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8. Chemical Analyses

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

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

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8.1 Moisture

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

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

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

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

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

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

and

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

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

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

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8.2 Ash

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

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

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

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

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8.3 Protein

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

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

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

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

The chemical needs for the procedure are as follows:

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

The procedure is as follows:

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

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

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

The calculation is:

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

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

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

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

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8.4 Protein quality

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

Table 9

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

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

Table 10

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

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

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

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8.4.1. Urease Index

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

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

Procedure:

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

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

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

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

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8.4.2. KOH Protein Solubility

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

Procedure:

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

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

Table 11

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

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

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

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

8.4.3. Protein Dispersibility Index (PDI)

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

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

Procedure:

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

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

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

Table 12

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

Urease Index

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

KOH Protein Solubility

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

Protein Dispersibility Index (PDI)

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

Nitrogen Solubility Index (NSI)

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

Absorbance at 420 nm

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

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

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

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8.4.4. Protein quality in ruminants

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

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

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

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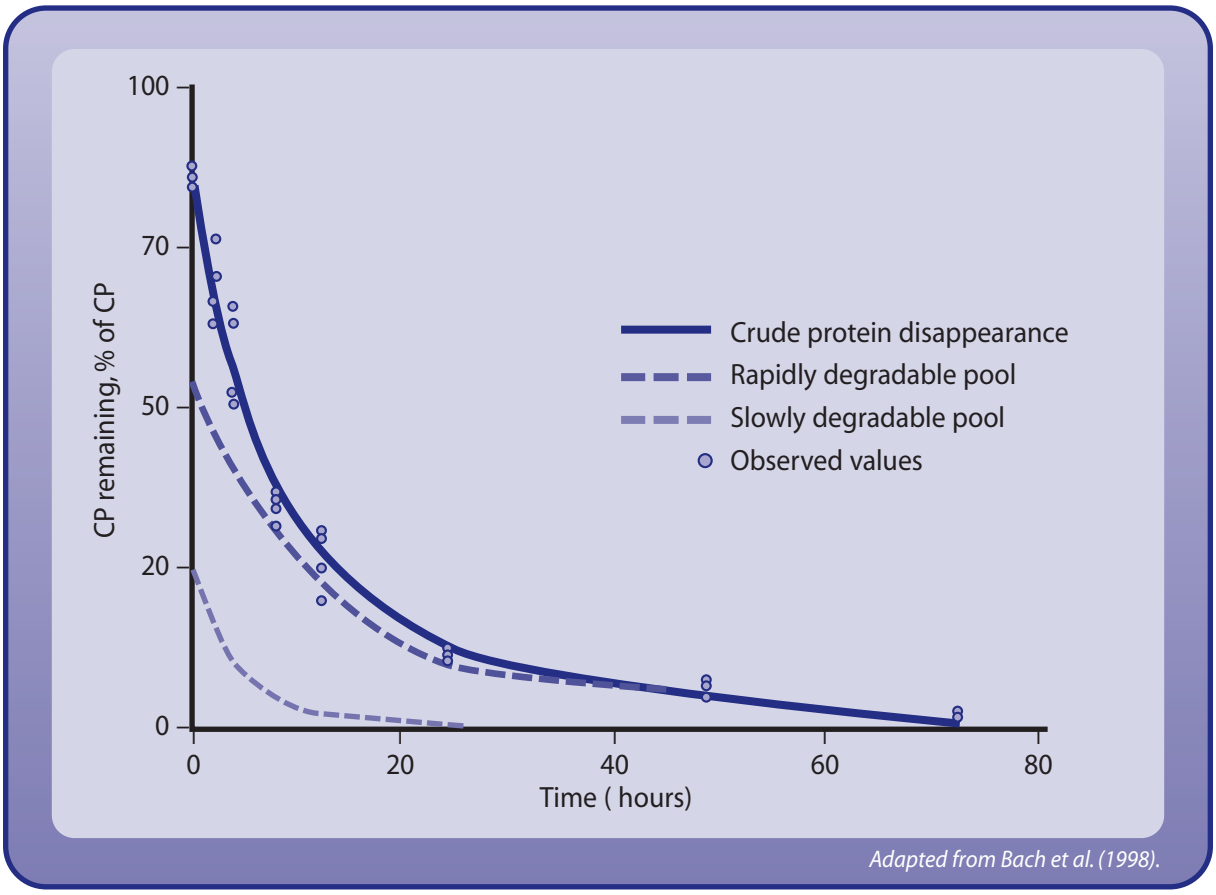
8.4.4.1. In situ technique

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

Figure 2

Protein disappearance from soybean meal and curve peeling processa



Adapted from Bach et al. (1998).

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

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8.4.4.2. In vitro technique

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

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

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

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

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8.5. Amino Acids

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

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

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

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

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

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8.6. Crude Fiber

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

The method requires the following reagents:

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

Equipment:

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

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

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

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

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8.7. Neutral Detergent Fiber (NDF)

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

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

The technique is as follows.

Reagents:

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

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

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

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

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

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

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

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

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

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8.8. Acid Detergent Fiber (ADF)

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

Reagents needed are:

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

Extraction of sample

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

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

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

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

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8.9. Lignin

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

There are two methods to determine lignin, the Klason lignin and the permanganate lignin. The method of choice is the Klason lignin.

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8.9.1 Klason lignin

Klason lignin requires 72% sulfuric acid and sintered glass crucibles.

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

Procedure:

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

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

Calculate Klason lignin (on dry matter basis) as:

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

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8.9.2. Permanganate lignin

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

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

Calculate Permanganate lignin (on dry matter basis) as:

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

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8.10. Starch

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

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

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

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8.10.1 Polarimatric starch determination

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

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

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

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

Total optical rotation determination:

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

Optical rotation determination of dissolved substances in 40% ethanol:

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

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

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

Table 13.

Optical rotation of various pure starch sources

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

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8.10.2. Enzymatic or colorimetric starch determination

The enzymatic method is much longer than the polarimetric one.

Reagents needed are:

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

The total procedure takes three days.

Day on:

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

Day two:

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

Day three:

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

Table 14.

Solutions to prepare standard curve for colorimetric starch determination

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

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

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

- Calculate the content of alpha linked glucose polymers:
  Alpha-linked glucose polymer, mg/g = Glucose concentration in standard x (V/100) x (1/sample weight)
  where, V is the flask volume difference (sample + flask weight - flask weight)

- Calculate starch content as:

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

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8.11. Non starch polysaccharides (NSP) and monosaccharides

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

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

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

The total NSP fraction can be estimated as follows:

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

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

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

After hydrolysis, the derivatization needs to be performed:

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

Chromatography:

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

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8.12. Ether Extract

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

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

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

The procedure is as follows:

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

The calculation of crude fat is as follows:

$$\text{Crude fat, \%} = \frac{\text{Final weight after extraction, g}}{\text{Original weight, g}} \text{ x100}$$

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8.13. Lipid quality

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

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

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8.13.1. Moisture

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

The procedure is following:

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

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

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

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8.13.2. Insoluble impurities

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

The method is as follows:

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

- The insoluble impurities are then calculated as:

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

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

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8.13.3. Unsaponifiable matter

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

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

The reagents for this method are:

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

The procedure is as follows:

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

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

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

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8.13.4. Iodine value

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

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

The procedure is as follows:

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

Determine the iodine value using the following equation:

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

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

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8.13.5. Acid value

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

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

The procedure is as follows:

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

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

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8.13.6. Lipid Oxidation

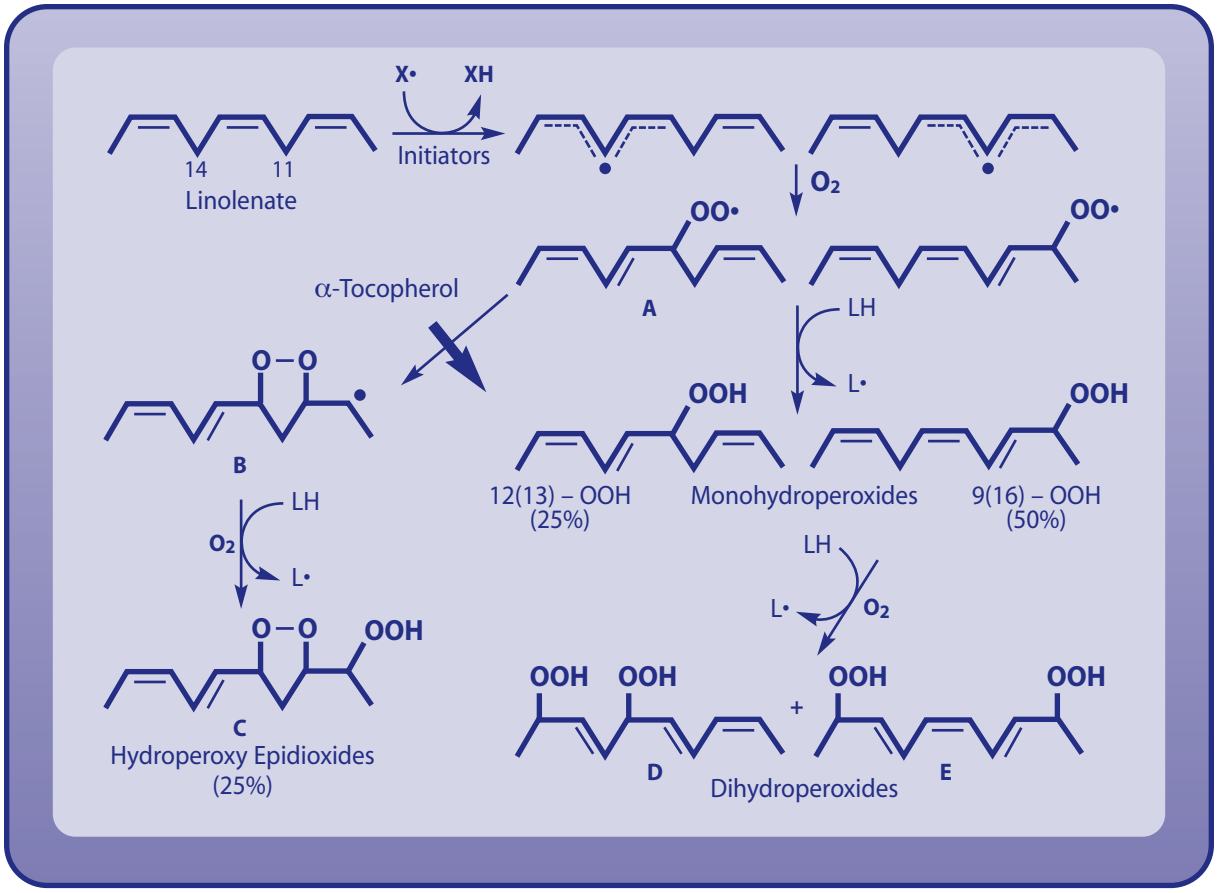
Lipids, especially oils, can undergo oxidation, leading to deterioration. In feeds, these reactions can lead to rancidity, loss of nutritional value, destruction of vitamins (A, D, and E) and essential fatty acids, and the possible formation of toxic compounds and changes in color of the product.

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

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

Figure 3

Auto-oxidation of linolenic acid



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

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

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

contents

8.13.6.1. Peroxide value

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

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

Procedure:

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

The calculations are as follows:

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

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

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8.13.6.2. Thiobarbituric acid (TBA)

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

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

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

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

The procedure is as follows:

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

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8.13.6.3. Anisidine value

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

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

8.13.6.4. Lipid Stability tests

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

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8.13.6.4.1. AOM (active oxygen method)

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

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

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8.13.6.4.2. OSI (oil stability index)

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

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8.13.7. Fatty acid profile

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

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

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

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

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

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

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8.14. Minerals

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

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

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

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8.14.1. Calcium

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

The following reagents are needed:

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

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

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

Calculation:

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

contents

8.14.2. Phosphorus

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

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

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

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

contents

8.14.3. Sodium chloride

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

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

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

Calculation:

$$NaCl(\%) = \frac{15 - \text{ml } 0.1 \text{ N } NH_4CNS \times 0.585}{\text{wt. of sample, g}}$$

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

contents

8.15. Isoflavones

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

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

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

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

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

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

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8.16. Antinutritional factors (ANF)

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

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

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

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8.16.1. Trypsin inhibitors

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

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

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

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

Reagents needed are:

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

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

The procedure is as follows:

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

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

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

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

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

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

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

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8.16.2. Soy antigens

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

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

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

Table 15

ELISA formats used for analysis of soy globulins

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

(from Tukur et al., 1996)

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8.16.3. Lectins

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

Table 16

Anti nutritional factor contents in various soy products

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

(adapted from Huisman and Tolman, 1992)

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

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

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

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

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

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8.17. Mycotoxins; rapid tests

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

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

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

General procedure:

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

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

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

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8.17.1. Ochratoxin

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

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8.17.2. Zearalenone

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

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8.17.3. Fumonisins

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

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8.17.4. Aflatoxins

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

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

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

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8.17.5. Deoxynivalenol

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

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

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

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8.18. Genetically modified organisms (GMO)

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

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

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

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

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