Afd. Diergeneesmiddelen 1982-07-16

VERSLAG 82.69 Pr.nr. 505.0600

Onderwerp: Verbetering van de methode voor identificatie van diergeneesmiddelen in diervoeders door middel van DLC.

Bijlage: 1

Verzendlijst: direkteur, sektorhoofd (3x), direktie VKA, afd. Diergeneesmiddelen (3x), afd. Normalisatie (Humme), Projektbeheer, Projektleider (Buizer).

8269

Afdeling Diergeneesmiddelen 1982-07-16

VERSLAG 82.69 Pr.nr. 505.0600

Projekt: Ontwikkeling methoden voor het aantonen en bepalen van diverse diergeneesmiddelen op niet microbiologische wijze

Onderwerp: Verbeteren van de methode voor identificatie van diergeneesmiddelen in diervoeders door middel van DLC

Bijlage: 1.

Doel:

Nagaan of het gebruikte loopmiddel bij analysemethode EEG 311/VI/77 vervangen kan worden door een minder schadelijk mengsel en of de zelfgemaakte kiezelgelplaten vervangen kunnen worden door pre-coated platen.

Samenvatting:

Bekeken is de mogelijkheid om benzeen te vervangen door tolueen, chloroform, methanol of ether, of dat een ander mengsel gebruikt kan worden.

De Rf-waarden van de diergeneesmiddelen werd bepaald op pre-coated platen in diverse mengsels en in enkele loopvloeistoffen op zelfgemaakte platen.

Conclusie:

Zelfgemaakte platen geven de beste resultaten. Benzeen kan wel vervangen worden door tolueen.

Verantwoordelijk: drs F.G. Buizer

Medewerkers/Samenstellers: Y. van Hemert, H. Roozendaal, M. Visser

Projektleider: drs F.G. Buizer

Inleiding:

Voor de kwalitatieve bepaling van diergeneesmiddelen wordt gewerkt volgens voorschrift EEG 311/VI/77.

Het gebruikte loopmiddel bevat benzeen. Dit is schadelijk voor de gezondheid; daarom is gezocht naar een vervanging hiervan. Tot nu worden voor deze bepaling zelfgemaakte kiezelgelplaten (dikte 0,8 mm) gebruikt. De dikte van de opgebrachte laag is afhankelijk van de snelheid, waarmee het opbrengapparaat over de platen wordt getrokken. Dientengevolge worden nooit exact dezelfde platen verkregen, terwijl pre-coated platen wel onderling even dik zijn.

Werkwijze:

Uitgegaan is van EEG-voorschrift 311/VI/77 (zie bijlage). De mogelijkheid om benzeen te vervangen door tolueen, chloroform, methanol of ether is onderzocht. Telkens zijn ook variaties in de samenstelling van de loopvloeistof bekeken.

Omdat men ook over wilde stappen op pre-coated platen zijn deze mengsels hierop getest.

De Rf-waarden van de meest gebruikte diergeneesmiddelen zijn telkens berekend.

Tenslotte zijn die mengsels die het meest geschikt waren ook op zelfgemaakte platen getest.

Resultaten:

De gevonden Rf-waarden, van de meest gebruikte diergeneesmiddelen, bij de verschillende systemen staan vermeld in onderstaande tabellen.

Tabel 1: Rf-waarden van de meest gebruikte diergeneesmiddelen op pre-coated platen.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
tolueen																	20
ether							60	60	60								
chloroform	60	60	60	60	45	60				60	60			30	45	60	40
ijsazijn	24	24	30	30	24	24	24	24	24	24	12	24	36	24	36	24	24
aceton	8	8	8	12	8	4	8	8	12	8	8	8	12	8	6	16	8
water	2,5	1,5	2,5	2,5	2,5	2,5	2,5	4	2,5	3,5	1,5	2,5	4	2,5	1,8	2,5	2,5
methanol												60	40				
carbadox	0,61	0,51	0,57	0,63	0,63	0,55	0,46	0,53	0,48	0,60	0,38	1,0	1,0	0,74	0,63	0,60	0,46
dimetridazol	0,80	0,71	0,66	0,78	0,76	0,76	0,61	0,62	0,60	0,67	0,40	1,0	1,0	0,67	0,60	0,55	0,62
amprolium	0	0	0	0	0	0	0	0	0	0	0	0,1	0,1	0	0	0	0
ethopabaat	0,85	0,82	0,83	0,87	0,88	0,81	0,80	0,84	0,81	0,82	0,72	1,0	1,0	0,93	0,84	0,88	0,71
furazolidon	0,49	0,44	0,46	0,52	0,51	0,44	0,42	0,56	0,44	0,46	0,38	1,0	1,0	0,65	0,52	0,53	0,41
ronidazol	0,53	0,49	0,50	0,58	0,60	0,51	0,65	0,70	0,65	0,94	0,72	1,0	1,0	0,74	0,63	0,56	0,45
sulfadimidine	0,81	0,78	0,76	0,81	0,82	0,77	0,79	0,84	0,80	0,79	0,67	1,0	1,0	0,87	0,73	0,79	0,67
sulfachinoxaline	0,83	0,80	0,78	0,83	0,85	0,79	0,87	0,90	0,87	0,82	0,66	1,0	1,0	0,89	0,80	0,80	0,73
nitrovin												1,0	1,0	0,66	0,41	0,30	0,27
olaquindox												1,0	1,0	0,52	0,37	0,29	0,26
metichlorpindol												1,0	1,0	0,62	0,46	0,45	0,32
arprinocid												1,0	1,0	0,92	0,84	0,85	0,66
sulfathiazole												1,0	1,0	0,66	0,53	0,54	0,45
D.O.T.												1,0	1,0	0,94	0,86	0,89	0,73
robenidine												1,0	1,0	1,0	0,95	0,95	0,87

Vervolg tabel 1: Rf-waarden van de meest gebruikte diergeneesmiddelen op pre-coated platen

	18	19	20	21	22	23	24	25	26	27	28	29	30	31
tolueen	40	30	20	30	30	30	35	20	20	40	40	40	30	60
ether														
chloroform	10	30	40	30	30	30	25	40	40	20	20	20	30	
ijsazijn	24	24	24	24	24	20	24	24	30	24	24	24	6	24
aceton	8	8	8	8	12	16	12	12	12	8	16	16	8	8
water	2,5	2,5	2,5	3,5	3,5	3,5	3,5	3,5	3,5	2,5	2,5	3,5	0,2	2,5
methanol														
carbadox	0,39	0,32	0,42	0,39	0,42	0,45	0,37	0,44	0,56	0,38	0,45	0,42	0,17	0,46
dimetridazol	0,59	0,47	0,65	0,52	0,61	0,63	0,56	0,64	0,70	0,57	0,61	0,61	0,46	0,65
amprolium	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ethopabaat	0,57	0,53	0,64	0,58	0,63	0,65	0,57	0,68	0,74	0,56	0,63	0,62	0,43	0,61
furazolidon	0,35	0,30	0,37	0,32	0,39	0,42	0,34	0,40	0,48	0,35	0,42	0,40	0,23	0,42
ronidazol	0,43	0,33	0,42	0,40	0,43	0,44	0,38	0,43	0,55	0,40	0,47	0,44	0,18	0,50
sulfadimidine	0,50	0,50	0,61	0,53	0,58	0,63	0,53	0,64	0,71	0,52	0,60	0,59	0,39	0,56
sulfachinoxaline	0,56	0,56	0,64	0,57	0,63	0,66	0,57	0,67	0,73	0,56	0,64	0,63	0,39	0,61
nitrovin	0,26	0,17	0,22	0,23	0,25	0,27	0,22	0,26	0,39	0,24	0,30	0,28	0,02	0,35
olaquindox	0,20	0,16	0,21	0,19	0,22	0,23	0,18	0,22	0,33	0,20	0,24	0,22	0,04	0,26
metichlorpindol	0,23	0,20	0,26	0,24	0,28	0,31	0,23	0,29	0,39	0,24	0,31	0,28	0,09	0,29
arprinocid	0,67	0,53	0,72	0,63	0,68	0,67	0,61	0,69	0,76	0,61	0,66	0,65	0,39	0,67
sulfathiazole	0,31	0,30	0,37	0,34	0,36	0,39	0,33	0,40	0,49	0,32	0,40	0,37	0,17	0,38
D.O.T.	0,64	0,60	0,71	0,63	0,69	0,68	0,64	0,69	0,75	0,62	0,67	0,66	0,44	0,69
robenidine	0,70	0,70	0,78	0,73	0,75	0,75	0,73	0,81	0,85	0,63	0,74	0,73	0,53	0,75

Tabel 2: Rf-waarden van de meest gebruikte diergeneesmiddelen op zelfgemaakte platen

	A	B	C	D
tolueen	60	60	60	60
ijsazijn	24	24	24	30
aceton	6	10	8	8
water	2,1	2,8	2,5	3,5
carbadox	0,14	0,24	0,23	0,25
dimetridazol	0,24	0,44	0,43	0,41
amprolium	0	0	0	0
ethopabaat	0,29	0,42	0,39	0,42
furazolidon	0,19	0,26	0,26	0,26
ronidazol	0,20	0,28	0,28	0,34
sulfadimidine	0,26	0,35	0,34	0,36
sulfachinoxaline	0,35	0,41	0,42	0,47
nitrovin	0,06	0,10	0,10	0,13
olaquindox	0,06	0,11	0,10	0,12
metichlorpindol	0,10	0,16	0,16	0,18
sulfathiazole	0,17	0,23	0,21	0,24
D.O.T.	0,53	0,54	0,58	0,61
robenidine	0,48	0,59	0,63	0,68

Conclusie:

Met verscheidene loopvloeistoffen werd een goede scheiding op pre-coated platen tussen de standaarden bereikt (tabel 1). Wanneer echter op deze platen niet alleen standaarden maar ook verschillende monsters werden opgebracht, bleek dat de verontreinigingen, welke na de voorzuivering nog altijd aanwezig zijn, te veel storing gaven. Door deze storing was het niet mogelijk om stoffen met een hoge Rf-waarde te kunnen onderscheiden. Het mengsel no. 31 gaf hierbij nog de beste resultaten. Wanneer men dit mengsel gebruikt bij zelfgemaakte platen heeft men geen last van deze storingen.

De kleine variaties op dit mengsel staan vermeld in tabel II.

Gezien dit feit, alsmede dat de elutietijd van pre-coated platen ongeveer 2 maal de tijd is van de zelfgemaakte platen moet geconcludeerd worden dat naar het werken met de zelfgemaakte platen nog steeds de voorkeur uitgaat waarbij het mengsel tolueen-ijsazijn-aceton-water (60-24-8-2,5) gebruikt kan worden.

EEC-number: 311/VI/77-E

Rijkslandbouwproefstation Maastricht
Draft Method
Author: Miss C.M.J. Stegen

BIJLAGE 1.

TRACING AND IDENTIFICATION OF COCCIDIOSTATS AND SOME OTHER CHEMOTHERAPEUTIC AGENTS IN ANIMAL FEEDING STUFFS BY THIN-LAYER CHROMATOGRAPHY

- - - - - - - - - - -

1) Scope and field of application.

This method permits the tracing and identification of the chemotherapeutic compounds listed below in animal feeding stuffs, if present in amounts down to about 2 mg/kg. Once an analyst has got sufficient experience to make a distinction between the spots originating from other feed components and the relevant spots by examination under UV-light, he will be able to screen about 3 to 10 samples a day.
The compounds concerned are: acetylenheptin, buquinolate, carbadox, decoquinate, dimetridazole, diaveridine, dinitolmide, ethopabate, furazolidone, methyl benzoquate, meticlorpindol (clopidol), nicarbazin, nifursol, nitrofurazone, nitrovin, pyrimethamine, robenidine, ronidazole, sulphaquinoxaline, sulphaguanidine, sulphacetamide, sulphanilamide, sulphadimidine, sulphapyridine, sulphadiazine, sulphadimethazine, sulphamerazine, sulphathiazole and sulphadimethoxine.

2) Principle.

The feed is extracted by refluxing with methanol. Interfering feed components are removed by pouring through an aluminium oxyde column. After concentration, the purified extract is spotted to two TLC-plate one with SiO_2, the other with Al_2O_3. After development, the plates are examined under UV-light. If desired, the plates are sprayed with Dragendorff reagent.

3) Reagents.

All chemicals should be of analytical reagent grade unless otherwise stated.

3.1. Methanol 100%
3.2. Ethanol 96%
3.3. Chloroform (containing 0.6 - 1.0 % ethanol)
3.4. Hydrochloric acid (sp. gr. 1.18)
3.5. Ammonia (sp. gr. 0.91)

3.6. Methanol - chloroform mixture: add 1 volume of 3.1. to 1 volume of 3.3. and mix well

3.7. Methanol - hydrochloric mixture: add 2 volumes of 3.4. to 98 volumes of 3.1. and mix well

3.8. Aluminium oxyde, standardized according to Brockmann, activity grade II-III (e.g. Merck GFR 1097)

3.9. Aluminium oxyde GF 254 for TLC (e.g. Merck GFR 1092)

3.10. Silica gel, according to Stahl, GF 254 (e.g. Merck GFR 7730)

3.11. Hydrochloric acid 6 N

3.12. Acetic acid 100%

3.13. Nitric acid 25%

3.14. Bismuth oxyde nitrate

3.15. Potassium iodide

3.16. Developing solvent mixtures:

3.16.1.	600	parts by volume	benzene
	240	,, ,, ,,	acetic acid (3.12.)
	60	,, ,, ,,	acetone
	24	,, ,, ,,	water
3.16.2.	90	,, ,, ,,	hydrochloric acid 1N
	10	,, ,, ,,	methanol (3.1.)
3.16.3.	95	,, ,, ,,	chloroform (3.3.)
	5	,, ,, ,,	methanol (3.1.)
3.16.4.	90	,, ,, ,,	chloroform (3.3.)
	10	,, ,, ,,	methanol (3.1.)
3.16.5.	90	,, ,, ,,	methanol (3.1.)
	10	,, ,, ,,	acetic acid (3.12.)
3.16.6.	80	,, ,, ,,	methanol (3.1.)
	20	,, ,, ,,	acetic acid (3.12.)

3.17. Detection (spraying) agents

3.17.1. Modified Dragendorff reagent

Stock solution: dissolve 8 g bismuth oxyde nitrate (3.14.) in 25 ml nitric acid (3.13.). Add this solution to a mixture of 5 ml hydrochloric acid (3.11) and 20 g potassium iodide (3.15.). This mixture must become a clear solution. If not, add more potassium iodide and hydrochloric acid (3.11.). Fill up with water to 100 ml, store in refrigerator.

Working solution: add to 20 ml water 5 ml hydrochloric acid (3.11.), 2 ml stock solution and 6 ml 6N NaOH solution. If a white precipitate might occur, add some HCl (3.11.), until a clear solution is obtained.

3.17.2. Saturated solution of bariumdiphenylamine-4-sulphonate in methanol (3.1.): dissolve about 0.2 g into several ml dimeth formamide and fill up to 100 ml with methanol (3.1.).

3.17.3. Reagent on sulphonamides: dissolve 500 mg 4-dimethylamino-benzaldehyde into 25 ml hydrochloric acid (3.4.) and 25 ml ethanol (3.2.).

4. Apparatus/equipment

4.1. Glass tube with restriction on one end, for chromatography, length about 300 mm, inner diameter 22 - 25 mm.

4.2. Magnetic stirrer with heating

4.3. Centrifuge, for tubes 100 ml, about 4000 rpm.

4.4. Thin-layer chromatography equipment

4.5. Syringe 0.1 ml, e.g. Hamilton threaded plunger syringe.

4.6. Rotary evaporator

4.7. Drying cabinet preferably with forced ventilation

4.8. Normal laboratory glasswork

5. Preparation of TLC - plates

5.1. Silica gel: for the preparation of 5 plates, shake 70 g silica ge GF 254 (3.10.) with 140 ml water for 1½ minute; spread the resul-ting suspension with spreading apparatus over 5 20x20 cm plates, thickness of layer 0.8 mm. Allow to dry in the laboratory atmo phere for at least 15 minutes, and afterwards for 45 minutes at 120°C in the drying cabinet (4.7.). Keep plates in exsiccator with blue silica gel.

5.2. Aluminium oxyde: for the preparation of 5 plates: shake 120 g aluminium oxyde (3.9) with 150 ml water for 1½ minute ; spread th resulting suspension with spreading apparatus over 5 20x20 cm plates, thickness of layer 0.8 mm. Leave the plates for at least 15 minutes to the laboratory atmosphere and dry afterwards for 45 minutes in the drying cabinet (4.7.) at 120°C. Keep the plates in an exsiccator with blue silica gel.

6. Extraction and purification

Weigh about 10 g of feed (or less if a higher than normal content is to be expected) into a conical flask of 200 ml. Add 50 ml methanol (3 and reflux for 5 minutes under stirring (4.2.). Cool down and filter. Stuff the restricted end of the glass tube (4.1.) with cotton wool, and pour 25 g aluminuium oxyde (3.8.)into the tube. The whole filtrat is transferred to the top of this column, that is rinsed again with60 ethanol (3.2.). The total throughput of the column is collected into a round-bottomed evaporation flask of 200 ml, evaporated in the rotary

evaporator (4.6.) at 50°C and the residue dissolved into 1 ml methanol chloroform (3.6.). This is solution 6.1..

After the rinsing with 60 ml ethanol (3.2.) the column still contains most of the eventually present sulphonamides. Elute the sulphonamides with about 70 ml methanol/HCl (3.7.), using the green/yellow feed substances as a guide: when all of the green/yellow band has been eluted, the sulphonamides also may be considered as eluted. Collect the eluate in a separate flask, add ammonia (3.5.) until a pH of about 6 is obtained, remove an eventually formed precipitate by filtration or centrifugation and evaporate the clear liquid in the rotary evaporator (4.6.) at 50°C. Dissolve the residue in about 3 ml methanol/chloroform (3.6.) and remove the remaining precipitate by centrifugation. This is solution 6.2..

N.B. Last-minute addition: the eventual presence of methylbenzoquate may render the reduction of the amount of aluminium oxyde (3.8.) in the column from 25 to 12 g necessary, since MBQ tends to remain in the 25 g, but leaves the column when 12 g has been used. This reduction might give rise to more spots from normal feed ingredients; this should, however, be no problem to an analyst with ample experience into the method.

7. Application of the purified extract to the plates.
Before the plates, both the SiO_2 and the Al_2O_3-plates, are spotted with the purified extracts, they have to be activated by taking them out of the exsiccator and heating them for half an hour at 100°C. Then apply, in a 1 cm line, 0.05 ml of 6.1. and 0.08 ml of 6.2. to both an SiO_2 and an Al_2O_3-plate. If already an indication of the nature of the additive(s) has been gained, apply also the relevant standard spots: dissolve 10 mg of the pure product into 10 ml (3.6.) and apply next to the sample spot 0.02 ml of this solution(s) to the plates.

8. Development of the plates.
   8.1 SiO_2-plates: to prevent the appearance of double front lines (separation of the developing solvent?) keep the plates after application of the extracts for 15 minutes at 50°C and place them afterwards immediately into the unsaturated developing trough, containing developing solvent 3.16.1. Develop until 3 cm from the upper edge, dry the plates at 50°C in the heating cabinet (4.7.) and check by nose that all developing solvent is gone.
   8.2 Al_2O_3-plates: equilibrate the developing trough with developing solvent3.16.4. To perform this, cover the walls of the trough with filter paper, pour solvent 3.16.4. into the trough, cover

the trough and wait for one hour before putting in the plate. Proceed further as under 8.1..

9. Detection.

For an analyst skilled in the method, the only necessary way of detection is scrutinizing the dried plates under UV-light of 254 nm and of 366 nm. The R_f-values, combined with fluorescence colour resp,. quenching, are sufficient to trace and identify the compounds listed under 1. , surely when spots of the pure substances are next to the sample spots. Of course, the R_f-values may vary, from laboratory to laboratory and even in the same laboratory, mainly due to variations in atmospheric conditions. Therefore, the given R_f-values have to be considered as guidelines. To provide oneself with more certainty, specially during the start of the work with the method, the spraying agents (3.17.1.), (3.17.2.) and (3.17.3.) may be applied.

The R_f-values, found in our laboratory and the colours under 254 nm and 366 nm are given in the table in 11.

10. Special remarks.

The R_f-value of a substance may be influenced by accompanying feed substances (e.g. by impairing the flow of the developing agent). If this leads to insecurity, a comparable amount of the suspected additi should be added to the extract, and the TLC repeated. If the added product is identical to the one already present, only one spot will appear; if different, two spots.

For amprolium a special remark has to be made: this molecule being a quaternary nitrogen compound, will adhere to the origin with the mentioned systems. When a spot, suspected to be amprolium, remains in the origin on the SiO_2-plate, then, after inspection under UV, cover the lowest 2 cm of the plate with filter paper and develop again, now in 3.16.2. The filter paper is necessary to prevent crumbling away of the layer in contact with the solvent. Now amprolium moves, to about R_f% 40, but the fluorescence indicator is destroyed. Therefore, after drying the plate must be sprayed with 3.17.1., giving an orange - red spot.

Normally, only the developing solvents 3.16.1.and 3.16.4. are used The solvents 3.16.3.,3.16.5. and 3.16.6. are only mentioned as alter atives in the cases where 3.16.4. does not give a satsfactory result on the Al_2O_3-plate.

The purified, concentrated extracts (6.1.) and (6.2.) may be kept for several days to repeat the TLC if desired.

311/VI/77-E

11. Table of R_f-values and colours under UV of the different spots.

Additive's name	R_f-value x 100 Si+3.16.1.	R_f-value x 100 Al+3.16.4	Colour at 254 nm	Colour at 366 nm
Acetylenheptin	77	59	dark brown	black
Amprolium	0	5	blue	---
Buquinolate	36	64	blue	blue
Carbadox	27	74	dark blue	yellow
Decoquinate	42	64	blue	blue
Dimetridazole	59	85	blue	black
Diaveridine	14	59	blue	---
Dinitolmide	75	55	blue	black
Ethopabate	51	76	blue	---
Furazolidone	36	72	black	blue
Furnicozone	21	58	black	black
Methylbenzoquate	27	40	blue	blue
Meticlorpindol	22	32	blue	---
Nicarbazin comp.1	8	--	light blue	---
,, ,, 2	64	67	yellow-browm	black with tai
Nifursol	16	0	dark brown	black with tai
Nitrofurazone	62	29	black	black with yellow rim
Nitrovin	16	57	dark brown	black
Pyrimethamine	20	70	brillant blue	light blue
Robenidine	77	86	brillant blue	light blue
Ronidazole	41	50	blue	dark blue
Sulphaquinoxalin	51	79	dark blue	black
Sulphaguanidine	11	6	blue	---
Sulphacetamide	23	3	blue	black
Sulphanilamide	23	24	blue	black
Sulphadimidine	43	20	blue	black
Sulphadiazine	35	19	blue	black
Sulphapyridine	43	4	blue	---
Sulphamerazine	46	6	blue	black
Sulphathiazole	28	0	blue	black
Sulphadimethoxine	60	7	blue	---