# ENTOMELA 50SL/ENT 50

## 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 |
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[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

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# CP 5 ANALYTICAL METHODS

## CP 5.1 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.

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 part of the Registration Report of ENTOMELA 50SL submitted in Greece on 2015.

### 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

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. 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:

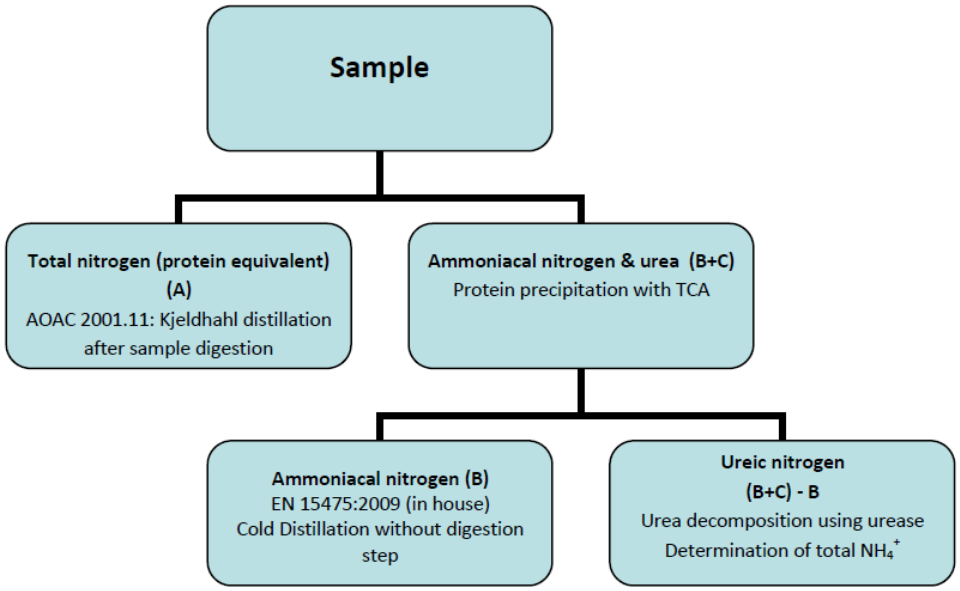
1) Total nitrogen determination (**A).** (Wet digestion, Kjeldahl distillation, Titrimetry)
2) Protein precipitation with TCA (trichloroacetic acid). (This step of protein precipitation with TCA based on AOAC 991.21).
3) Ammoniacal nitrogen determination **(B)** (Cold distillation, Titrimetry)
4) Urea 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 below 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**

## b) 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 for the "fresh" formulation (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<br><br>Similar method to<br><br>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 105$^o$ 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.

See also "Information in laboratory methods - AMINOACID INDEX TEST" document 5129.1/3.8.2015 and 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 $105^0$C.

**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 $25^0$C (without any dilution).

7) **The density** is determined by CIPAC MT 3.3.2 method at temperature of $20^0$C (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)<br>or expressed as<br>NH4Cl (% w/w) | | | 1.38<br><br>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.

The maximum pH value for the "fresh" formulation (within one month) is 7,10. The range in this case is 6.20-7.10.

### (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

***Not applicable***

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

***Not applicable***

## CP 5.1.2 Methods for the Determination of Residues

*Guidance from SANCO/12592/2012 'Template Assessment Report':*
*Cross reference as appropriate to the AS section.*

***Not applicable***

## CP 5.2 Methods for Post-Authorisation Control and Monitoring Purposes

***Not applicable***