Veterinary Drugs in food of animal origin: from single-class to multi-residue LC-MS analysis. How to improve reproducibility and recovery of some analytes

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Learning Objectives

1. Veterinary drugs: Classes
2. Towards a generic sample preparation for the simultaneous detection
   2.1 Composition of of foods of animal origin
   2.2 EU MRLs / physico-chemical properties: Tetracyclines (TCs), Sulfonamides (SAs), Fluoroquinolones (FLQs), penicillin beta-lactams (β LCs)

3. Generic Sample Preparation Alternatives:
   3.1 Solvent extraction (SE) without further purification
   3.2 Solid phase extraction (SPE)
   3.3 Dispersive-SPE (d-SPE)

4. Filtration before LC-MS injection

Veterinary drugs: Classes

- Commonly used in animal production to treat disease and promote growth. Active against bacteria, fungi and/or parasites
- Can lead to excessive residues in food of animal origin
- Can be toxic and development of antimicrobial resistance by pathogenic microorganisms

- Several classes

S. no,	Classes of veterinary drugs	Common drugs
1	Tetracyclines	Chlortetracycline, doxycycline, oxytetracycline, tetracycline
2	β-lactams	Cefacetrile, cefalexin, cefalotin, cefapyrin, cefazolin, cefalonium, cefuroxime, cefoperazone, ceftiofur, ceftriaxone, cefquinome, benethamine penicillin, benzylpenicillin, benzathine penicillin
3	Macrolides	Erythromycin, oleandomycin, gamithromycin, tulathromycin, carbomycin, josamycin, kitasamycin, spiramycin, tilmicosin, tylosin, mirosamycin, terdecamycin, tildipirosin, tylvalosin, sedecamycin
4	Aminoglycosides	Spectinomycin, streptomycin, dihydrostreptomycin, kanamycin, neomycin, framycetin, paromomycin, apramycin, fortimycin, gentamicin, tobramycin, amikacin
5	Amphenicols	Florphenicol, thiamphenicol
6	Quinolones	Ciprofloxacin, danofloxacin, difloxacin, enrofloxacin, marbofloxacin, norfloxacin, ofloxacin, orbifloxacin, sarafloxacin, flumequin, miloxacin, nalidixic acid, oxolinic acid
7	Sulfonamides	Sulfadiazine, sulfadimethoxine, sulfadoxine, sulfamerazine, sulfachloropyridazine, sulfadimidine, sulfafurazole, sulfaguanidine, sulfadimethoxazole, sulfamethoxine, sulfamonomethoxine, sulfanilamide, sulfapyridine, phthalylsulfathiazole, sulfaquinoxaline
8	Lincosamides	Pirlimycin, lincomycin

Source: World Organization for Animal health (2015)

Towards a Generic Sample Preparation

for the simultaneous detection of different classes

Non-selective procedures to ensure "good enough" LODs and recovery for many compounds with varying chemical structures and properties

Ideally, the **Generic Sample Preparation** should be:

- Capable of extracting a wide variety of target compounds
- Applicable to a variety of matrices

However, the selection of the suitable protocol depends on not only the target compounds but also **on the matrix composition**

- Acetonitrile (ACN) - water mixtures frequently used as extraction solvents.

- **Number of steps in the generic sample preparation**
  Generally, non-selective sample prep includes a few steps. Each step, (e.g. defatting with hexane, deproteination with ACN, and a SPE step), can generate errors and losses in an analytical routine

  Ex: the need for SPE. Can this step be avoided?
  Introduction of SPE limits compound expandability.

- **Acid in the extraction solvent or in the mobile phase**
  Some analytes interact with the matrix. This could be avoided by extracting at low pH. However, β-lactam ring unstable; nearly double concentrations for sulfonamides than when acid was present

- **Injection volume**
  should be as small as possible (1–5 µl are common)

Carbohydrates, Fat and Proteins in food of animal origin

Matrix	Food/feed composition (%)					
	moisture	total fat	total protein	total carbohydrates	carbohydrates total sugars	carbohydrates starch
Compound feed (horse feed) [1]	10.5	3.9	10.6	46.4	5.5	38.7
Maize flour [2]	11	2	7	79	0,8	69
Honey [2]	18	0	0.3	82	75.1	0
Raw milk [2]	88	3.6	3.5	4.5	3.8	0
Minced meat (pork/beef, low fat) [1,3]	72	3.1	23.6			
Minced meat (pork/beef) [4]		13				
Whole egg [2]	75	11.2	12.1	1.2	1	0

[1] composition data obtained after analysis of a sample from the lot used in this work
[2] composition taken from the Danish Food Composition Databank*
[3] used for extraction experiments with pesticides/mycotoxins/plant toxins
[4] used for extraction experiments with veterinary drugs, fat content as indicated on label

* Danish Food Composition Bank, National Food Institute, Dept. Nutrition, Technical University of Denmark, http://www.foodcomp.dk/fcdb_default.asp (January 2008)

Anal. Chem. 2008, 80, 9450–9459

Toward a Generic Extraction Method for Simultaneous Determination of Pesticides, Mycotoxins, Plant Toxins, and Veterinary Drugs in Feed and Food Matrixes

Hans G. J. Mol,*,† Patricia Plaza-Bolaños,‡ Paul Zomer,† Theo C. de Rijk,† Alida A. M. Stolker,† and Patrick P. J. Mulder†

Carbohydrates, Fat and Proteins in food of animal origin

Eggs	Cont. /100g	Unit
Protein, total	15.6	g
Carbohydrate, total	1.4	g
Fat, total	28.4	g

Kidney	Cont. /100g	Unit
Protein, total	16.0	g
Carbohydrate, total	1.7	g
Fat, total	3.0	g

Minced meat (low fat)	Cont. /100g	Unit
Protein, total	8.1	g
Carbohydrate, total	4.9	g
Fat, total	8.3	g

Liver ox, raw	Cont. /100g	Unit
Protein, total	20.3	g
Carbohydrates, total	3.4	g
Fat, total	3.0	g

Beef meat raw	Cont. /100g	Unit
Protein, total	21.5	g
Carbohydrate, total	0.0	g
Fat, total	6.5	g

Fish (cod)	Cont. /100g	Unit
Protein, total	17.6	g
Carbohydrate, total	0.0	g
Fat, total	0.6	

Milk	Cont. /100g	Unit
Protein, total	3.5	g
Carbohydrate, total	4.5	g
Fat, total	3.6	g

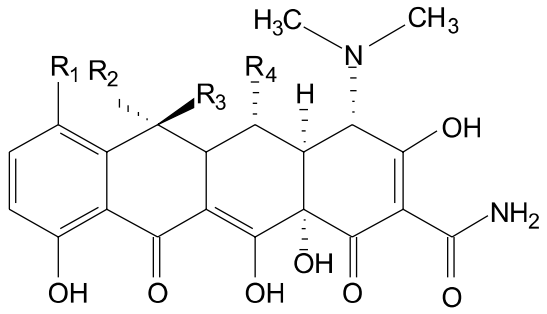
Honey	Cont. /100g	Unit
Protein, total	0.3	g
Carbohydrate, total	81.5	g
Fat, total	0.0	g

Danish food composition database FRIDA

https://frida.fooddata.dk/food/lists/alphabetic

Tetracyclines (TCs): chemical structure

- The class includes **Tetracycline (TC)**, **Oxytetracycline (OTC)**, **Chlortetracycline (CTC)**, **Doxycycline (DC)**

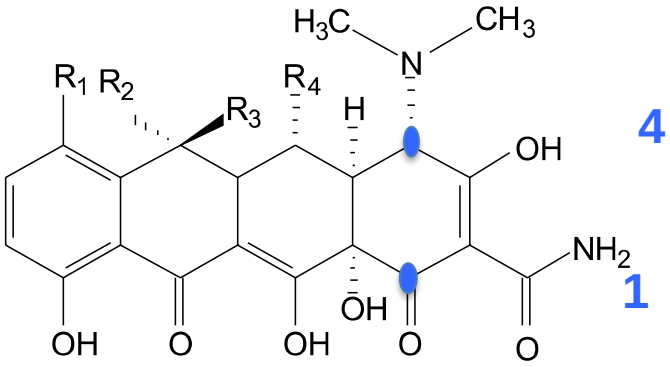


Compound	R_1	R_2	R_3	R_4
Tetracycline	H	CH_3	OH	H
Oxytetracycline	H	CH_3	OH	OH
Chlortetracycline	Cl	CH_3	OH	H
Doxycycline	H	CH_3	H	OH
Demeclocycline	Cl	H	OH	H

Figure 1. *Structure of tetracyclines*

Tetracyclines (TCs): EU Regulation on MRLs

- In the EU (Reg. N.37/2010), the MRLs for the parent drugs TC, CTC and OTC are fixed as a sum with their respective 4-epimer except for doxycycline



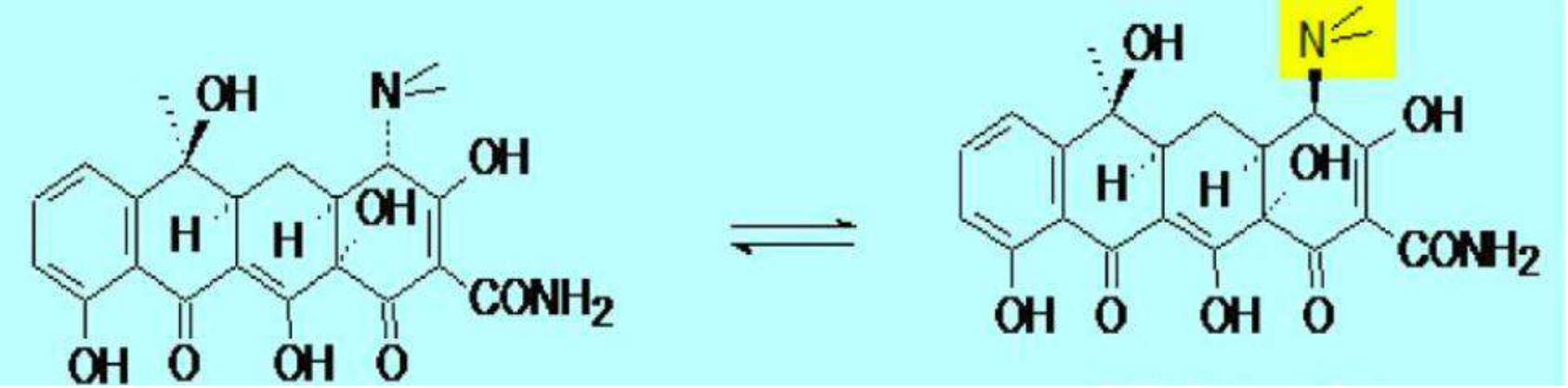
SUM is required for quantitative determination of TCs

Pharmacologically active substance(s)	Marker residue	Animal Species	MRLs	Target tissues
Tetracycline Oxytetracycline Chlortetracycline	Sum of parent drug and its 4-epimer	All food producing species	600 µg/kg	Kidney
			300 µg/kg	Liver
			100 µg/kg	Muscle
			100 µg/kg	Milk
			200 µg/kg	Eggs

Tetracyclines (TCs): **Physicochemical properties**

- Diluted acid promotes epimerization at C-4

➢ Epimers are diastereomers which differ from each other in the absolute configuration at only one chiral center (C4)



➢ In acid medium (pH 2-6), epimerization of the “natural” C-4 α-dimethylamino group to the C-4 β-epimer occurs.

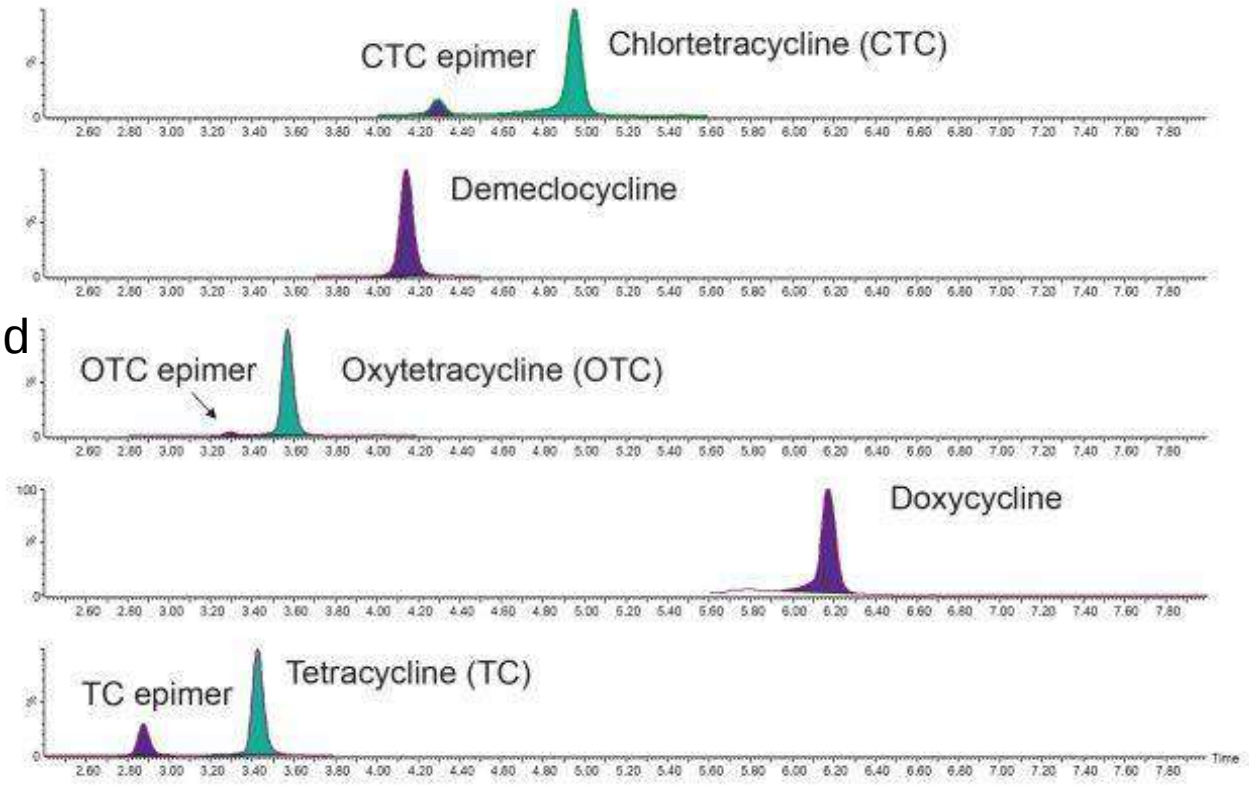
Chromatographic resolution of parent and epimeric forms

- Combination of C18 column with methanol rather than ACN and low column temperature (25 ° C) generally results in complete separation of the parent and epimeric forms

Mobile phase **A**
Water + 0.1 % formic acid

Mobile phase **B**
Methanol + 0.1 % formic acid

Time	Flow rate (mL/min)	% A	% B
0.00	0.4	90	10
6.00	0.4	50	50
7.50	0.4	0	100
9.00	0.4	90	10



From the Waters Application Note: *Analysis of Tetracycline and Sulfonamide Antibiotics in Shrimp Tissue using Liquid Chromatography Tandem Quadrupole Mass Spectrometry*

MS/MS resolution of the parent and epimeric forms

Table 1. Summary of multiple reaction monitoring parameters for all compounds determined.

	Parent ion (*m/z*)	1° daughter ion (*m/z*)	Cone voltage (V)	2° daughter ion (*m/z*)	3° daughter ion (*m/z*)	Collision (V)	ESI	RT (min)
Tetracycline	445.4	154.0	22	410.2	427.0	26,20,18	+	4.66
Epichlortetracycline	479.2	444.2	31	462.2		22,15	+	5.01
Epioxytetracycline	461.3	426.2	19	444.2		19,16	+	4.56
Epitetracycline	445.3	410.2	25	427.2		19,15	+	4.38
Doxycycline	445.2	154.0	25	428.2		28,20	+	5.73
Chlortetracycline	479.3	444.2	27	462.2		20,18	+	5.31
Oxytetracycline	461.2	426.2	22	443.1		19,13	+	4.72
Methacycline	443.0	201.0	28	381.0	426.0	25,20,16	+	5.55

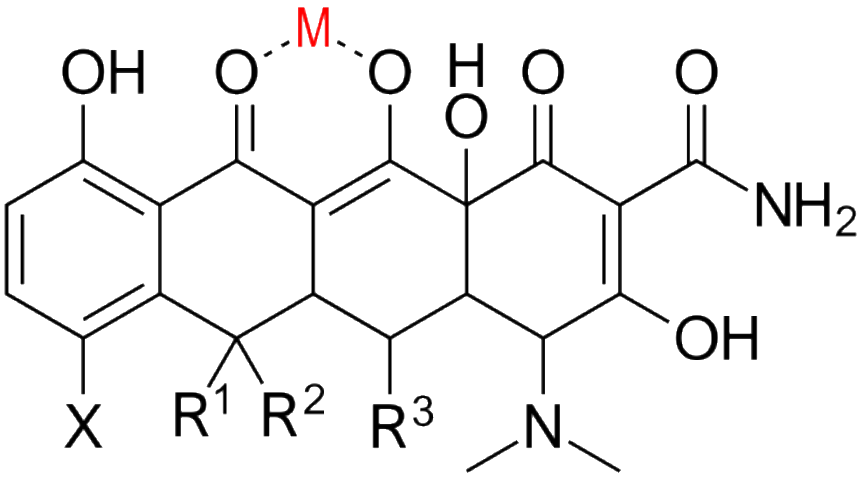
Development of a rapid method for the determination of antibiotic residues in honey using UPLC-ESI-MS/MS

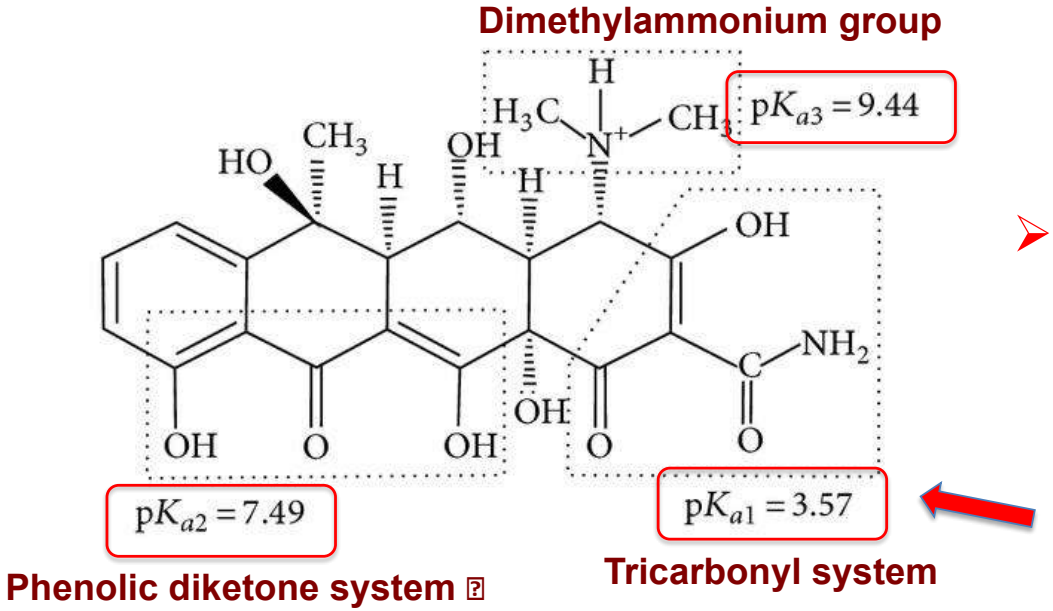
İbrahim KIVRAK[1,2*], Şeyda KIVRAK[3], Mansur HARMANDAR[4]

DOI: http://dx.doi.org/10.1590/1678-457X.0037

Tetracyclines (TCs): **Physicochemical properties**

- TCs have a strong ability to chelate the transition metal ions and alkaline earth metal salts and show high interaction with silanol groups of the SP





- The lower pKa value is attributed to tricarbonyl system and is between 3.0-3.6 in water

Tetracyclines (TCs): Optimizing a LC-MS method

- **Extraction solvents**: McIlvaine buffer (pH 4.0), also known as citrate-phosphate buffer, 0.1 M EDTA, methanol, acetonitrile, citrate buffer at pH 4.7, or mixtures of these solvents
  - McIlvaine Buffer solution containing 0.1 M EDTA is the most widely used
- Time of shaking has also been reported to influence the recovery (Gavilán et al. 2015). Carson et al. (2002) reported that increasing the shaking time to 2 h showed an increase in recovery by 5–10%

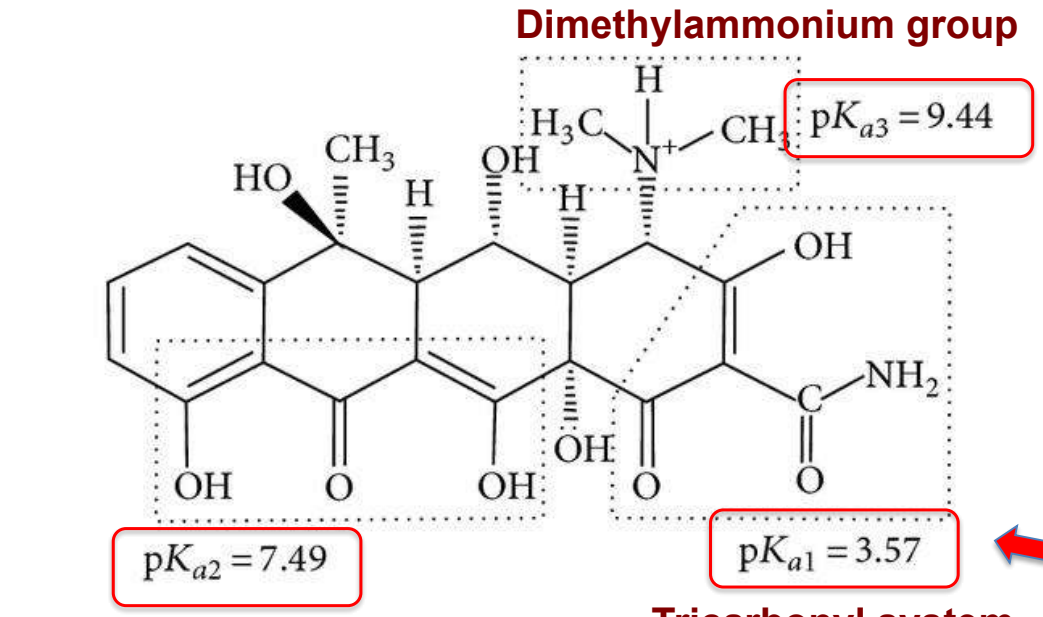
- **Clean-up**: C18 SPE cartridges (e.g. Oasis HLB, Strata-X 33U)

Multiresidue analysis of sulfonamides, quinolones, and tetracyclines in animal tissues by ultra-high performance liquid chromatography– tandem mass spectrometry.
Zhiwen Zhang et al. *Food Chemistry* 204 (2016) 252–262

Tetracyclines (TCs): Optimizing a LC-MS method

Mobile Phase (MP) Selection

- It is better to avoid buffers
- The MP pH should be < pKa_s of all analytes to keep them in a single form
- Reducing the MP pH will enhance the MS sensitivity
- Commonly used: oxalic acid, due to its ability to mitigate the effect of residual silanols on SP



The lower pKa value (3.0-3.6 in water) for all TCs

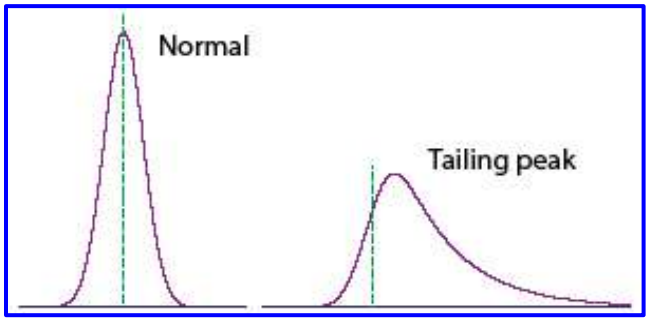
Tetracyclines (TCs): Optimizing a LC-MS method

Stationary Phase (SP) Selection

- Often reported: Peak Tailing

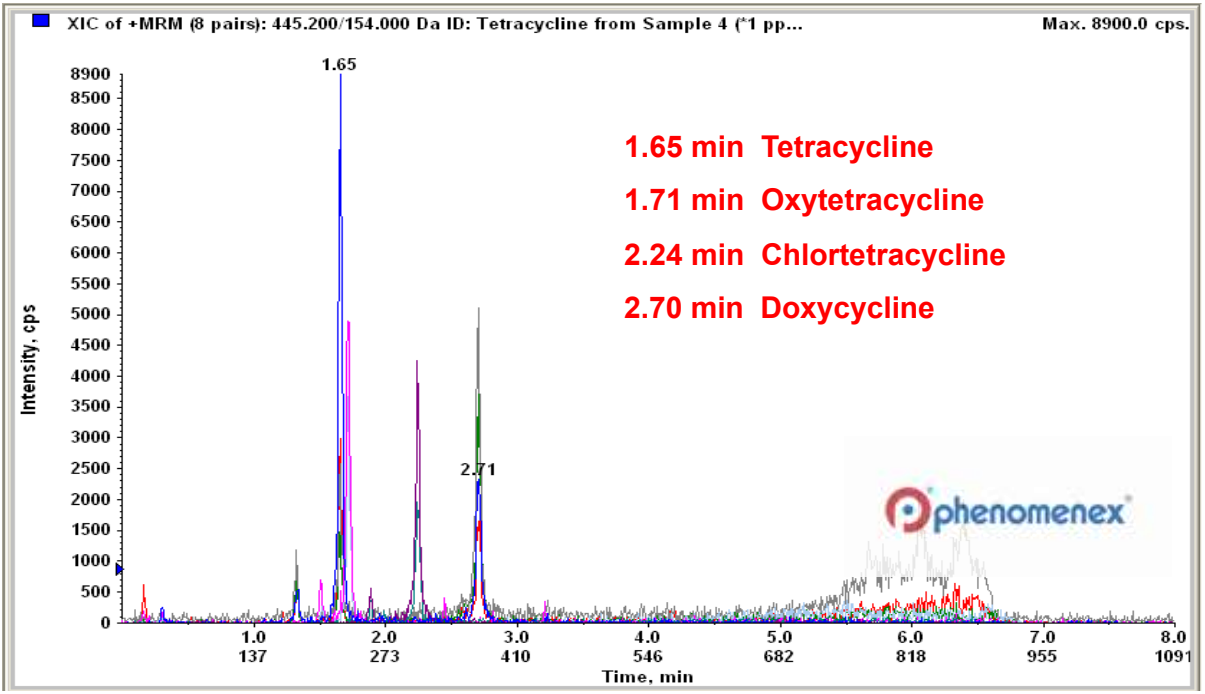
  Due to secondary interactions of TCs with anionic free silanol groups and trace metals present in silica-based SP (derivatized with C8 or C18 groups)

- How minimizing silanol ionization?
  - Use **low-pH** MP (with formic or oxalic acids)
  - Choose a reverse phase SP with **extremely low silanol activity** (high surface alkyl chains coverage)
- How minimizing metal chelation?
  - Choose a SP made from high-purity (e.g. **Type B**) silica



Tetracyclines (TCs): Optimizing a LC-MS method

LC Gradient



Time (min)	% B
0.01	5
5.00	90
6.00	90
6.01	5
8.00	5

Flow rate **0.7 mL/min**

Inj Vol 20 µL

Col Temp: 20-25 °C

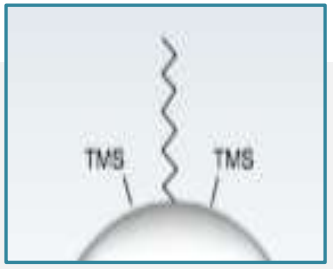
Luna **C8**, 50x2.0 mm, 3 µm

A: 0.1% formic acid in water

B: 0.1% formic acid in MeOH

MP pH: 2.7

Stationary phase with high alkyl surface coverage

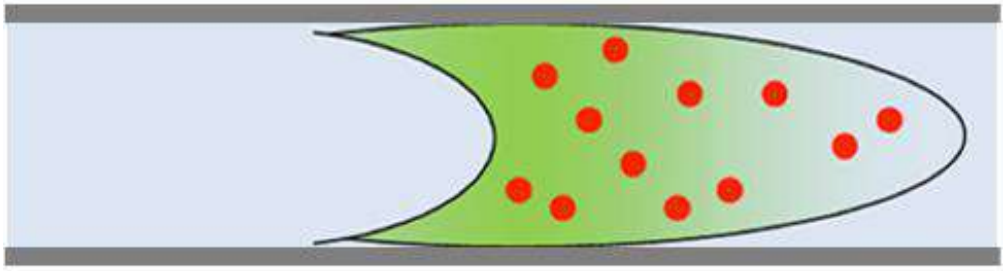


Flow rate

If your system can not handle a flow rate > 0.5 mL/min then split the post column flow

Tetracyclines (TCs): Optimizing a LC-MS method

Effect of sample solvent on the peak shape and signal intensities



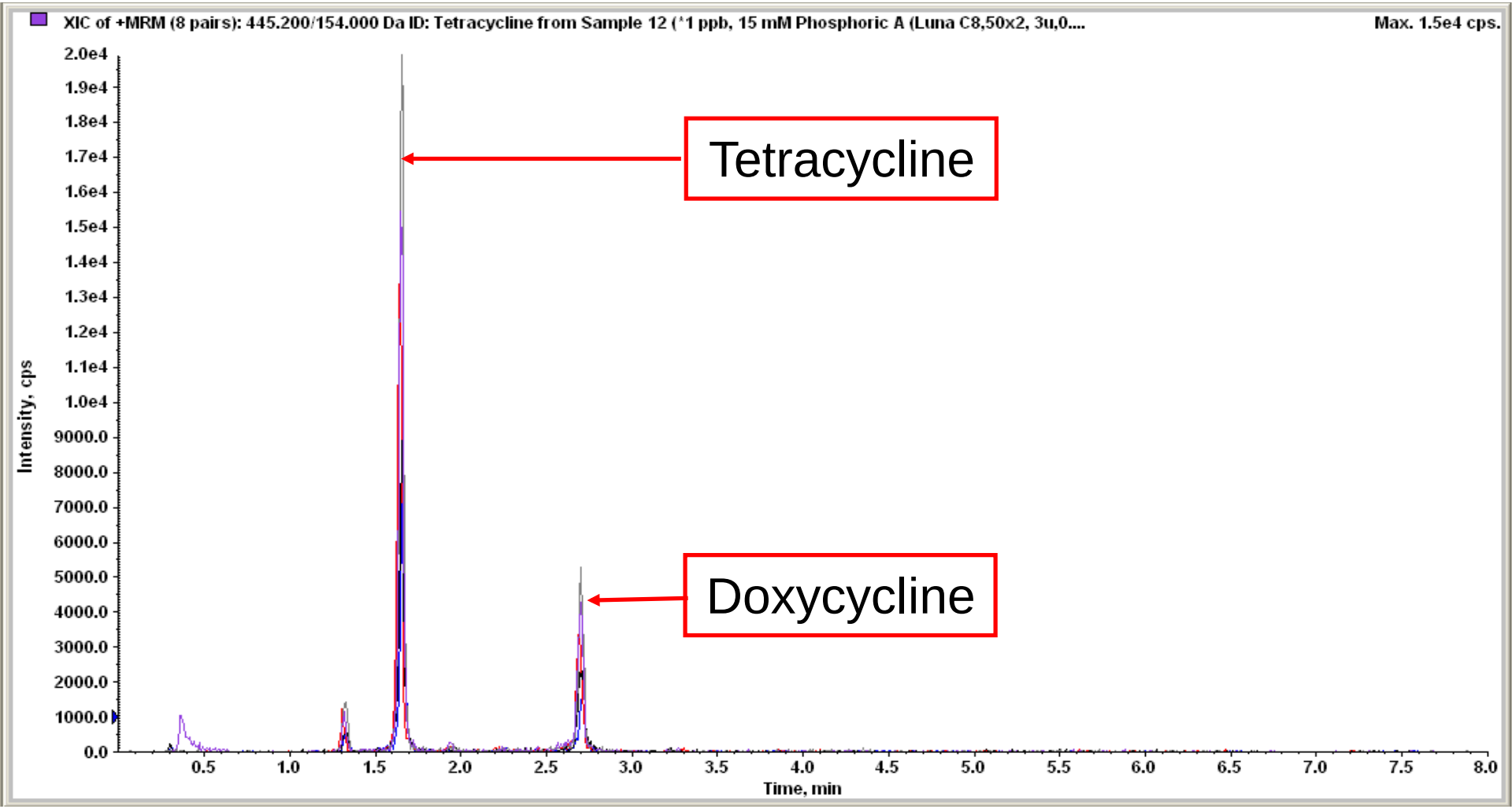
- 5 Sample diluent used
  - 0.1% Formic Acid
  - 0.01% TFA
  - 0.010 M Oxalic Acid
  - 0.015 M Phosphoric Acid
  - 0.3% Formic Acid
- Oxalic, TFA and Phosphoric acids have very low pH

✓ 5 injections of analytes mix with different diluents tested

Tetracyclines (TCs): Optimizing a LC-MS method

Effect of sample diluent on analyte intensity

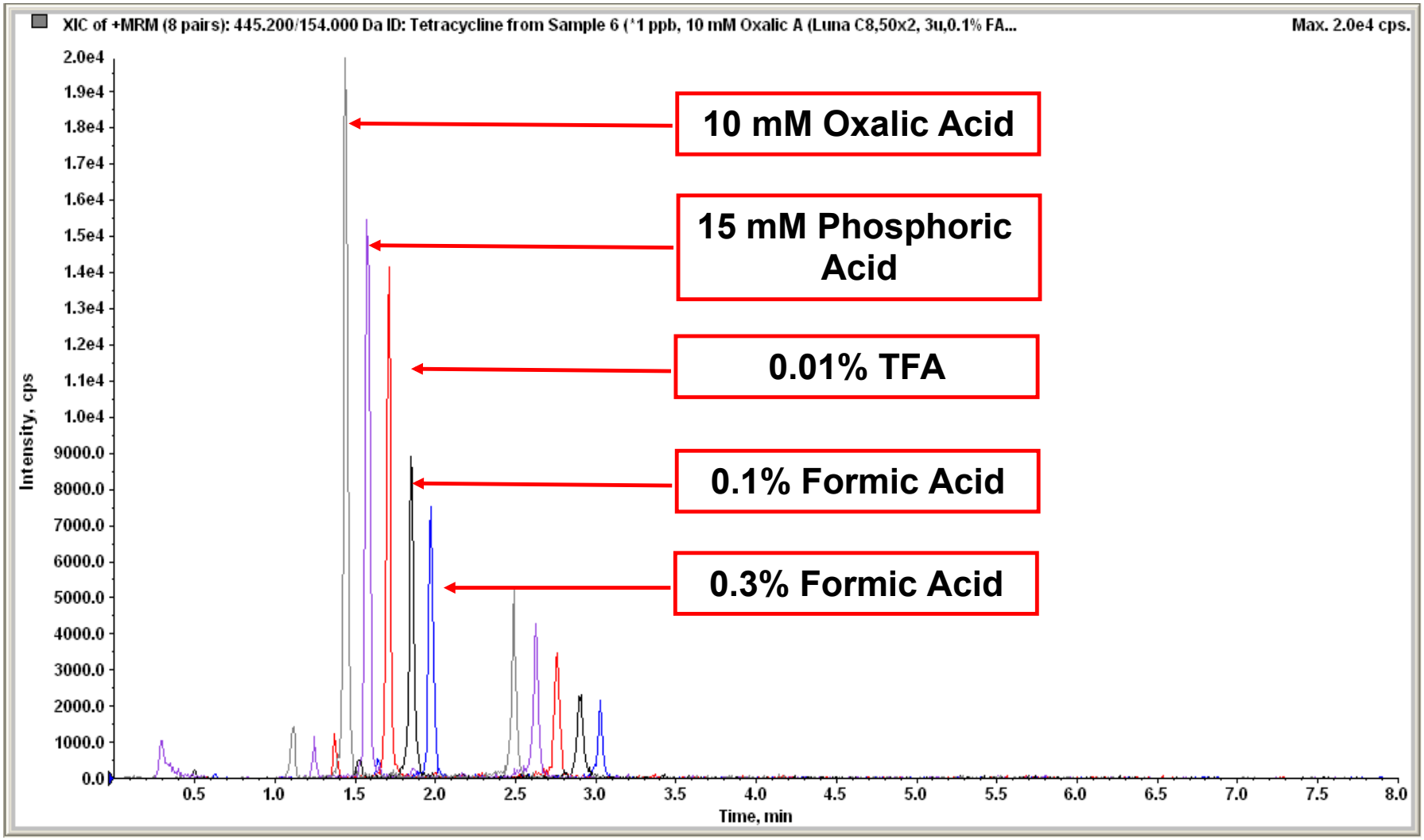
all chromatograms are overlaid (1 ng/mL)



Tetracyclines (TCs): Optimizing a LC-MS method

Effect of sample diluent on analyte intensity

shifted overlay chromatograms (1 ng/mL)



Tetracyclines (TCs): Optimizing a LC-MS method

- **Acidic MP** (no buffer)

  A: 0.1% formic acid in water

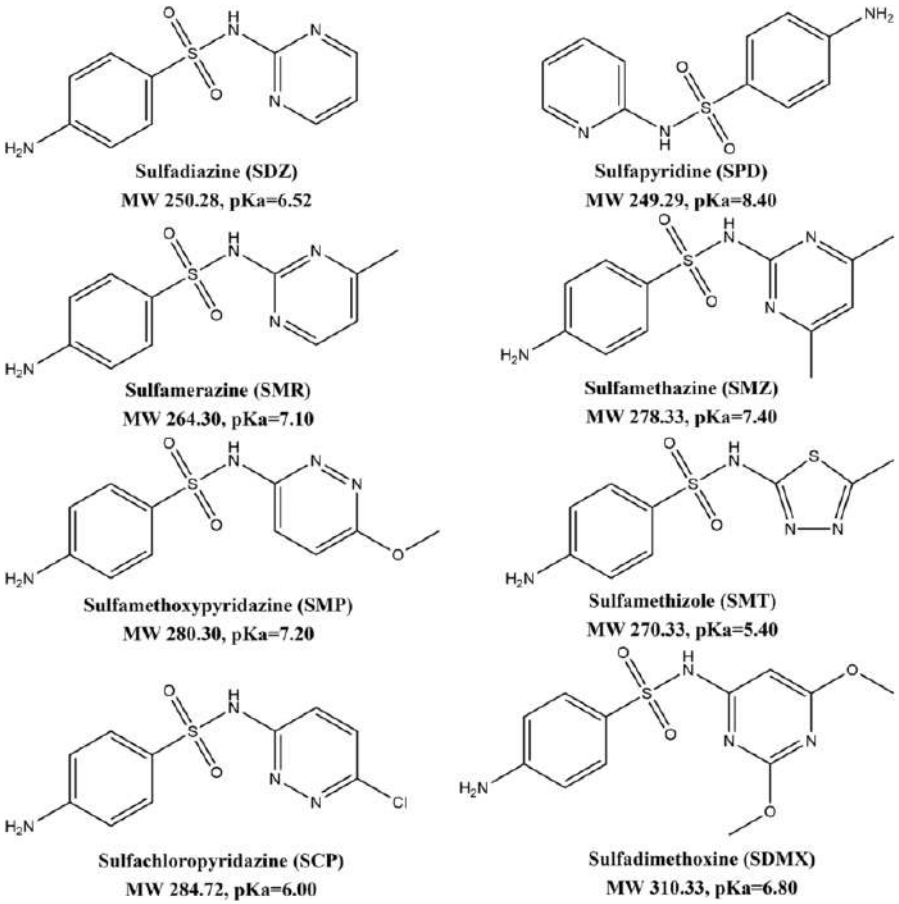
  B: 0.1% formic acid in MeOH

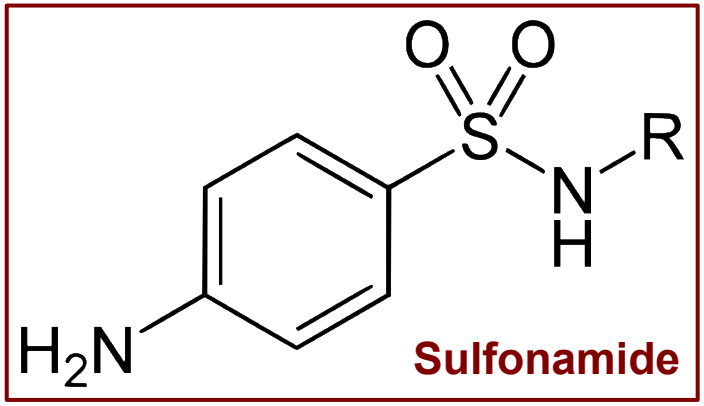
- **SP C8 (o C18) with extremely low silanol activity**

- **Sample diluent with low pH** (specifically oxalic acid) can further increase the stability and the sensitivity of the analysis

Sulfonamides (SAs): chemical structure

- SAs are sulfonamide derivatives that result from the substitution of the various R radicals of the −SO2-NH-R group





- pKa value in water:

  5.0-8.40

Sulfonamides (SAs): EU Regulation on MRLs

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
Sulfonamides (all substances belonging to the sulfonamide group)	Parent drug	All food-producing species	100 µg/kg 100 µg/kg 100 µg/kg 100 µg/kg	Muscle Fat Liver Kidney	The combined total residues of all substances within the sulfonamide group should not exceed 100 µg/kg. For fin fish the muscle MRL relates to 'muscle and skin in natural proportions'. MRLs for fat, liver and kidney do not apply to fin fish. Not for use in animals from which eggs are produced for human consumption.	Anti-infectious agents/ Chemotheurapeutics/
		Bovine, ovine, caprine	100 µg/kg	Milk		

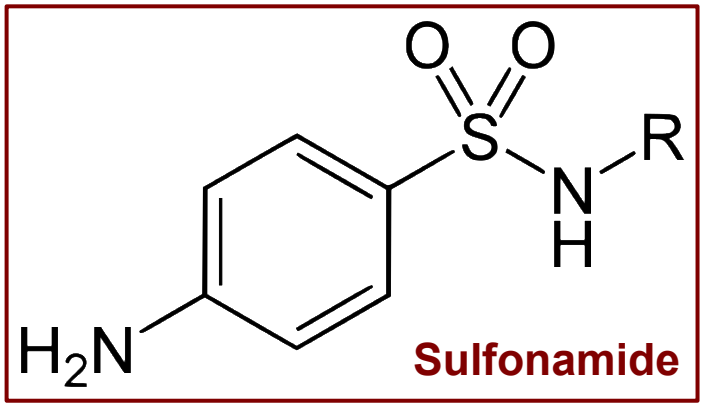
Sum of the SA residues

- In the EU (Reg. N. 37/2010), the MRLs for all SA is set at 100 µg/kg for muscle, fat, liver, and kidney from all food-producing species and bovine, ovine, and caprine milk, while **their use is prohibited for animals that produce eggs for human consumption**.

Sulfonamides (SAs): Physicochemical properties

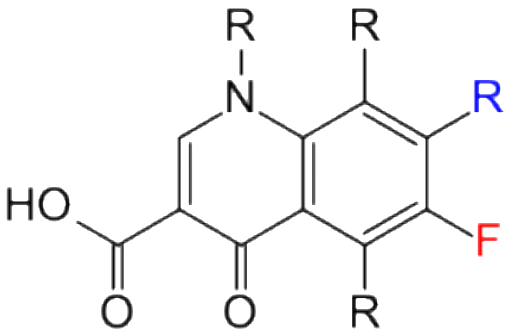
- pKa value in water: **5.0-8.40**
- Solubility: poor soluble in water at pH<7, with increasing solubility in alkaline pH. Their sodium salts are readily soluble
- Good solubility in polar solvents such as ACN, chloroform, acetone, and methanol
- Lehotay et al. (2002) reported that the absence of acid in the extraction solvent led to nearly double concentrations for sulfonamides than when acid was present in the extraction solvent.

- Liver and kidney are animal tissues with the highest concentrations of SAs and their metabolites

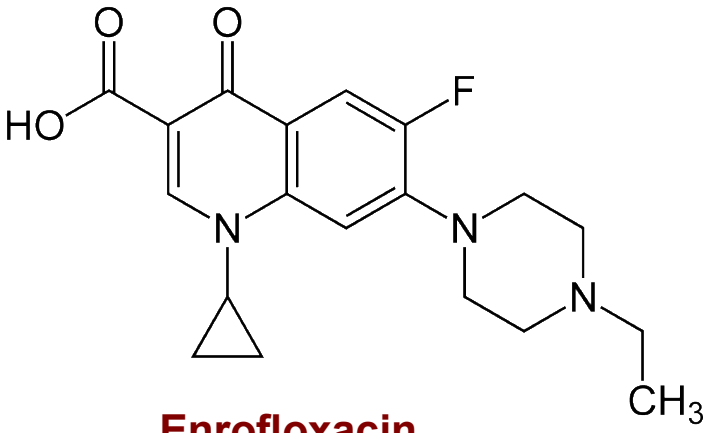


Fluoroquinolones (FLQs): chemical structure

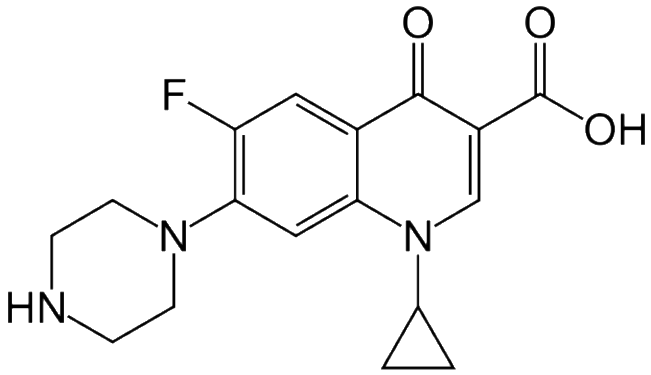
- The majority of quinolones, called **fluoroquinolones,** possess a fluorine atom attached to the central ring system, typically at the C-6 or C-7 positions



General structure: fluoroquinolone antibiotics, as a fluorine atom is present in the quinolone basic structure



Enrofloxacin



Ciprofloxacin (metabolite)

Fluoroquinolones (FLQs): EU Regulation on MRLs

Pharmacologically active substance(s)	Marker residue	Animal species	MRLs	Target tissues	Other provisions
Enrofloxacin	Sum of enrofloxacin and ciprofloxacin	Bovine, ovine, caprine	100 µg/kg 100 µg/kg 300 µg/kg 200 µg/kg 100 µg/kg	Muscle Fat Liver Kidney Milk	
		Porcine, rabbits	100 µg/kg 100 µg/kg 200 µg/kg 300 µg/kg	Muscle Fat** Liver Kidney	
		Poultry	100 µg/kg 100 µg/kg 200 µg/kg 300 µg/kg	Muscle Skin +fat Liver Kidney	Not for use in animals from which eggs are produced for human consumption
		All food producing species except bovine, ovine, porcine, caprine, rabbits and poultry	100 µg/kg 100 µg/kg 200 µg/kg 200 µg/kg	Muscle* Fat Liver Kidney	

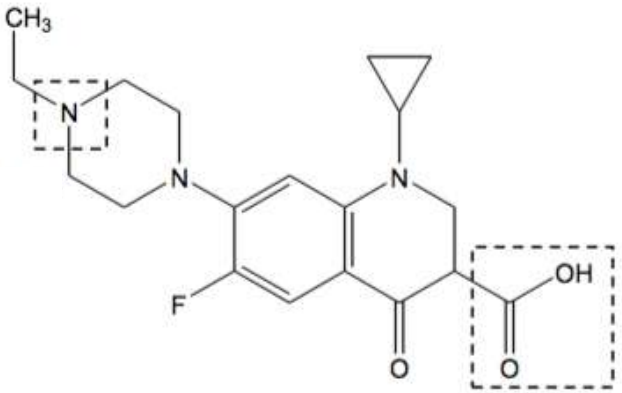
*For fin fish this MRL relates to "muscle and skin in natural proportions"

**For porcine species this MRL relates to "skin and fat in natural proportions"

- In the EU (Reg. N. 37/2010), the MRLs for Enrofloxacin was set at 100 µg/kg as sum of enrofloxacin and its metabolite ciprofloxacin. **Their use is prohibited for animals that produce eggs for human consumption**.

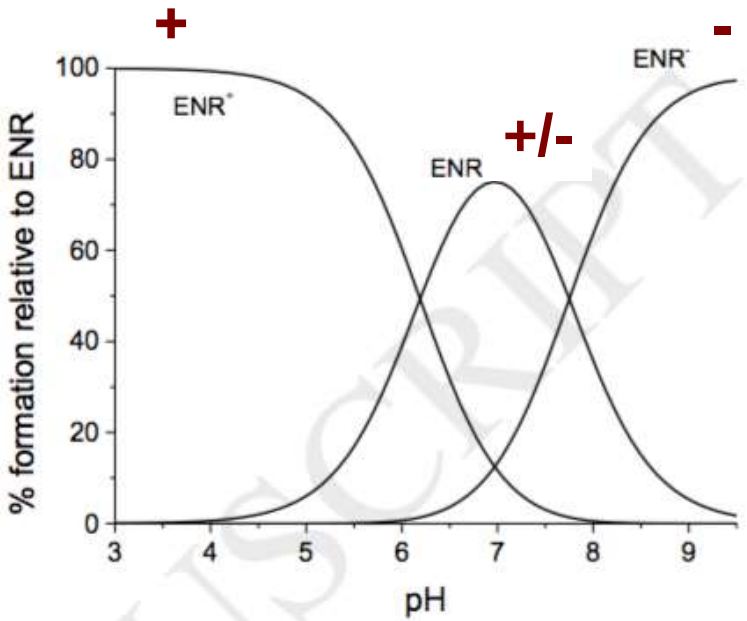
Enrofloxacin (ENR): Physicochemical properties

pKa2 (tertiary amine) = [7.70]



Enrofloxacin

pKa1 = [6.06]

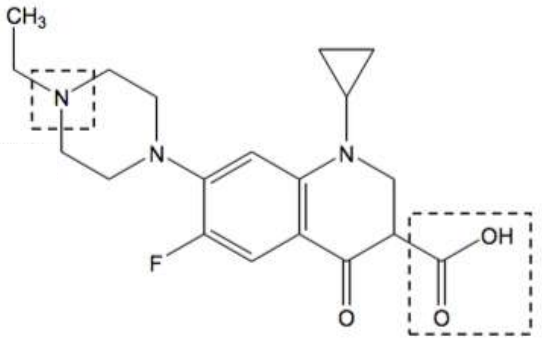


https://doi.org/10.1016/j.jece.2018.08.012

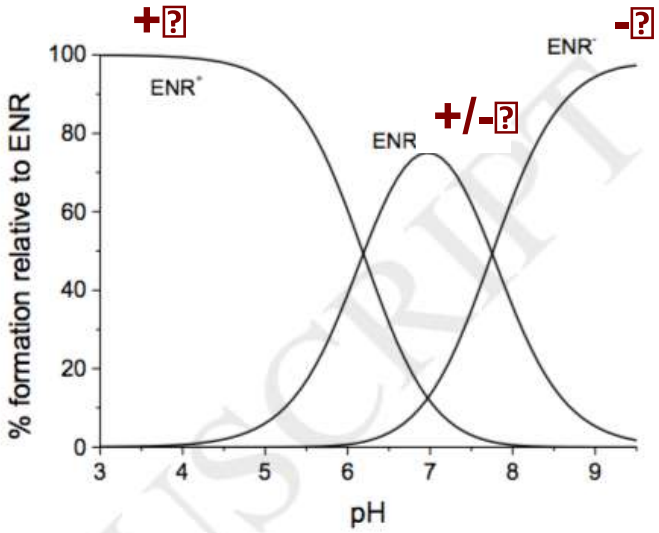
- Zwitterionic molecule with the lowest pKa due to the carboxyl acid group and the second to the basic tertiary amine. Enrofloxacin doesn't bear charge between these two pH values
- Can be extracted using ion-exchange sorbents:
- At **pH < 6** using cation-exchange, at **pH >8** using anion-exchange

Enrofloxacin (ENR): Physicochemical properties

pKa2 (tertiary amine) = [7.70]



Enrofloxacin pKa1 = [6.06]



https://doi.org/10.1016/j.jece.2018.08.012

- **Solubility**: **greatly depending on the solvent and the pH value!**
- Low water solubility (only 0.23 g/L). **Best water solubility** at pH=5.02 (*)
- Easily dissolved in: ACN, CH_2Cl_2, NaOH solution
- Slightly soluble in MeOH

(*) Lizondo M, Pons M, Gallardo M, et al. Physicochemical properties of enrofloxacin. *J Pharm Biomed Anal.* 1997;15(12):1845–1849.

Fluoroquinolones (FLQs):
Considerations on extraction solvent from tissues (ex. Kidney)

- FLQs antibiotics can be extracted from tissue at low or at neutral pH

- At low pH, extraction into ethanol is preferred; **higher matrix interference results from aqueous buffer extraction**
- At high pH extraction is difficult using either ethanol or aqueous buffer; high amount of matrix interference
- At neutral pH, extraction into aqueous buffer is preferred; **higher matrix interference results from ethanol extraction**
- A two-step extraction with ACN generally gives good results with acceptable recoveries between 60 and 95%.

Kidney	Cont. /100g	Unit
Protein, total	16.0	g
Carbohydrate, total	1.7	g
Fat, total	3.0	g

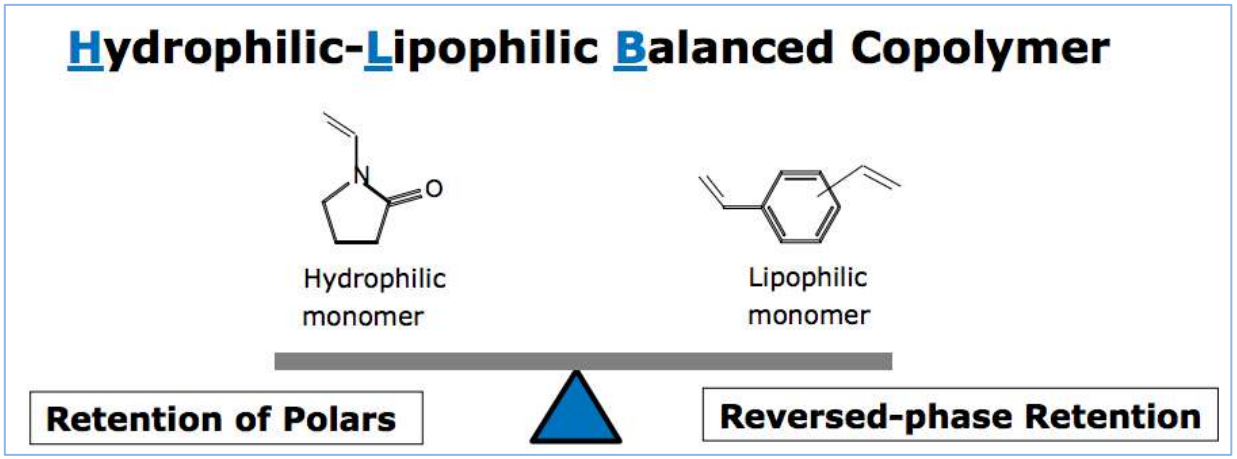
Initial Tissue Extraction: Extract 2 g of kidney with 30 mL of 50 mM phosphate buffer (pH 7.4) centrifuge at 10 000 rpm for 10 minutes.

Fluoroquinolones (FLQs): Considerations on SPE

- **Hydrophilic-lipophilic-balanced** polymeric sorbents

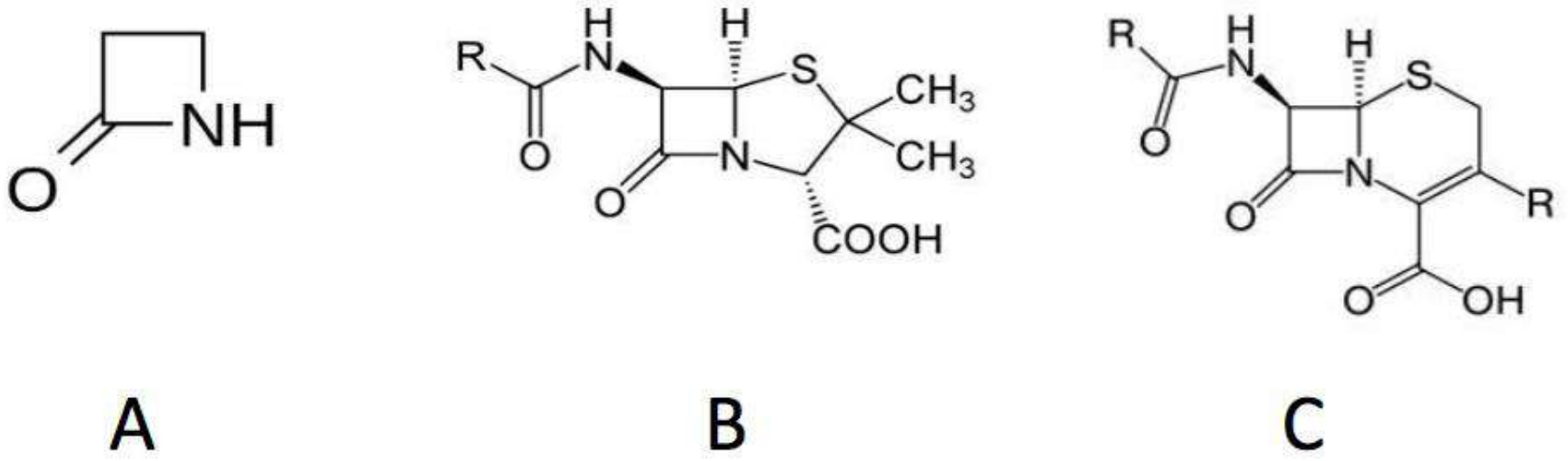
  SPE (e. g. Oasis HLB or Strata-X-PRO) cartridges retain analytes by different mechanism like hydrophilic, hydrophobic and π – π interaction; making it nearly universal SPE material for acidic, basic and neutral analytes

- **Elimination of condition and equilibration steps**



β-Lactams (β LC): chemical structure

- The ß-lactams family holds two main groups: penicillins and cephalosporins that have in common a four member cyclic amine



Structure of β-lactam ring (A); basic structure of penicillin (B), and basic structure of cephalosporin (C)

Penicillins: EU Regulation on MRLs

COMPOUND	EDIBLE TISSUES µg/kg	MILK µg/kg
Benzylpenicillin (Penicillin G)	50	4
Ampicillin	50	4
Amoxicillin	50	4
Oxacillin	300	30
Cloxacillin	300	30
Dicloxacillin	300	30

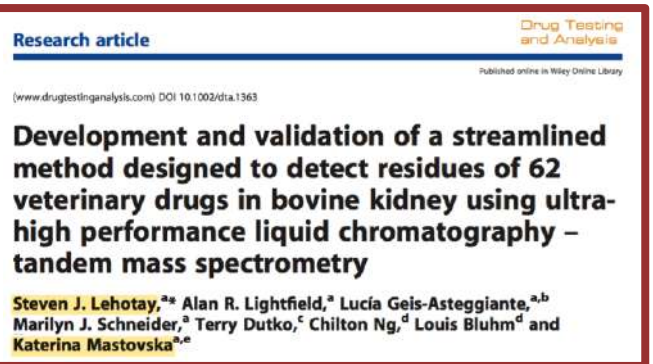
- In the EU (Reg. N. 37/2010), the MRLs for all Penicillins was set at 50 or 100 µg/kg for all edible tissues and at 4 µg/kg for milk, while **their use is prohibited for animals that produce eggs for human consumption**.

β-Lactams (β LCs): Physicochemical properties

- Penicillins and cephalosporins **easily degraded by heat and in organic extraction solvents in presence of acids**
- In order to overcome degradation **avoid acid in the extraction solvent !!!!!!! No more than 0.1 FA % in the mobile phase**

LC-MS/MS method for multi-residue analysis in bovine kidney using LC-MS/MS

Classes: penicillins, cephalosporins, sulfonamides, macrolides/ lincosamides, fluoroquinolones, and tetracyclines



Research article

Drug Testing and Analysis

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(www.drugtestinganalysis.com) DOI 10.1002/dta.1363

Development and validation of a streamlined method designed to detect residues of 62 veterinary drugs in bovine kidney using ultra-high performance liquid chromatography – tandem mass spectrometry

Steven J. Lehotay,[a]* Alan R. Lightfield,[a] Lucía Geis-Asteggiante,[a,b] Marilyn J. Schneider,[a] Terry Dutko,[c] Chilton Ng,[d] Louis Bluhm[d] and Katerina Mastovska[a,e]

- Very rapid degradation of all **β LCs** was observed in **MeOH** and a slower degradation rate in the 50:50 MeOH–water mixture

- Good stability in water, ACN, and ACN–water solutions; also suitable solvents for long-term storage of std solutions at low temperatures.

Generic Sample Preparation Alternatives

- Multi-residue method (MMM): Non-selective (generic) sample preparation to obtain sufficient **recovery** for all compounds

  - Solvent extraction (SE) without further purification
  - Very generic solid phase extraction (SPE)
  - Dispersive-SPE (modified QuEChERS approach)

Solvent extraction (SE) without further purification

L-L extraction and solid - liquid extraction of homogenized tissues

- The selection of the extraction solvent depends on not only the target compounds but also on the matrix
- Suppression effects are usually related to the retention time of the compounds and are most pronounced for early-eluting and late-eluting compounds due to co-elution of salts, carbohydrates, fatty acids and proteins
- Very extensive signal suppression effects are caused by co-eluting proteins or peptides.

Solvent extraction (SE) without further purification

L-L extraction and liquid extraction of homogenized tissues

- **ACN** is the most frequently reported extraction solvent
- **ACN** minimizes co-extraction of lipids and it is efficient for denaturation of proteins
- **β-Lactams**, and in particular penicillin G, degrade when acid is present during extraction
- **ACN alone** does not sufficiently extract **Tetracyclines**. Complexing agent (e.g. EDTA) should be added to the extraction solvent to prevent complexes forming.

Solvent extraction (SE) without further purification

- Mol et al. [ref] compared recoveries and MEs when using 1% formic acid in: ACN, MeOH and in acetone (ACE) to extract veterinary drugs, pesticides and toxins from **honey**, **milk**, **eggs** and **muscle**

Proposed Method A: Water/Acetonitrile/Formic Acid (MeCN)

To 2.5 g of sample 5 mL of water was added and mixed using a vortex. In case of dry matrixes the mixture was allowed to soak for 2 h. Then 15 mL of acetonitrile containing 1% formic acid was added, and the sample was extracted by end-over-end shaking for 1 h. The tube was centrifuged (10 min, 2000 rcf), and 0.5 mL of extract was transferred into an autosampler vial.

Proposed Method B: Water/Methanol/Formic Acid (MeOH)

This was the same as proposed method A but with methanol instead of acetonitrile.

Proposed Method C: Water/Acetone/Formic acid (ACE)

This was the same as proposed method A but with acetone instead of acetonitrile.

Recoveries and ME were determined for 86 veterinary drugs from different classes (macrolides, nitroimidazoles, NSAIDs, **quinolones**, **sulfonamides**, **tetracyclines**, tranquilizers).

- Water / ACN (75/25 v/v) with 1% formic acid
- Water /MeOH (75/25 v/v) with 1% formic acid
- Water / ACE (75/25 v/v) with 1% formic acid

Anal. Chem. 2008, 80, 9450–9459

Toward a Generic Extraction Method for Simultaneous Determination of Pesticides, Mycotoxins, Plant Toxins, and Veterinary Drugs in Feed and Food Matrixes

Hans G. J. Mol,*,† Patricia Plaza-Bolaños,‡ Paul Zomer,† Theo C. de Rijk,† Alida A. M. Stolker,† and Patrick P. J. Mulder†

Solvent extraction (SE) without further purification

- Although acetone (ACE) showed the highest recovery overall, it also showed more severe MEs compared to ACN

- When using ACN for the extraction of veterinary drugs, the overall MEs decrease for the matrixes in the order of:

feed > maize > meat > milk > egg > honey

	matrix effects				
	signal suppression		not significant	signal enhancement	
matrix	>50%	20–50%	80–120%	20–50%	>50%
feed	33	19	**31**	6	12
maize	7	17	**40**	22	14
honey	0	2	**67**	24	6
meat	2	2	**92**	2	1
egg	1	0	**79**	16	3
milk	0	5	**85**	6	5

[a] Taking phase partitioning into account.

- For milk, egg and honey, ACE was clearly preferable over ACN in terms of recovery, whereas matrix effects were considered acceptable for both solvents

- As a result, **ACN** -water /ACN (75/25 v/v) with 1% formic acid-was selected as the solvent for extraction of muscle and ACE for the extraction of milk, egg and honey.

Approaches to reduce ME in solvent extraction (SE) without additional clean-up

- Methods that only involve an SE procedure usually result in final extracts contain a large amount of matrix interferences, so matrix effects are pronounced

- Matrix effects can be abundant, especially for feed samples

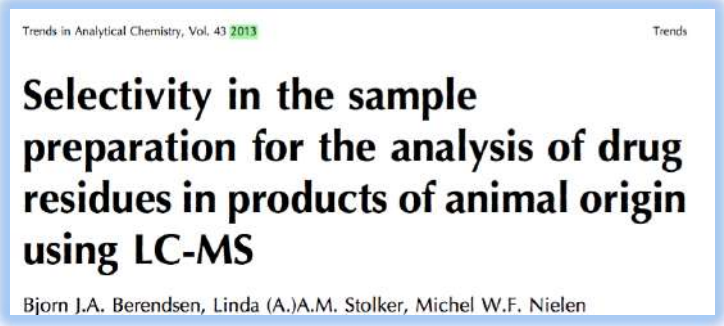
  - Small injection volumes
  - Dilute the final extract

Summary of applications using SE without further clean-up for the analysis of multi-class veterinary drugs in products of animal origin

Compound groups	Matrix	Method
A, AVM, B, Q, T, TMP	Meat-based baby food, powdered milk	Soak with water. Extraction with acidic ACN. Filtration (nylon). HRLC-QqQ-MS
Q, S, T	Kidney, liver	Extraction with acidic ACN/water (7:2 v/v). Partly evaporation of solvent and dilute with water/ACN (95:5 v/v). Filtration. HRLC-Orbitrap-MS
AG, C, L, M, P, Q, morantel	Milk	Extraction with ACN. Evaporation of solvent and dissolve in water/ACN (1:1 v/v). Filtration (PVDF). HRLC-QqQ-MS
C, P, M, S	Milk	Extraction with ACN. Evaporation of solvent and dissolve in NH4Ac. Filtration. LC-QqQ-MS
A, AG, M, P, S, T	Honey	Extraction with ACN. Extraction of the pellet with 10% TCA. Extraction of the pellet with nonafluoropropionic acid in ACN followed by neutralization. Hydrolysis of the pellet followed by extraction with ACN. Evaporation of the individual extracts and dissolve in water/MeOH (8:2 v/v). LC-QqQ-MS
B, COC, M, N, NIZ, Q, S, T, TQ	Honey, egg, milk, meat	Soak the sample with water. Extraction with acidic ACN, MeOH or acetone (ACE). HRLC-QqQ-MS
A, AG, C, M, NSAID, P, Q, T, S	Muscle	Extraction with ACN/water (86:14 v/v) at 60°C. Second extraction with water. Dilution of the combined extracts with water. Fat removal with hexane. LC-QqQ-MS
AG, COC, L, M, P, Q, S, T	Muscle	Extraction with 2% TCA/ACN (1:1 v/v). fat removal using hexane. Dilution with acidic water/ACN (1:9 v/v). Filtration (Nylon). HILIC-LC-QqQ-MS
AG, L, Q, T	Milk	Extraction with 5% TCA. Filtration (PVDF). LC-QqQ-MS
S, TMP, TPM	Shrimp	Extraction with 6 mL 20% TCA in ultrasonic bath. Evaporation of solvent and dissolve in MeOH/water (1:4 v/v). Filtration (PTFE). LC-TOF-MS
M, P, Q, S, T,	Muscle	Add EDTA solution. Extraction with MeOH/water (7:3 v/v). Dilution with water. LC-QqQ-MS
M, P, Q, S, T	Muscle	Extraction with MeOH/water (7:3 v/v) and EDTA. Dilution with water. Filtration. HRLC-QqQ-MS
C, L, M, NIZ, P, Q, S, T, TMP	Muscle	PLE with water and EDTA. Evaporation of solvent. LC-QqQ-MS
M, NIZ, P, Q, S	Muscle	PLE with water and EDTA. Evaporation of the solvent. LC-QqQ-MS

A, Amphenicols; AG, Aminoglycosides;
AVM, Avermectins; bA, b-agonists;
B, Benzimidazoles; BDP, Benzodiazepams;
COC, Coccidiostats;
CS, Corticosteroids; I, Ionophores;
L, Lincosamides; M, Macrolides;
NIZ, Nitroimidazoles;
NSAID, Non-steroidal anti-inflammatory drugs;
P, Penicillins; Q, Quinolones; S, Sulfonamides;
T, Tetracyclines; TMP, Trimethoprim;

TPM, Triphenylmethanes; TQ, Tranquilizers


Trends in Analytical Chemistry, Vol. 43 2013 Trends

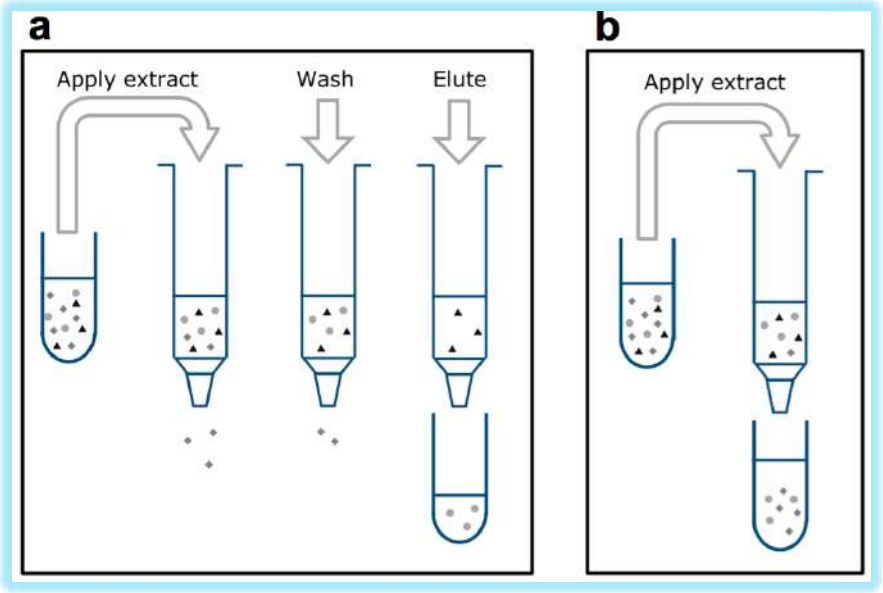
Selectivity in the sample preparation for the analysis of drug residues in products of animal origin using LC-MS

Bjorn J.A. Berendsen, Linda (A.)A.M. Stolker, Michel W.F. Nielen

Solid phase extraction (SPE): two approaches

(a)

To remove the most matrix **phospholipids** and **proteins** in sample aqueous solution



(b)

To quickly remove matrix interferences in sample solution contains a high amount of organic solvent (s)

(a) Retentive SPE: the extract is applied onto an SPE cartridge to retain the compounds of interest, followed by a wash step with 5% MeOH to remove matrix interferences and subsequent elution of analytes with 100 % ACN or MeOH [LSEP]
(b) Non-retentive SPE applying a highly organic raw extract onto an SPE cartridge and immediately collecting the eluent for further analysis.

➢ Retentive SPE: Which Sorbent ?

- C18 SPEs based upon hydrophilic-lipophilic interactions (Oasis HLB, Strata-X RP or Evolute ABN) are the mostly used

➢ To retain even the polar analytes:

- Apply onto the SPE cartridge a low organic content of the extract. The extraction solvent should have a high water content or change the solvent of the raw extract prior to SPE. Before SPE: evaporate organic solvent or dilute organic phase with an aqueous solvent

➢ Washing step solvents:
Water, formic acid, water/methanol (95:5 v/v) and hexane

➢ Elution solvent for polar or semi-polar compounds:
Pure ACN or MeOH or mixtures thereof at neutral or alkaline conditions.

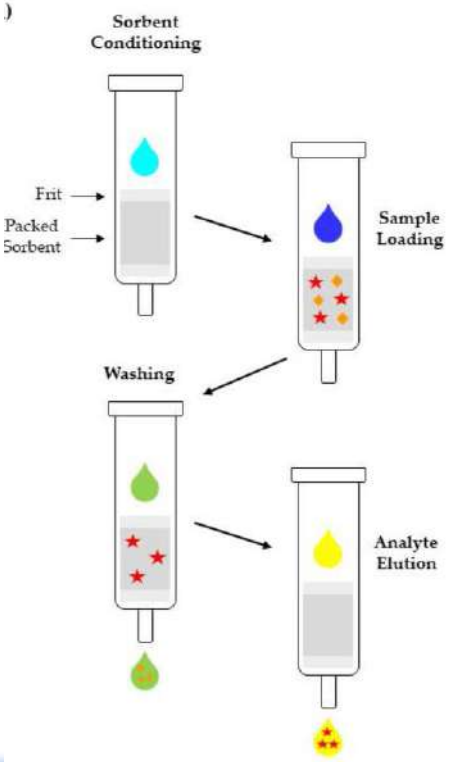
Solid phase extraction (SPE)

- RP SPE is somewhat limited towards polar compounds
  For **penicillins** and **tetracyclines**, enhanced results are achieved without SPE step

- For **milk**, the MEs after SPE are relatively variable throughout different samples, complicating quantitative analysis when no isotopically-labeled internal standards are available.

Summary of applications using SPE for the analysis of multi-class veterinary drugs in products of animal origin

Compound groups	Matrix	Method
S, TMP, TPM	Shrimp	Extraction with metaphosphoric acid in ACN (6:4 v/v). Filtrate (PTFE). Partially evaporate the solvent. SPE Oasis HLB, wash with ACN/water (1:4 v/v), elute with ACN. Partially evaporation of solvent. Filtration (PTFE). LC-TOF-MS
B, M, Q, S	Egg	Extraction with 0.1 M Na_2EDTA (pH = 4). Oasis HLB, wash with n-hexane, elution with ACN, MeOH, alkaline MeOH. Evaporation of solvent and dissolve in acidic MeOH/water (1:1 v/v). Filtration (nylon). HRLC-QqQ-MS
B, C, NIZ, P, Q, S, TMP, TQ	Meat	Extraction with water/ACN (1:1 v/v), EDTA, $(NH_4)_2SO_4$. Add $(NH_4)_2SO_4$. Evaporation of organic solvent. Adjust to pH = 6.5. SPE Oasis HLB, wash with water, elution with ACN and succinate buffer/ACN (1:1 v/v). Add DMSO. Evaporation of the organic solvent. Dilution with water. HRLC-TOF-MS
L, M, Q, T, TMP	Honey	Extraction with McIlvain, Na2EDTA. Filtration. SPE Oasis HLB, wash with water/MeOH (95:5 v/v), elution with MeOH. Evaporation of the solvent. Dissolve in 0.2 % formic acid.
C, M, P, Q, S, T, bacitracin	Milk	Extraction with ACN. Dilution in 0.1% formic acid. SPE Oasis HLB, wash with 0.1% formic acid, elution with ACN/MeOH (7:3 v/v). Add 0.1% formic acid. Partial evaporation of solvent. Ultrafiltration 30 kD
M, Q, S, T	Honey	Extraction with Na_2EDTA (pH = 4). SPE Oasis HLB
A, B, COC, I, M, P, NIZ, NSAID, Q, S, T, TMP, TQ	Egg, fish, meat	Extraction with ACN/water (6:4 v/v). Dilution of supernatant in water. SPE Strata-X RP, wash with water. Egg: elution with MeOH/ethyl acetate (EtAc) (1:1 v/v). Fish/meat: elution MeOH/ACN (1:1 v/v). Evaporation of solvent and dissolve in ACN, dilute in acidic water. HRLC-TOF-MS
A, AG, I, L, M, S, T, fumagillin	Honey	Extraction with water. Aliquot for streptomycin analysis. Filtration (nylon). SPE Strata-X RP, wash with water and hexane, elute with MeOH, ACN and alkaline MeOH. Partial evaporation of solvent. Dilution with water. LC-QqQ-MS
A, B, I, M, NIZ, NSAID, P, Q, S, T, TMP, TQ	Milk	Extraction with ACN. Dilution with water. SPE Strata-X RP, wash with water, elution with MeOH. Evaporation of the solvent. Dissolve in ACN. Dilute in acidic water. HRLC-TOF-MS
COC, CS, Q, S, TMP, griseofulvin	Milk	Extraction with ACN. Dilution with water. SPE Strata-X RP, elution with MeOH. Evaporation of the solvent. Dissolve in acidic MeOH
B, C, I, NIZ, P, Q, S, TMP, TPM, TQ	Honey, fish	Extraction with water/ACN (35:45 v/v), EDTA, $(NH_4)_2SO_4$. Add $(NH_4)_2SO_4$. Evaporation of organic solvent. Adjust to pH = 6.5. SPE Evolute ABN, wash with water, elution with ACN and succinate buffer/ACN (1:1 v/v). Partial evaporation of the solvent. Dissolve in water. HRLC-Orbitrap-MS
βA, CS, stimulants, narcotics	Urine	Hydrolysis at pH = 4.8 with β-glucuronidase/aryl sulfatase. SPE Oasis MCX, wash with 1 M acetic acid and NaAc buffer/ACE (85:15 v/v), elution with alkaline ethyl acetate. Evaporation of the solvent. Dissolve in water/ACN (5:95 v/v). HRLC-TOF-MS
B, βA, BDP, CS, NIZ, Q, S, T, TPM	Meat, milk, egg	Mix with anhydrous Na_2SO_4. Double extraction with acidic ACN. Extraction EtAc. Evaporate combined extract and dissolve in alkaline MeOH. Pass through SPE Oasis HLB, elute with alkaline MeOH. Evaporation of solvent and dissolve in water/ACN (7:3 v/v). HRLC-QTOF-MS
AVM, B, M, Q, T, S	Egg	Extraction with ACN, citric acid (pH = 4), 0.1 M Na_2EDTA. Pass through Oasis HLB cartridge, Partial evaporation of solvent and dilute with acidic MeOH/water (1:1 v/v). Filtrate (nylon). HRLC-QqQ-MS



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Selectivity in the sample preparation for the analysis of drug residues in products of animal origin using LC-MS

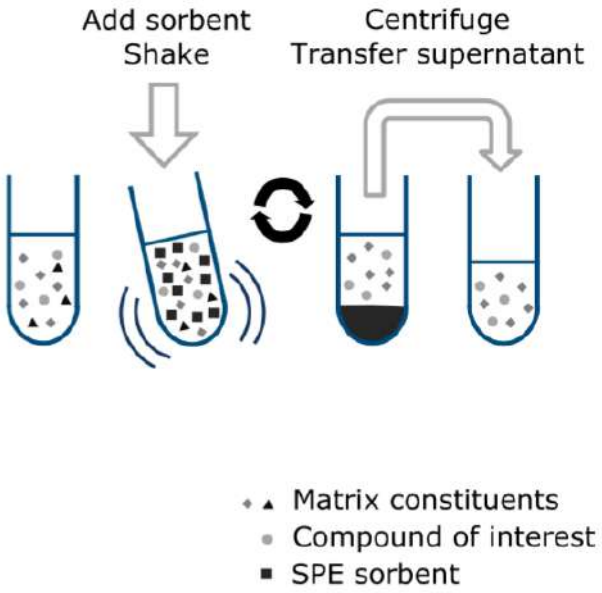
Bjorn J.A. Berendsen, Linda (A.)A.M. Stolker, Michel W.F. Nielen

Dispersive-SPE (modified QuEChERS approach)

- Dispersive solid phase extraction (d-SPE) involves a simple mixing of the crude extract (ex. solution in 4:1 ACN–water) with a sorbent that removes matrix interferences but does not retain the analytes

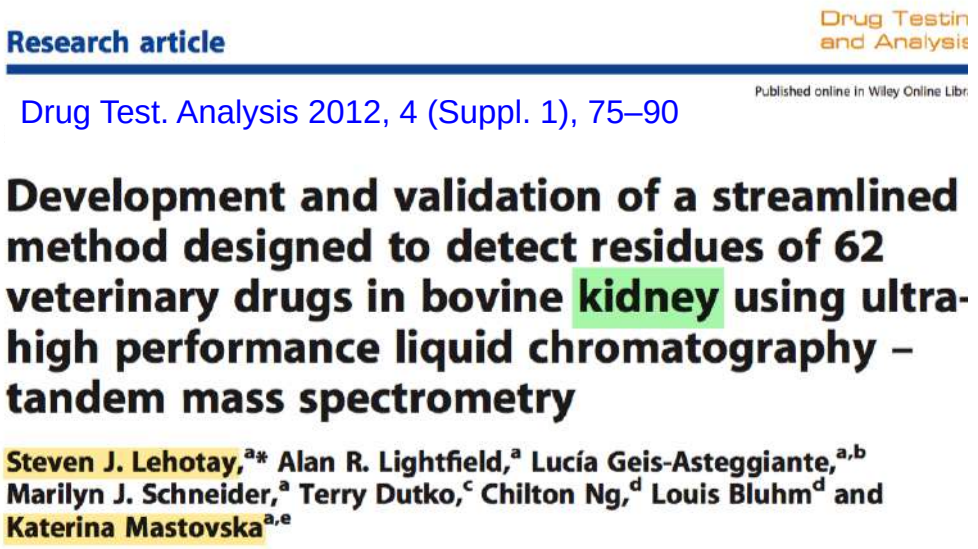
- Shaking facilitates the contact between sorbent and analytes, and centrifugation and subsequent isolation of the supernatant.

- ✓ **C18 sorbent**
- ✓ **Primary-secondary amine** (PSA)
- ✓ **Graphitised carbon black** (GPB)
  removal of removes highly lipophilic compounds, such fatty acids and pigments



MMM in kidney: *d*-SPE

- 16 **Sulfonamides**
- 10 **β-lactams** (cephalosporins and **penicillins)**
- 8 **Macrolides/ lincosamides**
- 7 **Fluoroquinolones**
- 3 **Tetracyclines**



Research article

Drug Testin and Analysis

Published online in Wiley Online Libr

Drug Test. Analysis 2012, 4 (Suppl. 1), 75–90

Development and validation of a streamlined method designed to detect residues of 62 veterinary drugs in bovine kidney using ultra-high performance liquid chromatography – tandem mass spectrometry

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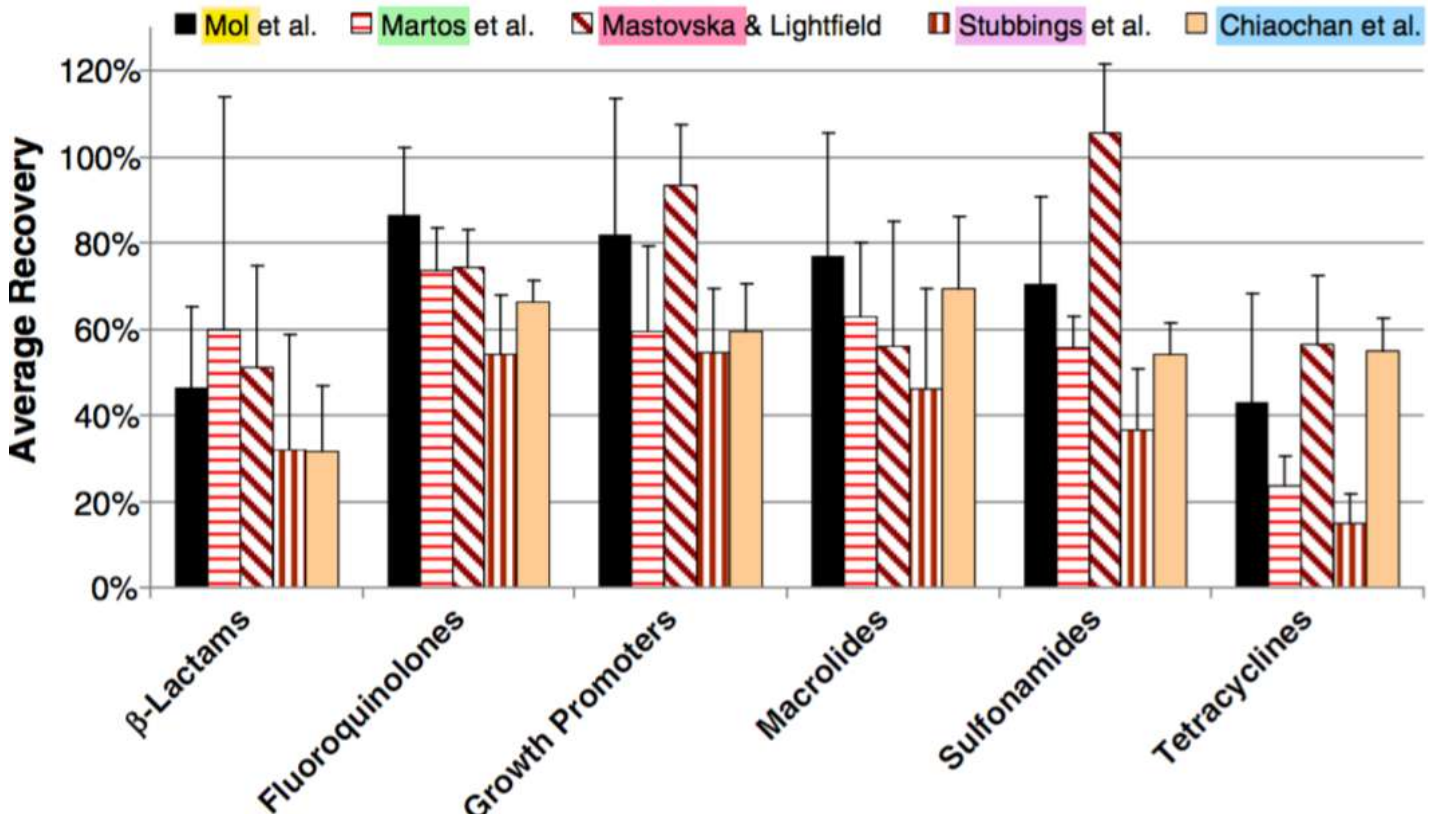
- **50** of the 62 drugs met qualitative MS identification criteria
- **30 drugs** gave ≥70% recoveries and ≤25% reproducibilities

Mol	Martos	Mastovska	Stubbings	Chiaochan
• ACN/water 75/25 with 1% formic acid • LL extraction with Hexane pre-saturated with ACN • Hexane aspirated to waste. Water extract was taken	• ACN/water 86/14 + formic acid • LL extraction with Hexane pre-saturated with ACN • Hexane aspirated to waste. Water extract was taken	• ACN/water 4/1 • dSPE with 500 mg C18 + hexane pre-saturated with ACN • Hexane aspirated to waste. Water extract was taken	• ACN with 1% acetic acid + anhydrous sodium sulfate • dSPE with 200 mg aminopropyl • Extract volume reduction + water	• ACN/water 1/1 with 2% trichloroacetic acid • LL extraction with Hexane pre-saturated with ACN • Hexane aspirated to waste. Extract was taken



Optimized sample preparation

- 5-min shake of 2 g homogenized kidney with 10 ml of 4/1 (v/v) ACN /water followed by simultaneous clean-up of the initial extract with 0.5 g C18 and 10 ml hexane presaturated with ACN

- The hėxane was aspirated to waste and 5 ml of extract was taken to 0.7 ml on the TurboVap at 40 C (45 C in the final method), and water was added to reach the 1-ml mark (1 g/ml sample equivalent)

- Acceptably screen for **50** of the 62 drugs met **qualitative MS identification criteria**

- Method gave ≥70% recoveries and ≤25% reproducibilities **for 30** of the drugs


Research article

Drug Testing and Analysis

Development and validation of a streamlined method designed to detect residues of 62 veterinary drugs in bovine kidney using ultra-high performance liquid chromatography – tandem mass spectrometry

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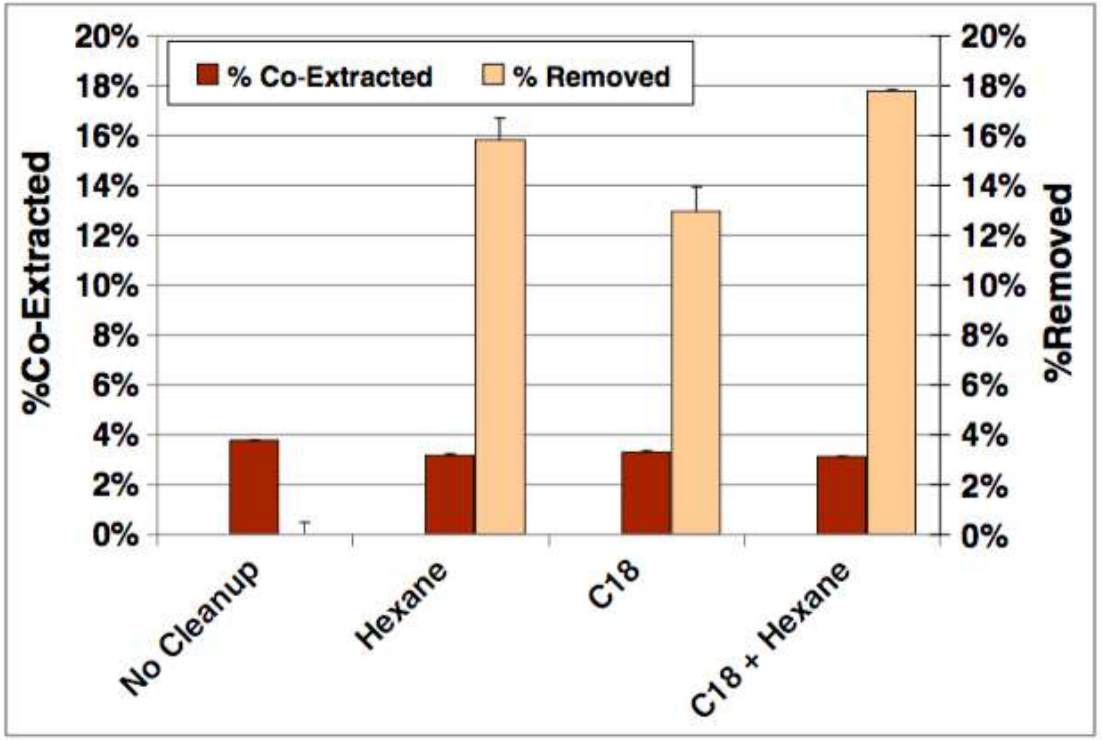
Clean-up effects by weight of co-extractives before and after d-SPE and/or hexane partitioning clean-up



Extraction and clean-up of bovine kidneys by weight of co-extractives using different clean-up. % Co-extracted is kidney weight equivalent remaining in the extract, %Removed is the difference in weights before and after clean-up.

Generic sample preparations in a MMM

The sample preparation must not use acidic conditions during extraction

- With respect to inclusion of 1% formic acid or not in the 4/1 ACN/water extraction solvent, the highest drug concentrations were found to occur in only 5 out of the 26 instances (analysis of real samples) when acid was present.
- The absence of acid during extraction led to nearly double concentrations for sulfonamides than when acid was present
- Most prominently and importantly, the β-lactams, penicillin G and DCCD, disappeared (degraded) when acid was present during extraction.
- Penicillin and ceftiofur (the parent drug of the DCCD metabolite) are two of the most widely used antibiotics in veterinary medicine, and the analytical screening method must be able to detect them.

molecules

MDPI

Article

Comparison of Sample Preparation and Determination of 60 Veterinary Drug Residues in Flatfish Using Liquid Chromatography-Tandem Mass Spectrometry

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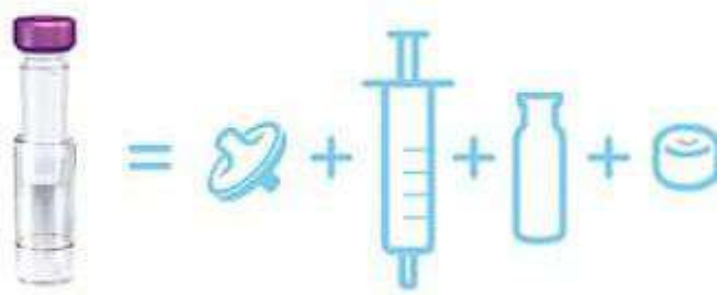
Filtration: Mini-UniPrep syringeless Filters

1. Place unfiltered sample (max. 0.5 mL) in chamber

2. Compress filter plunger into sample chamber

Clean filtrate fills reservoir bottom up

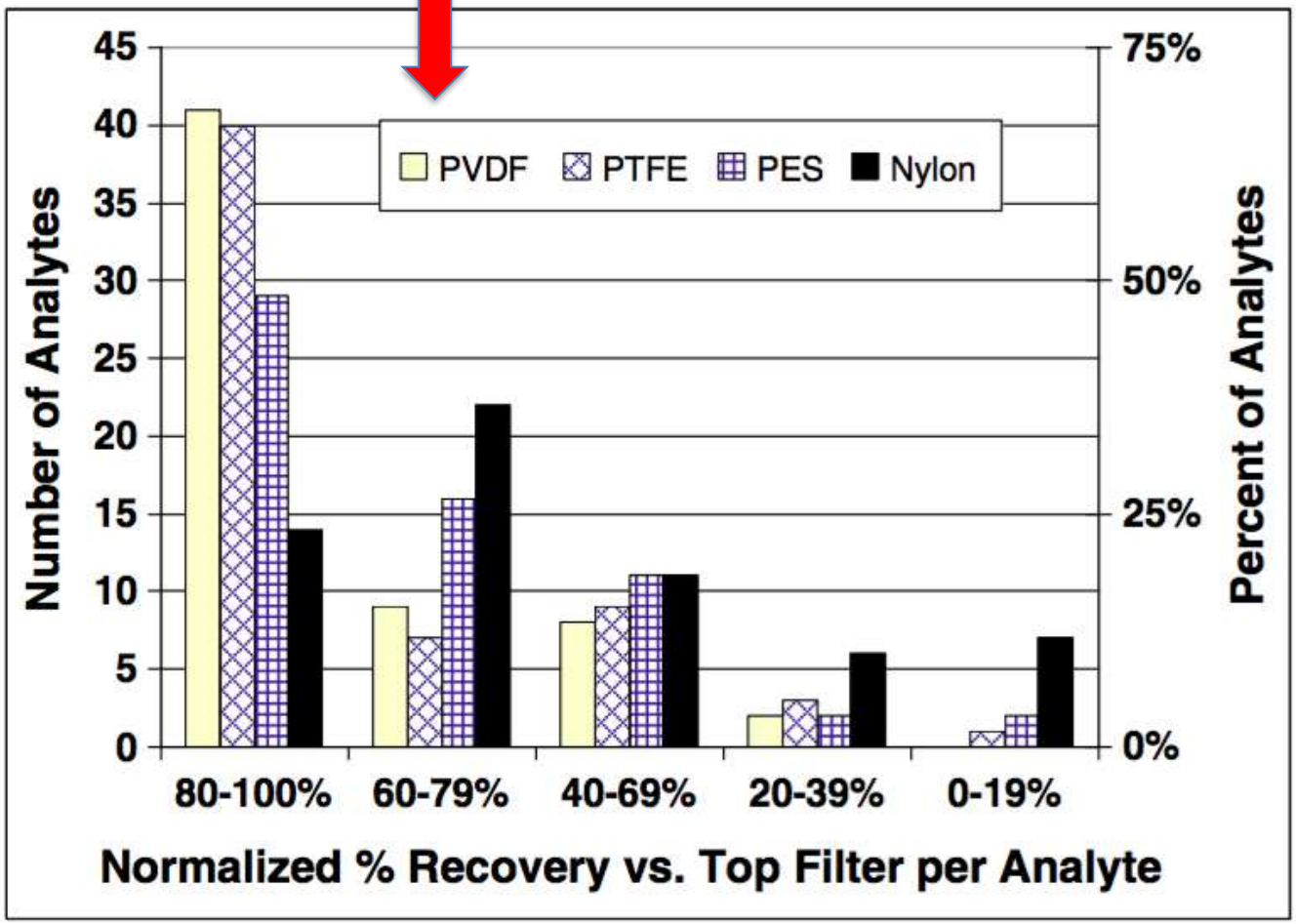
3. Place the Mini-UniPrep vial in an autosampler



- Nylon (polyamide)
- Polytetrafluoroethylene (PTFE)
- Polyvinylidene fluoride (PVDF)
- Polyethersulfone (PES)

For aqueous samples before LC-MS injection

0.2 µm PVDF



Research article

Drug Testing and Analysis

Development and validation of a streamlined method designed to detect residues of 62 veterinary drugs in bovine kidney using ultra-high performance liquid chromatography – tandem mass spectrometry

Steven J. Lehotay,*† Alan R. Lightfield,[a] Lucía Geis-Asteggiante,[a,b] Marilyn J. Schneider,[a] Terry Dutko,[c] Chilton Ng,[d] Louis Bluhm[d] and Katerina Mastovska[a,c]

Figure 1. Relative performances of different types of Mini-UniPrep filter vials in bovine kidney extracts spiked at 100 ng/g for the drug analytes.