Protocol No: 4780
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To: Phytofil – N. Stavrakis
Cc: Dr N. Stavrakis

Information in laboratory methods

Dear Dr. Stavrakis,

We would like to inform you concerning some methods that are referred as “modified” or “in house” in test reports, which steps of method protocols have been modified.

- **Urea nitrogen: AOAC 959.03**

This method is suitable for fertilizers. According to method a quota of 10g is weighed. For “ENTOMELA 50SL” and “ENTOMELA 75SL” samples a quota of 2-3g of sample was weighed.

Principle:
The urea nitrogen determination according to AOAC 959.03 is based on urea hydrolysis by urease and the determination of ammonia liberated in a slightly acid environment according to Kjeldhahl method. The samples tested are free from proteins that previously precipitated with trichloroacetic acid. After precipitation and centrifugation of sample, the supernatant is transferred to a distillation apparatus and after the addition of sodium hydroxide solution ammonia is liberated and it is captured to an acid media (boric acid). A final step of titration (with HCl) occurs similar to total nitrogen determination (AOAC 2001.11).

Results are corrected from free ammoniacal nitrogen of samples that is determined by EN 15475 in house method as previously described.

Apparatus:
Buchi distillation apparatus B-323

Reagents:
As referred in AOAC method 959.03

Preparation of analytical sample:
No preparation is needed

Determination:
In a centrifuge tube of 50ml is added 2-3g of sample (where contains approximately 1,23g N or 7,7g protein equivalent) and TCA solution for protein precipitation. After solution vortex, the sample is centrifuged for 10min at 3000rpm. After precipitation and centrifugation of sample, 25ml of supernatant is transferred and the sample solution is handled as AOAC 959.03 referred

Calculation:
Nitrogen is determined from the titration (expressed as %w/v) and the result is reduced to the original sample.
Urea is determined after the subtraction of ammoniacal nitrogen that was previously determined (according to EN 15475:2009 in house method) and using a factor 60/28 that is the molecular weight ratio for nitrogen of urea (Urea=Ureic nitrogen X 60/28)

- **Nitrogen ammoniacal: EN 15475:2009**

For ammoniacal nitrogen a step of precipitation of proteins with trichloroacetic acid was added.

Distillation apparatus of Buchi, model B323 was used for cold distillation of ammoniacal nitrogen, in accordance with Reg. 2003/2003/EC paragraph 2.6.2 section 7.5.

Principle:
The ammoniacal nitrogen determination according to EN 15475:2009 in house method is based on ammonia no thermal distillation in samples free from proteins and without any digestion step.
A primary step of protein precipitation using trichloroacetic acid (TCA) occurs. A sample solution portion free from protein is transferred to a distillation apparatus and after the addition of sodium hydroxide solution ammonia is liberated and it is captured to an acid media (boric acid). A final step of titration (with HCl) occurs similar to total nitrogen determination (AOAC 2001.11) but also other combinations of acid media (sulphuric acid) & indicators could be used e.g. as those referred in EN 15475.

Apparatus:
Buchi distillation apparatus B-323

Reagents:
As referred in method EN 15475:2009
Trichloroacetic acid (TCA)
Boric acid sln (according to AOAC method 2001.11)
Mix of bromocresol green sln & methyl red sln (according to AOAC method 2001.11)

Preparation of analytical sample:
No preparation is needed

Determination:
In a centrifuge tube of 50ml is added 10g of sample (where contains approximately 1,23g N or 7,7g protein equivalent) and TCA solution for protein precipitation. After solution vortex, the sample is centrifuged for 10min at 3000rpm. After precipitation and centrifugation of sample, 10ml of supernatant is transferred to Buchi distillation tube and 40ml of water is added. A no thermal distillation occurs according to 2.6.2 section 7.5 EC Reg. 2003/2003 and the liberated ammonia is captured by boric acid (as AOAC 2001.11 method, but also other compinations of acid media & indicators could be used e.g. those that are referred in EN 15475)

Calculation:

Nitrogen is determined from the titration as in AOAC method 2001.11 (expressed as %w/v) and the results is reduced to the original sample

Ammoniacal nitrogen is expressed as ammonium chloride using a factor 53.5/14 that is the molecular weight ratio for nitrogen of ammonium chloride (Ammonium chloride = Ammoniacal nitrogen X 53.5/14.

- **Chlorine salts as NaCl: ISO 457:1983**

This method is suitable for chlorine determination in soaps. Though, "ENTOMELA 50SL" and "ENTOMELA 75SL samples were easily tested for chlorine content using argentimetric titration.

A test portion was diluted in 50 ml of hot water and .transferred to the one-mark volumetric flask. 5 ml of nitric acid (1,42 g/ml) and immediately 25,0 ml of the silver 0.1N were added. The flask was placed on boiling water bath until the silver chloride formed had been collected in a mass. The sample solution was left to cool and then was diluted to the mark, with deionised water. A step of mixing by shaking and filtering through a dry, fluted filter Paper was occurred. The first 10 ml were discarded, and then it was collected at least 110 ml, of the filtrate. 100ml were transferred to a conical flask, 2 to 3 ml of the ammonium iron(lll) sulfate Solution (2,5%w/w) were added and the the sample was titrated with Standard volumetric ammonium thiocyanate solution 0.1mol/l until the appearance of a permanent reddish-brown coloration

The chlorine content (as % NaCl) is given by the type:

$$0{,}058\,5 \times (25\,c_1 - 2\,Vc_2) \times \frac{100}{m}$$

Where,

m is the mass, in grams, of the test Portion

V is the volume, of NH4SCN 0,1N solution

c1 is the actual concentration of the silver nitrate solution (0.1N)

c2 is the actual concentration of the ammonium thiocyanate solution (0.1N)

0,0585 is the mass, in grams, of sodium chloride corresponding to 1,00 ml of the silver nitrate Solution, c(AgN03) = 1,000 mol/l

- **Amino-acids index: AOAC 965.31**

This method is suitable for lemon juices. Though, "ENTOMELA 50SL" and "ENTOMELA 75SL" samples were tested for amino-acids index determination.

25g of samples are diluted with 25ml of water. A solution of NaOH was added dropwise to pH 7. A titration potentiometrically up to pH 8.4 was occurred, with NaOH 0,1M. After the addition of 10ml HCHO 37% a second titration with NaOH 0,1M was occurred up to pH 8.4

Total aminoacids (meq/100g) = 0.4 x ml NaOH 0.1M of second titration

- **Dry matter: ISO 2920:2004**

This method is suitable for cheese dry matter. Though, “ENTOMELA 50SL” and “ENTOMELA 75SL” samples were tested with this method, as sand added is necessary for satisfied water evaporation in syrup liquids as these samples.

In a glass weighing bottle of large diameter and low high (M) a quota of sample was added (M1) and then a quota of sea sand (m) previously dried. The sample was placed in the oven at 105^0C for at least 24h. Then the weighing bottle with the sample and the sand was weighed and it was place again in the oven and again after hours was weighed until of a stable indication in the balance (M3)

Dry matter = sample weigh - moisture

- **Insoluble in water: CIPAC MT 10.2**

A stoppered cylinder of 100ml was used and a quota of 10g of sample was weighed. Whatman 1827-047 Glass Microfiber Binder Free Filter, 1.5 Micron was used instead of sintered glass crucible p16

In the glass equipment of HELLASCHEM laboratory, there are not both stoppered measured cylinder of 200ml and sintered glass crucible with porosity P16 (pore size 10-16 μm). Though it is used a relative stoppered cylinder (of 100ml) and the concentration (10% or 0.1g/ml) was the same as CIPAC MT 10.2 referred. The filter that it is used (Whatman 1827-047 Glass Microfiber Binder Free Filter, 1.5μ) has much more less pore size than sintered glass crucible with porosity P16 (pore size 10-16 μm) that CIPAC MT 10.2 is referred.

Comparing the analysis results (of the same sample ENTOMELA) of CIPAC MT 10.2 and CIPAC MT 10.2 modified, as the pore size that it was used in the modified method was less than the one that it is mentioned in the standard method, the results of modified method should regard as a worst case/higher “insoluble in water”.

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