 mL beaker
- Add 9 mL of an acid mixture of 65% nitric acid (HNO_3) and 37% hydrochloric acid (HCl). Mix.
- Heat gently to digest in a water bath at 95 °C for 4-5 hours, or until the sample has completely dissolved.
- During the digestion procedures, the inner walls of the beakers are washed with 2 mL of deionized water to prevent sample loss.
- Following complete digestion, filter through Whatman 42 filter paper with 2.5 µm particle retention.
- Add deionized water to make the final volume up to 50 mL.

In AAS, the aqueous sample is aspirated in the flame atomizer by the nebulizer to measure the analyte concentration at parts per million (ppm) concentration. A standard is included to determine analyte concentrations.

Equipment recommended operating conditions need to be adjusted to the specific mineral to be determined. This refers specifically to the type of lamp, lamp current and wavelength, slit width of the burner and fuel, type of gas and support. Each mineral and equipment has its own specific operating conditions and consultation of the manual provided by the supplier is necessary.

For normal QC objectives, classical wet chemistry can be used to estimate the content of the most important minerals..

8.14.1. Calcium

The determination of Ca by wet chemistry requires the following equipment:

- A set of porcelain dishes
- 250 mL volumetric flasks
- 250 mL beakers
- Filter paper
- Funnels
- A burette

The following reagents are needed:

- Hydrochloric acid (1 to 3 v/v)
- Nitric acid (70%)
- Ammonium hydroxide (NH_4OH), 1 to 1 v/v
- Methyl red indicator. Dissolve 1 g in 200 mL alcohol
- Ammonium oxalate $(NH_4)_2C_2O_4$, 4.2% solution
- Sulfuric acid (H_2SO_4), 98%
- Standard potassium permanganate ($KMnO_4$) solution, 0.05 N

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 or 25 mL for mineral feeds, into a beaker, dilute to 100 mL and add two 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 sulfuric acid.
- Heat to 70 °C and titrate against the standard $KMnO_4$ solution.
- Calculate.

$$Ca\ (\%) = \frac{mL\ permanganate\ solution}{Weight\ of\ sample,\ g} \times \frac{Aliquot\ used\ (mL)}{250} \times 0.1$$

8.14.2. Phosphorus

P is one of the most critical nutrients in any ingredient or diet. All animals have a P requirement, and most ingredients are deficient in digestible P. Consequently, inorganic P or a phytase enzyme needs to be added to diets to increase its availability. Either solution adds to the cost associated with this essential mineral.

Excess P — excreted in manure — is considered a possible pollutant of soil and water, and many countries or regions have set limits to the excretion of P in manure. This has led to the establishment of upper limits for P in diets and/or restrictions on the number of animals per unit of available crop area, further stimulating the use of phytases. These restrictions, along with the relatively low levels of P in feed ingredients, have led to P being considered the second or third most expensive nutrient in least-cost formulations.

The method for P analysis requires the following equipment and reagents:

- A spectrophotometer that can read at 400nm
- 100 mL volumetric flasks
- Molybdovanadate reagent
- To obtain this, dissolve 40 g ammonium molybdate ($(NH_4)_2MoO_4$) 4H0 in 400 mL hot water and cool
- Dissolve 2 g ammonium metavanadate (NH_4VO_3) in 250 mL hot water, cool and add 450 mL 70% perchloric acid ($HClO_4$)
- Gradually add the molybdate to the vanadate solution with stirring and dilute to 2 L

P standards:

- Prepare stock solution by dissolving 8.788 g potassium dihydrogen orthophosphate (KH_2PO_4) in water and making up to 1 L.
- Prepare the working solution by diluting the stock 1 in 20, working concentration = 0.1 mg P/mL.

The procedure to determine P follows:

- Pipette an aliquot of the sample solution prepared as for the Ca determination into a 100 mL flask and add 20 mL of the molybdovanadate 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 P to 100 mL flasks and treat as above.
- Read sample at 400 nm setting the 0.5 mg standard at 100% transmission.
- Determine mg P in each sample aliquot from a standard curve.

8.14.2.1 Available and phytate phosphorus

The determination of the total amount of P in soy products, as well as in any other ingredient of plant origin, is only one analytical element in the quality assessment of the value of P, because a fraction of total P is not available to monogastric animals. In soy products, much of the P is present in the phytin molecule and is referred to as phytic or phytate phosphorus (PP). The P not bound to the phytin molecule is referred to as non-phytin P (NPP). Although there are different schools of thought on how to deal with the subject, the current recommendation is to estimate the available phosphorus (aP) in soy products and other ingredients of vegetable origin by subtracting analyzed PP from analyzed total P (Angel et al., 2002). There are now a number of assays available to determine PP or aP.

The original methods and those that are used for detailed analyses are tedious and require major investments in equipment, such as high-performance liquid chromatography (HPLC). However, more recently several quick tests have become available in the form of assay kits, each with their specific procedure.

Due to the cost of P and environmental restrictions, many monogastric diets are now formulated with the inclusion of phytase, which — according to the type and concentration — will allow variable amounts of PP to be converted to aP. The amount of phytase added depends on the total PP level and clearly represents an additional cost. Consequently, the PP level of all ingredients has a major effect on formulation cost. Lower PP levels combined with equal or higher aP concentrations add significantly to the value of an ingredient, including soy products. Substantial differences in these parameters have been shown to exist among soy products and soy products of different origin (Mateos, personal communication). These differences are considered in the formulation of diets and add significantly to the value differences of soy products of different origin.

8.14.2.2. Phytate analysis

One of the methods available to quantify PP is the anion exchange method (AOAC, 2012), in which phytate is extracted from ingredients of vegetable origin with dilute hydrochloric acid (HCl). The extract is then mixed with an ethylenediaminetetraacetic acid (EDTA)-sodium hydroxide (NaOH) solution and placed on an ion-exchange column. Phytate is eluted with 0.7 mol sodium chloride (NaCl) solution and wet-digested with a mixture of concentrated nitric acid (HNO_3)-sulfuric acid (H_2SO_4) to release P, which is measured colorimetrically in a similar fashion as 8.14.2 above.

- Hydrochloric acid, 2.4%. Add 5 4mL of hydrochloric acid to 1 L volumetric flask and make to volume with water.
- NaCl solutions 0.1 and 0.7 mol.
- Phosphate standard solution. 80 µg P/mL. Weigh 0.350 g dried, desiccated potassium acid phosphate (KH_2PO_4) primary standard into 1 L volumetric flask, add approximately 500 mL water and 10 mL 5 mol sulfuric acid dilute to volume with water. This solution is stable.
- Molybdate solution. 2.5% ammonium molybdate in 0.5 mol H_2SO_4. Dissolve 12.5 g ammonium molybdate in 200 mL water. Transfer to 500 mL volumetric flask, add 50 mL 5 mol sulfuric acid and dilute to volume with water. This solution is stable.
- Sulfonic acid reagent. Dissolve 0.16 g 1-amino-2-naphthol-4-sulfonic acid ($C_{10}H_9NO_4S$), 1.92 g sodium sulfite (Na_2SO_3), and 9.60 g sodium bisulfite ($NaHSO_3$) in 90 mL of water. Quantitatively transfer to 100 mL volumetric flask. Heat to dissolve if necessary. Store in brown bottle in refrigerator. Prepare fresh weekly.
- Na_2EDTA-NaOH reagent. In 250 mL flask, stir 10.23 g Na_2EDTA (0.11 mol) and 7.5 g NaOH (0.75 mol). Dilute to volume with water. This solution is stable.
- Phosphate standard curve. Adjust spectrophotometer to 640 nm and equilibrate at least 15 minutes. Pipet 1.0, 3.0 and 5.0 mL of P standard solution into 50 mL flasks. Add approximately 20 mL water. Mix thoroughly. Add 2 mL molybdate solution. Mix well. Wait 15 minutes and read in spectrophotometer at 640 nm. See Table 16 for the calculations for typical standard curve.

Table 16. Calculations for typical phosphate standard curve

ml, standard	µg P	A	Concentration/A (K)
1.0	80	0.1805	443.21
3.0	240	0.516	465.12
5.0	400	0.852	469.48
"Mean K"			459.27

(AOAC, 2012)

To determine phytate, steps follow:

- Weigh approximately 2.0000 g of test portion and place in 125 mL Erlenmeyer.
- Add 40 mL of the 2.4% HCl solution, 20 mL/g of test portion.
- Cover flask and shake vigorously for 3 hours at room temperature.
- Meanwhile, prepare columns. Glass barrel columns: 0.7 x 15 cm equipped with valve, Econo-columns, Bio-Rad Laboratories or equivalent.
- Add 3 mL water to empty mounted column and then pour water slurry of 0.5 g resin into column. Anion exchange resin: AG1-X4, 100-200 mesh, chloride form, Bio-Rad Laboratories. Check resin by measuring recovery of purified sodium phytate.
- Remove test solution from shaker and filter with vacuum through Whatman No. 1 paper. The extract is stable at least one week if refrigerated.
- Prepare blank by mixing 1 mL of the 2.4% hydrochloric acid solution with 1 mL of Na_2EDTA-NaOH reagent, dilute to 25 mL with water, and pour mixture onto column.
- Pipet 1 mL filtrate into 25 mL glass-stoppered graduate.
- Add 1 mL of Na_2EDTA-NaOH reagent. Dilute to 25 mL with water.
- Mix and quantitatively transfer to column. Discard eluate.
- Elute with 15 mL water. Discard eluate.
- Elute with 15 mL 0.1 mol NaCl. Discard eluate.
- Elute with 15 mL 0.7 mol NaCl; collect this 0.7 mol fraction in digestion vessel, micro-Kjeldahl flasks, 100 mL or 25 x 20 mm digestion tubes.
- Add 0.5 mL sulfuric acid and 3 mL nitric acid to flask.
- Add three glass beads.
- Before adding next test solution to column, pour 15 mL water through column. After one week or three test solutions, discard old resin and replace with fresh resin.
- Digest under hood on micro-Kjeldahl rack over medium heat until active boiling ceases and cloud of thick yellow vapor fills neck of flask. Heat contents five minutes more on medium heat, five minutes on low heat, then turn off burner.
- When flask is cool, add approximately 10 ml water, swirl or heat flask on low temperature setting if necessary to dissolve salt. Continue heating flask on low temperature 10 minutes. Let solution cool.
- Quantitatively transfer solution to 50 mL volumetric flask. Add 2 mL of the molybdate solution; mix well.
- Add 1.0 mL of the sulfonic acid reagent; mix well.
- Dilute to volume, mix well, let stand 15 minutes, and read A at 640 nm.
- Calculate phytate concentration in the soy product under study or the ingredient of vegetable origin.

Where:

A = absorbance; “mean K” = standard P (µg)/A/n (standards);
phytate = 28.2% P

8.14.3. Sodium chloride

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

- Standard 0.1 N silver nitrate ($AgNO_3$) solution.
- Standard 0.1 N ammonium thiocyanate (NH_4SCN) solution.
- Ferric indicator: Saturated aqueous solution of ferric aluminum.
- Potassium permanganate ($KMnO_4$) solution, 6% w/v.
- Urea solution, 5% w/v.
- Acetone, AR grade.
- The method consists of the following.
- Weigh a 2 g sample into a 250 mL conical flask.
- Moisten the sample with 20 mL water and then pipette 15 mL 0.1 N AgNO3 solution. Mix well.
- Add 20 mL concentrated nitric acid and 10 mL $KMnO_4$ 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 $AgNO_3$ with the 0.1 N thiocyanate solution to the red-brown end point.
- Calculate.

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

For rapid, routine Q.C. procedures, Quantabs, a benchtop test kit is used.

8.15. Isoflavones

In many diets, both human and animal, 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. Interest is also increasing 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., 2000; Kerley et al., 2003).

Two forms of isoflavones can be determined: the bound glucoside form in genistin, daidzin and glycitin, and the free aglycone form in genistein, daidzein and glycitein. Lee et al. (2003) reported that the total isoflavone contents in soybean cultivars grown in Korea ranged from 110-330 mg 100/g. The U.S. Department of Agriculture (USDA) and Iowa State University have developed a database on isoflavones from scientific articles (2002). 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 (C_2H_3N)
- Hydrochloric acid (HCl), 0.1 N, or phosphoric acid (H_3PO_4)
- Isoflavone standards, commercial source

Besides normal laboratory equipment, the assay requires an HPLC instrument with a YMC-pack ODS-AM-323 column with specification of 10 µm particle size, 25 cm long and an inner diameter of 10 mm.

The procedure consists of an isoflavone extraction and an HPLC quantification step:

- For the extraction, mix 2 g ground soybean products with 2 mL of hydrochloric acid and 10 mL of acetonitrile in a 125 mL flask, stir for two hours and filter.
- The filtrate is dried under vacuum at a temperature below -30 °C and then redissolved in 10 mL of 80% HPLC-grade methanol in distilled water.
- The sample is then filtered through a 0.45 µm filter unit and then transferred to 1mL 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 (H_3COOH) 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 minutes and then held at 35% for 10 minutes.
- The recommended flow rate is 1 mL/minute and the detection wavelength is 200-350 nm.

The content of each isoflavone is expressed on a w/w basis.

8.16. Anti-nutritional factors

One of the most important restrictions in the use of soybeans and their products in animal diets are the presence of a relatively large number of anti-nutritional factors (ANF). The presence of ANF is also the main reason different technological treatments are applied to soybeans or their products. The ANF in soybeans include compounds classified as protease inhibitors, phytohemagglutins (lectins), lipoxygenases and antivitamin factors, which can be destroyed relatively easily 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 not destroyed by heat are the poorly digested carbohydrates, saponins, estrogens, cyanogens and phytate (Liener, 2000). Likewise, heat treatment will also destroy urease in soybeans, although this compound is not generally considered an ANF. In the case of soybean products, trypsin inhibitor (TI) is the most abundant and best known ANF , followed by soybean lectins. The quality of technological treatment to destroy ANF is mostly related to residual TI.

To analyze for any ANF, many different methods and procedures are available, ranging from instrumental methods like high-performance liquid chromatography (HPLC), gas chromatography (GC) or capillary electrophoresis (CE) to thin-layer chromatography (TLC) and immunoassays. The reliability and accuracy of results obtained with these methods vary, and no preferred method has been defined for all ANF. When possible, and for practical routine quality control (QC) purposes, the use of enzyme-linked immuno-sorbent assay (ELISA) tests are recommended.

The ELISA tests rest on the principle that the compound of interest 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, which may be 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 that is inversely related to the antigen concentration. Thus, the deeper the color, the smaller the antigen, or 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.

8.16.1. Trypsin inhibitors

The residual TI in soy products combines with the trypsin in the small intestine and forms an inactive complex, reducing protein digestibility. In addition to the negative effect on protein digestibility, TI induces pancreatic hypertrophy in some species of poultry, but not in swine. This hypertrophy may lead to an increase in secretion of trypsin and losses of endogenous nitrogen (N). TI has been correlated with field observation of rapid feed passage (RFP) syndrome in broilers and broiler breeders (Ruiz et al., 2005). It has been proposed that this syndrome, which includes the presence of undigested feed and sloughing of intestinal tissue in the excreta, is the manifestation of an excess in TI intake. The combined effect on the animal is a reduction in N retention, growth and feed conversion. Detailed control laboratory work on the effects of excess TI intake and excess lectin intake has been conducted with growing swine and shows striking similarities with RFP syndrome in broilers (Schulze, 1994; Schulze et al., 1995). However, in swine it is simply referred to as a form of diarrhea.

The procedures described to determine trypsin inhibitor activity (TIA) are based on the ability of the inhibitors to form a complex with the enzyme and reduce enzyme activity. Uninhibited trypsin catalyzes the hydrolysis of a synthetic substrate α-Benzoyl-DL-arginine-p-nitroanilide (BAPNA), forming a yellow- colored product and producing a change in absorbance. The reference procedures proposed by the American Oil Chemists' Society (AOCS, 2011b), the French Association for Normalization (AFNOR, 1997), and the International Organization for Standardization (ISO, 2001) are based upon the work of Kakade et al. (1969; 1974). Here, the AOCS and the ISO procedures are summarized, but the only difference with the AFNOR (1997) procedure is the composition of the extraction buffer, which is alkaline rather than acid. Still, these procedures are not very well-adapted for routine QC use, and a well-equipped lab with a skilled technician is necessary.

For practical reasons, the methods described measure total TI. They reflect the concentration and effects of two distinctively different types of inhibitors, Kunitz trypsin inhibitor (KTI) and Bowman-Birk inhibitor (BBI).

The AOCS (2011b) method needs the following reagents:

- Hexane or petroleum ether.
- 0.01 N sodium hydroxide (NaOH) solution.
- Tris buffer: Dissolve 6.05 g hydroxyl-methyl)-amino-methane (Tris) and 2.94 g calcium chloride ($CaCl_2$) in 900mL 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 0.001 N hydrochloric acid (HCl).
- BAPNA solution: In a water bath, dissolve 40 mg BAPNA in 1 mL dimethyl sulfoxide ($(CH_3)_2SO$). Dilute to 100 mL with Tris buffer at 37 °C. Prepare new solution daily. Maintain at 37 °C for use.
- Acetic acid (H_3COOH) solution, 30%: Mix 30 mL glacial acetic acid and 70 mL water with caution.

Equipment required:

- A grinding mill with screen size 0.15 mm or smaller
- 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.
- 1 g of ground sample is subsequently weighed into a beaker containing a magnetic stirring bar. Add 50 mL NaOH solution and agitate the suspension slowly. After three hours, measure the pH. The pH should range between 8.4-10.0.
- Take an aliquot of suspension with a serological pipette and dilute with distilled water so that soybean TI concentration is sufficient for 40-60% TI. When it is not possible to estimate the expected trypsin inhibitor units (TIU), make more than one dilution.
- With serological pipettes, add 0, 0.6, 1.0, 1.4 and 1.8 mL of the diluted suspension to duplicate sets of test tubes. Then add water to bring the volume to 2 mL in each tube.
- With a regular time interval for the different tubes, add 2 mL trypsin solution to each tube, quickly mix on the Vortex stirrer and place in the 37 °C water bath. Add 5 mL BAPNA to each tube and mix on Vortex stirrer. The samples are incubated for 10 minutes at 37 °C. After exactly 10 minutes, the reaction is stopped by adding 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.
- Calculate TIA in TIU/g. one trypsin unit is arbitrarily defined as the amount of enzyme that will increase absorbance at 410 nm by 0.01 unit after 10 minutes of reaction for each 10 mL of reaction volume (Kakade et al., 1969). TIA is defined as the number of TIU.

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

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/g sample.

$$TIU(/g) = TIU\ (/ml)\ x\ d\ x\ 50$$

Where:

d = dilution factor, or 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.

The ISO (2012) method needs the following reagents:

- NaOH solution, 0.01 mol
- Hydrochloric acid solution, 6 mol
- Hydrochloric acid solution, 1 mol
- Hydrochloric acid solution, 0.1 mol
- Hydrochloric acid solution, 0.001 mol
- Acetic acid solution, 5.3 mol
- $CaCl_2$ dihydrate
- $CaCl_2$ solution in hydrochloric acid. Dissolve 735 mg of $CaCl_2$•H20 in 1 L 0.001 mol hydrochloric acid solution and check the pH. The pH should be 3.0 (± 0.1)

- Bovine trypsin, Merck No. 24579 or equivalent
- Trypsin stock solution: Allow the trypsin to reach room temperature. Dissolve 27 mg of trypsin in $CaCl_2$ solution in a 100 mL volumetric flask and dilute to the mark with the $CaCl_2$ solution. The solution can be used for five days maximum when stored in the refrigerator
- Trypsin working solution: Pipette 5 mL of the trypsin stock solution into a 100 mL volumetric flask and dilute to the mark with the $CaCl_2$ solution
- N α-benzoyl L-arginine p-nitroanilide (L-BAPNA)
- Tris-hydrochloride (HCl).
- Tris buffer/$CaCl_2$ solution: Dissolve 6.05 g Tris and 735 mg $CaCl_2$ in 900 mL water in a 1 L graduated measuring cylinder. Adjust the pH to 8.2 (± 0.1) with hydrochloric acid 6 mol and dilute to 1 L with water
- L-BAPNA reagent solution: Prepare this reagent on the day of use. Dissolve 60 mg L-BAPNA in 1 mL dimethylsulfoxide (DMSO) in a 100 mL volumetric flask and dilute to the mark with Tris buffer/$CaCl_2$ solution

Equipment required:

- A grinding mill with a screen size of 0.15 mm or smaller.
- A spectrophotometer capable to read at 410 nm.
- A water bath with circulation pump capable of being maintained at 37 °C (± 0.25 °C).
- A centrifuge operating at a radial acceleration of approximately 1,500 g.

The procedure is as follows:

- Sample extraction.
  - Weigh 1 g (± 0.001 g) ground soybean product in a 100 mL conical flask and add 50 mL of NaOH 0.01 mol. Completely suspend the sample.
  - Adjust the pH to 9.5 (± 0.1) with hydrochloric acid 1 mol and hydrochloric acid 0.1 mol. Rinse the electrode with as little water as possible.
  - Close the conical flask and store overnight, 15-24 hours, in the refrigerator at 4 °C (± 3 °C). Place in the refrigerator the quantity of water needed for making up the sample extracts.
- Dilution of sample extract. Estimate the TIA value of the sample and prepare three different dilutions based on the dilution scheme in Table 17 so that at least one of the three inhibition percentages obtained as a result of the TIA measurement will be within the 40-60% range.
- Measurement of the TIA of working solution. Check the TIA of each batch of trypsin. The difference between the absorbance of the working solution and the absorbance of the blank (Ar - Abr) should be 0.380 (± 0.050). If this is not the case check the quality of the trypsin. If necessary, take a fresh jar of trypsin.
- Pipette into two centrifuge tubes labeled as follows:
  - (1) Blank standard: 5 mL L-BAPNA reagent solution, 3 mL water and 1 mL acetic acid solution.
  - (2) Standard: 5 mL L-BAPNA reagent solution and 3 mL water.

- Mix on Vortex stirrer the contents of the tubes and place the tubes in the water bath for 10 minutes.
- Add 1 mL trypsin working solution to tubes (1) and (2). Mix on Vortex stirrer and place the centrifuge tubes back in the water bath.
- After 10 minutes (± 5 seconds) of incubation, add 1 mL acetic acid solution to the standard tube. Mix on Vortex stirrer.
- Centrifuge for 10 minutes at a radial acceleration of approximately 1,500 g. Measure the absorbance of the clear solutions relative to water in the spectrometer at 410 nm in a 10 mm cuvette. These solutions remain stable for at least two hours.
- Measuring the TIA of the sample. Prepare for each dilution of sample extract a corresponding blank solution. Sample extract solutions and corresponding blank solutions should be dealt with simultaneously in the procedure, including centrifuging.
    - Pipette 5 mL L-BAPA reagent solution into four centrifuge tubes labeled as follows:
        - (1) Blank standard.
        - (2) Standard.
        - (3) Blank sample.
        - (4) Sample.
    - Add to tubes (2) and (3) 1 mL diluted sample extract.
    - Add to tubes (1) and (2) 3 mL of water, and to tubes (3) and (4) 2 mL of water.
    - Add to tubes (1) and (3) 1 mL of acetic acid solution.
    - Mix on Vortex stirrer and place the tubes in the water bath for 10 minutes.
    - Add to the four tubes 1 mL of trypsin working solution. Mix on Vortex stirrer and place the tubes back in the water bath.
    - After 10 minutes (± 5 seconds) incubation, add to tubes (2) and (4) 1 mL of the acetic acid solution. Mix on Vortex stirrer.
    - Centrifuge for 10 minutes at a radial acceleration of approximately 1,500 g.
    - Measure the absorbance of the clear solutions relative to water in the spectrometer at 410 nm in a 10 mm cuvette. These solutions remain stable for at least two hours.
- Calculate mg TIA/g soy product.

  1) Inhibition percentage of sample extract solutions.

$$i = \frac{(Ar - Abr) - (As - Abs)}{(Ar - Abr)} X\ 100$$

Where:

i = Inhibition percentage.

Ar = Absorbance of the solution with standard.

Abr = Absorbance of the blank with standard.

As = Absorbance of the solution with sample.

Abs = Absorbance of the blank with sample.

2) Trypsin Inhibitor Activity (TIA).

$$TIA = \frac{i}{100\%} \; x \; \frac{m_1 f_1 f_2}{m_o}$$

Where:

TIA = Trypsin inhibitor activity, in milligrams per gram.

i = inhibition percentage.

m_o = Mass of the test sample, in grams.

m_1 = Mass of trypsin in milligrams.

f_1 = Dilution of the sample extract [(100 mL x 100 mL)/V, where V is the volume derived from Table 17, in mL].

f_2 = Conversion factor (2.8 x 10-4) based on the purity of trypsin of 56% (Kakade et al. 1969; Smith et al., 1980) and on the dilution of trypsin as described above under trypsin stock solution and trypsin working solution.

- Round the result to the nearest 0.1 mg/g.

Table 17. Dilution scheme to determine trypsin inhibitor activity by the ISO (2001) method

Expected TIA mg/g	Theoretical dilution at different inhibition percentages ml/100 ml		
	40%	50%	60%
0.5	61	76	91
1.0	30	38	45
1.5	20	25	30
2.0	15	19	23
2.5	12	15	18
3.0	10	13	15
3.5	8.6	11	13
4.0	7.6	9.5	11
4.5	6.7	8.4	10
5.0	6.0	7.6	9.1
6	5.0	6.3	7.6
7	4.3	5.4	6.5
8	3.8	4.7	5.7
9	3.4	4.2	5.0
10	3.0	3.8	4.5
11	2.7	3.4	4.1
12	2.5	3.2	3.8
13	2.3	2.9	3.5
14	2.2	2,7	3.2
15	2.0	2.5	3.0
16	1.9	2.4	2.8
17	1.8	2.2	2.7
18	1.7	2.1	2.5
19	1.6	2.0	2.4
20	1.5	1.9	2.3
25	1.2	1.5	1.8

An important difference between the two methods described above is the units in which the results are expressed: trypsin inhibitor units (TIU)/g used in the AOCS Method Ba 12-75 (2017) vs. mg inhibited trypsin/g used in the ISO (2012) method. Note that in some literature, TIU are also known as trypsin units inhibited (TUI), while inhibited trypsin is also known as trypsin inhibited. Therefore, TUI and TIU are used interchangeably, and inhibited trypsin and trypsin inhibited are also interchangeable. Historically, the only documented conversion factor of 1.9 TIU = 1 µg trypsin inhibited was initially determined by Kakade et al. (1969). Later, Smith et al. (1980) described a modified AOCS method and used the same conversion factor of 1.9 TIU = 1 µg trypsin inhibited, determined by Kakade et al. (1969), for expressing TIA as mg trypsin inhibited/g sample. At a similar time, Hamerstrand et al. (1981) suggested expressing TIA as mg trypsin inhibitor/g sample, again by using the same 1.9 conversion factor determined by Kakade et al. (1969). They also assumed that 1 µg trypsin inhibits the same weight of trypsin inhibitor.

However, in a series of four experiments, Liu (2021) at the U.S. Department of Agriculture (USDA) recently investigated the relationships among the different units of expression for TIA and demonstrated that the 1.9 factor assumption is flawed. This work also introduced a standardization procedure, which if applied for each method, makes it possible to compare TI results across methods. A brief summary of the work by Liu (2021) follows:

1. Three units have commonly been used for expressing measured TIA results: trypsin units inhibited (TUI)/mg (or g) sample, mg trypsin inhibited/g sample, and mg trypsin inhibitors/g sample. This makes comparison of results among studies difficult and in some cases impossible. The problem with using different units in expressing trypsin inhibitor activity (TIA) has bothered many analysts for years. Furthermore, in expressing TIA as absolute amounts, the terms "trypsin inhibited" and "trypsin inhibitor" become indistinguishable when both share the same abbreviation as "TI", even though they refer to two different things.

2. The original and most common unit of expression for TIA is TUI/mg sample, where 1 TUI is typically defined as an increase of 0.01 A410 (Kakade et al., 1969). This arbitrary unit is used in the AOCS (2017) and American Association of Cereal Chemists (AACC, 2009) methods. The advantage of using this arbitrary unit is that it is independent of purity of trypsin used in an assay. The results can be compared among laboratories if the same assay method is used. However, when different methods are used, results become incomparable.

3. To solve this problem, TIA has been expressed in terms of an absolute amount of pure trypsin inhibited. One common way to achieve this expression is to measure TIA as TIU and then convert it to µg trypsin inhibited with a predetermined conversion factor (Kadade et al., 1969; Smith et al., 1980). Alternatively, the expression as mg trypsin inhibited/g sample, or µg/mg, can be calculated directly by multiplying percent trypsin inhibition based on absorbance readings by the amount of pure trypsin used for an assay, as shown in the ISO method. TIA has also been expressed in terms of absolute amounts of pure TI, as suggested by Hammerstrand et al. (1981).

4. However, calculating TIA values in terms of absolute amounts of pure trypsin inhibited or pure TI is also problematic.

   First, in determining a conversion factor, there are two influential elements involved: the assay method itself and the enzymatic purity of the trypsin used. Both factors affect the reference reading, A410 in the absence of an inhibitor. Since most commercial preparations of crystalline trypsin are far from pure and expected to vary in purity, it is necessary to establish the enzymatic purity of trypsin before conversion factor determination. The problem is that in using the AOCS method or developing modified versions based on it, some workers simply bypassed the complex determination of trypsin purity, as well as a conversion factor for their methods, and treat the conversion factor of 1 µg trypsin = 1.9 TU, determined by Kadade et al. (1969), as universal (Smith et al., 1980; Hammerstrand et al., 1981). This approach overlooks the fact that these later methods for TIA assay differ from the original method of Kakade et al. (1969), and that their trypsin preparations differ in purity from that of Kakade et al. (1969) as well.

   Second, although the ISO method eliminates the need for conversion factor determination, it assumes that the enzymatic purity of trypsin to be a fixed value of 56%, originally determined by Kakade et al. (1969). Furthermore, TIA calculation based on percent trypsin inhibition and the amount of trypsin used for the assay makes the results more dependent on the specific activity of a trypsin reagent used and sample dilution levels than the calculation method based on the conversion factor.

Third, expression and calculation of TIA in terms of absolute amount of pure TI, as proposed by Hammerstrand et al. (1981), also has a problem: The assumption that 1µg trypsin = 1 µg trypsin inhibitor is wrong, not mentioning use of the same conversion factor of Kakade et al. (1969).

5. The study (Liu, 2021) showed a new way of expressing, calculating and standardizing TIA in absolute amounts of trypsin inhibited. It is based on a standardized conversion factor of 1 µg trypsin inhibited = 1.5 TUI, whereas the reference trypsin has a specific activity of 15,000 BAEE units/mg protein. BAEE stands for Nα-benzoyl-L-arginine ethyl ester. Here, 1 BAEE unit is defined as catalyzing a change in A253 nm of 0.001/minute at pH 7.6, 25 °C, using BAEE as substrate with 3.2 mL reaction volume and 1 cm light path cuvettes (Bergmeyer et al., 1974). Vendors of trypsin reagents typically provide information on their specific activity as BAEE units/mg protein in lieu of enzymatic purity.

6. The study also showed methods to express and calculate TIA in absolute amounts of pure TI. However, the study found that the conversion factor between TUI and TI varies with the type of TI, while the conversion factor between TUI and trypsin inhibited remains consistent when it is standardized against the reference trypsin. Therefore, expressing TIA as mg trypsin inhibited/g sample is preferred.

Furthermore, there have been some additional developments regarding TI assays. Research at the USDA on TI determination methods in the last few years has resulted in an improved method over the AOCS Method Ba 12-75 (Liu, 2019). The new method features use of the enzyme last sequence, as opposed to the substrate last sequence, the 5 mL total assay volume, as opposed to the 10 mL, and a single level of a dilute extract in duplicate, as opposed to series levels. Based on this improved method, a new AOCS Method Ba 12a-2020 has been proposed. A very recent international collaborative study supported the replacement of the AOCS Method Ba 12-75 by the AOCS Method Ba 12a-2020 (Liu et al., 2021). The new AOCS method calls for expression of TIA in two units, TUI/mg sample, whereas 1 trypsin unit (TU) is defined as an increase in 0.02 of A410, and mg trypsin inhibited (TId)/g sample, or µg/mg. The latter expression is based on a standardized conversion factor of 1 µg trypsin = 1.5 TU, or 1 µg trypsin = 0.03 A410, where the trypsin has a specific activity of 15,000 BAEE units/mg protein (Liu et al., 2021). When expressed as TUI/mg sample, defining 1 TU as an increase in 0.02 A410 for new AOCS Method Ba 12a-2020 (5 mL reaction volume) will make the result relatively comparable to AOCS Method Ba 12-75 (10 mL reaction volume) which defines 1 TU in 0.01 A410 (Liu, 2019).

8.16.2. Soy antigens

Immunoassay techniques are used to determine concentrations of soy antigens, glycinin and β-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 (see Table 18). To apply the different ELISA tests, the protein fraction of the soy product is first extracted in borate buffer, 100 mmol sodium perborate ($NaBO_3$), 0.15 mol sodium chloride (NaCl), pH 8, for 1.5 hours (Tukur et al., 1993). The level of glycinin and β-conglycinin can be measured by a specific competitive inhibition ELISA using anti-soy globulin Pabs (Heppell et al., 1987). Serial, 4-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 18. Enzyme-linked immuno-sorbent assay (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
β-conglycinin	Pab LJR J2	inhibition	intact β-conglycinin
	Mab IFRN 0089	inhibition	recognizes epitopes in acidic regions of α and α' subunits of β-conglycinin
	Mab IFRN 0089 & Pab R195b$_3$	two-site	recognition of thermally denatured β-conglycinin is 3-fold greater than native

(From Tukur et al., 1996)

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 are important ANF in raw soy products (Pusztai, 1991; Fasina et al., 2004).

Lectins are heat-sensitive and only present at residual levels in soybean products. Heat treatment to inactivate ANF in soy products is less efficient for antigens than for TI or lectins (see Table 19).

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

Table 19. Anti-nutritional factor (ANF) 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)

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

- 1 g sample is mixed with 20 mL Tris-hydrochloride buffer, 50 mmol, pH 8.2, and stirred for one hour.
- Extracts are centrifuged at 7,500 g for 15 minutes and the supernatant is used for serial dilutions. Lectins are determined in the supernatant.
- Pabs against the soybean lectin (ELISA) are coated to micro-titer plates overnight at 4 °C. The plates are then blocked with 0.5% bovine serum albumin (BSA) and 0.2% Tween-20 in TBS for one hour at 37 °C.
- Subsequently, the plates are washed, and samples are diluted at appropriate concentrations. A reference soybean lectin sample is run in parallel.

- All samples are transferred to micro-titer wells and incubated for two hours at 37 °C.
- The plates are washed, and peroxidase-conjugated anti-lectin antibodies are applied and incubated for two hours at 37 °C.
- Finally, the plates are washed again and bound conjugated antibodies are developed for peroxidase activity using 1,2-phenylendiamine (C6H8N2).
- 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 on a dry matter (DM) basis

8.17. Mycotoxins; rapid test kits

Mycotoxins are a major quality concern for the feed industry. Although soy products do not generally have the same level or range of mycotoxin contamination as cereal grains, soybeans and soybean products may occasionally be contaminated with mycotoxins. Routine quality control (QC) methods should be in place to monitor their presence. This is especially the case now that regulatory restrictions on mycotoxin levels are becoming more stringent. Among the most common mycotoxins occurring in feed ingredients are aflatoxins, deoxynivalenol (DON), zearalenone, ochratoxin and fumonisins. Ochratoxin A, produced by the molds Aspergillus ochraceous or Penicillium verrucosum under poor storage conditions, has been reported in soy products, specifically in soy protein infant formula (Cappozzo et al., 2017). zearalenone, produced by the fungus Fusarium graminearum, has also been reported (Schollenberger et al., 2007).

Like in the case of ANF, the analyses for mycotoxins and their metabolites can be carried out by a range of methods. Mycotoxins have quite diverse molecular structures. Therefore, a universal test method is not available for all of them. The test kits available in the market use different technologies such as enzyme linked immunosorbent assay (ELISA), monoclonal antibody (Mab) affinity chromatography, lateral flow strip or fluorescence technology. For practical QC purposes, test kits are recommended. However, mycotoxin analysis conducted by test kits should be considered screening tests, particularly with ELISA test kits, due to their high variability. Whitaker et al. (1996) determined the variability associated with three analytical methods, high-performance liquid chromatography (HPLC), thin-layer chromatography (TLC) and ELISA, in 11 commodities, for aflatoxin content. They found that the interlaboratory variability, or coefficient of variation (CV), to measure 100 ppb aflatoxins by HPLC, TLC and ELISA were 25, 34 and 43%, respectively. Therefore, positive results obtained with test kits should be confirmed by HPLC, or the current state-of-the-art, liquid chromatography-mass tandem mass spectrometry (LC- MS/MS), at a specialized laboratory.

Test kit users are advised to consult the list of FGIS approved mycotoxin rapid test kits (USDA, 2021); https://www.ams.usda.gov/sites/default/files/media/FGISApprovedMycotoxinRapidTestKits.pdf ". (USDA, 2021). This website provides the following information:

- Approved instructions for each specific kit.
- A list of commodities for which the kit has been approved. For instance, not all test kits are approved to be used with soy products.
- The test kit range for the specific mycotoxin is stated.
- Test kits may be available, particularly in the international market, that have not been approved by FGIS. For example, currently there are no FGIS approved test kits for T-2 toxin, diacetoxyscirpenol (DAS), or any other Type A trichothecenes.

Nevertheless, due to the many factors affecting the test kit results, the variation among laboratories and analyses 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 QC 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, sample collection and preparation are critical because the non-uniform distribution of mycotoxins in grains, including soybeans. Whitaker et al. (2005) demonstrated that a 4.54 kg sample of grain or soybean meal (SBM), rounded to 5 kg, should be collected from multiple sites in the load (truck, rail car, etc.) to detect a mycotoxin at the limit of 20 ppb. The entire sample must be ground and homogenized before a sub-sample for mycotoxin extraction is taken. This step requires a special mill, since a normal laboratory mill is not designed to grind a 5 kg original sample. This sampling procedure and handling of the sample before extraction of the mycotoxin to be analyzed is usually ignored by the industry. Most mycotoxins can be extracted by grinding the sub-sample to 0.6 mm mesh, then blending 25 g of that sub-sample with 125 mL of a 70% methanol solution (seven parts methanol and three parts deionized water). Stir vigorously in a high-speed blender for two to three minutes. The test kit should be performed as indicated by the manufacturer. 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

8.17.1. Ochratoxin

Ochratoxin A is often considered the most common mycotoxin in soybean products. It is thought to be principally produced during storage under humid and warm conditions, above 20 °C. 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. Current rapid tests for ochratoxins detect 5 ppb (GIPSA, 2018), with LC-MS/MS having a lower detection limit of 1 ppb (Trylogy Labs, 2017).

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 100 ppb (GIPSA, 2018), with LC-MS/MS having a lower detection limit of 12.5 ppb (Trylogy Labs, 2017).

8.17.3. Fumonisins

Fumonisins include 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 0.5ppm (GIPSA, 2018), with LC-MS/MS capable to detect 0.1 ppm for each of the three fumonisins (Trylogy Labs, 2017).

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 produce aflatoxin. 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. Aflatoxins are the only mycotoxins for which official maximum levels, also called action levels, have been defined. The Food and Drug Administration (FDA) and the European Economic Community (EEC) have established general maximum levels 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 5 ppb (GIPSA, 2018) and a limit of quantification by HPLC of 1ppb (Trylogy Labs, 2017).

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.

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. Currently, FDA does not have an advisory level for DON in raw wheat intended for milling purposes and will rely on processors to reduce the level in finished products for human consumption to a level that does not exceed 1ppm.

The FDA[1] advisory levels for DON are as follows:

- 1 ppm DON on finished wheat products, e.g. flour, bran, and germ, that may potentially be consumed by humans.
- 5 ppm DON on grains and grain by-products destined for swine provided that these ingredients do not exceed 20% of their diet.
- 5 ppm DON on grains and grain by-products destined for all other animals provided that these ingredients do not exceed 40% of their diet.
- 10 ppm DON on grains and grain by-products and 30 ppm in distillers grains, brewers grains, gluten feeds and gluten meals (all on an 88% dry matter basis) destined for ruminating beef and feedlot cattle older than 4 months and ruminating dairy cattle older than 4 months, with the added recommendations that the total ration[2] for ruminating beef and feedlot cattle older than 4 months not exceed 10 ppm DON, and the total ration for ruminating dairy cattle older than 4 months not exceed 5 ppm DON.
- 10 ppm DON on grains and grain by-products for chickens, with the added recommendation that these ingredients not exceed 50% of the diet of chickens.

The extraction of DON from soybeans should not be performed with ethanol. It should be conducted with about a 10 g of sub-sample ground to 0.6 mm. Shake vigorously in 50 mL of deionized water for three minutes. Then the sample is filtered, and the liquid fraction is kept for subsequent test kit analyses.

Usually, the rapid tests for DON have a lower limit of detection of 0.5 ppm (GIPSA, 2018) for the screening tests and 0.1 ppm by LC-MS/MS (Trylogy Labs, 2017).

[1]https://www.fda.gov/regulatory-information/search-fda-guidance-documents/guidance-industry-and-fda-advisory-levels-deoxynivalenol-don-finished-wheat-products-human

[2]The total ration includes grains, all grain by-products including distillers and brewers grains, hay, silage, and roughages.

8.18. Genetically modified organisms

Along with a few other agricultural crops or plants, certain soybean varieties have been genetically modified (GM). They are routinely referred to as GM plants or crops, or genetically modified organisms (GMOs). By definition, these plants have undergone a change in their genetic code that is detectable at the DNA or transcriptional level. However, a wide range of external conditions, such as growing stage, plant cell or tissue-type, among others, can influence transcription regulation, making detection of GMOs through this method less certain than detection through targeted DNA sequence analyses (Holst-Jensen et al., 2012).

As market demands for traceability and identification of GMO or non-GMO products increase, it is important to be able to separate soy products as well as other ingredients on the basis of their GM characteristics. In the United States, no mandatory GM labeling law applies, although there is an increased consumer demand for this information. Depending on the country, labels need to indicate the GM nature of the ingredients and official maximum limits exist on the presence of GMO material in non-GMO products. In the EU, these levels are now fixed at a maximum of 0.9%, on the assumption that their presence is adventitious or technically unavoidable. However, in the case of feed or food products, the list of ingredients must be provided and in the case of GM ingredients, it must be clearly indicated that they are "genetically modified" or "produced from genetically modified material, including the name of the GM organism" (EU Regulation No 1830/2003). Japanese legislation does not require food products containing less than 5% approved biotech crops, like corn and soybeans, to be labeled as GM foods. Above the 5% levels for the approved crops, labeling is mandatory (MAFF, 2013). Like the EU and Japan, most countries now have specific labeling requirements and laws for GM feeds and foods. Numerous exceptions exist, often without specification of a labeling threshold. Nevertheless, the minimal allowed levels of GM material in products labeled as GMO-free reflect not only the extensive use of GM soybeans and other plants, but also the practical risk of contamination and analytical variability.

GM varieties are characterized by the insertion of a new, functional gene or cluster of genes into their genomes. In the case of soybeans, the gene or genes normally originate from a bacterium or plant. The expression of these genes provides specific soybean varieties with some advantages, such as resistance to herbicides, pests or drought. In the United States, 25 GM soybean varieties have been approved, but only 17 are available. In the EU, 19 events have been approved, but only 12 are available (ISAAA, 2018). For details on the U.S. varieties, see Table 20.

Several commonly used GMO testing protocols exist. These include polymerase chain reaction (PCR) and enzyme-linked immuno-sorbent assay (ELISA) tests, as well as biological tests. Additional analytical methods such as mass spectrometry, chromatography, near-infrared reflectance spectroscopy (NIRS), micro fabricated devices and DNA chip technology, or microarrays, have been developed and are being used. However, the most widely used methods remain PCR and ELISA tests.

PCR tests, more sensitive than ELISA methods, are based on the detection of DNA sequences in the genome of the soybean product. The PCR is an extremely sensible technique and can identify and quantify different types of GMOs, single and stacked traits, at very low levels. It is also the only method that can effectively detect GMOs in heat-treated ingredients and feeds. This makes this method the preferred procedure in the case of most soy products. The PCR method is also the only method that is considered to have legal validity. However, due to the requirements for equipment, the 2-3-day delay in obtaining results and the level of expertise required, the test is not suited for routine quality control (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.

The ELISA methods are based on the same principle as described above for the detection of mycotoxins (see Section 8.17). In other words, the production of a stable antigen-antibody complex on a small plate that provokes a color reaction after addition of an enzyme-linked antibody specific for the substrate. This color shift can be observed visually or measured photometrically. By comparing the coloring to standard analyte concentrations, such as 0-10 ppb, or precisely measuring the optical density, a semi-quantitative measure of the analyte can be obtained. Since the procedure is simple and relatively quick, it is the most common QC method for the determination of GMO in soy products and other ingredients or feed. A more popular version of the ELISA test, used for screening purposes only, uses lateral flow strips that deliver results in a couple minutes. This makes this test especially suited for QC purposes in feed mills.

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, or extrusion. This limitation applies to all products in which application of high temperatures leads to substantial denaturation of soy proteins, making protein detection difficult.

Biological tests are mainly limited to herbicide-resistant soybean varieties and can only be applied to the untreated soybean. The advantage of this method is that it is relatively inexpensive and produces clear-cut results. In this test, 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.

Table 20. U.S. — genetically modified (GM) soybean events available

#	Available event	Trade name	GM trait	Gene introduced	Gene source
1	A2704-12	Liberty Link® soybean	Glufosinate herbicide tolerance	pat	[XStreptomyces viridochromogenes
2 ⊗	A2704-21	Liberty Link® soybean	Glufosinate herbicide tolerance	pat	[XStreptomyces viridochromogenes
3	A5547-127	Liberty Link® soybean	Glufosinate herbicide tolerance	pat	[XStreptomyces viridochromogenes
4 ⊗	A5547-35	Liberty Link® soybean	Glufosinate herbicide tolerance	pat	[XStreptomyces viridochromogenes
5	CV127	Cultivance	Sulfonylurea herbicide tolerance	csr1-2	Arabidopsis thaliana
6	DAS68416-4	Enlist™ soybean	Glufosinate herbicide tolerance 2,4-D herbicide tolerance	aad-12 pat	Delftia acidovorans; Streptomyces viridochromogenes
7	DP305423	Treus™, Plenish™	Sulfonylurea herbicide tolerance Modified oil/fatty acid	gm-hra gm-fad2-1	Glycine max Glycine max
8	DP356043	Optimum GAT™	Glyphosate herbicide tolerance Sulfonylurea herbicide tolerance	gm-hra gat4601	Glycine max Bacillus licheniformis
9	GTS 40-3-2 (40-3-2)	Roundup Ready™ soybean	Glyphosate herbicide tolerance	cp4 epsps	Agrobacterium tumefaciens
10 ⊗	GU262	Liberty Link™ soybean	Glufosinate herbicide tolerance Antibiotic resistance	pat bla	Streptomyces viridochromogenes Escherichia coli
11 ⊗	IND-ØØ41Ø-5	Verdeca HB4 soybean	Drought stress tolerance	Hahb-4	Helianthus annuus
12	MON87705	Vistive Gold™	Glyphosate herbicide tolerance, Modified oil/fatty acid	cp4 epsps fatb1-A fad2-1A	Agrobacterium tumefaciens Glycine max Glycine max
13	MON87708	Genuity® Roundup Ready™ 2 Xtend™	Glyphosate herbicide tolerance Dicamba herbicide tolerance	cp4 epsps dmo	Agrobacterium tumefaciens Stenotrophomonas maltophilia
14	MON89788	Genuity® Roundup Ready 2 Yield™	Glyphosate herbicide tolerance	cp4 epsps	Agrobacterium tumefaciens
15 ⊗	SYHTØH2	Herbicide-tolerant soybean line	Glufosinate herbicide tolerance Mesotrione herbicide tolerance	pat avhppd-03	Streptomyces viridochromogenes Oat (Avena sativa)
16 ⊗	W62	Liberty Link™ soybean	Glufosinate herbicide tolerance	bar	Streptomyces hygroscopicus
17 ⊗	W98	Liberty Link™ soybean	Glufosinate herbicide tolerance	bar	Streptomyces hygroscopicus

Source: International Service for the Acquisition of Agri-biotech Applications (ISAAA), 2018

Approval.

⊗ Not available in the EU.

9. NEAR-INFRARED REFLECTANCE SPECTROSCOPY ANALYSES

Almost 45 years have passed since the pioneering work by Hymowitz et al. (1974), Rinne et al. (1975), Williams (1975), and Norris et al. (1976) on the application of near-infrared reflectance spectroscopy (NIRS, also referred to as NIR) technology to animal feeds.

Steady development in this area, coupled with the tremendous progress in computer technology and its commercial accessibility, has increased the application of this technology. The important contribution of statisticians, mathematicians and chemists in the form of methods and software programs — particularly in the treatment of large data sets — has facilitated the use of NIRS at all levels of the food and feed industry. Equipment operation is not limited to specialized NIRS technicians or statisticians. A clear, basic understanding of the analytical capacity of NIRS — especially its limitations in terms of specific chemical components — is essential for good operation, and the interpretation or application of results.

One major advantage of NIRS is that it is a non-destructive analytical method and does not require chemical reagents. Once calibrations are in place, results for one or more constituents can be obtained in just a few minutes, when conventional chemistry may take hours or days.

Concerning soybean products, the largest and most evident application is in the rapid determination of proximate components, previously carried out exclusively by time-consuming conventional wet chemistry. Progress has been made in developing NIRS calibrations for more evolved analysis such as protein quality parameters, digestible amino acid (DAA) and anti-nutritional factors (ANF). However, coherence of the data in terms of sample set and methods of analysis is of paramount importance.

Given that different qualities of soy products are available in the global market and that some differences are critical for the success and profitability of animal nutrition businesses, it is imperative that NIRS applications be developed to determine — in real time — ANF such as trypsin inhibitors (TI) and in vivo DAA for different species. Today NIRS technology represents the best opportunity to develop in vitro/in vivo correlations for routine quality assurance and formulation.

Basically, NIRS is applicable to organic compounds rich in oxygen-hydrogen (O-H) bonds such as moisture, carbohydrates and fat; carbon-hydrogen (C-H) bonds such as organic compounds and petroleum derivatives; and nitrogen-hydrogen (N-H) bonds such as proteins and amino acids (AA). NIRS instruments operate by statistically correlating near-infrared signals in the near-infrared region of the electromagnetic spectrum, 800-2,500 nm, with the characteristics or properties intended to be measured (Jordon, 1996a). Consequently, in NIRS analysis, a sample is irradiated with near-infrared light and the reflection of each wavelength from the sample, called the spectrum, is measured and correlated with the analyte of interest in the sample. In other words, the near-infrared spectra are treated mathematically to “extract” the analytical information from the sample. For practical purposes, consider the complete compositional information of a feed sample is contained in the near-infrared spectrum.

The actual numerical value of a specific analyte in the sample, such as protein or lysine, is mediated by a calibration approach known as chemometrics. As explained by Jordon (1996b): It is “a discipline with one foot in chemistry and one in mathematics.” Chemometrics applies statistical methods such as multiple linear regression (MLR), partial least squares (PLS) and principle component analysis (PCA) to the spectral data and correlates them with a property that is determined directly. The primary method remains the direct chemical analyses, or wet chemistry.

A large set of samples, generally more than 40, is required to develop a NIRS calibration for each analyte. The precise number of samples depends on sample and analytical variation. Paired chemical analyses and NIRS readings are used to develop prediction equation or calibration. When found sufficiently robust, this equation is subsequently used to predict the analyte in a set of independent samples whose wet chemistry analysis is known for validation. It is critical to use independent samples in the validation procedure, i.e., that they have not been used to develop the calibration. Generally, a minimum of 20 samples is required for validation. In other words, only when a set of diverse independent samples are correctly predicted by the calibration is it possible to assert that a calibration is working correctly. Validation is essential in the development of NIRS calibrations. No magic number of samples develops a calibration, nor does having the highest regression coefficient (R^2) or lowest mean squared error (MSE) for the model that fits the data. Rather, it is the correct prediction of the analyte in samples unknown to the NIRS machine that is important. Therefore, the concept of a calibration in NIRS is quite different than the classical calibration curve in wet chemistry.

Several items interfere with the NIR spectra. The reflectance obtained from a sample is characterized by scatter due to instrument type and function, sample preparation, including grinding and particle size, temperature, water content and interference of reflectance from other compounds. Variations in sample water content are important because water strongly absorbs radiation. To increase the precision of NIRS analyses, the factors interfering with the NIR spectra need to be standardized when developing the calibration and analyzing an ingredient. Interfering factors can also be reduced or eliminated through application of mathematical corrections on the calibrations. Since standardization of sample preparation is not always practical and since it reduces a major benefit of NIRS analyses, time savings, some preference may be given to mathematical corrections such as MLR, PLS and PCA. The choice and application of these corrections differ considerably among the constituents to be analyzed. The range of mathematical tools available to treat spectral data is increasing rapidly, improving analysis quality and alleviating the requirements for sample preparation.

Before routine analyses can be carried out, calibrations need to be developed for each individual constituent and the individual ingredient. Sometimes, a common calibration can be developed for ingredients and their by-products. In the case of soy products, a single calibration can be developed for a group of soy products if they are sufficiently alike in composition and preparation. This is the case for all soybean meal (SBM) generated by the solvent-extraction process. However, generally, the larger the physical and chemical differences among ingredients, the greater the need to develop separate calibrations.

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. For instance, in the case of a calibration to measure crude protein (CP) in all SBM, the calibration data set should include samples of SBM ranging from 42-50% CP. Similarly, applying NIRS technology to more complex analytes like reactive lysine (Moughan et al., 1996), with the objective of predicting the bioavailable lysine content in SBM for monogastrics, implies obtaining enough variability in the contents of reactive lysine to mirror what is found in commercially available SBM.

Calibration sets should have the widest possible range in composition, but above all, they should represent all samples to be routinely analyzed with the particular calibration. It is generally not recommended to include samples with extreme values (Shenk et al., 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 (FFSB), SBM, soy protein concentrates (SPC), oils, etc.

The quality of a calibration depends greatly on the number and choice of samples. No definite numbers can be provided, as the size of the calibration data set is related to the variability within a set and the range of values that needs to be covered. Analysis of the statistical parameters associated with the calibration will indicate if more samples need to be added. The larger the set of well-prepared and selected samples, the stronger the calibration will be. Independent samples for validation, as defined above, are subject to the same criteria for representation as samples used to establish the equation. Generally, a smaller number is allowed when samples are representative of the population. Key in the quality of a functional calibration is the predictability of the independent sample set.

Routine procedures to verify the validity and quality of the calibration need to be established. The calibration can and should be strengthened through a process of continuous updating and expansion of the calibration set by adding critically selected samples. Some of those critical samples are the outliers, which are observations that are distant from the rest of the data. An outlier may occur due to the variability in the wet chemistry analysis, that is, the analytical error associated with the analysis, or it may indicate experimental error. If the latter is suspected, one way to deal with outliers is repeating the wet chemistry for those specific samples. Outliers should not be physically discarded; some outliers may stop being outliers when included in an expansion of the calibration. The software used by most NIRS machines is evolving rapidly and can define, within their specific statistical limitations, the samples that should undergo chemical analysis for potential use in future calibration expansion, thus adding to existing calibrations.

Several 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 MLR techniques used to develop the equations. Most common measures are the R^2, the standard error of prediction (SEP) or estimate 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 data other than the calibration set, and D is the average difference between the predicted and reference values. From a statistical standpoint, ideally R^2 should be as close as possible to 1.0 while SEP and D should be as small as possible.

Maintenance of NIRS equipment in prime operating conditions is clearly important. Analyses obtained by NIRS are potentially subject to a considerable 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 NIRS techniques and equipment. Users have learned to manage the equipment better and increased their understanding of the special requirements needed for NIRS analysis. While the routine use of the equipment is simple, the maintenance and development of calibrations require a high level of expertise. For proper operation and to reduce errors, clear protocols should be established and implemented at all levels of NIRS operations. These protocols must assure continuity between use of NIRS for routine analytical functions and development of new calibrations or expansion of existing calibrations. When used for routine QC analyses, it is important to provide a separate, dust-free environment. This is often difficult to realize in operations dealing with soybean products.

An important number of errors that can occur in NIRS analyses are related to equipment type. A relatively large variation exists among different NIRS equipment. In the case of monochromatic equipment, calibrations cannot be transferred directly from one NIRS 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 large data set that may cover different regions and years. Results of these calibrations are often less accurate than those of equipment-specific calibrations.

More recently, the concept of cloning or a network of NIRS has been developed. In these networks and through a series of mathematical corrections, NIRS machines are calibrated to provide identical spectral results. This facilitates enormously the transfer of calibrations and the verification of different NIRS in the network.

In principle, all organic compounds of a diet or feed ingredient can be analyzed by NIRS. For most ingredients and especially for soybean products, best results — in terms of accuracy and precision — are obtained for humidity, CP and lipids. NIRS results for fiber components and non-fiber carbohydrates like starch or sugars normally give larger SEP and D values and lower R^2 values. NIRS cannot be used for mineral analysis, although a rough estimate for ash and minerals may be obtained by relating the reflectance at specific wavelengths to the organic matter or its components normally associated with the sample (Givens et al., 1997).

Equipment required for NIRS analysis of soy products includes the following:

- Drying equipment, like a force draught oven.
- Wet chemistry laboratory to conduct analyses for reference values used in calibration development (see Chapter 8).
- Grinder, preferably a Retch grinder, but this is optional. Calibrations can be developed for un-ground, homogeneous material.
- NIRS equipment.

Calibration development procedure follows:

- Dry sample to constant weight (see Section 8.1).
- Grind (optional).
- Split sample into two sub-samples, one for reading on NIRS equipment and one for analysis by the wet chemistry reference methods.
- Fill sample holder as described in equipment-specific manual.
- Insert sample holder in NIRS and read reflectance or analyte concentration.
- Obtain analytical results for analyte of interest by reference method (see Chapter 8).
- Using a statistical software, perform MLR analysis between wavelength spectra, the independent variable, and results of chemical analysis, the dependent variable.
- Establish regression equation with high R^2 and low SEP; beware of over-parameterization, or use of too many wave lengths.
- Validate calibration with independent samples not used in developing the calibration.
- Re-evaluate calibration regularly and send for wet chemistry analysis those samples whose readings the NIRS software has considered unreliable or that statistical procedures indicate as being outliers, outside the borders of the normal population covered by the calibration.

Application procedure follows:

- Dry sample to constant weight (see Section 8.1).
- Grind (optional).
- Fill sample holder as described in manual.
- Insert sample holder in NIRS and record concentration of compound or nutrient.
- Duplicate readings. Two different samples and sample holders are recommended.

Today, the animal feed industry has access to a considerable amount of analytical information processed via NIRS, generated either by NIRS machines in the company's QC lab or through NIRS services by suppliers. Both approaches imply robust calibrations, meaning acceptable levels of prediction capability for specific analytes. NIRS analyses are now so prevalent that it may be useful to emphasize some basic concepts, particularly for laboratory analysts, formulators and nutritionists.

- Wet chemistry is the primary method. NIRS is the secondary method. This simply means that the wet chemistry method commands, not NIRS. Therefore, lab analysts and QC must be familiar with the wet chemistry method, its advantages, limitations, and particularly the analytical error associated with it.
- NIRS is just one tool in the arsenal. Not all analytes can be calibrated by NIRS. Consequently, actual wet chemistry methods need to be standardized, audited and realized for critical nutrients in ingredients such as SBM, FFSB, fermented soybean meal (FSBM) and feeds.
- NIRS cannot be more accurate than wet chemistry. Since the wet chemistry method is the basic analysis, and all wet chemistry methods have an analytical error associated with them, NIRS is limited by the analytical error of the wet chemistry method. Consequently, the larger the analytical error in the wet chemistry method the more compromised is the SEP in NIRS.
- NIRS can be more precise than wet chemistry. Spectrometers, due to their sophisticated design, yield data that are more repeatable than most chemical assay methods.
- Since the analysis of multiple samples of the same lot of a given ingredient or feed is less expensive by NIRS than replicate analysis by wet chemistry, it makes sense to have multiple samples analyzed, which in turn allows for a better estimate of the analyte's variability.

Finally, using NIRS analytical data coming from your own lab is not the same as data originating from an external source. The same applies to wet chemistry when analyses are contracted out. Analysts have control and detailed information on the primary method in their own laboratory. This is not the case with an external supplier. QC should establish a check of NIRS analytical data coming from an external source.

When looking for coherence in the data, do not get too impressed with the speed and automation with which the data are generated. Attention should be centered on expected results versus what is delivered. When a company's QC lab sends the spectrum of a sample via Internet to an external supplier who controls the NIRS calibration, and receives analytical data back, remember that the actual physical sample remains in the company's laboratory. Further tests can and should be carried out or repeated if the NIRS predictions are questionable.

Some feed industry suppliers have developed NIRS applications capable of assessing the critical quality parameters in commercial SBM. The service estimates via NIRS of protein dispersibility index (PDI), potassium hydroxide protein solubility (KOHPS), TI, and reactive lysine, which are then collectively interpreted and expressed in a single indicator value. Whereas an assessment of SBM quality in real time and in a single value may be valuable, such a value expresses different processing effects and does not necessarily reflect a coherent relationship among these quality parameters.

Meeting the objectives of a KOHPS value in the 78-85% range for an adequately processed SBM may not correlate with a range in TI values of 1.60-2.35 mg/g, or 3-4 TIU/mg.

In this context, it is important to note that not all parameters reflect the same processing effects, nor are they based on similar sets of supporting data in published research and industry practice. As discussed in Section 8.5.2, the reactive lysine concept — particularly the guanidination method — does not seem to qualify as a routine quality parameter for soy products (Hulshof et al., 2017). Likewise, except for the work by Frikha et al. (2012), no equations relating in vivo DAA with either KOHPS or PDI values have been published. A great leap ahead in QC and precision in formulation of soy products would be the ability to formulate poultry and swine diets based on DAA values adjusted for KOH or PDI values.

Having an NIRS-determined indicator value, that regroups the various quality parameters for SBM, providing a low, intermediate or high value, may not provide a clear understanding of the type of processing issue associated with the SBM in question. This is likely to complicate the use of the single indicator value in formulation.

10. FULL FAT SOYBEANS

Soybean meal (SBM) production started at the end of the 19th century in China. The processing of whole raw soybeans to generate full-fat soybeans (FFSB) for poultry feeding didn't happen until the end of the 1950s as an academic concept at the University of Arkansas. As defined in Table 1A, FFSB is the meal product resulting from the heat treatment of whole soybeans without removing any of the component parts. As in the case of SBM, the heat treatment is a necessary step to reduce the concentration of thermolabile anti-nutritional factors (ANF) in raw soybeans (see Table 4).

FFSB are generated by a variety of methods. These methods are generally part of the product description. For instance, Table 1A defines two different FFSB products on the basis of their processing methods:

- FFSB, extruded (IFN 5-14-005) involves dry or wet extrusion associated with various degrees of a steam treatment.
- FFSB, roasted (IFN 5-04-597) implies dry heating.

The complete range of processing conditions used to obtain FFSB include cooking, extrusion, toasting or roasting, flaking, jet-sploding, micronizing and enzymatic treatment. These processing methods and their variable effects on product quality have been described in detail (Monari et al., 1996) and they have been subject of more recent applied research (Azcona et al., 2012; Varga-Visi et al., 2009; Mirghelenj et al., 2013; Foltyn et al., 2013; Heger et al., 2016). However, each of these methods have been used in different commercial applications with different production efficiencies (MT/hour) and optimum processing conditions, mainly duration of heat treatment and temperature. For all production methods, particle size and humidity play a major role in the effectiveness of the treatment. Consequently, FFSB derived from different processes have different nutritional values that can vary considerably. A detailed chemical analysis is necessary for each product. Annex 1 summarizes the average nutrient composition of various soy products utilized in animal nutrition, among them extruded and roasted FFSB.

The standard chemical analyses described in Chapter 8 apply to FFSB, although care needs to be exercised in sample preparation and interpretation of results. This seems especially true for the use of potassium hydroxide protein solubility (KOHPS) (see Section 8.4.2) as detailed below. Also, analysis of the oil fraction deserves special attention. The correct determination of the level and availability of this fraction is obviously of major economic importance. The technological treatments applied to FFSB should assist in making all the oil available for digestion and utilization. The above-mentioned effects of processing methods on digestion of the ether extract (EE) fraction is in part a reflection of these treatments on rupturing the cell wall or cell structures that contain the oil stores in the seed. There is, however, no established quality control (QC) method to measure this effect. A potential short-cut method may be the determination of the EE fraction that results from the difference between an EE determination following an acid hydrolysis and a simple direct EE analysis. However, for routine description of nutritive value and formulation purposes concerning the EE or fatty acid (FA) fraction, a full acid hydrolysis preceding ether extraction is recommended. A more detailed analysis of the lipid fraction (see Section 8.13) may be considered to better evaluate its full nutritional value.

The heat treatment applied to raw soybeans that results in various types of FFSB has ANF destruction as its main objective. Besides destroying ANF, the heat treatment will also improve the nutritive value through its effect on the digestibility of the protein and oil fraction in the bean, rendering the oil more available and stable through the destruction of oxidative enzymes. The heating process is critical in terms of duration and temperature, especially when FFSB is used in poultry or swine diets. Consequently, the treatment must be applied under strictly controlled conditions. Insufficient heat treatment will result in under-processed FFSB with contents of ANF that remain too high, while excess heat treatment may result in over-processing and serious loss of digestibility or destruction of nutrients, especially the essential amino acids (AA).

Most studies have concentrated on dry roasting or extrusion. Specific recommendations are difficult — if not impossible — to provide, given that treatment conditions change with equipment type and FFSB preparation prior to treatment. The parameters associated with the extrusion process to obtain FFSB have been investigated on numerous occasions and are now sufficiently well-defined to provide general recommendations. Besides the references provided by Monari and Wiseman (1996), the work of Perilla et al. (1997) may be used as an example to produce FFSB under practical, or wet, extrusion conditions. The results provide an indicator of the effects of heat treatment on FFSB quality and as summarized in Table 21. They clearly show the dominant effect of temperature on the residual trypsin inhibitor activity (TIA) and broiler performance. Feeding broiler chicks FFSB processed in a commercial extruder at temperatures ranging between 118-140 °C and with a resident time of 20 seconds suggests that the optimal FFSB extrusion temperature in terms of residual ANF and broiler performance is close to 125 °C. Using the same FFSB samples generated from the experiment of Perilla et al. (1997), Ruiz et al. (2004) conducted in vivo digestible amino acid (DAA) assays in broilers to assess the effect of the above-mentioned temperatures on protein quality. Table 22 summarizes these results and demonstrates that the extrusion temperature that led to optimal broiler performance corresponds to the treatment that assures the lowest ANF levels associated with the highest DAA.

Table 21. Extrusion temperature of full-fat soybeans (FFSB), trypsin inhibitors (TI) and performance of broiler chickens

Treat-ment	Extrusion temp., ºC	Trypsin inhibitor, mg/g	Urease activity, pH units	Weight gain, 35 d, g	Feed Intake, g/h	F:G ratio
1 (Raw)	-	37.92	2.03	1502± 32	3815	2.53
2	118	9.41	1.08	1890± 40.9	3627	1.91
3	120	6.68	0.85	1897± 36.5	3565	1.87
4	122	1.66	0.10	2056± 28.6	3534	1.71
5	126	1.26	0.09	2068± 30.7	3594	1.73
6	140	N.D.	0.05	1988± 37.1	3403	1.71

Source: Adapted from Perilla et al., 1997.

Table 22. Extrusion temperatures of full-fat soybeans (FFSB), *in vivo* digestible amino acids (DAA) and potassium hydroxide protein solubility (KOHPS), %

Treat-ment	Extrusion temp., ºC	Lysine	Methionine	Cystine	Arginine	Threonine	KOHPS
1 (Raw)	-	73	72	69	78	73	98
2	118	81	79	68	81	76	87
3	120	85	82	67	81	77	89
4	122	87	88	80	88	85	89
5	126	94	91	81	90	90	88
6	140	84	92	80	87	87	79

Source: Adapted from Ruiz et al., 2004.

In a similar experiment, again working with a commercially roasted FFSB, Ordoñez and Palencia (1998) fed broiler chicks a diet that contained FFSB processed between 113-150°C. The results obtained with roasted soybeans confirmed the results obtained with wet-extruded FFSB, both in terms of chick performance and digestibility.

A series of research trials at the University of Nottingham confirmed the above results in terms of processing conditions of FFSB and their effects on the nutritional value for poultry (Table 23). In these trials, FFSB was produced using a Clextral laboratory model twin-screw extruder, or dry extrusion. The four different levels of TI in FFSB generated resulted in a clear improvement in apparent ileal digestibility (AID) in broiler chicks 19-25 days of age.

Table 23. Apparent ileal amino acid (AA) digestibility coefficients in broiler chickens fed full-fat soybeans (FFSB) containing different concentrations of trypsin inhibitor activity (TIA)

Amino acid	Trypsin inhibitor concentrations mg/g			
	14.8	9.6	4.5	1.9
	Apparent ileal digestibility coefficients, %			
Lysine	58.2	75.7	82.7	85.8
Methionine	54.5	74.3	81.1	87.2
Cystine	34.5	59.5	66.6	71.8
Valine	46.1	71.1	77.1	82.3
Barreltemperature during extrusion, ºC	90	110	130	160

Source: Adapted from Clarke and Wiseman, 2007.

The data referred to above confirm the concept that, within a well-defined set of operations, an optimum temperature exists to maximize in vivo DAA in both extrusion and roasting processes of FFSB. They also show that these treatment conditions correspond well with the lowest level of ANF as measured by TI.

However, in the above experiments, producing FFSB under commercial conditions (Perilla et al., 1997; Ordoñez et al.,1998), KOHPS was not correlated with bird performance nor the digestibility of lysine or any other AA (Ruiz et al., 2004). Similar observations have been made under a range of practical conditions. These results suggest an important difference between FFSB and SBM. In SBM, a close correlation between KOHPS and the decrease in total or digestible lysine is well-documented (Lee et al., 1991; Parsons et al., 1991). A similar relationship does not seem to exist for FFSB. Consequently, in the case of FFSB, KOHPS as a quality measure to evaluate heat treatment should be used with extreme caution.

New soybean cultivars low in ANF — especially TIA and oligosaccharides — have been developed. These cultivars have generally shown a superior nutritional value. In the case of low TIA cultivars, higher levels of DAA have been observed, underlining the inverse relationship between TIA levels and digestibility. However, other classical quality methods applied to SBM, such as the urease index (UI), are higher in the low TIA cultivars, demonstrating the importance of using caution when evaluating the treatment of FFSB with classical quality methods as applied to SBM (Wiseman, 2006).

11. DATA MANAGEMENT

11.1. Sample statistics

The physical, chemical and microbiological analyses performed on feed or soybean products provide information on the nutritional or sanitary 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. The collection of a representative sample is critical and has been discussed in detail in Chapter 6. Failure to collect a truly representative sample completely undermines any chemical or physical evaluation and thus any quality control (QC) procedure.

11.1.1. Basic assumptions

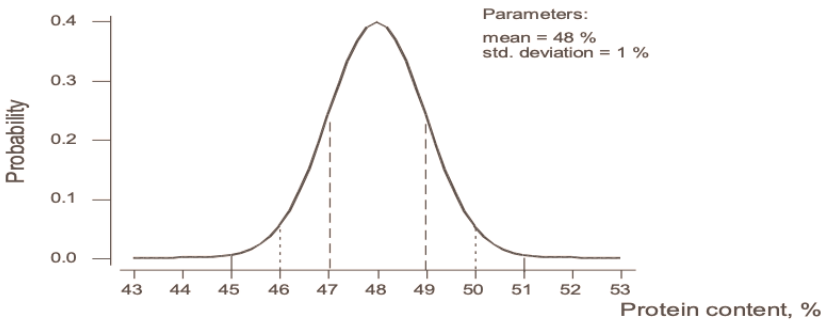
The distribution of a measured parameter, X; for instance, crude protein (CP), in the population of size N (example 20 MT) is assumed to be normal. In statistical terms, this is expressed as follows:

$X_i \sim N(\mu,\sigma^2)$.

Where: μ = population mean.

σ^2 = population variance.

Figure 4. Example of a density curve describing a normal distribution



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 ± 1σ of the mean μ.
- 95% of the observations fall within ± 2σ of the mean μ.
- 99.7% of the observations fall within ± 3σ of the mean μ.

The population can be characterized by the mean (m) and variance (s2), which is unknown. The normal distribution is the most common random distribution about the mean value. An example of this could be the distribution of CP content in a load of soybean meal (SBM) guaranteed to contain 48% CP (see Figure 4).

11.1.2. Parameter estimetes

Sample statistics are used to estimate the population parameters from a smaller sample size, n. In our SBM example, this would be the estimation of CP of all SBM in the load based on a set of SBM samples from that load. Main parameter estimates (see Table 23) can be calculated simply from the measured results on the samples.

Table 24. 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.

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

Where:

x_i = individual sample measurement.
n = number of samples.

Variability

The variability in the observations on the samples may be more important than the mean. Variability provides information about the spread in values within the population. In other words, it describes the homogeneity of a load for a particular parameter. For our example, how many samples have CP 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 in a set of measurements:

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

$$RPD = \frac{w}{\bar{x}} \cdot 100\ \%$$

- Variance (s^2) obtained from at least three replicates.

$$s^2 = \frac{\Sigma(x_i - \bar{x})^2}{n-1} \text{ or } s^2 = \frac{\Sigma x_i^2 - (\Sigma x_i)^2/n}{n-1}$$

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

11.1.3. Presentation of analytical results

In our example, a cargo of SBM was sampled and 14 samples were collected (n = 14 replicates) to determine protein content. The sampling was conducted to be representative of the entire load. The results of the analysis are presented in Table 25.

In this example, the mean protein content in the sample was 48.41% and the standard deviation was 3.04% on a dry matter (DM) basis.

Histograms can be helpful to visualize the data, like average value and range, and to determine if the collected samples follow or represent a normal distribution. Histograms are an important tool in QC because they help identify the cause of problems by the shape, i.e., uni- or bimodal, and the width of the distribution.

Table 25. Protein content of soybean meal (SBM): Calculation steps to determine mean and variance

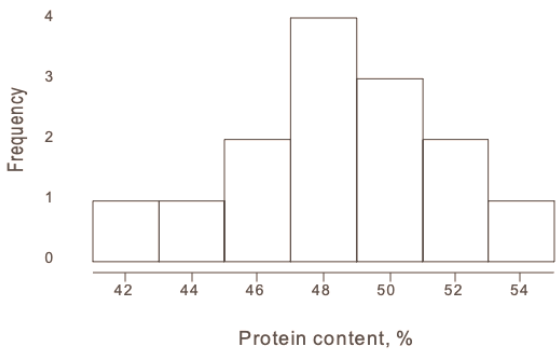
	n° sample, n	Measure-ment: x_i	$x_i - \bar{x}$	$(x_i - \bar{x})^2$
	1	50.2	1.79	3.19
	2	54	5.59	31.20
	3	48.7	0.29	0.08
	4	44.2	-4.21	17.76
	5	45.4	-3.01	9.09
	6	46.8	-1.61	2.61
	7	51.3	2.89	8.33
	8	49.7	1.29	1.65
	9	47.7	-0.71	0.51
	10	47.6	-0.81	0.66
	11	42.9	-5.51	30.41
	12	48	-0.41	0.17
	13	52.1	3.69	13.58
	14	49.2	0.79	0.62
Sum Σ	14	677.8	0	119.86
Σ / n		$\bar{x}$ = 48.41		
Σ / (n-1)				s^2 = 9.22
CV = (3.04/48.41)*100% = 6.3				s = 3.04

11.1.4. Creation of a histogram

Continuing with the above example, a histogram provides a picture of the load of SMB.

- Calculate the range of the values.
  w = 54.0 – 42.9 = 11.1%
- Choose a number of intervals, for instance, seven. The size of the interval is figured as shown.
  w/ number of intervals = 11.1/7 = 1.6
- For practical considerations, it is better to round the interval size, for this example, 2% of DM.
- Calculate the frequency of occurrences for each interval.
  Interval: 41-43 > occurrence: 1
  Interval: 45-47 > occurrence: 2
- Draw the corresponding figure (Figure 5).

Figure 5. Histogram of the data based on 7 intervals



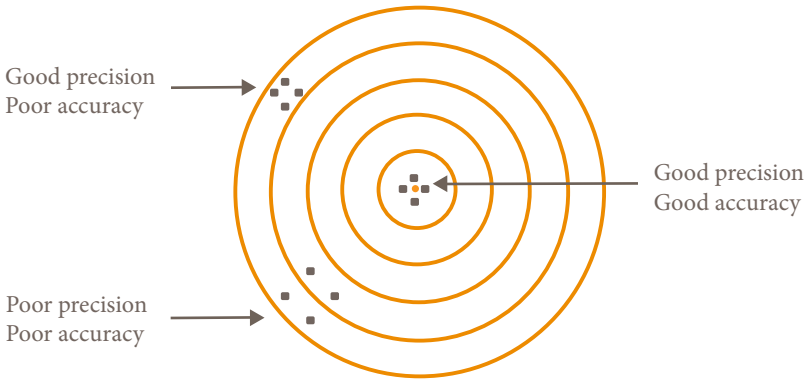
11.2. Quality indicators

The reliability of analytical results and the quality of any estimation concerning the population of soybean meal (SBM) depends on critical parameters. First, the analytical method should be specific for the compound to be measured, such as crude protein (CP). 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 (see Figure 6) and quality indicators can help evaluate these two measures.

11.2.1. Accuracy versus precision

In quality control (QC), it is important to distinguish between accuracy and precision. Accuracy is generally defined as the degree to which the result of a measurement conforms to the correct value or a standard. In our example, this would be how close the analysis of CP in the SBM samples approach the population CP level. Precision is defined as the degree to which repeated measurements under unchanged conditions show the same results. In our example, how close the CP values of the SBM samples are to each other.

Figure 6. Definition of accuracy versus precision



Source: Galyean, 1997.

Statistically speaking, accuracy and precision are expressed in different terms.

11.2.1.1. Accuracy

Accuracy is a measure of the difference or bias (D) 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 calculating the absolute error or the relative error.

Absolute error = $(X_i - X_t)$

Relative error = $100 \times (X_i - X_t) / X_t$

For example, if the CP value of SBM is 48% and the analytical result yields 50%, the method is not accurate: the absolute error for this result is 2% 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 whose properties are validated and reliable. The use of CRM is a powerful tool to assess the good performance of 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:

Q_S = Measured quantity in spiked sample.

QN= Measured quantity in unspiked sample.

S = the quantity of substance in spiked sample.

- Blank: A blank is a QC sample designed to check for contamination in the sampling and analytical procedure. A method blank should be free of the molecule to be measured.
- Interlaboratory comparisons: Interlaboratory comparisons programs should be conducted to compare accuracy of analytical results.

11.2.1.2. 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 be analyzed in the same conditions. Laboratory duplicates are intended to be identical to the original samples.

Table 26. Typical ranges and acceptable coefficients of variation (CV) for proximate analysis in feedstuffs

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

Source: Galyean, 1997.

11.3. Significance of parameter estimates

11.3.1. Hypothesis test

These tests can be performed to address the uncertainty of the sample estimates and to make decisions about the validity of the data (Feinberg, 1996). For example, they can help determine if an observed value of a statistic differs from a hypothesized value of a parameter. For our soybean meal (SBM) example, 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:

Ho: Null hypothesis. The population mean is equal to a reference value ($\mu - \mu_o = 0$). The mean value of CP in all SBM is equal to 48% (DM basis).

H_1: Alternative hypothesis. The population mean is different than the reference value ($\mu - \mu_o$ ¹ 0). The mean value of CP in all SBMs differs from 48% of DM.

Steps to follow:

- Select a level of significance (a). 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, at 10% vs. 5%.
- Calculate the test statistics. In other words, test the hypothesis for the sample data. The test procedure measures the compatibility between Ho and the data. Several statistical tests exist. The choice of the statistical test will depend on the sample size, the knowledge about the population parameters, such as variance, the accepted or 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's 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.

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

- Determine the probability value (P-value). The 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, for example 0.51, with values from the probability distribution, such as the Student distribution. The Student t-test and the Normal z tables are presented in Annexes 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 Annex 7: $t_{0.4}$ (13) < 0.51 < $t_{0.25}$ (13), therefore P ranges from 0.25-0.40. 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 0.50-0.80. 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 than or equal to the level of significance. In this case, it can be concluded that results are not due to chance and Ho can be rejected.

P-value > α

The difference is attributed to chance or to an error of measurement. In that case, the Ho cannot be rejected. Instead, Ho is accepted. In the above example, P-value is 0.62, p > 0.05, therefore it is concluded that CP content of the SBM load is not statistically different from 48% of DM.

Table 27. Error types in hypothesis testing

		Actual situation	
		Ho	H_1
Decision	Reject Ho	Type I error (*P: α*)	Correct (*P: 1-β*)
	Retain Ho	Correct (*P: 1- α*)	Type II error (*P: β*)

Two types of errors may occur (see Table 27). Ho is rejected when it is true, a type I error. Ho is accepted when H_1 is true, a 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.

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 test's ability to detect differences, P = 1-β, 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 it 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.

11.3.2 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), associated with the measured variable, 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 (see Annexes 7 and 8).

- The calculation for a small sample from a normal population follows.

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

- In the case of a large sample, n > 30, from a normal population, the calculation follows.

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

- The calculation for our example is as follows.

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

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

$$= [46.66 - 50.16]$$

Thus, we are 95% confident that the average CP content in the SBM load is 46.7–50.2% of DM.

11.3.3 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 or *P*-value, for example, α = 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.

- Calculate unknown population variance as shown.

$$n = \left(\frac{t_{(\alpha/2)} s}{H} \right)^2$$

- Calculate known population variance (σ) as shown.

$$n = \left(\frac{z_{(\alpha/2)} \sigma}{H} \right)^2$$

11.4. Control charts

Control charts are efficient devices to control an analytical method and to check its stability over time (Daudin et al., 1996). They are used to indicate the range of variability of a process and to decide if it is under statistical control. In certification schemes like Hazard Analysis Critical Control Point (HACCP), the International Organization for Standardization (ISO) and Good Manufacturing Practice (GMP) and solid quality control (QC) 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 a group develop an X or range chart.
- Individual measurements can be shown with an individual X or moving range (MR) chart.

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

Basic principles.

- 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 (s).
  - 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 the process is not operating properly. The analyst can try to assess the source of errors; however, no action is needed, provided 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 26).

The lines are then defined as follows.

Centerline $\bar{\bar{x}}$ 48.41

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

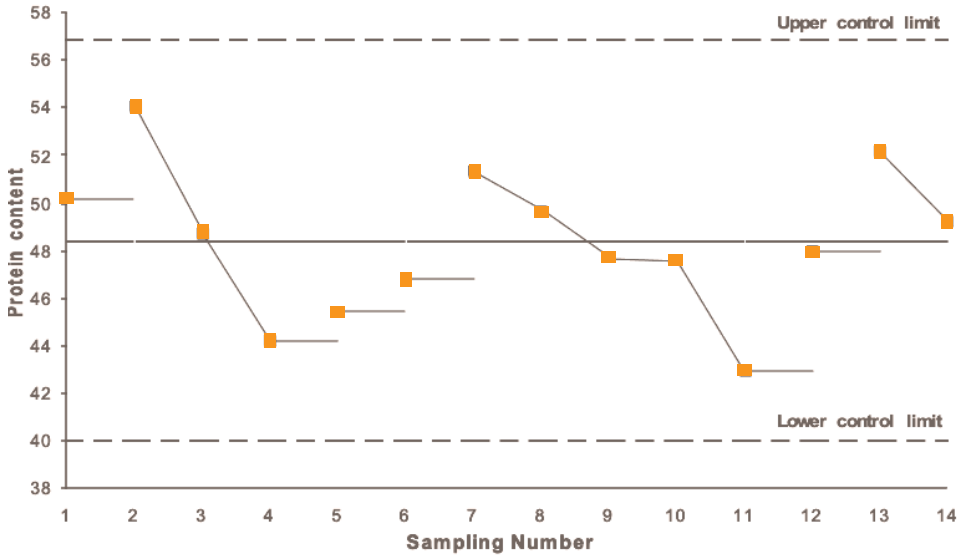
Action control limits $\bar{\bar{x}} \pm 3 \frac{\overline{MR}}{1.128}$

LCL = 39.98
UCL = 56.84

Table 28. Protein content as % of dry matter (DM) in soybean meal (SBM) 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 analysis by % of dry matter (DM) in soybean meal (SBM) samples



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

11.5. 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 errors and to estimate the reliability of analytical results. This should be part of a standard quality control (QC) procedure, and it needs to be developed through a joint effort between analysts and nutritionists. Each has specific responsibilities and tasks, which can be summarized as follows.

11.5.1. Analyst

- Perform the sampling and analysis correctly.
- Use proper Q.C. 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 concentration of the nutrient (see Table 3).
- Propose corrective actions (re-sampling, re-calibration ...) if and where needed.

11.5.2. 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; build in securities like mean ±a fraction standard deviation (s) for critical nutrients.
- Evaluate the cost:benefit ratio, or security for the above.
- Find an alternative ingredient if quality specifications are not met.

The objectives of the analyst and the nutritionist may be to reduce variation and increase quality of the results, but also to maximize the value of a raw material. Subjective judgment is associated with QC. The risk of type I or type II errors exists. It is possible to reduce these risks with 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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ANNEX

Annex 1. Average table values for nutrient composition of common soy protein products used in livestock diets[1,2]

Nutrient	Unit	SBM Mechanical Extracted	SBM Solvent Extracted 44	SBM Solvent Extracted 48	SBM Solvent Extracted 50	Soy bean Mill Feed	Soybean Oil
Int. Feed Number		5-04-600	5-04-604	5-04-612	5-04-612	4-04-594	4-07-983
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 extract	%	5.74	2.60	2.13	2.40	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[3]	%	<1.0	<1.0	1.10	0.80		
Sugars[4]	%	8.20	8.80	9.90	10.30		
NSP[5]	%	32.23	33.38	31.08	29.83		
Energy[6]							
GE	kcal/kg	4420	4165	4130	4120	2330	9400
DE-swine	kcal/kg	3880	3394	3446	3776	1167	8915
ME-swine	kcal/kg	3580	2986	3210	3299	1010	8400
NE-swine	kcal/kg	2275	1903	1955	1992	645	6760
AMEN - broiler	kcal/kg	2531	1929	1973	2147		8600
AMEN - adult	kcal/kg	2624	2171	2208	2464	774	8805
ME -ruminants	kcal/kg	2435	2831	2840	3010	1630	8180
NE-dairy	kcal/kg	2005	1706	1748	1826	1001	4520
NE - beef	kcal/kg	2186	1838	1847	1993	965	5022
Amino acids							
Lysine	%	2.65	2.85	2.89	3.00	0.65	
Threonine	%	1.70	1.80	1.84	1.90	0.30	
Methionine	%	0.59	0.62	0.63	0.67	0.13	
Cystine	%	0.65	0.68	0.73	0.73	0.14	
Tryptophane	%	0.59	0.56	0.63	0.65	0.13	
Isoleucine	%	1.97	2.26	2.17	2.30	0.41	
Valine	%	2.07	2.19	2.30	2.38	0.38	
Leucine	%	3.32	3.42	3.60	3.60	0.58	
Phenylalanine	%	2.21	2.16	2.37	2.37	0.38	
Tyrosine	%	1.58	1.61	1.68	1.64	0.23	
Histidine	%	1.15	1.64	1.21	1.21	0.18	
Arginine	%	3.21	2.99	3.48	3.53	0.75	
Alanine	%	1.88	2.53	2.05	2.04		
Aspartic acid	%	4.97	4.03	5.49	5.55		
Glutamine	%	7.81	6.29	8.62	8.52		
Glycine	%	1.87	1.88	1.97	2.09	0.48	
Serine	%	2.20	2.13	2.38	2.49	0.30	
Proline	%	2.23	2.17	2.37	2.43		

[1]As Fed basis; FF Soybean is Full Fat Soybeans; SBM=Soybean meal.
[2]Source: compilation of NRC, INRA-AFZ, CVB, Feedipedia, FEDNA and selected supplier.
[3]As determined by the Enyzmatic (amylo-glucosidase) method.
[4]Base; Reducing sugars as determined by the Luff-Schoorl method (Glucose, Galactose, Fructose, Mannose, Galactose).
[5]Estimated; basis: 100--(humidity, % + ash, % + protein, % + lipids, % + lignin, % + starch, %)
[6]DE: Digestible Energy; ME: Metabolizable Energy; NE: Net Energy; AMEN: Apparent Metabolizable Energy-corr. N Losses.

Annex 1 (Continued). Average table values for nutrient composition of soy protein products used in livestock diets[1,2]

Nutrient	unit	Full Fat Soybeans (FFSB)	Soy Protein Concentrate (SPC)	Soy Protein Isolate (SPI)	Soy Hulls	Fermented SBM (FSBM)
Int. Feed Number		5-04-597	5-32-183	5-24-811	1-04-560	
Minerals						
Calcium	g/kg	2.62	2.37	1.50	4.96	2.90
Phosphorus	g/kg	5.70	7.63	6.50	1.59	5.60
Magnesium	g/kg	2.80	1.85	0.80	2.23	3.33
Potasium	g/kg	15.93	12.35	2.75	12.15	23.10
Sodium	g/kg	0.29	0.55	2.85	0.10	0.19
Chloride	g/kg	0.33	0.20	0.20	0.25	0.39
Sulfur	g/kg	2.43	0.70	7.00	0.95	3.03
Manganese	mg/kg	31.79	27.50	5.00	10.67	47.42
Zinc	mg/kg	47.80	47.00	34.00	37.75	55.13
Copper	mg/kg	15.17	17.00	14.00	10.68	19.84
Iron	mg/kg	128.01	137.00	137.00	437.50	308.00
Selenium	mg/kg	0.17	0.14	0.14	0.16	0.33
Cobalt	mg/kg	0.00			0.06	0.18
Molybdenum	mg/kg	2.00			0.60	2.69
Fatty acids						
Myristic acid - C14:0	%	0.03			0.00	0.00
Palmitic acid - C16:0	%	1.95			0.24	0.15
Palmitoleic acid - C16:1	%	0.04			0.00	0.00
Stearic acid - C 18:0	%	0.71			0.09	0.06
Oleic acid - C18:1	%	3.96			0.50	0.30
Linoleic acid - C18:2	%	9.70			1.21	0.88
Linolenic acid - C18:3	%	1.40			0.17	0.13

[1]As Fed basis; FF Soybean is Full Fat Soybeans; SBM=Soybean meal.
[2]Source: compilation of NRC, INRA-AFZ, CVB, Feedipedia, FEDNA and selected supplier/analyses.
[3]As determined by the Enyzmatic (amylo-glucosidase) method.
[4]Base; Reducing sugars as determined by the Luff-Schoorl method (Glucose, Galactose, Fructose, Mannose, Galactose).
[5]Estimated; basis: 100--(humidity, % + ash, % + protein, % + lipids, % + lignin, % + starch, %)
[6]DE: Digestible Energy; ME: Metabolizable Energy; NE: Net Energy; AMEN: Apparent Metabolizable Energy-corr. N Losses.

Annex 2. Average table values for nutrient composition of soy protein products used in livestock diets - specialty products[1,2]

Nutrient	Unit	Full-at soybeans (FFSB)	Soy protein concentrate (SPC)	Soy protein isolate (SPI)	Soy hulls	Fermented soybean meal (FSBM)
International Feed Number (IFN)		5-04-597	5-32-183	5-24-811	1-04-560	
Dry matter (DM)	%	89.4	91.83	93.38	89.76	90.1
Crude protein (CP)	%	37.1	68.60	85.88	12.04	52.1
Crude fiber (CF)	%	5.1	1.65	1.32	34.15	3.6
Ether extract (EE)	%	18.4	2.00	0.62	2.25	2.7
Ash	%	4.9	5.15	3.41	4.53	6.6
Neutral detergent fiber (NDF)	%	13.0	13.50		56.91	9.0
Acid detergent fiber (ADF)	%	7.2	5.38		42.05	5.6
Acid detergent lignin (ADL)	%	4.3	0.40		2.05	0.3
Starch[3]	%	<0.5	0.00	0.00	0.70	<0.5
Sugars[4]	%	7.00	<0.5	0.00	1.40	<0.5
Non-starch polysaccharides (NSP)[5]	%	24.32	15.18	2.97	68.39	27.90
Energy[6]						
Gross Energy	kcal/kg	5013	4665	5370	3890	4700
DE - swine	kcal/kg	4088	4517	4545	1944	4224
ME - swine	kcal/kg	3714	3661	3955	1687	3486
NE - swine	kcal/kg	2803	2000	2000	1074	2165
AMEN - broiler	kcal/kg	3332		4060		2413
AMEN - adult	kcal/kg	3564	2472	3945	334	2890
ME - ruminants	kcal/kg	3400	2690		1241	2850
NE - dairy	kcal/kg	2097	1600		1544	1755
NE - beef	kcal/kg	2230	1750		1618	1900
Amino acids (AA)						
Lysine	%	2.26	4.59	5.26	0.73	3.02
Threonine	%	1.44	2.82	3.17	0.73	2.01
Methionine	%	0.52	0.87	1.01	0.14	0.65
Cystine	%	0.53	0.89	1.19	0.16	1.03
Tryptophane	%	0.49	0.81	1.08	0.12	0.66
Isoleucine	%	1.68	3.68	4.25	0.41	0.67
Valine	%	1.75	3.69	4.21	0.49	2.48
Leucine	%	2.76	5.41	6.65	0.75	3.99
Phenylalanine	%	1.87	3.60	4.35	0.47	2.43
Tyrosine	%	1.22	1.55	3.14	0.43	1.78
Histidine	%	0.96	2.41	2.25	0.29	1.33
Arginine	%	2.66	7.34	6.87	0.62	3.52
Alanine	%	1.52		3.33	0.51	3.10
Aspartic acid	%	4.06		8.29	1.14	5.51
Glutamine	%	6.35		12.00	1.49	8.99
Glycine	%	1.58	3.32	3.38	0.85	2.15
Serine	%	1.86	5.19	4.81	0.67	2.35
Proline	%	1.86		4.45	0.55	2.46

[1]As-fed basis.
[2]Sources: Compilation of National Research Council (NRC), National Institute of Agricultural Research – French Association for Animal Production (INRA-AFZ), Dutch Central Animal Feed Agency (CVB), Feedipedia, Spanish Foundation for the Development of Animal Nutrition (FEDNA) and selected suppliers.
[3]As determined by the enzymatic amyloglucosidase method.
[4]Base; reducing sugars as determined by the Luff-Schoorl method (glucose, galactose, fructose, mannose).
[5]Estimated; basis = 100 – (% humidity +% ash + % protein + % lipids + % li gnin + % starch).
[6]DE = Digestible energy; ME = Metabolizable energy; NE = Net energy; AMEn: Apparent metabolizable energy, corrected for N losses.

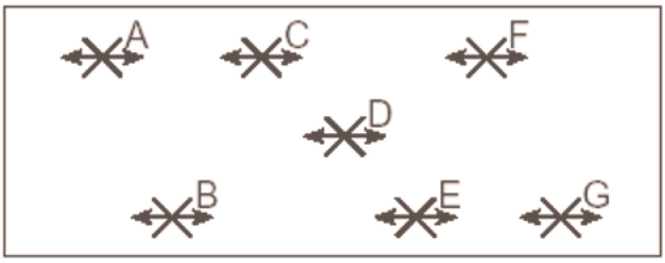
Annex 2 (Continued). Average table values for nutrient composition of soy protein products used in livestock diets - specialty products[1,2]

Nutrient	unit	Full Fat Soybeans (FFSB)	Soy Protein Concentrate (SPC)	Soy Protein Isolate (SPI)	Soy Hulls	Fermented SBM (FSBM)
Int. Feed Number		5-04-597	5-32-183	5-24-811	1-04-560	
Minerals						
Calcium	g/kg	2.62	2.37	1.50	4.96	2.90
Phosphorus	g/kg	5.70	7.63	6.50	1.59	5.60
Magnesium	g/kg	2.80	1.85	0.80	2.23	3.33
Potasium	g/kg	15.93	12.35	2.75	12.15	23.10
Sodium	g/kg	0.29	0.55	2.85	0.10	0.19
Chloride	g/kg	0.33	0.20	0.20	0.25	0.39
Sulfur	g/kg	2.43	0.70	7.00	0.95	3.03
Manganese	mg/kg	31.79	27.50	5.00	10.67	47.42
Zinc	mg/kg	47.80	47.00	34.00	37.75	55.13
Copper	mg/kg	15.17	17.00	14.00	10.68	19.84
Iron	mg/kg	128.01	137.00	137.00	437.50	308.00
Selenium	mg/kg	0.17	0.14	0.14	0.16	0.33
Cobalt	mg/kg	0.00			0.06	0.18
Molybdenum	mg/kg	2.00			0.60	2.69
Fatty acids						
Myristic acid - C14:0	%	0.03			0.00	0.00
Palmitic acid - C16:0	%	1.95			0.24	0.15
Palmitoleic acid - C16:1	%	0.04			0.00	0.00
Stearic acid - C 18:0	%	0.71			0.09	0.06
Oleic acid - C18:1	%	3.96			0.50	0.30
Linoleic acid - C18:2	%	9.70			1.21	0.88
Linolenic acid - C18:3	%	1.40			0.17	0.13

[1]As Fed basis; FF Soybean is Full Fat Soybeans; SBM=Soybean meal.
[2]Source: compilation of NRC, INRA-AFZ, CVB, Feedipedia, FEDNA and selected supplier/analyses.
[3]As determined by the Enyzmatic (amylo-glucosidase) method.
[4]Base; Reducing sugars as determined by the Luff-Schoorl method (Glucose, Galactose, Fructose, Mannose, Galactose).
[5]Estimated; basis: 100--(humidity, % + ash, % + protein, % + lipids, % + lignin, % + starch, %)
[6]DE: Digestible Energy; ME: Metabolizable Energy; NE: Net Energy; AMEN: Apparent Metabolizable Energy-corr. N Losses.

Annex 3. Sampling patterns for bulk carriers

A. Sampling pattern for bulk carriers containing a homogeneous load

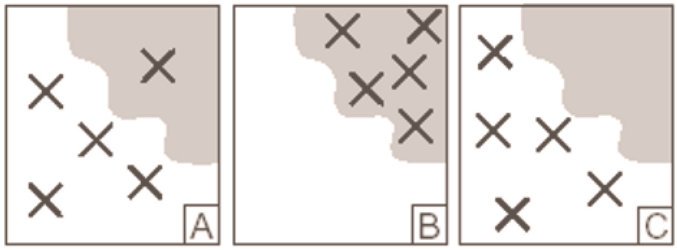


Sampling pattern as recommended by USDA (2021) for sampling bulk truck or rail shipments of soybean seeds or soybean meals (SBM) using a hand-held sampling device or an automatic sampler.

- Site A: Probe the grain approximately 0.6 m from the front and side.
- Site B: Probe approximately halfway between the front and center.
- Site C: Probe approximately three-quarters of the way between the front and center.
- Site D: Probe grain in the center of the carrier.
- Sites E, F and G: follow a similar pattern described above for the back part of the carrier.

Source: Herrman, 2001.

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



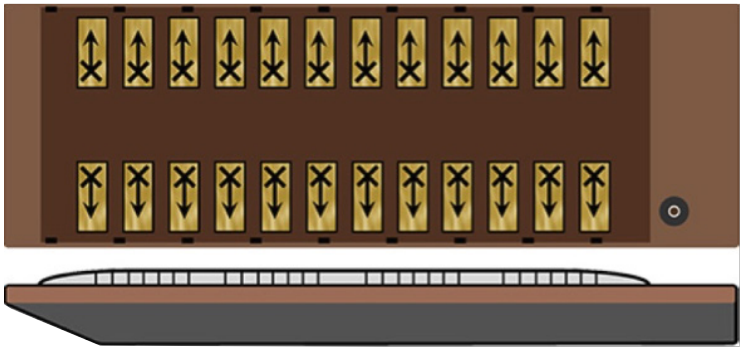
Recommended stratified sampling patterns for carriers containing inferior or damaged portions of soybean seeds or SBM. In this case a three-step procedure is recommended.

- A: Probe the carrier as a whole, both inferior and sound portions, as if the load was homogeneous.
- B: Probe the portion or portions containing the inferior grain thoroughly to obtain a representative cross-section 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.

(From: Herrman, 2001).

C. Sampling pattern for fiberglass hatch-top barges with soybeans (Fiberglass Hatch Top Barge).

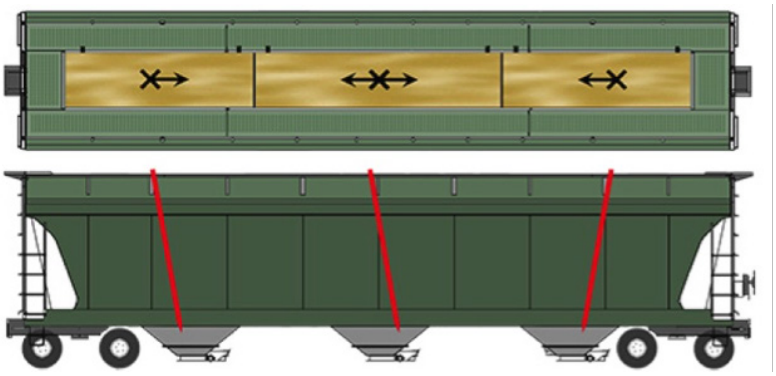
Draw one probe sample from each opening in the direction of the arrowhead. Insert the probe in the center of the opening, approximately 0.3 m, or 7 feet, from the side edge.



Source: USDA, 2014.

D. Sampling pattern for hopper cars with soybeans

Insert probe in the direction of the arrow at an approximately 10° angle. The probe may be inserted either in the center of each hopper or slightly off-center to miss the cross beam.

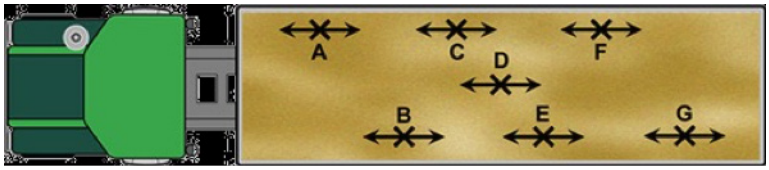


Source: USDA, 2014.

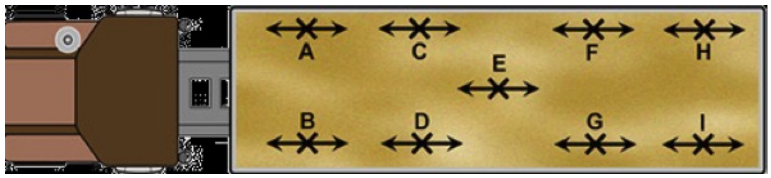
E. Sampling patterns for trucks with soybeans

Insert the probe at an approximately 10° angle in the direction of the arrows shown in the diagram. The probe pattern shown may also be used in reverse of the one shown.

a. Flat-bottom truck or trailer with soybeans more than 1.2 m, or 4 feet, deep or eight filled probe compartments.



b. Flat-bottom truck or trailer with soybeans less than 1.2 m, or 4 feet, deep or eight filled probe compartments.



Source: USDA, 2014.

F. Determination of the number of containers to be samples in a lot of containers.

- Determine the number of containers in the lot.
- Determine the minimum number of containers from which samples need to be drawn (see table below).
- Randomly select an appropriate number of containers from the lot.

Determination of sampling rate

Containers in lot	Sample size	Containers in lot	Sample size	Containers in lot	Sample size
100 or less	10	1,522 - 1,600	40	4,762 - 4,900	70
101 - 121	11	1,601 - 1,681	41	4,901 - 5,041	71
122 - 144	12	1,682 - 1,764	42	5,042 - 5,184	72
145 - 169	13	1,765 - 1,849	43	5,185 - 5,329	73
170 - 196	14	1,850 - 1,936	44	5,330 - 5,476	74
197 - 225	15	1,937 - 2,025	45	5,477 - 5,625	75
226 - 256	16	2,026 - 2,116	46	5,626 - 5,776	76
257 - 289	17	2,117 - 2,209	47	5,777 - 5,929	77
290 - 324	18	2,210 - 2,304	48	5,930 - 6,084	78
325 - 361	19	2,305 - 2,401	49	6,085 - 6,241	79
362 - 400	20	2,402 - 2,500	50	6,242 - 6,400	80
401 - 441	21	2,501 - 2,601	51	6,401 - 6,561	81
442 - 484	22	2,602 - 2,704	52	6,562 - 6,724	82
485 - 529	23	2,705 - 2,809	53	6,725 - 6,889	83
530 - 576	24	2,810 - 2,916	54	6,890 - 7,056	84
577 - 625	25	2,917 - 3,025	55	7,057 - 7,225	85
626 - 676	26	3,026 - 3,136	56	7,226 - 7,396	86
677 - 729	27	3,137 - 3,249	57	7,397 - 7,569	87
730 - 784	28	3,250 - 3,364	58	7,570 - 7,744	88
785 - 841	29	3,365 - 3,481	59	7,745 - 7,921	89
842 - 900	30	3,482 - 3,600	60	7,922 - 8,100	90
901 - 961	31	3,601 - 3,721	61	8,101 - 8,281	91
962 -1,024	32	3,722 - 3,844	62	8,282 - 8,464	92
1,025 - 1,089	33	3,845 - 3,969	63	8,465 - 8,649	93
1,090 - 1,156	34	3,970 - 4,096	64	8,650 - 8,836	94
1,157 - 1,225	35	4,097 - 4,225	65	8,837 - 9,025	95
1,226 - 1,296	36	4,226 - 4,356	66	9,026 - 9,216	96
1,297 - 1,369	37	4,357 - 4,489	67	9,217 - 9,409	97
1,370 - 1,444	38	4,490 - 4,624	68	9,410 - 9,604	98
1,445 - 1,521	39	4,625 - 4,761	69	9,605 - 9,801	99
				9,802 - 10,000	100

Source: USDA, 2014.

Annex 4. Sampling devices for soybean products

Figure 1A. Grain probes

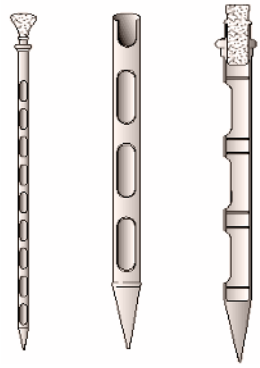


Figure 1B. Tapered bag triers

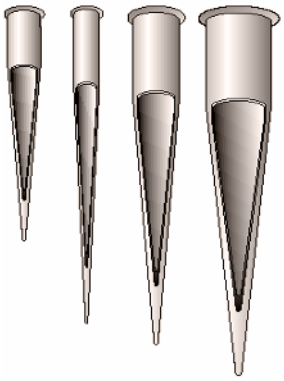
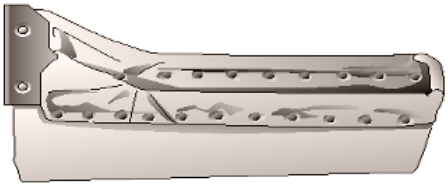


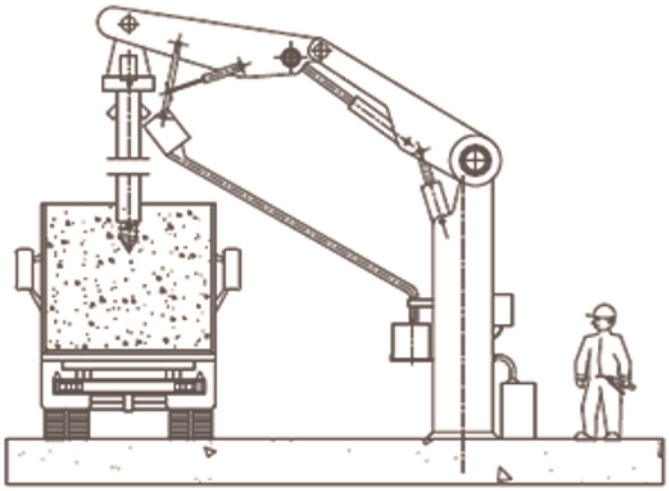
Figure 1C . Bomb

Figure 1D. Pelican grain probe sampler



Source: Hermann, 2001.

Figure 1E. Sampling with auger system



Annex 5A. Sampling guidelines for bagged material

- Sampling 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 sampled.
- 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.

Annex 5B. Sampling of soybean meal at origin

Automatic mechanical sampler system

a. Sampling of soybean meal (SBM) shall be done by an automatic mechanical sampler located in a spout or at the discharge of a belt conveyor, as appropriate. The sampler shall be designed to cut an increment from the entire cross-section of the SBM stream, perpendicular to the flow, at a location where the SBM is flowing freely and at a uniform rate, in order to obtain the most representative sample of the SBM flow. If the sampler is located in a spout, the spout slope must be 45° or more from horizontal and the flow must not be choked. When the diverter, or pelican, is stationary between cuts on either side of the meal stream, the opening shall be sealed to prevent dust from entering.

b. The sampler system shall be located at a point beyond which no blending or addition to the product may be introduced prior to its being loaded, and at a point where it is representative of each loadout.

c. The activation of the sampler shall be regulated by an adjustable timer. When the average SBM-flow rate through the sampler is less than 800 tons/hour, a sample, or cut, shall be taken for every 5 tons or less of meal flow. When the flow rate is between 800 and 1,200 tons/hour, a sample shall be taken for every 8 tons or less of SBM flow. When the flow rate is 1,200 tons/hour or greater, a sample shall be taken for every 12 tons or less of meal flow. A minimum of five samples shall be taken during the loading of any one vehicle.

d. The diverter opening for cross-cut samplers and swing-type samplers in which the diverter moves about a horizontal shaft, where the entire length of the diverter opening passes through the stream at the same speed, shall be of uniform width in the range of 0.8-2.2 cm, or 5/16-7/8 inch. For rotary-type samplers, in which the diverter moves about a vertical shaft and passes through the stream similar to a swinging door, with the outer end of the diverter moving at a higher speed than the inner end, the diverter opening width shall be a minimum of 0.8 cm, or 5/16 inch, at the end nearest the pivot and shall increase in width in proportion to the distance from the pivot point. In all cases, the diverter shall cut the meal stream at an average speed of approximately 30.5 m/minute, or 100 feet/minute.

e. The sample taken by the automatic sampler may be reduced in size by one or more mechanical dividers, but the reduced sample must still be representative of the SBM passing the sampler. The accuracy of the divider shall be equal in performance to a Jones- or Boerner-type divider. To comply with contract specifications, the entire sample may be further reduced through a Jones- or Boerner-type divider or its equivalent, and then each portion of the sample must be placed in an official National Oilseed Processors Association (NOPA) SMB sample bag and properly identified

f. An automatic sampler system manufactured and installed in accordance with the requirements stated in these Rules shall be considered "NOPA approved" upon completion of the following:

   i. Written certification of the installation by the NOPA Designated Licensed Professional Engineer on NOPA Form UCI-1, stating that the automatic sampler and mechanical divider installation meets the requirements set forth under these Rules. Certifications in effect prior to January 1, 1985, are exempted from this requirement.

   ii. Semi-annual written certification to NOPA by the operator of the origin loading facility that the sampler is in good working order and will be properly maintained and operated when an official method of sampling under these Rules is required by the terms of the sales contract.

Source: NOPA, 2017, Annex B.

Annex 6. Devices for splitting of samples

Figure 2A. Riffle sample splitter

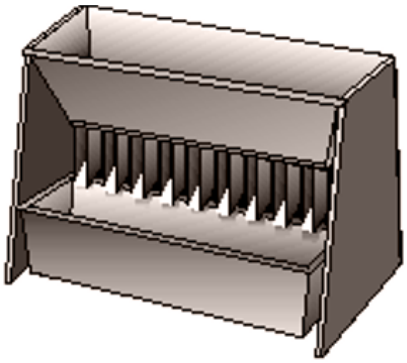
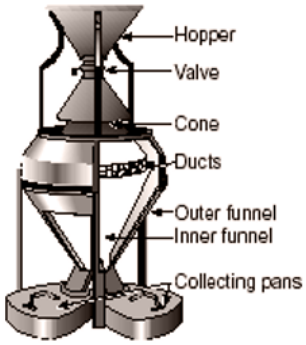


Figure 2B. Boerner divider



Source: Herrman, 2001.

Annex 7. Student's test table

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

Annex 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

THANK YOU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