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ISTITUTO ZOOPROFILATTICO SPERIMENTALE DELL'ABRUZZO E DEL MOLISE "G. CAPORALE"

I risultati della ricerca corrente condotta dall'Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise. Anno 2021

Teramo, 15 Maggio 2022

Scenari di esposizione alimentare ad alcuni contaminanti di interesse prioritario nella popolazione generale italiana (IZS AM 06/20 RC)
Responsabile scientifico: Gianfranco Diletti

Determinazione di residui di antibiotici nelle uova: valutazione del livello di contaminazione in Italia

Federica Castellani, Giorgio Saluti, Maria Novella Colagrande, Matteo Ricci, Gianfranco Diletti, Giampiero Scortichini

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SUMMARY

1.INTRODUCTION

- Official control of drug residues in European Union
- Legal framework
- Objectives of the project

→ Legal framework and objectives

2.EXPERIMENTAL

- Antibiotics detected
- Mass spectrometric conditions
- Chromatographic conditions
- Sample preparation

→ Our method

3.RESULTS AND DISCUSSION

- Optimization of sample preparation
- Real samples analysis
- Risk exposure
- Goals and dissemination

→ Method optimization and real sample analysis

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1.INTRODUCTION

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OFFICIAL CONTROL OF DRUG RESIDUES IN EUROPEAN UNION

NATIONAL RESIDUE PLANS

Residue drug definition: molecule present in edible material after pharmaceutical treatment. It includes the drug and every metabolite.

To detect drug residues in food chain and to assure consumer safety, regulations were established within the European Union from 1986 starting with the **Directive 86/469/EC** that imposed implementation of the annual National Residue Plan (PNR). Before this date, countries carried out not harmonized controls about the samples number and penalties, for instance.

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OFFICIAL CONTROL OF DRUG RESIDUES IN EUROPEAN UNION

PNR 1988
(Circolare Ministero della Sanità n.12, 8/2/1988)

Banned substances: **7** molecules/classes:
- ✓ Stilbenes
- ✓ Thyreostats
- ✓ Trenbolone
- ✓ Zeranol
- ✓ Estradiol
- ✓ Progesterone
- ✓ Testosterone

Matrices: bovine, swine and ovine (urine, faeces, plasma and muscle)

PNR 2022
Regulation (EU) 2017/625)

Banned substances: **22** molecules/classes:
- ➢Stilbenes
- ➢Thyreostats
- ➢Ethinylestradiol
- ➢Estradiol
- ➢Progesterone
- ➢Testosterone
- ➢Beta-agonists
- ➢Chloramphenicol
- ➢Nitroimidazoles
- ➢Metabolites of nitrofurans
- ➢…


REGULATIONS

REGULATION (EU) 2017/625 OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL

of 15 March 2017

on official controls and other official activities performed to ensure the application of food and feed law, rules on animal health and welfare, plant health and plant protection products, amending Regulations (EC) No 999/2001, (EC) No 396/2005, (EC) No 1069/2009, (EC) No 1107/2009, (EU) No 1151/2012, (EU) No 652/2014, (EU) 2016/429 and (EU) 2016/2031 of the European Parliament and of the Council, Council Regulations (EC) No 1/2005 and (EC) No 1099/2009 and Council Directives 98/58/EC, 1999/74/EC, 2007/43/EC, 2008/119/EC and 2008/120/EC, and repealing Regulations (EC) No 854/2004 and (EC) No 882/2004 of the European Parliament and of the Council, Council Directives 89/608/EEC, 89/662/EEC, 90/425/EEC, 91/496/EEC, 96/23/EC, 96/93/EC and 97/78/EC and Council Decision 92/438/EEC (Official Controls Regulation)

Matrices:
- ✧honey, milk, eggs, …
- ✧urine, muscle, liver, serum, plasma, kidney, …

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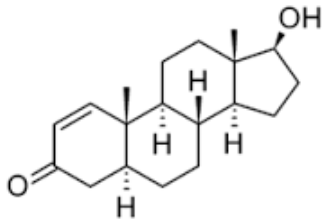
LEGAL FRAMEWORK

RESIDUE DRUG ANALYSIS IN FOODSTUFFS: THE EU STRATEGY

Regulation (EU) 2017/625

GROUP A (BANNED)

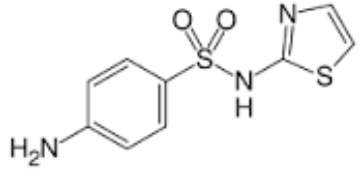
- Stilbenes, stilbene derivatives…
- Antithyroid agents
- Steroids
- Resorcylic acid lactones including zeranol
- Beta-agonists
- Chloramphenicol
- …



e.g. Alpha-trenbolone

GROUP B (PERMITTED)

- **Antibacterial substances, including sulfonamides, quinolones (B1)**
- Anthelmintics
- Sedatives
- Non-steroidal anti-infiammatory drugs (NSAIDs)
- Mycotoxins
- Chemical elements
- …



e.g. Sulfathiazole

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IMPORTANT

LEGAL FRAMEWORK

LIST OF MRLs: COMMISSION REGULATION (EU) No 37/2010 (GROUP B SUBSTANCES-VETERINARY DRUGS)

on pharmacologically active substances and their classification regarding maximum residue limits (**MRL**) in foodstuffs of animal origin

Pharmacologically active Substance	Marker residue	Animal Species	MRL	Target Tissues	Other Provisions (*according to Article 14(7) of Regulation (EC) No 470/2009*)	Therapeutic Classification
Tetracycline	Sum of parent drug and its 4- epimer	All food-producing species	100 µg/kg 300 µg/kg 600 µg/kg 100 µg/kg 200 µg/kg	Muscle Liver Kidney Milk Eggs	For fin fish the muscle MRL relates to 'muscle and skin in natural proportions'. MRLs for liver and kidney do not apply to fin fish.	Anti-infectious agents/Antibiotics

- Chlortetracycline, oxytetracycline and tetracycline
- Lincomycin (lincosamide), penicillin V (penicillin), tiamulin (pleuromutilin)
- Erythromycin A, tylvalosin and tylosin A (macrolides)
- Neomycin B (aminoglycoside)

→ **MRL**

Other regulated antibiotics such as **amphenicols**, **cephalosporins**, **doxycycline** (tetracyclines), **several β-lactams**, some **macrolides**, **quinolones**, and **sulfonamides** are prohibited in laying hens

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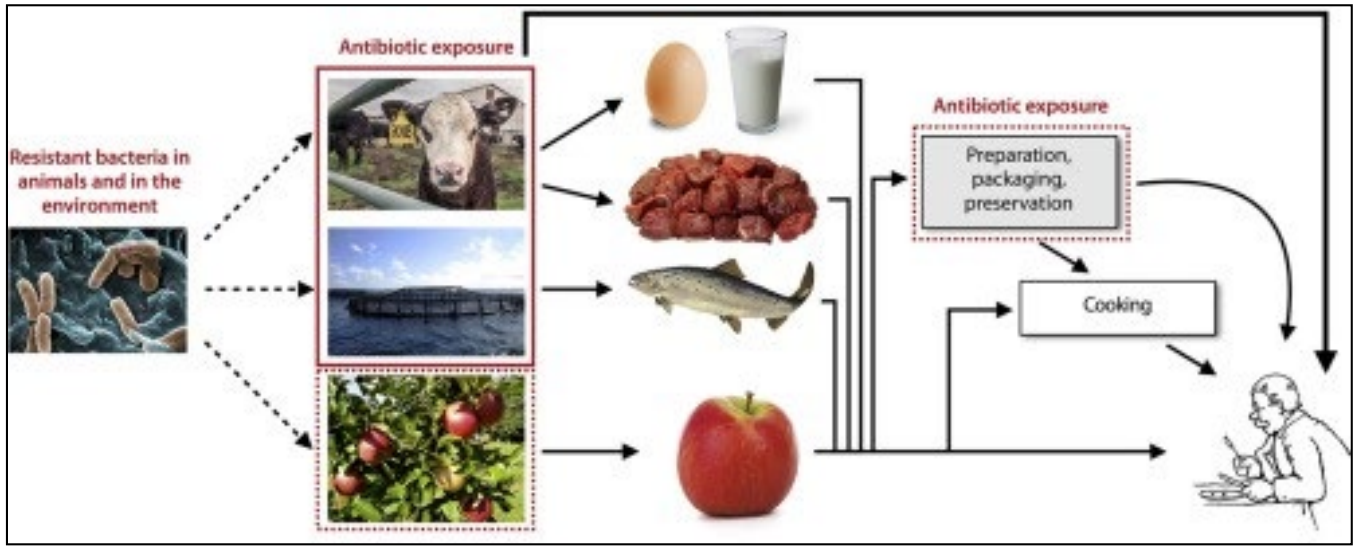
USE AND EFFECTS OF ANTIBIOTICS

Antibiotics have been widely administered in animal husbandry to treat and prevent diseases and to act as growth-promoting agents.

Their residues can become part of the food chain through various environmental pathways (i.e., water, soil, plant, and aquaculture), affecting human health

Allergic reactions and antibiotic resistance phenomena



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OBJECTIVES OF THE PROJECT

Title: «**Scenari di esposizione alimentare ad alcuni contaminanti di interesse prioritario nella popolazione generale italiana**»

1. Determination of BFR and PFAS in eggs, fish, molluscan shellfish, fruits and vegetables
2. Determination of rare-earth elements in vegetables, clams and mussels
3. Determination of antibiotic residues in eggs

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DELIBERAZIONE DEL DIRETTORE GENERALE

Immediatamente esecutiva

DELIBERAZIONE N 551 avente ad oggetto: Provvedimenti in merito al progetto di Ricerca Corrente anno 2020 dal titolo "Scenari di esposizione alimentare ad alcuni contaminanti di interesse prioritario nella popolazione generale italiana" (IZS AM 06/20 RC)

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OBJECTIVES OF THE PROJECT

Determination of antibiotic residues in eggs

- Development a confirmatory multiclass method for more than 70 of the regulated and most used antibiotics (except for aminoglycosides and colistin) in eggs using LC-HR-MR/MS
- Validation of the developed method according to **Commission Decision 2002/657/EC**
- Analysis of real samples: 200 Italian, commercial egg samples produced with conventional and organic approaches, collected during the years 2018–2021.

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2. EXPERIMENTAL

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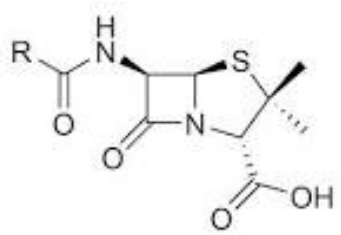
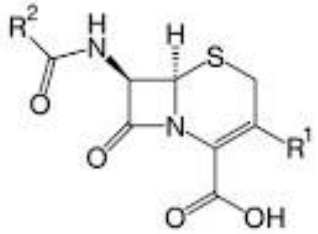
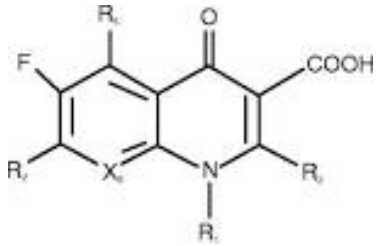
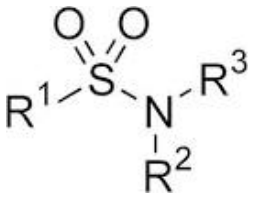
2. EXPERIMENTAL

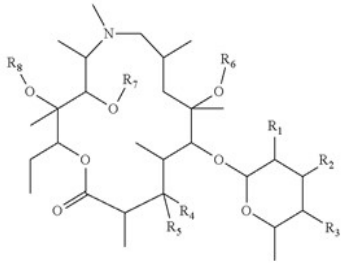
ANTIBIOTICS DETECTED
(SEVENTY-THREE➔11 FAMILIES)

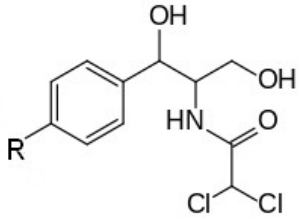

Penicillins

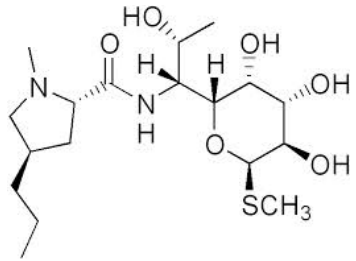

Cephalosporins

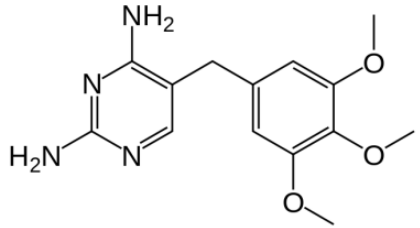

Quinolones

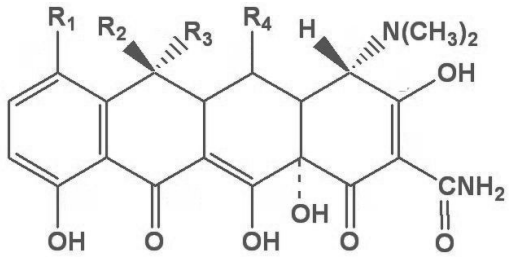

Sulfonamides

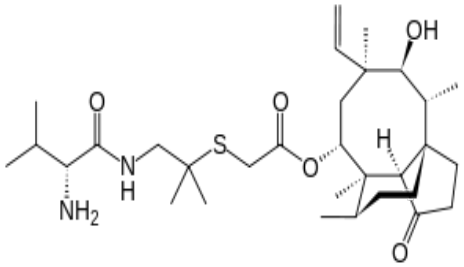

Macrolides

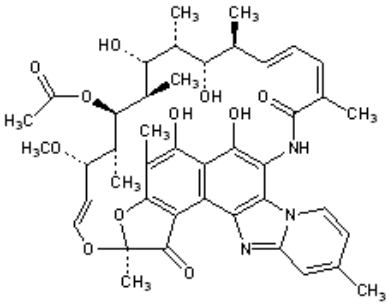

Amphenicols


Lincosamides
(Lincomycin - Pirlimycin)


Diaminopyrimidines
(Trimethoprim)


Tetracyclines


Pleuromutilins


Rifamicyns (Rifaximin)

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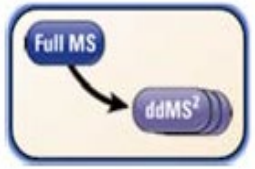
2. EXPERIMENTAL

MASS SPECTROMETRIC CONDITIONS

LC-HR-MS/MS: UHPLC Ultimate 3000 coupled to Q Exactive (Thermo): adducts

Class	Drug	Parent Exact m/z	Adduct
Quinolones	Difloxacin	400.1467	$+H^+$
Penicillins	Penicillin V	373.0829	$+Na^+$
Cephalosporins	Cephapirin	424.0632	$+H^+$
Macrolides	Erythromycin A	734.4685	$+H^+$
Sulfonamides	Sulfaquinoxaline	301.0754	+H+
Tetracyclines	Tetracycline	445.1605	+H+
Amphenicols	Thiamphenicol	358.0077	+H+
Diamino-pirimidines	Trimethoprim	479.1216	+H+
Lincosamides	Lincomycin	407.2210	+H+



Full MS / dd-MS²
(*m/z* 150-1200)

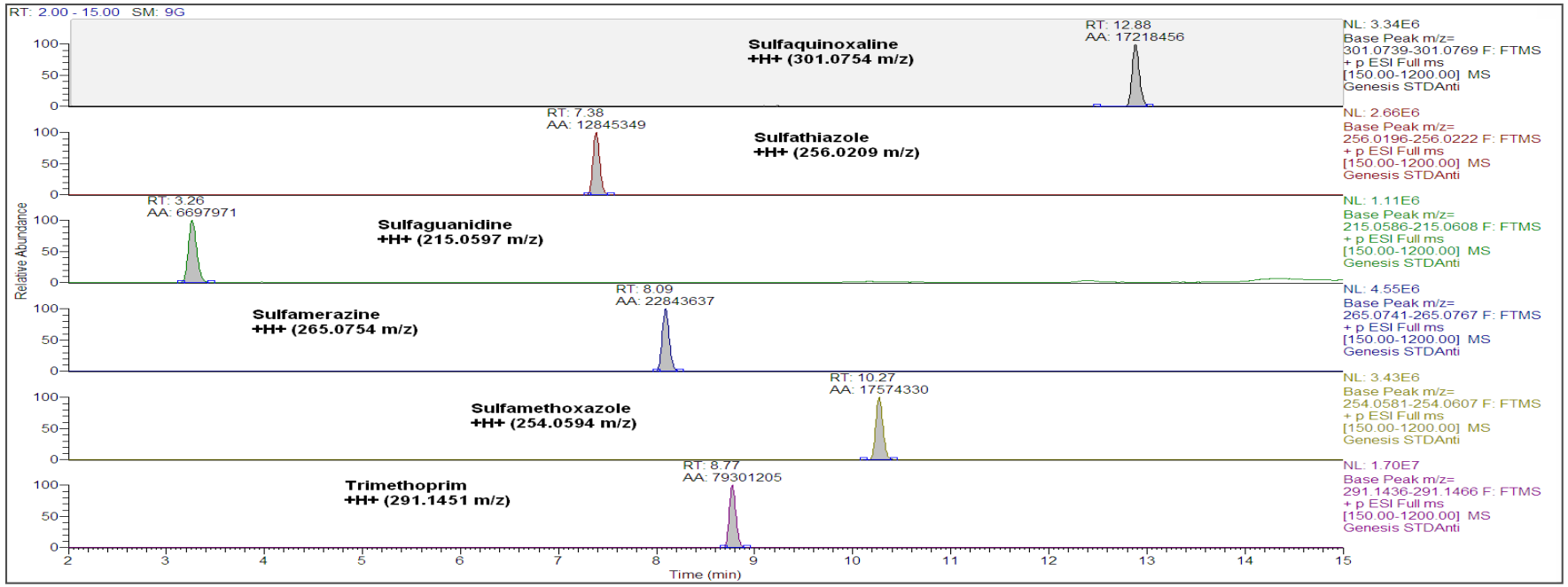
A **Full MS** scan (without collision energy) is followed by a set of **Data Dependent** Scan with a fragmentation energy applied.

DEFINITIVE CONFIRMATION OF THE IDENTITY AND QUANTITY OF THE ANTIMICROBIAL RESIDUE DETECTED

2. EXPERIMENTAL

CHROMATOGRAPHIC CONDITIONS

Column	Agilent Technologies Poroshell 120-EC-C18 (100 x 3.0 mm, 2.7 µm)
Mobile phase	HCOOH 0.1 % (A) / CH_3OH (B)
Mobile phase rate	0.25 mL min^{-1}
Gradient program	0-1 min 5 % [B]; 1-20 min 95 % [B]; 20-25 min 95 % [B]; 25-26 min 5 % [B]; 26-30 min 5 % [B]
Injection volume	5 µL



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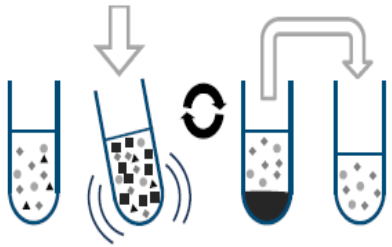
2. EXPERIMENTAL

SAMPLE PREPARATION



1,5 g of homogenized eggs

↓

Extraction with acetonitrile, in presence of 0,15 M EDTA, mixing and centrifuging

↓

Extraction with acetonitrile, mixing, sonication and centrifuging

↓

Reunited extracts: evaporation

↓

Resuspension in 200 mM aqueous ammonium acetate

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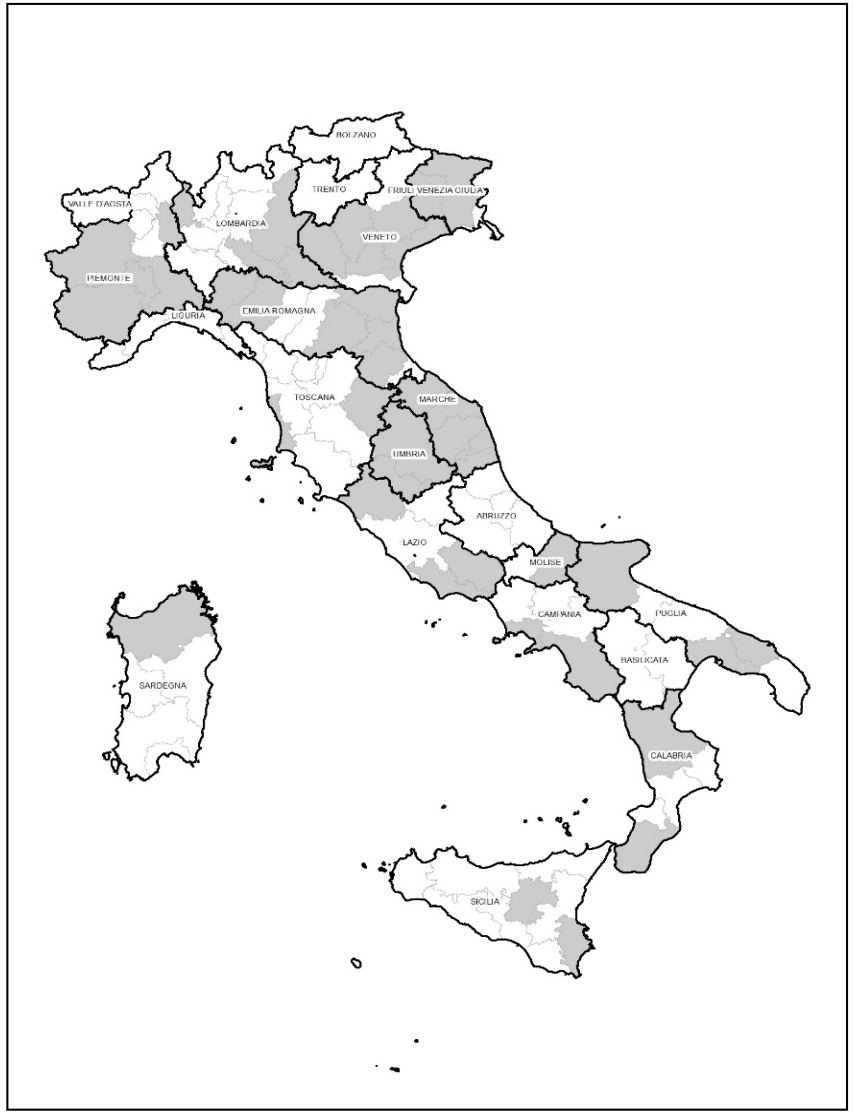
REAL SAMPLES ANALYSIS AND RISK ESPOSURE

- The validated method was applied to **200** real egg samples, taken from the Italian market during October 2018–June 2021.
- The risk exposure for Italian public health was determined according to Italian food consumption (*Leclercq et al., 2009).*



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3. RESULTS AND DISCUSSION

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METHODOLOGY: EVALUATION OF EXTRACTION/PURIFICATION YIELDS

SAMPLE →

Spiking operation with all analytes to the sample

→

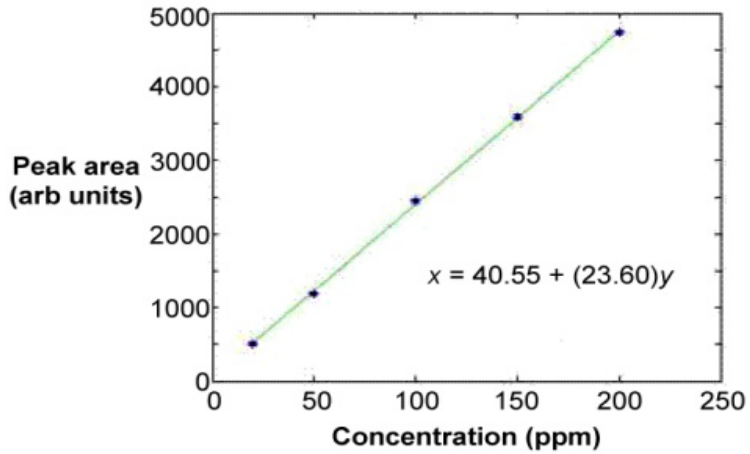
Extraction/purification protocol

→

Instrumental determination

Curve standard interpolation to calculate the concentration of each analyte after extraction/purification

$$\text{Recovery (\%)} = \frac{C_{OBSERVED}}{C_{SPIKING}} \cdot 100$$



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IMPORTANT

OPTIMIZATION OF SAMPLE PREPARATION

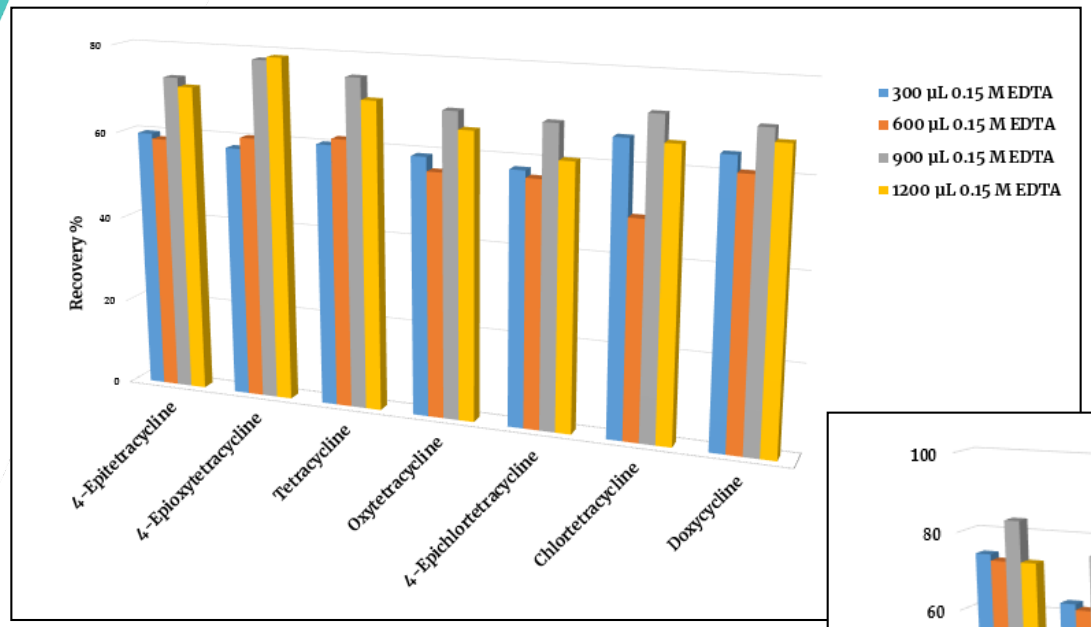
DEVELOPMENT OF A SAMPLE PROTOCOL ABLE TO ACHIEVE A COMPROMISE BETWEEN PERFORMANCE AND CLEANLINESS OF THE FINAL EXTRACT

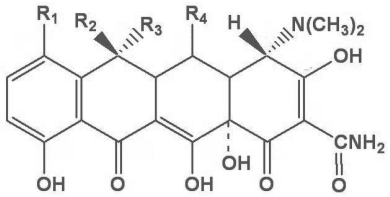
- Strong matrix effects for several analytes
- High concentrations of minerals in the matrix
- Chelating properties of quinolones, sulfonamides and tetracyclines

1. Volume of acetonitrile
2. **Concentration of the EDTA solution**
3. **Volume of the EDTA solution**

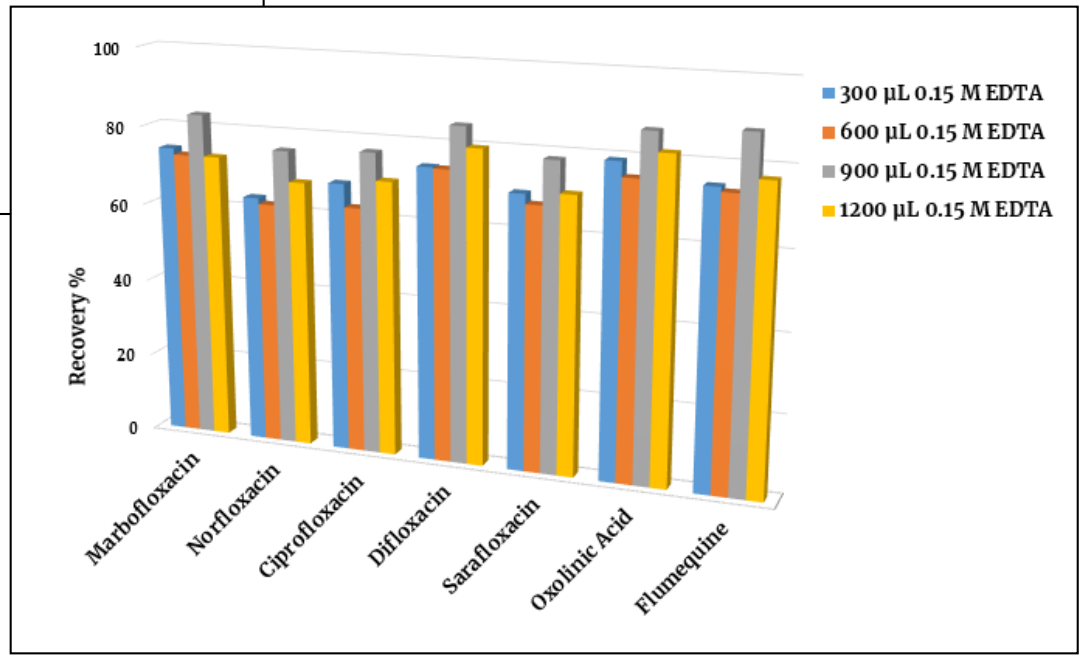
OPTIMIZATION OF SAMPLE PREPARATION

TETRACICLYNES

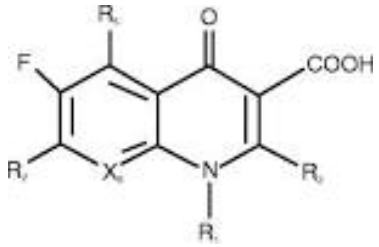




QUINOLONES



- Sulfonamides
- Macrolides
- Cephalosporins



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METHOD VALIDATION

COMMISSION DECISION

of 12 August 2002

implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results

(notified under document number C(2002) 3044)

(Text with EEA relevance)

(2002/657/EC)

3.1.2.1. *Recovery*

If there is no CRM available, the recovery has to be determined by experiments using fortified blank matrix using, for example, the following scheme:

— select 18 aliquots of a blank material and fortify six aliquots at each of 1, 1,5 and 2 times the minimum required performance limit or 0,5, 1 and 1,5 times the permitted limit,

3.1.2.2. *Repeatability*

— Prepare a set of samples of identical matrices, fortified with the analyte to yield concentrations equivalent to 1, 1,5 and 2 times the minimum required performance limit or 0,5, 1 and 1,5 times the permitted limit.

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METHOD VALIDATION

COMMISSION REGULATION (EU) No 37/2010

of 22 December 2009

on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin

(Text with EEA relevance)

Pharmacologically active Substance	Marker residue	Animal Species	MRL	Target Tissues	Other Provisions (*according to Article 14(7) of Regulation (EC) No 470/2009*)	Therapeutic Classification
Phenoxymethyl-penicillin	Phenoxymethyl-penicillin	Porcine	25 µg/kg 25 µg/kg 25 µg/kg	Muscle Liver Kidney	NO ENTRY	Anti-infectious agents/Antibiotics
		Poultry	25 µg/kg 25 µg/kg 25 µg/kg 25 µg/kg 25 µg/kg	Muscle Skin and fat Liver Kidney Eggs		
Tiamulin	Sum of meta-bolites that may be hydrolysed to 8-α-hydroxy-mutilin	Porcine, rabbit	100 µg/kg 500 µg/kg	Muscle Liver	NO ENTRY	Anti-infectious agents/Antibiotics
		Chicken	100 µg/kg 100 µg/kg 1 000 µg/kg	Muscle Skin and fat Liver		
		Turkey	100 µg/kg 100 µg/kg 300 µg/kg	Muscle Skin and fat Liver		
	Tiamulin	Chicken	1 000 µg/kg	Eggs		

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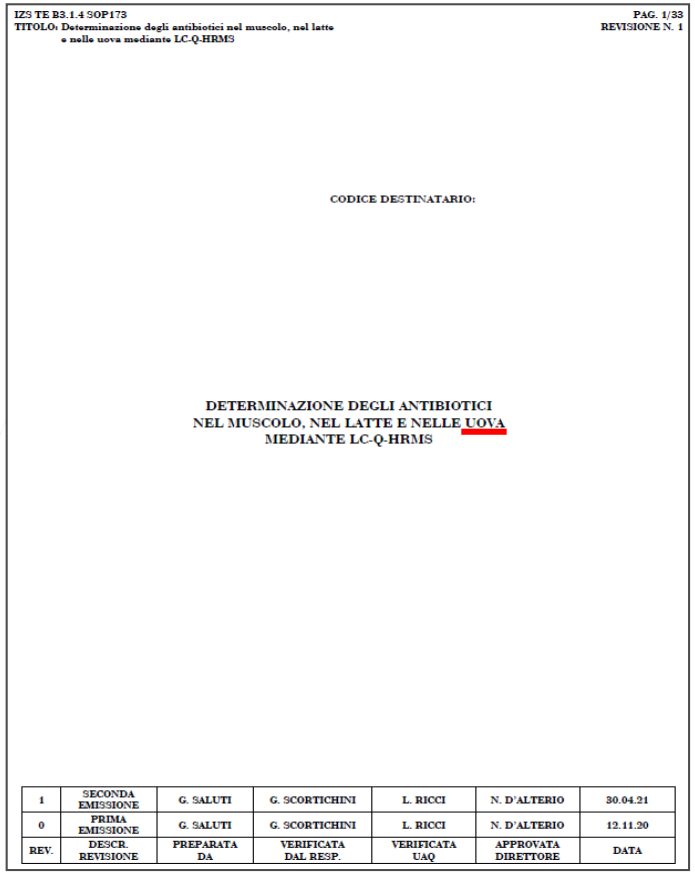
METHOD VALIDATION

3.1.3. **Validation according to alternative models**

When alternative validation procedures are applied, the underlying model and strategy with the respective prerequisites, assumptions and formulae shall be laid down in the validation protocol or at least references shall be given to their availability. In the following an example for an alternative approach is given. When applying e.g. the in-house validation model, the performance characteristics are determined in a manner that permits validation for major changes within the same validation procedure. This requires design of an experimental plan for validation.



IZS TE B3.1.4 SOP173 PAG. 1/33
TITOLO: Determinazione degli antibiotici nel muscolo, nel latte e nelle uova mediante LC-Q-HRMS REVISIONE N. 1

CODICE DESTINATARIO:

DETERMINAZIONE DEGLI ANTIBIOTICI NEL MUSCOLO, NEL LATTE E NELLE UOVA MEDIANTE LC-Q-HRMS

1	SECONDA EMISSIONE	G. SALUTI	G. SCORTICHINI	L. RICCI	N. D'ALTERIO	30.04.21
0	PRIMA EMISSIONE	G. SALUTI	G. SCORTICHINI	L. RICCI	N. D'ALTERIO	12.11.20
REV.	DESCR. REVISIONE	PREPARATA DA	VERIFICATA DAL RESP.	VERIFICATA UAQ	APPROVATA DIRETTORE	DATA

Validation level n°	Analyte spiking level (µg kg^{-1})	Concentration of analyte solution (µg mL^{-1})	Added volume of analyte solution (µL)	Number of fortified sample/day
1	3.3	0.1	50	4
2	10	1	15	4
3	33.3	1	50	4
4	100	1	150	4
5	333	10	50	4
6	1000	10	150	4
7	3333	100	50	4

The method has been **successfully** validated in eggs according to **Commission Decision 2002/657/EC** as confirmatory method and, on July 2021, the laboratory accredited it.

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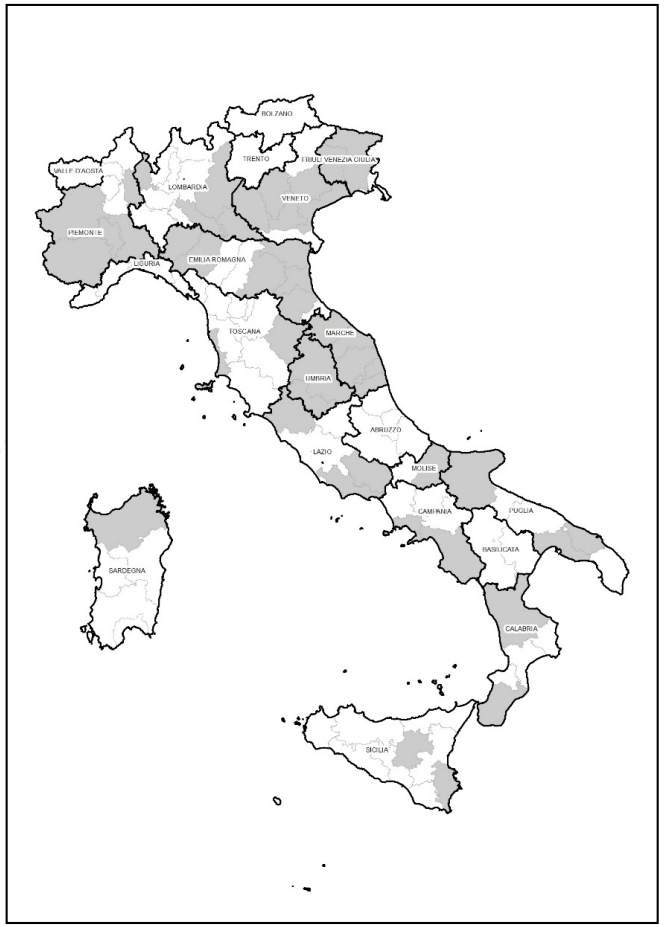
3. RESULTS AND DISCUSSION

REAL SAMPLES ANALYSIS



200 REAL EGG SAMPLES

60 ORGANIC

73 FREE-RANGE

67 BARN

RANDOM COLLECTION IN ITALIAN SUPERMARKET DURING 2018 – 2019 – 2020 -2021

119 FARMS LOCATED IN 45 PROVINCES

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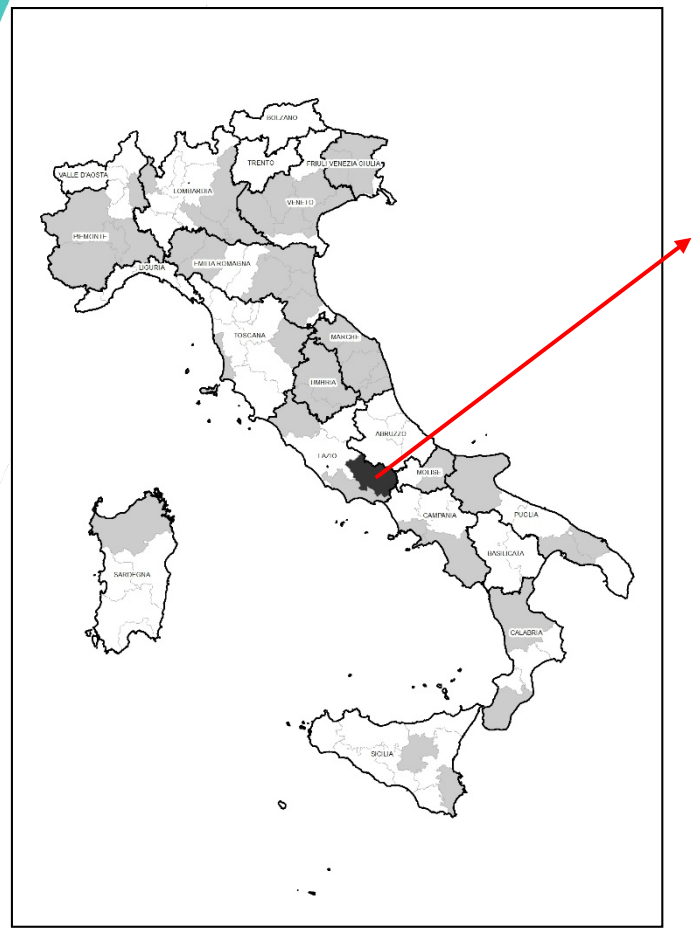
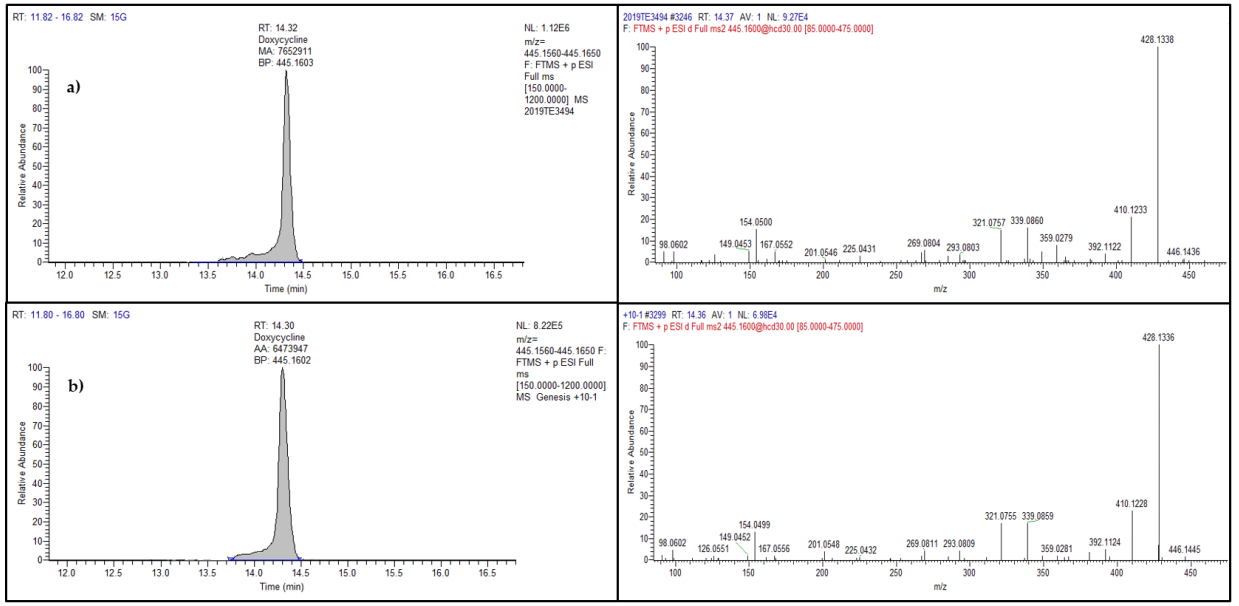
REAL SAMPLES ANALYSIS





Antibiotic residue was detected in **only one sample**, collected in 2019 (**0.5%**), belonged to the **free-range** farming method

DOXYCYCLINE = 22 µg kg^{-1}

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RISK EXPOSURE

The calculation of the daily intake and the consequent ADI percentage was based on egg consumption presented in the most recent published survey of the Italian diet.

Public Health Nutrition: 12(12), 2504–2532 doi:10.1017/S1368980009005035

The Italian National Food Consumption Survey INRAN-SCAI 2005–06: main results in terms of food consumption

Catherine Leclercq*, Davide Arcella, Raffaela Piccinelli, Stefania Sette, Cinzia Le Donne and Aida Turrini on behalf of the INRAN-SCAI 2005–06 Study Groupt
INRAN, National Research Institute for Food and Nutrition, Via Ardeatina 546, I-00178 Rome, Italy

Submitted 25 January 2008: Accepted 18 December 2008: First published online 12 March 2009

$$\text{ADI\% } (d^{-1}) = \frac{C\ (\mu g\ kg^{-1}) \cdot E\ (kg\ d^{-1})}{w\ (kg) \cdot ADI\ (\mu g\ kg^{-1}\ bw)} \cdot 100$$

Risk exposure based on the Italian diet

Detected Analyte	Detected Concentration (µg kg^{-1})	MRL (µg kg^{-1})	ADI (µg kg^{-1} b.w.)	ADI % (Mean)								ADI % (99th)							
				Infants	Children	Teenagers		Adults		Elderly		Infants	Children	Teenagers		Adults		Elderly	
						M	F	M	F	M	F			M	F	M	F	M	F
Doxycycline	22	Not fixed	3	0.4	0.6	0.3	0.3	0.2	0.2	0.2	0.2	2.9	3.6	1.5	1.7	1.2	1.1	1.1	0.8

M: Male, F: Female.

Toxicologically acceptable

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REAL SAMPLES ANALYSIS

Report for 2018 on the results from the monitoring of veterinary medicinal product residues and other substances in live animals and animal products

Report for 2019 on the results from the monitoring of veterinary medicinal product residues and other substances in live animals and animal products

Residues in live animals and animal products - Results 2018

Category	Group	Substance	Member State	Number of samples analysed [a]	Non-compliant results	% non-compliant results
		Total mercury	Germany	287	3	1
		Sub-total for B3c	**8**		**111**	
	B3d	Zearalenone	Spain	43	3	7
		Sub-total for B3d	**1**		**3**	
		Total for Bovines	**18**		**369**	
Eggs	A6	Chloramphenicol	Latvia	111	1	0.9
		Sub-total for A6	**1**		**1**	
	B1	Doxycycline	Italy	40	1	2.5
			Spain	242	1	0.4
		Enrofloxacin	Croatia	150	3	2
			Poland	259	1	0.4
		Oxolinic Acid	Spain	111	1	0.9
		Sulfadiazine	Spain	306	2	0.7
		Sulfadimethoxine	France	22	1	4.5
		Tilmicosin	Italy	27	1	3.7
		Trimethoprim	Spain	222	2	0.9
		Sub-total for B1	**5**		**13**	

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REAL SAMPLES ANALYSIS

efsa European Food Safety Authority Supporting Publications

Veterinary medicinal product residues in live animals and animal products – 2020 results

Product group	Residue group	Substance	Sampling country	Samples analysed	Non-compliant results	% Non-compliant
Eggs	**Group A6**	**Sub-total for Group A6**	**1**		**4**	
Eggs	Group B1	Doxycycline	Latvia	154	1	0.65
Eggs	Group B1	Doxycycline	Poland	305	1	0.33
Eggs	Group B1	Doxycycline	Spain	287	1	0.35
Eggs	Group B1	Sulfadiazine	Spain	339	3	0.88
Eggs	Group B1	Sulfadimethoxine	Italy	126	1	0.79

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4. CONCLUSIONS

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GOALS

- The developed and validated multiclass method for the determination of 73 antibiotic residues was applied to 200 egg samples.
- The monitoring showed the presence of antibiotic residues in 0.5% of the cases and the same percentage of noncompliant samples.
- The results of this wide survey are reassuring in relation to Italian public health, considering the acceptable toxicological level.

PERSPECTIVES

- The number of the monitored samples in Italy and use of multiclass methods should be increased to offer a better overwiew of egg contamination.

DISSEMINATION


separations

MDPI

Article

Survey on Antibiotic Residues in Egg Samples in Italy

Giorgio Saluti *, Maria Novella Colagrande, Federica Castellani, Matteo Ricci, Gianfranco Diletti and Giampiero Scortichini

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Thanks to:

Giorgio Saluti, Maria Novella Colagrande, Matteo Ricci, Gianfranco Diletti, Giampiero Scortichini

… AND THANKS FOR YOUR ATTENTION!!!!