Faculty of Agricultural Sciences | JHM | 12. December 2010
Department of Animal Health and Bioscience | J.nr.:

Ruggedness test of procedure for determination of soluble crude protein

Introduction

In 2007 the Danish Plant Directorate performed a proficiency test in which several of the new NorFor methods was included. The results for soluble crude protein were dissatisfactory. The calculated Horrat values was higher than what can be accepted for analytical procedures used for official feed control. The test included 12 different raw materials or compound feeds. The results from the eight participating laboratories were divided in two groups. DP and DIAS reported lower values than the remaining five laboratories. One laboratory was excluded for using an analytical procedure which is different from the specified NorFor procedure.
It was concluded that a ruggedness test of the procedure was necessary and that the method eventually should be improved according to the results of the ruggedness test.

An explanation of the high Horrat values was not obvious. After a very close inspection of the diffent steps in the procedure the following steps was suspected to influence the methods performance (the numbers refer to the different steps in the method description from 2006)

- Step 3 Sample preparation: Has the drying conditions an effect?
- Step 3 Sample preparation: What is the effect of the mill type used for grinding (hammer or knife mill)?
- Step 4.4: pH is specified to 6.75 ± 0.05. If the results of the method is highly dependent on pH, it should be specified to be used a 2-point calibration of pH meter before adjusting the pH of the buffer.
- Step 6.4: Hydration of sample material at the beginning of extraction. If hydration is not perfect, then according to the method section 7.3 it may be necessary to sieve the material by using a tea strainer before pipeting material for Kjeldal destruction. Hydration may be done more efficiently.
- Step 6.5. Time used for extract for is unclear. It must be assumed that the clock starts from the moment the temperature reaches the 39 ° C.
- Step 6.5. Is it important to shake every 15 minutes?
- Step 6.6 to 6.7. The centrifuged extract may be more or less ambiguous, depending on how good hydration is performed and how hard the material is spun. One might choose to specify that the solution must be filtered through a filter paper.

After the optimized of the method, the methods repeatability and reproducibility should be investigated. It should be focuses only on the method's ability to measure dissolved crude protein, ie, the outcome is not being compared with samples total crude protein content and the content of volatile ammonia in silage samples are not determined separately. This is done in order not to implicate the uncertainty in the measurement of crude protein in the characterization of the optimized methods uncertainty.
At a laboratory (DIAS) studied the method repeatability and reproducibility over 5 different days. Reproducibility of the method among the three laboratories investigated too.

Abbreviations

DLG: Dansk Landbrugs Grovvareselskabs Centrallaboratorium (Danish Cooperative Farm Supply, Central Lab.), Odense. This laboratory is now part of Eurofins

DIAS: Danish Institute of Agricultural Science, Department of Animal Health and Bioscience
Kungsengen: Uppsala
PD: Danish Plant Directorate, Sorgenfri
Eurofins: Eurofins Steins, Holstebro
sCP: Solubile crude protein

Participating laboratories
DLG, PD, Eurofins, Kungsengen (SLU Sweeden), DIAS

Materials and methods ruggedness test
Materials
Maize silage (from 2007), grass silage (from 2007), barley, hay (barn dried hay) and rapeseed cake were selected. The materials were dried at < 60 °C and grinded on a Fritch Pulverizette 15 (Fritch, Idar-Oberstein, Germany) knife mill fitted with a 6 x 6 mm screen. Each material were carefully mixed and subdivided in eight using a Retch PT subdivide (Retch, Haan, Germany)

Grinding of samples at the five different laboratories
A set of bags containing approximately 50 g of each of the samples was mailed to the laboratories. They were asked to grind all the material, as normally for this analytical procedure. They were also asked to record the temperature prior to and after the grinding of the material as well as the mill type and the size of the screen used.
After reception of the samples in Foulum, they were sieved on a sieve with a square mesh of 1 x 1 mm (ISO 3310-1 1 mm). The amount of material left on the screen was recorded. All material was carefully mixed again. Dry matter content was determined at < 60°C.

Method for determination of soluble crude protein
The materials were analyzed between three and five times for sCP content using the original NorFor procedure (Attachment 1).

Instruments
The nitrogen content was analyzed on a Kjedltec 1035 (the grinding test). The ruggedness test was analyzed on a Kjeldtec 2460.

Design of ruggedness test
In the ruggedness test the following parameters were investigated:
1. Filtration instead of centrifugation for clarification of the extract in the method step 6.6
2. Shaking interval on 20 instead of 15 minutes
3. Time of extraction 90 minutes instead of 60 minutes
4. pH of the extraction buffer varied between 6.65 – 6.95
5. Use of ultrasound for hydration of samples as alternative to shaking

The actual design is given in Table 1

Trial	Ref	Clarification	Interval shaking	Ekstraktion	pH	Ultrasound
1		Centrifugation	15 min	60 min	6,75	No
2	6.6	Filterpaper	15 min	60 min	6,75	No
3	6.5	Centrifugation	20 min	60 min	6,75	No
4	6.5	Centrifugation	15 min	90 min	6,75	No
5	4.4	Centrifugation	15 min	60 min	6,55	No
6	4.4	Centrifugation	15 min	60 min	6,95	No
7	6.4	Centrifugation	15 min	60 min	6,75	2 min

Table 1 Design of ruggedness test

Rapeseed cake, maize silage and grass silage were selected for the ruggedness test. All materials were analyzed in triplicates.

Statistical analysis
The data statically analyzed using the proc GLM in SAS (Version 9.2, SAS institute, XX). The model for analysis of the effect of handling and grinding at different laboratories were:

```
Proc GLM;
class foder lab mille sold;
model sCP=foder lab mille sold /ss1 ss2;
Lsmeans foder lab m_lle sold /stderr pdiff;
```

The model for investigation of the effect of the variation of the methods parameters were:

```
Proc Glm;
Class feed clarification ph interval time hydration;
Model sCP = feed clarification ph interval time hydration feed*clarification
feed*ph feed*interval feed*time feed*hydration/SS1 SS2;
Lsmeans  feed clarification ph interval time hydration feed*clarification
feed*interval/stderr pdiff;
```

Materials and methods for repeatability and reproducibility determination

Experimental materials: grass silage, corn silage, barley, rapeseed cake, soybean meal and lupine found at DIAS. Silage samples predrying at <60 ° C and milled in a knife mill fitted with a 6 mm sieve. Samples shredded on the rotary snow share and circulated to other laboratories.

The following laboratories apart from DIAS attend: Plant Directorate and Kungsengen in Uppsala.

Method for determination of soluble crude protein
The materials were analyzed using the optimized NorFor procedure (Attachment 2).

Instruments
The nitrogen content was analyzed on a Kjeldtec 2460.

Results and discussion

Ruggedness test
From Table 2 it can be seen that the five laboratories have a broad range of mills available. Kungsengen and DIAS both have grinded the samples on both hammer and knife mill to the specified particle size. In addition DIAS have tried several other mills which were available, and included the results in the grinding test to give a better view of the performance of the different mills and screens.

In "forordning 152/2009/EF" for analysis of animal feed, it is stated that dry material should be grinded prior to analysis. It is specified that the material should be capable of passing a sieve with a seize of 1 x 1 mm and that over grinding should be avoided. Heating during grinding should also be avoided.
Comparing the sieving rest with the screen size it is clear that grinding with a screen size of 1 mm will not necessarily give a material which is capable of passing a 1 x 1 mm sieve!

For barley as much as 12.5 % did not pass the sieve if a combination of a Fritch Pulverisette with a 1 mm screen is used! The sieving test of the barley grinded by PD and Eurofins showed that 5.5 and 6.4 % of the material did not pass sieve.
In praxis it is probably impossible to grind the materials so that all will pass the specified sieve with out over grinding of the material. A small rest may be acceptable.
It may be concluded that several laboratories dos not comply with 152/2009/EF. It is therefore recommended that the efficiency of the grinding equipment is verified regularly at the laboratories.

Several of the mills used is heating the materials considerably during grinding. Grinding maize silage on a Retch ZM 200 mill a temperature increase of 20 °C was observed. Grinding barley on the same mill, a temperature increase of 12 °C was observed. This could however be reduced to only 2 °C if the mill was fitted with a cyclone for collection of the material.
Looking at materials which, according to the participating laboratories, were ready for analysis, the falling number mill has the highest temperature increase (15 °C) for barley followed by the Retch ZM 200 grinding of maize silage (14 °C) and Retch knife mill for grinding of barley (12 °C).
The heat increase observed must be considered as an average increase, which implies that the temperature locally may have been even higher with a risk of heat damaging of the proteins which should be extracted with the method.
According to “forordning 152/2009/EF“ heating during grinding should also be avoided. From table 2 it is seen that it is possible to select a mill that only heat the materials marginally. Possibilities are the Retch SM 2000, Årslev Machin Factory and the knife and hammer mills used by Kungsengen.

Laboratory	Mill	Type	Screen size (mm)	Δ T (°C)	Sieving rest (%)	Analyses
Gras silage						
PD	Retch knife mill, SM 2000	Knife	1	4	0.9	no
Eurofins	Foss Cyclotec	Hammer	1	6	0.7	
DLG	Retch ZM 200	Hammer	1	9	1.5	
DIAS	Fritch Pulverizette	Knife	0.5	1	0.1	
DIAS	Årslev machine factory	Hammer	1	1	0.1	
Kungsengen	Knife mill	Knife	1	1	0.0	
Kungsengen	Hammer mill	Hammer	1	0	0.7	
Maize silage						
PD	Retch knife mill, SM 2000	Knife	1	4	1.1	No
Eurofins	Foss Cyclotec	Hammer	1	8	0.1	
DLG	Retch ZM 200	Hammer	1	14	2.1	
DIAS	Fritch Pulverizette	Knife	0.5	4	0.1	
DIAS	Årslev machine factory	Hammer	1	3	0.1	
DIAS	Retch ZM 200	Hammer	0.75	20	0.0	No
Kungsengen	Knife mill	Knife	1	2	0.0	
Kungsengen	Hammer mill	Hammer	1	2	0.0	
Hay						
PD	Retch knife mill, SM 2000	Knife	1	4	0.6	No
Eurofins	Foss Cyclotec	Hammer	1	4	1.0	
DLG	Retch ZM 200	Hammer	1	8	1.1	
DIAS	Fritch Pulverizette	Knife	0.5	2	0.1	
DIAS	Årslev machine factory	Hammer	1	2	0.1	
Kungsengen	Knife mill	Knife	1	1	0.5	
Kungsengen	Hammer mill	Hammer	1	1	2.0	
Rapeseed cake						
PD	Retch ZM 100	Hammer	1	3	0.0	No
Eurofins	Retch	Knife	1	6	0.3	
DLG	Retch ZM 200	Hammer	1	5	0.1	
DIAS	Fritch Pulverizette	Knife	0.5	4	0.0	
DIAS	Årslev machine factory	Hammer	1	3	0.0	
DIAS	Foss Cyclotec	Hammer	1	3	0.0	
Kungsengen	Knife mill	Knife	1	4	0.0	
Kungsengen	Hammer mill	Hammer	1	2	0.0	
Barley						
PD	Retch ZM 100	Hammer	1	1	5.5	
Eurofins	Retch	Knife	1	12	6.4	
DLG	Falling number laboratory mill	Hammer	0.8	15	0.1	
DIAS	Fritch Pulverizette	Knife	1	4	12.5	
DIAS	Fritch Pulverizette	Knife	0.5	4	0.2	
DIAS	Årslev machine factory	Hammer	1	3	0.2	
DIAS	Foss Cyclotec	Hammer	1	3	0.2	No
DIAS	Retch ZM 200	Hammer	0.75	12	0.0	No
DIAS	Retch ZM 200 (with cyclone)	Hammer	0.75	2	0.0	No
Kungsengen	Knife mill	Knife	1	5	2.0	
Kungsengen	Hammer mill	Hammer	1	4	5.3	

Table 2 Results of the grinding at five different laboratories. At DIAS several mill types as well as screen sizes have been used. Analysis indicate if the material was analyzed and part of the grinding study.

Parameter	**Probability**	**Effect**	**LSMEAN**
Lab	< 0.001	DIAS DLG Eurofins Kungsengen	0.853 0.938 0.935 0.905
Mill	0.130	Hammer Knife	0.923 0.893
Screen size	0.029	0.5 mm (0.8 mm) 1.0 mm	0.958 0.867) 0.899

Table 3 Effect of laboratory, mill type and screen size on the sCP content

The effect of having the different laboratories to grind the samples, shows a significant effect on the sCP content (Table 3). Grinding af DIAS gave the lowest sCP content while grinding at DLG gave the highest content. The material received from PD have been analyzed, but unfortunately on our laboratory's new Kjeldtec 2460 and not the Kjeldtec 1035 which have been used for all other samples in the grinding test.
The effect of the mill type is not significant although the P value is 0.13 indicating there is a small difference between knife and hammer mills, with the hammer mill giving the highest values (Table 3). This might be expected, if it is assumed that the hammer mill will tear up the material more efficiently than the knife mill.
The effect of the screen size, show that the smaller the screen the higher the sCP content. This might be expected, if it is assumed that the interior of the material is more accessible, if the particle size is smaller.

Parameter	**Probability**	**Effect**	**LSMEAN**
Clarification	< 0.001	Centrifugation Filtration	1.108 0.997
Hydration	0.0199	Shaking Ultrasound	1.025 1.080
Feed * clarification	0.0973	Grass (cent) Grass (filt) Mays (cent) Mays (filt) Rapeseed (cent) Rapeseed (filt)	1.198 1.125 0.873 0.793 1.255 1.071
Feed * shaking interval	0.0038	Grass (3) Grass (4) Mays (3) Mays (4) Rapeseed (3) Rapeseed (4)	1.180 1.142 0.775 0.891 1.176 1.150

Table 4. Results of parameters having the highest effects in the ruggedness test.

The effect of clarification is pronounced with centrifugation giving higher values than filtration. In the procedures step 6.6 a centrifugation at 3000 g for 10 min is specified. By experience the centrifuges solution very often appears unclear, indicating that sample particles is still suspended in the fluid. The amount of suspended particles left in the fluid will depend on how many small particles originally present in the material, the density difference between the solvent and the material and finally the g-force and time combination used during the centrifugation.

In the method's note 7.2 It is explained that particularly from forages particles may float on the surface after centrifugation, but if the supernatant is carefully pipetted the insoluble matter will not cause contamination. The particles may be removed with a spoon or a paper tissue. If still a problem the supernatant can carefully be poured into a beaker through a tea-strainer and then pipetted. By experience the recommendations on pipetting is very difficult to performed, a slight mistake and all the precipitate is "hvirvlet op" and the extract have to be centrifuged again. If a tea-strainer have an effect it indicates that the grinding of the material has not been sufficient.
During discussions of the results with other laboratories, several suggested that centrifugation was exchanged with filtration through nitrogen free filter paper. The advantages is easier pipetting, no need to decide on when to use a tea-strainer and much less fine particles suspended in the sample used for the Kjeldahl analysis.

The clarification and feed was slightly confounded.

Hydration by treatment with ultrasound gave slightly higher values than ordinary shaking. This is not surprising as the ultra sound may facilitate the extraction of soluble protein.

The feed type and shaking interval was also confounded, showing that four shakings gave a higher content compared to tree for grass and maize silage. For rapeseed cakes the opposite was the case.
During discussions of the results with other laboratories, several suggested that a continues shaking was used instead of interval shaking. This is a more cost effecctice procedure on a routine laboratory as thechnician don't have to handle the samples every 15 minutes.

Varying the pH of the extraction buffer to 6.55 or 6.95 had no effect on the sCP content.

Conclusion ruggednestest
It may be concluded that the laboratories should regularly verify that the grinding procedure is working as intented by performing a sieving test. Over grinding and excessive heat formation during grinding should be avoided.
Filtration should be used in stead of centrifugation.
Continues shaking should be used instead of interval shaking.

Reproducibility and repeatability

In table 5 the repeatability and reproducibility of the optimized method obtained at DIAS is shown. These analysis were performed on the same material grinded at DIAS. The highest CV (%) on 5.4 % was observed for soy cake. The CV's for the other materials is in the range of what can be expected for analysis performed at the same laboratory.

In table 6 the interlaboratory repeatability and reproducibility of the optimized method investigated on 3 different laboratories is shown. The repeatability of the method is generally god although the r-value for lupin is high (2.34). The reproducibility for barley, grass and maize silage is acceptable low. For rapeseed and soy R is 1,112 and 1.073 corresponding to CV values of 12 and 15% , this is values in the high end. For lupin R is 10,5 corresponding to a CV value of 36% which at present makes it almost impossible to compare results for solubile protein un lupin obtained at different laboratories. An explanation of the high R value may be that the 3 participating laboratories have used different mill types for grinding. This is in agreement with the ruggedness test, that showed that the mill type has some influence on the result. To clarify this a future ringtest should include a sample of lupin grinded as well as ungrinded.

DIAS	Maiz silage	Gras silage	Rapeseed cake	Soy cake	Lupin	Barley
Repeatability						
Averag	6,01	7,50	8,33	7,16	29,62	2,46
Stddev	0,10	0,10	0,28	0,39	0,99	0,09
CV (%)	1,7	1,4	3,4	5,4	3,3	3,7
N	11	10	12	10	8	10
Reproducibility						
Averag	5,91	7,43	8,25	7,35	29,64	2,38
Stddev	0,15	0,27	0,25	0,27	0,21	0,08
CV (%)	2,5	3,6	3,0	3,7	0,7	3,5
N	7	7	6	6	6	7
Drymatter (%)	96,56	96,18	95	91,87	91,27	91,33

Table 5. Repeatability and reproducibility at DIAS

DIAS	Maize silage	Gras silage	Rapeseed	Soy	Lupin	Barley
% sCP in DM	6,04	7,51	8,31	7,08	29,45	2,41
	5,89	7,49	8,08	7,14	29,77	2,35
Tørstof (%)	96,6	96,2	95,0	91,9	91,3	91,3
SLU						
% sCP in DM	5,82	7,36	8,84	7,14	33,53	2,41
	5,82	7,43	8,91	6,86	31,62	2,34
Tørstof (% ved 60C)	96,7	95,9	93,3	91,1	91,9	90,9
PD						
% sCP in DM	5,98	7,65	8,80	6,40	24,98	2,25
	6,10	7,70	8,90	6,30	25,60	2,13
DM (%)	93,7	92,2	90,7	89,1	87,6	86,6
P	3	3	3	3	3	3
N	2	2	2	2	2	2
S1	17,825	22,570	25,920	20,300	87,475	6,945
S2	105,936	169,842	224,246	137,625	2577,467	16,101
S3	0,037	0,008	0,068	0,156	4,135	0,023
s2r	0,0062	0,0013	0,0113	0,0260	0,6892	0,0038
s2L	0,0094	0,0194	0,1430	0,1178	13,0762	0,0098
m (avrage)	5,94	7,52	8,64	6,77	29,16	2,32
r (repeatability)	0,222	0,102	0,301	0,456	2,349	0,175
R (reproducibility)	0,353	0,407	1,112	1,073	10,500	0,330

Table 6. Repeatability and reproducibility calculated between 3 different laboratories

Conclusion on the repeatability and reproducibility test

It may be concluded that the repeatability and reproducibility for several of the tested materials like barley, grass- and maiz-silage is as low as could be expected. If feed material containing lupin is analyzed on different laboratories, the results for soluble crude protein may only be compared with great caution.

References

Licitra, G., Hernandez, T.M., Van Soest, P.J., 1996. Standardization of procedures for nitrogen fractionation of ruminant feeds. Anim. Feed Sci. Technol. 51, 347-358

152/2009/EF: COMMISSION REGULATION (EC) No 152/2009 of 27 January 2009 laying down the methods of sampling and analysis for the official control of feed