

3.2.S.4.2 Analytical procedure

3.2.S.4.2.1 - Characters

Examine visually the product for the above-mentioned characters.

3.2.S.4.2.2 - IR Identification

Test is performed according to Ph. Eur. current ed. (Monograph n° 2.2.24)

Routine method used for the acquisition of spectra: ATR (Attenuated Total Reflection)

Preparation of the sample and standard (for recording by Transmission or Absorption)

Grind the amount of sample necessary to cover the whole crystal surface (approximately 100mg) in an agate mortar, or other suitable material, for a time of about 30sec and then place it on top of the latter.

Method

Record the IR spectrum in the range between 4000 cm⁻¹ and 650 cm⁻¹.

Assessment of Spectrum

Compare the spectrum of the sample with the reference spectrum.

The spectrum of the sample should exhibit absorption maxima which correspond in position and relative intensity to those in the reference spectrum: The correlation factor between sample and reference spectra in library must be no less than 0.95.

Alternative method used for the acquisition of spectra: Transmission Mode

Preparation of the sample and standard (for recording by Transmission or Absorption)

Triturate approximately 1 mg of the sample with approximately 300 mg of finely powdered potassium bromide, previously dried in a muffle furnace at 250°C for 1 hour.

Carefully grind the mixture, spread uniformly in a suitable die, and submit it, under vacuum, to a pressure of about 10 Tons, for 5 minutes.

Preparation of the standard disc

Proceed as reported above, using a. 1 mg of Methisoprinol standard sample.

Method

Record the IR spectrum in the range between 4000 cm⁻¹ and 650 cm⁻¹.

Assessment of Spectrum

Compare the spectrum of the sample with the reference spectrum.

The spectrum of the sample should exhibit absorption maxima which correspond in position and relative intensity to those in the reference spectrum: The correlation factor between sample and reference spectra in library must be no less than 0.95.

3.2.S.4.2.3 - HPLC Identification

Method

Carry out the HPLC assay method (In House Method)

Assessment of Chromatograms

Compare the retention times of the main peak in the chromatograms obtained with the sample solution with the main peak in the chromatograms obtained with the Methisoprinol standard solution and assess whether they are concordant.

The retention time of the major peaks in the chromatogram corresponds to that of the Standard preparation.

3.2.S.4.2.4 – Clarity (10% w/v water solution)

Test is performed according to Ph. Eur. current ed. (Visual Method Monograph n° 2.2.1)

Method

Weight 10 g of Methisoprinol sample in a 100 ml Nessler cylinder. Dissolve and take to volume with purified water: the obtained solution must be clear and fully soluble.

Colour of the solution must be less intense than standard B5 (Ph. Eur. current ed. Monograph n°2.2.2)

3.2.S.4.2.5 – pH 10% w/v water solution

Test is performed according to Ph. Eur. current ed. (Monograph n° 2.2.3)

Method

Weight 10 g of Methisoprinol sample in a 100 ml Nessler cylinder. Dissolve and take to volume with Purified Water Carbon dioxide free (Ph. Eu 1095502): the pH must be between 6.0 and 7.0.

3.2.S.4.2.6 – Water content (by KF method)

Test is performed according to Ph. Eur. current ed. (Monograph n° 2.5.12)

Method

Place about 50 ml of Karl Fischer solvent into the titration vessel and titrate with standardised Karl Fischer titrant to the electrometric endpoint.

The Karl Fischer titrant (iodosulphurous reagent) is commercially available and is standardised as described in the Reagents section of the European Pharmacopoeia.

Place 0.5 g of sample into a suitable container and weigh (W1 mg).

Add the sample to the equilibrated Karl Fischer solvent and titrate to the electrometric endpoint (T1 ml). Reweigh the empty sample container (W2 mg)

Calculation

Calculate the water content using the following formula:

$$\text{Water, \%w/w} = \frac{T1 * F * 100}{(W1 - W2)}$$

where F is the factor (mg/ml) of the standardised Karl Fischer reagent.

3.2.S.4.2.7 – Related Substances HPLC (In house method)**Reference solution preparation (SOL ST)****Impurities**

In a 100.0 volumetric flask transfer about 250mg of Hypoxanthine WS161, 750mg of Guanosine WS182, 750mg of 4-Amino benzoic Acid WS122, 250mg of Adenosine WS181. Add about 30ml of Formic acid anhydrous (Ph. Eu. Reagent); dissolve and bring to volume with ultrapure water and mix. (SOL ST IMP 1)

Transfer 1.0 ml of SOL ST IMP 1 in a 100.0 ml volumetric flask and bring to volume with ultrapure water and mix. (SOL ST IMP 2)

Transfer 1.0 ml of SOL ST IMP 2 in a 100.0 ml volumetric flask and bring to volume with ultrapure water and mix. (SOL ST IMP 3)

Active ingredients

In a 20.0 volumetric flask transfer about 240.5mg of Inosine WS064 and 759.5mg of Methisoprinol salt WS115 and dissolve it with ultrapure water. Bring to volume with the same solvent and mix. (SOL ST ACI 1)

Transfer 1.0 ml of SOL ST ACI 1 in a 100.0 ml volumetric flask and bring to volume with ultrapure water and mix. (SOL ST ACI 2)

Sample solution preparation (SOL C)

In a 100.0 ml volumetric flask weigh about 200 mg of sample and dissolve with ultrapure water. Bring to volume with ultrapure water and mix. (SOL C 1)

Transfer 5.0 ml of SOL C 1 in a 20.0 volumetric flask. Bring to volume with ultrapure water and mix. (SOL C 2).

Operate in duplicate at room Temperature.

System Suitability solution preparation (SOL SS)

Transfer 1.0 ml of SOL ST IMP 2 and 1.0 ml of SOL ST ACI 1 solution in a 100.0 ml volumetric flask; add 0.5ml of Dimethylsulfoxide; bring to volume with ultrapure water and mix. (SOL SS)

Mobile phase preparation

Mobile phase A: Weight and transfer about 2.65 g of Potassium Dihydrogen Phosphate (S-65) in 1000 mL of water. Shake well to dissolve. Adjust to pH 3.5 with ortophosphoric acid 80% (L-44).

Mobile phase B: Methanol (L-02)

Chromatographic condition

Instrument	HPLC Line
Column	Pursuit RP 18 5µm, length 15 cm, Ø 4.6 mm.
Oven temperature	25°C
Sample temperature	20°C (room Temperature)
Detector	UV a 254nm
Flow rate	1 ml/min.
Injection volume	20 µl
Elution	gradient
Run time	40 minutes.

<u>Step</u>	<u>Time</u>	<u>Fm A</u>	<u>Fm B</u>	<u>Curve</u>
0	5 min	95	5	0
1	5 min	90	10	1
2	15 min	40	60	1
3	7 min	40	60	0
4	3 min	95	5	1
5	5 min	95	5	0

Injection sequence

Nº	Injection of solution
1	Blank (ultrapure water)
2	System Suitability solution (1 replicas)
3	Standard solution ST IMP 3 (3 replicas)
4	Standard solution ST ACI 2 (3 replicas)
5	Sample solution SOL C 2 (2 replicas)

Retention time

- HYPOXANTINE = 3,5 min
- INOSINE = 7.0 min
- GUANOSINE = 7,7 min
- 4-AMINOBENZOIC ACID = 11.0 min
- ADENOSINE = 11.7 min
- METHISOPRINOL SALT = 18.5 min

System Suitability

- Calculate the RSD % for Inosine and Methisoprinol salt peaks on the three standard ACI 2 injections. The test is conform if RSD % is less than 1.04 %.
- Calculate the RSD % for Hypoxanthine, Guanosine, 4-Aminobenzoic Acid and Adenosine peaks on the three standard IMP 3 injections. The test is conform if RSD % is less than 15 %.
- In SOL SS injection resolution factor between Inosine and Guanosine peaks; 4-AminoBenzoic acid and Adenosine peaks must be ≥ 1.5 .
- In SOL SS injection tailing factor for each component must be included between 0.8 and 1.5

Results evaluation

The evaluation of the impurities must be performed only on the first injection of the sample solution (SOL C 2)

Calculation for Known impurities.**Hypoxanthine - Guanosine - 4-Aminobenzoic Acid - Adenosine**

$$\text{Impurity content \%} = \frac{A_{\text{cimp}} * P_{\text{simp}} * 1 * 1 * T_{\text{simp}} * 100 * 20 * 100}{\bar{A}_{\text{STDimp}} * 100 * 100 * 100 * 100 * P_c * 5 * (100 - \text{KF})} * 100$$

Calculation for Known impurities (Simplified)

$$\text{Impurity content \%} = \frac{A_{\text{cimp}} * P_{\text{simp}} * T_{\text{simp}}}{\bar{A}_{\text{STDimp}} * P_c * (100 - \text{KF}) * 25}$$

Calculation for Unknow impurities

$$\text{Impurity content \%} = \frac{A_{\text{cimp}}}{\bar{A}_{\text{MTP}}} * 100$$

where:

A_{cImp} = area of impurity peak in sample solution (SOL C2)

P_{Simp} = weight in mg of know impurity in standard solution

T_{Simp} = assay % of know impurity in standard solution

$\bar{A}_{\text{STDimp}}$ = average between know impurity peak in standard solution (ST IMP 3)

P_c = weight in mg of sample

$\bar{A}_{\text{MTP}}$ = sum of Inosine and Methisoprinol salt peaks area in sample solution

Results evaluations

If the know impurity % value is under the relative LOD, the impurity must be not considered. Report "< LOD"

If the know impurity % value is between the relative LOD and LOQ the impurity, for total impurities calculation, must be consider at LOQ value. Report "<LOQ"

If the unknow impurity % value is < 0.03 % (Disregard limit), the impurity must be not considered.

The Hypoxantine maximum permitted concentration, in respect to Methisoprinol, is 0.05%. If applicable report the numerical result.

The Guanosine maximum permitted concentration, in respect to Methisoprinol, is 0.15%. If applicable report the numerical result.

The 4-AminoBenzoic acid maximum permitted concentration, in respect to Methisoprinol, is 0.15%. If applicable report the numerical result.

The Adenosine maximum permitted concentration, in respect to Methisoprinol, is 0.05%. If applicable report the numerical result.

The Unknow impurity maximum permitted concentration, in respect to Methisoprinol, is 0.05%. If applicable report the numerical result.

The sum of impurity maximum permitted concentration, in respect to Methisoprinol, is 0.50%. Report the numerical result.

3.2.S.4.2.8 – Assay by HPLC (Inosine and 4 acetamido benzoic acid salt) - In House Method

Method: See 3.2.S.4.2.7 – Related Substances HPLC

Calculation for component INOSINE

$$\text{INOSINE \% on decl. (s.s.)} = \frac{A_{ic} * P_{sti} * [I] * 100 * 20 * 100 * 100 * 100}{A_{ist} * 20 * 100 * 100 * P_c * 5 * (100 - KF) * 24,05}$$

Calculation for component INOSINE (Simplified)

$$\text{INOSINE \% on decl. (s.s.)} = \frac{A_{ic} * P_{sti} * [I] * 2000}{A_{ist} * P_c * (100 - KF) * 24,05}$$

Calculation for component SMP

$$\text{SMP \% on decl. (s.s.)} = \frac{A_{ac} * P_{sta} * [A] * 100 * 20 * 100 * 100 * 100}{A_{ist} * 20 * 100 * 100 * P_c * 5 * (100 - KF) * 75,95}$$

Calculation for component SMP (Simplified)

$$\text{SMP \% on decl. (s.s.)} = \frac{\text{Aac} * \text{Psta} * [\text{A}] * 2000}{\text{Aast} * \text{Pc} * (100 - \text{KF}) * 75,95}$$

where:

- Aic = area of Inosine peak in sample solution (SOL C 2)
- Aist = average area of Inosine peak in standard solution (ST ACI 2)
- [I] = Inosine reference standard assay %(as is)
- Aac = area of SMP peak in sample solution (SOL C 2)
- Aast = average area of SMP peak in standard solution (ST ACI 2)
- [A] = SMP reference standard assay %(as is)
- Pc = weight in mg of sample
- Psti = weight in mg of Inosine in standard solution
- Psta = weight in mg of SMP in standard solution
- KF = water KF assay % contained in sample
- 24,05 = % theoretical dry matter of inosine in Methisoprinol
- 75,95 = % theoretical dry matter of SMP in Methisoprinol

Sum of the active components calculation

$$\text{Sum of active components \%} = A + B$$

where:

$$A = (\text{Inosine assay \% on declared}) \times 0,2405$$

$$B = (\text{SMP assay \% on declared}) \times 0,7595$$

3.2.S.4.2.9 – Assay by Potentiometric (N,N Dimethylamino-2-propanol)
- In House Method
Method

Weigh exactly about 200 mg of product and dissolve it in about 50 ml of glacial acetic acid, and sonicate to allow dissolution, after adding 10 ml of Acetic anhydride and cool to RT

Titrate potentiometric with Perchloric acid 0.1 N.

Carry out the determination twice and give the average.

1 ml of titrator corresponds to 10,317mg of N,N-dimethylamino-2-propanol.

Calculation

$$\text{Assay, \%} = \frac{\text{ml of titration sol.} * 10.317 * \text{factor of titrating sol.} * 100 * 100}{\text{weigh (mg) of the sample} * (100 - \% \text{H}_2\text{O}) * 27.75} * 100$$

3.2.S.4.2.10 - Residual solvent (*In House Methods*)**Routine method applied by ABC Farmaceutici – Treocate site****Reference solution preparation (SOL ST)**

In a 50.0 ml flask add about 20 ml of ultrapure water. Then transfer about 200 mg of Acetone WS011 and about 1000mg of Isopropanol WS105. Take to volume with ultrapure water and mix. (SOL ST 1). Transfer 1.0 ml of SOL ST 1 solution in a 50.0 ml flask, take to volume with ultrapure water and mix. (SOL ST 2)

Sample solution preparation (SOL C)

In a 10.0 ml flask weight about 800 mg of sample and dissolve with ultrapure water. Then take to volume with ultrapure water and mix. Operate in duplicate.

Vials preparations

Blank: In a 20 ml head space vial transfer 1.0 ml of ultrapure water.

Standard solution: In a 20 ml head space vial transfer 1,0 ml of SOL ST 2. Operate in triple.

Sample solution: In a 20 ml head space vial transfer 1,0 ml of SOL C.

Injection sequence

- 1) Blank,
- 2) Standard solution (in triple)
- 3) Sample solution (in duplicate)

Retention time

ACETONE = 1,4 min

ISOPROPANOL = 2,2 min

GC Operative parameters

Equipment	Gas chromatograph with head space sampler
Column	Zebron ZB-WAX Plus 30m * 0,32 mm * 1,00 µm or similar
Detector	F.I.D.
Carrier Gas	Helium
Flow velocity	21 p.s.i.
Split factor	1:1
Injector temperature	180 °C
Detector temperature	220 °C
Column temperature	60 °C * 4 min. Increase of 25 °C/min to 160°C
Run time	8 min

Head space operative parameters

Oven sample bath temperature	80 °C
Syringe temperature	90 °C
Transfer line temperature	120 °C
Injection time	0.03 min
Withdraw time	0.5
Pressure time	1.5 min
Termostation time	30 min
Cycle time	13.5 min

System Suitability

- ✓ Calculate the RSD % for each component on the three standard injection. The test is conform if RSD % is less than 15 %.
- ✓ Resolution factor between Acetone and Isopropanol peaks in the first standard B injection must be > 1.5
- ✓ Tailing factor for each component must be included between 0.8 and 1.5

Results evaluation**Calculation for Acetone**

$$\text{Acetone (in ppm)} = \frac{A_{cAcc} * P_{SAcc} * 1 * 1 * 10}{\bar{A}_{STDAcc} * 50 * 50 * P_c * 1} * 1000000$$

Calculation for Acetone (Simplified)

$$\text{Acetone (in ppm)} = \frac{A_{cAcc} \times P_{SAcc}}{\bar{A}_{STDAcc} \times P_c} * 4000$$

Calculation for Isopropanol

$$\text{Isopropanol (in ppm)} = \frac{A_{cIso} * P_{SIso} * 1 * 1 * 10}{\bar{A}_{STDIso} * 50 * 50 * P_c * 1} * 1000000$$

Calculation for Isopropanol (Simplified)

$$\text{Isopropanol (in ppm)} = \frac{A_{cIso} * P_{SIso}}{\bar{A}_{STDIso} * P_c} * 4000$$

where:

A_{cAce} = area of Acetone peak in sample solution

$\bar{A}_{cIso}$ = area of Isopropanol peak in sample solution

P_{SAce} = weight in mg of Acetone in standard solution

P_{SIso} = weight in mg of Isopropanol in standard solution

$\bar{A}_{STD Ace}$ = average between Acetone peak in standard solution

$\bar{A}_{STD Iso}$ = average between Isopropanol peak in standard solution

P_c = weight in mg of sample

Results

If the Acetone (ppm) value is under the relative LOD, the impurity must be not considered. Report “< LOD”

If the Isopropanol (ppm) value is under the relative LOD, the impurity must be not considered. Report “< LOD”

If the Acetone (ppm) value is between the relative LOD and LOQ, must be consider at LOQ value. Report “<LOQ”

If the Isopropanol (ppm) value is between the relative LOD and LOQ, must be consider at LOQ value. Report “<LOQ”

The Acetone maximum permitted concentration is 1000 ppm. Report the numerical result.

The Isopropanol maximum permitted concentration is 5000ppm. Report the numerical result.

Method applied by ICE Pharma – Basaluzzo site

Solvent solution preparation (Blank)

In a 200.0 ml flask add about of DMSO and 80 ml of ultrapure water; for different volumes scale up or down the components keeping the ratio unchanged.

Reference solution preparation (SOL F)

In a 50.0 ml flask add about 10 ml of Blank. Then transfer about 100 mg of Acetone and about 500mg of Isopropanol. Take to volume with Blank and mix. (SOL E). Transfer 2.5 ml of SOL E solution in a 50.0 ml flask, take to volume with Blank and mix. (SOL F)

Sample solution preparation (SOL MTP)

In a 20.0 ml GC-HS vial weight about 1000 mg of sample; dissolve with 10.0 ml of Blank with aid of ultrasonic bath. Operate in double.

Vials preparations

Blank: In a 20 ml GC-HS vial transfer 10 ml of Blank solution.

Standard solution: In a 20 ml GC-HS vial transfer 100 µl of SOL F

Operate in sextuple.

Sample solution: Operate as describer previously for SOL MTP.

Injection sequence

- 1) Blank,
- 2) Standard solution (in triple)
- 3) Sample solution (in duplicate)
- 4) Standard solution (in triple)

GC Operative parameters

Equipment	Gas chromatograph with head space sampler
Column	TG-624 SilMS 30m * 0,32 mm * 1,8 µm
Detector	F.I.D.
Carrier Gas	Helium
Gas setting	Air flow control: 400 ml/min Makeup gas flow control: 20.0 ml/min H ₂ flow control: 30.0 ml/min
Injector temperature	250 °C
Detector temperature	250 °C
Column temperature	40 °C for 5 min. Increase of 10 °C/min to 150°C then increase of 20 °C /min to 240°C
Run time	25.5 min

Head space operative parameters

Syringe volume	2.5 ml
Needle lenght	65 mm
Sample draw	1 ml
Analysis time	30 min
Incubation temperature	100 °C
Incubation time	10 min
Incubation mode	Constant, no agitation
Syringe temperature	120 °C
Syringe standby temperature	120 °C
Syringe filling volume	1.3 ml
Filling counts	2
Filling delay	0
Syringe filling speed	6 ml/min
Syringe injenction speed	50ml/min

System Suitability

- ✓ Calculate the RSD % for each component on the six standard injection. The test is conformed if RSD % is less than 10 %.

Results evaluation**Calculation for Acetone**

$$\text{Acetone (in ppm)} = \frac{A_{cAce} * C_{Ace} * 10^6}{\bar{A}_{Ace} * C_{sAce}}$$

Calculation for Isopropanol

$$\text{Acetone (in ppm)} = \frac{A_{cIso} * C_{Iso} * 10^6}{\bar{A}_{Iso} * C_{sIso}}$$

where:

A_{cAce} = area of Acetone peak in sample solution

A_{cIso} = area of Acetone peak in sample solution

$\bar{A}_{cIso}$ = area of Isopropanol peak in sample solution

C_{Ace} = concentration in mg/ml of Acetone in standard solution

C_{Iso} = concentration in mg/ml of Isopropanol in standard solution

$\bar{A}_{Ace}$ = average Area of Acetone in standard solution

$\bar{A}_{Iso}$ = average Area of Isopropanol in standard solution

C_{sAce} = concentration in mg/ml of Acetone in standard solution

C_{sIso} = concentration in mg/ml of Isopropanol in standard solution

10^6 = multiplicative factor to convert mg/ml in ppm

Results

If the Acetone (ppm) value is under the relative LOD, the impurity must be not considered. Report "< LOD"

If the Isopropanol (ppm) value is under the relative LOD, the impurity must be not considered. Report "< LOD"

If the Acetone (ppm) value is between the relative LOD and LOQ, must be consider at LOQ value. Report "<LOQ"

If the Isopropanol (ppm) value is between the relative LOD and LOQ, must be consider at LOQ value. Report "<LOQ"

The Acetone maximum permitted concentration is 1000 ppm. Report the numerical result.

The Isopropanol maximum permitted concentration is 5000ppm. Report the numerical result.

3.2.S.4.2.11 – TAMC

Total viable aerobic count is determined as described under Ph. Eur. current ed. (membrane filtration method - Monograph n° 2.6.12).

Culture media and reagents

- Casein soya bean digest agar (TSA)
- Buffer NaCl-Peptone pH 7.0 (Diluent):
 - Peptone 1.0 g*
 - NaCl 4.3 g*
 - Potassium dihydrogen phosphate 3.6 g*
 - Disodium hydrogen phosphate dihydrate 7.2 g*
 - Purified water to 1000 mL*

Sample preparation

- 1) Dissolve about 2 g of product to analyse in 200 ml of Buffer NaCl-Peptone pH 7.0 (dilution 1:100), swirl container and wait a few minutes until complete dissolution (Sol.A).
- 2) A 20 ml of Sol.A sample is aseptically taken to check its pH that must range between 6 and 8. If necessary, the pH of the solution is adjusted with a few drops of sterile 0.1 N HCl or 0.1 N NaOH.
- 3) Set up the sterilized filtration ramp and place the filter.

- 4) Per-wet the membrane with 50 ml of Buffer NaCl-Peptone pH 7.0
- 5) Filtrate 10 ml of Sol. A (corresponding to 0.1 g of product)
- 6) Rinse the membrane with 150 ml of Buffer NaCl-Peptone pH 7.0
- 7) With sterile tweezers, remove the membrane and transfer it to a TSA plate.
- 8) Incubate for 3-5 day at 30 – 35 °C.

Negative control

At the end of the analytical session carry out the test as previously described using of Buffer NaCl-Peptone pH 7.0 instead of the sample.

Results

At the end of the incubation, read the plates and apply the following formula:

$$\text{UFC/g} = n^{\circ} \text{ of colonies} * 10$$

Acceptance Criteria

The expected acceptance criteria are:

- TAMC ≤ 500 ufc/g
- Negative control: absence of growth

3.2.S.4.2.12 – TYMC

Yeast and moulds are determined as described under Ph. Eur. current ed. (membrane filtration method - Monograph n° 2.6.12).

Culture media and reagents

- Sabouraud -dextrose agar (SDA)
- Buffer NaCl-Peptone pH 7.0 (Diluent):
Peptone 1.0 g
NaCl 4.3 g
Potassium dihydrogen phosphate 3.6 g
Disodium hydrogen phosphate dihydrate 7.2 g
Purified water to 1000 mL

Sample preparation

- 1) Dissolve about 2 g of product to analyse in 200 ml of Buffer NaCl-Peptone pH 7.0 (dilution 1:100), swirl container and wait a few minutes until complete dissolution (Sol.A).
- 2) A 20 ml of Sol.A sample is aseptically taken to check its pH that must range between 6 and 8. If necessary, the pH of the solution is adjusted with a few drops of sterile 0.1 N HCl or 0.1 N NaOH.

- 3) Set up the sterilized filtration ramp and place the filter.
- 4) Per-wet the membrane with 50 ml of Buffer NaCl-Peptone pH 7.0
- 5) Filtrate 10 ml of Sol. A (corresponding to 0.1 g of product)
- 6) Rinse the membrane with 150 ml of Buffer NaCl-Peptone pH 7.0
- 7) With sterile tweezers, remove the membrane and transfer it to a SDA plate.
- 8) Incubate for 3-5 day at 30 – 35 °C.

Negative control

At the end of the analytical session carry out the test as previously described using of Buffer NaCl-Peptone pH 7.0 instead of the sample.

Results

At the end of the incubation, read the plates and apply the following formula:

$$\text{UFC/g} = n^{\circ} \text{ of colonies} * 10$$

Acceptance Criteria

The expected acceptance criteria are:

- TYMC $\leq$ 50 ufc/g
- Negative control: absence of growth

3.2.S.4.2.13 – *Escherichia Coli*

Test for *Escherichia Coli* is performed according to Ph. Eur. current ed. (Monograph n° 2.6.13)

Culture media and reagents

- Casein soya bean digest broth (CASO B)
- MacConkey broth (MCB)
- MacConkey Agar (MCA)
- Buffer NaCl-Peptone pH 7.0 (Diluent):
Peptone 1.0 g
NaCl 4.3 g
Potassium dihydrogen phosphate 3.6 g
Disodium hydrogen phosphate dihydrate 7.2 g
Purified water to 1000 mL

Sample preparation

- 1) Dissolve about 10 g of product to analyse in 200 ml of Buffer NaCl-Peptone pH 7.0 (dilution 1:10), swirl container and wait a few minutes until complete dissolution (Sol.B).
- 2) For the pH value of this solution refer to Sol.A prepared for TAMC and TYMC determinataion.

- 3) 10 ml of Sol.B (correspondent to 1 g of product) inoculated with E.coli microbial suspension, will be transferred in 100 ml of Casein soya bean digest broth (CASO B) homogenised and incubated at $32.5 \pm 2.5^{\circ}\text{C}$ for 18-24 h.
- 4) Transfer 1 mL of Casein soya bean digest broth (CASO B) to 100 mL of MacConkey broth (MCB) and incubate at $42-44^{\circ}\text{C}$ for 24-48h.
- 5) Subculture to MacConkey agar plates (MCA) and incubate at $32.5 \pm 2.5^{\circ}\text{C}$ for 18-72 hours.

Negative control

At the end of the analytical session carry out the test as previously described using of Buffer NaCl-Peptide pH 7.0 instead of the sample.

Results

At the end of the incubation, read the plates: no presence of colonies must be observed

Acceptance Criteria

The expected acceptance criteria are:

- *E.Coli*: absent /1g
- Negative control: absence of growth