Hydrolysed proteins

DOCUMENT M-CA, Section 4

ANALYTICAL METHODS

Version history[1]

Date	Data points containing amendments or additions and brief description	Document identifier and version number
10-2-2020	4.1.1. Update of the methods used for analysing the active substance: Nitrogen species and amino acids determination.	M-CA 4

[1] It is suggested that applicants adopt a similar approach to showing revisions and version history as outlined in SANCO/10180/2013 Chapter 4 How to revise an Assessment Report

Table of Contents

CA 4 ANALYTICAL METHODS (BIO)....4
Methods used for the Generation of Pre-Approval Data....4
CA 4.1.1 Methods for the analysis of the active substance as manufactured....4
CA 4.1.2 Methods for risk assessment....13
Methods for Post-Approval Control and Monitoring Purposes....16
CA 4 ANALYTICAL METHODS (PHY)....18
CA 4.1 Methods used for the Generation of Pre-Approval Data....18
CA 4.1.1 Methods for the analysis of the active substance as manufactured....18
CA 4.1.2 Methods for risk assessment....26
CA 4.2 Methods for Post-Approval Control and Monitoring Purposes....26
CA 4 ANALYTICAL METHODS (SIC)....18
CA 4.1 Methods used for the Generation of Pre-Approval Data....27
CA 4.1.1 Methods for the analysis of the active substance as manufactured....27
CA 4.1.2 Methods for risk assessment....28
CA 4.2 Methods for Post-Approval Control and Monitoring Purposes....29

CA 4 ANALYTICAL METHODS (BIO)

Introduction

Methods used for the Generation of Pre-Approval Data

CA 4.1.1 Methods for the analysis of the active substance as manufactured

(a) Determination of the pure active substance in the active substance as manufactured and specified in the dossier submitted in support of approval under Regulation (EC) No 1107/2009

The methods used for the analysis of the active substance Hydrolysed proteins are the following:

-Spanish Royal Decree 1110/1991:
- -Method 8 for Total Nitrogen determination
- -Method 9 for Ureic Nitrogen determination
- -Method 10 for Nitric Nitrogen determination
- -Method 12 for Organic Nitrogen determination
- -Method 18 for Free and Total amino acids determination

The organic nitrogen is determined by the Method 12 of the Royal Decree. However, in fact, the first part of this method corresponds to the previous Method 8, because in order to determine the organic Nitrogen, the value of the total Nitrogen is needed.

The organic nitrogen is calculated with the following formula:

N organic = T – (N + A + U)

Being:
T = Total N
N = Nitric Nitrogen (Determination according to Robertson's method)
A = Ammonium Nitrogen (determination according to formaldehyde method)
U = Urea Nitrogen (determination according to urease method)

Total Nitrogen determination (Method 8 of Royal Decree 1110/1991)

The method described below is the official method for the determination of the total nitrogen. MAPA, 1994. - Determination of Total Nitrogen. Official Methods of Analysis, vol. III. Therefore, no validation data are required.

Principle of the Method:
Transform the organic nitrogen into ammonium sulphate by boiling it with concentrated sulphuric acid. Previously, the nitric nitrogen has to be reduced to ammonium and distil all the ammonium nitrogen in alkaline medium with an acid of known titration.

Material and tools:

- Kjeldahl Flasks from 500 to 800 ml.
- Distillation plant

Reagents:

- Concentrated sulphuric acid
- Salicylic acid / sulphuric acid: dissolve 25 g of salicylic acid in one litre of concentrated sulphuric acid
- Thiosulphate of solid sodium
- Catalytic blending: Blend closely 80 g of potassium sulphate, 20 g of copper sulphate and 2 g of selenium.
- Solution of sodium hydroxide at 30%
- Solution of phenolphthalein at 1 % in ethanol
- Aqueous solution of boric acid at 2%
- Indicator. Dissolve 0,125 g of methyl red and 0.080 g of methylene blue in 100 ml of ethanol
- Sulphuric acid or hydrochloric acid 0,1 N

Procedure:
Compound from 0.2 to 2 g of the sample, put them into a Kjeldahl Flask and add 10 ml of the salicylic-sulphuric reactant, stir it in order to wet all the sample and leave it rest for 30 minutes; add 1 g of solid sodium thiosulphate and stir it; wait for 15 minutes and add between 10 and 15 ml of concentrated sulphuric acid and 5 g approximately of catalytic blending.

Place the flask in a heater blanket. Heat it slowly for 5 minutes until the white smokes disappear. Stir it softly by rotation and elevate the temperature as much as possible. Then continue the digestion until the solution becomes clear (it usually happens in 60 minutes).

Cool it and then add carefully 200 ml of water; cool it again, then add 2 or 3 drops of phenolphthalein and solution of NaOH at 30% until getting the red colour.

Immediately after, connect the flask with the distillation plant always having the end of the adapter in an Erlenmeyer flask or in a glass that contains 20 ml of acid 0.1 N. The colour change goes from green into dark red.

Calculations:
Percentage N = V x 0.14 / W

Being:
V = volume, in ml, of acid 0,1 N consumed
W = weight, in grams, of the sample

Observations:
Some digestion equipment's with temperature regulation and equipment's of distillation with air entrainment or by water vapour with semiautomatic addition of reagents can be used.

Organic Nitrogen determination (Method 12 of Royal Decree 1110/1991)

The determination of the content of hydrolysed protein in samples is performed by the determination of the percentage of protein nature nitrogen.

The samples whose composition contains nitrogen of different nature (urea, nitric, ammonium, organic); the content of protein nitrogen is determined by the difference between the total nitrogen and the non-organic nitrogen by the following formula:

N organic = T – (N + A + U)

Principle of the Method:
This method is very similar to Robertson's, except that in the end, ammoniacal nitrogen and ureic nitrogen are also determined to deduce them, together with nitric nitrogen, from total nitrogen

Material:
- Kjeldahl Flasks from 500 to 800 ml.

Reagents:
The respective reagents needed for total Nitrogen, nitric Nitrogen and ureic Nitrogen determination.

Procedure:
- Determine the total N by the official method number 8
- Separate and determine the water insoluble N by the corresponding method
- In individual portions of the filtrate from the previous section, determine the nitric N (by Robertson's method), the ammoniacal N and the N urea (by the urease method).

Calculations:
The organic nitrogen is calculated with the following formula:

N organic = T – (N + A + U)

Being:
T = Total N
N = Nitric Nitrogen (Determination according to Robertson’s method)
A = Ammonium Nitrogen (determination according to formaldehyde method)
U = Urea Nitrogen (determination according to urease method)

Ureic Nitrogen determination (Method 9 of Royal Decree 1110/1991)

Principle of the Method:
This method is based on the enzymatic hydrolysis of urea to ammonium carbonate and its titration, prior removal of calcium and phosphates.

Material:
- Albet paper number 242 or similar
- Phimeter

Reagents:

- 0.1 N hydrochloric acid solution
- 0.1 N sodium hydroxide solution
- 2 N hydrochloric acid solution
- 10% sodium carbonate solution
- Saturated Barium Hydroxide Solution
- Lyophilized Ureasa MERCK Ref. 8489 or equivalent. Check its enzymatic activity periodically and store at a temperature below 4ºC. Use it in freshly prepared suspension
- Neutral urease solution. Prepare a urease suspension in 0.25 percent distilled water and neutralize at pH = 4.4

Procedure:
- Take 10 g of sample, weighed with precision of 1 mg and place it in a 250 ml volumetric flask, bring to volume with distilled water, stir fifteen minutes and filter on Albet 242 paper or similar. Take 50 ml of filtered solution with a pipette and transfer them to a 50 ml volumetric flask.
- Add enough saturated barium hydroxide solution to precipitate the phosphates, allow to settle and check if the precipitation was complete.
- Add sodium carbonate solution to precipitate excess barium and any soluble calcium salt. Let sediment and check again if the precipitation was complete.
- Mix and bring to volume. Filter on Albet 242 or similar dry paper and transfer 50 ml of filtrate to a 250 ml Erlenmeyer flask, neutralize with 2 N hydrochloric acid solution and add two or three drops in excess. Neutralize the solution with 0.1 N sodium hydroxide until pH = 4.4
- Add to each sample 20 ml of eurease suspension, cover with a rubber stopper and let stand for one hour at 20-25 ° C. Cool to 0 ° C and titrate with 0.1 N hydrochloric acid by adding an excess of this reagent and titrate back with 0.1 N sodium hydroxide to pH 4.4.
- Enter the total volume, in ml, added of 0.1 N hydrochloric acid (A) and 0.1 N sodium hydroxide (B).

Calculations:

$$\text{Percentage of ureic N} = \frac{(A-B)^* \cdot 0{,}1 \cdot 14{,}008 \cdot 100}{100 \text{ g of valued sample}}$$

Nitric Nitrogen determination (Method 10 of Royal Decree 1110/1991). Method of Robertson

Principle of the Method:

- Determine the total N and the water insoluble N. The difference between the two is soluble N.
- In the soluble Nd solution, remove the nitric N to the nitric oxide state by means of iron (II) sulfate. Once removed, determine the total N in the residue and the difference between the soluble N and the latter is nitric N.
- Applicable in the presence of calcium cyanamide and urea.

Material and tools:

- Kjeldahl Flasks from 500 to 800 ml.
- Distillation plant

Reagents:

- Iron (II) sulfate heptahydrate
- Mercury oxide or metallic mercury, free of N
- Anhydrous potassium or sodium sulfate, free of N
- Sulfuric acid from 93 to 98 percent, free of N
- Dissolution of sodium thiosulfate or sodium sulphide in 1 L of water
- Sodium hydroxide or in solution: 450 g of NaOH in water, cool and make up to 1 L. It must have a density of 1.36 or higher.
- Inert Boiling Regulator
- Methyl red: dissolve 1 g in 100 ml of ethanol
- 0.1 N sulfuric or hydrochloric acid solution
- 0.1 N sodium hydroxide solution

Procedure:

Modality A: General case in which it is necessary to determine the water insoluble N

- Separate and determine the water insoluble N by the corresponding method.
- In the solution obtained, remove the nitric N and determine the remaining N. To do this, place the filtrate from the previous section in a 500 ml flask and add 2 g of SO2Fe · 7H2O and 20 ml.
- Put it on the flame until the water evaporates and white fumes appear. Continue digestion for at least ten more minutes to expel all nitric N. If strong vaporization occurs, add 10 or 15 glass beads.
- Add 0.65 g of Hg or 0.7 of HgO and continue digestion until the organic matter has oxidized.
- Determine the total N by the official method 8 (*).

Modality B: Modification of Jones in the case that it is not necessary to determine the N insoluble in water because it is all soluble.

- Determine the total N by the official method 8.
- Remove the nitric N and determine the remaining N. To do this weigh 0.5 g of the problem, place them in a 500 ml flask, add 50 ml of water and shake gently.
- Add 2 g of SO4Fe · 7H2O and 20 ml of SO4H2, continuing as in the previous points;

- Put it on the flame until the water evaporates and white fumes appear. Continue digestion for at least ten more minutes to expel all nitric N. If strong vaporization occurs, add 10 or 15 glass beads.
- Add 0.65 g of Hg or 0.7 of HgO and continue digestion until the organic matter has oxidized.

Calculations:
Modality A:

Total N – insoluble N = Soluble N
N soluble – N obtained in the step (*) = **Nitric N**

Modality B:
Total N before eliminating nitric N minus total N after disposal = **Nitric N**

Free and total amino acids determination (Method 18 of Royal Decree 1110/1991)

Principle of the Method:

- The method described below is the official method for the determination of free and total amino acids. The method is based on the separation and determination of the different amino acids by high pressure liquid chromatography. Prior to chromatography, amino acids react with the OPA reagent (Ortoftalaldehyde) and FNIC-C1 (fluorenyl methyl chloro formate) to form fluorescent derivatives (Precolumn Derivation).
- It is an Official Method of Analysis; therefore, no validation data are required.

Material and tools:

- 30 ml glass tubes, SOVIREL type, or similar with screw cap.
- Round bottom flasks, approximately 100 ml, with frosted mouth.
- Dried out with two openings.
- Vacuum pump.
- Drying oven with automatic temperature regulation.
- Rotary evaporator
- Albet filter paper number 240, 242 or similar.
- 50 ml volumetric flasks
- 10 ml tubes
- 10 ml vials
- High pressure liquid chromatograph with fluorescence detector. OPA detection conditions: Excitation wavelength: 340 nm. Detection wavelength: 335 nm.
- C18 column, 5 u in particle size and 20 cm in length.
- Mobile phase:
- Reagent FMOC: dissolve 155 mg of FMOC in 40 ml of acetone
- 0.1 N sodium hydroxide
- Nitrogen (pure gas)

-Standard of amino acids: prepare a solution of amino acids in 0.1 N hydrochloric acid:

Amino acid	**(µg/ml)**
Aspartic acid	6
Glutamic acid	-
Serine	-
Histidine	8
Glycine	4
Threonine	6
Arginine	4
Alanine	4
Tyrosine	8
Methionine	8
Valine	6
Phenilalanine	8
Isoleucine	6
Proline	6
Leucine	6
Lysine	6

Procedure:
Total amino acids: hydrolysis with 6 N hydrochloric acid. Weigh a quantity of sample containing about 1.5 mg of nitrogen coming from amino acids and insert it in a 30 ml glass tube with a thread pin.
Add 15 ml of the phenol solution in 6 N hydrochloric acid.
Place in an apparatus where nitrogen can be injected in order to obtain an oxygen-free atmosphere. Carry out this operation 5 times.
Dry in a drier at 100-105 ºC for 24 hours. Evaporate at 40-50 ºC. Dilute the residue with 25 ml of distilled water and evaporate again (until the hydrochloric acid odour has disappeared).
Filter the residue through paper filter and dissolve it with water in a 50 ml volumetric water.
Free amino acids:
Weigh a quantity of sample containing about 1.5 mg of nitrogen coming from amino acids. Dilute to 50 ml with 0.1 N hydrochloric acid.
Derivation:
Mix 1 ml of sample solution (prepare as in total amino acids or free amino acids) with 0.1 ml of 0.1 N sodium hydroxide and 2.9 ml of distilled water. To 100 µl, add 100 µl of OPA and mix. Wait for a minute. Add 100 µl of FMOC. Wait for 40 minutes. Add 100 µl of pentane and stir. Allow to settle and inject 10 µl of the aqueous phase in the chromatograph.

Chromatographic conditions:
λexc = 340 nm (starting detector)
λem = 420 nm (detection with OPA, maintain until the peak corresponding to isoleucine is obtained)
λexc = 250 nm
λem 335 nm (detection with FMOC, maintain until the peak corresponding to proline is obtained)
λexc = 340
λem = 420 (detection with OPA)

Expression of the results
The results are expressed in percentage (w/w) by comparing the area of the peak of each amino acid to the one corresponding in the chromatogram of the standard solution.

Observations
-In the case of hydrolysis, tryptophan results are not accurate and the sensitivity for cystine is low.
-Tryptophan would be determined by subjecting the sample to alkaline hydrolysis.
-Cystine would be determined by subjecting the sample to oxidation with performic acid prior to hydrolysis.

Analytical Method for pH MEASUREMENT

REFERENCES

Ph. Eur. 3rd Edition, (2.2.3)

EQUIPMENT

- Crison pHmeter model 2001 (or equivalent), resolution: 0.01 pH unit, equipped with a glass-Ag/AgCl combined electrode, standardized with phosphate (pH : 7.00) and citrate

(pH : 4.00) buffers.

- Temperature probe connected to the equipment.

- Alternatively use pH-meter Metrohm 691, with temperature probe. Other pH-meters provided with a temperature compensation probe can be used.

PROCEDURE

Weigh the product (accurately to 1 mg) into a volumetric flask or beaker. Dissolve in 100 mL of carbon dioxide-free water. Read the pH with the pH-meter, recently standardized using the temperature compensation probe. Keep the sample stirring while measuring.

Analytical Method for density determination

REFERENCES

European Pharmacopoeia, method 2.2.5

EQUIPMENT

- Analytical balance

- 10-ml volumetric flasks

PROCEDURE

Weigh a 10-ml volumetric flask (A g). Fill the volumetric flask to volume with the liquid, which is at 20-30ºC. Weigh again the volumetric flask (B g)

(b) Determination of significant and relevant impurities and additives (such as stabilisers) in the active substance as manufactured

Due to the composition of the active substance Hydrolysed proteins, it was stated in the DAR that this substance does not have relevant impurities or additives.

Nevertheless, although impurities are not relevant in Hydrolysed Proteins according to final review report SANCO/2615/08 rev. 3, a new five-batch analysis of the active substance is being performed in the same GLP laboratories as the previous one submitted in 2015.

The determination will include the following components of the active substance: total protein content, amino acids content as well as other components as impurities (other kinds of nitrogen, inorganic components, and water). A letter of commitment from the external laboratory is attached with an estimated end date of the study.

CA 4.1.2 Methods for risk assessment

The biotic degradation of the hydrolysed proteins results in more simple metabolites called amino acids. These compounds are present in live cells; consequently, they are not considered real waste, since they can be used again by the same live cells in the protein synthesis.

The metabolites that come from the degradation of the formulated product are identical to those that exist in cells in a natural way. Therefore, any analysis of waste would not be capable of distinguishing them.

Effects of the industrial and/or domestic transformation on nature and magnitude of waste

Hydrolysed proteins are completely biodegradable, so waste is not expected to be found in harvested vegetable products treated with formulated products containing hydrolysed proteins.

Alterations in smell, taste or other quality aspects due to the presence of waste

Hydrolysed proteins do not modify the organoleptic characteristics of the treated crops or their fruits.

Waste estimation in animal products, if appropriate

In treated vegetables Hydrolysed proteins are degraded without any waste accumulation, waste estimation in animal products is thus not necessary.

Furthermore, it would not be possible to distinguish the proteins brought in an artificial way from the ones already existing in the same animal tissues.

Waste data in crop rotations

Hydrolysed proteins are quickly degraded being soluble in water. Therefore, once the substance has been degraded, it is not probable that it has an effect on next crops, in case waste could be quantified.

Proposed terms of security

Due to the nature and behaviour of waste and the toxicity of this substance, establishing a term of security is thought to be unnecessary.

Proposed maximum limits of waste

The hydrolysed proteins do not accumulate in treated vegetables, nor they produce active or toxic metabolites, so establishing maximum limits of waste is not necessary.

In any country of the EU, Asia or America establishing maximum limits of waste has not been necessary for this kind of substance.

(a) Methods In soil, water, sediment, air and any additional matrices used in support of environmental fate studies

Not required. In the initial dossier version the following justification was provided and was found acceptable, no further data requests have been received.

Hydrolysed proteins are natural compounds of degradation from the hydrolysis of living organism's tissues, that can have vegetable or animal origin. Proteins are the most abundant organic molecules in cells, constitute the 50% of the dry weight of cells or even more. They can be found in every single cell, since they are fundamental in all aspects of the cell structure and function (Lehninger, 1983).

Hydrolysed proteins are quickly degraded to more simple metabolites, which do not have any insecticide activity either. Waste is only superficial and it easily disappears with a simple wash or with the rainfall action. The biotic degradation of the hydrolysed proteins results in more simple metabolites called amino acids. These compounds are present in live cells; consequently, they are not considered real waste, since they can be used again by the same live cells in the protein synthesis.

Not only the hydrolysed proteins but also the metabolites (peptides, amino acids) coming from the biodegradation are compounds that can be found in animal and vegetal tissues. Therefore, if any analysis of residues is performed, the part that has been artificially incorporated could not be distinguished from the natural one, and then, this kind of analysis is not necessary.

Hydrolysed proteins are included in the positive plant protection products list of the Regulation EC 889/2008, annex 2, to be used in Organic Farming. This list is the most restrictive in terms of use of products that may cause environmental dangers.

Moreover, the overall conclusion from the draft assessment report, the recommendations by the rapporteur Member State and the result of the examination in accordance with the provisions of Article 24a of Regulation 2229/2004 is that there are clear indications that it may be expected that hydrolysed proteins does not have any harmful effects on human or animal health or on groundwater or any unacceptable influence on the environment, as set out in Annex VI of regulation (EC) 2229/2004 as last amended by Regulation (EC) 1095/2007.

(b) Methods in soil, water and any additional matrices used in support of efficacy studies

Not required. See explanation in part a).

(c) Methods in feed, body fluids and tissues, air and any additional matrices used in support of toxicological studies

Since Hydrolysed proteins are not classified as toxic, no analytical method is required for its determination in body fluids and tissues.

(d) Methods in body fluids, air and any additional matrices used in support of operator, worker, resident and bystander exposure studies

Since Hydrolysed proteins is not classified as toxic, no analytical method is required for its determination in body fluids, air or any additional matrices.

(e) Methods in or on plants, plant products, processed food commodities, food of plant and animal origin, feed and any additional matrices used in support of residues studies

Not required. See explanation in part a).

(f) Methods in soil, water, sediment, feed and any additional matrices used in support of ecotoxicology studies

Not required. Due to the nature of the hydrolysed proteins and its characteristics regarding its fate and behaviour in the Environment, it could be considered very unlikely the existence of relevant residues of hydrolysed proteins in the soil derived from the application of formulated products containing hydrolysed proteins. In addition, it is unlikely that leaching of hydrolysed proteins can occur or that residues can reach groundwater under the proposed conditions of use. For this reason,

it is not necessary to carry out the evaluation of the fate and behaviour in the environment of Hydrolysed proteins.

(g) Methods in water, buffer solutions, organic solvents and any additional matrices resulting from the physical and chemical properties tests

Not required. See explanation in part e).

Methods for Post-Approval Control and Monitoring Purposes

(a) Methods for the determination of all components included in the monitoring residue definition as submitted in accordance with the provision of point 6.7.1 in order to enable Member States to determine compliance with established maximum residue levels (MRLs); they shall cover residues in or on food and feed of plant and animal origin

As stated in the Draft Assessment Report submitted by Greece to EFSA and still valid, there is no MRL's stablished for hydrolysed proteins at the community or member State level. The argumentation was based on these two points:

a) A residue definition of hydrolysed protein for plants is not considered relevant for the uses intended in EU.

b) No supervised trials were conducted since hydrolysed proteins is exempted from the requirements of data residues.

By default, a MRL of 0.01 mg/kg was set according to Article 18 (1) (b) of the Regulation 396/2005.

(b) Methods for the determination of all components included for monitoring purposes in the residue definitions for soil and water as submitted in accordance with the provisions of point 7.4.2

Not applicable. The biotic degradation of the hydrolysed proteins results in more simple metabolites called amino acids. These compounds are present in live cells; consequently, they are not considered real waste, since they can be used again by the same live cells in the protein synthesis. Because all this, BIOIBERICA requested the exemption of hydrolysed proteins from the requirement of residue data and the argumentation was considered acceptable. Therefore, no residue data was deemed necessary.

(c) Methods for the analysis in air of the active substance and relevant breakdown products formed during or after application, unless the applicant shows that exposure of operators, workers, residents or bystanders is negligible

As previously stated, hydrolysed proteins are natural compounds derived by the hydrolysis of tissues from living organisms. The biotic degradation of the hydrolysed proteins results in more simple metabolites called amino acids. These compounds are present in live cells; consequently, they are not considered real waste, since they can be used again by the same live cells in the protein synthesis.

As stated in the EFSA conclusions, hydrolysed proteins per se are likely to be of low toxicological concern provided hydrolysed proteins of animal origin are pathogen-free, since it complies with the Regulation (EC) No 1069/2009 of the European Parliament and of the Council laying down health rules as regards animal by-products and derived products not intended for human consumption.

(d) Methods for the analysis in body fluids and tissues for active substances and relevant metabolites

Not applicable, see answer to point b).

CA 4 ANALYTICAL METHODS (PHY)

Introduction

In first inclusion of hydrolysed protein DACONA (registered in Spain) was the representative ppp because it was the registered ppp with the higher content of beet molasses and the lower content of urea. In this renewal we replaced DACONA with ENTOMELA 50SL as the representative ppp because this is now the registered ppp with the same characteristics means the registered ppp with the highest beet molasses protein content.

In case of Hydrolysed proteins the a.s. and the ppp are identical, the physical and chemical properties of a.s. are similar to the properties of formulated product. For more detailed information refer on formulation process on Document J - PHY and for detailed physical and chemical properties refer to MC-P Section 2 – ENT50.

In this section we are presenting all the new analytical methods used for the determination of hydrolysed proteins (as crude protein equivalent) and urea content and for the determination of the Product Specifications (physicochemical properties). All these are also part of the Registration Report of ENTOMELA 50SL.

CA 4.1 Methods used for the Generation of Pre-Approval Data

CA 4.1.1 Methods for the analysis of the active substance as manufactured

(a) Determination of the pure active substance in the active substance as manufactured and specified in the dossier submitted in support of approval under Regulation (EC) No 1107/2009

Analytical methods for determination of hydrolysed proteins (as crude protein equivalent) and urea content and for the determination of the Product Specifications (physicochemical properties) of ENT50 were not evaluated as part of the EU review of urea and hydrolysed proteins but evaluated as part of the registration report of ENT50 . Therefore all relevant data are provided now and are considered adequate.
Below is the table with the methods used for all parameters of the Product Specification and the limits.

Test parameter	**Method of analysis**	**Minimum**	**Maximum**
Total nitrogen (x 6.25 = Crude protein equivalent)	AOAC 2001.11	80 gr/kg (500gr/kg)	92.4gr/kg (577.5 gr/kg)
Ureic nitrogen (x 60/28=Urea)	Modified AOAC 959.03	74.6gr/kg (159.8gr/kg)	84.1gr/kg (180.2gr/kg)
Ammoniacal Nitrogen as NH_4Cl	Modified EN 15475:2009 - Similar method to 2.6.2 section 7.5 EC Reg. 2003/2003		5.30 % w/w
Chlorine salts expressed as NaCl	In house ISO 457/1983		2.00 % w/w
Amino-acids index	Modified AOAC 965.31		2.00 meq/10gr
Dry matter	In house ISO 2920 at 105^{o}C	74%w/w	82%w/w
Insoluble in water	Modified CIPAC MT.10.2		0.7 % w/w

PH	CIPAC 75.3	6.20	7.30 after 1 year 8.00 after 2 years
Density	CIPAC 3.3.2	1.31 g/ml	1.39 g/ml
Appearance	Macroscopic examination	Surupy liquid	
Color	Macroscopic examination	Deep reddish-brown	
Odor	Sensory evaluation	Characteristic	

Description of the analytical methods for the determination of the active substance in the plant protection product

Beet molasses – Urea Hydrolysates as ENT50 has two active substances Urea and Hydrolysed Protein.
In this kind of natural and complex mixtures the determination that is used is total nitrogen and ureic nitrogen determination. Specific methods cannot be applied due to high density of the product that makes other methods not applicable.

Method

An analytical method has been developed for the determination of the two active substances urea and hydrolysed protein (as crude protein equivalent) in ENT50 based on determination of different nitrogen types.
The analysis steps which lead us to determine the two types of nitrogen (total and ureic) is given below:

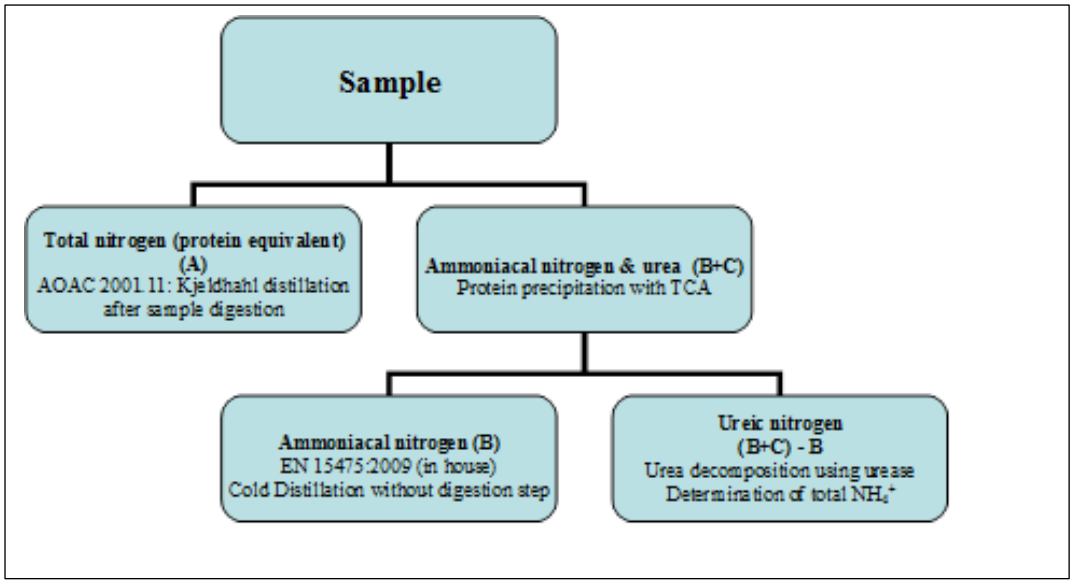
1) Total nitrogen determination (**A).** (Wet digestion, Kjeldahl distillation, Titrimetry)
2) Protein precipitation with TCA (trichloroacetic acid). (This step of protein precipitation with TCA based on AOAC 991.21).
3) Ammoniacal nitrogen determination **(B)** (Cold distillation, Titrimetry)
4) Urea hydrolysis with urease and nitrogen determination **(B+C)** (Titrimetry, Kjeldahl)
5) Ureic nitrogen **(C)**is calculated as the difference **(C) = (B+C) – (B)**

Finally the two types of nitrogen are expressed as:

1) Crude protein equivalent = Total nitrogen (A) x 6.25.
2) Urea = Ureic nitrogen (D) x 60/28.

The schematic of analysis steps is given at figure 1.

Figure 1.



The methods used in nitrogen analysis are all based on AOAC or EN methods or EC Reg. 2003/2003 methods which are acceptable and used in chemical analysis of similar products according to the table:

Test parameter	**Method of analysis**	**Principle**
Total nitrogen (A) - Crude protein equivalent	AOAC 2001.11	Sample digestion, distillation & titrimetry (Kjeldahl)
Ureic nitrogen (C)	AOAC 959.03 modified	Urea decomposition with urease, Titrimetry
Ammoniacal Nitrogen (B)	EN 15475:2009 (in house) Similar method to 2.6.2 section 7.5 EC Reg. 2003/2003	Sample cold distillation & titrimetry (Kjeldahl) without sample digestion

Below is the summary description of these methods used

1) **Total Nitrogen** (AOAC 2001.11) The total nitrogen determination according to AOAC 2001.11 method is based on Kjeldahl method. Nitrogen (all forms) derived from the product, is oxidized to ammonium nitrogen using catalyst (copper sulfate – potassium sulfate), sulfuric acid and *high temperature* for the reaction. After the digestion, a distillation is occurred. The ammonia is captured by a boric acid solution, forming 1:1 complex by ammonia and boric acid. The boric acid captures the ammonia gas, forming an ammonium-borate complex. As the ammonia collects, the color of the receiving solution changes. The addition of an acid solution by titration (as HCl 0,1N) exactly neutralizes the ammonium borate complex, and a reverse color change is produced.

   Equivalent crude protein is determined by multiplying total nitrogen with a factor 6.25 as method refers.

   For detailed description see

   a)“Method for urea and hydrolysed protein content determination in beet molasses urea hydrolysates” of 29.12.2014.

See also AOAC official method 2001.11, published in J.AOAC INTERNATIONAL of 2002.

2) **Ammoniacal nitrogen** (EN 15475:2009 (in house) similar method to 2.6.2 section 7.5 EC Reg. 2003/2003). 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.

Modification of the original method: 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.

For detailed description see

a)"Information in laboratory methods" Document 4780/24.12.2014 and

b)"Method for urea and hydrolysed protein content determination in beet molasses urea hydrolysates" of 29.12.2014.

See also EN 15475:2009, Reg. 2003/2003/EC paragraph 2.6.2, AOAC Official method 991.21 and AOAC 2001.11.

3) **Ureic nitrogen** (Modified AOAC 959.03). The urea nitrogen determination according to AOAC 959.03 modified 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 and the sample solution is handled as AOAC 959.03 referred. *Results are corrected from free ammoniacal nitrogen of samples* that is determined by EN 15475 in house method as previously described.

Urea is determined using a factor 60/28 that is the molecular weight ratio for nitrogen of urea (Urea=Ureic nitrogen X 60/28).

Modification of the original method: This method is suitable for fertilizers. According to method a quota of 10g is weighed. For "ENTOMELA 50SL" samples a quota of 2-3g of sample was weighed. For ureic nitrogen, a step of precipitation of proteins with trichloroacetic acid was added (based on AOAC 991.21) and results are corrected for free ammoniacal nitrogen of the sample, previously determined (by modified EN 15475:2009)".

For detailed description see

a)"Information in laboratory methods" Document 4780/24.12.2014 and

b)"Method for urea and hydrolysed protein content determination in beet molasses urea hydrolysates" of 29.12.2014.

See also AOAC 950.03 and AOAC 991.21

In next table are the specifications and the analysis limits of active ingredients for the product ENTOMELA 50SL.

Active ingredients	gr/kg	Limits	
		Min	Max
Ureic nitrogen	79.33	74.57	84.09
Urea (1)	170.00	159.80	180.20
Average Normal Value of Total Nitrogen content	84.0 gr/kg	80.0 gr/kg*	92.4 gr/kg
Hydrolysed protein(2)	**Min500gr/kg**	500gr/kg	577.5gr/kg **

*This value is according to minimum guaranteed hydrolysed protein content and not FAO tolerances for total nitrogen content.

* *Down limit is the minimum guaranted value for crude protein equivalent. Upper limit for crude protein equivalent is calculated from the upper limit of total Nitrogen content (which is 92.40 gr/kg) multiplied by 6.25.
This upper limit value (92.4 gr/kg) of total nitrogen content comply with the FAO limit +10% for the average normal value of total nitrogen content (84.00gr/kg) verified by 5 batches analysis of the formulation.

(1) Urea is calculated as a result of ureic nitrogen content multiplied by factor 60/28 (molecular weight ratio for nitrogen of urea).

Urea=Ureic nitrogen X 60/28

(2) **Hydrolysed protein as crude protein equivalent** is calculated as a result of total nitrogen content multiplied by 6.25 (protein factor).

Crude protein equivalent = Total nitrogen X 6.25

Other/Special Studies - Methods

Beet molasses – Urea Hydrolysates as ENT50 has two active substances Urea and Hydrolysed Protein.
In this kind of natural and complex mixtures for the identification of the product except the active ingredient content for ensuring the identity and the quality of the product it is very important the determination of the physicochemical properties.

Physicochemical characteristics-properties of ENTOMELA 50SL(PRODUCT SPECIFICATION):

Ammonium salts (as NH4Cl): max 5.30 % w/w

Chlorine salts (as NaCl): max 2.0 % w/w

Amino-acids index: max 2.0 meq/10gr

Dry matter: 74-82 % w/w

Insoluble in water: max 0.7%w/w

pH normal value: 6.75

pH range: 6.2-8.0 (7.3) *

Density: 1.31-1.39 g/ml (Average normal value: 1.35g/ml)

Appearance: Syrupy liquid

Color: Deep reddish brown

Odor: Characteristic

* pH maximum value 7.3 may appear after 1 year of storage. The maximum pH value 8.0 may appear after two years of storage with no other effect on specifications and no significant effect on application as when diluted in application rates gives lower pH.
The maximum pH value of the preparation immediately after preparation (within one month) is 7,10. The range in this case is 6.20-7.10.

The methods used in physicochemical properties analysis of Product Specification are all based on AOAC or CIPAC or EN or ISO or EC Reg. 2003/2003 methods which are acceptable and used in chemical analysis of similar products according to the table:

Test parameter	**Method of analysis**	**Principle**
Ammoniacal Nitrogen expressed as NH_4Cl	Modified EN 15475:2009 Similar method to 2.6.2 section 7.5 EC Reg. 2003/2003	Titrimetric with cold distillation without digestion step
Chlorine salts expressed as NaCl	In house ISO 457/1983	Argentimetric titration
Amino-acids index	Modified AOAC 965.31	Volumetric (Modified Sorensen Method)
Dry matter	In house ISO 2920:2004 at 105º C	Gravimetric
Insoluble in water	Modified CIPAC MT.10.2	Gravimetric
pH	CIPAC 75.3	Potentiometric
Density	CIPAC 3.3.2	Gravimetric

Appearance	Macroscopic examination	Surupy liquid
Color	Macroscopic examination	Deep reddish-brown
Odor	Sensory evaluation	Characteristic

Below is the summary description of these methods used:

1) **The ammoniacal nitrogen** 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.

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.

Modification of the original method: For ammoniacal nitrogen a step of precipitation of proteins with trichloroacetic acid was added (based on AOAC 991.21). 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.

For detailed description see

a)"Information in laboratory methods" Document 4780/24.12.2014 and

b)"Method for urea and hydrolysed protein content determination in beet molasses urea hydrolysates" of 29.12.2014

See also EN 15475:2009, Reg. 2003/2003/EC paragraph 2.6.2, AOAC Official method 991.21 and AOAC 2001.11.

2) **The chloride content (**expressed as NaCl) determination according to ISO 457:1983 by argentimetric titration, is based on precipitation of chlorides with the addition of a silver nitrate standard solution and the back titration of dilutes silver nitrate with ammonium thiocanate standard solution.

Modification of the original method: This method is suitable for chlorine determination in soaps. Though, "ENTOMELA 50SL samples were easily tested for chlorine content using argentimetric titration.

For detailed description see "Information in laboratory methods" Document 4780/24.12.2014.

See also the ISO 457:1983.

3) **The amino acids index** determination according to AOAC 965.31 modified, is based on the potentiometric titration up to pH 9 after the addition of neutralized formol solution (titrated potentiometrically up to pH 9) in a sample solution neutralized (titrated potentiometrically up to

pH 9). In this method maybe used pH 8.5 or pH 9.0 see also Taylor 1957 "Formol titration. An evaluation of his various modifications."

Modification of the original method: This method is suitable for lemon juices. Though, "ENTOMELA 50SL" samples were tested for amino-acids index determination.

For detailed description see "Information in laboratory methods" Document 4780/24.12.2014

and Information in AMINOACID INDEX TEST 5129.1/03.08.2015

See also AOAC 965.31.

4) **The dry matter** determination according to ISO 2920:2004 is based on the determination of loss of water content after the addition of sand (previously dried) and the sample drying in an oven at 105^0C.

Modification of the original method: This method is suitable for cheese dry matter. Though, "ENTOMELA 50SL" samples were tested with this method, as sand added is necessary for satisfied water evaporation in syrup liquids as these samples.

For detailed description see "Information in laboratory methods" Document 4780/24.12.2014.

See also ISO 2910:2004

5) **The insoluble matter in water** is determined by modified CIPAC MT 10.2 method, based on cold water dissolution of sample and the filtration and weighting of insoluble matter.

Modification of the original method: 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.

For detailed description see "Information in laboratory methods" Document 4780/24.12.2014

See also CIPAC MT 10.2

6) **PH** is determined by CIPAC MT 75.3 method, at temperature of 25^0C (without any dilution).

7) **The density** is determined by CIPAC MT 3.3.2 method at temperature of 20^0C (gravimetrically, using density bottles).

In next table are the normal value and limits of physicochemical properties for the product Specifications of ENTOMELA 50SL.

Physicochemical characteristics-properties	**Average Normal Value**	**Min**	**Max**
Ammoniacal Nitrogen (% w/w) or expressed as NH4Cl (% w/w)			1.38 5.30
Chlorine salts expressed as NaCl (%w/w)			2.00
Total amino-acids (meq/10gr)			2

Dry matter % w/w	78	74	82
Insoluble in water % w/w			0.7
pH	6.75	6.2	8.0(7.3)*
Density g/mL	1.35	1.31	1.39

* pH maximum value 7.3 may appear after 1 year of storage. The maximum pH value 8.0 may appear after two years of storage with no other effect on specifications and no significant effect on application as when diluted in application rates gives lower pH.

(b) Determination of significant and relevant impurities and additives (such as stabilisers) in the active substance as manufactured

Not applicable

CA 4.1.2 Methods for risk assessment

Not applicable

CA 4.2 Methods for Post-Approval Control and Monitoring Purposes

Not applicable

CA 4 ANALYTICAL METHODS (SIC)

Introduction

CA 4.1 Methods used for the Generation of Pre-Approval Data

CA 4.1.1 Methods for the analysis of the active substance as manufactured

(a) Determination of the pure active substance in the active substance as manufactured and specified in the dossier submitted in support of approval under Regulation (EC) No 1107/2009

Principle: The determination of organic nitrogen is made subtracting the ammonium nitrogen content to total nitrogen content.
The method of determination of total nitrogen provides for the sample digestion with sulphuric acid at high temperatures using copper sulfate as catalyst to convert organic nitrogen to ammonia, which is distilled after alkalinization into a boric acid solution. The concentration is determined by acid-base titration(nitrogen determination according to Kjeldahl method).
The method of ammonium nitrogen determination provides for the ammonia distillation from a solution buffered at pH=7,4 into a boric acid solution. The concentration is determined by acid-base titration.

Apparatus:
Digestion unit for Kjeldahl method.
Distillation unit for Kjeldahl method.
Kjeldahl flasks.

DETERMINATION OF TOTAL NITROGEN:

Procedure :
A weighed sample (0,5g) is put in a Kjeldahl digestion flask, 20 mL of phosphosulfuric acid, the tablet containing potassium and copper sulphate are added. The sample is digested until solution clears (2 hours) and cooled.
50 mL water and 50 mL sodium hydroxide 32% w/w are added and the ammonia is distilled in 100 ml of receiving solution until neutrality.
The ammonia is titrated with standard sulphuric acid solution 0,1N until the colour change of indicator.

Calculation relative to total nitrogen content:
Total Nitrogen (% w/w)=((a-b)xNx1,4)/m
where:
a=ml mL standard sulphuric acid used to titrate the sample
b=ml mL standard sulphuric acid used to titrate the blank
N=normality relative to the sulphuric acid
m=quantity of sample (g)

DETERMINATION OF AMMONIUM NITROGEN

Procedure :
A weighed sample (1,5g) are put in a Kjeldahl digestion flask, 50 mL of buffered solution are added and the ammonia is distilled in 100 ml of receiving solution until neutrality.
The ammonia is titrated with standard sulphuric acid solution 0,1N until the colour change of indicator.

Calculation relative to total nitrogen content:
Ammonium Nitrogen (% w/w)=((a-b)xNx1,4)/m
where:
a=ml mL standard sulphuric acid used to titrate the sample
b=ml mL standard sulphuric acid used to titrate the blank
N=normality relative to the sulphuric acid
m=quantity of sample (g)

(b) Determination of significant and relevant impurities and additives (such as stabilisers) in the active substance as manufactured

Methods for the determination of impurities: not required.

CA 4.1.2 Methods for risk assessment

The hydrolysed protein produced by SICIT is obtained by hydrolysis of animal tissues. Hydrolysed protein is used also as a foliar fertiliser and does not cause negative transformations in the environment, if it is used following the suggested conditions. The product is constituted by natural substances and consequently is completely biodegradable. Reasonably there are not negative effects on the environment.

(a) Methods In soil, water, sediment, air and any additional matrices used in support of environmental fate studies

(b) Methods in soil, water and any additional matrices used in support of efficacy studies

(c) Methods in feed, body fluids and tissues, air and any additional matrices used in support of toxicological studies

(d) Methods in body fluids, air and any additional matrices used in support of operator, worker, resident and bystander exposure studies

(e) Methods in or on plants, plant products, processed food commodities, food of plant and animal origin, feed and any additional matrices used in support of residues studies

(f) Methods in soil, water, sediment, feed and any additional matrices used in support of ecotoxicology studies

(g) Methods in water, buffer solutions, organic solvents and any additional matrices resulting from the physical and chemical properties tests

CA 4.2 Methods for Post-Approval Control and Monitoring Purposes

Hydrolysed proteins are a naturally occurring as low toxic active substance, and no MRLs are set in plants. Thus, no analytical methods are required in plants, soil, water and air, according to guideline SANCO 825/00 rev. 8.1.

The product is constituted by natural substances, is completely biodegradable and it’s used also as foliar fertiliser. It does not cause negative transformations in the environment, if it is used following the suggested dosages and the suggested conditions.

(a) Methods for the determination of all components included in the monitoring residue definition as submitted in accordance with the provision of point 6.7.1 in order to enable Member States to determine compliance with established maximum residue levels (MRLs); they shall cover residues in or on food and feed of plant and animal origin

Hydrolysed proteins are a naturally occurring as low toxic active substance, and no MRLs are set in plants. Thus, no analytical methods are required in plants, soil, water and air, according to guideline SANCO 825/00 rev. 8.1.

The product is constituted by natural substances, is completely biodegradable and it’s used also as foliar fertiliser. It does not cause negative transformations in the environment, if it is used following the suggested dosages and the suggested conditions.

(b) Methods for the determination of all components included for monitoring purposes in the residue definitions for soil and water as submitted in accordance with the provisions of point 7.4.2

The product is constituted by natural substances, is completely biodegradable and it's used also as foliar fertiliser. It does not cause negative transformations in the environment, if it is used following the suggested dosages and the suggested conditions.

(c) Methods for the analysis in air of the active substance and relevant breakdown products formed during or after application, unless the applicant shows that exposure of operators, workers, residents or bystanders is negligible

Hydrolysed proteins per se are likely to be of low toxicological concern provided hydrolysed proteins of animal origin are pathogen-free and hydrolysed proteins from plant origin do not have sensitisation potential.
No risks to human health could be expected from its use as a plant protection product and data waivers for specific toxicological studies were initially supported. no risk is anticipated for the worker entering the application area directly after treatment when the same personal protective equipment with the operator is used.

(d) Methods for the analysis in body fluids and tissues for active substances and relevant metabolites