STANDARDISED METHODS FOR THE TESTING OF EDIBLE COLLAGEN PEPTIDES (synonyms: hydrolysed collagen and collagen hydrolysate)

COLLAGEN PEPTIDES MONOGRAPH

Final Version 3 – August 2023

GME
GELATINE MANUFACTURERS OF EUROPE

NOTICE

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*****All GME members are obliged to use the GME methods as they are written in the GME monograph. Alternative methods may be used, only in case if they have been validated against the GME method.

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Follow-up Supplements

It is recommended that follow-up supplements be recorded on this sheet. Supplements are numbered and the chapter/pages they replace indicated upon distribution.

Supplement no.	Date	Amendments
2.6 Molecular Weight Determination	May 2022	Addition: the columns should be changed after 250 injections.
2.4.1 Viscosity, point 9.5	May 2022	Cleaning of pipettes
2.1.3 Hydroxyproline determination	Dec 2022	Update on ISO reference
2.2.2 Ash (Residue on ignition)	August 2023	Updated – with point 7

1. FOOD GRADE COLLAGEN PEPTIDES

1.1. DEFINITION

Collagen peptides is a purified protein obtained from collagen of healthy animals (e.g. bovine, porcine, fish and poultry) used for food by humans by alkaline hydrolysis, acid hydrolysis, enzymatic hydrolysis, and/or thermal hydrolysis. The hydrolysis leads to cold water soluble, non-gelling grades of collagen peptides in a powder form, distinguished from gelatin that is gel-forming. This monograph covers non-gelling collagen peptides, further classified as Type I/III collagen for commercial applications.

1.2. IDENTIFICATION

1.2.1. Protein determination

Test for protein according to Biuret method.

1.2.2. Gelling/non gelling character

1.2.3. Hydroxyproline determination

Test for hydroxyproline, the characteristic amino acid of collagen. Edible collagen of bovine and porcine raw material typically contains a minimum of 11% hydroxyproline.

1.3. QUALITY STANDARDS

1.3.1. Description

Specific health conditions for collagen peptides intended for human consumption are laid down in Regulation (EC) No 853/2004 of the European Parliament and of the Council (See Annex to this document).

This decision specifies requirements for raw materials, their transport and storage, collagen manufacturing processes, collagen quality, packaging, storage and transport.

The chemical and microbiological quality requirements for finished collagen peptides are listed in 1.3.2 and 1.3.3.

1.3.2. Chemical quality

Maximum values for collagen peptides:

		Regulation (EC) No 853/2004	USP-NF, Ph.Eur. (Gelatin Monograph)	GME requirements for collagen peptides	Ref.
		Edible	**Pharma**		
Moisture	%		≤ 15	≤ 15	2.2.1
Ash	%			≤ 2	2.2.2
pH (1%, 55°C)			3.8 - 7.6		2.4.4
Conductivity (1%, 30°C)	mS/cm		≤ 1		2.4.5
SO_2	ppm; mg/kg	≤ 50	≤ 50	≤ 50(*)	2.2.4
H_2O_2	ppm; mg/kg	≤ 10	≤ 10	≤ 10	2.2.5
As	ppm; mg/kg	≤ 1		≤ 1	2.2.6
Pb	ppm; mg/kg	≤ 5		≤ 5	2.2.6
Cu	ppm; mg/kg	≤ 30		≤ 30	2.2.6
Zn	ppm; mg/kg	≤ 50	≤ 30	≤ 30	2.2.6
Cr	ppm; mg/kg	≤ 10	≤ 10	≤ 10	2.2.6
Hg	ppm; mg/kg	≤ 0.15		≤ 0.15	2.2.7
Cd	ppm; mg/kg	≤ 0.5		≤ 0.5	2.2.6
Fe	ppm; mg/kg		≤ 30	≤ 30	2.2.6

(*) Edible collagen peptides must contain neither preservatives nor inhibitors.

1.3.3. Microbiological quality

Maximum values for edible collagen peptides are described in Regulation (EC) No 2073/2005, Annex I, Chapter 1, point 1.10. Only Salmonella testing is required for food grade collagen peptides.
The GME Members agreed to extend the bacteriological testing for edible collagen peptides with Total Aerobic Microbial Count, E. coli and Anaerobic sulphite-reducing spores.

	Regulation (EC) No 2073/2005	**European Pharmacopoeia (Gelatin Monograph)**	**GME requirements for collagen peptides**	**Ref.**
Salmonella	(n=5) Absence/25g	Absence/10g	Absence/25g	2.3.4
Total aerobic microbial count	-	max. 1000 CFU/g	< 1000 CFU/g	2.3.1
E.coli	-	Absence/g	Absence/10g	2.3.2
Anaerobic sulphite-reducing spores	-	-	< 10 CFU/g	2.3.3
Yeast and moulds	-	max. 100 CFU/g	-	-

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GELATINE MANUFACTURERS OF EUROPE

GME STANDARD CODE
BACTERIOLOGICAL SPECIFICATION
FOOD GRADE GELATINE AND COLLAGEN PEPTIDES

All Members of GME are committed to comply with the food hygiene requirements, specified by the EU regulations EC/853/2004 and EC/2073/2005. Although only the salmonella testing is required for food grade gelatine and collagen peptides, all the Members of GME agreed to extend the bacteriological testing.

The GME standard for bacteriological specification is therefore defined as follows:

- Total aerobic microbial count: < 1000 cfu/g
- E. coli: absence in 10 g
- Anaerobic sulphite-reducing spores: < 10 cfu/g
- Salmonella: absence in 25 g

Validation period: until 31 December 2022.

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European Chemical Industry Council - Cefic aisbl
EU Transparency Register n° 64879142323-90

1.4. PHYSICAL TESTING OF COLLAGEN PEPTIDES

In addition to the chemical and microbiological requirements for edible collagen peptides listed in 1.3, physical properties are also generally of importance for fitness for use and standardised methods are listed below.

- Viscosity (See 2.4.1.)
- Colour (See 2.4.2.)
- Clarity (See 2.4.3.)
- pH (See 2.4.4.)
- Conductivity (See 2.4.5.)
- Molecular weight (See 2.4.6)

2. TEST METHODS

REMARK: For all tests purified water should be used, unless the quality of the water is defined differently in the respective test description.
Purified water is prepared by distillation, ion exchange, reverse osmosis or by any other suitable method that complies with the regulations on water intended for human consumption laid down by the competent authority.

2.1. IDENTIFICATION

(Other methods may be used provided that correlation has been established with the reference methods. In case of disagreement, the reference method is the one described in this document)

2.1.1. Biuret Method

1. Principle

Reaction between proteins (or peptides) and cupric ions in alkaline medium produces a complex with a violet colour.

2. Reference(s)

European Pharmacopoeia (current edition) – Gelatin Monograph - Identification test A

3. Reagents

- Cupric sulphate ($CuSO_4$ 5 H_2O) 12,5 g/100 ml purified water
- Sodium hydroxide (NaOH) 8,5 g/ 100 ml purified water
- Purified water (demin. or distilled water)

4. Apparatus

- Water bath at approximately 55 °C
- Standard laboratory glassware

5. Procedure

5.1. Dissolve 1 g of the sample in purified water at about 55 °C, dilute to 100 ml with the same solvent and keep the solution at this temperature.

5.2. To 2 ml of the sample solution, add 0.05 ml of cupric sulphate solution, mix, and add 0,5 ml of sodium hydroxide solution.

6. Result

A violet colour is produced if the test is positive**.**

2.1.2. Gelling / non-gelling character

1. Principle:

Gelatine is forming a gel. Collagen peptides and other types of proteins are not able to form a gel under the described test conditions.

2. Reference(s)

European Pharmacopoeia (current edition) – Gelatin Monograph - Identification test B

3. Reagents

- Purified water (demineralized or distilled water)

4. Apparatus

- Water bath at approximately 60°C
- Refrigerator, ice cubes
- Standard laboratory glassware

5. Procedure and result

In a test-tube about 15 mm in internal diameter, place 0.5 g of the substance to be examined and add 10 mL of water. Allow to stand for 10 min, heat at 60 °C for 15 min and keep the tube upright at 2-8 °C for 6 h. Invert the tube; the contents flow out immediately for non-gelling grades and do not flow out immediately for gelling grades.

2.1.3. Hydroxyproline content

1. Principle

The sample is first hydrolysed in acid medium to liberate hydroxyproline from the sample. The hydrolysate is oxidised with chloramine-T. The oxidised hydroxyproline is measured by colourimetry using p-dimethylaminobenzaldehyde.

2. Reference(s)

European Pharmacopoeia (current edition) – Gelatin Monograph - Identification test C
ISO - TC 34 - 3496 (1994); Meat and meat products - Determination of L-hydroxyproline content (Reference method).

3. Reagents and solutions

- All reagents are analytical grade. Water is purified water or equivalent.
- Hydrochloric acid 6N, alternatively sulfuric acid 6N
- Sodium hydroxide 10N
- 1-Propanol
- 2-Propanol
- Buffer solution pH 6: dissolve in water 50 g citric acid monohydrate, 12 ml glacial acetic acid, 120 g sodium acetate trihydrate, 34 g sodium hydroxide. Make up to volume 1 litre with water. Mix with 200 ml water and 300 ml 1-propanol.
- Oxidant reagent: dissolve 1.4 g chloramine-T in 10 ml water, add 10 ml 1-propanol and 80 ml buffer solution pH 6.
- Colourimetric reagent: (*Prepare fresh each day*) dissolve 10 g p-dimethylamino-benzaldehyde in 35 ml perchloric acid 60%, add slowly 65 ml 2-propanol.
- Hydroxyproline standard solutions: dissolve 100 mg in water, add 1 drop HCl 6 N and dilute to 100 ml. For use, dilute 5 ml of the solution to 500 ml. Prepare three standard solutions by diluting 10, 20 and 40 ml of this solution to 100 ml with water.
- Stability of the reagents, e.g.:
  - Buffer solution stable for 4 weeks in refrigerator Oxidant reagent prepare fresh each day.
  - Colourimetric reagent prepare fresh each day.
  - Hydroxyproline standard solutions stable for one week stored in the refrigerator.

4. Apparatus

- Digestion vessels
- Paper filters
- Water bath at 60 ± 0.5 °C.
- Spectrophotometer and optical cells (path 1 cm).
- Analytical balance.

5. Procedure

5.1. Accurately weigh, in duplicate, 1 g of sample and introduce into digestion vessels. Add 30 ml HCl 6 N (or H_2SO_4 6 N) and hydrolyse in a drying oven for 22 to 24 hours at 105 °C (do not screw the caps of the digestion vessels tightly).

Cool the hydrolysate to room temperature and transfer to a 1000 ml volumetric flask through a funnel containing a filter. Wash the digestion vessel with water. Make up to 1000 ml with water.

Dilute the hydrolysate with a factor of 1 / 10: put 10 ml of the hydrolysate solution in a 100 ml flask and make up until 100 ml with water.

5.2. Pipette 2 ml of diluted hydrolysate sample solution (5.1) in a test tube and add 2 ml of oxidant reagent (Chloramine-T solution). Mix and allow to stand for 20 minutes at room temperature.

Add 2 ml of colourimetric reagent, mix and close the tube. Place the tube in a water bath at 60°C for 15 minutes. Cool to room temperature within 3 minutes (cooling water bath).
Make up to 10 ml with water and mix the tube. Allow to stand for 30 min at room temperature.

Mix again and measure the absorbance at 558 nm against a blank.

5.3. Blank preparation: proceed in the same way from 5.2 using 2 ml of water instead of the hydrolysate.

5.4. Calibration curve: proceed in the same way from 5.2 using 2 ml of the three standard solutions instead of the hydrolysate. Plot the curve.

6. Result

6.1 If “h” is the hydroxyproline content in µg/ml read from the calibration curve, “m” is the weight of the sample in mg then:

$$\% \text{ hydroxyproline} = \frac{1000 \text{ x h}}{\text{m}}$$

Express the result to 0.1 %.

6.2 Repeatability: the difference between two determinations should not be larger than 10 %.

7. Hydroxyproline content proposed conversion factors (@8%hum)

7.1 Mammalian hide gelatine (porcine/bovine): ~ 7,7
7.2 Mammalian bone gelatine (porcine/bovine): ~ 7,3
7.3 Fish gelatine (warm-water fish): between 8,5 and 11,5
7.4 Fish gelatine (cold-water fish): between 11,5 and 14

8. Remark

8.1. The determination of Hydroxyproline may also be used as the identification test C in accordance to the Gelatine Monograph of the European Pharmacopoeia (current edition). The method described in the Pharmacopoeia is a qualitative test for Hydroxyproline based on the same chemical reaction.

8.2 The hydrolysation step of the protocol can be shortened using high speed digestion equipment such as:

- Hach High temperature thermostat HT 200S (part no. LTV077)
- Hach Reaction tubes with screw cap (part no. LZC924)

In this case, step 5.1 can be modified to read:

- Accurately weigh, in duplicate, **0,25g** of sample and introduce into the **reaction tube**. Add 7,5 ml HCl 6N (or H2SO4 6N) and hydrolyse **in a high temperature thermostat for one hour at 150 °C (screw the caps of the reaction tube tightly).**

  Cool the hydrolysate to room temperature and transfer to a **250 ml** volumetric flask through a funnel containing a filter. Wash the digestion vessel with water. Make up to **250 ml (V)** with water.

  Dilute the hydrolysate with a factor of 1 / 10 : put 10 ml of the hydrolysate solution in a 100 ml flask and make up until 100 ml with water.

While following the rest of original protocol, step 6.1 should then read:

- If “h” is the hydroxyproline content in µg/ml read from the calibration curve, “m” is the weight of the sample in mg, **and factor “V” in ml** then:

  % hydroxyproline = $V \times h / m$

This method is only for internal use by GME members.

2.2. CHEMICAL CHARACTERISTICS

(Other methods may be used provided that correlation has been established with the reference methods. In case of disagreement, the reference method is the one described in this document)

2.2.1. Loss on drying (moisture)

1. Principle

A weighed sample of collagen peptides is maintained for 16 to 18 hours at 105 +/- 2 °C and is then reweighed. The moisture content is defined as the percentage loss in weight of the sample.

2. Reference(s)

AFNOR NF V 59-003
European Pharmacopoeia – Gelatin Monograph (current edition)

3. Apparatus

- E.g.Pyrex evaporating dishes, 45 mm in diameter and 30 mm high
- Drying oven, set at 105 +/- 2 °C
- Dessicator containing $CaCl_2$ or silicagel
- Analytical balance

4. Procedure

4.1. SAMPLE PREPARATION

Wash the evaporating dish very carefully in hot water, place it in the drying oven at 105 +/- 2 °C for at least one hour, then leave it to cool in the dessicator until room temperature is reached. Weigh approximately 5 g of the sample to the nearest milligram and note the weight of the test sample (m0) and the weight of the sample together with the evaporating dish (m1).

4.2. DETERMINATION

Place the evaporating dish containing the sample in the drying oven at 105 +/- 2 °C and leave for 16 to 18 hours. Leave to cool in the dessicator until room temperature is reached and weigh to the nearest milligram (m2).

5. Result

THE MOISTURE CONTENT, EXPRESSED AS A PERCENTAGE BY WEIGHT, IS EQUAL TO :

$$\frac{m_1 - m_2}{m_0} \times 100$$

where:

m_0 is the weight in grams of the test sample

m_1 is the weight in grams of the test sample and the evaporating dish before drying

m_2 is the weight in grams of the test sample and the evaporating dish after drying

2.2.2. Ash (Residue on ignition)

1. Principle

Collagen peptides is incinerated in a crucible and ashing is finished in a muffle furnace at 550 °C. The residue is determined by differential weighing and the result expressed as a weight percentage of the sample.

2. Reference(s)

USP 35 (The United States Pharmacopoeia 35); The National Formula (30) = USP35/NF30 – Gelatin Monograph

3. Reagents and Solutions

Paraffin
Purified water (demin. or distilled water)

4. Apparatus

Muffle furnace 550°C (± 25°C)
Analytical balance
Desiccator
Crucible

5. Procedure

5.1. SAMPLE PREPARATION

Weigh approx. 5.0 g of collagen peptides to 0.001 g in a suitable crucible that previously has been ignited, cooled and weighed. Add 1.5 to 2.0 g of paraffin to avoid loss due to swelling.

Heat, gently at first on a low flame, electric hot plate or muffle furnace, until the substance is thoroughly charred and finish ashing in a muffle furnace at 550 °C for 15 to 20 hours.

5.2. DETERMINATION

Cool in a desiccator, weigh and calculate the percentage of residue.

(*Do not touch dried crucibles without gloves or crucible tongs*).

6. Results

THE ASH CONTENT, EXPRESSED AS % ASH, IS EQUAL TO:

$$= \frac{m_1}{m_0} \times 100$$

Where: m_0 is the quantity weighed in

m_1 is the quantity weighed out

7. Remarks

The described ash determination is a limit test. If the aim of the analysis is just to examine whether the ash is below or above a certain limit, the above defined method is applicable.

If the amount of the residue obtained exceeds the specified limit additional procedure steps are recommended to reach a constant weight.

After weighing out the product, the sample should be wetted with some drops of purified water and heated again in the muffle furnace at 550°C at least for another 30 minutes.

Repeat the moistening with purified water, heating and igniting as before, using at least a 30-minute ignition period, until two consecutive weighings of the residue do not differ by more than 5,0 mg or until the percentage of residue complies with the limit in the individual monograph.

This procedure is also highly recommended if more detailed information about the ash content is needed, e.g. for exact comparison of lab results in ring tests or similar.
Also ashing of bone gelatins often requires an additional treatment of the sample as described above.

2.2.3. Nitrogen

1. Principle

Digestion of a test portion with concentrated sulfuric acid in the presence of catalysts to convert the organic nitrogen into ammonium sulphate. Excess sodium hydroxide is added to the cooled digest to release the ammonia. The released ammonia is distilled into an excess of boric acid solution and then titrated with a standard solution of sulfuric or hydrochloric acid. The nitrogen content is calculated from the quantity of ammonia produced.

2. Reference(s)

ISO 1871:2009(E) General guidelines for the determination of nitrogen by the Kjeldahl method.

3. Reagents

Use only reagents of recognized analytical grade free from any residual Nitrogen, unless otherwise specified, and distilled or demineralized water or water of equivalent purity.

- **Sulfuric acid**, minimum 95 %, virtually free from nitrogenous compounds.

- **Catalysts,** ready-to-use composite catalysts are available on the market, and are preferably used.

  It is important to differentiate between the substances used to raise the boiling point of the liquid during digestion and the catalysts themselves that facilitate digestion. The former are usually potassium sulphate or, possibly, sodium sulphate. The most commonly used catalyst is copper in the form of copper sulphate alone or mixed with titanium oxide or Selenium.

- **Boric acid solution** (10 g/l to 40 g/l depending on the apparatus used). If using the colorimetric end-point titration, boric acid solution shall contain indicator (the pH or colour of this mixed solution shall be adjusted before use).

- **Standard sulfuric acid solution** (0,01 mol/l to 0,25 mol/l). The titre of the solution, c_t, shall be known to at least within 0,001 mol/l.

- **Indicators**, which should change colour between pH 4 and pH 5.

- **Sodium hydroxide solution**, min. 30 % mass fraction.
- **Ammonium sulphate** or **ammonium chloride** (minimum purity 99,9 %). Ready to use liquids are available.
- **Tryptophan, acetanilide**, **lysine hydrochloride or L-aspartic acid** (minimum purity 99 % mass fraction).

4. Apparatus

- **Analytical balance**, capable of weighing to the nearest 0,0001 g (Kjeldahl ***weighing*** boats can be used).
- **Digestion, steam distillation and titration systems**

5. Procedure

5.1. TEST PORTION

The test portion, the quantity of which depends on the presumed nitrogen content determined by the Kjeldahl method, shall be representative of the sample and contain between 0,001 g and 0,2 g of nitrogen.

The test portion can be obtained by weighing 0,5 – 1 g collagen peptides with the analytical balance into a test tube (Kjeldahl weighing boats can be used), to give mass, *m*, in grams

5.2. DIGESTION

A. Catalysts

Catalysts are added to raise the boiling point of the acid to between 380°C and 430°C, and facilitate digestion.

Preferably ready-to-use composite catalysts are used. They should meet the requirements of the blank and control tests.

B. Addition of acid

It is important to use a sufficient quantity of sulfuric acid to ensure digestion after:

- acid consumption by the organic matter of the sample, bearing in mind the fact that 1 g of fat consumes 10 ml of sulfuric acid, 1 g of protein consumes 5 ml of sulfuric acid, 1 g of carbohydrate consumes 4 ml of sulfuric acid
- acid consumption by the reagents (salts)
- acid losses by evaporation

The addition of 20 ml to 25 ml of acid is generally sufficient for good digestion and to maintain excess acid at the end of the reaction.

C. Heating

The manufacturer's instructions relating to the use of the equipment should generally be followed.

In all cases, the digestion temperature and time should be determined to meet the requirements of the digestion control test. Heating at 420 °C for two hours is appropriate for numerous matrices.

The tubes can be placed directly in a preheated unit

Acid fumes shall be removed with an extraction system suitable for the equipment used. Excessive extraction may cause crystallization and a loss of nitrogen.

The digest obtained should be clear and free from black particles.

At the end of the digestion process, allow the tubes to cool away from any possible contamination. At this stage, the test portions can be stored and distilled later.

5.3. AMMONIA DISTILLATION

A. Alkalinisation

Dilute the digest with water and then alkalise by adding at least 3,5 ml of sodium hydroxide solution per millilitre of sulfuric acid used for the digestion process. (The volume of the added sodium hydroxide solution may be lower if its mass fraction is higher than 30 %.). Stage of alkalisation can be checked by adding indicator.

B. Distillation

Perform the distillation with the apparatus under consideration in its usual condition. Collect the distillate in the boric acid solution, which shall contain the indicator. Adjust the pH until there is a change of colour in accordance to the used indicator before beginning the distillation.

- Ensure that in compliance with the control tests, the ammonia distillation is complete and that there is no excess by entrainment of the alkaline liquid (see also instructions of the manufacturer).

5.4. TITRATION

The distillate obtained is titrated with sulfuric acid; this can be done simultaneously or after distillation. Post-distillation titration should be performed as soon as possible after distillation.

There are two methods of detecting the end point.

- By visual colorimetry or using an optical measurement system: The end point is reached when the indicator changes colour. In the case of visual colorimetry, it is important to titrate each test referring to the conditions obtained in the blank test.

- By potentiometric analysis with a pH measurement system: Depending on the equipment or operating methods, the end point may be a fixed pH (generally pH 4,6, which corresponds to the inflection point of the titration curve), the pH obtained in the blank test, or the original pH of the boric acid solution.

Record the volumes of titrant obtained: V_0 for the blank test and V_1 for the samples.

5.5. CONTROL TESTS

One blank test and at least one digestion control test should be included in each set of nitrogen determination tests.
Additionally, distillation control tests should be performed on a regularly base.

A. Blank test

Perform a blank test using the operating method described above, replacing the liquid test

B. Digestion test

Perform a digestion control test using the operating method described above, replacing the test portion with the same quantity of tryptophan, acetanilide, lysine hydrochloride or L-aspartic acid as the quantity of nitrogen in the samples.
Calculate the percentage mass fraction of nitrogen recovered, which should be between 98 % and 102 %.

C. Distillation-titration tests

Perform a blank distillation-titration test using the operating method described in 5.3, but without a test portion. The volume obtained should be subtracted from that of the distillation-titration test.

Perform a distillation-titration control test under the same conditions on a test portion of ammonium salt corresponding to the quantity of nitrogen in the samples.

Calculate the percentage mass fraction of nitrogen recovered, which should be between *98 % and 102 %.*

7. Result

The nitrogen content, expressed as a percentage mass fraction or in grams per 100 ml, is equal to:

$$\frac{(V_1\text{-}V_0)\times c_t\times 28\times 100}{m\times 1000} \; or \; \frac{(V_1\text{-}V_0)\times c_t\times 28\times 100}{V_t\times 1000}$$

where

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

c_t is the titre, in moles per litre, of sulfuric acid
V_0 is the volume, in millilitres, of sulfuric acid used in the blank test titration
V_1 is the volume, in millilitres, of sulfuric acid used in the test portion titration
V_t is the volume, in millilitres, of the test portion

Calculation of collagen protein: N x 5.55

For plausibility check of the results obtained for moisture, ash and collagen protein (N x 5.55) it is recommended to calculate the sum of all items.
The result of this calculation should be within 100 +/- 3 %. In case of non-compliance repeat tests should be carried out.

8. Remarks

The following potential sources of error can occur

Faults noted	Causes	Solutions proposed
DURING DIGESTION		
Too much spray or foam	Too rapid a rise in temperature	Reduce the heating rate or adjust the steps
Black particles in the digest	Inappropriate digestion time/temperature	Optimize the conditions: check the digestion test Check the sample/acid/catalyst proportions
Pellet crystallization	Loss of acid due to a too powerful fume extraction system	Reduce the extraction rate: it can be reduced as soon as the white fumes disappear Check the sample/acid/catalyst proportions
DURING DISTILLATION AND NITROGEN CONTENT DETERMINATION		
Distillation-titration test result too low	Loss of ammonia	Check the apparatus for tightness (seals and glass instruments)
	Insufficient boric acid	Increase the concentration or volume of the boric acid solution
	Ammonia entrainment incomplete	Increase the distillation time
	Incorrect acid titre measurement	Titrate the acid
	Blank distillation-titration test result too high	Perform a new blank test
Distillation-titration test result too high	Incorrect acid titre measurement	Titrate the acid
	Pollution due to ammonia vapour	Avoid handling ammonia in the vicinity
	Entrainment of sodium hydroxide in the distillate	Reduce the volume of water added before distillation

Digestion test result too low	Inappropriate digestion time/temperature	Optimize the conditions: check the digestion test
		Check the sample/acid/catalyst proportions

2.2.4 Sulfur Dioxide

2.2.4.1. Distillation - Method

1. Principle

Sulfur dioxide is set free from the collagen peptides sample by boiling it with the hydrochloric acid. The created sulphur dioxide is distilled into a hydrogen peroxide solution with a stream of carbon dioxide.
Sulfuric acid is formed that can be titrated with sodium hydroxide.

2. Reference(s)

European Pharmacopoeia (current edition) – Gelatin Monograph

3. Reagents and solutions (for additional details see also Eur.Pharm.)

- Carbon dioxide : CO2 > 99,5 % V/V
- Dilute Hydrogen peroxide solution : H2O2, 3 % in water (+/- 0,5 %)
- Bromophenol blue solution : 1 g/L in ethanol (20 % V/V) Ethanol: 96 % C2H5OH (V/V)
- Dilute hydrochloric acid : HCl, 2 mol/l (73 g / l)
- Sodium hydroxide : NaOH, 0.1 mol / l or 0.01 mol / l
- Water: Purified water

4. Apparatus

Apparatus for the determination of sulfur dioxide.

5. Procedure

5.1. SAFETY INSTRUCTIONS

When working with HCl, NaOH or H_2O_2, avoid contact with eyes and skin; do not breathe vapours.

Wear protective glasses and gloves.

5.2. PREPARATION OF APPARATUS

Introduce 150 ml of water into the flask (A, see figure) and pass carbon dioxide through the whole system for 15 min. at a rate of 100 ml +/- 5 ml/min

To 10 ml of dilute hydrogen peroxide solution add 0,15 ml of a 1 g/L solution of bromophenol blue in ethanol (20 % V/V). Add 0,1M sodium hydroxide until a violet-blue colour is obtained, without exceeding the end-point. Place the solution in the test-tube (D).

5.3. SAMPLE PREPARATION

Without interrupting the stream of carbon dioxide, remove the funnel (B) and introduce through the opening into the flask 25.0 g (m in g) of the collagen peptides sample with the aid of 100 ml water. Replace the funnel.

Close the tap of the funnel and add 80 ml of dilute hydrochloric acid to the funnel. Open the tap of the funnel to allow the hydrochloric acid solution to flow into the flask, making sure that no sulfur dioxide escapes into the funnel by closing the tap before the last few millilitres of hydrochloric acid solution drain out. Boil for 1 h.

Open the tap of the funnel and stop the flow of carbon dioxide and also the heating and the cooling water. Transfer the contents of the test-tube with the aid of a little water to a 200 ml wide-necked, conical flask. Heat on a water-bath for 15 min and allow to cool.

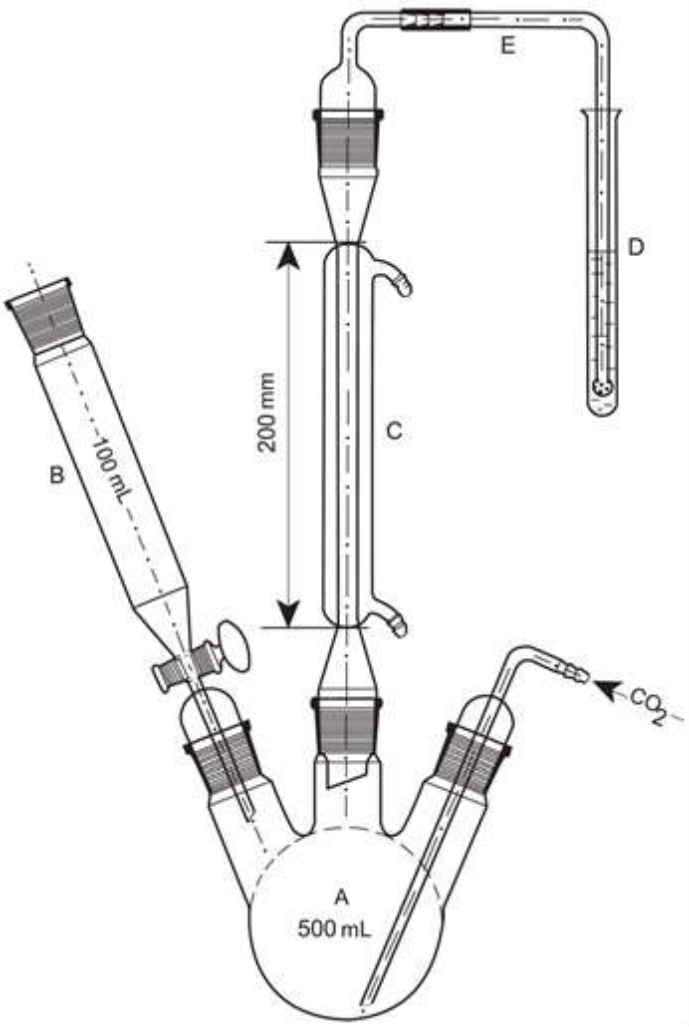
5.4. DETERMINATION

Add 0.1 ml of a 1 g/L solution of bromophenol blue R in ethanol (20 per cent V/V) R and titrate with 0.1 M sodium hydroxide until the colour changes from yellow to violet-blue (V1 mL). Carry out a blank titration (V2 mL).

6. Result

6.1. Calculate the content of sulfur dioxide in mg / kg collagen peptides using the following expression:

32030 x (V1 – V2) x n / m
n = molarity of the sodium hydroxide solution used as titrant.



Apparatus for the determination of sulfur dioxide

6.2 Remarks: The sensitivity of the described method is improved by increasing the sample weight to 50 g and the use of a 0,01 mol/L NaOH as the titrant.
The official EP - Method 2.5.29 defines 25.0 g sample amount and 0,1 M NaOH as a titrant.

2.2.4.2. Annex I - Büchi method – SO_2 Determination

The Büchi method is an internal method and should only be used as a rapid test!

1. Principle

Add HCl 4 N to the sample and distil the released SO_2 by means of water steam distillation. Absorb the SO_2 in a receiving solution of 0.1 N Iodine solution. Titrate the remaining Iodine solution with 0.1 N Sodium thiosulphate.

$$SO_2 + 2H_2O + I_2 \longrightarrow 2\,HI + H_2SO_4$$

2. Interference

Eventually, other reducing volatile components will be determined as well.

3. Reagents

- Iodine solution 0.1 N
- Sodium thiosulfate solution 0.1 N
- Hydrochloric acid solution 4 N

4. Instruments

- BÜCHI distillation unit (K-314, B-324 o B-334 or similar)
- BÜCHI digestion tube 500 ml
- Receiving vessel 600 ml
- Magnetic stirrer, place underneath receiving vessel
- 10 ml volumetric pipette
- Titrator

5. Sample weight

The weight of the sample depends on the quantity of SO_2 expected, according to the following table

SO_2 content	**Sample weight**
0 – 50 mg/kg; ppm	**100 g**
50 – 200 mg/kg; ppm	**50 g**
200 – 500 mg/kg; ppm	**25 g**
500 – 1000 mg/kg; ppm	**10 g**

6. Determination of blanc

Introduce 10.0 ml of 0.1N Iodine solution and 190 ml of deionised water in the receiving vessel. Place magnetic stirrer rod in the receiving vessel.
Fill 50 ml of 4N HCl into the digestion tube and distil for 7 minutes. After completion of distillation titrate the receiving solution with 0.1 N Sodium thiosulfate solution until the colour becomes yellowish. Add 1 ml of starch solution 1 % and continue titration until the solution becomes colourless.

7. Procedure for samples

Weight collagen peptides sample according to SO_2 content directly into digestion tube. Add 50 ml of HCl 4 N and continue according to charter F.

8. Calculations

$$\text{mg } SO_2\text{/kg Sample (ppm)} = \frac{V_{\text{Iodine}}(\text{ml}) - V_{\text{thiosulphate}}(\text{ml}) \times 3200}{\text{Weight Sample (g)}}$$

2.2.4.3. Annex II – EU Regulatory limits

- Max. 50ppm: for edible (food hygiene regulation) and pharma (European Pharmacopoeia – Gelatin Monograph (current edition) gelatine.
- if more than 10 ppm: mandatory allergen labelling on final consumer products in accordance with regulation EC N° 1169/2011.

2.2.5. Peroxides

2.2.5.1 Semi-Quantitative Method using peroxide test strips

1. Principle

Peroxidase transfers oxygen from the peroxide to an organic redox indicator which is converted to a blue-coloured oxidation product.

The intensity of the colour obtained is proportional to the quantity of peroxide and can be compared with a colour scale, provided with the test strips, to determine the peroxide concentration.

2. Reference

European Pharmacopeia (current edition) – Gelatine Monograph

3. Reagents

Test strips for the detection and semi-quantitive determination of peroxides:

- *MERCKOQUANT 10011*
- *BOERHRINGER Nº 123266.*

4. Apparatus

Water bath 40°C ± 2

5. Procedure

5.1. PREPARATION OF A 20% COLLAGEN PEPTIDES SOLUTION

Weigh 20.0 g ± 0.1 of collagen peptides in a beaker, add 80 ml ± 0.2 of deionised water. Stir to moisten all the collagen peptides and allow the sample to stand at room temperature for 1-3 hours. Cover the beaker with a watch glass.

Place the beaker in the 40°C water bath for about 20 min ± 5 for dissolving the sample. To achieve a homogeneous solution, the contents of the beaker should be stirred with a glass rod.

5.2. CARRYING OUT THE TEST

Take a test strip and dip it into the solution for one second, such that the reaction zone is properly wetted.

Remove the test strip, shake off excess liquid and compare the reaction zone with the colour scale after 15 seconds.

6. Result

The result of the colour scale is expressed in mg/l H_2O_2.

To obtain the concentration of H_2O_2 in mg/kg of collagen peptides, this result must be multiplied by a dilution factor equal to 5.

7. Remarks

7.1 Suitability test using 2 ppm H_2O_2 solution:

Dip a test strip for 1 s into hydrogen peroxide standard solution (2 ppm H_2O_2), such that the reaction zone is properly wetted. Remove the test strip, shake off excess liquid after 15 s. Compare the reaction zone with the colour scale provided. The test strips are suitable if the colour matches that of the 2 ppm concentration.

7.2 Storage of test strips: see supplier instructions of the test strips.

2.2.6. Arsenic, Cadmium, Chromium, Copper, Iron, Lead and Zinc

1. Principle

Determination by Atomic Spectrometry or ICP-OES (Inductively Coupled Plasma Optical Emission Spectrometry) after adequate acidolysis (digestion) of the sample using the principle of standard addition (further details see references).

2. Reference

EN ISO 15586 Water quality – Determination of trace elements using atomic absorption spectrometry with graphite furnace, 2003

EN ISO 11885 Water quality – Determination of selected elements by inductively coupled plasma optical emission spectrometry “, 2007

European Pharmacopeia (current edition) Method 2.2.23

3. Reagents and apparatus

Only use purified water and reagents of analytical quality or even better quality.
Use of appropriate plastic labware is recommended wherever possible.
After cleaning procedure finally rinse the labware with pure water or acidic water for the analysis of trace elements. Besides other cleaning steps the use of acidic washing water is recommended.
Analytical balance

4. Instrument parameters

Element	Recommended wavelength (nm)	
	AAS	ICP-OES
• As	193,7	193,696
• Cd	228,8	228,802
• Cr	357,9	267,719
• Cu	324,7	327,396
• Fe	248,3	238,204
• Pb	283,3	220,353
• Zn	213,9	213,857

It is recommended to verify the selected measuring wavelength for any interference caused by reagents or sample matrices (analytical specificity).

5. Blank test

A blank test always should be performed to check the influence and the purity of the reagents.

6. Sampling and sample preparation

Use an adequate amount of a representative sample for the digestion.
The digestion may be performed using e.g. hydrochloric acid or/and nitric acid in combination with a heating step in order to clear up the sample matrix and to decrease carbon-containing material.
The concentration of the sample solution should be within the calibration range. If necessary, the measuring solution has to be diluted. Typically, the collagen peptides concentration in the measuring solution is about 0,5 to 2,0 %.

7. Calibration

The influence of the sample matrix has to be verified.
Generally, the standard addition technique is recommended.
The composition of the calibration solutions and the sample solutions should be identical regarding the concentration of reagents and sample amount.

8. Validation

Analytical specificity, linearity, accuracy (e.g. performing recovery tests), repeatability and limit of quantification must be checked (compare references, Eur.Pharm. method 2.2.23).
It must be provided proof that the specified limits can be checked by the established test procedures.

2.2.7. Mercury

1. Principle

Determination by Atomic Spectrometry. Cold Vapour Technique

The sample is digested under adequate oxidizing conditions to form Hg2+. Then the mercury atoms are generated by chemical reduction with stannous chloride or sodium borohydride and the atomic vapour is swept by a stream of an inert gas into a cold quartz cell mounted in the optical path of the instrument to measure the atomic absorption (further details see references).

2. Reference (s)

EN ISO 12846 Water quality – Determination of mercury – Method using atomic absorption spectrometry (AAS) with and without enrichment, 2012.

EN 13346 Characterization of sludges – Determination of trace elements and phosphorus – Aqua regia extraction methods (sample digestion).

European Pharmacopeia (current edition) Method 2.2.23

3. Reagents and apparatus

Only use purified water and reagents of analytical quality or even better quality.

After cleaning procedure finally rinse the labware with pure water or acidic water for the analysis of trace elements. Besides other cleaning steps the use of acidic washing water is recommended.

Analytical balance.

4. Instrument parameters

Follow instructions of the instrument manufacturer.

5. Blank test

A blank test always should be performed to check the influence and the purity of the reagents.

6. Sampling and sample preparation

Use an adequate amount of a representative sample for the digestion.

The digestion may be performed using e.g. hydrochloric acid or/and nitric acid together with oxidizing reagents to generate Hg2+. Typically, the digestion is carried out under heating in order to clear up the sample matrix and to decrease carbon-containing material.
The concentration of the sample solution should be within the calibration range. If necessary, the measuring solution has to be diluted.
Typically, the collagen peptides concentration in the measuring solution is about 0,5 to 2,0%.

7. Calibration

The influence of the sample matrix has to be verified.
The composition of the calibration solutions and the sample solutions should be identical regarding the concentration of reagents and occasionally sample amount.

8. Validation

Analytical specificity, linearity, accuracy (e.g. performing recovery tests), repeatability and limit of quantification have to be checked (compare references, Eur.Pharm. method 2.2.23.).
It must be provided proof that the specified limits can be checked by the established test procedures.

2.3. MICROBIOLOGICAL CHARACTERISTICS

(Other methods and media may be used if officially recognised in other standards. In case of disagreement, the reference method is the one described in this document).

2.3.0. Preparation of the initial collagen peptides test solution

1. Principle

An initial suspension is prepared to obtain as uniform a distribution as possible of the microorganisms contained in the test sample.
A pre-enrichment or enrichment suspension is prepared in the same way, using the medium recommended by the method of analysis concerned.

2. Reference(s)

European Pharmacopeia (current edition)
US Pharmacopeia (current edition)
ISO 6887-4 Preparation of test samples, initial suspension and decimal dilutions for microbiological examination

3. Diluents[1]

3.1. CASEIN SOYA BEAN DIGEST BROTH

Pancreatic digest of casein	17.0 g
Papaic digest of soya bean	3.0 g
Sodium chloride (NaCl)	5.0 g
Dipotassium hydrogen phosphate (K_2HPO_4)	2.5 g
Glucose monohydrate	2.5 g

Use ready-to-use media or prepare by the following instructions.

- Dissolve the ingredients in the water (1000 ml).
- If necessary, adjust the pH so that after sterilization it is 7.3 ± 0.2 at 25°C.
- Divide 90 ml (180 ml) of the medium into flasks.
- Sterilize for at least 15 min in an autoclave set at 121°C.

3.2 BUFFERED SODIUM CHLORIDE-PEPTONE SOLUTION (PH 7.0)

Potassium dihydrogen phosphate	3.6 g
Disodium hydrogen phosphate dehydrate (Na_2HPO_4)	7.2 g
Sodium chloride (NaCl)	4.3 g
Peptone (meat or casein)	1.0 g

Use ready-to-use media or prepare by the following instructions.

- Dissolve the ingredients in the water (1000 ml).
- If necessary, adjust the pH so that after sterilization it is 7.3 ± 0.2 at 25°C.

[1] The following reagents and culture media have been found satisfactory for the purposes for which they are prescribed in the test. Other media may be used if they have similar nutritive and selective properties for the test micro-organisms.

- Divide 90 ml (180 ml) of the medium into flasks.
- Sterilize for at least 15 min in an autoclave set at 121°C.

3.3 BUTTERFIELD'S PHOSPHATE BUFFERED DILUTION WATER (PH 7.2)

Potassium dihydrogen phosphate	34.0 g

Stock solution: Transfer potassium dihydrogen phosphate to a 1000 ml volumetric flask, dissolve in 500 ml of purified water, adjust with sodium hydroxide 1N to a pH of 7.2 ± 0.2, add purified water to 1000 ml, and mix. Dispense in containers and sterilize. Store in refrigerator.

Phosphate buffer solution: Prepare a mixture of purified water and stock buffer solution (800:1 v/v, e.g. 1.25 ml stock solution in 1000 ml water) and sterilise.

3.4 PHOSPHATE BUFFER SOLUTION (PH 7.0)

Disodium hydrogen phosphate dodecahydrate ($Na_2HPO\ 12H_2O$)	9.0 g
Potassium dihydrogen phosphate (KH_2PO_4)	1.5 g

Use ready-to-use media or prepare by the following instructions:

- Dissolve the ingredients in the water (1000 ml).
- If necessary, adjust the pH so that after sterilization it is 7.0 ± 0.2 at 25°C.
- Divide 90 ml (180 ml) of the medium into flasks.
- Sterilize for at least 15 min in an autoclave set at 121 °C.

3.5 BUFFERED PEPTONE WATER

Peptone	10.0 g
Sodium chloride (NaCl)	5.0 g
Disodium hydrogen phosphate dodecahydrate ($Na_2HPO4\ 12\ H_2O$)	9.0 g
Potassium dihydrogen phosphate (KH_2PO_4)	1.5 g

Use ready-to-use media or prepare by the following instructions:

- Dissolve the ingredients in the water (1000 ml).
- If necessary, adjust the pH so that after sterilization it is 7.0 ± 0.2 at 25 °C.
- Divide 225 ml of the medium into flasks.
- Sterilize for at least 15 min in an autoclave set at 121 °C.

4. Apparatus and equipment

- Conventional microbiological laboratory equipment.
- 40±2°C water bath
- Autoclave

5. Procedure

Under aseptic conditions, prepare a 1:10 solution of not less than 10 g collagen peptides in specified diluent, and shake to disperse. Leave the collagen peptides to absorb the diluent for 30-60 min at room temperature, then place the flask in the 20-40°C water bath, occasionally shake gently to assure dissolution (maximum 1 hour) and obtain the test solution (S1).

2.3.1. Total Aerobic Microbial Count (TAMC)

(Pour-plate method)

1. Principle

Use of soybean casein digest agar media to enumerate in 3 to 5 days at 30-35°C the mesophilic micro-organisms (bacteria and some fungi) that may grow under aerobic condition by counting of the colony forming units (cfu) per 1 g of collagen peptides.

2. Reference(s)

European Pharmacopeia (current edition)
US Pharmacopeia (current edition)

3. Definition

For the purpose of this method "micro-organisms" are taken to mean the mesophilic bacteria, developing in the operating conditions described: if colonies of fungi are detected on this medium, they are counted as part of the TAMC.

4. Reagents and culture media[1]

4.1 DILUENT

See chapter 2.3.0. All described diluents can be used.

4.2 CASEIN SOYA BEAN DIGEST AGAR

Pancreatic digest of casein	15.0 g
Papaic digest of soya bean	5.0 g
Sodium chloride (NaCl)	5.0 g
Agar	15.0 g

Use ready-to-use media or prepare by the following instructions.

- Dissolve the ingredients in the water (1000 ml).
- If necessary, adjust the pH so that after sterilization it is 7.3 ± 0.2 at 25°C.
- Sterilize for at least 15 min in an autoclave set at 121°C.
- Before use, melt the media e.g. in a boiling water bath, then cool to not more than 45°C.

5. Apparatus and equipment

[1] The following reagents and culture media have been found satisfactory for the purposes for which they are prescribed in the test. Other media may be used if they have similar nutritive and selective properties for the test micro-organisms.

- Conventional microbiological laboratory equipment.
- Autoclave
- Boiling water bath or microwave oven
- 45°C regulated water bath or incubator
- 30-35 °C regulated incubator

6. Procedure

6.1. PREPARATION OF 1/10 TEST SOLUTION (S1)

Prepare a 10 % collagen peptides solution as described in chapter 2.3.0.

6.2. INOCULATION

- Transfer 1 ml of (S1) in each of two sterile Petri dishes
- add to each dish approx. 15-20 ml of casein soya bean digest agar being at not more than 45°C.
- Mix gently and leave to cool on a flat surface.

6.3. INCUBATION

Invert the dishes and incubate at 30-35°C for 3 to 5 days.

7. Result

- After incubation, count the colonies in each plate. For correct enumeration, the number of colonies per plate should comprise between 30 and 300.
- Take the arithmetical average of the counts, and multiply by 10 (inverse of the dilution factor of (S1) solution).
- Report the enumeration as colony forming units (cfu) per g of collagen peptides.

2.3.2. E. coli

1. Principle

Detection of the presence or absence of E. coli in 10 g of collagen peptides, after pre-enrichment, selective enrichment, isolation and biochemical identification.

2. Reference(s)

European Pharmacopeia (current edition)
US Pharmacopeia (current edition)

3. Definition

For the purpose of this method 'E. coli' means the micro-organisms which develop at 42 – 44°C in the selective enrichment medium (after pre-enrichment at 30 – 35°C) and forming characteristic brick red colonies on MacConkey agar.

4. Reagents and culture media[1]

4.1 Pre- enrichment medium: Casein soya bean digest broth.
See chapter 2.3.0. Casein soya bean digest broth.

4.2 Selective enrichment medium: MacConkey agar

Pancreatic digest of collagen peptides	20.0 g
Lactose Monohydrate	10.0 g
Dehydrated Ox Bile	5.0 g
Bromocresol Purple	10 mg

Use ready-to-use media or prepare by the following instructions:

- Dissolve the ingredients in the water (1000 ml).
- If necessary, adjust the pH so that after sterilization it is 7.3 ± 0.2 at 25°C.
- Sterilize in an autoclave using a validated cycle.

4.3 Isolation medium: MacConkey agar

Pancreatic digest of collagen peptides	17.0 g
Peptones (meat and casein)	3.0 g
Lactose Monohydrate	10.0 g
Sodium Chloride	5.0 g

[1] The following reagents and culture media have been found satisfactory for the purposes for which they are prescribed in the test. Other media may be used if they have similar nutritive and selective properties for the test micro-organisms.

Bile Salts	1.5 g
Agar	13.5 g
Neutral Red	30 mg
Crystal Violet	1 mg

Use ready-to-use media or prepare by the following instructions:

- Dissolve the ingredients in the water (1000 ml).
- If necessary, adjust the pH so that after sterilization it is 7.1 ± 0.2 at 25°C.
- Boil for 1 minute with constant shaking
- Sterilize in an autoclave using a validated cycle.
- Dispense 15 ml in petri dishes

5. Apparatus and equipment

- Conventional microbiological laboratory equipment.
- Autoclave
- 30-35°C regulated incubator
- 42-44°C regulated incubator

6. Procedure

6.1. Pre-enrichment

Prepare a 10% collagen peptides solution in Casein soya bean digest broth as described in chapter 2.3.0. starting from 10 g collagen peptides.
Incubate at 30 – 35°C for 18 to 24 hours..

6.2. Selective enrichment

Shake the container and transfer 1 ml to 100 ml of MacConkey Broth.
Incubate at 42 – 44°C for 24 to 48 hours.

6.3. Isolation

Subculture on a plate of MacConkey agar at 30 – 35°C for 18 – 72 hours.

7. Result

After incubation, examine for the presence or absence of characteristic colonies: growth of brick red gram negative colonies, maybe surrounded by a zone of precipitated bile, indicates the possible presence of E. coli. Proceed with further identification by transferring the suspect colonies on other suitable selective media or using biochemical test to confirm the presence of E. coli.

If characteristic colonies are confirmed as e. coli, the result is given in the form: **Presence of E. coli in 10 g of collagen peptides**

If there are no characteristic colonies present or their confirmation was negative, the result is given in the form:

Absence of E. coli in 10 g of collagen peptides

2.3.3. Sulphite-Reducing Anaerobic Spores

(Anaerobic colony count technique at 37°C)

1. Principle

Use of iron sulphite medium to enumerate in 1 g of collagen peptides the spores of anaerobic sulphite-reducing micro-organisms by detection, after 24-48 hours at 37 ± 1°C, of black surrounded colonies.
Most clostridia possess sulphite reductase and are able to reduce sulphite to sulphide. The black colour of the colonies and the surrounding zone is due to the formation of iron(II) sulphide as a result of the reaction between sulphide ions and trivalent iron [Fe(III)] present in the medium.

2. Reference(s)

NEN-ISO 15213 – Microbiology of food and animal feeding stuffs – Horizontal method for the enumeration of sulphite-reducing bacteria growing under anaerobic conditions.

3. Definition

For the purpose of this method "sulphite-reducing anaerobic spores" are taken to mean all spores giving black colonies in the operating conditions described.

4. Reagents and culture media

4.1 DILUENT

See chapter 2.3.0. All described diluents can be used.

4.2 IRON SULPHITE AGAR MODIFIED (TSC BASE)

Enzymatic digest of casein	15.0 g
Pancreatic digest of soya	5.0 g
Yeast extract	5.0 g
Disodium di-sulphite ($Na_2S_2O_5$)	1.0 g
Iron(III) ammonium citrate	1.0 g
Agar	9 – 18 g (1)

(1) Depending on the gel strength of the agar

Use ready-to-use media or prepare by the following instructions:

- Dissolve the ingredients in the water (1000 ml) by heating.
- If necessary, adjust the pH so that after sterilization it is 7.6 ± 0.2 at 25°C.
- Divide 20-25 ml of the medium into tubes.
- Sterilize for 15 min in an autoclave set at 121°C.

Before use, liquefy the medium in boiling water meanwhile the medium is de-aerated. Keep warm till use.

5. Apparatus and equipment

- Conventional microbiological laboratory equipment.
- Boiling water bath or microwave oven
- 40 ± 2 °C water bath
- 80 ± 1 °C water bath
- Regulated incubator 37 ± 1 °C

6. Procedure

6.1. PREPARATION OF THE 1/10 TEST SOLUTION (S1)

Under aseptic conditions, weigh out 10 g (20 g) of collagen peptides, transfer to 90 ml (180 ml) of diluent, and swirl to disperse. Leave the collagen peptides to absorb the diluent for 30-60 min at room temperature, then place the flask in the 40°C water bath, occasionally shake gently to assure dissolution (maximum 1 hour) and obtain the test solution (S1).

6.2. TREATMENT OF THE TEST SOLUTION

To activate spores and destroy vegetative cells of spore-forming bacteria and/or non-spore-forming bacteria heat treatment is necessary.

This is preferably done on the initial test solution, but validation tests showed no difference between results when pasteurisation was done after inoculation.

Make sure the temperature of the pasteurised solution is 10 min at 80° C. The total residence time is depending on the total volume of the pasteurised solution. Validate the total residence time for the used sample volume and tube diameter. Collagen peptides surface must be completely below the water surface.

After pasteurisation, cool rapidly in 40°C water bath.

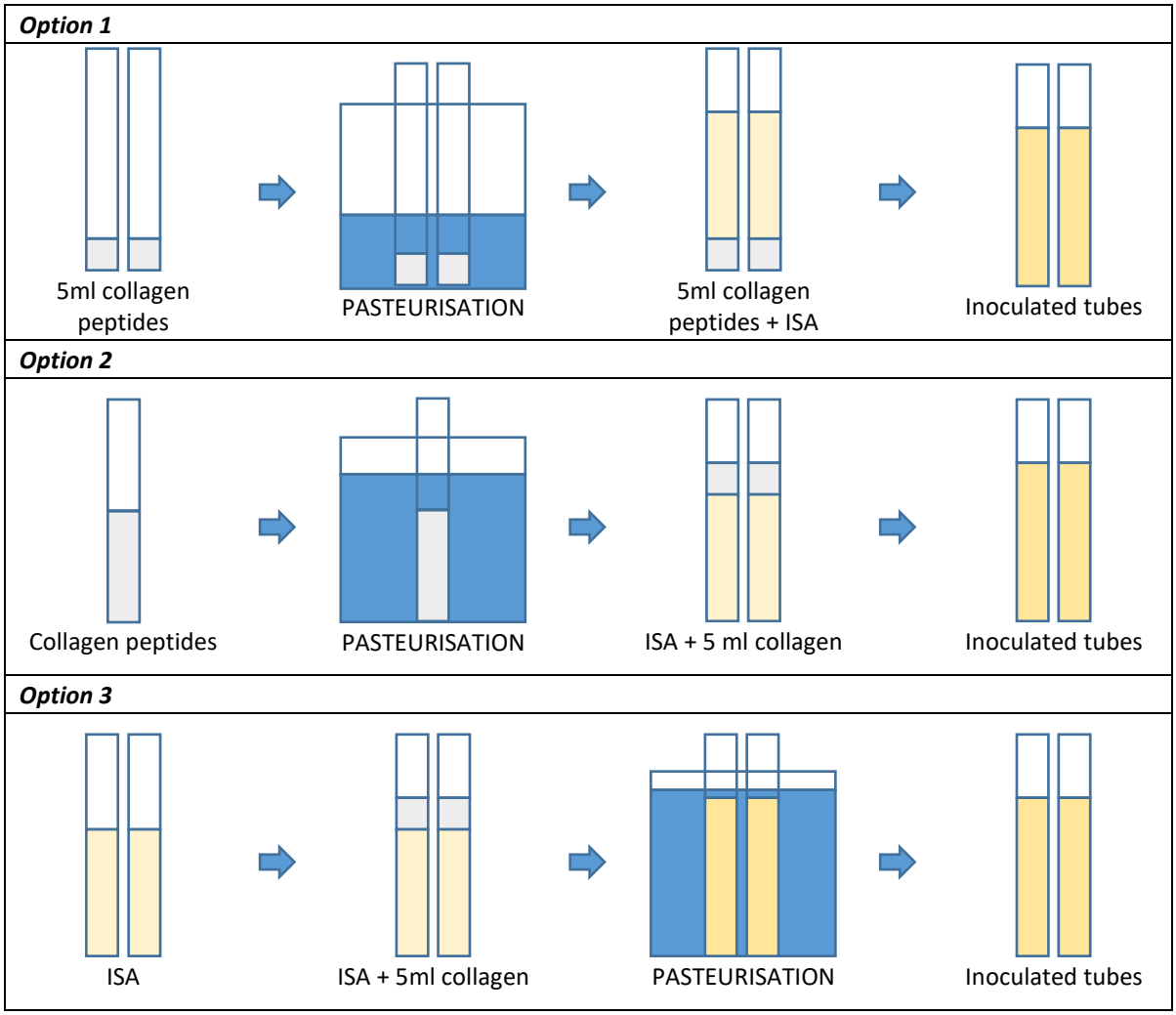
6.3. INOCULATION

Two times 5 ml of the (pasteurised) test solution (6.2) and two times 20-25 ml modified iron sulphite agar are brought together in tubes.

As follows, assure medium and test solution are mixed well:
- After pipetting collagen peptides test solution into modified iron sulphite agar, use circular movements to avoid formation of air bubbles
- Or, pour iron sulphite medium to the test solution, this immediately guarantees a good mixing without formation of air bubbles.

If the collagen peptides solution was not pasteurised before inoculation, it still can be pasteurized after inoculation. The same rules for time and temperature are applicable. Make sure the temperature of the pasteurised solution is 10 min at 80°C. The total residence time is depending on the total volume of the pasteurised solution. Validate the total residence time for the used sample volume and tube diameter. Surface must be completely below the water surface.

A schematic overview is given in the next table:



6.4. INCUBATION

After solidification, incubate the 2 tubes for 24-48 h at 37 ± 1°C.
In case of tubes, incubation in anaerobic jars is not necessary.

7. Result

Count the black halo surrounded colonies which are present in the 2 tubes and totalise.
Report the enumeration of sulphite-reducing anaerobic micro-organisms spores in 1g of collagen peptides.

NOTE 1: With some colonies the black halo may invade the culture medium. To avoid complication, record the results daily during the incubation period after 24 & 48 hours.

NOTE 2: Diffuse, unspecific blackening of the medium may occur. The growth of bacteria which only produce hydrogen (not H2S), may also reduce the sulphite present and lead to a general blackening of the medium.

2.3.4. Salmonella

1. Principle

The detection of salmonella includes the four successive classical stages:
Pre-enrichment, double enrichment, isolation, and biochemical-serological identification, in compliance with the more recent techniques.

2. Reference(s)

ISO 6579:2002: Microbiology of food and animal feeding stuffs – Horizontal method for the detection of Salmonella spp. and Technical corrigendum 1 to ISO 6579:2002 dated 2004-04-01

3. Definition

For the purpose of this method "Salmonella detection" means the determination (on a definite quantity) of the presence/absence of micro-organisms forming typical colonies on selective solid media and having the biochemical and serological characteristics described.

4. Reagents and culture media[1]

4.1 PRE-ENRICHMENT MEDIUM - BUFFERED PEPTONE WATER
See chapter 2.3.0. buffered peptone water.

Two liquid selective media are used: Rappaport-Vassiliadis soya peptone broth and Muller Kauffmann tetrathionate novobiocine broth.

4.2 FIRST SELECTIVE ENRICHMENT MEDIA: RAPPAPORT-VASSILIADIS SOYA PEPTONE BROTH
Use ready-to-use media or prepare by the following instructions:

Base medium (Solution A):

Soya peptone	5.0	g
Sodium chloride (NaCl)	8.0	g
Potassium dihydrogen phosphate (KH_2PO_4)	1.4	g
Dipotassium monohydrogen phosphate (K_2HPO_4)	0.2	g

Dissolve in 1000 ml of distilled water

Magnesium chloride solution (Solution B):

[1] The following reagents and culture media have been found satisfactory for the purposes for which they are prescribed in the test. Other media may be used if they have similar nutritive and selective properties for the test micro-organisms.

Magnesium chloride ($MgCl_2$, $6H_20$) 400.0 g

Dissolve in 1000 ml of distilled water

This solution, hold in a brown flask, is stable for 2 years max.

Malachite green solution (Solution C):

Malachite green 0.4 g

Dissolve in 100 ml of distilled water:

This solution, hold in a brown flask, is stable for 8 months max.

Final solution: Rappaport-Vassiliadis soya peptone broth

- Add to 1000 ml of solution A, 100 ml of solution B and 10 ml of solution C.
- If necessary, adjust the pH so that the final value after sterilisation will be 5.2 ± 0.2 at 25°C
- Dispense 10 ml in tubes
- Sterilise for 15 min in autoclave set at 121°C,

Store for 1 month maximum at 3 ± 2°C.

4.3 SECOND SELECTIVE ENRICHMENT MEDIA: MULLER KAUFFMANN TETRATHIONATE NOVOBIOCINE BROTH

Use ready-to-use media or prepare by the following instructions:

Base medium (Solution A):

Meat extract	4.3 g
Enzymatic digest of casein	8.6 g
Sodium chloride (NaCl)	2.6 g
Calcium carbonate ($CaCO_3$)	38.7 g
Sodium thiosulphate pentahydrate ($Na_2S_2O_3$, $5H_20$)	47.8 g
Ox bile	4.78 g
Brilliant green	9.6 mg

Dissolve in 1000 ml of distilled water:
Suspend the powders in water and bring to boil for 5 minutes.
Cool below 45°C and adjust the pH to 8.0 ± 0.2 at 25°C.

This solution hold at 3 ± 2°C is stable for 4 weeks.

Iodine (Solution B):

Iodine	20 g
Potassium iodide	25 g

- Dissolve in 100 ml sterile distilled water in a sterile flask
- Dissolve the potassium iodide and gently warm the solution to completely dissolve it
- Make up the volume to 100 ml with distilled water
- Do not sterilise.

Novobiocine (Solution C):

Novobiocine monosodium salt	0.04 g

- Dissolve in 5 ml sterile distilled water in a sterile flask
- Add the Novobiocine monosodium salt solution to the water
- Sterilise by filtration.

Final solution: Muller-Kauffmann tetrathionate novobiocin broth

- Add 5 ml Novobiocine solution to 1000 ml base medium.
- Mix and then add 20 ml of Iodine solution.
- Mix well and fill out into 10 ml tubes.

Use this medium the day of preparation.

4.4 FIRST ISOLATION MEDIA: XYLOSE-LYSINE-DESOXYCHOLATE AGAR (XLD AGAR)

Yeast extract	3.0	g
Sodium chloride (NaCl)	5.0	g
Xylose	3.75	g
Lactose monohydrate	7.5	g
Sucrose	7.5	g
L-Lysine HCl	5.0	g
Sodium thiosulphate	6.8	g
Ferric ammonium citrate	0.8	g
Sodium desoxycholate	1.0	g
Phenol red	0.08	g
Agar	13.5	g

Use ready-to-use media or prepare by the following instructions:

- Dissolve in 1000 ml of distilled water
- Adjust the pH so that the final value will be 7.4 ± 0.2 at 25°C
- Boil gently to dissolve the components
- Do not sterilise
- Cool to 50 °C.
- Dispense 15 ml in Petri dishes

- Cool to room temperature and dry the plates in the oven before use
- Do not heat in an autoclave.

4.5 SECOND ISOLATION MEDIUM

At discretion of the laboratory.

4.6 CONFIRMATION REAGENTS

Biochemical confirmation: miniaturised identification kits (i.e. API-System 20 E).

Serological confirmation: commercially available "poly O" and "Vi" anti-sera.

5. Apparatus and equipment

- Conventional microbiological laboratory equipment.
- Autoclave
- Boiling water bath
- 40°C water bath
- 37 ± 1°C regulated incubator
- 41.5 ± 1°C regulated incubator (or regulated water bath)

6. Procedure

6.1. Pre-enrichment

Prepare a 10 % collagen peptides solution in Buffered peptone water as described in chapter 2.3.0. starting from 25 g collagen peptides.

Incubate at 37 ± 1°C for at least 16 hours but no more than 20 hours.

6.2. Selective enrichment

Seed 10 ml of Rappaport-Vassiliadis soya broth with 0.1 ml of the culture (6.1), and incubate at 41.5 ± 1°C for 24 ± 3 hours.

Seed 10 ml of Muller Kauffmann Tetrathionate Novobiocine broth with 1 ml of the culture (6.1), and incubate at 37 ± 1°C for 24 ± 3 hours.

6.3. ISOLATION

After 24 h, transfer a loopful of the two tubes (6.2) and streak on the surface of the XLD agar and of the optional agar. Invert the plates and incubate at 37 ± 1°C for 24 ± 3 hours.

7. Result

After incubation, examine the plates for the presence of characteristic colonies (depending of the nature of the isolation medium).
Any colony having a characteristic (or doubtful) appearance should be submitted to confirmatory analysis, by means of purification by sub-culture, biochemical identification, and serological confirmation, in accordance with the procedure described in the standard ISO 6579:2002 and the specific instructions of the commercial products.

Depending on the conclusion of the analysis, the result is given in the form:

Presence/absence of salmonella in 25 g of collagen peptides.

GME
GELATINE MANUFACTURERS OF EUROPE

SALMONELLA TESTING ACCORDING TO THE FOOD HYGIENE REGULATION EC/853/2004 AND EC/2073/2005

EU Regulation 2073/2005 requests

Testing to be performed 5 times 25 g on the gelatine placed on the market during their shelf-life.

The number of sample units of the sampling plans may be reduced if the food business operator can demonstrate by historical documentation that he has effective HACCP-based procedures.

GME position

1. A batch is defined as a daily production of finished products.
2. The testing will be performed on a mixed sample of all final blends.
3. The sample will be taken proportionally. See the following example:

10 ton blend	= 100g
5 ton blend	= 50g
8 ton blend	= 80g
4 ton blend	= 40g
Total	**= 270g**

For the testing, 5 times 25g will be taken from the 270g mixed sample and analysed for Salmonella.

GME members will ensure the traceability of the composition of these blends versus the daily production.

Validation period: until 31 December 2022.

GME
Rue Belliard 40 -1040 Brussels Belgium
Tel. +32.2.436.93.00 lje@cefic.be www.gelatine.org

A sector group of Cefic
European Chemical Industry Council - Cefic aisbl
EU Transparency Register n° 64879142321-90

2.4. PHYSICAL PROPERTIES

(Physical properties are of importance for fitness for use. Other methods may be used if officially recognized in other standards. In case of disagreement, the reference methods are the ones described in this document).

2.4.1. Viscosity

1. Principle

The viscosity of a 20 % solution of collagen peptides is determined at 25°C by measuring the flow time of 100 ml of the solution through a standard pipette. The measurement of the viscosity using other sample concentrations or/and temperatures is after adaption of this method also possible.

2. Definition

The viscosity of a collagen peptides is defined as its dynamic viscosity, expressed in mPa.s when tested as a 20% solution in water at 25°C.

3. References

GME	Gelatine Manufacturere of Europe, Standardised methods for the testing of edible gelatine
GMIA	Gelatin Manufacturers Institute of America, Standard Methods for the Sampling and Testing of Gelatins, 1986
JIS	Japanese Industrial Standard, K 6503-1970
PAGI	Methods for Testing Photographic Gelatine; Photographic and Gelatine Industries, Japan; Seventh Edition, 1992
BSI	British Standards Institution, BS 757:1975 (U-tube method)

4. Calibrations oils

Two standard (reference) viscosity oils calibrated at 25° C, having viscosities within the approximate range 2-10 *cSt (=mm^2/s).* One oil should have a viscosity at least twice that of the other oil.

5. Apparatus

5.1. Pipette (see Annex 1):
100 ml pipette with a precision capillary outlet and an upper and lower mark on the glass.

5.2. Thermostatic bath for the pipette with a thermostatic device for stirring and heating (25°C ± 0.1).

5.3. Precision thermometer (mercury or electronic thermometer), graduated in 0.1°C, with a long slim stem for measuring temperature inside the pipette.

5.4. Stop watch, accurate to 0.1 sec.

5.5. Thermostatic water bath for sample preparation, held at 40°C ± 2.

5.6. Balance with a sensitivity of 0.01 g

6. Procedure

6.1. Weigh 100 g (± 0.1) of water into a 250 ml bottle, add 25 g (± 0.01), of collagen peptides, stir, so that all is moistened, cover the bottles with a rubber stopper or a watch glass.

6.2. Dissolve the hydrolysed collagen completely at room temperature. To achieve a homogenous solution, the bottles should be shaken (when covered with stoppers) or stirred gently with a glass rod (when covered with watch glasses) occasionally. Take care not to build up too much froth. Dissolve the samples with stirring gently with a glass rod (when covered with watch glasses) or shaking (when covered with stoppers). The collagen peptides has to be dissolved completely. Take care not to build up too much froth. If necessary, the preparation of the solution can be accelerated by heating the sample solution up to 40°C.

6.3. Before starting the measurement, the temperature of the sample solution should achieve about 26.5°C.

6.4. Sufficient solution is poured into the pipette to bring its level about 1 cm above the upper mark while a finger closes the capillary end. The bottle with the residual solution is placed directly under the exit tube.

The temperature of the solution in the pipette can be checked with the thermometer and when it has reached 25°C the measurement can be started by removing the finger and determining the flow time between upper mark and lower mark.

6.5. Read and record the time required for the 100 ml of solution to pass through the capillary tube of the pipette.

6.6. After each determination and also before use the pipette is washed out with about 25 ml of water heated to about 28°C and allowed to drain thoroughly.

7. Results

The viscosity of any sample (V) with the efflux time t may be calculated from the equation:

$$V = (A \times t - B / t) \times d$$

where V = dynamic viscosity in mPa.s

For collagen peptides at 20 %, 25°C d = 1.060

8. Calibration

8.1. Pipettes can be calibrated using two standard oils of different viscosity (determination of constants A and B).

8.2. The pipette must be thoroughly cleaned before use and dried with reagent grade acetone.

8.3. Preheat each oil in a constant temperature bath set at 27-28°C by stirring and transfer to the pipette, holding a finger over the pipette outlet until the oil temperature is exactly 25°C as read on the immersed thermometer.

8.4. Measure the efflux time of the oil between the upper and lower graduation of the pipette using the stopwatch. Repeat the measurement at least three times with each oil.

8.5. Clean the pipette thoroughly between different oils using suitable organic solvents for removing the oil, wash out the solvent with acetone and dry.

8.6. Calculation of the constants A and B:
(t = efflux time in sec; d = oil density at 25°C; A and B = pipette constants)

$$V = (A\,t - B / t) \times d$$

$$B = \frac{t1 \times t2\,(V2 \times t1 - V1 \times t2)}{t2^2 - t1^2}$$

$$A = \frac{V1 + B / t1}{t1} = \frac{V2 + B / t2}{t2}$$

V1 = kinematic viscosity of lower viscosity oil in cSt
V2 = kinematic viscosity of higher viscosity oil in cSt
t1 = average efflux time of lower viscosity oil in sec
t2 = average efflux time of higher viscosity oil in sec

9. Remarks

9.1. ***Standard collagen peptides:***

To ensure that the equipment and procedure is satisfactory it is recommended that standard collagen peptides of established viscosity are prepared and tested regularly as reference collagen peptides.

The standard collagen peptides must be stored under suitable conditions so that the moisture does not change with the time.

9.2. Correction of the results regarding to the moisture:

Moisture content of the samples influences the viscosity result for a 20% solution, so that with all viscosity results also the moisture content should be indicated.

The viscosity value that corresponds to a certain moisture content can be calculated approximately using a graph (annex 3) or the following formula:

$$V2 = V1 + V1 \times 0.02 \times (M1 - M2)$$

(V1 = viscosity at moisture M1, V2 = viscosity at moisture M2)

9.3. Test report:

In addition to the viscosity value and the moisture also details of the method used should be given, if any parameters are different form this procedure.

9.4. Other viscometers:

If other viscometers are used, e.g. U-tube viscometer (BSI), Ostwald viscometer or Ubbelohde viscometer, this must be indicated in the results.

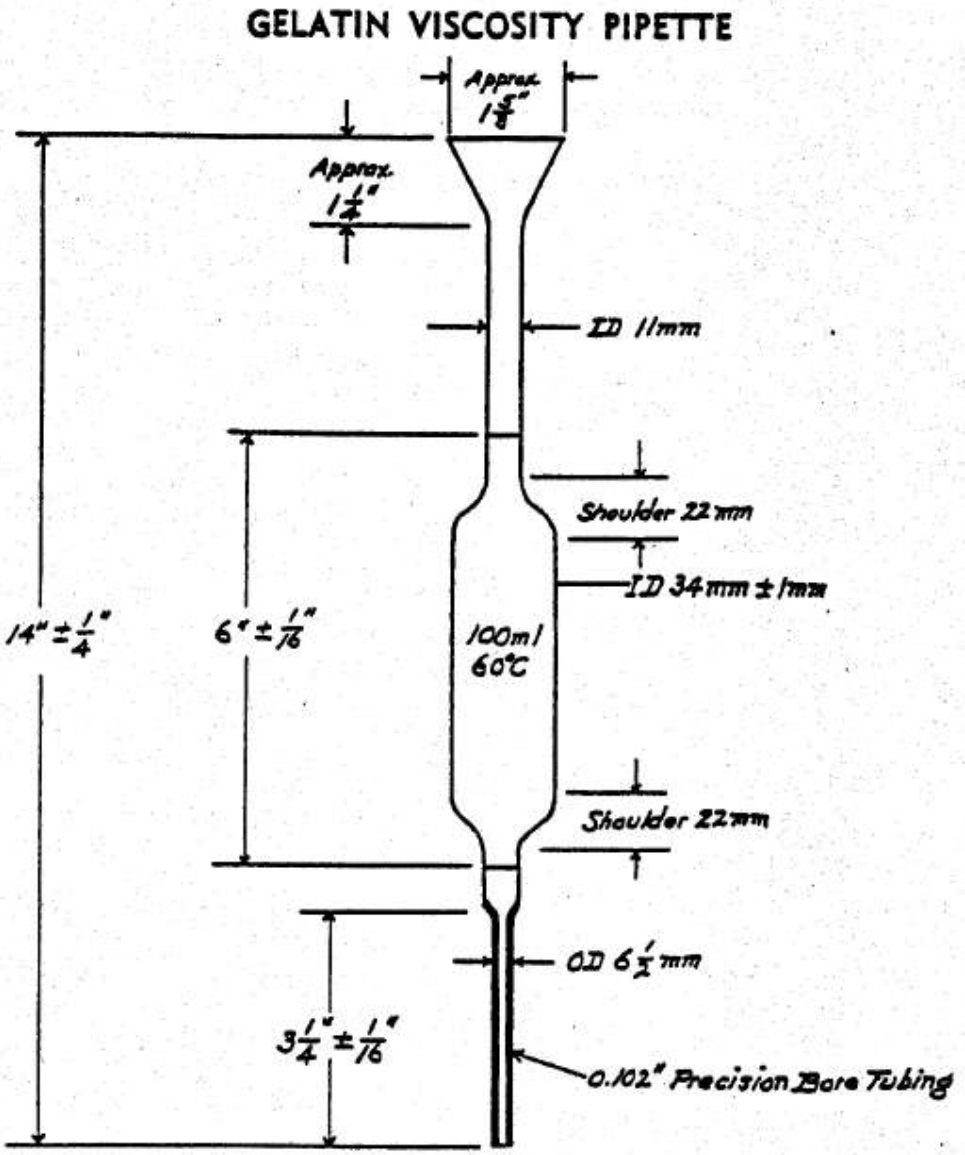
9.5. Cleaning of pipette:

The pipette must be thoroughly cleaned before use, preferably by washing with soap and hot water, and rinsed with distilled water. When not in use it is best to keep the pipette filled with distilled water.
Optional: Specific periodic cleaning of glass pipettes can be done with strong acid like Chromosulfuric acid.

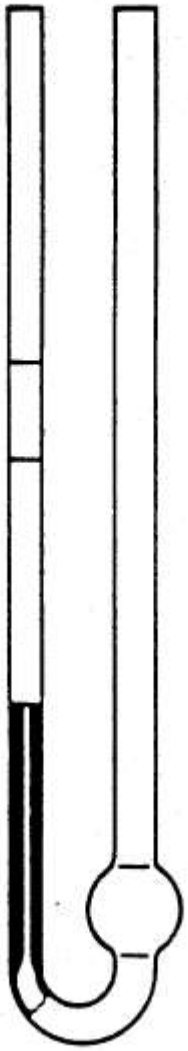
10. Annexes

Annex 1: Viscosity pipette
Annex 2: U-tube viscometer (BSI)

Annex 1


GELATIN VISCOSITY PIPETTE
Approx. 1 5/8"
Approx. 1 1/4"
ID 11mm
Shoulder 22mm
ID 34mm ± 1mm
14" ± 1/4"
6" ± 1/16"
100ml 60°C
Shoulder 22mm
OD 6 1/2 mm
3 1/4" ± 1/16"
0.102" Precision Bore Tubing

Annex 2



2.4.2. Colour

Note

The colour of a collagen peptides solution can be important for certain applications and this characteristic is frequently assessed for collagen peptides. However, unlike the testing of viscosity, there is no universally accepted standard procedure for measuring the colour of a collagen peptides solution. Visual assessment of collagen peptides solutions (normally at 20% concentration) against standard collagen peptides is common practice.

Turbidity of the collagen peptides solution can influence the perceived colour and interfere with instrumental methods for colour measurement. Provided the turbidity of collagen peptides is not too high the spectrophotometric method described in this standard may be used. Other suitable methods may be adopted.

1. Principle

The absorbance at 450 nm of a 20 % collagen peptides solution is measured in 1 cm cuvettes against water.

2. Definition

Results of spectrophotometric measurements can be expressed as absorbance (E) or transmission (T). Conversion of results is possible using the following formulas:

$$E = \log 1 / T \qquad T = 1 / 10^{E}$$

3. Apparatus

3.1. Spectrophotometer

3.2. Cuvettes 1 cm (glass or plastic without optical defects as scratches etc)

3.3. Thermostatic water bath, held at 25°C ± 5 (sample preparation)

3.4 Balance with a sensitivity of 0.01 g

4. Procedure

4.1. Weigh 25 g (± 0.01) collagen peptides into a 250 ml bottle, add 100 ml (± 0.2) water, stir so that all the collagen peptides is moistened cover the bottles with a rubber stopper or a watch glass and allow the sample to stand at room temperature (25°C ± 5) for 1 - 4 h.

(The solution prepared for viscosity measurement could also be used).

4.2. When the sample is completely dissolved about 3 ml of the solution is transferred to a cuvette.

4.3. The absorbance or transmission at 450 nm is measured at room temperature against deionised water.

5. Results

The absorbance or transmission (%) can be read from the spectrophotometer.

7. Remarks

7.1. Standard collagen peptides:

To ensure that the equipment and procedure is satisfactory it is recommended that standard collagen peptides of established absorbance are prepared and tested regularly as reference collagen peptides

The standards should not exceed ± 2 % from the expected value.

The standard collagen peptides must be stored under suitable conditions so that the moisture does not change with the time.

7.2. Moisture:

Moisture content of the samples influences the colour measurement, so that with all results also the moisture content should be indicated.

7.3. Test report:

In addition to the absorbance value and the moisture also details of the method used should be given, if any parameters are different form this procedure.

7.4. Cuvettes:

If plastic cuvettes are used it has a favourable effect on the result using three cuvettes for each sample instead of one and calculating the average of the three results.

7.5. The spectrophotometer should be switched on about 15 min. before measurement

7.6. The measurement of the colour using other sample concentrations is after adaption of this method also possible.

2.4.3. Clarity

Note

The clarity (i.e. inverse of turbidity) of a collagen peptides solution can be important for certain applications and this characteristic is frequently assessed for collagen peptides. However, unlike the testing of Bloom or viscosity, there is no universally accepted standard procedure for measuring the clarity of a collagen peptides solution.

Visual assessment of collagen peptides solutions (normally at 20 % concentration) against standard collagen peptides is common practice. Turbidity measurement may also be performed using a nephelometer or spectrophotometer. Any suitable method may be adopted but it is recommended that a correlation be established between the assigned turbidity values and NTU values based on formazine standard dispersions. This enables turbidity to be recorded in standard NTU units, if required.

A nephelometer method and a spectrophotometer method are described in this standard but other suitable methods may be adopted.

1. Principle

The clarity / turbidity of a 20 % collagen peptides solution is measured using a nephelometer. The result is expressed in NTU (Nephelometric Turbidity Units).

- or -

The absorbance at 620 nm of a 20 % collagen peptides solution is measured in 1 cm cuvettes against water using a spectrophotometer.

2. Definition

Results of spectrophotometric measurements can be expressed as absorbance (E) or transmission (T). Conversion of results is possible using the following formulas:

$$E = \log 1 / T \qquad T = 1 / 10^{E}$$

3. References

ISO 7027, EN 27027,1994; Water quality; Determination of turbidity.

4. Reagents and solutions

Formazine stock dispersion (400 NTU):

- 1.00 g of hydrazine sulphate is dissolved in deion. water and made up to 100 ml.
- 10.0 g of hexamethylenetetramine are dissolved in deion. water and made up to 100 ml.
- 5.0 ml of each dispersion are mixed, left to stand for about 24 h and made up to 100 ml with deion. water. This standard dispersion 400 NTU can be used for about 4 weeks (stored at room temperature and in the dark).

Other dilutions are also possible, e.g. 25 ml stock dispersion made up to 100 ml gives a standard dispersion 100 NTU. These diluted dispersions can be used for about 1 week.

5. Apparatus

- Nephelometer
- Spectrophotometer
- Glass tubes adapted to the nephelometer or cuvettes 1 cm (glass or plastic without optical defects as scratches etc)
- Balance with a sensitivity of 0.01 g

6. Procedure

6.1. Weigh 25 g (± 0.01) collagen peptides into a 250 ml bottle, add 100 ml (± 0.2) water, stir so that all the collagen peptides is moistened, cover the bottles with a rubber stopper or a watch glass and allow the sample to stand at room temperature (25°C ± 5) for 1 - 4h.

(The solution prepared for viscosity measurement could also be used).

6.2. When the sample is completely dissolved the solution is transferred to the glass tubes or the cuvettes.

6.3. The absorbance at 620 nm or turbidity using a nephelometer is measured at room temperature against deionized water.

7. Results

The clarity in NTU can be read from the nephelometer.

The absorbance / transmission (%) can be read from the spectrophotometer.

8. Remarks

8.1 Standardisation:

Standardisation can be done using formazine dispersions (dilutions of formazine stock dispersion) to give NTU values in the range 10 - 400, from which a calibration curve can be constructed.

8.2 Standard collagen peptides:

To ensure that the equipment and procedure is satisfactory it is recommended that standard collagen peptides of established turbidity / absorbance are prepared and tested daily as reference collagen peptides.

The standards should not exceed ± 2 % from the aim.

The standard collagen peptides must be stored under suitable conditions so that the moisture does not change with the time.

8.3. Moisture:

Moisture content of the samples influences the clarity / transmission measurement so that with all results also the moisture content should be indicated.

8.4. Test report:

In addition to the turbidity / absorbance value and the moisture also details of the method used should be given, if any parameters are different form this procedure.

8.5. The nephelometer / spectrophotometer should be switched on about 15 min. before measurement.

8.6. The measurement of the clarity using other sample concentrations is after adaption of this method also possible.

2.4.4. pH

1. Principle

The pH is a number, which represents conventionally the hydrogen ion concentration of an aqueous solution. The pH of a 20 % collagen solution is determined by potentiometry at a temperature of 25°C using a pH meter. The pH may also be determined on a 1 % solution at 55°C like described in the European Pharmacopoeia.

2. Reference(s)

- European Pharmacopoeia (current edition) – Gelatin Monograph
- GME, Gelatine Manufacturers of Europe, Standardised methods for the testing of edible gelatine
- GMIA, Gelatin Manufacturers Institute of America, Standard Methods for the Sampling and Testing of Gelatins, 1986; (1.5% solution at 25°C)
- PAGI, Methods for Testing Photographic Gelatin; Photographic and Gelatin Industries, Japan, Seventh Edition 1992; (5% solution at 35°C)

3. Reagents and solutions

Carbon dioxide free water i.e. distilled water or equivalent
pH 4 reference solution
pH 7 reference solution

4. Apparatus

Analytical balance
Glasswork
pH meter:

The measuring apparatus is a voltmeter with an input resistance of at least 100 times that of the electrodes used. The sensitivity must allow discrimination of at least 0.05 pH units (better 0.01) within a region of 0 - 14. The pH meter consists of a thin glass H^+-selective membrane, an internal reference electrode, an external reference electrode and a meter with control electronics and display. Commercial pH electrodes usually combine all electrodes into one unit that are then attached to the pH meter. An automatic temperature compensation is nowadays available as standard.

5. Calibration

Calibration should be done at the same temperature as the pH measurement.

The calibration is performed by using the 2 reference solutions at a pH of 7 (i.e. potassium dihydrogen phosphate 0.025 M + disodium hydrogen phosphate 0.025 M, pH 6.87 at 20-25°C) and a pH of 4 (i.e. Potassium hydrogen phthalate 0.05 M, pH 4.01 at 20-25°C).

Calibration is performed according the pH meter-instructions. Swirl well the solution to fully saturate the electrode with buffer and rinse well with distilled water or equivalent afterwards. Electrodes are regularly cleaned by using an enzyme solution.

6. Procedure

6.1 Weigh 25 g (± 0.01) collagen peptides into a 250 ml bottle, add 100 ml (± 0.2) water, stir so that all the collagen peptides is moistened, cover the bottles with a rubber stopper or a watch glass and allow the sample to stand at room temperature (25°C ± 5) for 1 - 4 hours.

(The solution prepared for viscosity or for clarity determination could also be used).

6.2 Determine the pH of the collagen peptides solution according the pH-meter instructions.
Swirl well the solution to fully saturate the electrode and rinse well with warm distilled water or equivalent afterwards.

7. Result

The pH result is determined with an accuracy of ± 0.1.

2.4.5. Conductivity

1. Principle

The conductivity of a 20% collagen peptides solution is determined with a conductivity meter at a temperature of 25.0°C ± 1.0 without temperature compensation.

The conductivity may also be determined on a 1% collagen peptides solution at 30°C ± 1.0 without temperature compensation like described in the European Pharmacopoeia.

2. Reference(s)

European Pharmacopoeia (current edition) – Gelatin Monograph

3. Apparatus

Analytical balance
Glasswork
Water bath at 30°C

4. Procedure

4.1. Weigh 1.00 ± 0.01 g of collagen peptides in a beaker, add 99.0 ml ± 0.1 deionised water, cover and swell at room temperature for around 1 h.

4.2 Place the beaker in a water bath at 30°C and let the solution cool down.

4.3 Determine the conductivity of the solution at 30.0°C ± 1.0 according to the conductivity meter's instructions without temperature compensation.

Stir the solution using the probe before measuring and rinse the probe with deionised water before and after usage.

5. Result

The conductivity is read directly from the conductivity meter in mS/cm or µS/cm.

6. Remarks

Use a conductivity cell that is appropriate to the range of the solution to be analysed.
Use a certified reference solution to calibrate the constant K of the cell.
A correlation curve can be used for the correlation between the conductivity at 1% and 30°C and the conductivity at 20% and 25°C.

2.5 SODIUM

Recommendations for Sodium testing:

1. Ion chromatographic determination of Sodium, Calcium and Magnesium in 0.5% aqueous gelatine solution. The cations are separated on a special cation exchange column with conductivity detection after chemical suppression.
   Column = Dionex CS12A.

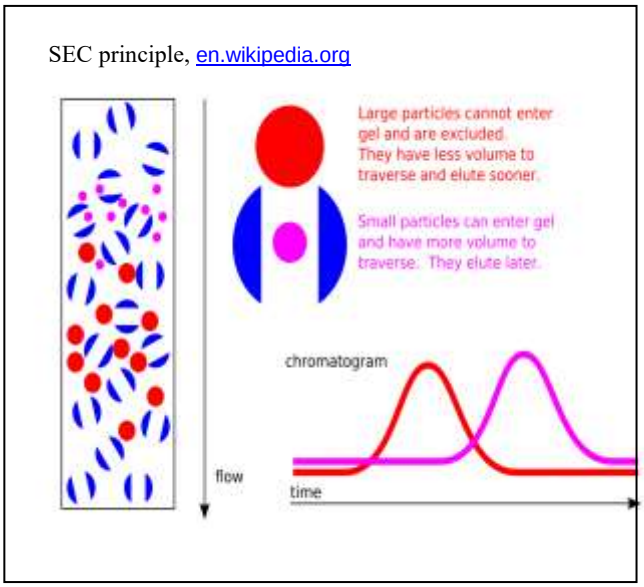
2. Atomic-emission spectrometry (AES); also known as Flame Emission Spectroscopy (VES).

3. Ion meter with a sodium selective electrode.

2.6. MOLECULAR WEIGHT DETERMINATION

1. Principle

The molecular weight profile is realized by high performance size exclusion chromatography (HPSEC).This chromatographic method separates molecules on the basis of their size, or in more technical terms, their hydrodynamic volume. The separation of molecules takes place in a chromatographic column (or set of columns) filled with beds of a rigid porous material characterized by a certain range of pore size.



At a constant flow rate, the mobile phase is pumped through the column. A defined volume of the dissolved sample is applied to the column via a switching valve (a sample of a dilute polymer solution is introduced into the eluent at the top of the column).
As the dissolved polymer molecules flow passes the porous beads, they can diffuse into the internal pore structure to an extent depending on their size and the pore distribution of the material. Molecules larger than the pore size cannot enter the pores and elute together as the first peak in the chromatogram. This condition is called total exclusion.
Molecules that can enter a partial fraction of the pores will have an average retention time in the particles that can depend on the molecules size and shape.

Molecules with different size show different total transit times through the columns
This portion of a chromatogram is called the selective permeation region. The molecules that are smaller than the pore size can enter all pores and have the longest retention time in the column and elute together as the last peak in the chromatogram. The concentration of molecules drawing out of the column is monitored by using UV-detector.
Molecular weights are determined by comparing the retention time (RT) or elution volume (Ve) of a sample with those given by a calibration curve.

2. Materials and method

- KH_2PO_4 anhydrous Pro analysis
- Na_2HPO_4 anhydrous Pro analysis
- NaH_2PO_4 anydrous Pro analysis (as alternative)
- NaCl rectapur
- Benzoic acid Normapur PA
- NaOH to adjust pH
- Collagen fragments (FILK CNBr-peptides of acid-soluble collagen, Ref. A001587, A001588 or A001406)
- GME broad calibration collagen standards
- Ultrapure water (resistivity ≥ 18MOhm)

2.2. Equipment
2.2.1. The size-exclusion chromatography (HPSEC):

- HPLC isocratic pump (e.g. Model 510. Waters or equivalent)
- Vacuum membrane degasser (e.g. TSP, SCM 1000 or equivalent) or helium to degas eluents
- Auto sampler with a cooler at 10°C if possible (e.g. AS 100. Thermo Separation Products or equivalent)
- Column oven (constant temperature between 25°C and 40°C)
- UV detector (214 nm (e.g. UV 100 Thermo Separation Products or equivalent)
- Computer with a Software for GPC acquisition (e.g. Cyrrus, PSS WinGPC, Breeze or equivalent)

2.2.2. Columns:

- Column :TSK G 2000 SW_{XL} 5µm (Ref. 0008540 Tosoh Bioscience GmbH)
- Guard column: TSK SW_{XL} Guardcol 7µm (Ref. 0008543, Tosoh Bioscience GmbH)
- The columns should be changed after 250 injections

2.2.3. Materials:

- Balance with sensitivity of 1 mg (Sartorius or equivalent)
- Precise balance with sensitivity of 0.1 mg (Mettler AE240 or equivalent)
- Automatic pipettes : 200-1000 µl; 20-200 µl
- Magnetic stirrer and small magnetic stirring bars.
- Filters : 0.1 µm and 0.22 µm (Gelman or Wathman or equivalent)
- pH meter

3. Method

All the solutions will be done with ultra-pure water previously filtered on the 0.2 µm filter and degassed.

3.1. Eluent preparation
Weighed 13.27 +/- 0.01 g KH_2PO_4, 0.445 +/- 0,005 g Na_2HPO_4 and 11.69 +/- 0.01 NaCl and transfer the salts into a 1L volumetric flask. Fill the bottle with ultra-pure water up to the mark.
Alternative buffer solutions should be used like :

- 200 mM NaH_2PO_4 or
- 200 mM KH_2PO_4

Use 0.1 M NaOH to adjust the pH to 5.3.
Filter the eluent on 0.2 µm filter and degasse.

3.2. Calibration standards
3.2.1. Narrow calibration
The columns are calibrated with standards with a well determined molecular weight covering the entire range that can be measured by the column and the range of samples to be analysed.
It is performed with FILK standards.

- FILK calibration solution (CNBr bovine) : Prepare solutions containing 10 mg/mL ultra-pure filtered and degassed water.

- Internal standard (correction of flow rate): Benzoic acid 0.01 % (shelf life of solution: 1 month).
- Mix 30 µl of calibration standard solution + 600 µl mobile phase + 30 µl benzoic acid solution

3.2.2. Broad calibration
A broad calibration is needed in order to increase the range of the narrow calibration obtained with the FILK standard.
It is performed with the following GME porcine collagen peptides broad calibration standards:

- 893614 standard (Mw = 1920 Da, Mn obtained with the narrow calibration)
- 899598 standard (Mw = 2850 Da, Mn obtained with the narrow calibration)
- 899154 standard (Mw = 3310 Da, Mn obtained with the narrow calibration)
- 891727 standard (Mw = 4560 Da, Mn obtained with the narrow calibration)
- 890482 standard (Mw = 7765 Da, Mn obtained with the narrow calibration)

The standards can be obtained at the Gelita Eberbach lab.

Prepare 1,0 % solutions of each GME broad calibration standard:

- Weigh 1 g +/- 0.1 g of broad calibration standard and add ultra-pure filtered and degassed water to 100 g
- Mix 30 µl from each standard solution with 30 µl benzoic acid 0.01 % and 600 µl of eluent
- Homogenize the solution

3.3. Sample preparation
The sample preparation for injection is as follows:

- Weigh 1 g +/- 0.1 g of collagen hydrolysate and add ultra-pure water to 100 g
- Mix 30 µl from this with 30 µl benzoic acid 0.01 % and 600 µl of eluent
- Homogenize the solution

A quality control standard (collagen hydrolysate) will be prepared following above procedure.

4. Analysis

The column must be conditioned before analysis with the eluent as long as necessary to achieve a stable base line.

4.1. HPLC conditions

- Flow : 0.5 ml/min
- Injection volume : 20 µl
- Runtime per injection: 40 min
- Detector : UV 214 nm

4.2. Injections
The following injections are done:

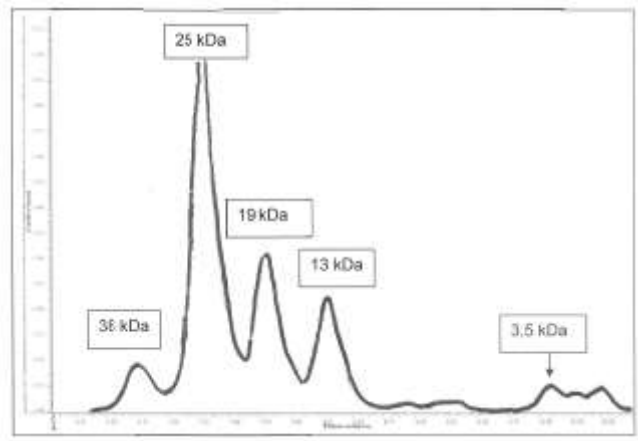
- Blank (solvent which is used for preparation of the sample solutions),
- FILK standard,
- GME broad calibration standards,
- Control standard,
- Samples to be analysed

After a complete run the entire system is washed with ultra-pure water with 0.02% or 0.05% NaN_3.

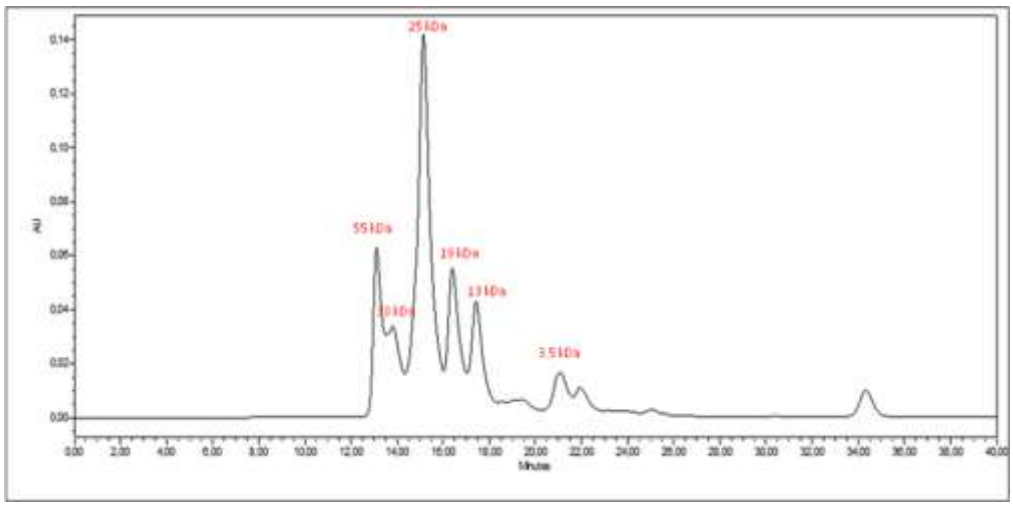
4.3. Calibrations

4.3.1. Narrow calibration

First, the narrow calibration is performed with the FILK standard, using the below assignment for the peaks:



In some cases, an additional peak at 55 kDa is visible. In this case, the below assignment should be used:



For the peaks indicated above, the elution volume is determined at the peak maximum, V_p and plotted against the corresponding molar mass ($\log M_p$).

The data points are then adapted via a polynom 3th order.

4.3.2. Broad calibration

The broad calibration is based on the measurement of standards (5 GME broad calibration standards), which are evaluated like samples. The elugrams of the various broad standards are used to modify the previously created narrow calibration. This is usually done by an iterated software routine. Then the data points are fitted with a third-order polynomial (order 3).

The broad calibration is performed with the 5 GME broad calibration standards using the Mw mentioned in 3.2.2., and, if needed by the software, the Mn obtained by the narrow calibration.

The calibration is tested every 12 to 15 injections of samples.

5. Calculation and Documentation

The relevant samples are measured and integrated after a flow correction using internal standard. It must be ensured that the broad calibration is used for the calculation of the average molar mass of the sample.

ANNEXES:

Annex 1: Regulation (EC) No 853/2004 of the European Parliament and of the Council

Link: http://ec.europa.eu/food/safety/biosafety/food_hygiene/legislation_en

Annex 2: Commission Regulation (EC) No 2073/2005 on microbiological criteria for foodstuff