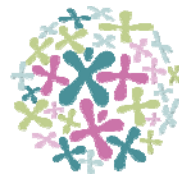


# Fluoro-quinolones in honey by turbulent flow chromatography - MS/MS

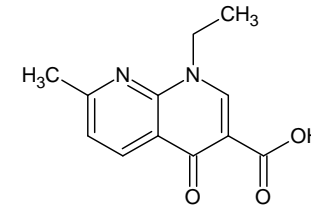
**S. Bruce**  
Nestlé Research Centre  
Lausanne  
Switzerland



- ~1.2 million tons of honey are produced worldwide annually
- ~400,000 tons of this is traded internationally
- Honey is a product used widely within the food industry (e.g. cereal based products, baby foods, chocolate, pastries)
- Within the last 8 years, the findings of veterinary drug residues (e.g. aminoglycosides, tetracyclines, sulfonamides etc) in batches of honey have resulted in rejection and even destruction of batches
- EU regulations for maximum residual limits of these drugs exist for many different foods, but currently not for quinolones / fluoro-quinolones in honey!



- 1st quinolone (nalidixic acid, “NegGram”) introduced in 1962
- Fluoro-quinolones introduced for clinical use in the 1980’s
  - Improved activity
  - Greater bacterial resistance
- They inhibit DNA synthesis – promoting cleavage of bacterial DNA-enzyme complexes – resulting in rapid bacterial death
- All types of quinolones are well absorbed orally
- Quinolones and fluoro-quinolones belong to the current arsenal of antibiotics used in animal therapy



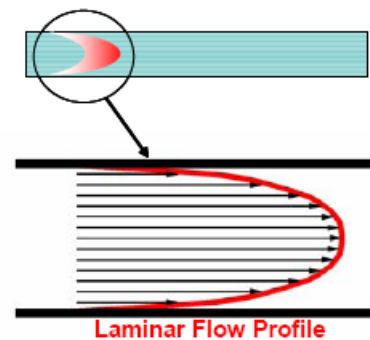
*Oliphant & Green. Clinical Pharmacology, 65:3 455-464 2002*

- The continuous expansion within the global trade of food leads to a growing numbers of samples to be analysed
- High sample through-put has become mandatory within this area
- Previously, either ELISA kit or liquid-liquid extraction (LLE) followed by solid phase extraction (SPE) has been the choice preparation procedure – good quality data, but time consuming, labour intensive, high organic solvent consumption, and manual procedures not as reproducible as automated ones
- Automated sample preparation by means of turbulent flow chromatography (TFC) could help fulfil this requirement

# Turbulent flow chromatography (TFC)

- TFC utilises particle size columns ~40 – 150  $\mu\text{m}$  long, with a pore size ~60  $\text{\AA}$
- By applying flow rates of ~1.0 – 6.0 mL/min cause to behave in the turbulent manner
- The altered flow profile is caused by the packed bed particles, which form eddies and in turn cause flow equalization across the column (below)

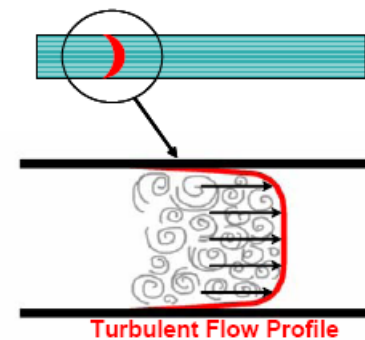
## Conventional Column (3 – 5 $\mu\text{m}$ particle size)



### Laminar flow

Distinct streams of fluid  
rely on diffusion in a radial  
direction

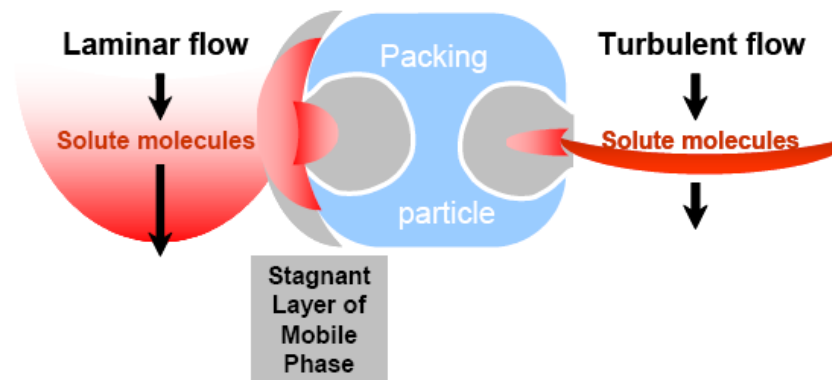
## TurboFlow Column (40 – 150 $\mu\text{m}$ particle size)



### Turbulent flow

The linear fluid streams break  
apart and form counter  
rotating vortices or eddies

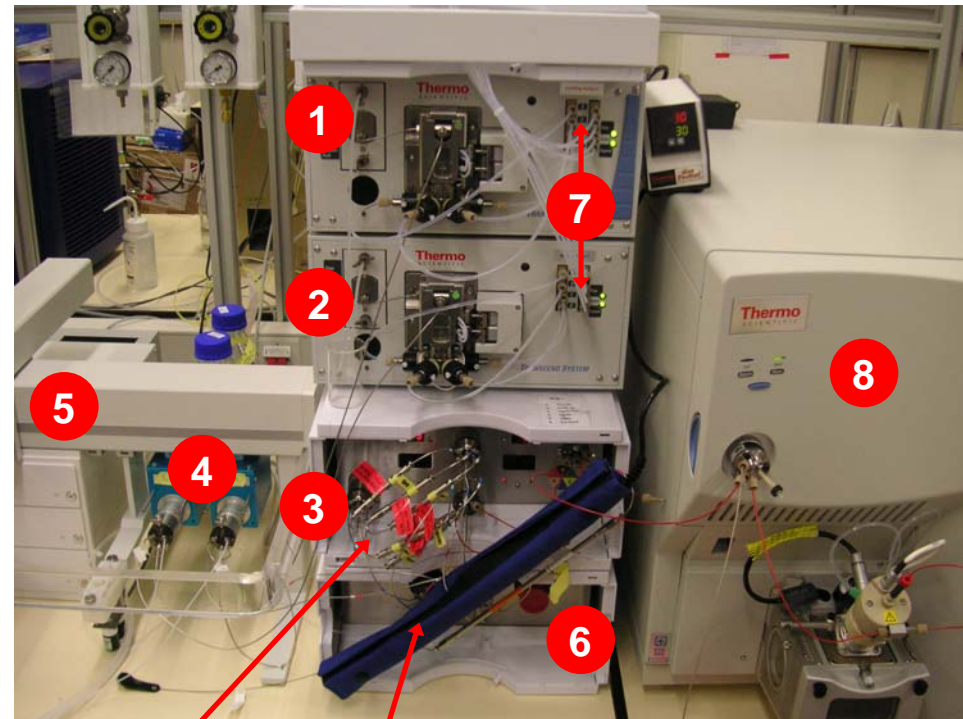
- Under laminar conditions – solutes must diffuse through the stagnant layer of mobile phase in order to enter the pores of the packing material = peak broadening and tailing
- Under turbulent flow conditions – solute molecules are forced rapidly in and out of the particle pores, thus controlling the effective diffusion of the targeted solute
- Larger molecules (i.e. proteins, carbohydrates etc) at the higher flow rate diffuse much slower than smaller molecules, and therefore are flushed through the column



- TFC also combines simultaneously size exclusion, and analyte selectivity – due to the chemistry of the stationary phase

# The TLX1 System (coupled to MS)

- 1 Loading pump
- 2 Eluting pump
- 3 Multiple Column Module (MCM)
- 4 Two-valve sample injection unit
- 5 PAL autosampler
- 6 3-valve switching device unit
- 7 Degassers (above = loading, below = eluting lines)
- 8 Quantum Ultra AM triple-quad MS

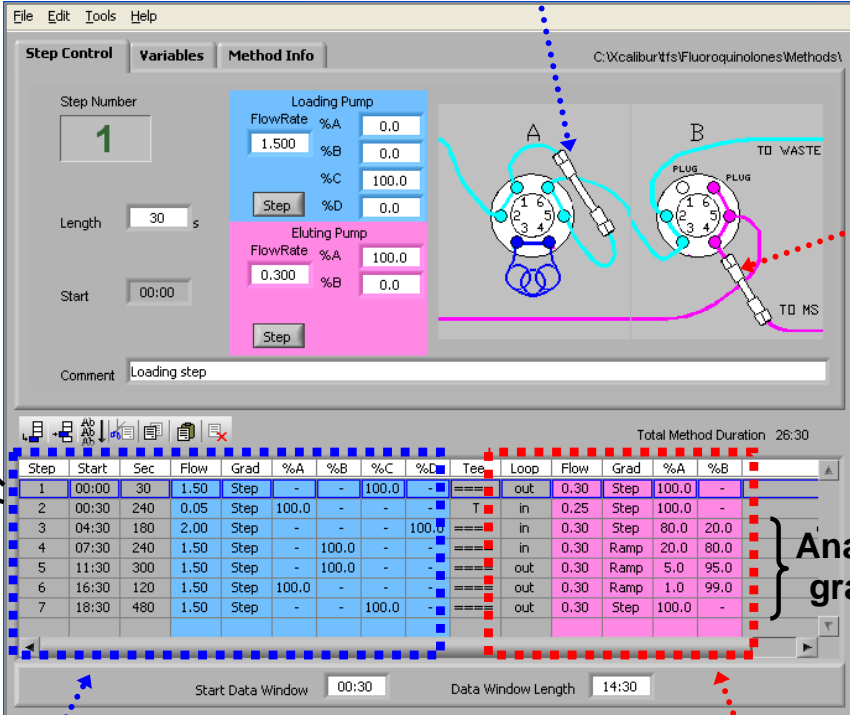


TurboFlow columns  
(loading flow)

Analytical column  
(eluting flow)

# The TLX1 System (through Aria™ software)

**TFC column**



**Analytical column**

**Analytical gradient**

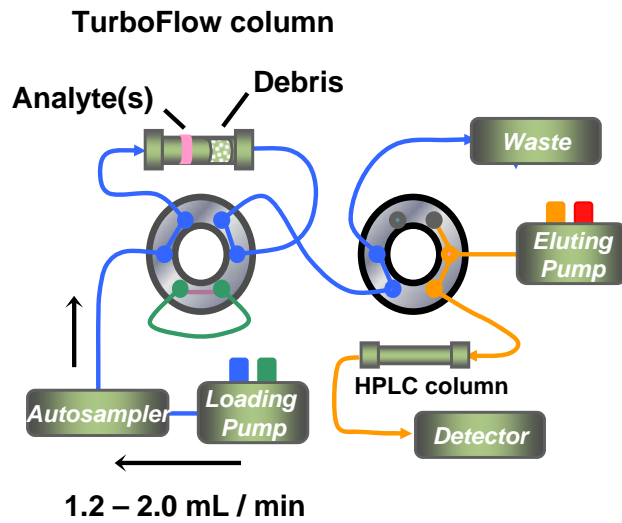
**TFC gradient conditions (loading pump)**

**Analytical gradient conditions (eluting pump)**

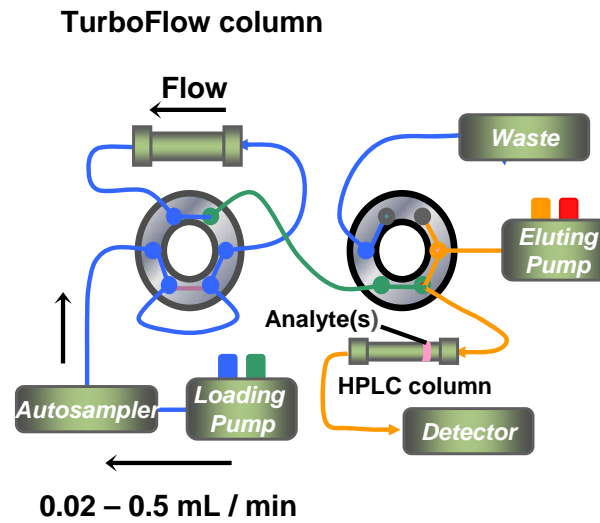
Step	Start	Sec	Flow	Grad	%A	%B	%C	%D	Tee	Loop	Flow	Grad	%A	%B
1	00:00	30	1.50	Step	-	-	100.0	-	====	out	0.30	Step	100.0	-
2	00:30	240	0.05	Step	100.0	-	-	-	T	in	0.25	Step	100.0	-
3	04:30	180	2.00	Step	-	-	-	100.0	====	in	0.30	Step	80.0	20.0
4	07:30	240	1.50	Step	-	100.0	-	-	====	in	0.30	Ramp	20.0	80.0
5	11:30	300	1.50	Step	-	100.0	-	-	====	out	0.30	Ramp	5.0	95.0
6	16:30	120	1.50	Step	100.0	-	-	-	====	out	0.30	Ramp	1.0	99.0
7	18:30	480	1.50	Step	-	-	100.0	-	====	out	0.30	Step	100.0	-

# TFC system (focus mode)

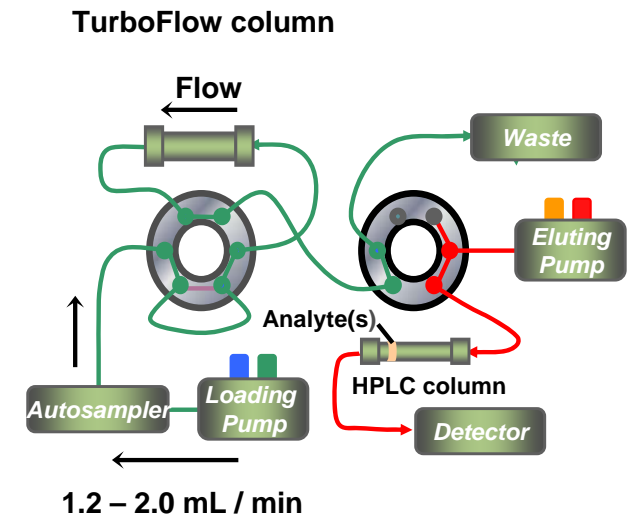
(a) Loading step



(b) Transfer step

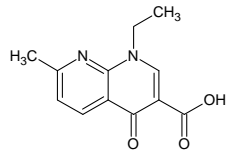


(c) Cleaning / eluting

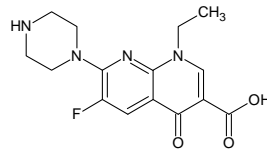


- Honey samples (at least 50 g) heated in a water bath (40 °C), and mixed until achieving a homogenous consistency
- 1.0 g of this mixture was weighed out into a micro-centrifuge tube, 1 mL of water added
- Solution vortexed (1 min) and gently heated again until a homogenous slurry was achieved
- Solution then filtered (0.22 µm polyethersulfone membrane syringe filter) directly into sample vial for analysis

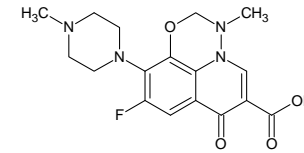
# Quinolones & fluoro-quinolones for quantitation



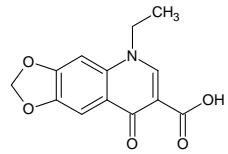
Nalidixic acid



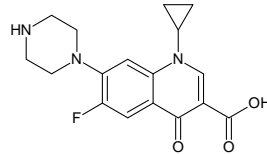
Enoxacin



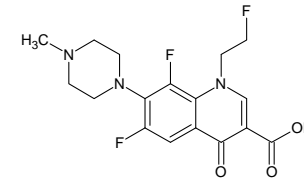
Marbofloxacin



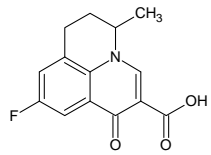
Oxolinic acid



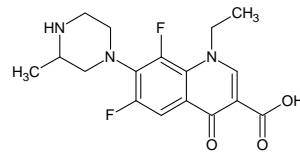
Ciprofloxacin



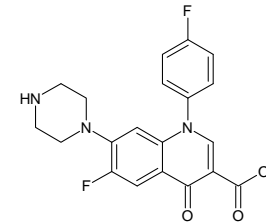
Fleroxacin



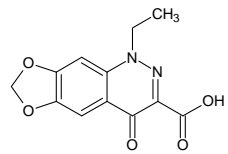
Flumequine



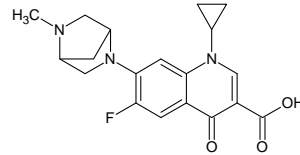
Lomefloxacin



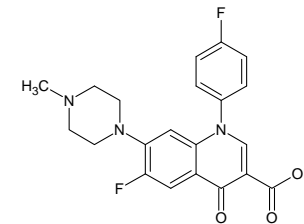
Sarafloxacin



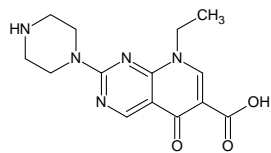
Cinoxacin



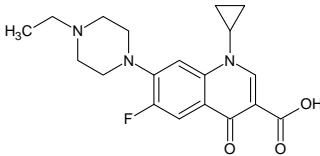
Danofloxacin



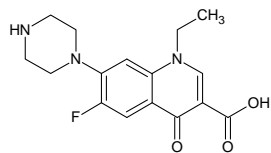
Difloxacin



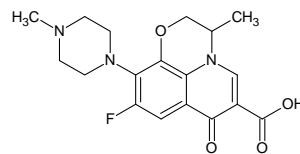
Pipemidic acid



Enrofloxacin



Norfloxacin



Ofloxacin

# MS conditions & SRM parameters

Data was acquired in positive ionisation and SRM mode using a TSQ Quantum Ultra™ AM MS/MS system

## MS Conditions

Spray Voltage:	3000 V
Vaporizer Temperature:	350 °C
Sheath Gas Pressure (N <sub>2</sub> ):	40 units
Auxiliary Gas Pressure (N <sub>2</sub> ):	35 units
Capillary Temperature:	325 °C
Collision Gas (Ar):	1.5 mTorr
Q1/Q3 Peak Resolution:	0.7 u (unit mass resolution)
Scan Time:	0.025 s
Scan Width:	0.010 <i>m/z</i>

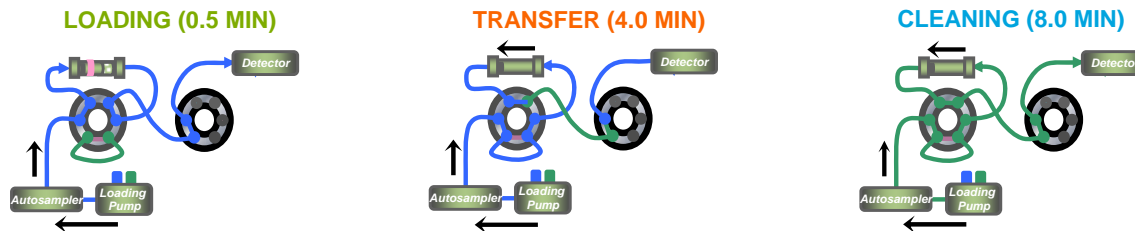
## Transition reactions (*m/z*) used for

Analyte	Quantification	Analyte confirmation		TL (V)	Scan Time (msec)
	SRM 1 (CE)	SRM 2 (CE)	SRM 3 (CE)		
Pipemidic acid	304 → 215 (33)	304 → 217 (22)	304 → 286 (21)	82	25
Cinoxacin	263 → 105 (37)	263 → 189 (29)	263 → 217 (22)	59	25
Marboflaxin	363 → 72 (23)	363 → 276 (15)	363 → 320 (15)	67	25
Enoxacin	321 → 206 (29)	321 → 234 (22)	321 → 257 (18)	65	25
Fleroxacin	370 → 223 (36)	370 → 269 (27)	370 → 326 (19)	112	25
Ofloxacin	362 → 205 (40)	362 → 261 (27)	362 → 318 (19)	109	25
Norfloxacin	320 → 233 (25)	320 → 276 (17)	320 → 302 (21)	70	25
Oxolinic acid	262 → 118 (36)	262 → 130 (33)	262 → 158 (30)	82	25
Ciprofloxacin	332 → 231 (36)	332 → 245 (24)	332 → 288 (17)	74	25
Danofloxacin	358 → 82 (39)	358 → 283 (25)	358 → 314 (18)	75	25
Lomefloxacin	352 → 251 (25)	352 → 265 (23)	352 → 308 (17)	78	25
Enrofloxacin	360 → 204 (31)	360 → 245 (26)	360 → 315 (19)	72	25
Difloxacin	400 → 299 (29)	400 → 356 (20)	400 → 382 (23)	75	25
Sarafloxacin	386 → 299 (28)	386 → 342 (18)	386 → 368 (22)	105	25
Nalidixic acid	233 → 104 (40)	233 → 131 (35)	233 → 187 (25)	78	25
Flumequine	262 → 146 (46)	262 → 200 (34)	262 → 202 (33)	61	25

## Online extraction steps:

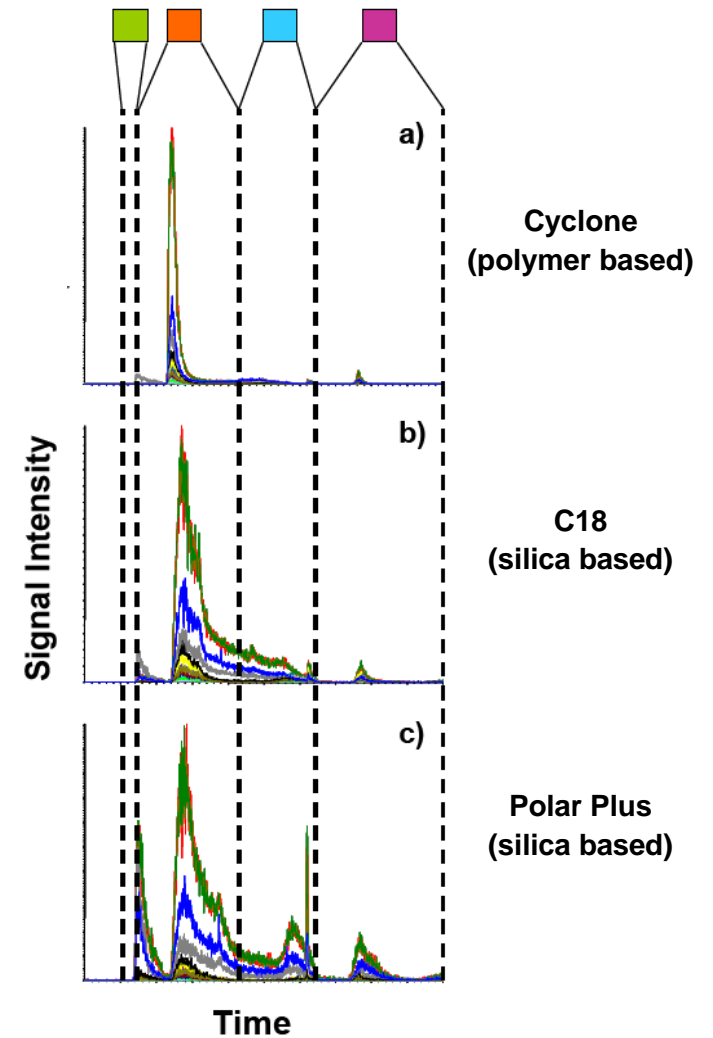
- Loading (0.5 min)
- Transfer (4.0 min)
- Cleaning 1 (3.0 min)
- Cleaning 2 (5.0 min)

Performed using the quick elute mode



## Selected variables that can be optimised through Aria™ software (method development)

- Mobile phase (composition, pH)
- Flow rates (likely that transfer flow rate is a sensitive parameter)
- Column choice – TurboFlow & analytical (via the MCM unit)
- Washing and equilibration steps



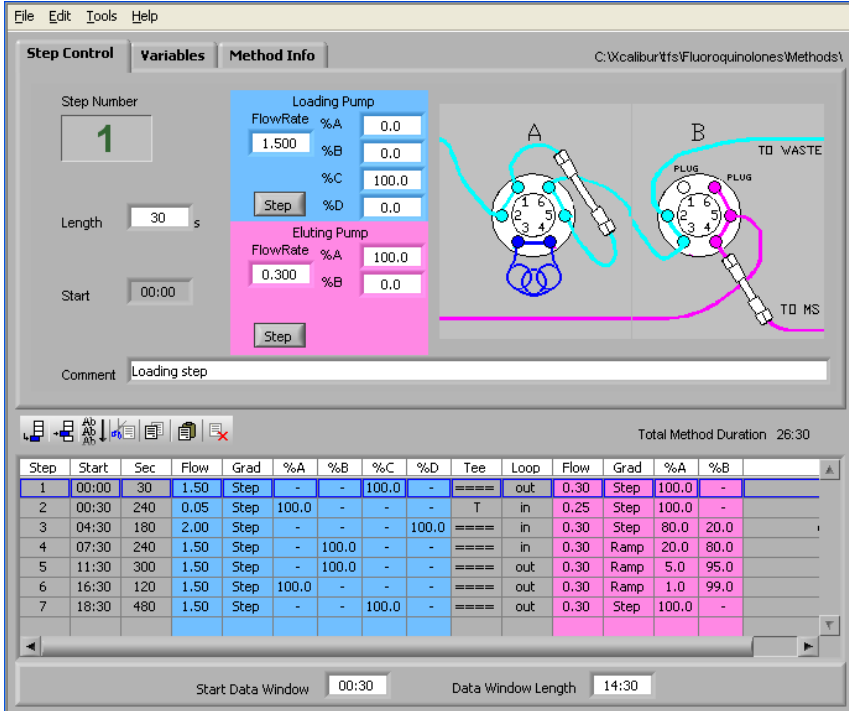
# TFC & LC final conditions

## TurboFlow Conditions (Sample injection volume = 90 µL)

System:	Thermo Scientific Aria TLX-1 controlled by Aria™ software
Online Extraction:	TurboFlow Cyclone (50 x 0.5 mm)
Mobile Phase A:	0.1 % formic acid in water
Mobile Phase B:	0.1 % formic acid in acetonitrile
Mobile Phase C:	10 mM % ammonium formate in water
Mobile Phase D:	acetonitrile / isopropanol / acetone (4:3:3 v/v/v)

## HPLC Conditions

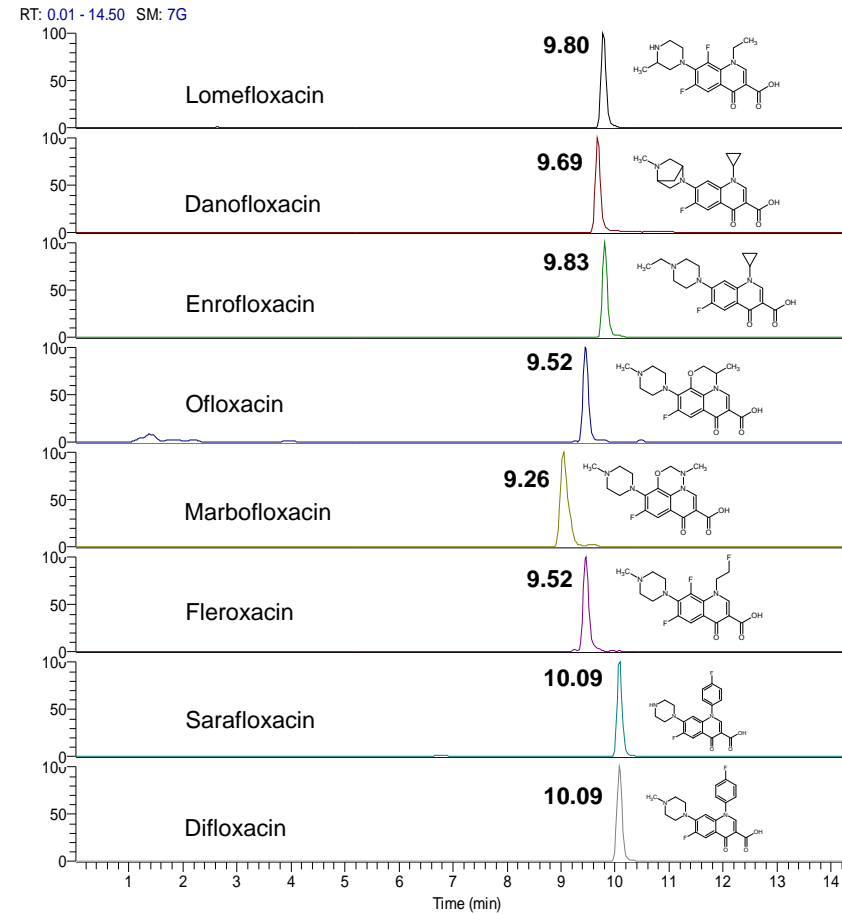
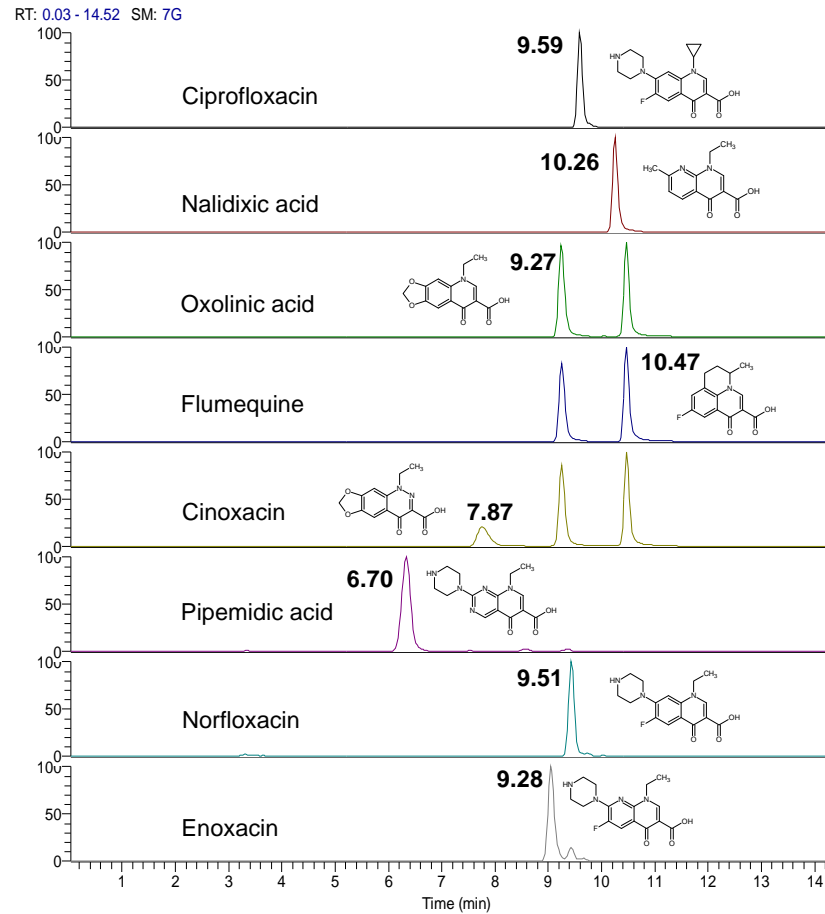
Analytical Column:	Thermo Scientific Hypersil GOLD 2.1 x 50 mm (3 µm, 40 °C)
Solvent A:	0.5 % formic acid in water
Solvent B:	0.5 % formic acid in methanol / acetonitrile (1:1 v/v)



The screenshot shows the 'Step Control' window of the HPLC software. It displays parameters for a 'Loading Pump' and an 'Eluting Pump'. The 'Loading Pump' is set to 1.500 flow rate with 100.0% mobile phase C. The 'Eluting Pump' is set to 0.300 flow rate with 100.0% mobile phase A. A schematic diagram of the HPLC system is shown, including two rotary valves (A and B) and connections to waste and MS. Below the schematic is a table of method steps.

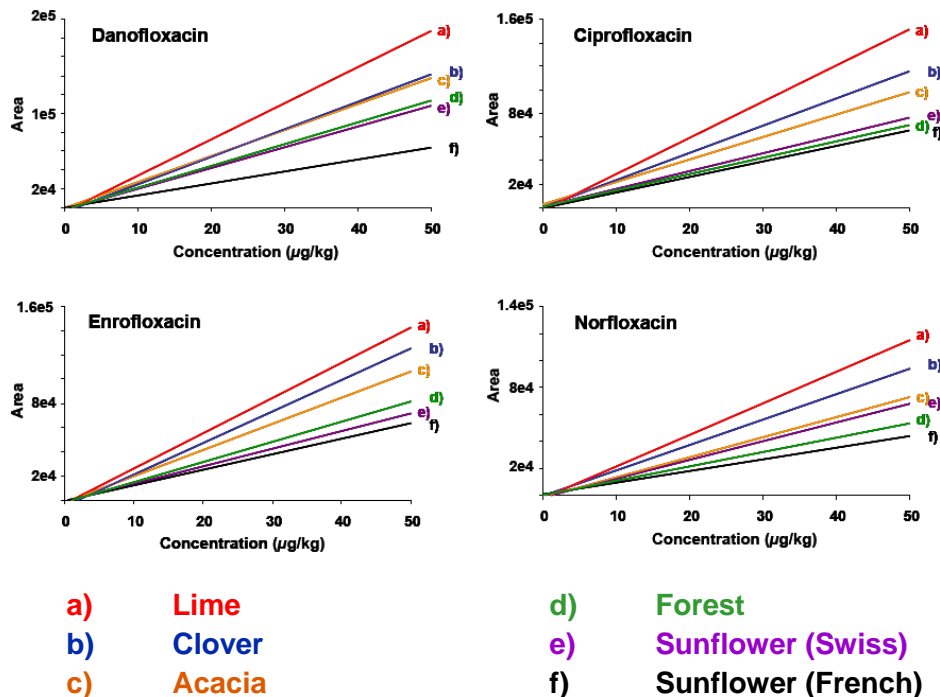
Step	Start	Sec	Flow	Grad	%A	%B	%C	%D	Tee	Loop	Flow	Grad	%A	%B
1	00:00	30	1.50	Step	-	-	100.0	-	=====	out	0.30	Step	100.0	-
2	00:30	240	0.05	Step	100.0	-	-	-	T	in	0.25	Step	100.0	-
3	04:30	180	2.00	Step	-	-	-	100.0	=====	in	0.30	Step	80.0	20.0
4	07:30	240	1.50	Step	-	100.0	-	-	=====	in	0.30	Ramp	20.0	80.0
5	11:30	300	1.50	Step	-	100.0	-	-	=====	out	0.30	Ramp	5.0	95.0
6	16:30	120	1.50	Step	100.0	-	-	-	=====	out	0.30	Ramp	1.0	99.0
7	18:30	480	1.50	Step	-	-	100.0	-	=====	out	0.30	Step	100.0	-

# Results: SRM chromatograms



# Results: Method performance / quantification

- Manual sample preparation – heating step assessed: samples spiked at 10 µg/kg and heated at 85 °C in an oven for 0, 15, 30, and 60 mins – no significant decrease in peak areas was observed
- Time related variations of the observed retention time during validation, RSD of the mean RT < 0.5 % (n = 243, over a 1 month period).
- Accuracy of quantitation – without labelled standards, evaluate “matrix matched calibration curves”



- No isotopically labelled standards used (cost and availability)
- The TFC clean up of 6 honeys from different origins was assessed
- 4 Fluoro-quinolones were spiked between the range 0 – 50 µg/kg

Same result as seen previously in the literature (e.g. Verzeznassi *et al*, Khong *et al*)

Some matrix effects are still present, room for improvement in the method

- Calibration eventually performed using the standard addition procedure, using one-point calibration

Drawbacks to this approach:

- The level of contamination should fall within the calibration range (no prior knowledge of this)
  - Several extractions for 1 result (not with one-point approach)
- 
- Samples spiked at either 10 µg/ kg or 25 µg/ kg at beginning of sample work-up
  - 16 (fluoro)quinolones in 3 types of honey considered:
    - Within day precision <20 % (min 2 %, max 18 %, median 6 %) for all fortification levels
    - Between day precision: min 2 %, max 42 %, median 9 % (all levels) Marbofloxacin responsible for the high variability
- 
- Stability of the 16 analytes assessed: One honey sample spiked with 10 µg/ kg (all analytes) injected once a day for 10 days kept in the autosampler (8 °C), and an additional sample kept at -20 °C
    - No decrease in peak area observed for any analyte
    - In water – observed that analytes were not stable, even at -20 °C (<2 days)

- The method proved linear in the range studied
- For the data table below – 3 replicates were used for each point of the calibration levels
- The calibration replicates along with the RSD values clearly demonstrate the precision of the method

Analyte	Range (µg/kg)	LOD (µg/kg)	RSD (%)	R2 (1/x)
Pipemidic acid	1 - 100	0.9	1.5 – 10.1	0.9964
Cinoxacin	2 - 100	2.0	4.3 – 7.7	0.9918
Marboflaxin	1 - 100	2.6	2.4 – 13.9	0.9939
Enoxacin	1 - 100	1.9	2.1 – 11.7	0.9928
Fleroxacin	1 - 50	1.5	6.0 – 16.8	0.9903
Ofloxacin	2 - 100	1.3	2.1 – 11.5	0.9945
Norfloxacin	1 - 100	2.3	2.7 – 11.5	0.9925
Oxolinic acid	1 - 50	1.4	0.3 – 10.6	0.9909
Ciprofloxacin	1 - 100	1.4	2.4 – 11.6	0.9967
Danofloxacin	1 - 100	1.1	2.3 – 13.6	0.9961
Lomefloxacin	1 - 100	0.5	0.2 – 13.7	0.9954
Enrofloxacin	1 - 100	0.8	1.5 – 16.9	0.9907
Difloxacin	1 - 100	0.8	1.9 – 10.4	0.9947
Sarafloxacin	1 - 100	1.1	1.1 – 11.2	0.9966
Nalidixic acid	1 - 50	0.8	1.3 – 4.7	0.9943
Flumequine	1 - 100	0.9	1.7 – 8.9	0.9902

- A rapid, sensitive, and reliable automated on-line preparation method for the quantitation of 12 fluoro-quinolones and 4 quinolones was developed using a TurboFlow method (TLX1 system) coupled to a TSQ Quantum Ultra™ mass spectrometer.
- The TFC approach greatly reduces sample preparation time, reduces organic solvent consumption, and produces accurate results
- The instrumentation / approach described here could be used for the quantification of a large number of different compounds in food samples

# Acknowledgements

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Switzerland***

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4817 BL Breda  
The Netherlands***

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M&B group &  
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