Rapid Methods of Proximate Analyses

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The format of this session will be to spend 10 to 15 minutes reviewing the methodologies which are available at the present time for proximate analysis and at the end of that period to open the floor for discussion. As was indicated yesterday, the people leading the discussions are not necessarily experts, so we will depend upon the audience for input. We should keep several things in mind for the reciprocation portion of this session. First, if you have any specific questions we should try to answer them. Second, we should share personal experiences about certain methodologies – personal pros and cons for certain methods. Third, we may want to assist people by suggesting the type of methodology most suited for their laboratory. Fourth, perhaps we can discuss the possibility of AMSA assisting in having certain methods approved by AOAC. AOAC certainly carries a lot of weight. But as many of us know, in order to get a method approved by AOAC, considerable effort is required. The USDA labs in Philadelphia have taken the lead in having several procedures approved by AOAC. Bob Benedict of that lab is here and is willing to share their experiences.

Moisture

Moisture can be determined by oven drying, by convection heating and by the preferred method, infrared heating. The infrared method is preferable because drying can be conducted at a lower temperature and can be done faster. Most of us use the vacuum oven method. It requires a lower temperature and results are obtained in five to six hours. The microwave oven method originated with the USDA in Philadelphia in 1975. Joe Pettinati reported good results with a microwave oven. One modification of this method involves sandwiching of samples. Each sample is weighed between two filter papers and dried two to four minutes in the microwave. When using microwave ovens, care must be exercised to avoid incomplete drying due to uneven heating in the oven.

Chemical dessication provides precise results for special cases. Most of us in meat science do not have to have results which are accurate to the third or fourth decimal, which this method provides.

Moisture can also be determined by the azeotropic method involving the distillation of toluene and water, the distillate coming off at 85°C. The ratio of water to toluene is 20 to 80. The amount of water is measured volumetrically. The Karl Fischer method is the most common titration method. This method is extremely precise and sensitive. However, it is not suitable for routine purposes. Gas chromatography (GC) is an instrumental method which involves extracting water with a solvent and running it on a GC. This method is not practical. Paul Addis (Minnesota) devised a refractive index method wherein moisture is extracted with solvent and is determined by the change in refractive index. The near infrared method, in my opinion, is one of the most frustrating methods. In theory, it is really an attractive procedure where one can determine protein, moisture and fat almost instantaneously on a single sample. However, in practice, people have had a lot of trouble with this method when attempting to analyze meat. For grain it has worked quite well and is used routinely in the field.

Fat

You are all familiar with the ether extraction procedure (soxhlet). It is relatively slow, and ether is explosive. The chloroform-methanol method is a lab procedure which works quite well. I have provided the references for Folch et al. (1957) and Bligh & Dyer (1959) because several people have

Table 1. Methods of Moisture Analysis

1. Drying
a. Oven-drying (Infrared heating)
b. Vacuum oven drying
c. Microwave oven drying
d. Chemical dessication (P_2O_5, $CaCl_2$, etc.)
2. Distillation
a. Azeotropic method
3. Titration
a. Karl Fischer method
4. Instrumental
a. Gas chromatography
b. Refractive index (Addis and Chudgar)
c. Near-Infrared

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asked me about the differences between these two methods. These two methods originated in Canada in the late 1950's. The Folch method uses a salt in the aqueous fraction in order to reduce the interface problem as well as to get some of the more polar lipids into the organic phase. It does a more complete extraction of lipids. The Bligh & Dyer method is faster, uses less solvent but yields slightly lower total lipid values because some lipids are lost into the aqueous phase. Thus, it depends on what one's needs are. If you are concerned about some specific fatty acids or phospholipids, then I would recommend the Folch method.

The next major fat category is the heating method, which basically involves rendering. This method includes the Babcock and microwave procedures where the samples are heated and the amounts of melted fat are measured. Next is the specific gravity method which includes the popular Foss-Let method. A sample is extracted and the change in specific gravity is measured. The Foss-Let technique is an official AOAC method. As with many other methods, it suffers as a result of the use of a small sample size.

The X-Ray or Anyl-Ray, used in large commercial operations, is very popular. The IR reflectance and transmission methods have problems. They work well for moisture and fat analyses under controlled conditions, but considering the investment and the care required, I am not sure that many people are investing in the equipment at the present time. The electrical inductance method requires more work before people will feel comfortable using it. Finally, the chromatography method will be considered. This method, which has been buried in the literature for several years, was first reported by Maxwell et al. (1980). They devised a method in which the fat sample was ground with sodium sulfate and celite, applied on the column and eluted with solvent. Polar and non-polar lipids could be eluted separately or together. For total lipids, 125 ml solvent are required and results can be obtained in two hours. Multiple samples can be run, so it is quite adaptable. Fresh as well as processed meat can be analyzed, so I think we should give a little more attention to this method.

Table 2. Methods of Fat Analysis

1. Extraction
   a. Ether (Soxhlet)
   b. Chloroform: methanol (Folch et al.; Bligh & Dyer)
2. Heating
   a. Babcock
   b. Microwave
3. Specific gravity
   a. Foss-Let
4. Instrumental
   a. X-ray (Anyl-Ray)
   b. IR reflectance and transmission
   c. Electrical inductance (EMME)
5. Chromatography
   a. Dry column method

Protein

The classical Kjeldahl method involves a bank of digestion heating units, distillation units and a titration unit. In recent years these units have become more automated. Technicon, Kjel-Foss and Tecator units are operational. The Tecator units were quite troublesome initially, but most of the problems have been resolved. It is rapid and in our laboratory, about 80 samples a day are completed without difficulty.

The combustion method for protein determination is not as familiar to most people. The sample is combusted in the presence of a catalyst, and the volume of nitrogen or changes in conductivity due to nitrogen are detected. One of the newer methods measures chemiluminescence in which the sample is combusted and the nitrogen compounds are reacted with ozone, converting the nitrogen compounds to an activated form of nitrogen dioxide. As the activated forms drop to a lower energy level, the emitted photons are detected by a spectrophotometer. It works quite well, and one result I have seen recently on some meat samples indicates that it does have some promise. About 2 minutes are required per analysis. The disadvantage of the system is that it cannot accommodate multiple samples.

The dye-binding method for protein is useful, but it has been superseded by some of the new automated Kjeldahl methods. This method is based on the fact that certain dyes bind with basic amino acids so that when a dye and protein are blended, the bound dye is removed with the protein. The amount of remaining dye, which is determined spectrophometrically, is related to the amount of protein which was present. Pettinati (1980) has reported that this method works well for screening purposes. One note of caution is that samples containing high connective tissue content tend to give low values because of their unique amino acid compositions.

The near-infrared method for protein has been evaluated extensively. I would say that under well-defined conditions, the near-IR method works well for moisture and fat but needs additional refinement for protein determination.

Table 3. Methods of Protein Analysis

1. Kjeldahl
   a. Classical
   b. Automated (Technicon, Kjel-Foss, Tecator)
2. Combustion – Nitrogen Analyzer
   a. Measure gaseous volume
   b. Thermal conductivity
   c. Chemiluminescence
3. Dye binding
4. Instrumental
   a. Near infrared

Combination Approaches

People have tried combining protein, moisture and fat analyses on one sample by instrumental methods. The in-

strumental method by near-IR has been one approach that has resulted in only partial success. Also, the microwave gravimetric method has been used where moisture is determined first and on the residue, fat is determined by rendering. Then, by using a factor, protein is determined. This approach offers some possibilities.

Table 4. Methods of Moisture:Protein:Fat Combination Analysis

1. Instrumental
   a. Infrared
   b. Microwave-Gravimetric (CEM, Hobart)

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Discussion

Steve Smith: In biochemical journals, almost everyone determines protein by Lowry, Biuret, or in some cases, dye binding. Why are not Lowry or Biuret methods discussed here?

Katz Ono: The Lowry and Biuret require soluble proteins and many of our samples really are not soluble.

Bob Benedict: There was a report last year from some European laboratories where they did a collaborative study among eight countries. They did moisture fat, protein, hydroxyproline, pH and, I think, one other thing because the European market is more interested in the fat-free protein and in collagen-free protein. They found that the most reliable or reproducible analyses were moisture and pH. The least was hydroxyproline, and if one took the values that were reported there by the various laboratories and calculated out this non-collagen protein, I think the values ranged from 18.8 to 21.2 for the same samples. This indicates that we have a lot of work to do to get reproducible or better analytical methods than we now have.

I think that one of the things that has to be done is to examine other methods and see if we can test them on meat. For example, the Biuret method (as pointed out earlier) measures soluble protein. However there is a method which, if you divide up the Biuret solvents, treat the meat first with alkaline reagent and then add the copper, provides a good test for meat proteins because the high pH dissolves the meat proteins (breaks them up). The Lowry method measures aromatics, so it is not going to be effective at all against connective tissue, which is very low in aromatic amino acids. Thus, one has to know the aromatic amino acid makeup of the sample.

We really need a good test for collagen. Collagen has a different nitrogen value than skeletal muscle proteins, so if you had 100% collagen, then you have 120% protein if the Kjeldhal method is used. If you wanted to obtain a higher protein, you could throw connective tissue in. I would like to comment on Maxwell's procedure for fat (dry column method). I have used it for five years and it is fantastic. You obtain an undenatured sample and you can do all sorts of fatty acid, sterol and cholesterol analyses, and you can obtain a separate phospholipid fraction. It is a test which I think we should get behind and have a collaborative study, if possible.

Ono: I agree with you. There is great difficulty in getting people interested in developing analytical methods. It is not a very glamourous type of research to develop methods, and this is reflected in the attitude in both academic and govern-