

Commission Regulation (EC) No 152/2009 of 27 January
2009 laying down the methods of sampling and analysis
for the official control of feed (Text with EEA relevance)

- Article 1 Sampling for the official control of feed, in particular as...
Article 2 Preparation of samples for analysis and expression of results shall...
Article 3 Analysis for the official control of feed shall be carried...
Article 4 The energy value of compound poultry feed shall be calculated...
Article 5 The methods of analysis to control illegal presence of no...
Article 6 Directives 71/250/EEC, 71/393/EEC, 72/199/EEC, 73/46/EEC, 76/371/EEC, 76/372/EEC, 78/633/EEC, 81/715/EEC, 84/425/EEC,...
- Article 7 This Regulation shall enter into force on the twentieth day...
Signature

ANNEX I
METHODS OF SAMPLING

1. PURPOSE AND SCOPE
2. DEFINITIONS
3. GENERAL PROVISIONS
4. APPARATUS
 - 4.1. The sampling apparatus must be made of materials which cannot...
 - 4.2. Apparatus recommended for the sampling of solid feed
 - 4.2.1. Manual sampling
 - 4.2.1.1. Flat-bottomed shovel with vertical sides
 - 4.2.1.2. Sampling spear with a long split or compartments. The dimensions...
 - 4.2.2. Mechanical sampling
 - 4.2.3. Divider
5. QUANTITATIVE REQUIREMENTS AS REGARDS NUMBER OF INCREMENTAL SAMPLES
 - 5.1. Quantitative requirements as regards incremental samples in relation to the...
 - 5.1.1. Loose solid feed
 - 5.1.2. Loose liquid feed
 - 5.1.3. Packaged feed
 - 5.1.4. Feed blocks and mineral licks
 - 5.1.5. Roughages/forage
 - 5.2. Quantitative requirements as regards incremental samples in relation to the...
 - 5.3. Quantitative requirements as regards the incremental samples in the case...

6. QUANTITATIVE REQUIREMENTS AS REGARDS AGGREGATE SAMPLE
7. QUANTITATIVE REQUIREMENTS AS REGARDS FINAL SAMPLES
Final samples
8. METHOD OF SAMPLING FOR VERY LARGE LOTS OR LOTS STORED...
 - 8.1. General principles
 - 8.2. Large lots transported by ship
 - 8.2.1. Dynamic sampling of large lots transported by ship
 - 8.2.2. Sampling of lots transported by ship by static sampling
 - 8.3. Sampling of large lots stored in warehouses
 - 8.4. Sampling of storage facilities (silos)
 - 8.4.1. Sampling of silos (easily) accessible from above
 - 8.4.2. Sampling of silos not accessible from above (closed silos)
 - 8.4.2.1. Silos not accessible from above (closed silos) with size >...
 - 8.4.2.2. Silos not accessible from above (closed silos) with size <...
 - 8.5. Sampling of loose feed in large closed containers
9. INSTRUCTIONS FOR TAKING, PREPARING AND PACKAGING THE SAMPLES
 - 9.1. General
 - 9.2. Incremental samples
 - 9.2.1. Loose feed
 - 9.2.2. Packaged feed
 - 9.2.3. Homogeneous or homogenisable liquid or semi-liquid feed
 - 9.2.4. Non-homogenisable, liquid or semi-liquid feed
 - 9.2.5. Feed blocks and mineral licks
 - 9.3. Preparation of aggregate samples
 - 9.4. Preparation of final samples
 - 9.5. Packaging of samples
 - 9.6. Sending of samples to the laboratory
10. SAMPLING RECORD

ANNEX II

GENERAL PROVISIONS ON METHODS OF ANALYSIS FOR FEED

- A. PREPARATION OF SAMPLES FOR ANALYSIS
 1. Purpose
 2. Precautions to be taken
 3. Procedure
 - 3.1. General procedure
 - 3.1.1. Feed which can be ground as such
 - 3.1.2. Feed which can be ground after drying
 - 3.1.3. Liquid or semi-liquid feed
 - 3.1.4. Other feed
 - 3.2. Specific procedure in case of examination by visual inspection or...
 4. Storage of samples
- B. PROVISIONS RELATING TO REAGENTS AND APPARATUS USED IN METHODS OF...
 1. Unless otherwise specified in the methods of analysis, all analytical...

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2. Any operation involving preparation of solutions, dilution, rinsing or washing,...
 3. In view of the equipment normally found in control laboratories,...
- C. APPLICATION OF METHODS OF ANALYSIS AND EXPRESSION OF THE RESULTS...
1. Extraction procedure
 2. Clean-up procedure
 3. Number of determinations
 4. Reporting of the method of analysis used
 5. Reporting of the analytical result
 6. Measurement uncertainty and recovery rate in case of analysis of...

ANNEX III

METHODS OF ANALYSIS TO CONTROL THE COMPOSITION OF FEED MATERIALS AND COMPOUND FEED

- A. DETERMINATION OF MOISTURE
1. Purpose and Scope
 2. Principle
 3. Apparatus
 - 3.1. Crusher of non-moisture-absorbing material which is easy to clean, allows...
 - 3.2. Analytical balance, accurate to 1 mg.
 - 3.3. Dry containers of non-corrodible metal or of glass with lids...
 - 3.4. Electrically heated isothermal oven (± 2 oC) properly ventilated and...
 - 3.5. Adjustable electrically heated vacuum oven fitted with an oil pump...
 - 3.6. Desiccator with a thick perforated metal or porcelain plate, containing...
 4. Procedure
 - 4.1. Preparation
 - 4.1.1. Feed other than those coming under 4.1.2 and 4.1.3
 - 4.1.2. Cereals and groats
 - 4.1.3. Feed in liquid or paste form, feed predominantly composed of...
 - 4.2. Drying
 - 4.2.1. Feed other than those coming under 4.2.2 and 4.2.3
 - 4.2.2. Cereals, flour, groats and meal
 - 4.2.3. Compound feed containing more than 4 % of sucrose or...
 - 4.3. Preliminary drying
 - 4.3.1. Feed other than those coming under 4.3.2
 - 4.3.2. Cereals
 5. Calculation of results
 - 5.1. Drying without preliminary drying
 - 5.2. Drying with preliminary drying
 - 5.3. Repeatability
 6. Observation
- B. DETERMINATION OF MOISTURE IN ANIMAL AND VEGETABLE FATS AND OILS...
1. Purpose and scope

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2. Principle
 3. Apparatus
 - 3.1. Flat-bottomed dish, of a corrosion-resistant material, 8 to 9 cm...
 - 3.2. Thermometer with a strengthened bulb and expansion tube at the...
 - 3.3. Sand bath or electric hot-plate.
 - 3.4. Desiccator, containing an efficient drying agent.
 - 3.5. Analytical balance.
 4. Procedure
 5. Calculation of results
 - Repeatability
- C. DETERMINATION OF THE CONTENT OF CRUDE PROTEIN
1. Purpose and scope
 2. Principle
 3. Reagents
 - 3.1. Potassium sulphate.
 - 3.2. Catalyst: copper (II) oxide CuO or copper (II) sulphate pentahydrate,...
 - 3.3. Granulated zinc.
 - 3.4. Sulphuric acid, $\rho_{20} = 1,84$ g/ml.
 - 3.5. Sulphuric acid, standard volumetric solution, $c(\text{H}_2\text{SO}_4) = 0,25$ mol/l.
 - 3.6. Sulphuric acid, standard volumetric solution, $c(\text{H}_2\text{SO}_4) = 0,1$ mol/l.
 - 3.7. Sulphuric acid, standard volumetric solution, $c(\text{H}_2\text{SO}_4) = 0,05$ mol/l.
 - 3.8. Methyl red indicator; dissolve 300 mg of methyl red in...
 - 3.9. Sodium hydroxide solution (Technical grade may be used) $\beta = \dots$
 - 3.10. Sodium hydroxide, standard volumetric solution $c(\text{NaOH}) = 0,25$ mol/l.
 - 3.11. Sodium hydroxide, standard volumetric solution $c(\text{NaOH}) = 0,1$ mol/l.
 - 3.12. Granulated pumice stone, washed in hydrochloric acid and ignited.
 - 3.13. Acetanilide (m.p. = 114 °C, N-content = 10,36 %).
 - 3.14. Sucrose (nitrogen free).
 - 3.15. Boric acid (H_3BO_3).
 - 3.16. Methyl red indicator solution: dissolve 100 mg methyl red in...
 - 3.17. Bromocresol green solution: dissolve 100 mg bromocresol green in 100...
 - 3.18. Boric acid solution (10 g/l to 40 g/l depending on...
 - 3.19. Hydrochloric acid standard volumetric solution $c(\text{HCl}) = 0,1$ mol/l.
 4. Apparatus
 5. Procedure
 - 5.1. Digestion
 - 5.2. Distillation
 - 5.2.1. Distillation into sulphuric acid
 - 5.2.2. Distillation into boric acid
 - 5.3. Titration
 - 5.3.1. Sulphuric acid
 - 5.3.2. Boric acid
 - 5.4. Blank test
 6. Calculation of results
 - 6.1. Calculation for titration according to 5.3.1
 - 6.2. Calculation for titration according to 5.3.2
 - 6.2.1. Titration with hydrochloric acid
 - 6.2.2. Titration with sulphuric acid
 7. Verification of the method
 - 7.1. Repeatability

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- 7.2. Accuracy
- 8. Observations
 - 8.1. Apparatus may be of the manual, semi-automatic or automatic type....
 - 8.2. If the digest solidifies, recommence the determination using a larger...
 - 8.3. For products with a low nitrogen content, the volume of...
 - 8.4. For routine analysis, alternative methods of analysis can be applied...

D. DETERMINATION OF UREA

- 1. Purpose and scope
- 2. Principle
- 3. Reagents
 - 3.1. Solution of 4-dimethylaminobenzaldehyde: dissolve 1,6 g of 4-DMAB in 100...
 - 3.2. Carrez solution I: dissolve in water 21,9 g of zinc...
 - 3.3. Carrez solution II: dissolve in water 10,6 g of potassium...
 - 3.4. Active carbon which does not absorb urea (to be checked)...
 - 3.5. Urea, 0,1 % solution (w/v).
- 4. Apparatus
 - 4.1. Mixer (tumbler): approximately 35 to 40 r.p.m.
 - 4.2. Test tubes: 160 × 16 mm with ground-glass stoppers.
 - 4.3. Spectrophotometer.
- 5. Procedure
 - 5.1. Analysis of sample
 - 5.2. Calibration curve
- 6. Calculation of results
- 7. Observations
 - 7.1. In the case of contents of urea exceeding 3 %,...
 - 7.2. In the case of low contents of urea, increase the...
 - 7.3. If the sample contains simple nitrogenous compounds such as amino...

E. DETERMINATION OF VOLATILE NITROGENOUS BASES

I. BY MICRODIFFUSION

- 1. Purpose and scope
 - 2. Principle
 - 3. Reagents
 - 3.1. Trichloroacetic acid, solution 20 % (w/v).
 - 3.2. Indicator: dissolve 33 mg of bromocresol green and 65 mg...
 - 3.3. Boric acid solution: in a 1 litre graduated flask dissolve...
 - 3.4. Saturated potassium carbonate solution: dissolve 100 g of potassium carbonate...
 - 3.5. Sulphuric acid 0,01 mol/litre.
 - 4. Apparatus
 - 4.1. Mixer (tumbler): approximately 35 to 40 r.p.m.
 - 4.2. Glass or plastic Conway cells (see diagram).
 - 4.3. Microburettes graduated in 1/100 ml.
 - 5. Procedure
 - 6. Calculation of results
 - Repeatability
 - 7. Observation
- CONWAY CELL

II. BY DISTILLATION

- 1. Purpose and Scope
- 2. Principle

3. Reagents
 - 3.1. Trichloroacetic acid, solution 20 % (w/v).
 - 3.2. Magnesium oxide.
 - 3.3. Anti-foaming emulsion (e.g. silicone).
 - 3.4. Sulphuric acid 0,05 mol/litre.
 - 3.5. Sodium hydroxide solution 0,1 mol/litre.
 - 3.6. Methyl red solution 0,3 % in 95 %-96 % (v/v)...
4. Apparatus
 - 4.1. Mixer (tumbler): approximately 35 to 40 r.p.m.
 - 4.2. Distilling apparatus of the Kjeldahl type.
5. Procedure
6. Calculation of results
 - Repeatability

F. DETERMINATION OF AMINO ACIDS (EXCEPT TRYPTOPHANE)

1. Purpose and scope
2. Principle
 - 2.1. Free amino acids
 - 2.2. Total amino acids
3. Reagents
 - 3.1. Hydrogen peroxide, w (w/w) = 30 %.
 - 3.2. Formic acid, w (w/w) = 98 %-100 %.
 - 3.3. Phenol.
 - 3.4. Sodium disulphite.
 - 3.5. Sodium hydroxide.
 - 3.6. 5-Sulfosalicylic acid dihydrate.
 - 3.7. Hydrochloric acid, density approximately 1,18 g/ml.
 - 3.8. tri-Sodium citrate dihydrate.
 - 3.9. 2,2'-Thiodiethanol (thiodiglycol).
 - 3.10. Sodium chloride.
 - 3.11. Ninhydrin.
 - 3.12. Light petroleum, boiling range 40-60 oC.
 - 3.13. Norleucine, or other compound suitable for use as internal standard...
 - 3.14. Nitrogen gas (< 10 ppm oxygen).
 - 3.15. 1-Octanol.
 - 3.16. Amino acids.
 - 3.16.1. Standard substances listed under paragraph 1. Pure compounds containing no...
 - 3.16.2. Cysteic acid.
 - 3.16.3. Methionine sulphone.
 - 3.17. Sodium hydroxide solution, c = 7,5 mol/l:
 - 3.18. Sodium hydroxide solution, c = 1 mol/l:
 - 3.19. Formic acid — phenol solution:
 - 3.20. Hydrolysis mixture, c = 6 mol HCl/l containing 1 g...
 - 3.21. Extraction mixture, c = 0,1 mol HCl/l containing 2 %...
 - 3.22. 5-Sulfosalicylic acid, β = 6 %:
 - 3.23. Oxidation mixture (Performic acid — phenol):
 - 3.24. Citrate buffer, c = 0,2 mol Na⁺/l, pH 2,2:
 - 3.25. Elution buffers, prepared according to conditions for the analyser used...
 - 3.26. Ninhydrin reagent, prepared according to conditions for the analyser used...
 - 3.27. Standard solutions of amino acids. These solutions shall be stored...

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- 3.27.1. Stock standard solution of amino acids (3.16.1).
 - 3.27.2. Stock standard solution of cysteic acid and methionine sulphone, c...
 - 3.27.3. Stock standard solution of the internal standard e.g. norleucine, c...
 - 3.27.4. Calibration solution of standard amino acids for use with hydrolysates,...
 - 3.27.5. Calibration solution of standard amino acids for use with hydrolysates...
 4. Apparatus
 - 4.1. 100 or 250 ml round bottomed flask fitted with a...
 - 4.2. 100 ml borosilicate glass bottle with screw cap with rubber/teflon...
 - 4.3. Oven with forced ventilation and a temperature regulator with an...
 - 4.4. pH-meter (three decimal places).
 - 4.5. Membrane filter (0,22 µm).
 - 4.6. Centrifuge.
 - 4.7. Rotary vacuum evaporator.
 - 4.8. Mechanical shaker or magnetic stirrer.
 - 4.9. Amino acid analyser or HPLC equipment with ion exchange column,...
 5. Procedure
 - 5.1. Preparation of the sample
 - 5.2. Determination of free amino acids in feed and premixtures
 - 5.3. Determination of total amino acids
 - 5.3.1. Oxidation
 - 5.3.2. Hydrolysis
 - 5.3.2.1. Hydrolysis of oxidised samples
 - 5.3.2.2. Hydrolysis of unoxidised samples
 - 5.3.2.3. Open hydrolysis
 - 5.3.2.4. Closed Hydrolysis
 - 5.3.3. Adjustment of pH
 - 5.3.3.1. For Chromatographic Systems (4.9) requiring a low sodium concentration
 - 5.3.3.2. For all other Amino Acid Analysers (4.9)
 - 5.3.4. Sample solution for chromatography
 - 5.4. Chromatography
 6. Calculation of results
 - 6.1. The total dilution volume of extracts (F) for determination of...
 7. Evaluation of the method
 - 7.1. Repeatability
 - 7.2. Reproducibility
 8. Use of reference materials
 9. Observations
 - 9.1. Because of differences between amino acid analysers the final concentrations...
 - 9.2. Where high performance liquid chromatographic equipment is used to analyse...
 - 9.3. By applying the method to feed containing more than 1...
- G. DETERMINATION OF TRYPTOPHAN
1. Purpose and scope
 2. Principle
 3. Reagents
 - 3.1. Double distilled water or water of equivalent quality must be...
 - 3.2. Standard substance: tryptophan (purity/content ≥ 99 %) dried under vacuum...

- 3.3. Internal standard substance: α -methyl-tryptophan (purity/content ≥ 99 %), dried under...
- 3.4. Barium hydroxide octa-hydrate (care shall be taken not to expose...
- 3.5. Sodium hydroxide.
- 3.6. Ortho-phosphoric acid, w (w/w) = 85 %.
- 3.7. Hydrochloric acid, $\rho_{20,19}$ g/ml.
- 3.8. Methanol, equivalent to HPLC grade.
- 3.9. Light petroleum, boiling range 40-60 oC.
- 3.10. Sodium hydroxide solution, c = 1 mol/l:
- 3.11. Hydrochloric acid, c = 6 mol/l:
- 3.12. Hydrochloric acid, c = 1 mol/l:
- 3.13. Hydrochloric acid, c = 0,1 mol/l:
- 3.14. Ortho-phosphoric acid, c = 0,5 mol/l:
- 3.15. Concentrated solution of tryptophan (3.2), c = 2,5 μ mol/ml:
- 3.16. Concentrated internal standard solution, c = 2,5 μ mol/ml:
- 3.17. Calibration standard solution of tryptophan and internal standard:
- 3.18. Acetic acid
- 3.19. 1,1,1-trichloro-2-methyl-2-propanol.
- 3.20. Ethanolamine w (w/w) > 98 %.
- 3.21. Solution of 1 g 1,1,1-trichloro-2-methyl-2-propanol (3.19) in 100 ml methanol...
- 3.22. Mobile phase for HPLC: 3,0 g acetic acid (3.18) +...
4. Apparatus
 - 4.1. HPLC equipment with a spectrofluorometric detector.
 - 4.2. Liquid chromatographic column, 125 mm x 4 mm, C18, 3...
 - 4.3. pH-meter.
 - 4.4. Polypropylene flask, capacity 125 ml, with wide neck and screw...
 - 4.5. Membrane filter, 0,45 μ m.
 - 4.6. Autoclave, 110 (\pm 2) oC, 1,4 (\pm 0,1) bar.
 - 4.7. Mechanical shaker or magnetic stirrer.
 - 4.8. Vortex mixer.
5. Procedure
 - 5.1. Preparation of samples
 - 5.2. Determination of free tryptophan (extract)
 - 5.3. Determination of total tryptophan (hydrolysate)
 - 5.4. HPLC determination
6. Calculation of results
7. Repeatability
8. Results of a collaborative study
9. Observations
 - 9.1. Following special chromatographic conditions may give better separation between tryptophan...
 - 9.2. The chromatography will vary according to the type of HPLC...
 - 9.3. Barium hydroxide

H. DETERMINATION OF CRUDE OILS AND FATS

1. Purpose and scope
 - 1.1. Procedure A — Directly extractable crude oils and fats
 - 1.2. Procedure B — Total crude oils and fats
 - 1.3. Interpretation of results
2. Principle
 - 2.1. Procedure A
 - 2.2. Procedure B

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3. Reagents
 - 3.1. Light petroleum, boiling range: 40 to 60 oC. The bromine...
 - 3.2. Sodium sulfate, anhydrous.
 - 3.3. Hydrochloric acid, $c = 3 \text{ mol/l}$
 - 3.4. Filtration aid, e.g. Kieselguhr, Hyflo-supercel.
4. Apparatus
 - 4.1. Extraction apparatus. If fitted with a siphon (Soxhlet apparatus), the...
 - 4.2. Extraction thimbles, free of matter soluble in light petroleum and...
 - 4.3. Drying oven, either a vacuum oven set at $75 \pm \dots$
5. Procedure
 - 5.1. Procedure A (see point 8.1)
 - 5.2. Procedure B
6. Expression of result
7. Repeatability
8. Observations
 - 8.1. For products with a high content of oils and fats,...
 - 8.2. For products low in oils and fats the test sample...
 - 8.3. Pet foods containing a high content of water may need...
 - 8.4. In paragraph 5.2 it may be more effective to use...
 - 8.5. The drying time of 1,5 h may need to be...
 - 8.6. Pre-extraction by Procedure A prior to hydrolysis and re-extraction by...

I. DETERMINATION OF CRUDE FIBRE

1. Purpose and scope
2. Principle
3. Reagents
 - 3.1. Sulphuric acid, $c = 0,13 \text{ mol/l}$.
 - 3.2. Anti-foaming agent (e.g. n-octanol).
 - 3.3. Filter aid (Celite 545 or equivalent), heated at 500 oC...
 - 3.4. Acetone.
 - 3.5. Light petroleum boiling-range 40 to 60 oC.
 - 3.6. Hydrochloric acid, $c = 0,5 \text{ mol/l}$.
 - 3.7. Potassium hydroxide solution, $c = 0,23 \text{ mol/l}$.
4. Apparatus
 - 4.1. Heating unit for digestion with sulphuric acid and potassium hydroxide...
 - 4.2. Glass filter crucible with fused sintered glass filter plate pore...
 - 4.3. Cylinder of at least 270 ml with a reflux condenser,...
 - 4.4. Drying oven with thermostat.
 - 4.5. Muffle furnace with thermostat.
 - 4.6. Extraction unit consisting of a support plate for the filter...
 - 4.7. Connecting rings to assemble the heating unit (4.1), crucible (4.2)...
5. Procedure
6. Calculation of results
7. Repeatability
8. Observations
 - 8.1. Feed containing more than 10 % crude fat must be...
 - 8.2. Feed containing fats which cannot be extracted directly with light...
 - 8.3. If the feed contains over 5 % of carbonates, expressed...
 - 8.4. If an apparatus in the form of a stand is...
 - 8.5. If after boiling it is difficult to filter the acidic...
 - 8.6. The temperature for ashing shall not be higher than 500...

J. DETERMINATION OF SUGAR

1. Purpose and scope
2. Principle
3. Reagents
 - 3.1. Ethanol solution 40 % (v/v) density: 0,948 g/ml at 20...
 - 3.2. Carrez solution I: dissolve in water 21,9 g of zinc...
 - 3.3. Carrez solution II: dissolve in water 10,6 g of potassium...
 - 3.4. Methyl orange, solution 0,1 % (w/v).
 - 3.5. Hydrochloric acid 4 mol/litre.
 - 3.6. Hydrochloric acid 0,1 mol/litre.
 - 3.7. Sodium hydroxide solution 0,1 mol/litre.
 - 3.8. Luff-Schoorl reagent:
 - 3.8.1. Copper sulphate solution: dissolve 25 g of copper sulphate, Cu...
 - 3.8.2. Citric acid solution: dissolve 50 g of citric acid, C₆H₈O₇·H₂O...
 - 3.8.3. Sodium carbonate solution: dissolve 143,8 g of anhydrous sodium carbonate...
 - 3.9. Sodium thiosulphate solution 0,1 mol/litre.
 - 3.10. Starch solution: add a mixture of 5 g of soluble...
 - 3.11. Sulphuric acid 3 mol/litre.
 - 3.12. Potassium iodide, solution 30 % (w/v).
 - 3.13. Granulated pumice stone boiled in hydrochloric acid, washed in water...
 - 3.14. 3-methylbutan-1-ol.
4. Apparatus
5. Procedure
 - 5.1. Extraction of sample
 - 5.2. Determination of reducing sugars
 - 5.3. Determination of total sugars after inversion
 - 5.4. Titration by the Luff-Schoorl method
6. Calculation of results
7. Special procedures
 - 7.1. In the case of feed which are rich in molasses...
 - 7.2. In the case of molasses and feed materials which are...
8. Observations
 - 8.1. In order to prevent foaming it is advisable to add...
 - 8.2. The difference between the content of total sugars after inversion,...
 - 8.3. In order to determine the content of reducing sugars, excluding...
 - 8.3.1. For an approximate calculation, multiply by 0,675 the lactose content...
 - 8.3.2. For an accurate calculation of reducing sugars, excluding lactose, the...
Example:

K. DETERMINATION OF LACTOSE

1. Purpose and scope
2. Principle
3. Reagents
 - 3.1. Suspension of *Saccharomyces cerevisiae*: suspend 25 g of fresh yeast...
 - 3.2. Carrez solution I: dissolve in water 21,9 g of zinc...
 - 3.3. Carrez solution II: dissolve in water 10,6 g of potassium...
 - 3.4. Luff-Schoorl reagent:
 - 3.4.1. Copper sulphate solution: dissolve 25 g of copper sulphate Cu...
 - 3.4.2. Citric acid solution: dissolve 50 g of citric acid C₆H₈O₇·H₂O...
 - 3.4.3. Sodium carbonate solution: dissolve 143,8 g of anhydrous sodium carbonate...

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- 3.5. Granulated pumice stone boiled in hydrochloric acid, washed in water...
- 3.6. Potassium iodide, solution 30 % (w/v).
- 3.7. Sulphuric acid 3 mol/litre.
- 3.8. Solution of sodium thiosulphate 0,1 mol/litre.
- 3.9. Starch solution: add a mixture of 5 g of soluble...
4. Apparatus
5. Procedure
6. Calculation of results
7. Observation

L. DETERMINATION OF STARCH

POLARIMETRIC METHOD

1. Purpose and scope
2. Principle
3. Reagents
 - 3.1. Hydrochloric acid, solution 25 % (w/w) density: 1,126 g/ml.
 - 3.2. Hydrochloric acid. solution 1,13 % (w/v)
 - 3.3. Carrez solution I: dissolve 21,9 g of zinc acetate $Zn(CH_3COO)_2$...
 - 3.4. Carrez solution II: dissolve 10,6 g of potassium ferrocyanide K_4 ...
 - 3.5. Ethanol, solution 40 % (v/v), density: 0,948 g/ml at 20...
4. Apparatus
 - 4.1. 250 ml Erlenmeyer flask with standard ground-glass joint and with...
 - 4.2. Polarimeter or saccharimeter.
5. Procedure
 - 5.1. Preparation of the sample
 - 5.2. Determination of the total optical rotation (P or S) (see...
 - 5.3. Determination of the optical rotation (P' or S') of substances...
6. Calculation of results
 - 6.1. Measurement by polarimeter
 - 6.2. Measurement by saccharimeter
 - 6.3. Repeatability
7. Observations
 - 7.1. If the sample contains more than 6 % of carbonates,...
 - 7.2. In the case of products with a high lactose content,...
 - 7.3. The following feed materials, where they are present in significant...

M. DETERMINATION OF CRUDE ASH

1. Purpose and Scope
2. Principle
3. Reagents
4. Apparatus
 - 4.1. Hot-plate.
 - 4.2. Electric muffle-furnace with thermostat.
 - 4.3. Crucibles for ashing made of silica, porcelain or platinum either...
5. Procedure
 - 5.1. Put the crucible into the calibrated muffle furnace set at...
 - 5.2. Put the crucible into the calibrated muffle-furnace set at 550...
6. Calculation of results

7. Observations
 - 7.1. The ash of substances which are difficult to ash must...
 - 7.2. In the case of substances resistant to the treatment described...
 - 7.3. In the case of oils and fats, weigh accurately a...

- N. DETERMINATION OF ASH WHICH IS INSOLUBLE IN HYDROCHLORIC ACID
 1. Purpose and Scope
 - 1.1. Method A: applicable to organic feed materials and to most...
 - 1.2. Method B: applicable to mineral compounds and mixtures and to...
 2. Principle
 - 2.1. Method A: the sample is ashed, the ash boiled in...
 - 2.2. Method B: the sample is treated with hydrochloric acid. The...
 3. Reagents
 - 3.1. Hydrochloric acid 3 mol/litre.
 - 3.2. Trichloroacetic acid, solution 20 % solution (w/v).
 - 3.3. Trichloroacetic acid, solution 1 % (w/v).
 4. Apparatus
 - 4.1. Hot plate.
 - 4.2. Electric muffle-furnace with thermostat.
 - 4.3. Crucibles for ashing made of silica, porcelain or platinum, either...
 5. Procedure
 - 5.1. Method A
 - 5.2. Method B
 6. Calculation of results
 7. Observation

- O. DETERMINATION OF CARBONATES
 1. Purpose and Scope
 2. Principle
 3. Reagents
 - 3.1. Hydrochloric acid, density 1,1 g/ml.
 - 3.2. Calcium carbonate.
 - 3.3. Sulphuric acid, approximately 0,05 mol/litre, coloured with methyl red.
 4. Apparatus
 5. Procedure
 6. Calculation of results
 7. Observations
 - 7.1. When the portion of the sample weighs more than 2...
 - 7.2. If the apparatus used has a different volume from that...

- P. DETERMINATION OF TOTAL PHOSPHORUS
PHOTOMETRIC METHOD
 1. Purpose and Scope
 2. Principle
 3. Reagents
 - 3.1. Calcium carbonate.
 - 3.2. Hydrochloric acid, $\rho_{20} = 1,1$ g/ml (approx 6 mol/litre).
 - 3.3. Nitric acid, $\rho_{20} = 1,045$ g/ml.
 - 3.4. Nitric acid, $\rho_{20} = 1,38$ to 1,42 g/ml.
 - 3.5. Sulphuric acid, $\rho_{20} = 1,84$ g/ml.
 - 3.6. Molybdovanadate reagent: mix 200 ml of ammonium heptamolybdate solution (3.6.1),...

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- 3.6.1. Ammonium heptamolybdate solution: dissolve in hot water 100 g of...
- 3.6.2. Ammonium monovanadate solution: dissolve 2,35 g of ammonium monovanadate NH_4VO_3 ...
- 3.7. Standard solution of 1 mg phosphorus per ml: dissolve 4,387...
4. Apparatus
 - 4.1. Silica, porcelain or platinum ashing crucibles.
 - 4.2. Electric muffle-furnace with thermostat set at 550 °C.
 - 4.3. 250 ml Kjeldahl flask.
 - 4.4. Graduated flasks and precision pipettes.
 - 4.5. Spectrophotometer.
 - 4.6. Test tubes about 16 mm in diameter, with stoppers graded...
5. Procedure
 - 5.1. Preparation of the solution
 - 5.1.1. Usual procedure
 - 5.1.2. Samples containing organic substances and free from calcium and magnesium...
 - 5.2. Development of coloration and measurement of optical density
 - 5.3. Calibration curve
6. Calculation of results
 - Repeatability

Q. DETERMINATION OF CHLORINE FROM CHLORIDES

1. Purpose and Scope
2. Principle
3. Reagents
 - 3.1. Solution of ammonium thiocyanate 0,1 mol/litre.
 - 3.2. Solution of silver nitrate 0,1 mol/litre.
 - 3.3. Saturated solution of ammonium ferric sulphate $(\text{NH}_4)\text{Fe}(\text{SO}_4)_2$.
 - 3.4. Nitric acid, density: 1,38 g/ml.
 - 3.5. Diethyl ether.
 - 3.6. Acetone.
 - 3.7. Carrez I solution: dissolve in water 21,9 g of zinc...
 - 3.8. Carrez II solution: dissolve in water 10,6 g of potassium...
 - 3.9. Active carbon, free from chlorides and not absorbing them.
4. Apparatus
5. Procedure
 - 5.1. Preparation of the solution
 - 5.1.1. Samples free from organic matter
 - 5.1.2. Samples containing organic matter, excluding the products listed under 5.1.3....
 - 5.1.3. Cooked feed, flax cakes and flour, products rich in flax...
 - 5.2. Titration
6. Calculation of results
7. Observations
 - 7.1. Titration may also be carried out by potentiometry.
 - 7.2. In the case of products which are very rich in...
 - 7.3. In the case of fish-meal, titration may be carried out...

ANNEX IV

METHODS OF ANALYSIS TO CONTROL THE
LEVEL OF AUTHORISED ADDITIVES IN FEED

A. DETERMINATION OF VITAMIN A

1. Purpose and Scope
2. Principle
3. Reagents
 - 3.1. Ethanol, $\sigma = 96\%$
 - 3.2. Light petroleum, boiling range 40 °C-60 °C
 - 3.3. Methanol
 - 3.4. Potassium hydroxide solution, $c = 50\text{ g}/100\text{ ml}$
 - 3.5. Sodium ascorbate solution, $c = 10\text{ g}/100\text{ ml}$ (see 7.7...)
 - 3.6. Sodium sulphide, $\text{Na}_2\text{S} \cdot x\text{H}_2\text{O}$ ($x = 7-9$)
 - 3.6.1. Sodium sulphide solution, $c = 0,5\text{ mol}/\text{l}$ in glycerol, B...
 - 3.7. Phenolphthalein solution, $c = 2\text{ g}/100\text{ ml}$ in ethanol (3.1)...
 - 3.8. 2-Propanol
 - 3.9. Mobile phase for HPLC: mixture of methanol (3.3) and water,...
 - 3.10. Nitrogen, oxygen free
 - 3.11. All-trans-vitamin A acetate, extra pure, of certified activity, e.g. 2,8...
 - 3.11.1. Stock solution of all-trans-vitamin A acetate: Weigh to the nearest...
 - 3.12. All-trans-vitamin A palmitate, extra pure, of certified activity, e.g. 1,8...
 - 3.12.1. Stock solution of all-trans-vitamin A palmitate: Weigh to the nearest...
 - 3.13. 2,6-Di-tert-butyl-4-methylphenol (BHT) (see 7.5 observations)
4. Apparatus
 - 4.1. Vacuum rotary evaporator
 - 4.2. Amber glassware
 - 4.2.1. Flat bottom or conical flasks, 500 ml, with ground-glass socket...
 - 4.2.2. Graduated flasks with ground-glass stoppers, narrow-necked, 10, 25, 100 and...
 - 4.2.3. Separating funnels, conical, 1 000 ml, with ground-glass stoppers
 - 4.2.4. Pear shaped flasks, 250 ml, with ground-glass sockets
 - 4.3. Allihn condenser, jacket length 300 mm, with ground-glass joint, with...
 - 4.4. Pleated filter paper for phase separation, diameter 185 mm (e.g....)
 - 4.5. HPLC equipment with injection system
 - 4.5.1. Liquid chromatographic column, 250 mm x 4 mm, C18, 5...
 - 4.5.2. UV or fluorescence detector, with variable wavelength adjustment
 - 4.6. Spectrophotometer with 10 mm quartz cells
 - 4.7. Water-bath with magnetic stirrer
 - 4.8. Extraction apparatus (see figure 1) consisting of:
 - 4.8.1. Glass cylinder of 1 l capacity fitted with a ground...
 - 4.8.2. Ground glass insert equipped with a side-arm and an adjustable...
5. Procedure
 - 5.1. Preparation of the sample
 - 5.2. Saponification
 - 5.3. Extraction
 - 5.3.1. Extraction using a separating funnel (4.2.3)
 - 5.3.2. Extraction using an extraction apparatus (4.8)
 - 5.4. Preparation of the sample solution for HPLC
 - 5.5. Determination by HPLC
 - HPLC conditions

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- 5.6. Calibration
 - 5.6.1. Preparation of the working standard solutions
 - 5.6.2. Preparation of the calibration solutions and calibration graph
 - 5.6.3. UV standardisation of the standard solutions
 - 5.6.3.1. Vitamin A acetate stock solution
 - 5.6.3.2. Vitamin A palmitate stock solution
 - 5.6.3.3. Vitamin A working standard solution
- 6. Calculation of the results
- 7. Observations
 - 7.1. For samples with low vitamin A concentration it may be...
 - 7.2. The weight of the sample taken for the analysis shall...
 - 7.3. If phase separation does not occur add approximately 10 ml...
 - 7.4. With cod-liver oil and other pure fats the saponification time...
 - 7.5. Hydroquinone can be used instead of BHT.
 - 7.6. Using a normal phase-column the separation of retinol isomers is...
 - 7.7. Approximately 150 mg ascorbic acid can be used instead of...
 - 7.8. Approximately 50 mg EDTA can be used instead of sodium...
 - 7.9. In cases of analysis of vitamin A in milk replacers,...
- 8. Repeatability
- 9. Results of a collaborative study

B. DETERMINATION OF VITAMIN E

- 1. Purpose and Scope
- 2. Principle
- 3. Reagents
 - 3.1. Ethanol, $\sigma = 96 \%$.
 - 3.2. Light petroleum, boiling range 40 °C-60 °C.
 - 3.3. Methanol.
 - 3.4. Potassium hydroxide solution, $c = 50 \text{ g}/100 \text{ ml}$.
 - 3.5. Sodium ascorbate solution, $c = 10 \text{ g}/100 \text{ ml}$ (see 7.7...
 - 3.6. Sodium sulphide, $\text{Na}_2\text{S} \cdot x \text{H}_2\text{O}$ ($x = 7-9$).
 - 3.6.1. Sodium sulphide solution, $c = 0,5 \text{ mol}/\text{l}$ in glycerol, β ...
 - 3.7. Phenolphthalein solution, $c = 2 \text{ g}/100 \text{ ml}$ in ethanol (3.1)...
 - 3.8. Mobile phase for HPLC: mixture of methanol (3.3) and water,...
 - 3.9. Nitrogen, oxygen free.
 - 3.10. DL- α -tocopherol acetate, extra pure, of certified activity.
 - 3.10.1. Stock solution of DL- α -tocopherol acetate: Weigh to the nearest 0,1...
 - 3.11. DL- α -tocopherol, extra pure, of certified activity.
 - 3.11.1. Stock solution of DL- α -tocopherol: Weigh to the nearest 0,1 mg,...
 - 3.12. 2,6-Di-tert-butyl-4-methylphenol (BHT) (see 7.5 observations).
- 4. Apparatus
 - 4.1. Rotary film evaporator.
 - 4.2. Amber glassware.
 - 4.2.1. Flat bottom or conical flasks, 500 ml, with ground-glass socket....
 - 4.2.2. Graduated flasks with ground-glass stoppers, narrow-necked, 10, 25, 100 and...
 - 4.2.3. Separating funnels, conical, 1 000 ml, with ground-glass stoppers.
 - 4.2.4. Pear shaped flasks, 250 ml, with ground-glass sockets.
 - 4.3. Allihn condenser, jacket length 300 mm, with ground-glass joint, with...
 - 4.4. Pleated filter paper for phase separation, diameter 185 mm (e.g....
 - 4.5. HPLC equipment with injection system.
 - 4.5.1. Liquid chromatographic column, 250 mm \times 4 mm, C18, 5...

-
- 4.5.2. Fluorescence or UV detector, with variable wavelength adjustment.
 - 4.6. Spectrophotometer with 10 mm quartz cells.
 - 4.7. Water-bath with magnetic stirrer.
 - 4.8. Extraction apparatus (see figure 1) consisting of:
 - 4.8.1. Glass cylinder of 1 l capacity fitted with a ground...
 - 4.8.2. Ground glass insert equipped with a side-arm and an adjustable...
 5. Procedure
 - 5.1. Preparation of the sample
 - 5.2. Saponification
 - 5.3. Extraction
 - 5.3.1. Extraction using a separating funnel (4.2.3)
 - 5.3.2. Extraction using an extraction apparatus (4.8)
 - 5.4. Preparation of the sample solution for HPLC
 - 5.5. Determination by HPLC
 - HPLC conditions
 - 5.6. Calibration (DL- α -tocopherol acetate or DL- α -tocopherol)
 - 5.6.1. DL- α -tocopherol acetate standard
 - 5.6.1.1. Preparation of the working standard solution
 - 5.6.1.2. Preparation of the calibration solutions and calibration graph
 - 5.6.1.3. UV standardisation of the DL- α -tocopherol acetate stock solution (3.10.1)
 - 5.6.2. DL- α -tocopherol standard
 - 5.6.2.1. Preparation of the working standard solution
 - 5.6.2.2. Preparation of the calibration solutions and calibration graph
 - 5.6.2.3. UV standardisation of the DL- α -tocopherol stock solution (3.11.1)
 6. Calculation of the results
 7. Observations
 - 7.1. For samples with low vitamin E concentration it may be...
 - 7.2. The weight of the sample taken for the analysis shall...
 - 7.3. If phase separation does not occur add approximately 10 ml...
 - 7.4. After the spectrophotometric measurement of the DL- α -tocopherol acetate or DL- α -tocopherol...
 - 7.5. Hydroquinone can be used instead of BHT.
 - 7.6. Using a normal phase-column the separation of α -, β -, γ -...
 - 7.7. Approximately 150 mg ascorbic acid can be used instead of...
 - 7.8. Approximately 50 mg EDTA can be used instead of sodium...
 - 7.9. Vitamin E acetate hydrolyses very fast under alkaline conditions and...
 8. Repeatability
 9. Results of a collaborative study
- C. DETERMINATION OF THE TRACE ELEMENTS IRON, COPPER, MANGANESE AND ZINC...
1. Purpose and scope
 2. Principle
 3. Reagents
 - Introductory comments
 - 3.1. Hydrochloric acid (d:1,19 g/ml).
 - 3.2. Hydrochloric acid (6 mol/litre).
 - 3.3. Hydrochloric acid (0,5 mol/litre).
 - 3.4. Hydrofluoric acid 38 % to 40 % (v/v) having an...

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- 3.5. Sulphuric acid (d: 1,84 g/ml).
- 3.6. Hydrogen peroxide (approximately 100 volumes of oxygen (30 % by...
- 3.7. Standard iron solution (1 000 µg Fe/ml) prepared as follows...
- 3.7.1. Working standard iron solution (100 µg Fe/ml) prepared by diluting...
- 3.8. Standard copper solution (1 000 µg Cu/ml) prepared as follows...
- 3.8.1. Working standard copper solution (10 µg Cu/ml) prepared by diluting...
- 3.9. Standard manganese solution (1 000 µg Mn/ml) prepared as follows...
- 3.9.1. Working standard manganese solution (10 µg Mn/ml) prepared by diluting...
- 3.10. Standard zinc solution (1 000 µg Zn/ml) prepared as follows...
- 3.10.1. Working standard zinc solution (10 µg Zn/ml) prepared by diluting...
- 3.11. Lanthanum chloride solution: dissolve 12 g of lanthanum oxide in...
4. Apparatus
 - 4.1. Muffle furnace with temperature regulation and preferably recorder.
 - 4.2. Glassware must be of resistant borosilicate type and it is...
 - 4.3. Atomic absorption spectrophotometer meeting the requirements of the method with...
5. Procedure
 - 5.1. Samples containing organic matter
 - 5.1.1. Ashing and preparation of the solution for analysis
 - 5.1.1.1. Place 5 to 10 g of sample weighed to the...
 - 5.1.1.2. If the residue in the filter appears black (carbon), put...
Notes:
 - 5.1.2. Spectrophotometric determination
 - 5.1.2.1. Preparation of calibration solutions
Iron
Copper
Manganese
Zinc
 - 5.1.2.2. Preparation of solution for analysis
 - 5.1.2.3. Blank experiment
 - 5.1.2.4. Measurement of the atomic absorption
 - 5.2. Mineral feed
6. Calculation of results
7. Repeatability
8. Observation

D. DETERMINATION OF HALOFUGINONE

1. Purpose and scope
2. Principle
3. Reagents
 - 3.1. Acetonitrile, equivalent to HPLC grade.
 - 3.2. Amberlite XAD-2 resin.
 - 3.3. Ammonium acetate.
 - 3.4. Ethyl acetate.
 - 3.5. Acetic acid, glacial.
 - 3.6. Halofuginone standard substance (DL-trans-7-bromo-6-chloro-3-[3-hydroxy-2-piperidyl]acetyl] quinazoline-4-(3H)-one hydrobromide, E 764).
 - 3.6.1. Halofuginone stock standard solution, 100 µg/ml
 - 3.6.2. Calibration solutions
 - 3.7. Hydrochloric acid (ρ20 approximately 1,16 g/ml).
 - 3.8. Methanol.

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- 3.9. Silver nitrate.
 - 3.10. Sodium ascorbate.
 - 3.11. Sodium carbonate.
 - 3.12. Sodium chloride.
 - 3.13. EDTA (ethylenediaminetetraacetic acid, disodium salt).
 - 3.14. Water, equivalent to HPLC grade.
 - 3.15. Sodium carbonate solution, $c = 10 \text{ g/100 ml}$.
 - 3.16. Sodium chloride-saturated sodium carbonate solution, $c = 5 \text{ g/100 ml}$...
 - 3.17. Hydrochloric acid, approximately $0,1 \text{ mol/l}$.
 - 3.18. Ammonium acetate buffer solution, approximately $0,25 \text{ mol/l}$.
 - 3.19. Amberlite XAD-2 resin preparation.
 - 3.20. Silver nitrate solution, approximately $0,1 \text{ mol/l}$.
 - 3.21. HPLC Mobile phase.
 4. Apparatus
 - 4.1. Ultrasonic bath
 - 4.2. Rotary film evaporator
 - 4.3. Centrifuge
 - 4.4. HPLC equipment with variable wavelength ultraviolet detector or diode-array detector...
 - 4.4.1. Liquid chromatographic column, $300 \text{ mm} \times 4 \text{ mm}$, C18, 10...
 - 4.5. Glass column ($300 \text{ mm} \times 10 \text{ mm}$) fitted with a...
 - 4.6. Glass-fibre filters, diameter 150 mm
 - 4.7. Membrane filters, $0,45 \mu\text{m}$
 - 4.8. Membrane filters, $0,22 \mu\text{m}$
 5. Procedure
 - 5.1. General
 - 5.1.1. A blank feed shall be analysed to check that neither...
 - 5.1.2. A recovery test shall be carried out by analysing the...
 - 5.2. Extraction
 - 5.3. Clean up
 - 5.3.1. Preparation of the Amberlite column
 - 5.3.2. Sample clean up
 - 5.4. HPLC determination
 - 5.4.1. Parameters
 - 5.4.2. Calibration graph
 - 5.4.3. Sample solution
 6. Calculation of results
 7. Validation of the results
 - 7.1. Identity
 - 7.1.1. Co-chromatography
 - 7.1.2. Diode-array detection
 - 7.2. Repeatability
 - 7.3. Recovery
 8. Results of a collaborative study
- E. DETERMINATION OF ROBENIDINE
1. Purpose and scope
 2. Principle
 3. Reagents
 - 3.1. Methanol.
 - 3.2. Acidified methanol.
 - 3.3. Acetonitrile, equivalent to HPLC grade.

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- 3.4. Molecular sieve.
 - 3.5. Aluminium oxide acidic activity grade I for column chromatography.
 - 3.6. Potassium dihydrogen phosphate solution, $c = 0,025$ mol/l.
 - 3.7. Di-sodium hydrogen phosphate solution, $c = 0,025$ mol/l.
 - 3.8. HPLC mobile phase.
 - 3.9. Standard substance.
 - 3.9.1. Robenidine stock standard solution: 300 µg/ml
 - 3.9.2. Robenidine intermediate standard solution: 12 µg/ml
 - 3.9.3. Calibration solutions
 - 3.10. Water equivalent to HPLC grade.
 4. Apparatus
 - 4.1. Glass column.
 - 4.2. Mechanical shaker or magnetic stirrer.
 - 4.3. Rotary film evaporator.
 - 4.4. HPLC equipment with variable wavelength ultraviolet detector or diode array...
 - 4.4.1. Liquid chromatographic column: 300 mm x 4 mm, C18 10...
 - 4.5. Glass fibre filter paper (Whatman GF/A or equivalent).
 - 4.6. Membrane filters, 0,22 µm.
 - 4.7. Membrane filters, 0,45 µm.
 5. Procedure
 - 5.1. General
 - 5.1.1. A blank feed shall be analysed to check that neither...
 - 5.1.2. A recovery test shall be carried out by analysing the...
 - 5.2. Extraction
 - 5.3. Purification
 - 5.3.1. Preparation of the aluminium-oxide column
 - 5.3.2. Sample purification
 - 5.4. HPLC determination
 - 5.4.1. Parameters
 - 5.4.2. Calibration graph
 - 5.4.3. Sample solution
 6. Calculation of results
 7. Validation of the results
 - 7.1. Identity
 - 7.1.1. Co-chromatography
 - 7.1.2. Diode-array detection
 - 7.2. Repeatability
 - 7.3. Recovery
 8. Results of a collaborative study
- F. DETERMINATION OF DICLAZURIL
1. Purpose and scope
 2. Principle
 3. Reagents
 - 3.1. Water, equivalent to HPLC-grade
 - 3.2. Ammonium acetate
 - 3.3. Tetrabutylammonium hydrogen sulphate (TBHS)
 - 3.4. Acetonitrile, equivalent to HPLC grade
 - 3.5. Methanol, equivalent to HPLC grade
 - 3.6. N, N-dimethylformamide (DMF)
 - 3.7. Hydrochloric acid, $\rho_{20} = 1,19$ g/ml

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- 3.8. Standard substance: diclazuril II-24: (+)-4-chlorophenyl [2,6-dichloro-4-(2,3,4,5-tetrahydro-3,5-dioxo-1,2,4-triazin-2-yl) phenyl] acetonitrile with guaranteed...
 - 3.8.1. Diclazuril stock standard solution, 500 µg/ml
 - 3.8.2. Diclazuril standard solution, 50 µg/ml
- 3.9. Internal standard substance: 2,6 dichloro- α -(4-chlorophenyl)-4-(4,5 dihydro-3,5-dioxo-1,2,4-triazine-2 (3H) — yl) α -methylbenzene-acetonitrile...
 - 3.9.1. Internal stock standard solution, 500 µg/ml
 - 3.9.2. Internal standard solution, 50 µg/ml
 - 3.9.3. Internal standard solution for premixtures, p/1 000 mg/ml
- 3.10. Calibration solution, 2 µg/ml.
- 3.11. C18 solid phase extraction cartridge, e.g. Bond Elut, size: 1...
- 3.12. Extraction solvent: acidified methanol.
- 3.13. Mobile phase for HPLC
 - 3.13.1. Eluent A: ammonium acetate — tetrabutylammonium hydrogen sulphate solution.
 - 3.13.2. Eluent B: acetonitrile (3.4).
 - 3.13.3. Eluent C: methanol (3.5).
4. Apparatus
 - 4.1. Mechanical shaker
 - 4.2. Equipment for ternary gradient HPLC
 - 4.2.1. Liquid chromatographic column, Hypersil ODS, 3 µm packing, 100 mm...
 - 4.2.2. UV detector with variable wavelength adjustment or diode array detector...
 - 4.3. Rotary film evaporator
 - 4.4. Membrane filter, 0,45 µm
 - 4.5. Vacuum manifold
 - 4.6. Ultrasonic bath
5. Procedure
 - 5.1. General
 - 5.1.1. Blank feed
 - 5.1.2. Recovery test
 - 5.2. Extraction
 - 5.2.1. Feed
 - 5.2.2. Premixtures
 - 5.3. HPLC determination
 - 5.3.1. Parameters
 - 5.3.2. Calibration solution
 - 5.3.3. Sample solution
6. Calculation of the results
 - 6.1. Feeds
 - 6.2. Premixtures
7. Validation of the results
 - 7.1. Identity
 - 7.1.1. Co-chromatography
 - 7.1.2. Diode-array detection
 - 7.2. Repeatability
 - 7.3. Recovery
8. Results of a collaborative study
9. Observations

G. DETERMINATION OF LASALOCID SODIUM

1. Purpose and scope
2. Principle
3. Reagents
 - 3.1. Potassium dihydrogen phosphate (KH₂PO₄).
 - 3.2. Orthophosphoric acid, w (w/w) = 85 %.
 - 3.3. Orthophosphoric acid solution, c = 20 %.
 - 3.4. 6-Methyl-2-heptylamine (1,5-dimethylhexylamine), w (w/w) = 99 %.
 - 3.5. Methanol, equivalent to HPLC grade.
 - 3.6. Hydrochloric acid, density = 1,19 g/ml.
 - 3.7. Phosphate buffer solution, c = 0,01 mol/l.
 - 3.8. Acidified methanol.
 - 3.9. HPLC mobile phase, phosphate buffer-methanol solution 5 + 95 (V...
 - 3.10. Lasalocid sodium standard substance with guaranteed purity, C₃₄H₅₃O₈Na (sodium salt...
 - 3.10.1. Lasalocid sodium stock standard solution, 500 µg/ml
 - 3.10.2. Lasalocid sodium intermediate standard solution, 50 µg/ml
 - 3.10.3. Calibration solutions
 - 3.11. Water, equivalent to HPLC grade.
4. Apparatus
 - 4.1. Ultrasonic bath (or shaking water-bath) with temperature control.
 - 4.2. Membrane filters, 0,45 µm.
 - 4.3. HPLC equipment with injection system, suitable for injecting volumes of...
 - 4.3.1. Liquid chromatographic column 125 mm x 4 mm, reversed-phase C₁₈,...
 - 4.3.2. Spectrofluorometer with variable wavelength adjustment of excitation and emission wavelengths....
5. Procedure
 - 5.1. General
 - 5.1.1. Blank feed
 - 5.1.2. Recovery test
 - 5.2. Extraction
 - 5.2.1. Feed
 - 5.2.2. Premixtures
 - 5.3. HPLC determination
 - 5.3.1. Parameters
 - 5.3.2. Calibration graph
 - 5.3.3. Sample solution
6. Calculation of results
 - 6.1. Feed
 - 6.2. Premixtures
7. Validation of the results
 - 7.1. Identity
 - 7.1.1. Co-chromatography
 - 7.2. Repeatability
 - 7.3. Recovery
8. Results of a collaborative study

ANNEX V

METHODS OF ANALYSIS TO CONTROL UNDESIRABLE SUBSTANCES IN FEED

A. DETERMINATION OF FREE AND TOTAL GOSSYPOL

1. Purpose and scope
2. Principle
3. Reagents
 - 3.1. Propan-2-ol-hexane mixture: mix 60 parts by volume of propan-2-ol with...
 - 3.2. Solvent A: Place in a 1 litre graduated flask approximately...
 - 3.3. Solvent B: Pipette 2 ml of 3-aminopropan-1-ol and 10 ml...
 - 3.4. Aniline: If the optical density in the blank test exceeds...
 - 3.5. Standard gossypol solution A: Place 27,9 mg of gossypol acetate...
 - 3.6. Standard gossypol solution B: Place 27,9 mg of gossypol acetate...
4. Apparatus
 - 4.1. Mixer (tumbler): approximately 35 r.p.m.
 - 4.2. Spectrophotometer.
5. Procedure
 - 5.1. Test sample
 - 5.2. Determination of free gossypol
 - 5.3. Determination of total gossypol
6. Calculation of results
 - 6.1. From the specific optical density
 - 6.2. From a calibration curve
 - 6.2.1. Free gossypol
 - 6.2.2. Total gossypol
 - 6.3. Repeatability

B. DETERMINATION OF THE LEVELS OF DIOXINS (PCDD/PCDF) AND PCBs

CHAPTER I

Methods of sampling and interpretation of analytical results

1. Scope and definitions
2. Compliance of the lot or subplot with the maximum level...
 - 2.1. As regards non-dioxin-like PCBs
 - 2.2. As regards PCDD/Fs and dioxin-like PCBs
3. Results exceeding action thresholds as laid down in the CMU...

CHAPTER II

Sample preparation and requirements for methods of analysis used in...

1. Field of application
2. Background
3. Quality assurance requirements
 - 3.1. Measures shall be taken to avoid cross-contamination at each stage...
 - 3.2. The samples shall be stored and transported in glass, aluminum,...
 - 3.3. The sample storage and transportation shall be performed in a...
 - 3.4. Insofar as relevant, each laboratory sample shall be finely grinded...

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- 3.5. Control of reagents, glassware and equipment for possible influence of...
- 3.6. A blank analysis shall be performed by carrying out the...
- 3.7. For bioanalytical methods, all glassware and solvents used in analysis...
- 3.8. Sample quantity used for the extraction shall be sufficient to...
- 3.9. The specific sample preparation procedures used for the products under...
4. Requirements for laboratories
 - 4.1. In accordance with the provisions of Regulation (EU) 2017/625 ,...
 - 4.2. Laboratory proficiency shall be proven by the continuous successful participation...
 - 4.3. Laboratories applying screening methods for the routine control of samples...
5. Basic requirements to be met by analytical procedure for dioxins...
 - 5.1. Low working range and limits of quantification
 - 5.2. High selectivity (specificity)
 - 5.2.1. A distinction is required between PCDD/Fs and dioxin-like PCBs and...
 - 5.2.2. Bioanalytical methods shall be able to detect the target compounds...
 - 5.3. High accuracy (trueness and precision, bioassay apparent recovery)
 - 5.3.1. For GC-MS methods, the determination shall provide a valid estimate...
 - 5.3.2. For bioanalytical methods, the bioassay apparent recovery shall be determined....
 - 5.4. Validation in the range of maximum level and general quality...
 - 5.4.1. Laboratories shall demonstrate the performance of a method in the...
 - 5.4.2. Regular blank controls and spiking experiments or analysis of control...
 - 5.5. Limit of quantification
 - 5.5.1. For a bioanalytical screening method, the establishment of the limit...
 - 5.5.2. The LOQ for a confirmatory method shall be about one...
 - 5.6. Analytical criteria
 - 5.7. Specific requirements for screening methods
 - 5.7.1. Both GC-MS and bioanalytical methods may be used for screening....
 - 5.7.2. Laboratories applying screening methods for the routine control of samples...
 - 5.7.3. Performance verification of the screening method is required during routine...
 - 5.7.4. Check on possible suppression of the cell response and cytotoxicity:...
 - 5.7.5. Quality control on compliant samples:
 - 5.7.6. Determination of false-compliant rates from quality control data:
 - 5.7.7. Potential non-compliant samples from screening shall always be verified by...
 - 5.7.8. Under validation conditions, bioanalytical methods shall provide a valid indication...
6. SPECIFIC requirements for GC-MS methods to be complied with for...

- 6.1. Acceptable differences between upper-bound and lower-bound WHO-TEQ results
- 6.2. Control of recoveries
 - 6.2.1. Addition of 13 C-labelled 2,3,7,8-chlorine-substituted internal PCDD/F standards and of...
 - 6.2.2. Relative response factors shall also be determined for those congeners...
 - 6.2.3. For feed of plant origin and feed of animal origin...
 - 6.2.4. Prior to GC-MS analysis, 1 or 2 recovery (surrogate) standard(s)...
 - 6.2.5. Control of recovery is required. For confirmatory methods, the recoveries...
- 6.3. Removal of interfering substances
- 6.4. Calibration with standard curve
- 6.5. Specific criteria for confirmatory methods
7. Specific requirements for bioanalytical methods
 - 7.1. Evaluation of the test response
 - 7.1.1. General requirements
 - 7.1.2. Calibration
 - 7.1.2.1. Calibration with standard curve
 - 7.1.2.2. Calibration with reference samples
 - 7.1.3. Separate determination of PCDD/Fs and dioxin-like PCBs
 - 7.1.4. Bioassay apparent recoveries
 - 7.1.5. Control of recoveries for clean-up
 - 7.1.6. Reporting limit
 - 7.2. Use of reference samples
 - 7.2.1. Reference samples shall represent sample matrix, congener patterns and concentration...
 - 7.2.2. A matrix blank, and where it is not possible, a...
 - 7.2.3. Reference samples chosen to perform a recovery correction shall be...
 - 7.2.4. Extra reference samples at e.g. 0,5x and 2x the maximum...
 - 7.3. Determination of cut-off values
 - 7.3.1. Use of the lower band of the 95 % prediction...
 - 7.3.2. Calculation from bioanalytical results (corrected for blank and recovery) of...
 - 7.3.3. Calculation as mean value of bioanalytical results (in BEQ, corrected...
Figure 1
 - 7.3.4. Restrictions to cut-off values
 - 7.4. Performance characteristics
 - 7.4.1. Since no internal standards can be used in bioanalytical methods,...
 - 7.4.2. As part of the validation process, the test shall be...
 - 7.4.3. Target compounds, possible interferences and maximum tolerable blank levels shall...
 - 7.4.4. The percent standard deviation in the response or concentration calculated...
 - 7.4.5. The uncorrected results of the reference sample(s) expressed in BEQ...
 - 7.4.6. Quality control charts for procedure blanks and each type of...
 - 7.4.7. The results from the confirmatory methods of suspected samples and...

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- 7.4.8. Successful method performance may also be demonstrated by participation in...
- 7.4.9. During incidents, the cut-off values may be re-evaluated, reflecting the...
- 8. Reporting of the results
 - 8.1. Confirmatory methods
 - 8.1.1. The analytical results shall contain the levels of the individual...
 - 8.1.2. The report shall include the method used for extraction of...
 - 8.1.3. The recoveries of the individual internal standards shall be made...
 - 8.1.4. As the expanded measurement uncertainty is to be taken into...
 - 8.1.5. The results shall be expressed in the same units and...
 - 8.2. Bioanalytical screening methods
 - 8.2.1. The result of the screening shall be expressed as 'compliant'...
 - 8.2.2. In addition, an indicative result for PCDD/Fs and/or dioxin-like PCBs...
 - 8.2.3. Samples with a response below the reporting limit shall be...
 - 8.2.4. For each type of sample matrix, the report shall mention...
 - 8.2.5. The report shall mention the type of the test applied,...
 - 8.2.6. The report shall include the method used for extraction of...
 - 8.2.7. In case of samples suspected to be non-compliant, the report...
 - 8.2.8. Non-compliant results shall only be reported from confirmatory analysis.
 - 8.3. Physico-chemical screening methods
 - 8.3.1. The result of the screening shall be expressed as 'compliant'...
 - 8.3.2. For each type of sample matrix, the report shall mention...
 - 8.3.3. In addition, levels for individual PCDD/F and/or dioxin-like PCB congeners...
 - 8.3.4. The recoveries of the individual internal standards shall be made...
 - 8.3.5. The report shall mention the GC-MS method applied.
 - 8.3.6. The report shall include the method used for extraction of...
 - 8.3.7. In case of samples suspected to be non-compliant, the report...
 - 8.3.8. Non-compliance can only be decided after confirmatory analysis.

CHAPTER III

Sample preparation and requirements for methods of analysis used in...

- 1. Field of application
- 2. Applicable detection methods
- 3. Identification and confirmation of analytes of interest
 - 3.1. Relative retention time in relation to internal standards or reference...
 - 3.2. Gas chromatographic separation of the non-dioxin-like PCBs from interfering substances,...
 - 3.3. Requirements for GC-MS techniques
 - 3.4. Requirements for GC-ECD techniques
- 4. Demonstration of performance of method
- 5. Limit of quantification
- 6. Quality control
- 7. Control of recoveries

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- 7.1. Suitable internal standards with physico-chemical properties comparable to analytes of...
- 7.2. Addition of internal standards:
- 7.3. Requirements for methods using all six isotope-labelled non-dioxin-like PCB congeners...
- 7.4. Requirements for methods using not all six isotope-labelled internal standards...
- 7.5. The recoveries of unlabelled congeners shall be checked by spiked...
- 8. Requirements for laboratories
- 9. Performance characteristics: criteria for the sum of non-dioxin-like PCBs at...
- 10. Reporting of the results
 - 10.1. The analytical results shall contain the levels of the individual...
 - 10.2. The report shall include the method used for the extraction...
 - 10.3. The recoveries of the individual internal standards shall be made...
 - 10.4. As the expanded measurement uncertainty is to be taken into...
 - 10.5. The results shall be expressed in the same units and...

ANNEX VI

METHODS OF ANALYSIS FOR THE DETERMINATION OF CONSTITUENTS OF ANIMAL ORIGIN FOR THE OFFICIAL CONTROL OF FEED

- 1. PURPOSE AND SCOPE
- 2. METHODS
 - 2.1. Light microscopy
 - 2.1.1. Principle
 - 2.1.2. Reagents and equipment
 - 2.1.2.1. Reagents
 - 2.1.2.1.1. Concentrating agent
 - 2.1.2.1.1.1. Tetrachloroethylene (specific gravity 1,62)
 - 2.1.2.1.1.2. Staining reagent
 - 2.1.2.1.1.2.1. Alizarin Red solution (dilute 2,5 ml 1M hydrochloric acid in...)
 - 2.1.2.1.1.3. Mounting media
 - 2.1.2.1.1.3.1. Glycerol (undiluted, viscosity: 1 490 cP) or a mounting medium...
 - 2.1.2.1.1.3.2. Norland® Optical Adhesive 65 (viscosity: 1 200 cP) or...
 - 2.1.2.1.1.4. Mounting media with staining properties
 - 2.1.2.1.1.4.1. Lugol solution (dissolve 2 g potassium iodide in 100 ml...)
 - 2.1.2.1.1.4.2. Cystine reagent (2 g lead acetate, 10 g NaOH/100 ml...)
 - 2.1.2.1.1.4.3. Fehling's reagent (prepared before use from equal parts (1/1) of...)
 - 2.1.2.1.1.4.4. Tetramethylbenzidine/Hydrogen peroxide. (dissolve 1 g 3,3',5,5' tetramethylbenzidine (TMB) in 100...)
 - 2.1.2.1.1.5. Rinsing agents
 - 2.1.2.1.1.5.1. Ethanol ≥ 96 % (technical grade)

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- 2.1.2.1.5 ~~A~~ Acetone (technical grade)
 - 2.1.2.1.6 ~~B~~ Bleaching reagent
 - 2.1.2.1.6.1 ~~C~~ Commercial sodium hypochlorite solution (9 - 14 % active chlorine)...
 - 2.1.2.2. Equipment
 - 2.1.2.2.1 ~~A~~ Analytical balance with an accuracy of 0,001 g
 - 2.1.2.2.2 ~~X~~ Grinding equipment: knife or rotor mill. If a rotor mill...
 - 2.1.2.2.3 ~~S~~ Sieves with square meshes of 0,25 mm and 1 mm...
 - 2.1.2.2.4 ~~C~~ Conical glass separation funnel with a content of 250 ml...
 - Separation funnel
 - 2.1.2.2.5 ~~S~~ Stereomicroscope covering at least a 6,5× to 40× final magnification...
 - 2.1.2.2.6 ~~C~~ Compound microscope covering at least a 100× to 400× final...
 - 2.1.2.2.7 ~~S~~ Standard laboratory glassware
 - 2.1.2.2.8 ~~E~~ Equipment for slide preparation: classical microscope slides, hollow slides, coverslips...
 - 2.1.2.2.9 ~~L~~ Laboratory oven
 - 2.1.2.2.10 ~~O~~ Centrifuge
 - 2.1.2.2.11 ~~F~~ Filter paper: qualitative cellulose filter (pore size 4-11 µm)
 - 2.1.3. Sampling and sample preparation
 - 2.1.3.1. Sampling
 - 2.1.3.2. Precautions to be taken
 - 2.1.3.3. Preparation of samples other than fat or oil
 - 2.1.3.3.1 ~~S~~ Sample drying : samples with a moisture content > 14...
 - 2.1.3.3.2 ~~S~~ Sample pre-sieving : in order to collect information on possible...
 - 2.1.3.3.3 ~~S~~ Sub-sampling and grinding : at least 50 g of the...
 - 2.1.3.3.4 ~~E~~ Extraction and preparation of the sediment : a portion of...
 - 2.1.3.3.5 ~~E~~ Extraction and preparation of the flotata : after recovery of...
 - 2.1.3.3.6 ~~P~~ Preparation of raw material : a portion of at least...
 - 2.1.3.4. Preparation of samples consisting of fat or oil
 - 2.1.3.5. Use of staining reagents
 - 2.1.4. Microscopic examination
 - 2.1.4.1. Slide preparation
 - 2.1.4.2. Observation flowchart for the detection of animal particles in compound...
 - 2.1.4.3. Number of determinations
 - 2.1.5. Expression of the results
- 2.2. PCR
- 2.2.1. Principle
 - 2.2.2. Reagents and equipment
 - 2.2.2.1. Reagents
 - 2.2.2.1.1 ~~R~~ Reagents for DNA extraction step
 - 2.2.2.1.2 ~~R~~ Reagents for genetic amplification step
 - 2.2.2.1.2.1 ~~P~~ Primers and probes
 - 2.2.2.1.2.2 ~~M~~ Master Mix

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- 2.2.2.1. ~~Decontamination reagents~~
 - 2.2.2.1.1. ~~Hydrochloric acid solution (0,1 N)~~
 - 2.2.2.1.2. ~~Bleach (solution of sodium hypochlorite at 0,15 % of active...)~~
 - 2.2.2.1.3. ~~Non-corrosive reagents for decontaminating costly devices like analytical balances (e.g....)~~
- 2.2.2.2. Equipment
 - 2.2.2.2.1. ~~Analytical balance with an accuracy of 0,001 g~~
 - 2.2.2.2.2. ~~Grinding equipment~~
 - 2.2.2.2.3. ~~Thermocycler enabling real-time PCR~~
 - 2.2.2.2.4. ~~Microcentrifuge for microfuge tubes~~
 - 2.2.2.2.5. ~~Set of micropipettes allowing to pipet from 1 µl up...~~
 - 2.2.2.2.6. ~~Standard molecular biology plastic-ware: microfuge tubes, filtered plastic tips for...~~
 - 2.2.2.2.7. ~~Freezers to store samples and reagents~~
- 2.2.3. Sampling and sample preparation
 - 2.2.3.1. Sampling
 - 2.2.3.2. Sample preparation
- 2.2.4. DNA extraction
- 2.2.5. Genetic amplification
- 2.2.6. Interpretation and expression of results
 - 2.2.6.1. Negative result
 - 2.2.6.2. Positive result

ANNEX VII

METHOD OF CALCULATING THE ENERGY VALUE OF POULTRYFEED

1. Method of calculation and expression of energy value
2. Tolerances applicable to declared values
3. Expression of result
4. Sampling and analysis methods

ANNEX VIII

METHODS OF ANALYSIS TO CONTROL ILLEGAL PRESENCE OF NO LONGER AUTHORISED ADDITIVES IN FEED

Important notes:

- A. DETERMINATION OF METHYL BENZOQUATE
 1. Purpose and scope
 2. Principle
 3. Reagents
 - 3.1. Dichloromethane
 - 3.2. Methanol, equivalent to HPLC grade
 - 3.3. HPLC mobile phase
 - 3.4. Methanesulfonic acid solution, c = 2 %

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- 3.5. Hydrochloric acid solution, $c = 10 \%$
 - 3.6. Cation-exchange resin Amberlite CG-120 (Na), 100 to 200 mesh
 - 3.7. Standard substance: pure methyl benzoate (7-benzyloxy-6-butyl-3-methoxycarbonyl-4-quinolone)
 - 3.7.1. Methyl benzoate stock standard solution, $500 \mu\text{g/ml}$
 - 3.7.2. Methyl benzoate intermediate standard solution, $50 \mu\text{g/ml}$
 - 3.7.3. Calibration solutions
 4. Apparatus
 - 4.1. Laboratory shaker
 - 4.2. Rotary film evaporator
 - 4.3. Glass column ($250 \text{ mm} \times 15 \text{ mm}$) fitted with a...
 - 4.4. HPLC equipment with variable wavelength ultraviolet detector or diode-array detector...
 - 4.4.1. Liquid chromatographic column: $300 \text{ mm} \times 4 \text{ mm}$, C18, 10...
 - 4.5. Membrane filters, $0,22 \mu\text{m}$
 - 4.6. Membrane filters, $0,45 \mu\text{m}$
 5. Procedure
 - 5.1. General
 - 5.1.1. A blank feed shall be analysed to check that neither...
 - 5.1.2. A recovery test shall be carried out by analysing the...
 - 5.2. Extraction
 - 5.3. Liquid-liquid partition
 - 5.4. Ion-exchange chromatography
 - 5.4.1. Preparation of the cation-exchange column
 - 5.4.2. Column chromatography
 - 5.5. Liquid-liquid partition
 - 5.6. HPLC determination
 - 5.6.1. Parameters
 - 5.6.2. Calibration graph
 - 5.6.3. Sample solution
 6. Calculation of results
 7. Validation of the results
 - 7.1. Identity
 - 7.1.1. Co-chromatography
 - 7.1.2. Diode-array detection
 - 7.2. Repeatability
 - 7.3. Recovery
 8. Results of a collaborative study
- B. DETERMINATION OF OLAQUINDOX**
1. Purpose and scope
 2. Principle
 3. Reagents
 - 3.1. Methanol.
 - 3.2. Methanol, equivalent to HPLC grade.
 - 3.3. Water, equivalent to HPLC grade.
 - 3.4. Mobile phase for HPLC.
 - 3.5. Standard substance: pure olaquinox 2-[N-2'-(hydroxyethyl)carbamoyl]-3-methylquinoxaline-N1,N4-dioxide, E 851.
 - 3.5.1. Olaquinox stock standard solution, $250 \mu\text{g/ml}$
 - 3.5.2. Olaquinox intermediate standard solution, $25 \mu\text{g/ml}$
 - 3.5.3. Calibration solutions

4. Apparatus
 - 4.1. Ultrasonic bath
 - 4.2. Mechanical shaker
 - 4.3. HPLC equipment with variable wavelength ultraviolet detector or diode array...
 - 4.3.1. Liquid chromatographic column, 250 mm × 4 mm, C18, 10...
 - 4.4. Membrane filters, 0,45 µm
5. Procedure
 - 5.1. General
 - 5.1.1. A blank feed shall be analysed to check that neither...
 - 5.1.2. A recovery test shall be carried out by analysing the...
 - 5.2. Extraction
 - 5.3. HPLC determination
 - 5.3.1. Parameters:
 - 5.3.2. Calibration graph
 - 5.3.3. Sample solution
6. Calculation of the results
7. Validation of the results
 - 7.1. Identity
 - 7.1.1. Co-chromatography
 - 7.1.2. Diode array detection
 - 7.2. Repeatability
 - 7.3. Recovery
8. Results of a collaborative study
9. Observation

C. DETERMINATION OF AMPROLIUM

1. Purpose and Scope
2. Principle
3. Reagents
 - 3.1. Methanol.
 - 3.2. Acetonitrile, equivalent to HPLC grade.
 - 3.3. Water, equivalent to HPLC grade.
 - 3.4. Sodium dihydrogen phosphate solution, c = 0,1 mol/l.
 - 3.5. Sodium perchlorate solution, c = 1,6 mol/l.
 - 3.6. Mobile phase for HPLC (see observation 9.1).
 - 3.7. Standard substance: pure amprolium, 1-[(4-amino-2-propylpyrimidin-5-yl)methyl]-2-methyl-pyridinium chloride hydrochloride, E 750 (see...)
 - 3.7.1. Amprolium stock standard solution, 500 µg/ml
 - 3.7.2. Amprolium intermediate standard solution, 50 µg/ml
 - 3.7.3. Calibration solutions
 - 3.8. Extraction solvent.
4. Apparatus
 - 4.1. HPLC equipment with injection system, suitable for injection volumes of...
 - 4.1.1. Liquid chromatographic column 125 mm × 4 mm, cation exchange...
 - 4.1.2. UV detector with variable wavelength adjustment or diode array detector....
 - 4.2. Membrane filter, PTFE material, 0,45 µm.
 - 4.3. Membrane filter, 0,22 µm.
 - 4.4. Ultrasonic bath.
 - 4.5. Mechanical shaker or magnetic stirrer.

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5. Procedure
 - 5.1. General
 - 5.1.1. Blank feed
 - 5.1.2. Recovery test
 - 5.2. Extraction
 - 5.2.1. Premixtures (content < 1 % amprolium) and feed
 - 5.2.2. Premixtures (content ≥ 1 % amprolium)
 - 5.3. HPLC determination
 - 5.3.1. Parameters:
 - 5.3.2. Calibration graph
 - 5.3.3. Sample solution
6. Calculation of the results
7. Validation of the results
 - 7.1. Identity
 - 7.1.1. Co-chromatography
 - 7.1.2. Diode array detection
 - 7.2. Repeatability
 - 7.3. Recovery
8. Results of a collaborative study
9. Observations
 - 9.1. If the sample contains thiamine, the thiamine peak in the...
 - 9.2. According to the British Pharmacopoeia, the spectrum of an amprolium...
 - 9.3. The extract must always be diluted with the mobile phase,...

D. DETERMINATION OF CARBADOX

1. Purpose and scope
2. Principle
3. Reagents
 - 3.1. Methanol.
 - 3.2. Acetonitrile, equivalent to HPLC grade.
 - 3.3. Acetic acid, w = 100 %.
 - 3.4. Aluminium oxide: neutral, activity grade I.
 - 3.5. Methanol-acetonitrile 1 + 1 (v + v).
 - 3.6. Acetic acid, σ = 10 %.
 - 3.7. Sodium acetate.
 - 3.8. Water, equivalent to HPLC grade.
 - 3.9. Acetate buffer solution, c = 0,01 mol/l, pH = 6,0....
 - 3.10. Mobile phase for HPLC.
 - 3.11. Standard substance.
 - 3.11.1. Carbadox stock standard solution, 100 µg/ml (see Note 5. Procedure):...
 - 3.11.2. Calibration solutions
 - 3.12. Water-[methanol-acetonitrile] (3.5) mixture, 300 + 700 (v + v).
4. Apparatus
 - 4.1. Laboratory shaker or magnetic stirrer.
 - 4.2. Glass fibre filter paper (Whatman GF/A or equivalent).
 - 4.3. Glass column (length 300 to 400 mm, internal diameter approximately...
 - 4.4. HPLC equipment with injection system, suitable for injection volumes of..
 - 4.4.1. Liquid chromatographic column: 300 mm x 4 mm, C18, 10...
 - 4.4.2. UV detector with variable wavelength adjustment or diode array detector...

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- 4.5. Membrane filter, 0,22 µm.
- 4.6. Membrane filter, 0,45 µm.
- 4.7. Ultrasonic bath.
5. Procedure
 - 5.1. General
 - 5.1.1. Blank feed
 - 5.1.2. Recovery test
 - 5.2. Extraction
 - 5.2.1. Feed
 - 5.2.2. Premixtures (0,1 %-2,0 %)
 - 5.2.3. Preparations (> 2 %)
 - 5.3. Purification
 - 5.3.1. Preparation of the aluminium oxide column
 - 5.3.2. Sample purification
 - 5.4. HPLC determination
 - 5.4.1. Parameters
 - 5.4.2. Calibration graph
 - 5.4.3. Sample solution
6. Calculation of the results
 - 6.1. Feed
 - 6.2. Premixtures and preparations
7. Validation of the results
 - 7.1. Identity
 - 7.1.1. Co-chromatography
 - 7.1.2. Diode-array detection
 - 7.2. Repeatability
 - 7.3. Recovery
8. Results of a collaborative study

ANNEX IX

CORRELATION TABLES REFERRED TO IN ARTICLE 6

1. Directive 71/250/EEC
2. Directive 71/393/EEC
3. Directive 72/199/EEC
4. Directive 73/46/EEC
5. Directive 76/371/EEC
6. Directive 76/372/EEC
7. Directive 78/633/EEC
8. Directive 81/715/EEC
9. Directive 84/425/EEC
10. Directive 86/174/EEC

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11. Directive 93/70/EEC
12. Directive 93/117/EC
13. Directive 98/64/EC
14. Directive 1999/27/EC
15. Directive 1999/76/EC
16. Directive 2000/45/EC
17. Directive 2002/70/EC
18. Directive 2003/126/EC

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- (1) OJ L 165, 30.4.2004, p. 1, corrected by OJ L 191, 28.5.2004, p. 1.
- (2) OJ L 155, 12.7.1971, p. 13.
- (3) OJ L 279, 20.12.1971, p. 7.
- (4) OJ L 123, 29.5.1972, p. 6.
- (5) OJ L 83, 30.3.1973, p. 21.
- (6) OJ L 102, 15.4.1976, p. 1.
- (7) OJ L 102, 15.4.1976, p. 8.
- (8) OJ L 206, 29.7.1978, p. 43.
- (9) OJ L 257, 10.9.1981, p. 38.
- (10) OJ L 238, 6.9.1984, p. 34.
- (11) OJ L 130, 16.5.1986, p. 53.
- (12) OJ L 234, 17.9.1993, p. 17.
- (13) OJ L 329, 30.12.1993, p. 54.
- (14) OJ L 257, 19.9.1998, p. 14.
- (15) OJ L 118, 6.5.1999, p. 36.
- (16) OJ L 207, 6.8.1999, p. 13.
- (17) OJ L 174, 13.7.2000, p. 32.
- (18) OJ L 209, 6.8.2002, p. 15.
- (19) OJ L 339, 24.12.2003, p. 78.

Changes to legislation:

There are currently no known outstanding effects for the Commission Regulation (EC) No 152/2009.