

Joint Research Centre (JRC)



Methods for cross-contamination of coccidiostats from target feed to non-target feed

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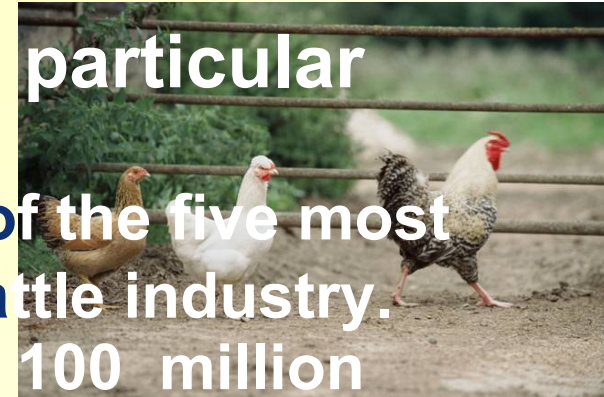
IRMM - Institute for Reference Materials and Measurements

Geel - Belgium

<http://irmm.jrc.ec.europa.eu/>

<http://www.jrc.ec.europa.eu/>

- **Coccidiosis is a disease affecting in particular poultry and cattle.**
 - **Economics: Coccidiosis in cattle is one of the five most economically important diseases of the cattle industry.**
 - **Estimated annual cost for the industry: \geq 100 million EUR**
- **Council Regulation No 2821/98 amending Directive 70/524/EEC concerning additives in feedingstuffs: Ban of certain antibiotics.**
- **Commission Regulation (EC) No 1831/2003**
 - **Only 11 authorised anticoccidial substances remaining**
 - **need for control methods at authorised and cross-contamination levels**



Cross-contamination

- assessment of the **risks** involved for animal and public **health** as a consequence of **unavoidable cross-contamination** of frequently used **coccidiostats** authorised as **feed additive** into **non-target feeds** and consequently the presence of such residues in **food** of animal origin.

Directive 2009/8/EC
applicable as from
01 July 2009

Maximum levels of unavoidable carry-over of
coccidiostats in non-target feed (**mg of active
substance/kg feed**)

	poultry	pig	cattle	calf
narasin	0.50	1.50	1.50	1.50
lasalocid	1.25	3.75	1.25	1.25
semduramicin	0.25	0.75	0.75	0.75
maduramicin	0.05	0.15	0.15	0.15
salinomycin	0.50	1.50	1.50	1.50
monensin	1.20	3.60	1.20	3.60
robenidine	0.70	2.10	2.10	2.10
decoquinate	0.40	1.50	1.50	1.50
halofuginone	0.03	0.09	0.09	0.09
diclazuril	0.01	0.03	0.03	0.03
nicarbazin	0.50	1.50	1.50	1.50

- **LC-MS/MS methods: e.g. developed and in-house validated at JRC-IRMM**

- **Rapid methods: multiplex immunoassays**
→ **Collaborative European project CONffIDENCE, WP2a**

- * **CONffIDENCE is funded by the European Community's Seventh Framework Programme (FP7/2007-2013) under grant agreement n° 211326 – RIKILT: co-ordinator**

➤ Collaborative European project CONffIDENCE, WP2a

➤ Partners: RIKILT, CER, FERA, QUB, NUTRECO, JRC

➤ Objectives:

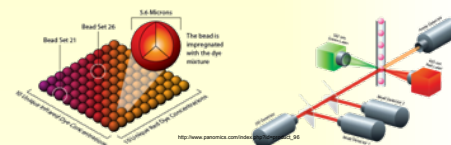
- ✓ Validated **flow cytometry based multiplex immunoassay** for residues of **lasalocid A, monensin, salinomycin, narasin, nicarbazin** and **diclazuril** in **eggs**
- ✓ **Cross- contamination** in **non-targeted feed** (laying hens feed)
- ✓ **Simplified sample preparation protocols** for **eggs and feed**
- ✓ **Carry-over study** of **lasalocid** from **laying hens feed** to **eggs** aiming at contribution to a **predictive hazard behaviour model**.

* CONffIDENCE is funded by the European Community's Seventh Framework Programme (FP7/2007-2013) under grant agreement n° 211326; Project co-ordinator: Dr. Jacob de Jong



➤ **Methodology principle: flow cytometry based multiplex immunoassay (FCI) using the Luminex™ platform.**

➤ **Simultaneous detection of coccidiostats: FCI combined MultiAnalyte Profiling (xMAP) technology.**



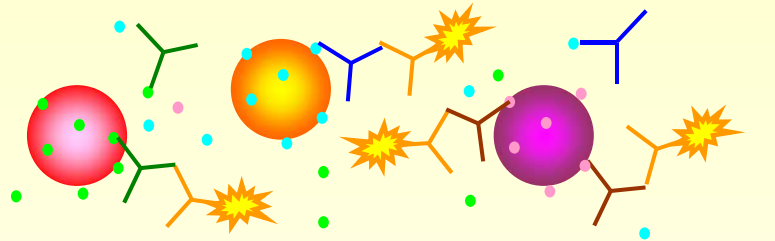
➤ **Antigens (drugs or drug-protein conjugates) covalently coupled on carboxylated polystyrene microspheres (beads) internally dyed with a red and an orange fluorophore.**

➤ **The Luminex contains a red laser for identification of the bead set by its characteristic colour and a green laser for the quantification of the amount of fluorescent dye corresponding with the amount of antibodies bound to the beads.**

➤ **This combination makes it possible to simultaneously measure up to 100 different biomolecular reactions in a single well.**



➤ Method under development



Principle of the multiplex inhibition assay in a well. The 3 different antibodies are represented in green, blue and red.

Production of conjugates: QUB



Production of antibodies: CER



Production of eggs materials: CER, FERA

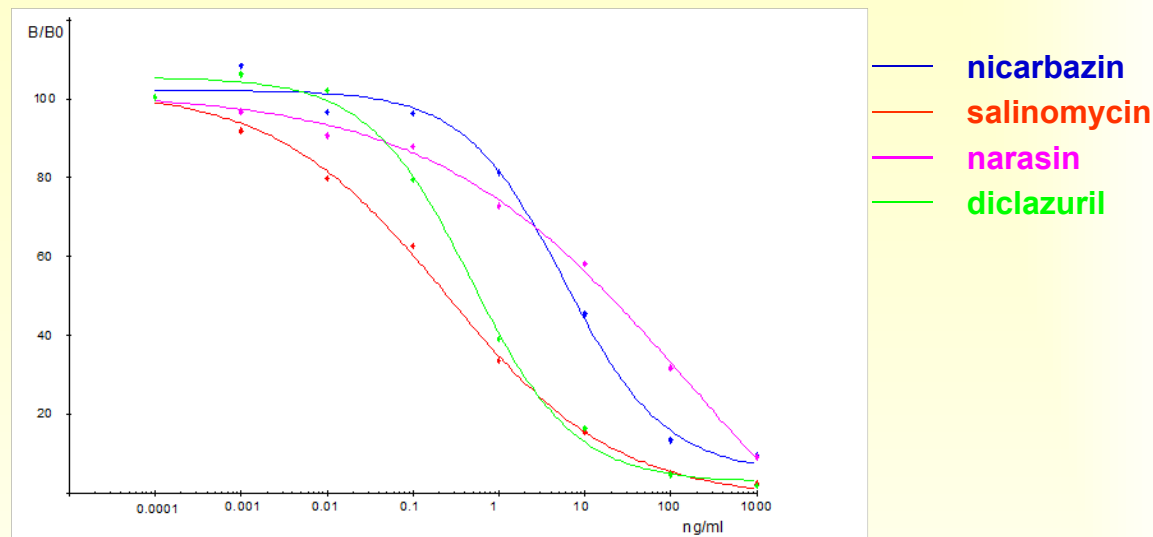


Production of feed materials: NUTRECO, CER



➤ First results

Calibration curves of nicarbazin, diclazuril, salinomycin and narasin in buffer.



- **Sensitivities** of nicarbazin, diclazuril and salinomycin assays promising
- Performance in egg and feed extracts to be tested
- Narasin assay needs further **optimization**



Follow-up:

- Development/optimisation of individual flow cytometry immunoassays, including bead-coupling in buffer
- Development/optimisation of the multiplex assay format for all coccidiostats in sample (eggs and feed) extracts
- Validation (single-laboratory and small-scale inter-laboratory) of the assay
- Comparison of performance characteristics with existing methods.
- Carry-over study of lasalocid from laying hens feed to eggs - assessment of fit for purpose of the new assay



Sample preparation:

- Liquid/solid extraction with MIBK (or ACN)
- Centrifugation
- Filtration on Nylon filters
- Evaporation to dryness and re-dissolution in ACN (with IS)
- Agitation, sonication and centrifugation

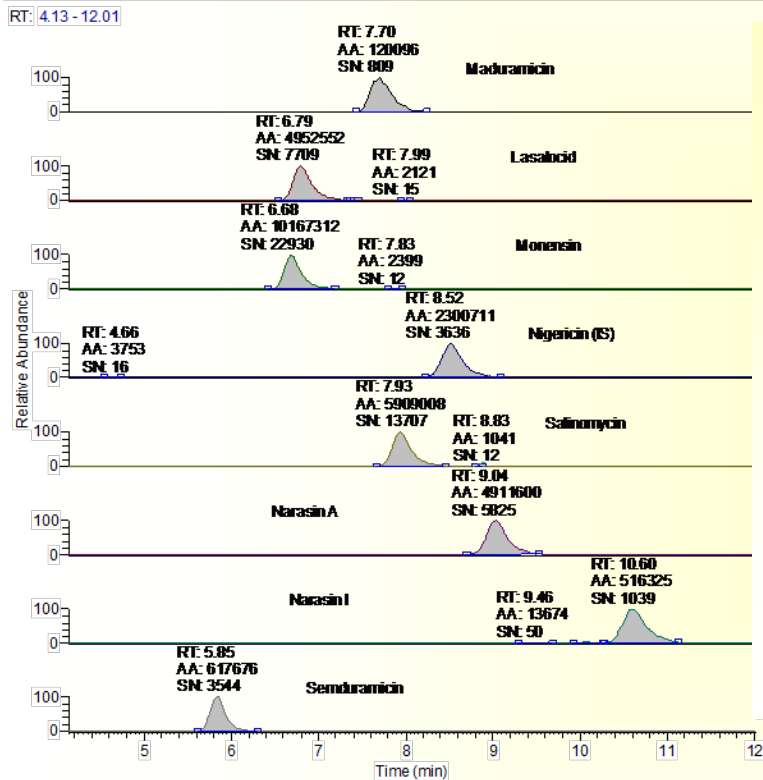
Note: for calf feed, an extra-step of de-fattening the extracts with n-hexane is introduced

LC-MS/MS determination:

- Isocratic RP-HPLC
- ESI+ in multi-reaction monitoring mode
- Main transitions used for the quantification

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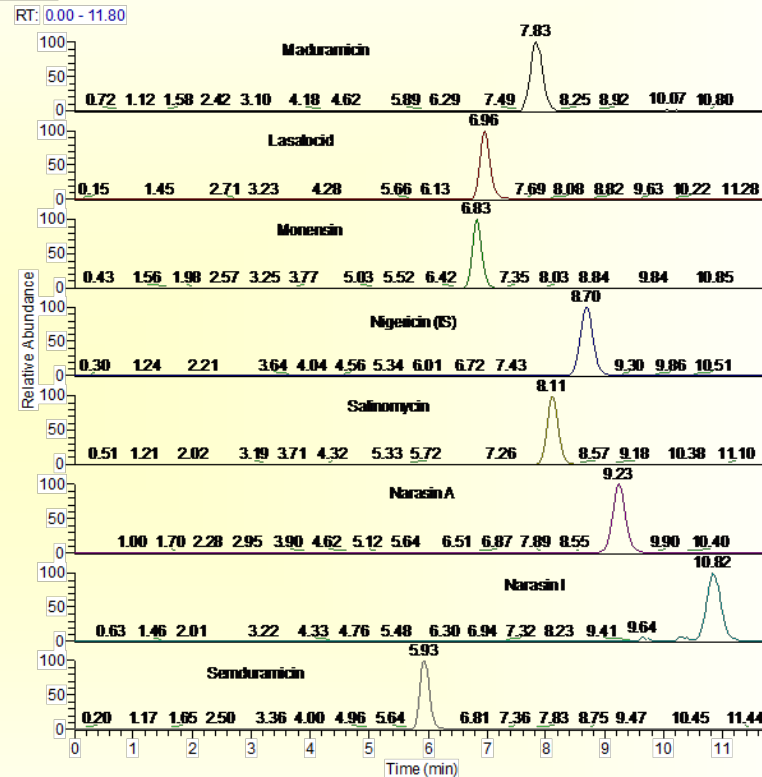
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Calf feed

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Cattle feed

Maduramicin
Lasalocid
Monensin
Nigericin (IS)
Salinomycin
Narasin A
Narasin I
Semduramicin

Maduramicin
Lasalocid
Monensin
Nigericin (IS)
Salinomycin
Narasin A
Narasin I
Semduramicin

- **3 days scheme**

- **3 concentrations**
 - $\frac{1}{2}$ target level – C1,
 - target level – C2,
 - 2*target level – C3

- **3 replicates per concentration and per day**

- **6 injections per sample**

- **Quantification by standard addition**

	Target value (mg kg ⁻¹)	Mean value (mg kg ⁻¹)	S _r	S _R	RSD _r %	RSD _R %	RR%	S _r	S _R	Horrat ratio
Maduramicin	0.08	0.07	0.004	0.005	5.98	7.47	89	5	7	0.31
	0.15	0.13	0.006	0.006	4.54	4.54	88	4	4	0.21
	0.3	0.29	0.03	0.03	10.88	10.88	95	10	10	0.56
Semduramicin	0.38	0.37	0.02	0.02	5.72	6.54	98	6	6	0.35
	0.75	0.71	0.04	0.04	5.08	5.64	95	5	5	0.33
	1.5	1.36	0.07	0.09	5.37	6.62	91	5	6	0.43
Narasin	0.75	0.78	0.05	0.05	6.68	6.68	104	7	7	0.40
	1.5	1.52	0.05	0.05	3.02	3.02	101	3	3	0.20
	3	2.96	0.06	0.07	2.09	2.33	99	2	2	0.17
Salinomycin	0.75	0.78	0.03	0.03	4.25	4.25	103	4	4	0.26
	1.5	1.52	0.03	0.03	1.65	1.65	101	2	2	0.11
	3	3.00	0.07	0.07	2.23	2.23	100	2	2	0.16
Lasalocid	0.63	0.70	0.04	0.08	5.44	12.02	112	6	13	0.71
	1.25	1.30	0.11	0.11	8.38	8.38	104	9	9	0.54
	2.50	2.77	0.16	0.16	5.77	5.77	111	6	6	0.42
Monensin	1.8	1.88	0.14	0.16	7.29	8.30	104	8	9	0.57
	3.6	3.68	0.14	0.14	3.84	3.84	102	4	4	0.29
	7.2	7.52	0.29	0.29	3.80	3.84	104	4	4	0.33

S_r, standard deviation for repeatability, S_R, standard deviation for intermediate precision

RSD_r (%), relative standard deviation for repeatability, RSD_R (%), relative standard deviation for intermediate precision

RR%: Recovery

	Target value (mg kg ⁻¹)	Mean value (mg kg ⁻¹)	S _r	S _R	RSD _r %	RSD _R %	RR%	S _r	S _R	Horrat ratio
Maduramicin	0.08	0.08	0.01	0.01	6.56	11.81	102	7	12	0.50
	0.15	0.15	0.01	0.01	5.35	6.02	100	6	22	0.28
	0.3	0.27	0.02	0.02	6.39	8.27	89	6	7	0.42
Semduramicin	0.38	0.32	0.02	0.03	5.92	7.79	86	5	7	0.41
	0.75	0.61	0.02	0.03	2.63	4.10	81	2	24	0.24
	1.5	1.16	0.12	0.17	10.71	14.50	77	8	11	0.93
Narasin	0.75	0.73	0.11	0.11	14.65	14.65	97	14	14	0.87
	1.5	1.43	0.04	0.08	2.44	5.30	96	2	5	0.35
	3	2.97	0.13	0.19	4.37	6.32	99	4	6	0.47
Salinomycin	0.75	0.77	0.02	0.04	3.10	5.04	103	3	5	0.30
	1.5	1.44	0.06	0.04	3.82	2.71	96	4	7	0.18
	3	2.93	0.05	0.08	1.74	2.73	98	2	3	0.20
Lasalocid	1.88	1.41	0.07	0.10	4.68	7.02	75	4	5	0.46
	3.75	2.69	0.16	0.23	5.83	8.58	72	4	6	0.62
	7.5	6.94	0.27	0.27	3.85	3.85	93	4	4	0.32
Monensin	1.8	1.89	0.07	0.16	3.86	8.67	105	4	9	0.60
	3.6	3.54	0.10	0.29	2.74	8.29	98	3	8	0.63
	7.2	8.11	0.24	0.43	2.96	5.34	113	3	6	0.46

S_r, standard deviation for repeatability, S_R, standard deviation for intermediate precision

RSD_r (%), relative standard deviation for repeatability, RSD_R (%), relative standard deviation for intermediate precision

RR%: Recovery

	Poultry feed		Pig feed		Cattle feed		Calf feed	
	RR (%)	RSDInt. (%)	RR (%)	RSDInt. (%)	RR (%)	RSDInt. (%)	RR (%)	RSDInt. (%)
Mad	94	13	101 97	13 17	96	6	96 91	6 8
Sem	93	16	96 87	14 21	94	6	94 94	6 7
Nar A	100	4	99 97	7 9	98	4	98 101	4 5
Sal	100	4	101 99	6 7	99	4	99 102	4 3
Las	103	7	111 80	12 12	108	5	108 109	5 11
Mon	102	4	107 105	9 11	101	5	101 104	5 7

RR (%): Mean percentage recovery rate of the recovery rates obtained on the three concentrations. RSDInt. (%): Relative standard deviation for intermediate precision. -: extraction solvent: ACN, -: extraction solvent: MIBK

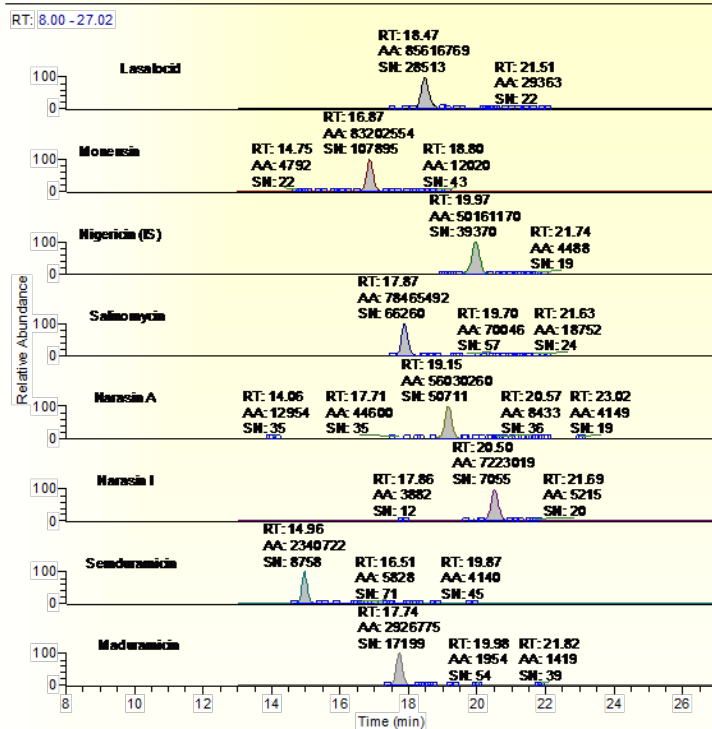
Sample preparation:

- Liquid/solid extraction with an ACN:MeOH mixture
- Centrifugation
- Filtration on Nylon filters
- Standard additions into filtrates
- De-fattening with n-hexane
- Evaporation to dryness and re-dissolution in ACN:MeOH
- Agitation

LC-MS/MS determination:

Step gradient RP-HPLC

- ESI+ in multi-reaction monitoring mode
- Main transitions used for the quantification



Lasalocid

Monensin

Nigericin (IS)

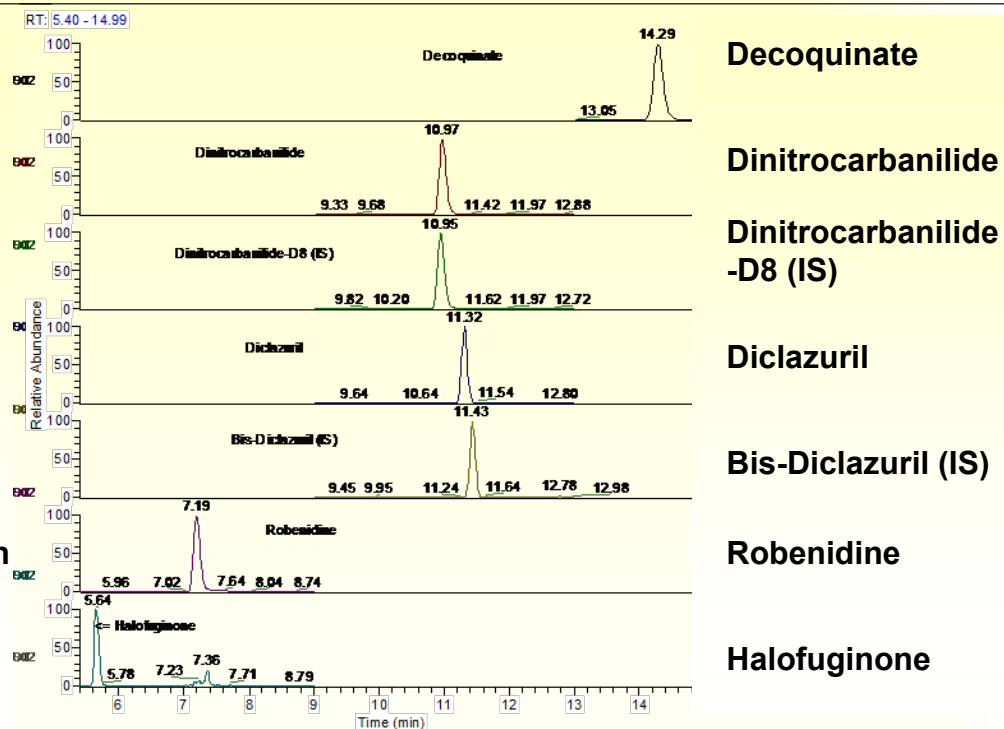
Salinomycin

Narasin A

Narasin I

Semsduramicin

Maduramicin



Decoquinate

Dinitrocarbanilide

Dinitrocarbanilide
-D8 (IS)

Diclozauril

Bis-Diclozauril (IS)

Robenidine

Halofuginone

Poultry feed

Pre-validation results

	Conc. mg kg⁻¹	Recovery %	R²
Maduramicin	0.05	110	0.997
Semduramicin	0.25	107	0.993
Narasin A	0.70	100	0.998
Salinomycin	0.70	105	0.999
Lasalocid	1.25	108	0.994
Monensin A	1.25	115	0.998
Halofuginone	0.03	95	0.960
Robenidine	0.70	93	0.999
Dinitrocarbanilide (DNC)	0.50	86	0.995
Diclazuril	0.01	85	0.975
Decoquinate	0.40	110	0.992

Follow-up:

- **Single-laboratory validation of the LC-MS method 2**
- **Collaborative study for the LC-MS method 2**
- **Submission for CEN standard (CEN TC 327 – WG3)**

➤ IRMM methods:

➤ Zigmantas Ezerskis, Mostafa Chedin

➤ CONFIDENCE partners:

➤ RIKILT: Monique Bienenmann-Ploum, Mirjam van Aalderen, Willem Haasnoot, Michel Nielen

➤ CER: Anne-Catherine Huet, Philippe Delahaut

➤ QUB: Katrina Campbell, Terence Fodey, Chris Elliott

➤ FERA: Matthew Sharman, Sara Stead

➤ NUTRECO: Albert Swinkels



Thanks for your attention