BIOCEBO/BIO

DOCUMENT M-CP, Section 5

ANALYTICAL METHODS

Version history[1]

Date	Data points containing amendments or additions and brief description	Document identifier and version number
10-2-2020	5.1.1. Update of the methods used for analysing the active substance: Nitrogen species and amino acids determination.	M-CP 5

[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

CP 5 ANALYTICAL METHODS

CP 5.1 Methods for the Generation of Pre-Authorisation Data

CP 5.1.1 Analysis of the Plant Protection Product

(a) Methods for the determination of the active substance and/or variant in the plant protection product

The methods used for the determination of the active substance in the plant protection product 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) Methods for determination of relevant impurities identified in the technical material or which may be formed during manufacture of the plant protection product or from degradation of the plant protection product during storage

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.

(c) Methods for the determination of relevant co-formulants or components of co-formulants, where required by the national competent authorities

Not applicable since Biocebo is composed by the active substance Hydrolysed proteins and water.

CP 5.1.2 Methods for the Determination of Residues

BIOCEBO is an aqueous solution of natural nature hydrolysed proteins that is used as an attractant trophic of diptera and it is applied in combination with insecticides to create baits to control this insect pests in deciduous fruit trees, citrus and olive grove.

The basic components of the formulated product are the hydrolysed proteins. Because of the organoleptic attributes of the hydrolysed properties, the insects become attracted by it. Therefore, when a fly eats a bait, then they die because of the insecticide action.

BIOCEBO is easily degraded in simple metabolites, like peptides and amino acids. The residues are only superficial and they easily disappear with a quick wash or by the rainfall action, because the formulated product is very soluble in water.

Proteins are the most common organic molecules in cells. They constitute the 50% of their dry weight, or even more. Can be found in all parts of each animal or vegetable cells, because they are essential in all aspects of cellular structure and the cell function. (LEHNINGER, 1983).

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 and useful at all.

Hydrolysed proteins, which are the basic components of BIOCEBO, 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.

The formulated products constituted by Hydrolysed Proteins were authorised in Spain more than ten years ago and no proposed maximum limits of residue have been established for these kinds of substances. In other countries such as the United States and the European Union, similar formulations than BIOCEBO have been authorised without any maximum limits of residues.

Due to all mentioned above, the non-inclusion of analytical methods for determination of residues of Hydrolysed Proteins was duly justified and found satisfactory during the Assessment Review.

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

Not applicable. Hydrolysed proteins are natural substances also naturally found on the environment. Thus, it will be impossible to distinguish proteins coming from the product BIOCEBO from those naturally present on the environment.

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

Not applicable

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

Not applicable

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

Not applicable

(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 applicable

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

Not applicable

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

Not applicable

CP 5.2 Methods for Post-Authorisation Control and Monitoring Purposes

Methods for the determination of residues in or on plants, plant products, processed food commodities, food and feed of plant and animal origin

Not applicable. Hydrolysed proteins are natural substances also naturally found on the environment. Thus, it will be impossible to distinguish proteins coming from the product BIOCEBO from those naturally present on the environment.

Methods for the determination of residues in body fluids and tissues

Not applicable

Methods for the determination of residues in soil

Not applicable

Methods for the determination of residues in water

Not applicable

Methods for the determination of residues in air, unless the applicant shows that exposure of operators, workers, residents or bystanders is negligible

Not applicable