A.L. 93 ta' l-2005

ATT DWAR IS-SERVIZZI VETERINARJI (KAP. 437)

Regoli ta' l-2005 dwar l-Analiżi għall-Kontroll Uffiċjali ta' l-Għalf

BIS-SAĦĦA tas-setgħat mogħtija bl-artiklu 25 ta' l-Att dwar is-Servizzi Veterinarji, il-Ministru għall-Affarijiet Rurali u l-Ambjent għamel dawn ir-regoli li ġejjin:–

Titolu, skop u applikabilità.

1. (1) It-titolu ta' dawn ir-regoli hu Regoli ta' l-2005 dwar l-Analiżi għall-Kontroll Uffiċjali ta' l-Għalf.

(2) L-iskop ta' dawn ir-regoli hu l-implementazzjoni tar-regoli mniżżla taħt id-Direttiva tal-Kummissjoni ta' l-Unjoni Ewropea 72/199/KEE dwar metodi għal analiżi għall-kontroll uffiċjali ta' l-għalf.

(3) Malta teħtieġ li l-analiżi għall-kontroll uffiċjali ta' l-għalf, fir-rigward tal-kontenut tagħhom ta' lamtu, proteina nejja, proteina nejja li tinħall bil-*Pepsin* u *hydrochloric acid*, ta' *gossypol* totali u liberu u fir-rigward ta' l-attività tal-*Pepsin* għandhom isiru skond il-metodi mniżżla fl-Ewwel Skeda ta' dawn ir-regoli.

(4) Malta teħtieg li l-analiżi għall-kontroll uffiċjali ta' l-Għalf, biex jiġu determinati l-livelli ta' *tylosin* u *Virginiamycin* fl-għalf, isiru skond il-metodi mniżżla fit-Tieni Skeda li tinsab f'dawn ir-regoli.

L-EWWEL SKEDA
DETERMINAZZJONI TAL-KONTENUT TA' LAMTU

METODU POLARIMETRIKU

1. **Għan u Skop**
Dan il-metodu jippermetti d-determinazzjoni tal-kontenut ta' lamtu u ta' *high molecular weight* lamtu *degradation products* bIl-għan li tiġi ivverifikata l-konformita` mad-disposizzjonijiet tad-Direttiva tal-Kummissjoni ta' l-Unjoni Ewropea 86/174/EEC u tad-Direttiva tal-Kunsill ta' l-Unjoni Ewropea 96/25/KEE.

2. **Principju**
Dan il-metodu jinkorpora żewg determinazzjonijiet. Fl-ewwel determinazzjoni, il-kampjun għandu jiġi trattat meta jkun jahraq ma' hydrochloric acid dilwit. Wara li jiċċara u jiġi iffiltrat l-*optical rotation* tat-taħlita tiġi mkejla bil-*polarimetry*.

Fit-tieni determinazzjoni l-kampjuni jiġu estratti b' 40% *ethanol*. Wara li l-filtrat jiġi acidifikat bil-hydrochloric acid, jiċċara u jiġi iffiltrat, l-*optical rotation* trid titkejjel bhal qabel.

Id-differenza bejn iz-żewg qifsien immultiplikata b'fattur magħruf jagħti l-kontenut ta' lamtu fil-kampjun.

3. **Reaġenti**

3.1 25 % (w/w) hydrochloric acid, d: 1,126 g/ml.

3.2 1. 28 % (w/v) hydrochloric acid.

Il-konċentrazzjoni trid tiġi verifikata b' *titration* permezz ta' soluzzjoni ta' *sodium hydroxide* ta' 0,1 mol/litru fil-preżenza ta' 0,1 % (w/v) *methyl red* f' 94 % (v/v) *ethanol*. 10 ml = 30.94 ml of NaOH 0.1 mol/litru

3.3 Carrez solution I: ħoll 21,9 g zinc acetate $Zn(CH_3COO)_2\ 2H_2O$ u 3 g *glacial acetic acid* fl-ilma. Żid sakemm ikollok 100ml ilma.

3.4 Carrez solution II: ħoll 10,6 g *potassium ferrocyanide* $[K_4(Fe(CN)_6].3H_2O$ fl-ilma. Żid sal- marka ikollok 100 ml ilma.

3.5 40 % (v/v) ethanol, d: 0,948 g/ml f'temperatura ta' 20 ºC.

4. **Apparat**

4.1 *erlenmeyer flask* ta' 250ml b' *standard ground glass joint* u *reflux condenser*

4.2 *polarimeter* jew *saccharimeter*

5. Proċedura

5.1 *Preparazzjoni tal-Kampjun*

Farrak il-kampjun sakem ikun biżżejjed fin biex jgħaddi kollu minn għarbiel b'xibka b'toqob tondi ta' 0.5mm.

5.2 Determinazzjoni tat- *total optical rotation (P jew S) ara osservazzjoni (7.1)*

Izen 2.5 grammi tal-kampjun midhun sa l-eqreb milligram u poggih ġo *graduated flask* tal-100ml. Żid 25ml hydrochloric acid (3.2) u ħawwad bizzejjjed biex il-kampjun jitħallat sew u erga' żid 25ml hydrochloric acid . Għaddas il-*flask* f' *water bath* jagħli waqt li tħawwad bil-qawwa għall-ewwel tlett minuti biex tiġi evitata l-formazzjoni tal-agglomerati. Irid ikun hemm biżżejjed ilma fil-*water bath* biex jibqa; jagħli meta l-*flask* jitpoġġa fih. Il-*flask* m'għandux jitneħħa mil-banju waqt li tkun qed tħawwad. Wara 15-il minuta bl-ezatt neħħi l-*flask* minn ġol-banju u żid 30ml ilma kiesaħ u kessah mil-ewwel sa 20 ºC.

Żid 5ml Carrez *Solution I* (3.3) u ħawwad għal minuta. Wara żid 5ml Carrez *Solution II (3.4)* u erġa' ħawwad għal minuta. Żid l-ilma sal-marka ta' 100ml, ixxejkja u ffiltra. Jekk il-filtrat ikun għadu mdardar (li hu rari) irrepeti d-determinazzjoni billi tuza kwantita` akbar ta' Carrez *Solution I* u *II*, per eżempju 10ml.

Kejjel l-*optical rotation* tas-solution f'tubu ta' 200mm bil-*polarimeter* jew *saccharimeter.*

5.3 *Determinazzjoni ta' l-Optical Rotation (P jew S) ta' Sustanzi li Jinhallu f'40% Ethanol*

Izen 5grammi mil-kampjun sa l-eqreb milligram u poggih f' *graduated flask* tal-100 ml u żid xi 80ml *ethanol* (3.5) (ara osservazzjoni 7.2). Ħalli l-*flask* joqgħod għal xi siegħa f'temperatura ambjentali filwaqt li tħawwad bis-sahha għal xi sitt darbiet biex il-kampjun ittestjat jiġi mħallat sewwa mal-*ethanol* . Żid sal-marka b'40% *ethanol*, ħallat u iffiltrah. Iġbed b'pipetta 50ml mil-filtrat (=2.5g mill-kampjun) u poggih f' *Erlenmeyer flask* ta' 250ml, żid 2.1ml hydrochloric acid u ħawwad bis-sahha. Qabbad *reflux condenser* ma' l-*erlenmeyer flask* u għaddas il-*flask* ġo banjo mishun jagħli. Wara eżattament 15-il minuta, neħħi l-*erlenmeyer flask* mill-banju, laħalħu ġo ftit ilma kiesah, u keshu sa20 ºC. Saffi permezz ta'Carrez *Solution I* (3.3) u Carrez *Solution II (3.4)*u żid l-ilma sal-volum meħtieg, ħawwad, iffiltra u kejjel l-*optical rotation* kif indikat fit-tieni u t-tielet paragrafu ta' 5.2.

6. **Kalkolu tar-Riżultati**

Il-kontenut ta' lamtu (%) huwa kalkulat kif imniżżel hawn taħt:

Miżura bi *polarimeter*:

$$\text{Kontenut ta' lamtu (\%)} = \frac{2000(P - P')}{[\alpha]_D^{20^\circ}}$$

Fejn:

P = *Optical rotation* totali fi *angle degrees*

P' = *Optical rot*ation f' *angle degrees* tas-sustanza solubbli fi 40% (V/V) ethanol

$[\alpha]_D^{20^\circ}$ = *optical rotation* specifika tal-*pure starch*. Il-valuri numeriċi konvenzjonalment aċċettati għall-fattur huma dawn li ġejjin:

+ 185.9º: lamtu tar-ross
+ 185.4º: lamtu tal-patata
+ 184.6º: lamtu tal-qamħirrum
+ 182.7º: lamtu tal-qamħ
+ 181.5º: lamtu ax-xgħir
+ 181.3º: lamtu tal-ħafur
+ 184.0º: tipi ohra ta' lamtu u taħlitiet ta' lamtu f' għalf kompost.

6.2. Miżuri bis-*saccharimeter*

$$\text{Kontenut ta' lamtu (\%)} = \frac{2000}{[\alpha]_D^{20^\circ}} \times \frac{(2N \times 0{,}665) \times (S - S')}{100} - \frac{26.6N \times (S - S')}{[\alpha]_D^{20^\circ}}$$

Fejn:

S = *optical rotation* totali f' *saccharimeter degrees*

S′ = *optical rotation fi saccharimeter degrees* tas-sustanzi solubbli fi 40% (V/V) *ethanol*

N = piż (g) ta' saccgarose fi 100ml ta' ilma u li jagħti *optical rotation* ta' 100 *saccharimeter degrees* meta mkejjel permezz ta' 200mm *tube*

16,29 g għal *saccharimeters*

26,00 g għal *saccharimeters* Ġermaniżi

20,00 g għal *saccharimeters* mħallta.

6.3 Kif jiġi ripetut

Id-differenza bejn ir-riżultati ta' żewg determinazzjonijiet paralleli meħudin mill-istess kampjun ma jridux jeċċedu 0.4 tal-valur assolut għall-kontenut tal-lamtu inqas minn 40% u 1.1% relattivament għall-kontenut ta' lamtu ta' 40% jew aktar.

7. **Osservazzjonijiet:**

7.1 Jekk il-kampjun ikun fih aktar minn 6% c*arbonates*, ikkalkulati fis-sens ta' *calcium carbonate* dawn iridu jiġu distrutti b'trattament b' kwantita` eżatta u xierqa ta' *dilute sulphuric acid* qabel ma tiġi mkejla t-*total optical rotation*.

7.2 Fil-kaz ta' prodotti li għandhom kontenut għoli ta' *lactose* bħal trab tas- *serum* tal-ħalib jew trab tal-ħalib xkumat imxi hekk:

Wara li żżid 80ml ta' *ethanol* (3.5) qabbad *reflux condenser* mal-*flask* u għadsu ġo *water bath* f' 50 C għal nofs siegħa. Ħallih jiksah u kompli l-analiżi skund paragrafu 5.3.

7.3 F' każ fejn il-materjali tal-Għalf segwenti jkunu preżenti f'ammonti kbar f'Għalf imħallat, jistgħu jkunu ta' tfixkil fid-determinazzjoni tal-kontenut ta' lamtu bil-metodu polarimetriku u allura johorgu riżultati inkorretti:

- prodotti ta' *sugar beet* bħal *(sugar) beet pulp*, (*sugar) beet molasses, (sugar) beet pulp-molassed, (sugar) beet vinasse, (beet) zokkor,*
- polpa taċ-ċitru,
- kittien*, expeller* tal-kittien*, extraxtor tal-kittien,*
- *rape seed; rape seed expeller; rape seed extracted; rape seed hulls,*
- *żerriegħa tal-ġirasol; żerriegħa tal-ġirasol estratti; żerriegħa tal-ġirasol,* parzjalment dekortikati u estratti*,,*
- *copra expeller; copra extracted,*
- *polpa tal-patata,*
- ħmira mnixxfa*,*
- prodotti mimlijin *in inulin (eż. chips* u *meal tal-qaqoċċ ta' Jerusalem);*
- *greaves.*

2. **DETERMINAZZJONI TAL- PROTEINA KRUDA**

1.Għ**an u Skop:**
Dan il-metodu jiddetermina l-kontenut tal-proteina kruda tal-għalf fuq il-bazi tal-kontentut nitroġenu determinat skond il-metodu *Kjeldahl.*

2. **Principju**
Il-kampjun jiġi iddiġerit mis-*sulphuric acid* fil-preżenza ta' katalist. Is-soluzzjuni aċiduza ssir alkalina b'soluzzjoni ta' *sodium hydroxide*. L-*ammonia* tiġi distillata u miġbura f'kwantita` mkejla ta' *sulphuric acid*, iz-żejjed jgħaddi minn *titration* f'soluzzjoni *standard* ta' *sodium hydroxide.*

3 **Reaġenti**

3.1 *Potassium sulfate*.

3.2 Katalista*: copper (II) oxide* CuO jew *copper (II) sulfate pentahydrate*, $CuSO_4 \cdot 5H_2O$

3.3 Żingu granulat.

3.4 *Sulfuric acid*, ρ_{20} = 1,84 g/ml.

3.5 *Sulfuric acid* c(½H_2SO_4) = 0,5 mol/l.

3.6 *Sulfuric acid* c(½H_2SO_4) = 0,1 mol/l.

3.7 *Methyl red indicator*; ħoll 300 mg *methyl red* ġo 100 ml *ethanol*, σ 95-96 % (v/v)

3.8 Soluzzjoni ta' *sodium hydroxide (Technical grade* tista' tintuża) β = 40 g/100 ml (m/v: 40 %).

3.9 Soluzzjuni ta' *sodium hydroxide* c = 0,25 ml/l

.

3.10 Soluzzjoni ta' *sodium hydroxide* c = 0,1 mol/l.

3.11 Ħaffiefa granulata, maħsula fil- *hydrochloric acid* u maħruqa f'nar qawwi.

3.12 *Acetanilide (*m.p. = 114 ºC, N = 10,36 %)

3.13 *Sucrose* (bla nitroġenu).

4 Apparat

Dan l-apparat huwa adattat biex jitwettqu id-diġestjoni, id-distillazzjoni u t-*titration* skond il-proċedura *Kjeldahl.*

5.Proċedura

5.1 Diġestjoni

Iżen 1g tal-kampjun sa l-eqreb 0.001 g u poġġi l- kampjun ġol-*flask* tal-apparat tad-digestjoni. Żid 15g *potassium sulfate* (3.1), kwantita` xierqa ta' katalizzatur (3.2) (0.3 sa 0.4 gr *copper II oxide* jew 0.9g sa 1.2 g *copper II sulfate pentahydrate*, 25ml *sulphuric acid* (3.4), u ftit granuli ta' ħaffiefa (3.11) u ħallat kollox. Għal-ewwel saħħan il-*flask* moderament, ħawwad minn hin għall-iehor sakemm it-taħlita tikkarbonizza u r-ragħwa tkun għebet; wara saħħan fuq nar iktar qawwi sakemm jibda jagħli sewwa. Ikun issaħħan biżżejjed jekk l-acidu jagħli jibda jikkondensa mal-gnub tal-*flask*. Tħallix li l-ġnub jisħnu ż-żejjed u li l-partiċelli organiċi jeħlu

magħhom. Meta s-soluzzjoni tiċċara u ssir tixgħel ħadra, kompli għalli għal sagħtejn u wara ħalliha tiksaħ.

5.2 Distillazzjoni

Żid attentament biżżejjed ilma biex tassigura li s-*sulphates* idubu kompletament. Ħalli t-taħlita tiksah u żid ftit granuli taż-żingu (3.3).

Poggi kwantita` preċiza *sulphuric acid* (3.5) jew (3.6) fil-*collecting flask* tal-apparat tad-distillazzjoni skond il-kontenut prezunt ta' nitroġenu li hemm fih. Żid ftit qtar ta' methyl red indicator (3.7).

Għaqqad id-*digestion flask* mal-kondensatur tal-apparat tad-distillazzjoni u għarraq it-tarf tal-kondensatur fil-likwidu li hemm fil-*collecting flask* għal fond ta' mill-inqas 1cm (ara osservazzjoni 8.3) Ferra bil-mod 100ml soluzzjoni ta' *sodium hydroxide* (3.8) fid-digestion *flask* mingħajr telf ta' *ammonia.*

Saħħan il-*flask* sakemm l-*ammonia* kollha tiddistilla ruħha.

5.3 *Titration*

Għamel *titration* b'soluzzjoni ta' *sodium hydroxide* (3.9) jew (3.10) tas-*sulphuric acid* żejjed li hemm fil-collecting *flask*, skond il-konċentrazzjoni ta' *sulphuric acid* użata, sakemm jidher il-qiegħ.

5.*4 Blank test*

Biex tikkonferma li r-reaġenti huma ħielsa min-nitroġenu għamel *blank test* (digestjoni, distillazzjoni u *titration*) billi tuża 1gr *sucrose* (3.13) minflok il-kampjun.

6 Kalkolu tar-Riżultati

Il-kontenut tal-proteina kruda huwa ikkalkolat skond il-formula segwenti:

$$\frac{(V_0 - V_1) \times c \times 0.014 \times 100 \times 6.25}{m}$$

Fejn

V_0 = volum (ml) ta' NaOH (3.9 jew 3.10) li gie uzat fil-*Blank test*

V_1 = volum (ml) ta' NaOH (3.9 jew 3.10) li gie uzat fit-*titration* tal-kampjun

c = Konċentrazzjoni (mol/l) tas- *sodium hydroxide* (3.9 jew 3.10)

m = massa (gr) tal-kampjun

7.**Verifikazzjoni tal-Metodu**

7.1 Kif jiġi ripetut

Id-differenza bejn iz-żewg riżultati meħuda fl-istess hin ma tistax taqbez:

0.2% tal-valur assolut, fil-kaz ta' kontenut ta' proteina kruda ta' inqas minn 20%;

1% relattiv għal-ogħla valur, fil-kaz ta' kontenut ta' proteina kruda minn 20% so 40%;

0.4% tal- valur assolut, fil-kaz ta' kontenut ta' proteina kruda ta' iktar minn 40%.

7.2 Eżattezza

Għamel l-analiżi (digestjoni, distillazzjoni u *titration*) fuq 1.5gr sa 2gr *acetanilide* (3.12) fil-preżenti ta' 1gr *sucrose* (3.13); 1gr *acetanilide* jikkonsma 14.80ml i*sulphuric acid* (3.5). L-irkupru għandu jkun ta' mill-inqas 99%.

8. **Osservazzjonijiet**

8.1 L-apparat jista' jkun ta' tip manwali, semi-awtomatiku jew awtomatiku. Jekk l-apparat jeħtieġ transference bejn id-diġestjoni u d-distillazzjoni , it-trasferiment irid isir bla telf. Jekk il-*flask* tal-apparat tad-distillazzjoni mhux mgħammar b'*dropping funnel*, mill-ewwel żid is-*sodium hydroxide* qabel ma tqabbad il-*flask* mal-*condenser* waqt li tferra l-likwidu minn mal-genb.

8.2 Jekk id-digest jagħqad, erga ibda d-determinazzjoni billi tuza ammont akbar ta' *sulphuric acid* (3.4) milli kien specifikat l-ewwel darba.

8.3 Fi-kaz ta'prodotti li għandhom kontenut ta' nitroġenu baxx il-valur ta' *sulphuric acid* (3.6) li jrid jitpogga fil-*collecting flask* jista' jekk ikun hemm bzonn jiġi mnaqqas għal 10ml sa 15ml u miżjud sa 25ml bl-ilma.

3.DETERMINAZZJONI TAL-PROTEINA KRUDA MAĦLULA BIL-PEPSIN U L-*HYDROCHLORIC ACID*

1.**Għan u Skop**

Dan il-metodu jiddetermina l-frazzjoni ta' proteina kruda maħlula bil-pepsin u *hydrochloric acid* taħt kundizzjonijiet iddeterminati. Japplika għal materjali tal-Għalf kollha.

2.**Principju**

Il-kampjun irid jissaħħan għal 48 siegħa f'soluzzjoni ta' Pepsin *hydrochloride* f' 40 C. Is-suspensjoni trid tiġi iffiltrata u l-kontenut ta' nitroġenu tal-filtrat irid jiġi iddeterminat skond il-metodu għad-determinazzjoni tal-proteina kruda.

3.**Reaġenti**

3.1 Hydrochloric acid, d: 1:25

3.2 hydrochloric acid 0.075 N

3.3 2.0 U/mg Pepsin*;* l-attivita` tal-Pepsin hi definite fil-metodu deskritt f'Parti 4 ta' din l-Iskeda

3.4 Xi 2% w/v soluzzjoni ippreparata friska ta' Pepsin fil-hydrochloric acid (3.2): attivita` 400U/l.

3.5 *anti-foaming emulsion*

3.6 Ir-reaġenti kollha fil-lista taħt 3 fil-metodu tad-determinazzjoni tal-proteina kruda.

4.**Apparat**

4.1 *Water bath* jew inkubatur, issettjat fuq 40 ºC ± 1 C,

4.2 apparat tal-*Kjeldahl digestion* u *distillation*.

5.**Proċedura**

5.1 Preparazzjoni tas-soluzzjoni (ara osservazzjoni 7.2)

Izen 2gr tal-kampjun sa l-eqreb mg u poggih ġo *graduated flask* tal-500ml. Żid 450ml ta' soluzzjoni ta' Pepsin hydrochloride (3.4) li qabel tkun issaħnet sa 40C u ixxejkja biex ma jkunx hemm mil-formazzjoni ta' *agglomerate*. Ara li l-pH tas-suspensjoni tkun inqas minn 1.7. Poggi l-*flask* ġo *water bath* jew inkubatur u ħallih hemm għal 48 siegħa. Ixxejkja wara 8, 24 u 32 siegħa. Wara 48 siegħa, żid 15ml hydrochloric acid (3.1), mkessah sa 20 ºC , żid sal-marka bl-ilma u iffiltrah.

5.2 Digestjoni

Hu 250ml tal-filtrat u poġġih fil-*flask* tal-apparat tad-distillazzjoni (4.2). Żid ir-reaġenti meħtiega għad-digestjoni indikati fit-tieni sentenza ta' 5.1 tal-metodu għad-determinazzjoni tal-proteina kruda. Omogenizzah u għallih. Jekk tifforma xi ragħwa, żid xi qtar ta' *anti-foaming emulsion* (3.5). Kompli għallih bil-qawwa sakemm kwazi l-ilma kollu jkun evapora. Naqqas in-nar u elimina b'attenzjoni l-aħħar traċċi ta' ilma.

Meta s-soluzzjoni tiċċara u tkun bla kulur (jew aħdar ċar jekk intuża katalista ibbażat fuq iċ-ċomb), kompli għallih għal siegħa oħra. Ħalliha tiksaħ.

5.3 Distillazzjoni u *titration*

Ipproċedi kif indikat f' 5.2 u 5.3 tal-metodu tal-proteina kruda.

5.4 *Blank test*

Għamel *Blank test* bl-istess proċedura izda mingħajr il-kampjun għal-analiżi.

5 Kalkolu tar-Riżultati

Naqqas il-volum ta' *sulphuric acid* ikkunsmat fil-*Blank test* minn dak ikkunsmat mill-kampjun tat-test. 1ml *sulphuric acid* 0.1N jikkorrispondu għal 1.4mg nitroġenu.

Immoltiplika l-kwantita` ta' nitroġenu bil-fattur 6.25. Esprimi r-riżultat bħala fattur ta' l-eżempju.

*6.*Kif jiġi ripetut

Id-differenza bejn ir-riżultati taz-żewg determinazzjoniiet paralleli magħmula fuq l-istess kampjun ma jistgħux jeccedu:

- 0.4%, valur assolut, fil-każ ta' kontenut inqas minn 20%;
- 2.0%, valur relattiv, fil-każ ta' kontenut ta' mhux inqas minn 20% u mhux inqas minn 40%;
- 0.8, valur assolut, fil-każ ta' kontenut ta' iktar minn 40%

7.**Osservazzjonijiet**

7.1 Il-valuri miksubin b'dan il-metodu m'għandhom ebda konnessjoni diretta mad-digestibilita` *in vivo*.

7.2 Prodotti b'kontenut ta' żejt jew xaħam ogħla minn 10% iridu l-ewwel jgħaddu minn process ta' *defatting* permezz ta' estrazzjoni bil-*petroleum ether*. (B.P. 40 to 60 ºC)

4.**ESTIMAZZJONI TA' *PEPSIN ACTIVITY***

1. **Għan u Skop**

Dan il-metodu jistabbilixxi l-attivita` tal-Pepsin użata fil-determinazzjoni tal-proteina kruda maħlula bil-Pepsin u l-*hydrochloric acid*..

2.**Principju**

Il-*haemoglobin* tiġi ittrattata bil-Pepsin ġo *hydrochloric acid medium* taħt kundizzjonijiet definiti. Il-*non-hydrolyzed fraction* tal-Pepsin tiġi preċipitata ġo *trichloroacetic acid*. Reaġenti ta' *Sodium hydroxide* u Folin-Ciocalteu huma mizjuda

mal-filtrat. L-*optical density* tas-soluzzjoni hija mkejla f' 750nm u l-kwantita` korrispondenti ta' *tyrosine* tinqara minn *calibration curve*.

Definizzjoni: L-unita` tal-Pepsin hija definita b]ala l-kwantita` ta' dak l-enżima li, taħt il-kundizzjonijiet ta' dan il-metodu, tillibera kull minuta, numru ta' *hydroxyl groups* li, meta jittebbgħu bil-*Folin-Ciocaltue reaġent*, għandhom *optical density* li tikkorrispondi ma' umole *tyrosine* imtebba' fl-istess mod.

3.**Reaġenti**

3.1 Hydrochloric acid 0.2 N

3.2 Hydrochloric acid 0.06 N

3.3 Hydrochloric acid 0.025 N

3.4 5% soluzzjoni (w/v) *trichloroacetic acid*

3.5 soluzzjoni ta' *sodium hydroxide* 0.5N

3.6 Folin-Ciocalteu reaġent. Poġġi 100gr *sodium tunsgate* ($Na_2WO_4 . 2H_2O$), 25gr *sodium molybdate* ($Na_2MoO_4 . 2H_2O$) u 700 ml ilma ġo *flask* bil-qiegħ tond ta' 2 litri iffittjat b'*standard ground glass joint*. Żid 50ml *phosphoric acid* (d:1.71) u 100ml *concentrated hydrochloric acid* (d:1.19), qabbad *reflux condenser* mal-*flask*, għallih u żomm is-soluzzjoni tagħli bil-mod għal 10 sigħat. Ħalliha tiksah, aqla' r-*reflux condenser*, żid 175gr *lithium sulphate* ($Li_2SO_4 . 2H_2O$), 50ml ilma u 1ml *bromine*. Għallih għal 15-il minuta biex telimina *bromine* żejjed.

3.7 Soluzzjoni tal-*haemoglobin*: Iżen kwantita` ta' *haemoglobin* (xi 2gr substratum tal-proteina iddeterminat skond Anson) li jikkorrispondu għal 354mg nitroġenu [a] u poġġih ġo *flask* tal-200ml iffittjat b'*standard ground glass joint*. Żid ftit ml hydrochloric acid (3.2), qabbad il-*flask* mal-*vacuum* pump u ixxejkja sakemm il-*haemoglobin* tinħall kompletament. Itlaq il-*vacuum* u waqt lit kun qed tixxejkja, żid hydrochloric acid (3.2) sa 100ml. *Ippreparah eżatt qabel ma tuzah.*

3.8 Standard *tyrosine* solution: Ħoll 181.2 mg *tyrosine* fil-hydrochloric acid (3.1) u żid bl-istess aċdu sakemm ikollok 1l (stick solution). Hu 20.0ml u ħallat sa 1ooml bil-hydrochloric acid (3.1). 1ml ta' din is-soluzzjoni fih 0.2 µmole *tyrosine*.

4.**Apparat**

4.1 *Water bath* issetjat fuq 25 ºC ± 0·1 ºC fuq l-*ultrathermostat*

[a] Iddetermina il-kontenut ta' nitroġenu permezz tal- metodu semi-micro ta' Kjeldahl (kontenut teoretiku: 17.7% nitroġenu)

4.2 *spectrophotometer*

4.3 Kronometru, eżattezza: sekonda

5.2 pH-*meter*

5.**Proċedura**

5.1 Preparazzjoni tas-Soluzzjoni (ara osservazzjoni 12)

Ħoll 150mg pepsin f'1ooml *hydrochloric acid* (3.2). Permezz ta' pipetta itfa' 2ml mis-soluzzjoni ġo *graduated flask* tal-50ml u imla sal-marka bil-hydrochloric acid (3.3). Il-pH, meta tiġi eżaminata bil-*pH meter* trid tkun 1·6 ± 0·1. Għaddas il-*flask* ġol-*water bath*.

5.2 Hydrolysis

Itfa b'pipetta 5ml soluzzjoni ta'*haemoġlobin* (3.7) ġo test tube, saħħan sa 25C ġo *water bath* (4.1), żid 1ml soluzzjoni tal-Pepsin meħuda minn 5.1 u ħawwad b'ġlass rod għandha tarf wieħed eħxen mill-ieħor, b'qisu 10 daqqiet minn naħa għal-oħra. Ħalli t-test tube ġol-*water bath* f'25C għal għaxar minuti eżatti, ikkalkulati miż-żieda tas-soluzzjoni tal-pepsin (durazzjoni u temperatura jridu jkunu osservati attentament). Żid 10ml soluzzjoni ta' *trichloroacetic acid* (3.4) li tkun qabel issaħnet sa 25ºC, omogenizza u għaddih minn filter niexef.

5.3 *Żvilupp tal-Kulur u Tkejjil tal-Optical density*

Itfa b'pipetta 5.0 ml mill-filtrat ġo *Erlenmeyer Flask* tal-50ml, żid 10ml soluzzjoni tas-*sodium hydroxide* (3.5), u waqt li tixxejkja mingħajr ma tieqaf, 3.0ml *Folin-Ciocalteu reaġent* imħallat (3.6). Wara 5 sa 10 minuti, determina l-*optical density* tas-soluzzjoni b'*spectrophometer* fuq 750nm f'ċellulli ħoxnin 1cm kontra l-ilma.

5.4 *Blank test*

Għal kull determinazzjoni, għamel *Blank test* f'dan il-mod:

Itfa' b'pipetta 5ml soluzzjoni tal-*haemoglobin* (3.7) ġo *test tube,* saħħan sa 25ºC ġo *water bath* (4.1), żid 10ml soluzzjoni tat-*trichloroacetic acid* (3.4) li qabel tkun issaħnet sa 25C, omogenizza u żid 1.0ml soluzzjoni tal-Pepsin meħuda minn 5.1. Ħawwad b'*glass rod* u ħalli t-*test tube* ġol-*water bath* (4.1) f'25ºC għal għaxar minuti eżatti. Omogenizza u għaddi minn filter niexef. Imxi mal-proċedura f'5.3

5.5*Calibration curve*

Itfa' 1.0, 2.0, 3.0, 4.0, 5.0 ml *aliquots* soluzzjoni tat-*tyrosine standard* (3.8) li tikkorrispondi għal 0.2, 0.4, 0.6, 0.8 and 1.0 *µmoles* tat-*tyrosine* repettivament

ġo *Erlenmeyer flask*s tal-50ml. Ikkompleta s-serje b' *reference solution* mingħajr *tyrosine*. Tella l-volumi sa 5.0ml bil-hydrochloric acid (3.1). Żid 10.0ml soluzzjoni tas-*sodium hydroxide* (3.5) u filwaqt li tixxejkja mingħajr waqfien, 3.0ml *Folin-Ciocalteu reaġent* imħallat (3.6). Kejjel l-*optical density* kif indikat fl-aħħar sentenza ta' 5.3. Sib il-*calibration curve* billi tipplottja l-*optical densities* kontra l-kwantitajiet tat-*tyrosine*.

6.**Kalkolazzjoni tar-Riżultati**

Mill-*Calibration curve* aqra l-kwantita` tat-*tyrosine*, f'µmoles, li tikkorrispondi mal-*optical density* tas-soluzzjoni ikkulurita, korretta fuq il-bazi tal-*blank value*.

L-attivita` tal-Pepsin, f' *µmoles* ta' *tyrosine* f'25C, f'mg u kull minuta, tiġi ikkalkulata permezz tal-formula:

$$\text{Units għal kull mg (U/mg)} = \frac{0 \cdot 32a}{P}$$

Fejn

a = kwantita` ta' *tyrosine*, f' µmoles, moqrija mil-*Calibration curve*;

p= massa f'mg tal-kwantita` ta' Pepsin miżjuda f'5.2

7.**Osservazzjonijiet**

7.1 Il-kwantita` ta' Pepsin li trid tinħall trid tkun tali li, fit-tkejjil fotometriku tal-aħħar, *optical density* ta' 0.35 +/- 0.035 tiġi miksuba.

7.2 Żewg *units* għal kull mg meħuda b'dan il-metodu jikkorispondu għal:

3.64 *Anson milliunits*/mg (*µmoles tyrosine*/mg . min f'35.5 C)jew 36400 *commercial units*/g (*µmoles tyrosine*/g f'10 minuti f'35.5 C).

6 DETERMINAZZJONI TAL-*FREE AND TOTAL GOSSYPOL*

1.**Għan u Skop**

Dan il-metodu jiddetermina l-livelli ta' *free gossypol, total ġossypol* u sustanzi kimiċi ohra li huma relatati ma' dawn fil-*cottonseed, cottonseed meal* u l-*cottonseed cakes* fl-għalf kompost li fihom dawn is-sustanzi fejn iktar minn 20ppm huma preżenti.

2.**Principju**

Il-gossypol jiġi estratt fil-preżenti ta' *3-aminopropane-1-ol,* jew b'taħlita ta' *propan-2-ol* u *hexane*, għad-determinazzjoni tal-*free ġossypol,* jew bid-*dimethylformamide,* għad-

determinazzjoni tat-*total ġossypol.* Il-*ġossypol* jiġi konvertit bl-anilina f' *ġossypol dianiline*, li l-*optical density* tagħha hija mkejla bħala 440 nm.

3.**Reaġenti**

3.1 Taħlita ta' *propan-2-ol* u *hexane,* ħallat 60 parti volum ta' *propan-2-ol A.R.* ma' 40 parti volum ta' *n-hexane*.

3.2 *Solvent A:* Poggi qisu 500ml taħlita ta' *propan- 2-ol-hexane* ġo *graduated flask* tal-1l (3.1), 2ml *3-aminopropan-1-ol*, 8ml *glacial acetic acid* u 50ml ilma. Żid sal-marka bis-soluzzjoni tal-*propan-2-ol-hexane* (3.1) Dan ir-*reaġent* huwa stabbli għal gimgħa.

3.3*Solvent B:* Itfa' b'pipetta 3ml ta' 3-*aminopropan-1-ol* u 10ml *glacial acetic acid* fi *graduated flask* tal-100ml. Kessaħ sat-temperatura ambjentali u imla sal-marka b'N, *N-dimethylformamide*. Dan ir-reaġent huwa stabbli għal gimgħa.

3.4 Anilina *A.R.*: Jekk *l-optical density* fil-*blank test* teċċedi *0.022,* iddistilla l-anilina bit-trab taż-żingu, u armi l-ewwel u l-ahhar 10% tad-distillat. Jekk tinżamm fil-friġġ ġo *stoppered flask* tal-ħġieġ kannella, dan ir-*reaġent* għandu jżomm għal xhur.

3.5 Soluzzjoni Standard tal-*ġossypol A:* Poggi 27.9 mg *ġossypolacetate* f'*graduated flask* tal-250ml. Ħoll u imla sal-marka bis-*solvent* (3.2). Itfa' b'pipetta 50ml min din is-soluzzjoni ġo *graduated flask* tal-250ml u imla sal-marka b'*solvent A*. Il-konċentrazzjoni ta' *ġossypol* ta' din is-soluzzjoni hi 0.02 mg għal kull ml. Ħalliha toqgħod għal siegħa f'temperatura ambjentali qabel ma tużaha.

3.6 Soluzzjoni *Standard* tal-*ġossypol B:* Poġġi 27.9 mg *ġossypol acetate* fi *graduated flask* tal-50 ml u imla sal-marka b'*solvent A*. Il-konċentrazzjoni ta' *ġossypol f'din is-soluzzjoni* hija 0.5mg għal kull ml.

Is-soluzzjonijiet *standard* tal-*ġossypol* A u B jibqgħu stabbli għal 24 siegħa jekk dawn ma jarawx dawl.

4**. Apparat**

4.1 *Mixer (tumbler*): madwar 35rpm.

4.2 *Spectrophotometer.*

5. **Proċedura**

5.1 *Kampjun tat-test*

L-ammont ta' kampjun għat-test li jiġi użat jiddependi fuq il-kontenut tal-*gossypol* preżunt fil-kampjun. Preferibilment għandu jintużaw kampjun żgħir u

parti *aliquot* mil-filtrat relattivament żghira, biex jiġi miksub biżżejjed *gossypol* biex ikun possibli tkejjil fotometriku preċiż. Għad-determinazzjoni *tal-free gossypol,* fil-*cottonseed meal* u l-*cottonseed cake*, il-kampjun ghat-test ma għandux jeċċedi 1gr; fil-każ ta' għalf kompost, jista' jkun daqs 5gr. Parti *aliquot* mill-filtrat ta'10ml hija adegwata f'ħafna mil-każijiet; għandu jkun fiha 50 sa 100 µg *gossypol.* Għad-determinazzjoni tat-*total gossypol,* il-kampjun ghat -test għandu jkun bejn 0.5 u 5gr. Parti *aliquot* mill-filtrat fiha 40 to 200 µg *gossypol.*

L-analiżi għandha ssir f'temperatura ambjentali ta' madwar 20C.

5.2 Determinazzjoni tal-*free gossypol*

Poġġi l-kampjun tat-test ġo *ground-necked flask* tal-250ml, bil-qiegħ tal-*flask* miksi bil-ħgieg imkisser. Permezz ta' pipetta żid 50ml *solvent A* (3.2), poġġi *stopper* fuq il-*flask* u ħallat għal siegħa bil-*mixer.* Iffiltrah b'filter niexef u iġbor il-filtrat ġo *ground-necked flask* żgħir. Waqt il-filtrazzjoni, għatti l-*funnel* b' *watch glass*. Itfa' b'pipetta partijiet ta'l-*aliquot* identiċi tal-filtrat li fihom 50 sa 100 µg *gossypol* f'żewg *graduated flasks* tal-25ml (A u B). Jekk neċessarju, imla sal-marka ta' 10ml b'*solvent A* (3.2). Mbaghad imla l-kontenut ta' *flask A(3.2)* sal-marka bit-taħlita tal-*propan-2-ol-hexane(3.1).* Din is-soluzzjoni tiġi użata bħala soluzzjoni ta' referenza li magħha titkejjel is-soluzzjoni tal-kampjun.

ItFa' b'pipetta 10ml *solvent A* (3.2) ġo żewġ *graduated flasks* oħra tal-25 ml (C u D). Imla l-kontenut ta'*flask C* sal-marka bit-taħlita tal-*propanol-2-ol-hexane*(3.1). Din is-soluzzjoni tiġi użata bhla soluzzjoni ta' referenza ma xiex titkejjel is-soluzzjoni tal-*blank test.*

Żid 2ml anilina (3.4) ma' żewġ *flasks (*D u B). Saħħan għal 30 minuta fuq *water bath* jagħli biex jiżviluppa l-kulur. Kessaħ sat-temperatura ambjentali, żid sal-marka bit-taħlita tal-*propan-2-ol-hexane* (3.1), omoġenizzaha u ħalliha toqghod għal siegħa.

Iddetermina l-*optical density* tas-soluzzjoni tal-*blank test* (D) billi tqabbilha mas-soluzzjoni ta' referenza (C) u l-*optical density* tas-soluzzjoni tal-kampjun (B) li titqabbel mas-soluzzjoni ta' referenza (A) , fil-*spectrophotometer* f' 440nm b' ċelluli tal-ħġieġ ta' 1cm.

Naqqas l-*optical density* tas-soluzzjoni tal-*blank test* minn dik tas-soluzzjoni tal-kampjun (= *corrected optical density).* Minn dan il-valur ikkalkula il-kontenut tal-*free gossypol* kif indikat f'6.

5.3 Determinazzjoni tat-*total gossypol*

Poġġi l-kampjun tat-test li fih 1 to 5mg *gossypol* ġo *graduated flask* tal-5-ml u żid 10ml *solvent B* (3.3). Fl-istess ħin, ipprepara *blank test*, u itfa' 1oml *solvent B* (3.3) ġo graduated flsak ieħor tal-50ml. Saħħan iż-żewġ *flasks* fuq *water bath* jagħli għal

30 minuta. Kessaħ sat-temperatura ambjentali u żid il-kontenut taż-żewġ *flasks* sal-marka bit-taħlita tal-*propan-2-ol-hexane* (3.1). Omoġenizzah u ħallih joqgħod bejn 10 u 15-il minuta, iffiltra u iġbor il-filtrati fi *ground-necked flasks.*

Itfa' b'pipetta 2ml tal-filtrat tal-kampjun ġo żewġ *graduated flasks* tal-25ml, u 2ml *filtrate* tal-*blank test* ġo żewġ *graduated flasks* oħra tal-25ml. Imla l-kontenut ta' *flask* minn kull serje sal-marka ta' 25ml bit-taħlita tal-*propan-2-ol-hexane* (3.1). Dawn is-soluzzjonijiet jintużaw bħala soluzzjonijiet ta' referenza.

Żid 2ml anilina (3.4) f'żewġ *flasks* oħra. Saħħanhom għal 30 minuta fuq *waer bath* jagħli biex jiżviluppa l-kulur. Kessaħ sat-temperatura ambjentali, żid sal-marka ta' 25ml tat-taħlita tal-*propan-2-ol-hexane*, omoġenizzhom u ħallihom joqogħdu għal siegħa.

Iddetermina l-*optical density kif indikat* f'5.2 għal *free gossypol*. Minn dan il-valur ikkalkula l-kontenut tat-*total gossypol* kif indikat f'6.

6.**Kalkolu tar-Riżultati:**

Ir-riżultati jistgħu ikunu ikkalkulati jew mil-*optical density* (6.1), jew b'referenza għal *Calibration Curve* (6.2).

6.1 Mis-*specific optical density*

Is-*specific optical densities,* taħt il-kundizzjonijiet deskritti, ikunu dawn li ġejjin:

free gossypol: $E\ \frac{1\%}{1\,cm} = 625$

total gossypol: $E\ \frac{1\%}{1\,cm} = 600$

$$\%\ gossypol = \frac{E \cdot 1250}{E_{1\,cm}^{1\%} \cdot p \cdot a}$$

fejn:

E = *corrected optical density*, skond kif imnizzel fi 5.2;
p= *test sample* fi g;
a = *aliquot part* tal-*filtrate* f' ml.

6.2 Minn *calibration curve*

6.2.1 *Free gossypol*

Ipprepara żewġ serje ta' ħames *graduated flasks* tal-25ml.Itfa' b'pipetta *aliquots* ta' 2.0, 4.0, 6.0, 8.0 u 10.0 soluzzjoni tas-*standard gossypol A* (3.5) ġo kull serje

ta' *flasks.* Żid il-volum sal-marka ta' 10ml b'solvent A (3.2). Ikkompleta kull serje bi *graduated flask* b'10ml solvent A (3.2) (*blank test).*

Żid il-volum tal-*flasks* fl-ewwel serje (inkluż il-*flask tal*-blank test) sal-marka ta'25ml bit-taħlita tal-*propan-2-ol-hexane* (3.1) (serje ta' referenza).
Żid 2ml anilina (3.4) f'żewġ *flasks* oħra. Saħħanhom għal 30 minuta fuq *water bath* jagħli biex jiżviluppa l-kulur. Kessaħ sat-temperatura ambjentali, żid sal-marka ta' 25ml tat-taħlita tal-*propan-2-ol-hexane*, omoġenizzhom u ħallihom joqogħdu għal siegħa. (serje *standard*)

Iddetermina kif indikat f'5.2 l-*optical density* tas-soluzzjonijiet fis-serje *standard* mqabbla mas-soluzzjonijiet korrispondenti tas-serje ta' referenza. Sib il-*calibration curve* billi tipplottja l-*optical densities* kontra l-kwantitajiet ta' *gossypol.* (f'µg)

6.2.2 *Gossypol* totali

Ipprepara 6 *graduated flasks* tal-50ml. Fl-ewwel *flask* poġġi 10ml solvent B (3.3), u fl-oħrajn 2.0, 4.0, 6.0, 8.0, 10.0 ml soluzzjoni tas-*standard gossypol* (3.6) respettivament. Żid il-kontenut ta' kull flask sal-marka ta' 10ml b'solvent B(3.3). Saħħan għal 30 minuta fuq *water bath* jagħli. Kessaħ sat-temperatura ambjentali, żid il-kontenut taż-żewġ *flasks* sal-marka bit-taħlita tal-*propan-2-ol-hexane* (3.1) u omoġenizzah.

Itfa' 2ml ta' dawn is-soluzzjonijiet f'żewġ serje ta' sitt *graduated flasks* tal-25ml. Imla l-kontenut ta' kull *flask* ta' l-ewwel serje sal-marka bit-taħlita tal-*propan-2-ol-hexane* (3.1). (soluzzjonijiet ta' referenza.)

Żid 2ml anilina (3.4) f'kull *flask fit-tieni serje*. Saħħanhom għal 30 minuta fuq *water bath* jagħli. Kessaħ sat-temperatura ambjentali, żid sal-marka bit-taħlita tal-*propan-2-ol-hexane*, omoġenizzhom u ħallihom joqogħdu għal siegħa (serje *standard*)

Iddetermina kif indikat f'5.2 l-*optical density* tas-soluzzjonijiet fis-serje standard kif imqabbla mas-soluzzjonijiet korrispondenti fis-serije ta' referenza. Sib il-*calibration curve* billi tipplottja l-*optical densities* kontra l-kwantitajiet ta' *gossypol.* (f'µg)

6.3 Kif jiġi ripetut

Id-differenza bejn ir-riżultati ta' żewġ determinazzjonijiet paralleli meħuda mill-istess kampjun ma jistgħux jeċċedu:

- 15%, valur relattiv, fil-każ ta' kontenut ta' *gossypol* ta' inqas minn 500ppm;
- 75 ppm, valur relattiv, fil-każ ta' kontenut ta' mhux inqas minn 500ppm u mhux iktar minn 750ppm;

- 10%, valur relattiv, fil-każ ta' kontenut ta' iktar minn 750ppm.

SKEDA II

4.**DETERMINAZZJONI TA' *TYLOSIN***
- bid-diffużjoni fuq iz-zokkor-

1. **Għan u Skop**

Dan il-metodu jiddetermina il-kontenut ta' *tylosin* fl-għalf, koncentrati ta' l-għalf u *premixes* fejn hemm iktar minn 2ppm.

2. **Principju**

Il-kampjun jiġi trattat b'pH 8 *buffer solution*, imsaħna minn qabel sa 80C, u mbagħed estratt bil-*methanol* . Wara is-*centrifuging*, l-estratt jiġi mħallat u l-attivita` antibjotika tiegħu tiġi determinata billi titkejjel id-diffużjoni tat-*tylosin* fuq *agar medium* miżrugħ bis-*Sarcina lutea*. Id-diffużjoni hija eveidenti mil-formazzjoni ta' *inhibition zones* fil-preżenza ta' mikro-organiżmu. Id-diameru ta' dawn iz-zoni huwa direttament proporzjonali mal-*logarithm* tal-konċentrazzjoni ta' l-antibjotiku.

3. **Mikro-organiżmu: *Sarcina lutea* ATCC Nu 9341**

3.1 Manutenzjoni *tal-Parent Strain*

Inokula bis-*sarcina lutea tube* bis-*sloped agar* meħud mil-*culture medium* (4.1), adattah fuq pH 7.0. Matul il-lejl żommu inkubat f'madwar 35 ºC . Żomm il-*culture* f' friġġ u erġa' inokula is-*sloped agar* bih kull xahar.

3.2 Preparazzjoni tas-soluzzjoni tal-*bacteria*

Iġbor il-*bacteria* minn *tube sloped agar* ippreparat reċentement (3.1) b' 2 sa 3 ml *physiological saline* (4.4). B'din is-sospensjoni għammar *Roux flask* b'250ml *culture medium* (4.1) adattat fuq pH 7. Inkubah għal 24 siegħa f'35 ºC , mbagħad

iġbor il-*bacteria* f'25ml *physiological saline* (4.4). Omoġenizza u ħallat is-sospensjoni biex ikollok qisu 75% trasmissjoni tad-dawl f' 650nm.

Jekk tinżamm fil-friġġ, din is-sospensjoni tista' tintuża għal gimgħa.

B'testijiet preliminari fuq *plates* bil-*medium* bażiku għad-determinazzjoni (3.4), stabilixxi l-ammont ta' *inoculum*, li għal-konċentrazzjonijiet differenti ta' *tylosin* użat, jagħti l-ikbar *inhibition zones* possibli li għadhom ċari. Il-*culture medium* jigi inokulat minn 48 sa 50ºC.

4.***CULTURE MEDIA*** **U REAĠENTI**

4.1 *Medium* bażiku għad-determinazzjoni[1]

Glukosju	1g
Tryptic peptone	10g
Estratt tal-laħam	1.5g
Estratt tal-ħmira	3g
Agar, skond il-kwalita`	10 sa 20g
Ilma distillat	1000ml

Adattah eżattament qabel ma tużah fuq pH 7.0 għal-manutenzjoni tal-*parent strain* u l-preparazzjoni tas-suspensjoni tal-*bacteria,* u fuq pH 8.0 ghad-determinazzjoni

4.2 Soluzzjoni tal--*phosphate buffer,* pH 8

Potassium dihydrogen phosphate KH_2PO_4 A.R.	0·523 g
diPotassium hydrogen phosphate K_2HPO_4 A.R.	16·730 g
ilma distillat	1000 ml

4.3 Soluzzjoni tal-*Phosphate buffer*, pH 7

Potassium dihydrogen phosphate KH_2PO_4 A.R.	5.5g
Di potassium hydrogen phosphate K_2HPO_4 A.R.	13.6g
Ilma iddistillat	1000ml

[1] Jista' jinutża kull *culture medium* kummercjali b'komposizzjoni simili u li jagħti l-istess riżultati.

4.4 *Sterile physiological saline*

4.5 *Methanol* pur

4.6 40% (v/v) *methanol*

4.7 taħlita ta' soluzzjoni tal-*phosphate buffer* (4.2) / *methanol:* 60/40 bil-volum

4.8 *standard substance*: *tylosin* ta' attivita` magħrufa

5. Soluzzjonijiet *standard*

Nixxef is-sustanza niexfa (4.8)n għal 3 sighat f' 60 C ġo forn tal-*vacuum* (5mm mercury). Iżen 10 sa 50mg ġo *graduated flask*, ħoll f'75 ml *methanol* (4.5) u ħallat is-soluzzjoni bis-soluzzjoni tal-*phosphate buffer* pH7 (4.3), biex ikollok konċentrazzjoni ta' *tylosin-base* ta' 1000 ug għal kull ml.

Ipprepara *standard working solution* S8 b' 2 ug għal kull 1ml *tylosin base* minn din il-*stock solution* billi tħawwadha mat-taħlita (4.7).

Għal kontenuti ta' inqas minn 10ppm, evapora l-estratt sakemm jinxef f' *rotary evaporator* f' 35 C u ħoll ir-residwu f'40% *methanol* (4.6).

7.Metodu ta' determinazzjoni

7.1 Tilqima tal-*culture medium*

Laqqam f'temperatura bejn 48C U 50c il-*medium* bażiku għad-determinazzjoni (4.1), adatta fuq pH 8.0, bis-suspensjoni tal-*bacteria* (3.2).

7.2 Preparazzjoni tat-*trays*

Id-diffużjoni fuq l-*agar* ssir fuq *trays* b'4 konċentrazzjinijiet tas-soluzzjoni *standard* (S_8, S_4, S_2, S_1) u 4 konċentrazzjonijiet ta' l-estratt (U_8, U_4, U_2, U_1). L-4 konċentrazzjonijiet tas-*standard solution* u ta' l-estratt iridu jitqegħdu fit-*trays*.

Għalhekk għandhom jintgħażlu *trays* li huma kbar biżżejjed biex ikunu jistgħu isiru mill-inqas 8 toqob fl-*agar medium*. Ikkalkula l-kwantita` ta' *culture medium* imlaqqam (7.1) li hemm bżonn biex ikun hemm għata uniformi ta' madwar 2 mm ħxuna. Preferibilment it-test għandu jsir fuq *trays* ċatti li jikkonsistu f'*plates* tal-ħġieġ mimlijin b'ħolqa tal-aluminju jew tal-plastik perfettament livellata, b'diametru ta' 200mm u għoli ta' 20mm.

Permezz ta' pipetta qattar fit-toqob kwantitajiet preċiżi ta' bejn 0.10 u 0.15ml ta' soluzzjoni antibiotika, skond id-diametru tat-toqob.

Għal kull kampjun irrepeti id-diffużjoni mill-inqas 4 darbiet b'kull konċentrazzjoni biex kull determinazzjoni jkun fiha 32 *inhibition zones.*

7.3 *Inkubazzjoni*
Inkuba t-*trays* matul il-lejl f'temperatura bejn il-35 to 37 ºC..

8. **Evalwazzjoni**

Kejjel id-diametru ta' l-*inhibition zones,* preferibilment bi projezzjoni. Żomm rendikont tar-riżultati fuq *semi-logarithmic paper*, u ipplottja il-*logarithm* tal-konċentrazzjonijiet kontra d-diametru tal-*inhibition zones*. Ittrejsja l-linji tas-soluzzjoni *standard* u ta' l-estratt. Sakemm ma jkun hemm interferenza iż-żewġ linji għandhom ikunu paralleli.

Il-*logarithm* ta' l-attivita` relattiva tista' tiġi ikkalkulata permezz tal-formula segwenti:

$$\frac{(U_1 + U_2 + U_4 + U_8 - S_1 - S_2 - S_4 - S_8) \cdot 0 \cdot 602}{U_4 + U_8 + S_4 + S_8 - U_1 - U_2 - S_1 - S_2}$$

*Real activity = presumed activity * relative activity*

9. **Kif jiġi ripetut**

Id-differenza bejn ir-riżultati ta'żewġ determinazzjonijiet paralleli meħuda mill-istess kampjun ma jistgħux jeċċedu 10% f'valur relattiv.

5. DETERMINAZZJONI TAL-VIRGINIAMYCIN
- b'diffużjoni f' *agar medium*-

1. **Għan u skop**

Dan huwa metodu għad-determinazzjoni ta' *Virginiamycin* fl-għalf u *premixes*. Il-*lower limit* ta' determinazzjoni hu 2mg/kg (2ppm)[1].

2. **Prinċipju**

Il-kampjun jiġi estratt b' soluzzjoni metanolika ta' *Tween 80* . Poġġi l-estratt f' *decanter* jew *centrifuge* u ħalltu bl-ilma. Lattivita` antibiotika hija determinata billi tikejjel i-diffużjoni ta' *Virginiamycin* f' *agar medium* imlaqqam bil-*Micrococcus luteus.* Id-diffużjoni tidher bil-formazzjoni ta' żoni ta' inibizzjoni tal-mikro-organiżmu. Id-diametru ta' dawn iż-żoni huwa direttement proporzjonali mal-

[1] 1mg *Virginiamycin* huwa ekwivalenti għal 1000 UK *units*

logarithm tal-konċentrazzjoni antibiotika fuq il-medda ta' konċentrazzjonijiet antibiotiċi użati.

3.**Mikro-organiżmu: *Micrococcus luteus* ATCC 9341 (NCTC 8340, NCIB 8553)**

3.1 *Manutenzjoni ta' stock culture*

Laqqam *tubes* bil-*Micrococcus luteus* bis-*sloped agar* meħud mil-*culture medium* (4.1), u żommu inkubat għal 24 siegħa f' 30ºC. Żomm il-kultura fi friġġ li għandha temperature ta' madwar 4ºC u erġa' laqqam kull ġimagħtejn.

3.2 Preparazzjoni tas-soluzzjoni tal-*bacteria*
Aħsad il-*bacteria* minn *agar slope* ippreparata reċentement (3.1) b' 2 sa 3 ml soluzzjoni ta' *sodium choride* (4.4). B'din is-suspensjoni laqqam 250ml *culture medium* (4.1) f' *Roux flask* u inkubah bejn 18 u 20 siegħa f'30ºC , imbagħad iġbor il-*bacteria* f'25ml soluzzjoni tas- *sodium chloride* (4.3) u ħallat. Ħallat is-suspensjoni sa 1/10 b' soluzzjoni tas-*sodium chloride*.It- trażmissjoni tad-dawl għandha tkun madwar 75%, imkejla bħala 650nm f' ċellula ta' 1cm kontra soluzzjoni ta' *sodium chloride* (4.3). Is-suspensjoni tista' tinżamm għal ġimgħa f' madwar 4C.

4. ***Culture** media* u reaġenti

4.1 *Culture u assay medium*[a]

Meat peptone	6.0g
Tryptone	4.0g
Estratt tal-ħmira	3.0g
Estratt tal-laħam	1.5g
Glucose	1.0g
Agar	10 sa 20g
Ilma pH6.5 (wara s-sterilizzazzjoni)	1000ml

4.2 *Phosphate buffer pH6*

Potassium hydrogen phosphate, K_2HPO_4	2.0g
Potassium dihydrogen phosphate, KH_2PO_4	8.0g
Ilma	1000ml

4.3 Soluzzjoni tas-*sodium chloride* 0.8% (w/v): ħoll 8g *sodium chloride* fl-ilma u ħallat sa 1000ml, sterilizza.

4.4 *methanol*

4.5 taħlita ta' *phosphate buffer* (4.2) / *methanol* (4.4): 80/20

[a] Kull *culture medium* li għandu komposizzjoni simili u li jagħti l-istess riżultati jista' jiġi użat.

(v/v)

4.6. Soluzzjoni *Tween 80* metalonika 0.5% (w/v); ħoll 5g *Tween 80* f'*methanol* (4.4) u holl bil- *methanol* sa 1 000ml.

4.7 Sustanza *standard e: Virginiamycin* ta' attivita` magħrufa.

5. **Soluzzjonijiet standard**

Ħoll kwantita` preċiża tas-sustanza *standard* (4.7) fil-*methanol* (4.4) u ħallat bil-*methanol* (4.4) biex ikollok *stock solution* b' 1000 µg *Virginiamycin* għal kull ml.

Miżmuma fi *stopper flask* f' temperature ta' 4ºC din is-soluzzjoni tibqa' stabbli sa hamest ijiem.

Minn din is-*stock solution* ipprepara b'taħlita suċċessiva bit-taħlita (4.5) is-soluzzjonijiet segwenti:

S_8	1	µg/ml
S_4	0,5	µg/ml
S_2	0,25	µg/ml
S_1	0,125	µg/ml

6. **Preparazzjoni ta' l-** ***extract and assay solutions:***

6.1 Estrattazzjoni

6.1.1 Prodotti b'kontenut *ta' viriginiamycin* sa 100mg/kg

Iżen kwantita` ta' 50g mil-kampjun, żid 200ml soluzzjoni (4.6) u ixxejkja għal 30 minuta. Ħalliha toqgħod jew uża *centrifuge* , ħu 20ml mis-*supernatant solution* u evapora sa 5ml f'*rotary evaporator* f'temperatura mhux iktar minn 40ºC. Ħallat ir-residwu mat-taħlita (4.5) biex ikollok il-mistenni kontenut ta' *Virginiamycin* ta' 1µg/ml (= u_8).

6.1.2 Prodotti b'kontenut ta' *Virginiamycin* ta' aktar minn 100mg/kg

Iżen kwantita` mill-kampjun ta' mhux iktar minn 10.0g u b'kontenut ta' bejn 1 u 50mg *Virginiamycin*, żid 100ml soluzzjoni (4.6) u ixxejkja għal 30 minuta. Ħalliha toqgħod jew uża *centrifuge,* mbagħad ħallat is-*supernatant solution* mat-taħlita (4.5) biex ikollok il-mistenni kontenut ta' *Virginiamycin* ta' 1 µg/ml (= u_8).

6.2 *Assay solutions*

Minn soluzzjoni u_8 ippepara soluzzjonijiet u_4 (kontenut mistenni: 0.5 µg/ml), u_2 (kontenut mistenni:0.25ug/ml), u u_1 (kontenut mistenni : 0.125 µg/ml) permezz ta' diluzzjoni suċċessiva (1+1) mat-taħlita (4.5).

7. **Proċedura ta' l-*Assay***

7.1 Tilqim ta' l-assay medium

Laqqam l-*assay medium* (4.1) bil-*bacterial suspension* (3.1) f'madwar 50ºC. Permezz ta' testijiet preliminari fuq *plates* bil-*medium (4.1)* determina l-kwantita` ta' *bacterial suspension* meħtieġa biex ikollok iż-żoni ta' inibizzjoni kemm jista' jkun ċari u kbar mad-diversi konċentrazzjonijiet ta'*Virginiamycin.*

7.2 Preparazzjoni tal-*plates*

Id-diffużjoni fuq l-*agar* ssir fuq *plates* bl-4 konċentrazzjonijiet tas-*standard solution* (s_8, s_4, s_2 u s_1) u l-4 konċentrazzjonijiet ta' l-*assay solution* (u_8, u_4, u_2 and u_1). L-4 konċentrazzjonijiet tas-*standard solution* u ta' l-*assay solution* iridu jitpoġġew fil-*plates*. Għalhekk għandhom jintgħażlu plates li huma kbar biżżejjed biex ikunu jistgħu isiru mill-inqas 8 toqob fl-*agar medium* b'diametru ta' 10 sa 13mm u mhux inqas minn 30mm bejn iċ-ċentri. Ikkalkula l-kwantita` ta' *culture medium* imlaqqam (7.1) li hemm bżonn biex ikun hemm għata uniformi ta' madwar 2 mm ħxuna. It-test jista' jsir fuq plates li jikkonsisti f'lastra tal-ħġieġ b' ħolqa tal-aluminju jew tal-plastik fil-wiċċ, b'diametru ta' 200mm u għoli ta' 20mm.

Ferra ġol-*plates* kwantita` tal-*medium* (4.1) imlaqqma kif jidher f' 7.1, biex ikollok strat ta' xi 2mm ħxuna (60mm għal *plate* b' diametru ta'200mm). Ħallih joqgħod f'pożiżżjoni illivellata, għamel it-toqob u poġġi fihom volumi preċiżi ta'*assay* u *standard solutions* (bejn 0.10 u 0.15ml, skond id-diametru). Applika kull konċentrazzjoni mill-inqas erba' darbiet biex kull determinazzjoni tkun soġġetta għal determinazzjoni ta' 32 *inhibition zones.*

7.3 **Inkubazzjoni**

Żomm il-*plates* inkubati minn 16 sa 18-il siegħa f' 30 +/- 2 ºC

8. Evalwazzjoni

Kejjel id-diametru tal-*inhibition zones* sa l-eqreb 0.1mm. Żomm rendikont tal-miżuri medji ta' kull konċentrazzjoni fuq *semi-logarithmic graph paper* bil-*logarithm* tal-konċentrazzjonijiet relatati mad-diametri taz-zoni ta' inibizzjoni. Ipplottja il-*best fit lines* kemm tas-soluzzjoni *standard* kif ukoll tal-estratt, per eżempju:

Determina il-'*best fit' point* għal- *standard lowest level (SL)* permezz tal-formula:

(a) $SL = \frac{7_{S_1} + 4_{S_2} + _{S_4} - 2_{S_8}}{10}$

Determina il- *'best fit' point* għal- *standard highest level* (SH) permezz tal-formula:

(b) $SH = \frac{7_{S_8} + 4_{S_4} + _{S_2} - 2_{S_1}}{10}$

F'mod simili ikkalkula il-*'best fit' points* għal-*extract lowest level* (UL) u l-*extract highest level* (UH) billi tissostitwixxi u_1, u_2, u_4 u u_8 għal s_1, s_2, s_4 u s_8 fil-formuli ta' fuq.

Żomm rendikont tal-valuri kalkulati SL u SH fuq l-istess *graph paper* u għaqqadhom biex ikollok il-'*best fit' line* għas-*standard solution*. Żomm rendikont ta' UL u UH u għaqqadhom biex ikollok il-*'best fit' line* għall-estratt.

Jekk ma jkunx hemm interferenza il-linji għandhom ikunu paralleli. Għal skopijiet prattiċi il-linji għandhom jiġu ikkunsidrati paralleli jekk il-valuri (SH-SL) u (UH-UL) ma fihomx differenza ta' iktar minn 10% tal-valur tagħhom medju.

Jekk il-linji ma jkunux paralleli, u1, s1, u8 jew s8 jista' jintrema u SL, SH, UL u UH ikkalkulati permezz ta' dawn il-formuli alternattivi, biex ikollok '*best fit' lines* alternattivi:

(a′) $SL = \frac{5_{S_1} + 2_{S_2} - _{S_4}}{6}$ jew $\frac{5_{S_2} + 2_{S_4} - _{S_8}}{6}$

(b′) $SH = \frac{5_{S_4} + 2_{S_2} - _{S_1}}{6}$ jew $\frac{5_{S_8} + 2_{S_4} - _{S_2}}{6}$

U l-istess għal UL u UH. L- istess kriterji ta' paralleliżmu għandhom jiġu sodisfatti. Il-fatt li r-riżultat ikun ġie ikkalkulat fuq tlett livelli għandu jiġi annotati fuq ir-rapport finali.

Meta l-linji huma kunsidrati paralleli, ikkalkula il-*logarithm* ta' l-attivita` relattiva (log A) permezz ta' waħda minn dawn il-formuli, skond jekk tliet jew erba' livelli jkunux intużaw fl-istima tal-paralleliżmu.

Għal erba' livelli

$$\text{(c) Log A} = \frac{(U_1 + U_2 + U_4 + U_8 - S_1 - S_2 - S_4 - S_8) \times 0{,}602}{U_4 + U_8 + S_4 + S_8 - U_1 - U_2 - S_1 - S_2}$$

Ghal tlett livelli

$$\text{(d) Log A} = \frac{(U_1 + U_2 + U_4 - S_1 - S_2 - S_4 - S_8) \times 0{,}401}{U_4 + S_4 - U_1 - S_1}$$

jew

$$\text{(d′) Log A} = \frac{(U_2 + U_4 + U_8 - S_2 - S_4 - S_8) \times 0{,}401}{U_8 + S_8 - U_2 - S_2}$$

Attivita` ta' kampjun estratt = attivita` ta' *standard* rilevanti × A

$$(U_8 = s_8 \times A)$$

Jekk l-attivita` relattiva tkun barra l-medda ta' bejn 0.5 sa 2.0, irrepeti l-adattamenti tal-proċedura biex issir l-*assay* sa l-konċentrazzjonijiet eżatti, jew jekk mhux possibbli, sas-soluzzjonijiet *standard*.. Meta l-attivita` relattiva ma tistax tinġieb fil-medda meħtieġa, kull riżultat għandu jiġi kunsidrat bħala approssimattiv u dan għandu jitniżżel fir-rapport finali.

Meta l-linji huma ikkunsidrati mhux paralleli, irrepeti d-determinazzjoni. Jekk il-paralleliżmu ma jirriżultax, id-determinazzjoni għandha tiġi kunsidrata insodisfaċenti.

Esprimi r-riżultat f'milligrammi ta' *Virginiamycin* għal kull kilogram *ta'* għalf.

9. **Kif jiġi ripetut**

Id-differenza bejn ir-riżultati ta' żewġ determinazzjonijiet paralleli li jsiru fuq l-istess kampjun mill-istess analista ma għandhomx jeċċedu:

-2mg/kg, valur relattiv, fil-każ ta' kontenut ta' *Virginiamycin* sa 10mg/kg;
-20% relatat ma' l-ogħla valur fil-każ ta' kontenut ta' 10 sa 25mg/kg,
-5mg/kg, valur assolut, fil-każ ta, kontenut minn 25 sa 50mg/kg,
-10% relatat ma' l-ogħla valur fil-każ ta' kontenut ta' iktar minn 50mg/kg.

L.N. 93 of 2005

VETERINARY SERVICES ACT
(CAP. 437)

Methods for Analysis in the Official Control of Feedingstuffs Rules, 2005

IN exercise of the powers conferred by article 25 of the Veterinary Services Act, the Minister for Rural Affairs and the Environment has made the following rules:–

Title, scope and applicability.

1. (1) The title of these rules is the Methods for Analysis in the Official Control of Feedingstuffs Rules, 2005.

(2) The scope for these rules is to implement the rules found under EU Commission Directive 72/199/EEC establishing methods of analysis for the official control of feedingstuffs.

(3) Malta shall require that analyses for official controls of feedingstuffs, as regards their level of starch, crude protein, crude protein which can be dissolved by Pepsin and hydrochloric acid, of free and total gossypol and as regards pepsin activity be carried out using the methods described in Schedule I to these rules.

(4) Malta shall require that analyses for official controls of feedingstuffs for the purpose for determining the levels of tylosin, and virginiamycin in feedingstuffs, shall be carried out using the methods described in Schedule II to these rules.

SCHEDULE I

1. DETERMINATION OF STARCH

POLARIMETRIC METHOD

1. **Purpose and scope**

This method makes it possible to determine the levels of starch and of high molecular weight starch degradation products in feedingstuffs for the purpose of checking compliance with EU Commission Directive 86/174/EEC and EU Council Directive 96/25/EC.

2. **Principle**

The method comprises two determinations. In the first, the sample is treated when hot with dilute hydrochloric acid. After clarification and filtration the optical rotation of the solution is measured by polarimetry.

In the second, the sample is extracted with 40% ethanol. After acidifying the filtrate with hydrochloric acid, clarifying and filtering, the optical rotation is measured as in the first determination.

The difference between the two measurements, multiplied by a known factor, gives the starch content of the sample.

3. **Reagents**

3.1. 25 % (w/w) hydrochloric acid, d: 1,126 g/ml.

3.2 1. 28 % (w/v) hydrochloric acid.

The concentration must be checked by titration using a sodium hydroxide solution 0,1 mol/litre in the presence of 0,1 % (w/v) methyl red in 94 % (v/v) ethanol. 10 ml = 30.94 ml of NaOH 0.1 mol/lite.

3.3 Carrez solution I: dissolve 21,9 g of zinc acetate $Zn\ (CH_3COO)_2\ 2H_2O$ and 3 g of glacial acetic acid in water. Make up to 100 ml with water.

3.4 Carrez solution II: dissolve 10,6 g of potassium ferrocyanide $[K_4(Fe(CN)_6].3H_2O$ in water. Make up to 100 ml with water.

3.5 40 % (v/v) ethanol, d: 0,948 g/ml at 20 ºC.

4. **Apparatus**

4.1. 250 ml erlenmeyer flask with standard ground-glass joint and with reflux condenser.

4.2. Polarimeter or saccharimeter.

5. **Procedure**

5.1. *Preparation of the sample*

Crush the sample until it is fine enough for all of it to pass through a 0,5 mm round-meshed sieve.

5.2. *Determination of the total optical rotation (P or S) (see observation 7.1)*

Weigh 2,5 g of the crushed sample to the nearest mg and place in a 100 ml graduated flask. Add 25 ml of hydrochloric acid (3.2), shake to obtain even distribution of the test sample and add a further 25 ml of hydrochloric acid (3.2). Immerse the flask in a boiling water bath shaking vigorously and steadily for the first three minutes to prevent the formation of agglomerates. The quantity of water in the water bath must be sufficient for the bath to remain at boiling point when the flask is introduced into it. The flask must not be taken out of the bath whilst being shaken. After exactly 15 minutes, remove from the bath, add 30 ml of cold water and cool immediately to 20 ºC.

Add 5 ml of Carrez solution I (3.3) and shake for one minute. Then add 5 ml of Carrez solution II (3.4) and shake again for one minute. Make up to volume with water, mix and filter. If the filtrate is not perfectly clear (which is rare), repeat the determination using a larger quantity of Carrez solutions I and II, for example 10 ml.

Measure the optical rotation of the solution in a 200 mm tube with the polarimeter or saccharimeter.

5.3. *Determination of the optical rotation (P′ or S') of substances soluble in 40 % ethanol*

Weigh 5 g of the sample to the nearest mg, place in a 100 ml graduated flask and add about 80 ml of ethanol (3.5) (see observation 7.2). Leave the flask to stand for 1 hour at room temperature; during this time, shake vigorously on six occasions so that the test sample is thoroughly mixed with the ethanol. Make up to volume with ethanol (3.5), mix and filter. Pipette 50 ml of the filtrate (= 2,5 g of the sample) into a 250 ml erlenmeyer flask, add 2,1 ml of hydrochloric acid (3.1) and shake vigorously. Fit a reflux condenser to the erlenmeyer flask and immerse the latter in a boiling water bath. After exactly 15 minutes, remove the erlenmeyer flask from the bath, transfer the contents to a 100 ml graduated flask , rinsing with a little cold water, and cool to 20 ºC. Clarify Using Carrez solutions I (3.3) and II (3.4), make up to volume with water, mix, filter and measure the optical rotation as indicated in the second and third paragraphs of 5.2.

6. **Calculation of results**

The starch content (%) is calculated as following:

6.1. *Measurement by polarimeter*

$$\text{Starch content (\%)} = \frac{2000(P - P')}{[\alpha]_D^{20^\circ}}$$

Where:

P = total optical rotation in angle degrees

P′ = optical rotation in angle degrees of the substances soluble in 40 % (V/V) ethanol

$[\alpha]_D^{20^\circ}$ = specific optical rotation of pure starch. The numerical values conventionally accepted for factor are the following

+ 185.9º: rice starch
+ 185.4º: potato starch
+ 184.6º: maize starch
+ 182.7º: wheat starch
+ 181.5º: barley starch
+ 181.3º: oat starch
+ 184.0º: other types of starch and starch mixtures in compound feedingstuffs

6.2. *Measurement by saccharimeter*

$$\text{Starch content (\%)} = \frac{2000}{[\alpha]_D^{20^\circ}} \times \frac{(2N \times 0{,}665) \times (S - S')}{100} - \frac{26.6N \times (S - S')}{[\alpha]_D^{20^\circ}}$$

Where:

S = total optical rotation in saccharimeter degrees

S′ = optical rotation in saccharimeter degrees of the substances soluble in 40 % (V/V) ethanol

N = weight (g) of saccharose in 100 ml of water yielding an optical rotation of 100 saccharimeter degrees when measured using a 200 mm tube

16,29 g for the French saccharimeters

26,00 g for the German saccharimeters

20,00 g for mixed saccharimeters.

$[\alpha]_D^{20^\circ}$ = specific optical rotation of pure starch (see 6.1)

6.3. *Repeatability*

The difference between the results of two parallel determinations carried out on the same sample must not exceed 0,4 in absolute value for a starch content lower than 40 % and 1,1 % relative for starch contents equal or higher than 40 %

7. **Observations**

7.1. If the sample contains more than 6% of carbonates, calculated in terms of calcium carbonate, they must be destroyed by treatment with an exactly appropriate quantity of dilute sulphuric acid before determination of the total optical rotation.

7.2. In the case of products with high lactose content, such as powdered milk serum or skimmed milk powder, proceed as follows after adding 80 ml of ethanol (3.5). Fit a reflux condenser to the flask and immerse the latter in a water bath at 50 ºC for 30 minutes. Leave to cool and continue the analysis as indicated in 5.3.

7.3. Following feed materials, in case they are present in significant amount in feedingstuffs, are known to give rise to interferences when determining the starch content by polarimetric method and whereby incorrect results could be yielded:

- (sugar) beet products such as (sugar) beet pulp, (sugar) beet molasses, (sugar) beet pulp-molassed, (sugar) beet vinasse, (beet) sugar, citrus pulp,
- linseed; linseed expeller; linseed extracted,
- rape seed; rape seed expeller; rape seed extracted; rape seed hulls, sunflower seed; sunflower seed extracted; sunflower seed, partially decorticated, extracted,
- copra expeller; copra extracted,
- potato pulp,
- dehydrated yeast,
- products rich in inulin (e.g. chips and meal of Jerusalem artichokes);
- greaves.

2. DETERMINATION OF CRUDE PROTEIN

1. **Purpose and scope**

This method makes it possible to determine the crude protein content of feedingstuffs on the basis of the nitrogen content, determined according to the Kjeldahl method.

2. **Principle**

The sample is digested by sulfuric acid in the presence of a catalyst. The acid solution is made alkaline with sodium hydroxide solution. The ammonia is distilled and collected in a measured quantity of sulfuric acid, the excess of which is titrated with a standard solution of sodium hydroxide.

3. **Reagents**

3.1 Potassium sulfate.

3.2 Catalyst: copper (II) oxide CuO or copper (II) sulfate pentahydrate, $CuSO_4 \cdot 5H_2O$

3.3 Granulated zinc.

3.4. Sulfuric acid, ρ_{20} = 1,84 g/ml.

3.5 Sulfuric acid c($\frac{1}{2}H_2SO_4$) = 0,5 mol/l.

3.6 Sulfuric acid c($\frac{1}{2}H_2SO_4$) = 0,1 mol/l.

3.7 Methyl red indicator; dissolve 300 mg of methyl red in 100 ml of ethanol, σ 95-96 % (v/v)

3.8 Sodium hydroxide solution (Technical grade may be used) β = 40 g/ 100 ml (m/v: 40 %).

3.9 Sodium hydroxide solution c = 0,25 ml/l

3.10 Sodium hydroxide solution c 0,1 mol/l.

3.11 Granulated pumice stone, washed in hydrochloric acid and ignited.

3.12 Acetanilide (m.p. = 114 ºC, N = 10,36 %)

3.13 Sucrose (nitrogen free).

4. **Apparatus**

Apparatus suitable for performing digestion, distillation and titration according to the Kjeldahl procedure.

5. **Procedure**

5.1 *Digestion*

Weigh 1 g of the sample to the nearest 0,001 g and transfer the sample to the flask of the digestion apparatus. Add 15 g of potassium sulfate (3.1.), an appropriate quantity of catalyst (3.2) (0,3 to 0,4 g of copper (II) oxide or 0,9 to 1,2 g of copper (II) sulfate pentahydrate), 25 ml of sulfuric acid (3.4) and a few granules of pumice stone (3.11) and mix. Heat the flask moderately at first, swirling from time to time if necessary until the mass has carbonized and the foam has disappeared; then heat more intensively until the liquid is boiling steadily. Heating is adequate if the boiling acid condenses on the wall of the flask. Prevent the sides from becoming overheated and organic particles from sticking to them. When the solution becomes clear and light green continue to boil for another two hours, then leave to cool.

5.2 *Distillation*

Add carefully enough water to ensure complete dissolution of the sulfates. Allow to cool and then add a few granules of zinc (3.3).

Place in the collecting flask of the distillation apparatus an exactly measures quantity of 25 ml of sulfuric acid (3.5) or (3.6) depending on the presumed nitrogen content. Add a few drops of methyl red indicator (3.7).

Connect the digestion flask to the condenser of the distillation apparatus and immerse the end of the condenser in the liquid contained in the collecting flask to a depth of at least 1 cm (see observation 8.3). Slowly pour 100 ml of sodium hydroxide solution (3.8) into the digestion flask without loss of ammonia (see observation 8.1).

Heat the flask until the ammonia has distilled over.

5.3 *Titration*

Titrate the excess sulfuric acid in the collecting flask with sodium hydroxide solution (3.9) or (3.10) depending on the concentration of the sulfuric acid used, until the end point is reached.

5.4 *Blank test*

To confirm that the reagents are free from nitrogen, carry out a blank test (digestion, distillation and titration) using 1 g of sucrose (3.13) in place of the sample.

6. **Calculation of results**

The content of crude protein is calculated according to the following formula:

$$\frac{(V_0 - V_1) \times c \times 0.014 \times 100 \times 6.25}{m}$$

Where:

V_0 = Volume (ml) of NaOH (3.9 or 3.10) used in the blank test.

V_1 = Volume (ml) of NaOH (3.9 or 3.10) used in the sample titration.

c = Concentration (mol/l) of sodium hydroxide (3.9 or 3.10).

m = Mass (g) of sample.

7. **Verification of the method**

7.1 *Repeatability*

The difference between the results of two parallel determinations carried out on the same sample must not exceed:

0,2 % in absolute value, for crude protein contents of less than 20 %;

1,0 % relative to the higher value, for crude protein contents from 20 % to 40 %;

0,4 % in absolute value, for crude protein contents of more than 40 %.

7.2 *Accuracy*

Carry out the analysis (digestion, distillation and titration) on 1,5 to 2,0 g of acetanilide (3.12) in the presence of 1 g of sucrose (3.13); 1 g acetanilide consumes 14,80 ml of sulfuric acid (3.5). Recovery must be at least 99 %.

8. **Observations**

8.1 Apparatus may be of the manual, semi-automatic or automatic type. If the apparatus requires transference between the digestion and distillation steps, this transfer must be carried out without loss. If the flask of the distillation apparatus is not fitted with a dropping funnel, add the sodium hydroxide immediately before connecting the flask to the condenser, pouring the liquid slowly down the side.

8.2 If the digest solidifies, recommence the determination using a larger amount of sulfuric acid (3.4) than that specified above.

8.3 For products with a law nitrogen content, the volume of sulfuric acid (3.6) to be placed in the collecting flask may be reduced, if necessary, to 10 or 15 ml and make up to 25 ml with water.

3. DETERMINATION OF CRUDE PROTEIN DISSOLVED BY PEPSIN AND HYDROCHLORIC ACID

1. Purpose and scope

This method makes it possible to determine the fraction of crude protein dissolved by pepsin and hydrochloric acid under defined conditions. It is applicable to all feedingstuffs.

2. Principle

The sample is heated for 48 hours at 40 ºC in a solution of pepsin hydrochloride.

The suspension is filtered and the nitrogen content of the filtrate determined according to the method for the determination of crude protein.

3. Reagents

3.1 Hydrochloric acid, d: 1·125.

3.2 Hydrochloric acid 0·075 N.

3.3 2·0 U/mg pepsin; pepsin activity is defined in the method described in Part 4 of this Annex and must be established according to that method.

3.4 About 0·2 % (w/v) freshly prepared solution of pepsin in hydrochloric acid (3.2): activity: 400 U/l.

3.5 Anti-foaming emulsion (eg. silicone).

3.6 All the reagents listed under 3 in the method for the determination of crude protein.

4. Apparatus

4.1 Water bath or incubator, set at 40 ºC ± 1 ºC.

4.2 Kjeldahl digestion and distillation apparatus.

5. Procedure

5.1 *Preparation of solution* (see observation 7.2)

Weigh 2 g of the sample to the mearest mg and place in a 500 ml graduated flask. Add 450 ml of pepsin hydrochloride solution (3.4) previously heated to 40 ºC and shake to prevent the formation of agglomerates. Check that the pH of the suspension is less than 1·7. Place the flask in the water bath or incubator (4.1) and leave there for 48 hours. Shake after 8, 24 and 32 hours. After 48 hours, add 15 ml of hydrochloric acid (3.1), cool to 20 ºC, make up to volume with water and filter.

5.2 *Digestion*

Take 250 ml of the filtrate and place in the flask of the distillation apparatus (4.2). Add the reagents necessary for digestion indicated in the second sentence of 5.1 of the method for the determination of crude protein. Homogenize and bring to the boil. If any foam should form, add a few drops of anti-foaming emulsion (3.5). Continue to boil vigorously until the water

has been almost completely evaporated. Reduce the heat and carefully eliminate the last traces of water.

When the solution becomes clear and colourless (or light green if a copper-based catalyst is used), continue to boil for another hour. Leave to cool.

5.3 *Distillation and titration*

Proceed as indicated in 5.2 and 5.3 of the method for the determination of crude protein.

5.4 *Blank test*

Carry out a blank test applying the same procedure but omitting the sample to be analyzed.

6. **Calculation of results**

Subtract the volume of sulphuric acid consumed in the blank test from that consumed by the test sample. 1 ml of sulphuric acid 0·1 N corresponds to 1·4 mg of nitrogen.

Multiply the quantity of nitrogen by the factor 6·25. Express the result as a percentage of the sample.

Repeatability

The difference between the results of two parallel determinations carried out on the same sample must not exceed:

— 0·4, in absolute value, for contents of less than 20 %;

— 2·0 %, in relative value, for contents of not less than 20 % and not more than 40 %;

— 0·8, in absolute value, for contents of more than 40 %.

7. **Observations**

7.1 The values obtained by this method have no direct connection with digestibility *in vivo*.

7.2 Products with an oil or fat content exceeding 10 % must first be defatted by extraction with petroleum ether (B.P. 40 to 60 ºC).

4. **ESTIMATION OF PEPSIN ACTIVITY**

1. **Purpose and scope**

This method makes it possible to established the activity of the pepsin used in the determination of crude protein dissolved by pepsin and hydrochloric acid.

2. **Principle**

Haemoglobin is treated with pepsin in a hydrochloric acid medium under defined conditions. The non-hydrolyzed fraction of the protein is precipitated in trichloroacetic acid. Sodium hydroxide and Folin-Ciocalteu reagent are added to the filtrate. The optical density of this

solution is measured at 750 nm and the corresponding quantity of tyrosine is read from a calibration curve.

Definition: The unit of pepsin is defined as being the quantity of that enzyme which, under the conditions of the method, liberates per minute, a quantity of hydroxyaryl groups which, when stained with the Folin-Ciocalteu reagent, has an optical density corresponding to that of one µmole of tyrosine stained in the same manner.

3. **Reagents**

3.1 Hydrochloric acid 0·2 N.

3.2 Hydrochloric acid 0·06 N.

3.3 Hydrochloric acid 0·025 N.

3.4 5 % solution (w/v) of trichloroacetic acid.

3.5 Sodium hydroxide solution 0·5 N.

3.6 Folin-Ciocalteu reagent. Place 100 g of sodium tungstate ($Na_2WO_4 . 2H_2O$), 25 g of sodium molybdate ($Na_2MoO_4 . 2H_2O$) and 700 ml of water in a 2 litre round-bottomed flask fitted with a standard ground-glass joint. Add 50 ml of phosphoric acid (d: 1·71) and 100 ml of concentrated hydrochloric acid (d: 1·19), connect a reflux condenser to the flask, bring to the boil and keep the solution gently boiling for 10 hours. Leave to cool, detach the reflux condenser, add 175 g of lithium sulphate ($Li_2SO_4 . 2H_2O$), 50 ml of water and 1 ml of bromine. Boil for 15 minutes to eliminate excess bromine.

Leave to cool, transfer the solution to a 1 litre graduated flask, make up to volume with water, homogenize and filter. No greenish coloration must remain. Before use, dilute 1 volume of the reagent with 2 volumes of water.

3.7 Haemoglobin solution: Weigh a quantity of haemoglobin (approx. 2 g of protein substratum determined according to Anson) corresponding to 354 mg of nitrogen[1] and place in a 200 ml flask fitted with a standard ground-glass joint. Add a few ml of hydrochloric acid (3.2), connect the flask to the vacuum pump and shake until the haemoglobin has completely dissolved. Release the vacuum and, while shaking, add hydrochloric acid (3.2) to make up to 100 ml. *Prepare immediately before use*.

3.8 Standard tyrosine solution: Dissolve 181·2 mg of tyrosine in the hydrochloric acid (3.1) and make up to 1 litre with the same acid (stick solution). Take 20·0 ml and dilute to 100 ml with hydrochloric acid (3.1). 1 ml of this solution contain 0·2 µmole of tyrosine.

4. **Apparatus**

4.1 Water bath set at 25 ºC ± 0·1 ºC by ultrathermostat.

4.2 Spectrophotometer.

4.3 Chronometer, accuracy: 1 second.

[1] Determine the nitrogen content by the semi-micro Kjehdahl method (theoretical content: 17.7 % of nitrogen).

4.4 pH-meter.

5. **Procedure**

5.1 *Preparation of the solution* (see observation 7.1)

Dissolve 150 mg of pepsin in 100 ml of hydrochloric acid (3.2). Pipette 2 ml of the solution into a 50 ml graduated flask and make up to volume with hydrochloric acid (3.3). The pH, checked with the pH-meter, must be 1·6 ± 0·1. Immerse the flask in the water bath (4.1).

5.2 *Hydrolysis*

Pipette 5·0 ml of haemoglobin solution (3.7) into a test tube, heat to 25 ºC in the water bath (4.1), add 1·0 ml of the pepsin solution obtained in 5·1 and mix with a glass rod thickened at one end, with about 10 back-and-forth movements. Leave the test tube in the water bath at 25 ºC for exactly 10 minutes, timed from the addition of the pepsin solution (duration and temperature must be strictly observed). Then add 10·0 ml of trichloroacetic acid solution (3.4) previously heated to 25 ºC, homogenize and filter through a dry filter.

5.3 *Development of coloration and measurement of optical density*

Pipette 5·0 ml of the filtrate into a 50 ml Erlenmeyer flask, add 10·0 ml of sodium hydroxide solution (3.5) and, shaking constantly, 3·0 ml of dilute Folin-Ciocalteu reagent (3.6). After 5 to 10 minutes, determine the optical density of the solution with the spectrophotometer at 750 nm in cells 1 cm thick against water.

5.4 *Blank test*

For each determination, carry out a blank test as follows:
Pipette 5·0 ml of haemoglobin solution (3.7) into a test tube, heat to 25 ºC in the water bath (4.1), add 10·0 ml of trichloroacetic acid solution (3.4) previously heated to 25 ºC, homogenize, then add 1.0 ml of the pepsin solution obtained in 5.1. Mix with a glass rod and leave the test tube in the water bath (4.1) at 25 ºC for exactly 10 minutes. Homogenize and filter through a dry filter. Follow the procedure indicated in 5.3.

5.5 *Calibration curve*
Place 1·0, 2·0, 3·0, 4·0 and 5·0 ml aliquots of standard tyrosine solution (3.8), corresponding to 0·2, 0·4, 0·6, 0·8 and 1·0 µmoles of tyrosine respectively in 50 ml Erlenmeyer flasks. Complete the series with a reference solution free from tyrosine. Make up the volumes to 5·0 ml with hydrochloric acid (3.1). Add 10·0 ml of sodium hydroxide solution (3.5) and, shaking constantly, 3·0 ml of dilute Folin-Ciocalteu reagent (3.6). Measure the optical density as indicated in the last sentence of 5.3. Trace the calibration curve by plotting the optical densities against the quantities of tyrosine.

6. **Calculation of results**

From the calibration curve read the quantity of tyrosine, in µmoles, corresponding to the optical density of the coloured solution, corrected on the basis of the blank value.

The pepsin activity, in µmoles, of tyrosine at 25 ºC, per mg and per minute, is calculated by using the formula:

$$\text{Units per mg (U/mg)} = \frac{0 \cdot 32a}{P}$$

Where:

a = quantity of tyrosine, in µmoles, read from the calibration curve;
p = weight in mg of the quantity of pepsin added in 5.2.

7. **Observations**

7.1 The quantity of pepsin to be dissolved must be such that, on final photometric measurement, an optical density of 0·35 ± 0·035 is obtained.

7.2 Two units per mg obtained by this method correspond to:

3·64 Anson milliunits /mg (µmoles of tyrosine/mg · min at 35·5 ºC) or
36 400 commercial units/g (µmoles of tyrosine/g in 10 min at 35·5 ºC).

5. DETERMINATION OF FREE AND TOTAL GOSSYPOL

1. **Purpose and scope**
This method makes it possible to determine the levels of free gossypol, total gossypol and chemically related substances in cottonseed, cottonseed meal and cottonseed cake and in compound feedingstuffs containing these substances where more than 20 ppm are present.

2. **Principle**

The gossypol is extracted in the presence of 3-aminopropan-1-ol, either with a mixture of propan-2-ol and hexane, for the determination of free gossypol, or with dimethylformamide, for the determination of total gossypol. The gossypol is converted by aniline into gossypol-dianiline, the optical density of which is measured at 440 nm.

3. **Reagents**

3.1 Propan-2-ol-hexane mixture: mix 60 parts by volume of propan-2-ol A.R. with 40 parts by volume of *n*-hexane.

3.2 Solvent A: Place in a 1 litre graduated flask approximately 500 ml of propan-2-ol-hexane mixture (3.1), 2 ml of 3-aminopropan-1-ol, 8 ml of glacial acetic acid and 50 ml of water. Make up to volume with the propan-2-ol-hexane mixture (3.1). This reagent is stable for one week.

3.3 Solvent B: Pipette 2 ml of 3-aminopropan-1-ol and 10 ml of glacial acetic acid into a 100 ml graduated flask. Cool to room temperature and make up to volume with N, N-dimethylformamide. This reagent is stable for one week.

3.4 Aniline A.R.: *If the optical density in the blank test exceeds 0·022*, distil the aniline over zinc dust, discarding the first and last 10 % fractions of the distillate. Refrigerated and stored in a brown, stoppered glass flask, this reagent will keep for several months.

3.5 Standard gossypol solution A: Place 27·9 mg of gossypol acetate in a 250 ml graduated flask. Dissolve and make up to volume with solvent A (3.2). Pipette 50 ml of this solution into a 250 ml graduated flask and make up to volume with solvent A. The gossypol concentration of this solution is 0·02 mg per ml. Leave to stand for one hour at room temperature before use.

3.6 Standard gossypol solution B: Place 27·9 mg of gossypol acetate in a 50 ml graduated flask, Dissolve and make up to volume with solvent B (3.3). The gossypol concentration of this solution is 0·5 mg per ml.

Standard gossypol solutions A and B will remain stable for 24 hours if protected from the light.

4. **Apparatus**

4.1 Mixer (tumbler): approximately 35 rpm.

4.2 Spectrophotometer.

5. **Procedure**

5.1 *Test sample*
The amount of test sample used depends on the presumed gossypol content of the sample. It is preferable to work with a small test sample and a relatively large aliquot part of the filtrate, so as to obtain sufficient gossypol for precise photometric measurement to be possible. *For the determination of free gossypol* in cottonseed, cottonseed meal and cottonseed cake, the test sample should not exceed 1 g; for compound feedingstuffs, it may be as much as 5 g. A 10 ml aliquot part of filtrate is suitable in most cases; it should contain 50 to 100 µg of gossypol. *For the determination of total gossypol,* the test sample should be between 0·5 and 5 g, that a 2 ml aliquot part of filtrate will contain 40 to 200 µg of gossypol.

The analysis should be carried out at a room temperature of about 20 ºC.

5.2 *Determination of free gossypol*

Place the test sample in a ground-necked 250 ml flask, the bottom of the flask having been covered with crushed glass. Using a pipette, add 50 ml of solvent A (3.2), stopper the flask and mix for one hour in the mixer. Filter through a dry filter and collect the filtrate in a small ground-necked flask. During filtration, cover the funnel with a watch glass.

Pipette identical aliquot parts of filtrate containing 50 to 100 µg of gossypol into each of two 25 ml graduated flasks (A and B). If necessary, make up the volume to 10 ml with solvent A (3.2). Then make the contents of flask (A) up to volume with the propan-2-ol-hexane mixture (3.1). This solution will be used as a reference solution against which to measure the sample solution.

Pipette 10 ml of solvent A (3.2) into each of two other 25 ml graduated flasks (C and D). Make the contents of flask (C) up to volume with the propan-2-ol-hexane mixture (3.1). This solution will be used as a reference solution against which to measure the blank test solution.

Add 2 ml of aniline (3.4) to each of flasks (D) and (B). Heat for 30 minutes over a boiling water bath to develop the colour. Cool to room temperature, make up to volume with the propan-2-ol-hexane mixture (3.1), homogenize and leave to stand for one hour.

Determine the optical density of the blank test solution (D) by comparison with the reference solution (C), and the optical density of the sample solution (B) by comparison with the reference solution (A), in the spectrophotometer at 440 nm using 1 cm glass cells.

Subtract the optical density of the blank test solution from that of the sample solution (= corrected optical density). From this value calculate the free gossypol content as indicated in 6.

5.3 *Determination of total gossypol*

Place a test sample containing 1 to 5 mg of gossypol in a 50 ml graduated flask and add 10 ml of solvent B (3.3). At the same time, prepare a blank test, placing 10 ml of solvent B (3.3) in another 50 ml graduated flask. Heat the two flasks for 30 minutes over a boiling water bath. Cool to room temperature and make the contents of each flask up to volume with the propan-2-ol-hexane mixture (3.1). Homogenize and leave to settle for 10 to 15 minutes, then filter and collect the filtrates in ground-necked flasks.

Pipette 2 ml of the sample filtrate into each of two 25 ml graduated flasks, and 2 ml of the blank test filtrate into each of two other 25 ml flasks. Make the contents of one flask from each series up to 25 ml with the propan-2-ol-hexane mixture (3.1). These solutions will be used as reference solutions.

Add 2 ml of aniline (3.4) to each of the other two flasks. Heat for 30 minutes over a boiling water bath to develop the colour. Cool to room temperature, make up to 25 ml with the propan-2-ol-hexane mixture (3.1), homogenize and leave to stand for one hour.

Determine the optical density as indicated in 5.2 for free gossypol. From this value calculate the total gossypol content as indicated in 6.

6. **Calculation of results**

Results may be calculated either from the specific optical density (6.1), or by reference to a calibration curve (6.2).

6.1 *From the specific optical density*

The specific optical densities, under the conditions described, will be the following:

free gossypol: $E\,\frac{1\,\%}{1\,\mathrm{cm}} = 625$

total gossypol: $E\,\frac{1\,\%}{1\,\mathrm{cm}} = 600$

$$\%\ \text{gossypol} = \frac{E \cdot 1250}{E_{1\,\mathrm{cm}}^{1\,\%} \cdot p \cdot a}$$

where:

E = corrected optical density, determined as indicated in 5.2;
p= test sample in g;
a = aliquot part of the filtrate in ml.

6.2 *From a calibration curve*

6.2.1 *Free gossypol*

Prepare 2 series of five 25 ml graduated flasks. Pipette aliquots of 2·0, 4·0, 6·0, 8·0 and 10·0 ml of standard gossypol solution A (3.5) into each series of flasks. Make up the volumes to 10 ml with solvent A (3.2). Complete each series with a 25 ml graduated flask containing only 10 ml of solvent A (3.2) (blank test). Make the volume of the flasks in the first series (including the flask for the blank test) up to 25 ml with the propan-2-ol-hexane mixture (3.1) (reference series).

Add 2 ml of aniline (3.4) to each flask in the second series (including the flask for the blank test). Heat for 30 minutes over a boiling water bath to develop the colour. Cool to room temperature, make up to volume with the propan-2-ol-hexane mixture (3.1), homogenize and leave to stand for one hour (standard series).

Determine as indicated in 5.2 the optical density of the solutions in the standard series by comparison with the corresponding solutions in the reference series. Trace the calibration curve by plotting the optical densities against the quantities of gossypol (in µg).

6.2.2 *Total gossypol*

Prepare six 50 ml graduated flasks. In the first flask place 10 ml of solvent B (3.3), and in the others 2·0, 4·0, 6·0, 8·0 and 10·0 ml of standard gossypol solution B (3.6) respectively. Make the contents of each flask up to 10 ml with solvent B (3.3). Heat for 30 minutes over a boiling water bath. Cool to room temperature, make up to volume with the propan-2-ol-hexane mixture (3.1) and homogenize. Place 2.0 ml of these solutions in each of two series of six 25 ml graduated flasks. Make the contents of the flasks in the first series up to 25 ml with the propan-2-ol-hexane mixture (3.1) (reference series).

Add 2 ml of aniline (3.4) to each flask in the second series. Heat for 30 minutes over a boiling water bath. Cool to room temperature, make up to volume with the propan-2-ol-hexane mixture (3.1), homogenize and leave to stand for one hour (standard series).

Determine as indicated in 5.2 the optical density of the solutions in the standard series by comparison with the corresponding solutions in the reference series. Trace the calibration curve by plotting the optical densities against the quantities of gossypol (in µg).

6.3 *Repeatability*
The difference between the results of two parallel determinations carried out on the same sample must not exceed:

- 15 %, in relative value, for gossypol contents of less than 500 ppm;

- 75 ppm, in absolute value, for contents of not less than 500 ppm and not more than 750 ppm;

- 10 %, in relative value, for contents of more than 750 ppm.

SCHEDULE II

4. DETERMINATION OF TYLOSIN
— by diffusion on agar —

1. Purpose and scope

This method makes it possible to determine the tylosin content of feedingstuffs, concentrates and premixes where more than 2 ppm are present.

2. Principle

The sample is treated with a pH 8 phosphate buffer solution, previously heated to 80 ºC, and then extracted with methanol. After centrifuging, the extract is diluted and its antibiotic activity determined by measuring the diffusion of the tylosin on an agar medium seeded with

Sarcina lutea. The diffusion is made evident by the formation of inhibition zones in the presence of the micro-organism. The diameter of these zones is directly proportional to the logarithm of the antibiotic concentration.

3. **Micro-organism: Sarcina lutea ATCC No. 9341**

3.1 *Maintenance of the parent strain*

Inoculate with *Sarcina lutea* a tube of sloped agar taken from the culture medium (4.1), adjust to pH 7·0. Incubate overnight at approximately 35 ºC. Keep the culture in a refrigerator and re-inoculate sloped agar with it every month.

3.2 *Preparation of the bacteria suspension*

Collect the bacteria from a recently prepared tube of sloped agar (3.1) using 2 to 3 ml of physiological saline (4.4). With this suspension seed a Roux flask containing 250 ml of the culture medium (4.1), adjusted to pH 7·0. Incubate for 24 hours at 35 ºC, then collect the bacteria in 25 ml of physiological saline (4.4). Homogenize, and dilute this suspension to obtain approximately 75 % light transmission at 650 nm.

If kept in a refrigerator this suspension may be used for one week.

By preliminary tests on plates with the basic medium for the determination (4.1), establish the quantity of inoculum which, for the different concentrations of tylosin used, will give the largest possible inhibition zones that are still clear. The culture medium is inoculated at 48 to 50 ºC.

4. **Culture media and reagents**

4.1 *Basic medium for the determination*)[1]

Glucose	1 g
Tryptic peptone	10 g
Meat extract	1·5 g
Yeast extract	3 g
Agar, according to quality	10 to 20 g
Distilled water to	1000 ml

Adjust immediately before use to pH 7.0 for maintenance of the parent strain and preparation of the bacteria suspension, and to pH 8·0 for the determination.

4.2 *Phosphate buffer solution, pH 8*

Potassium dihydrogen phosphate KH_2PO_4 A.R. 0·523 g

[1] Any commercial culture medium of similar composition and giving the same results may be used

*di*Potassium hydrogen phosphate K_2HPO_4 A.R.	16·730 g
Distilled water to	1000 ml

4.3 *Phosphate buffer solution, pH 7*

Potassium dihydrogen phosphate KH_2PO_4 A.R.	5·5 g
di Potassium hydrogen phosphate K_2HPO_4 A.R.	13·6 g
Distilled water to	1000 ml

4.4 Sterile physiological saline.

4.5 Pure methanol.

4.6 40 % (v/v) methanol.

4.7 Mixture of phosphate buffer solution (4.2)/ pure methanol: 60/40 by volume.

4.8 Standard substance: tylosin of known activity.

5. **Standard solutions**

Dry the standard substance (4.8) for 3 hours at 60 ºC in a vacuum oven (5 mm of mercury). Weigh 10 to 50 mg into a graduated flask, dissolve in 5 ml of methanol (4.5) and dilute the solution with the phosphate buffer solution, pH 7 (4.3), to obtain a tylosin-base concentration of 1000 µg per ml.

Prepare a standard working solution S_8 containing 2 µg per ml of tylosin base from this stock solution by diluting with the mixture (4.7).

Then prepare by successive dilutions (1 + 1), using the mixture (4.7), the following concentrations:

S_4	1 µg/ml
S_2	0·5 µg/ml
S_1	0·25 µg/ml

6. **Extraction**

For concentrates, take a 10 g test sample; for premixes and feedingstuffs, a 20 g test sample. Add 60 ml of phosphate buffer solution, pH 8 (4.2), previously heated to 80 ºC, and homogenize for 2 minutes (domestic mixer, Ultra-turrax, etc.).

Leave to stand for 10 minutes, add 40 ml of methanol (4.5) and homogenize for 5 minutes. Centrifuge the extract and dilute an aliquot part with the mixture (4.7) to obtain a presumed tylosin concentration of 2 µg per ml (= U_8). Then prepare the concentrations U_4, U_2 and U_1 by successive dilutions (1 + 1) using the mixture (4.7).

For contents of less than 10 ppm, evaporate the extract until dry in a rotary evaporator at 35 ºC and dissolve the residue in 40 % methanol (4.6).

7. **Determination method**

7.1 *Inoculation of the culture medium*

Inoculate at 48 to 50 ºC the basic medium for the determination (4.1), adjust to pH 8·0, with the bacteria suspension (3.2).

7.2 *Preparation of the trays*

Diffusion on agar is carried out in trays using 4 concentrations of the standard solution (S_8, S_4, S_2, S_1) and 4 concentrations of the extract (U_8, U_4, U_2, U_1). The 4 concentrations of standard solution and of extract must be placed in each tray.

Choose trays, therefore, which are large enough to allow at least 8 holes 10 to 13 mm in diameter to be made in the agar medium. Calculate the quantity of inoculated culture medium (7.1) needed to provide a uniform covering approximately 2 mm thick. The test should preferably be carried out on flat trays consisting of glass plates filled with a perfectly level aluminium or plastic ring, 200 mm in diameter and 20 mm high.

Pipette into the holes accurately measured quantities of between 0·10 and 0·15 ml of antibiotic solution, depending on the diameter of the holes.

For each sample repeat the diffusion at least 4 times with each concentration so that each determination comprises an evaluation of 32 inhibition zones.

7.3 *Incubation*

Incubate the trays overnight at 35 to 37 ºC.

8. **Evaluation**

Measure the diameter of the inhibition zones, preferably by projection.

Record the measurements on semi-logarithmic paper, plotting the logarithm of the concentrations against the diameter of the inhibition zones. Trace the lines of the standard solution and of the extract. Provided there is no interference the two lines will be parallel.

The logarithm of the relative activity is calculated by using the following formula:

$$\frac{(U_1 + U_2 + U_4 + U_8 - S_1 - S_2 - S_4 - S_8) \cdot 0 \cdot 602}{U_4 + U_8 + S_4 + S_8 - U_1 - U_2 - S_1 - S_2}$$

Real activity = presumed activity × relative activity.

9. **Repeatability**

The difference between the results of two parallel determinations carried out on the same sample must not exceed 10% in relative value.

5. DETERMINATION OF VIRGINIAMYCIN
— by diffusion in an agar medium —

1. **Purpose and scope**

The method is for the determination of virginiamycin in feedingstuffs and premixes. The lower limit of determination is 2 mg/kg (2 ppm) .[1]

2. **Principle**

The sample is extracted with a methanolic solution of Tween 80. The extract is decanted or centrifuged and diluted. Its antibiotic activity is determined by measuring the diffusion of virginiamycin in an agar medium inoculated with Micrococcus luteus. Diffusion is shown by the formation of zones of inhibition of the micro-organism. The diameter of these zones is taken to be in direct proportion to the logarithm of the antibiotic concentration over the range of antibiotic concentrations employed.

3. **Micro-organism: Micrococcus luteus ATCC 9341 (NCTC 8340, NCIB 8553)**

3.1. *Maintenance of stock culture*

Inoculate tubes containing slopes of culture medium (4.1) with Micrococcus luteus and incubate for 24 hours at 30 ºC. Store the culture in a refrigerator at about 4 ºC. Reinoculate every two weeks.

3.2. *Preparation of the bacterial suspension* ([a])

Harvest the growth from a recently prepared agar slope (3.1) with 2 to 3 ml of sodium cloride solution (4.3). Use this suspension to inoculate 250 ml of culture medium (4.1) contained in a Roux flask and incubate for 18 to 20 hours at 30 ºC. Harvest the growth in 25 ml of sodium chloride solution (4.3) and mix. Dilute the suspension to 1/10 with sodium chloride solution (4.3). The light transmission of the suspension must be about 75 %, measured at 650 nm in a 1 cm cell against sodium chloride solution (4.3). This suspension may be kept for one week at about 4 ºC.

4. **Culture media and reagents**

4.1. *Culture and assay medium* [a]

Meat peptone	6,0 g
Tryptone	4,0 g

[1] 1mg virginiamycin is equivalent to 1000 UK units

([a]) Other methods may be used provided that it has been established that they give similar bacterial suspensions.

[a] Any commercial culture medium of similar composition and giving the same results may be used.

Yeast extract	3,0 g
Meat extract	1,5 g
Glucose	1,0 g
Agar	10,0 to 20,0 g
Water	1 000ml
pH 6.5 (after sterilization)	

4.2. *Phosphate buffer, pH 6*

Potassium hydrogen phosphate, K_2HPO_4	2,0 g
Potassium dihydrogen phosphate, KH_2PO_4	8,0 g
Water to	1 000 ml

4.3. Sodium chloride solution 0,8 % (w/v): dissolve 8 g sodium chloride in water and dilute to 1 000 ml; sterilize.

4.4. Methanol.

4.5. Mixture of phosphate buffer (4.2)/methanol (4.4): 80/20 (v/v).

4.6. Tween 80 methanolic solution 0,5 % (w/v): dissolve 5 g Tween 80 in methanol (4.4) and dilute with methanol to 1 000 ml.

4.7. Standard substance: virginiamycin of known activity.

5. **Standard solutions**

Dissolve an accurately weighed quantity of the standard substance (4.7) in methanol (4.4) and dilute with methanol (4.4) to give a stock solution containing 1 000 µg virginiamycin per ml.

Stored in a stoppered flask at 4 ºC this solution is stable for up to five days.

From this stock solution prepare by successive dilution with the mixture (4.5) the following solutions:

S_8	1	µg/ml
S_4	0,5	µg/ml
S_2	0,25	µg/ml
S_1	0,125	µg/ml

6. **Preparation of the extract and assay solutions**

6.1. *Extraction*

6.1.1. *Products with a virginiamycin content up to 100 mg/kg*

Weigh out a quantity of sample of 50 g, add 200 ml of solution (4.6) and shake for 30 minutes. Leave to settle or centrifuge, take 20 ml of the supernatant solution and evaporate to about 5 ml in a rotary evaporator at a temperature not exceeding 40 ºC. Dilute the residue with the mixture (4.5) to obtain an expected virginiamycin content of 1 µg/ml (= u_8).

6.1.2. *Products with a virginiamycin content greater than 100 mg/kg*

Weigh out a quantity of sample not exceeding 10,0 g and containing between 1 and 50 mg virginiamycin, add 100 ml of solution (4.6) and shake for 30 minutes. Leave to settle or centrifuge, then dilute the supernatant solution with the mixture (4.5) to obtain an expected virginiamycin content of 1 µg/ml (= u_8).

(6.2. *Assay solutions*

From solution u_8 prepare solutions u_4 (expected content: 0,5 µg/ml), u_2 (expected content: 0,25 µg/ml) and u_1 (expected content: 0,125 µg/ml) by means of successive dilution (1 + 1) with the mixture (4.5).

7. **Assay procedure**

7.1. *Inoculation of the assay medium*

Inoculate the assay medium (4.1) with the bacterial suspension (3.2) at about 50 ºC. By preliminary trials on plates with the medium (4.1) determine the quantity of bacterial suspension required to give the largest and clearest zones of inhibition with the various concentrations of virginiamycin.

7.2. *Preparation of the plates*

Diffusion through agar is carried out in plates with the four concentrations of the standard solution (s_8, s_4, s_2 and s_1) and the four concentrations of the assay solution (u_8, u_4, u_2 and u_1). These four concentrations of extract and standard must necessarily be placed in each plate. To this effect, select plates big enough to allow at least eight holes with a diameter of 10 to 13 mm and not less than 30 mm between centres to be made in the agar medium. The test may be carried out on plates consisting of a sheet of glass with a faced aluminium or plastic ring placed on top, 200 mm in diameter and 20 mm high.

Pour into the plates a quantity of the medium (4.1) inoculated as in point 7.1, to give a layer about 2 mm thick (60 ml for a plate of 200 mm diameter). Allow to set in a level position, bore the holes and place in them exactly measured volumes of assay and standard solutions (between 0,10 and 0,15 ml per hole, according to the diameter). Apply each concentration at least four times so that each determination is subject to an evaluation of 32 zones of inhibition.

7.3. *Incubation*

Incubate the plates for 16 to 18 hours at 30 ± 2 ºC.

8. **Evaluation**

Measure the diameter of the zones of inhibition to the nearest 0,1 mm. Record the mean measurements for each concentration on semi-logarithmic graph paper showing the logarithm

of the concentrations in relation to the diameters of the zones of inhibition. Plot the best fit lines of both the standard solution and the extract, for example as below:

Determine the 'best fit' point for the standard lowest level (SL) using the formula:

$$\text{(a) SL} = \frac{7_{S_1} + 4_{S_2} + {}_{S_4} - 2_{S_8}}{10}$$

Determine the 'best fit' point for the standard highest level (SH) using the formula:

$$\text{(b) SH} = \frac{7_{S_8} + 4_{S_4} + {}_{S_2} - 2_{S_1}}{10}$$

Similarly calculate the 'best fit' points for the extract lowest level (UL) and the extract highest level (UH) by substituting u_1, u_2, u_4 and u_8 for s_1, s_2, s_4 and s_8 in the above formulae.

Record the calculated SL and SH values on the same graph paper and join them to give the 'best fit' line for the standard solution. Similarly record UL and UH and join them to give the 'best fit' line for the extract.

In the absence of any interference the lines should be parallel. For practical purposes the lines can be considered parallel if the values (SH—SL) and (UH—UL) do not differ by more than 10 % from their mean value.

If the lines are found to be non-parallel either u1 and s1 or u8 and s8 may be discarded and SL, SH, UL and UH calculated, using the alternative formulae, to give alternative 'best fit' lines:

$$\text{(a′) SL} = \frac{5_{S_1} + 2_{S_2} - {}_{S_4}}{6} \text{ OR } \frac{5_{S_2} + 2_{S_4} - {}_{S_8}}{6}$$

$$\text{(b′) SH} = \frac{5_{S_4} + 2_{S_2} - {}_{S_1}}{6} \text{ OR } \frac{5_{S_8} + 2_{S_4} - {}_{S_2}}{6}$$

and similarly for UL and UH. The same criteria of parallelism should be satisfied. The fact that the result has been calculated from three levels should be noted on the final report.

When the lines are considered as being parallel, calculate the logarithm of the relative activity (log A) by means of one of the following formulae, depending upon whether three or four levels have been used for the assessment of parallelism.

For four levels

$$\text{(c) Log A} = \frac{(U_1 + U_2 + U_4 + U_8 - S_1 - S_2 - S_4 - S_8) \times 0{,}602}{U_4 + U_8 + S_4 + S_8 - U_1 - U_2 - S_1 - S_2}$$

For three levels

$$\text{(d) Log A} = \frac{(U_1 + U_2 + U_4 - S_1 - S_2 - S_4 - S_8) \times 0{,}401}{U_4 + S_4 - U_1 - S_1}$$

or

$$\text{(d′) Log A} = \frac{(U_2 + U_4 + U_8 - S_2 - S_4 - S_8) \times 0{,}401}{U_8 + S_8 - U_2 - S_2}$$

Activity of sample extract = activity of relevant standard × A

$$(U_8 = s_8 \times A)$$

If the relative activity is found to be outside the range of 0,5 to 2,0, then repeat the assay making appropriate adjustments to the extract concentrations or, if this is not possible, to the standard solutions. When the relative activity cannot be brought into the required range, any result obtained must be considered as approximate and this should be noted on the final report.

When the lines are considered as not being parallel, repeat the determination. If parallelism is still not achieved, the determination must be considered as unsatisfactory.

Express the result in milligrams of virginiamycin per kilogram of feedingstuff.

9. **Repeatability**

The difference between the results of two parallel determinations carried out on the same sample by the same analyst should not exceed:

— 2 mg/kg, in absolute value, for contents of virginiamycin up to 10 mg/kg,

— 20 % related to the highest value for contents of 10 to 25 mg/kg,

— 5 mg/kg, in absolute value, for contents of 25 to 50 mg/kg,

— 10 % related to the highest value for contents above 50 mg/kg.

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