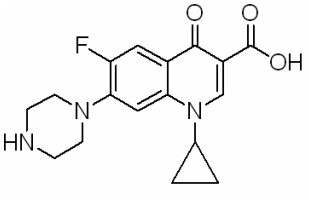
Quinolones and Macrolides in Honey – Simultaneous determination of 22 substances with Symbiosis Pico System coupled with API 3200

Introduction

A fast and very easy to use method for the determination of 22 veterinary drugs in honey is developed. For the laborious steps to separate quinolones and macrolides (see figure 1) from the sugars a Symbiosis Pico system is used. To reach the nearly "zero tolerance level" of this drugs in honey [1, 2] the lowest determinable content (limit of quantification, LOQ) was determined by the sensitive detection method using tandem mass spectrometry (MS/MS).

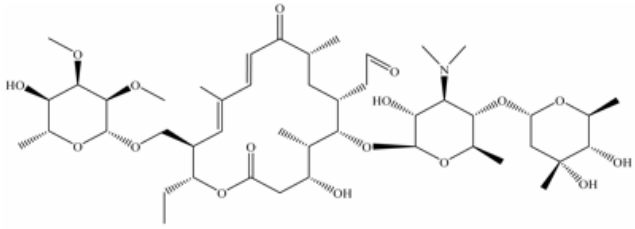
Ciprofloxacin



With the method analysable quinolones:

Cinoxacin	Lomefloxacin
Cirprofloxacin	Marbofloxacin
Danofloxacin	Nalidixinic acid
Difloxacin	Norfloxacin
Enoxacin	Ofloxacin
Enrofloxacin	Oxolinic acid
Flumequin	Sarafloxacin

Tylosin



With the method analysable macrolides:

Clindamycin	Spiramycin
Erythromycin	Tiamulin
Josamycin	Tilmicosin
Roxithromycin	Tylosin

Figure 1: Chemical structure of Ciprofloxacin and Tylosin and the list of analytes that can be determined with the Pico System and LC-MS/MS

Symbiosis Pico System

The online-SPE is directly automatically executed prior to LC-MS/MS analysis. The samples are placed in the autosampler and the XLC-program (XLC = eXtraction Liquid Chromatography) is selected. It consists of 2 parts: the extraction that finally elutes the

analytes from the cartridge onto the analytical column of the following LC-MS/MS (liquid chromatography coupled with MS/MS detection).

In this case a mass spectrometer (ABI 3200, Applied Biosystems, USA) is hyphenated with the online-SPE unit Symbiosis Pico (Spark Holland, The Netherlands). The online-SPE-LC-system consists of an autosampler, high pressure LC gradient pumps, a high pressure dispenser (HPD), valves and the XLC-unit for the solid phase extraction. Figure 2 demonstrates the principle of online-SPE.

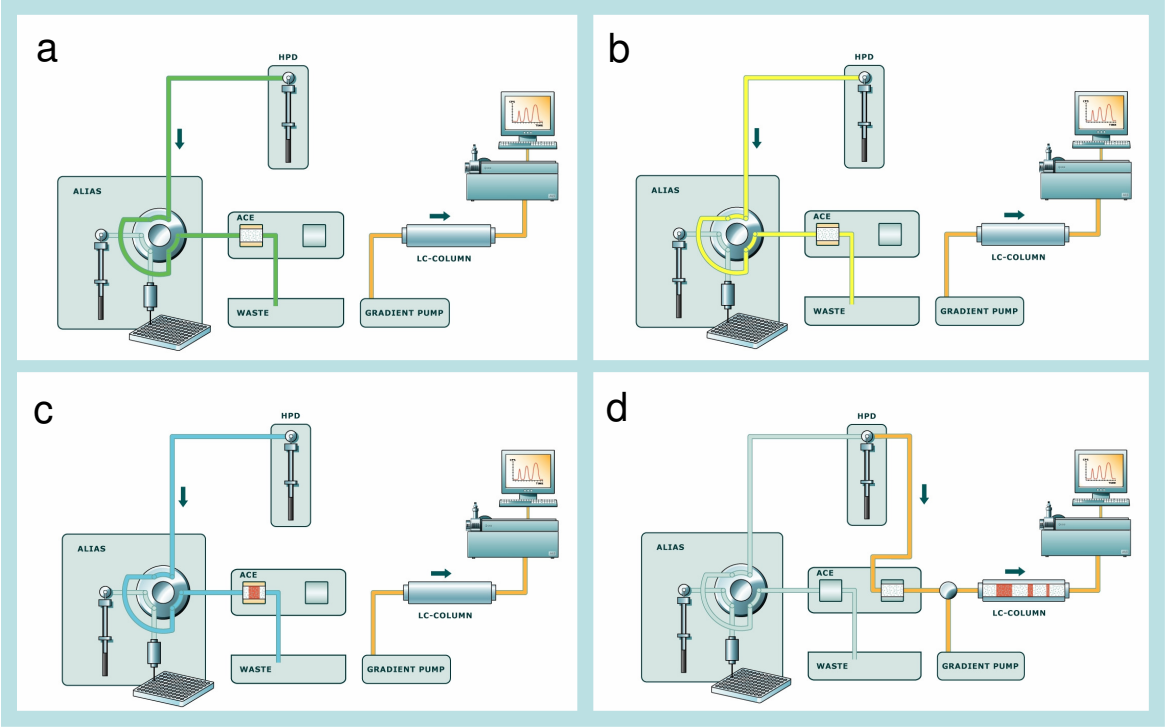


Figure 2 Principles of the online-SPE at the Pico system

1. Gripper inserts a cartridge into the left clamp of the XLC-unit (figure1: a = equilibrate, b = extraction, c = washing step to remove the matrix compounds)
2. Gripper transfers SPE-cartridge to right clamp of XLC-unit (figure 2: d = focussing: elution using the high pressure dispenser (HPD) directly on the analytical column). Focussing is a concentration step of the analytes at the bottom of the SPE-cartridge in a small spot. Here the focusing is used to achieve an enrichment and a better chromatographic behavior of the very different analytes (see figure 1) on the analytical column.

Method development XLC

For the development of the extraction method the automated method development (AMD) software package of the Pico system has been applied. In the first step a series of widely used sorbent materials are tested for suitability (sorbent screening). The following second step optimizes the solvents and solvent mixtures for conditioning, washing and elution. These optimization routines usually run overnight and after interpretation by the analyst result in an almost final purification method. Only a few changes to the method have been applied by adding further wash and cleaning steps. The final optimization of the chromatographic parameters such as analytical column, eluents and eluent additives has been performed by the analyst.

Sample pre-treatment

The honey samples (2g) are weight and 2 mL in succiante puffer buffer (pH 4) and internal standard solution are added. After shaking for 15 min 1 mL of this sample solution is filled for measurement.

XLC-MS protocol

SPE conditions

Cartridge:	HySphere-resin GP (10 x 2 mm, 10 µm, 13.74 mg, Spark Holland)	
Conditioning:	1000 µL acetonitrile (ACN) / water, 15/85	5 mL/min
Sample loading:	500 µL	2 mL/min
Wash step (three times):	1.000 µL water / ACN, 98.5/1.5	5 mL/min
Clamp flush:	500 µL ACN / water, 25/75 with 0.1% formic acid (FA)	5 mL/min
Elution:	1.000 µL ACN / water, 25/75 with 0.1% formic acid (FA)	0.1 mL/min

LC conditions

Analytical column:	Aqua 5µ C18 125A (150 x 3 mm, Phenomenex, USA)
Column temperature:	40°C
Eluent A:	ACN (0.1% FA)
Eluent B:	water (0.2% FA)

Time	Low (µL/min)	Eluent A (%)	action
0	500	95	focussing
1.5	500	95	focussing
2.0	700	95	Analytical run
9.0	700	10	Analytical run
9.5	700	10	Column flush and equilibration
10	700	95	Column flush and equilibration
15	700	95	Column flush and equilibration

MS settings

Measurement device: Sciex API 3200, used in positive mode

Q1 Parameters:

CUR: 25
IS: 5500
TEM: 450
GS1: 30
GS2: 70
EP: 10
Resolution Q1: Unit

Q3 Parameters

quinolone/macrolide	M H+	DP	EP	D1	CE	CXP	D2	CE	CXP
Ciprofloxacin	332.1	41	7.5	288.2	27	9	245.2	33	7
Danofloxacin	358.4	32	7	314.2	26	11	283.2	32	8
Difloxacin	399.9	38	6	356.1	38	9	299.1	40	6
Enoxacin	321	38	7	234	28	9.5	205.8	30	7
Enrofloxacin	360	41	7	316	36	7	244.8	44	5
Lomefloxacin	352.2	34	7.5	264.9	25	9	308.2	21	9
Marbofloxacin	363.1	25	5	320.3	20	7	277.2	25	5
Norfloxacin	320.1	41	8	276.1	33	7	233	37	7
Ofloxacin	362.4	38	5	261.3	28	5	318.3	40	6
Sarafloxacin	385.9	41	6	341.9	36	9	299	25	8
Spiramycin (half mass)*1	422.4	22.3	4	174.2	25.4	4	142.1	16.6	4
Cinoxacin	262.0	31	5	217.2	31	6	189.2	38	6
Flumequine	261.9	28	7	202.3	54	5	174.4	32	5
Nalidixinsäure	233	29	8	187.1	43	4	159.2	25	8
Oxolinsäure	262.3	41	6	216.3	52	4	160	25	4
Roxithromycin	837.7	73	4	679.4	30	22	158.2	42	27
Tilmicosin	869.4	108	4	174.3	58	16	696.5	23	7
Tylosin	916.6	65	5	772.5	41	29	174.2	45	29
Erythromycin	734	55	5	576.2	28	20	158.2	39	16
Erythromycin C2	736.6	55	5	578.2	28	20	160.2	39	4
Tiamulin	494.3	55	7	192.2	25	16	119.2	56	7.8
Clindamycin	425.3	45	6	126.1	30	6	377.2	24	8

*1 M+2H+; D1=daughter 1, D2=daughter 2

Results

One cycle consisting of SPE-purification and LC-MS/MS analysis has a runtime of 20 min in total. Immediately after the end of the extraction of sample #1 (in the right clamp) and the cleaning of the XLC-unit the conditioning of the next cartridge and injection of sample #2 starts (in the left clamp). Applying this overlapping procedure only 6 hours are

required for the total analysis of 20 samples. This results into a gain of 5 hours versus the classical (offline) SPE purification.

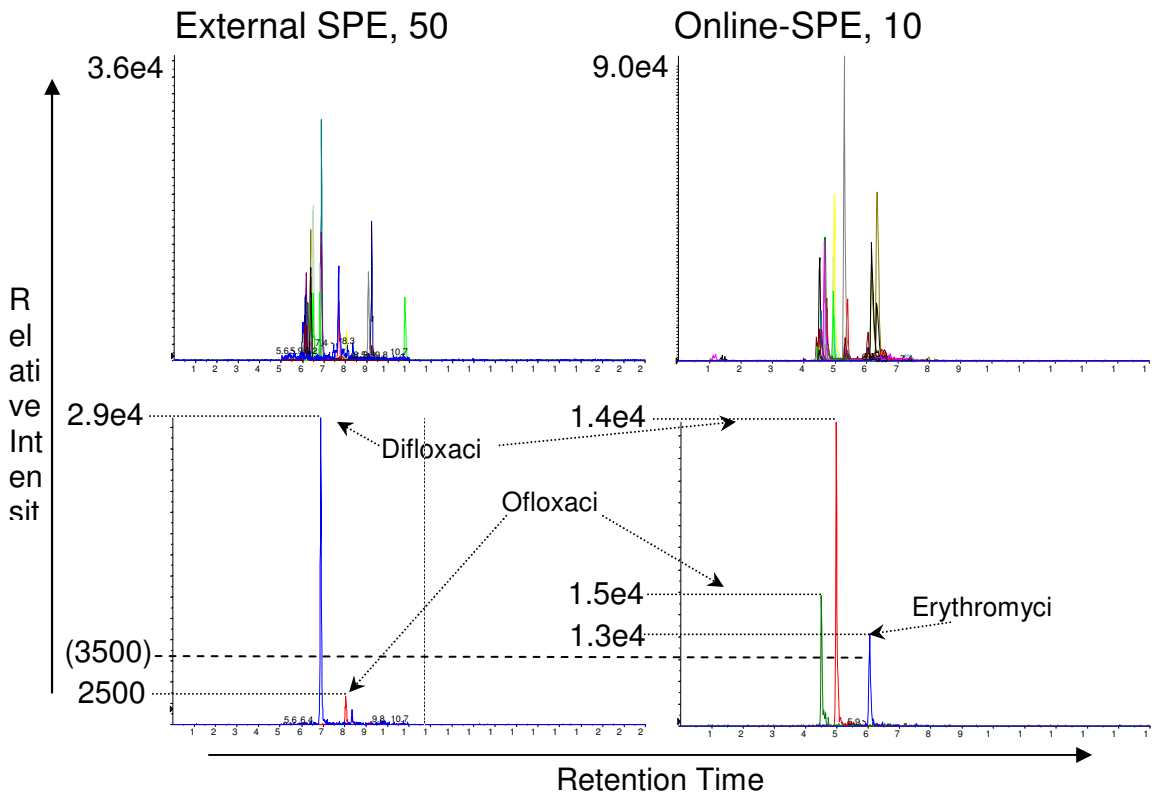


Figure 3: Chromatograms (and several mass traces) of spiked honey samples; Left side: quinolones at 50 µg/kg, analyzed by offline-SPE and API 4000 MS; Right side: online-SPE with Symbiosis Pico and API 3200 MS, quinolones and macrolides each at 10 µg/kg.

In figure 3 it becomes obvious that the samples analyzed with the Pico system show larger peak heights. Despite the use of the less sensitive API 3200 MS the combination with online-SPE-purification gives a higher sensitivity and a reduction of the detection limit lower than 5 µg/kg for the majority of the analytes.

Performance parameters:

substance	correlation	LOD [µg/kg]	reproducibility, n=6
Ciprofloxacin	0.99	3.3	16.5
Danofloxacin	0.97	4.4	18.9
Difloxacin	0.97	4.8	13.2
Enoxacin	0.98	3.3	27.9
Enrofloxacin	0.98	3.9	15.5
Lomefloxacin	0.99	3	15.8

Marbofloxacin	0.98	3.7	26.4
Norfloxacin	0.97	4.8	27
Ofloxacin	0.94	3.4	25
Sarafloxacin	0.95	6.2	6.9
d5 Norfloxacin (IS)			6.4*
Spiramycin	0.98	2.6	22
Spiramycin (half mass)	0.99	2.3	14.1
Cinoxacin	0.94	6.6	6.9
Flumequine	0.93	7.3	
Nalidixinsäure	0.69	17.6	
Oxolinsäure	0.94	6.6	5.7
Roxithromycin	0.95	5.9	8.1
Tilmicosin	0.98	3.5	7.9
Tilmicosin (half mass)	0.99	3.9	9.5
Tylosin	0.99	2	5
Erythromycin	0.99	3	10.3
Erythromycin C2 (IS)			9.5
Tiamulin	0.95	5.3	2.4
Clindamycin	0.97	5	7.8

*n=5

Conclusion

The analysis method described in this application note using online-SPE followed by LC-MS/MS offers a simple and time saving possibility for the combined analysis of quinolones and macrolides in honey. In addition to the simplified sample preparation a higher sensitivity can be achieved even when using the lower performing but also less expensive API 3200 mass spectrometer. The increase in sensitivity results from removing the matrix compounds and from the enrichment of the analytes.

[1] EC-directive 2001/82/EC
[2] EC-regulations 2377/90

Reference

Schittko, S., Online-SPE-LC-MS/MS, Bestimmung von 22 Chinolonen und Makroliden in Honig. GIT Special – Separation 1/2008, p. 8-11

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