Joint Research Centre (JRC)

CROSS-CONTAMINATION OF COCCIDIOSTATS FROM TARGET FEED TO NON-TARGET FEED: AN ANALYTICAL CHALLENGE

Dr. Ursula VINCENT

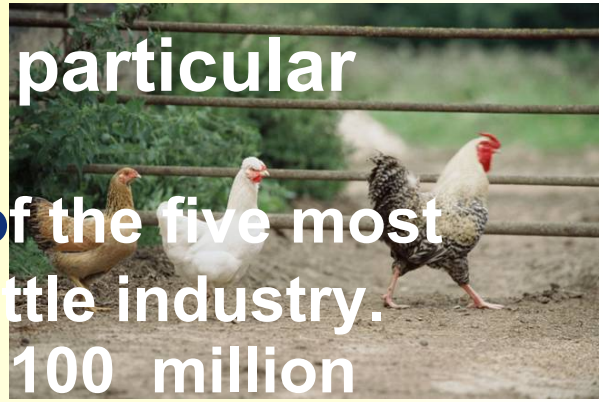
IRMM - Institute for Reference Materials and Measurements

Geel - Belgium

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

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

Scope



- 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: ≥ 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 11authorised 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.

→ Commission Directive 2009/8/EC: maximum levels of unavoidable carry-over of coccidiostats or histomonostats in non target feed

→Maximum levels (ML's) in eggs have been set by the European Union (Commission Regulation (EC) No 124/2009)

Scope *ctd.* - Need of sensitive methods!

	Maximum levels of unavoidable carry-over of coccidiostats in non-target feed (mg of active substance/kg feed)			
	poultry	pig	cattle	calf
narasin	**0.70**	2.10	2.10	2.10
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	**0.70**	2.10	2.10	2.10
monensin	**1.25**	3.75	1.25	1.25
robenidine	0.70	2.10	2.10	2.10
decoquinate	0.40	1.20	1.20	1.20
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

Scope *ctd.* - Need of sensitive methods!

	Maximum content of coccidiostats in eggs (ML's) (μg/kg wet weight)
narasin	2
lasalocid	5*
semduramicin	2*
maduramicin	2*
salinomycin	3
monensin	2*
robenidine	25
decoquinate	20*
halofuginone	6
diclazuril	2
nicarbazin	100

*** in other food stuff of animal origin**

Methods and challenges

- **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 ; Project co-ordinator: RIKILT - Dr. Jacob de Jong**

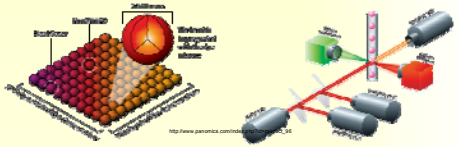
CONffIDENCE multiplex immunoassay

- 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 multiplex immunoassay

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

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

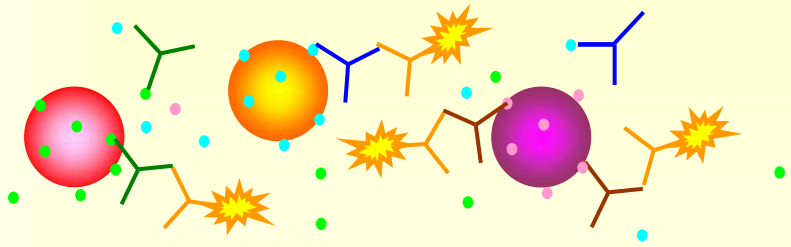


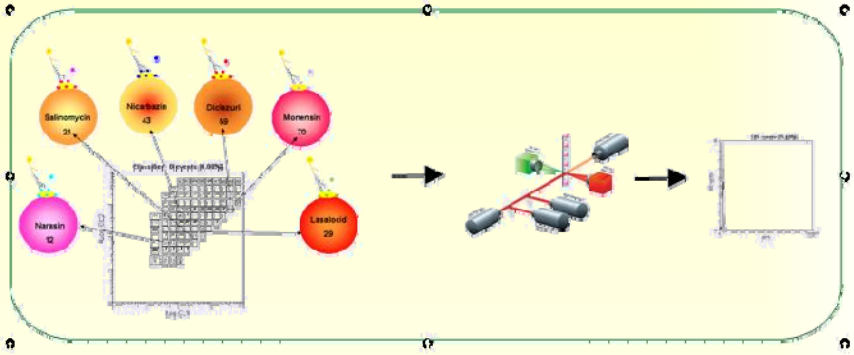
- Antigens (drugs or drug-protein conjugates) covalently coupled on carboxylated polystyrene microspheres (beads) internally dyed with a red and an orange fluorophore.
- The Luminex contains a red laser for identification of the bead set by its characteristic colour and a green laser for the quantification 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.

CONffIDENCE multiplex immunoassay
Poster 19

RIKILT INSTITUTE OF FOOD SAFETY WAGENINGEN UR

➢ Method under development





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

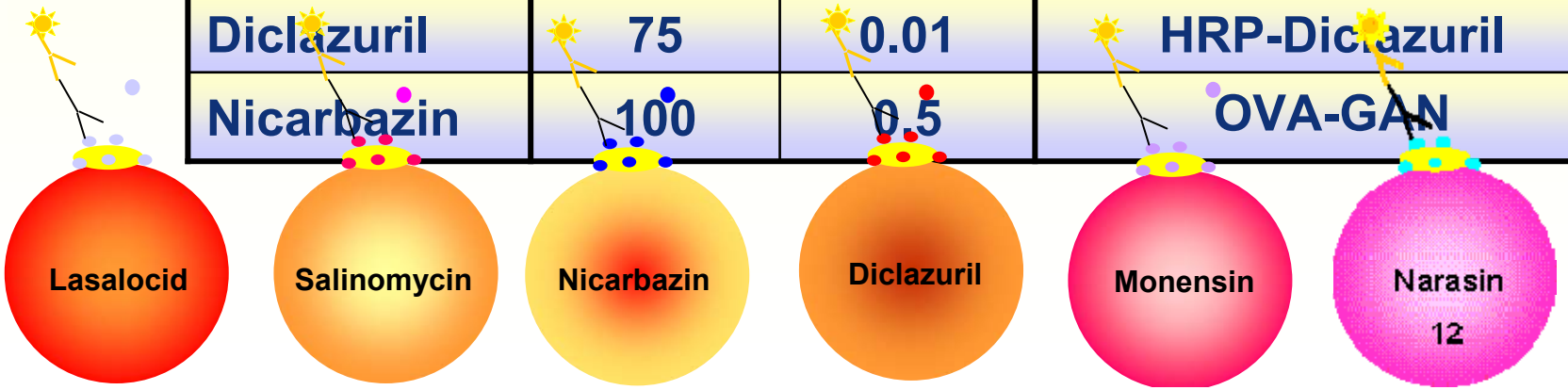
Production of conjugates: QUB
Production of antibodies: CER
Production of eggs materials: CER, FERA
Production of feed materials: NUTRECO, CER

Queen's University Belfast
CER Groupe
fera The Food and Environment Research Agency
nutreco
CONffIDENCE

Multiplex immunoassay in buffer
Antibodies production and characterisation

no cross-reaction, including with the other authorised coccidiostats: antibodies are specific

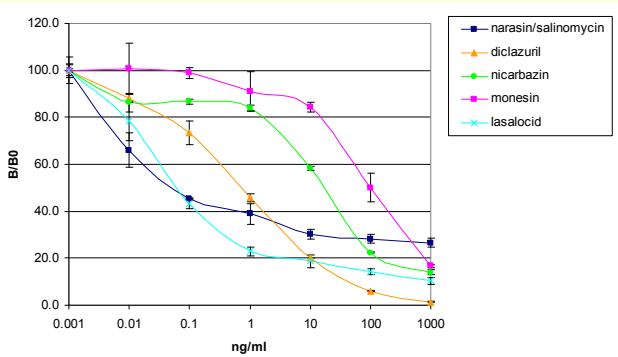
	LOD (DoW)		Conjugate on the bead	IC_{50}
	Egg (µg kg^{-1})	Feed (mg kg^{-1})		(ng ml^{-1})
Monensin	75	1.25	Monensin	25*
Salinomycin	75	0.7	OVA-Salinomycin	0.3
Narasin	75	0.7	OVA-Narasin	0.3
Lasalocid	75	1.25	OVA-Lasalocid	1
Diclazuril	75	0.01	HRP-Diclazuril	0.6
Nicarbazin	100	0.5	OVA-GAN	10



Multiplex immunoassay in buffer

Developed multiplex	Assay involved	IC_{50} (ng ml^{-1})
5-Plex	Salinomycin	0.1
	Nicarbazin	9
	Diclazuril	0.4
	Lasalocid	0.3
	Monensin	34
3-Plex	Narasin/ Salinomycin	0.1 / -
	Nicarbazin	11
	Diclazuril	0.5
2-Plex	Lasalocid	0.1
	Monensin	39*

Calibration curves of the 3-plex assay (nicarbazin, diclazuril, salinomycin and narasin) and of the 2-plex assay (monensin and lasalocid) in buffer.



Egg materials

	Target concentration ($\mu g\ kg^{-1}$)	Max. Exp. incurred concentration ($\mu g\ kg^{-1}$)	Amount of material available (kg)
Diclazuril	75	158	2
Narasin	75	30	2 (9 - 30 $\mu g\ kg^{-1}$)
Nicarbazin	75	1900	2
Monensin	75	37	2 (13 - 37 $\mu g\ kg^{-1}$)
Salinomycin	75	191	2
Lasalocid	75	7416	2

CONFIDENCE

Multiplex immunoassay in matrix

Feed materials

	Target concentration (mg kg^{-1})	Mean Exp. Concentration (mg kg^{-1})	Amount of available material (kg)
Blank	0	0	5
Diclazuril	0.01	0.007	1
	0.20	0.17	0.5
Narasin	0.70	0.58	1
	14.00	14.18	0.5
Nicarbazin	0.50	0.57	1
	10.00	11.57	0.5
Monensin	1.25	1.09	1
	25.00	22.42	0.5

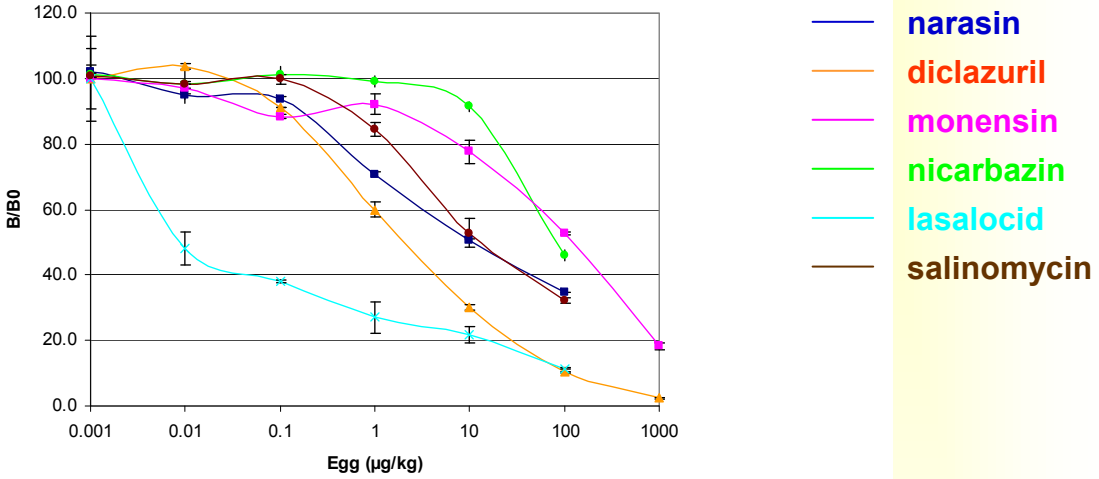
Multiplex immunoassay in matrix

Feed materials

	Target concentration (mg kg^{-1})	Mean Exp. Concentration (mg kg^{-1})	Amount of available material (kg)
Salinomycin	0.70	0.92	1
	14.00	15.30	0.5
Lasalocid	1.25	1.29	1
	25.00	21.30	0.5
	3.13	-	5
	12.50	-	5

First results in eggs

Calibration curves of nicarbazin, diclazuril, salinomycin, monensin, lasalocid and narasin in egg



Sensitivity of the combined 3-plex and 2-plex assays promising

	ML's in eggs (µg/kg)
narasin	2
salinomycin	3
nicarbazin	100
diclazuril	2
lasalocid	5*
monensin	2*

Developed multiplex	Assay involved	IC_{50} (ng ml^{-1}) in buffer
3-Plex	Narasin/ Salinomycin	0.1 / -
	Nicarbazin	11
	Diclazuril	0.5
2-Plex	Lasalocid	0.1
	Monensin	39*

JRC – IRMM: LC-MS/MS method

Poster 203

Simultaneous determination of the 11 authorised coccidiostats in poultry, cattle, pig and calf feed at both authorised and cross-contamination levels

Sample preparation:

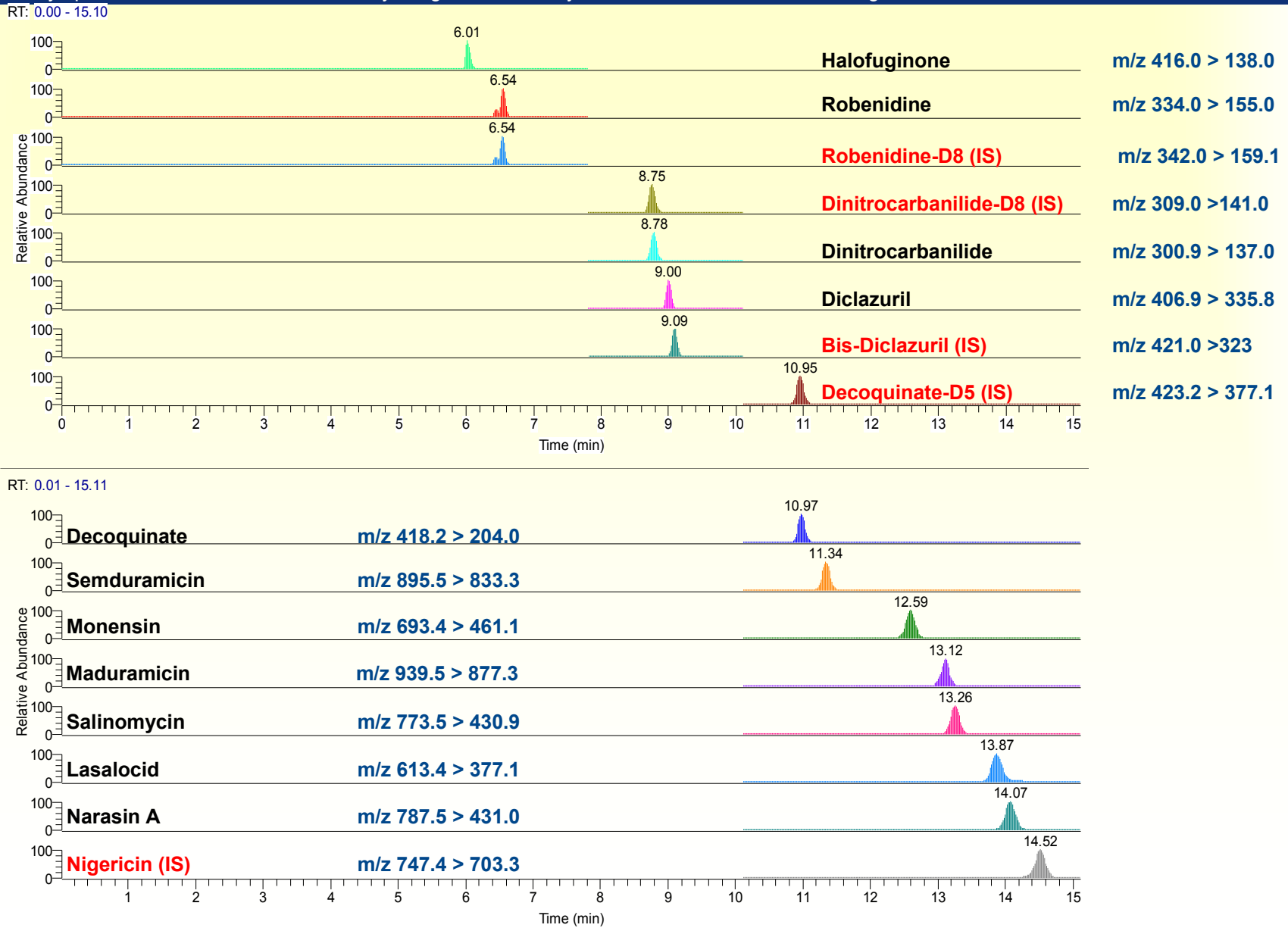
- Liquid/solid extraction with an ACN:MeOH mixture
- Sonication, agitation and centrifugation
- Filtration on Nylon filters
- Standard addition ("universal approach") in the extracts

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

LC-MS/MS determination:

- Step-gradient RP-HPLC on a C8 column, mixed ACN:H2O, MeOH:ACN mobile phases, 0.1% formic acid, 350 μl min^{-1}, analysis time: 20 min
- ESI+, ESI- in multi-reaction monitoring mode
- Main transitions used for the quantification

An example: poultry feed



Single-laboratory validation

- 3 days scheme
- 3 concentrations
  - ½ target level – C1,
  - target level – C2,
  - 2*target level – C3
- 3 replicates per concentration and per day
- 3 injections per sample
- Quantification by standard addition

JRC Performance characteristics poultry feed

EUROPEAN COMMISSION

Institute for Reference Materials and Measurements

S_r, standard deviation for repeatability,
$S_{Int.}$, standard deviation for intermediate precision
RSD_r (%), relative standard deviation for repeatability,
$RSD_{Int.}$, (%), relative standard deviation for intermediate precision
RR%: Recovery

Analyte	Target value (mg kg^{-1})	Mean value (mg kg^{-1})	S_r (mg kg^{-1})	Sint. (mg kg^{-1})	RSD_r%	$RSD_{Int.}$ %	RR %	S_r %	$S_{Int.}$ %	Horrat value
Maduramicin	0.03	0.03	0.002	0.002	7.51	7.51	107	8.72	0.00	0.27
	0.05	0.06	0.004	0.004	7.02	7.02	114	8.01	8.01	0.29
	0.10	0.10	0.007	0.007	7.04	7.04	99	7.08	7.08	0.31
Semduramicin	0.13	0.13	0.014	0.017	10.54	12.80	106	10.87	13.84	0.49
	0.25	0.30	0.01	0.014	3.34	4.67	120	3.91	5.46	0.17
	0.50	0.50	0.052	0.052	10.39	10.39	100	10.47	10.47	0.59
Narasin	0.35	0.38	0.015	0.015	3.91	3.91	110	4.27	4.27	0.21
	0.70	0.73	0.018	0.018	2.46	2.46	105	2.52	2.58	0.15
	1.40	1.47	0.039	0.059	2.66	4.02	105	2.82	4.21	0.18
Salinomycin	0.35	0.36	0.019	0.02	5.26	5.54	103	5.57	5.63	0.28
	0.70	0.71	0.021	0.021	2.97	2.97	101	2.98	2.98	0.18
	1.40	1.37	0.037	0.037	2.69	2.69	98	2.65	2.65	0.18
Lasalocid	0.63	0.72	0.034	0.046	4.70	6.35	116	5.44	7.39	0.28
	1.25	1.43	0.035	0.039	2.45	2.72	115	2.80	3.13	0.16
	2.50	3.09	0.095	0.131	3.08	4.24	123	3.80	5.25	0.23
Monensin	0.63	0.68	0.047	0.047	6.95	6.95	108	7.45	7.45	0.41
	1.25	1.31	0.155	0.155	11.83	11.83	105	12.44	12.44	0.77
	2.50	2.83	0.282	0.282	9.98	9.98	113	11.27	11.27	0.73
Halofuginone	0.02	0.01	0.001	0.001	8.31	8.31	80	6.34	6.34	0.27
	0.03	0.03	0.004	0.004	14.84	14.84	90	11.69	11.69	0.54
	0.06	0.05	0.007	0.007	13.73	13.73	85	10.93	10.93	0.55
Robenidine	0.35	0.34	0.007	0.01	2.07	2.96	96	2.13	2.85	0.11
	0.70	0.65	0.012	0.012	1.85	1.85	93	1.71	1.71	0.11
	1.40	1.33	0.033	0.071	2.48	5.33	95	2.37	5.10	0.16
DNC (Nicarbazin)	0.25	0.26	0.008	0.008	3.08	3.08	104	3.34	3.34	0.16
	0.50	0.50	0.007	0.009	1.40	1.81	100	1.37	1.88	0.08
	1.00	1.01	0.016	0.019	1.59	1.88	101	1.64	1.92	0.10
Diclazuril	0.005	0.004	0.0010	0.0010	22.35	22.35	89	12.88	12.88	0.62
	0.010	0.010	0.0010	0.0010	10.36	10.36	97	7.15	7.15	0.32
	0.020	0.020	0.0010	0.0010	5.07	5.07	99	6.76	6.76	0.18
Decoquinate	0.20	0.20	0.005	0.005	2.54	2.54	98	2.48	2.48	0.12
	0.40	0.39	0.007	0.007	1.81	1.81	97	1.78	1.78	0.10
	0.80	0.76	0.019	0.022	2.49	2.88	95	2.40	2.75	0.15

Performance characteristics poultry feed – Overall recovery

	Mean RR%	S_r %	$S_{Int.}$%
Maduramicin	107	10	10
Semduramicin	109	14	14
Narasin	106	5	5
Salinomycin	101	4	4
Lasalocid	118	7	8
Monensin	109	11	11
Halofuginone	85	10	10
Robenidine	95	3	4
DNC (nicarbazin)	102	3	3
Decoquinate	97	3	3

RR (%): Mean percentage recovery rate of the recovery rates obtained on the three concentrations. Sr %, standard deviation for repeatability, $S_{Int.}$ %, standard deviation for intermediate precision

Follow-up

- CON*ff*IDENCE project:
  - Performance to be increased for monensin
  - Sample preparation to be improved
  - Performance in egg extracts to be confirmed
  - Performance in feed extracts to tested
- JRC – IRMM LC-MS/MS method:
  - Collaborative study
  - Submission for CEN standard (CEN TC 327 – WG3)

- **JRC - IRMM:**
  - **Zigmas Ezerskis, Mostafa Chedin, Christoph von Holst**

- **CON*ff*IDENCE 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