蜂蜜中的喹诺酮和大环内酯类抗生素的检测

背景：

随着生活水平的提高，食品质量和安全问题越来越受到人们的关注。蜂蜜是一种广受人民欢迎的天然保健食品，在蜜蜂饲养中，为了降低蜜蜂死亡率，提高蜂蜜产量，多种药物从而被使用，其中喹诺酮和大环内酯类药物就是经常被使用的两种。在欧盟，已经建立了对蜂蜜中兽药残留“零容忍”的检测标准。要做到“零容忍”检测标准，必须使检测限尽可能的降低。目前使用串联质谱的方式已经可以使检测线降低到 ppb 范围以下。而为了保持蜂蜜中兽残分析的灵敏性，必须除去其中的糖类，以避免污染。

一般蜂蜜的兽残分析都需要将蜂蜜通过 SPE 进行前处理，传统手工的 SPE 方法包括：SPE 填料的筛选，将蜂蜜加入 SPE 柱中并选择合适的淋洗剂将基质去除并洗脱分析产物，有些情况下，还需要旋蒸和重新定容。

实验过程：

传统蜂蜜中兽残的分析方法主要是将 2g 蜂蜜加入水中，通过 SPE 净化，稀释样品后进入 LC-MS/MS 方法进行分析，检测限在 10μg/kg，而通过全自动在线 SPE 方法串联 LC-MS/MS 法进行检测，不仅可以省去与 SPE 相关的所有人工步骤，节约时间，还能将检测限降至 10μg/kg 以下，同时喹诺酮和大环内酯类药物可以在同一批次检出。通过全自动在线 SPE 方法，只要将蜂蜜加入 SPE 柱即可，后续即能自动完成（图 1）。

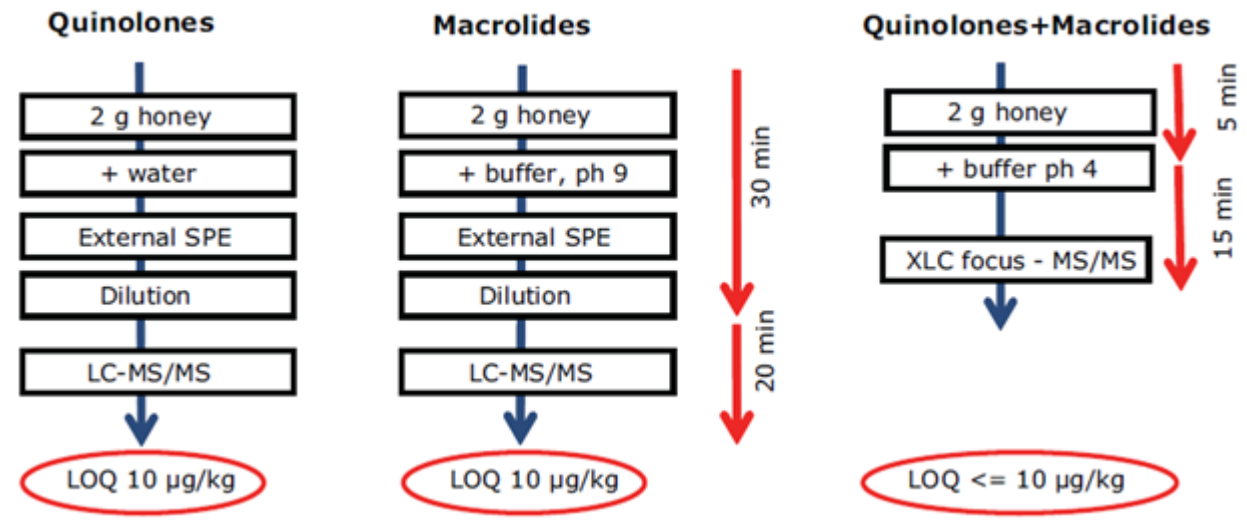

图 1 手动 SPE（左）和自动 SPE（右）方法流程比较

首先我们要进行在线萃取方法的开发，因此我们需要用到 Pico 系统的自动方法开发软件（AMD）。起初我们会筛选 SPE 应用到的吸附剂，以及进行 pH 值调节（图 2，3），随后我们会筛选分离时使用的溶剂，以及溶剂的比例（图 4），从而确定最佳淋洗条件。通常条件筛选需要一天时间，智能化软件能够记录并给出最佳分析条件。

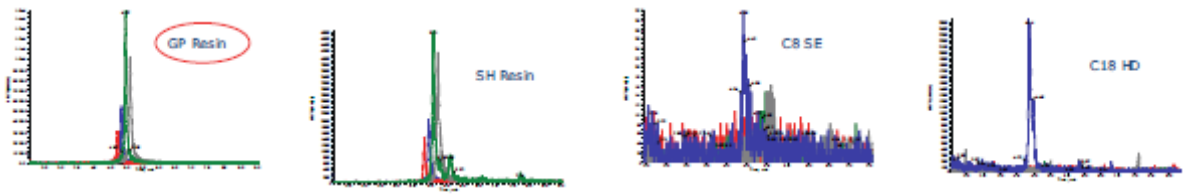

图 2 吸附剂的选择

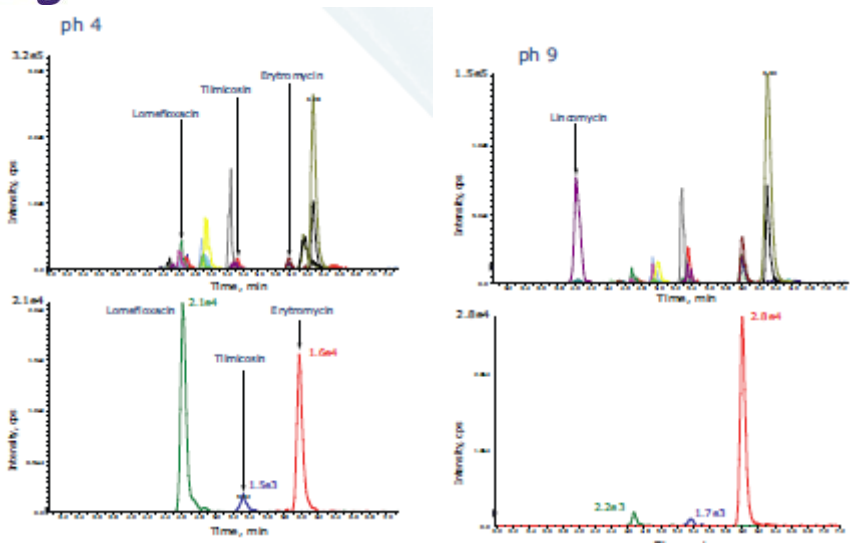

图 3 pH 值的调节

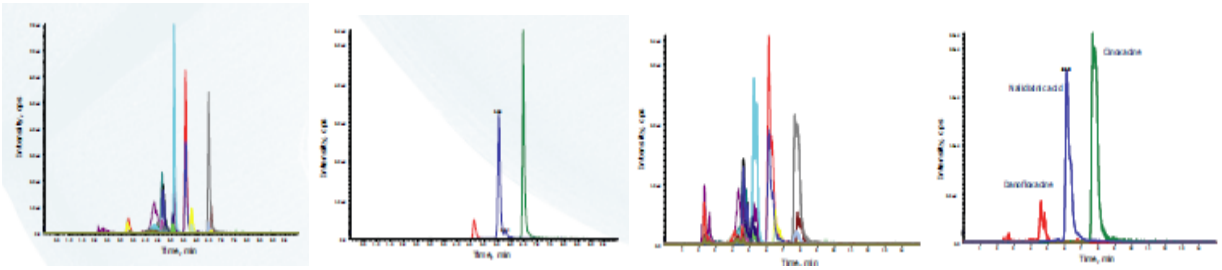

图 4 淋洗液的选择，峰聚焦

当条件确定好之后，就可以进入 XLC（萃取，淋洗，过柱分离）方法进行批量分析了(图 5)，产物结果通过 MS/MS 方法鉴定。

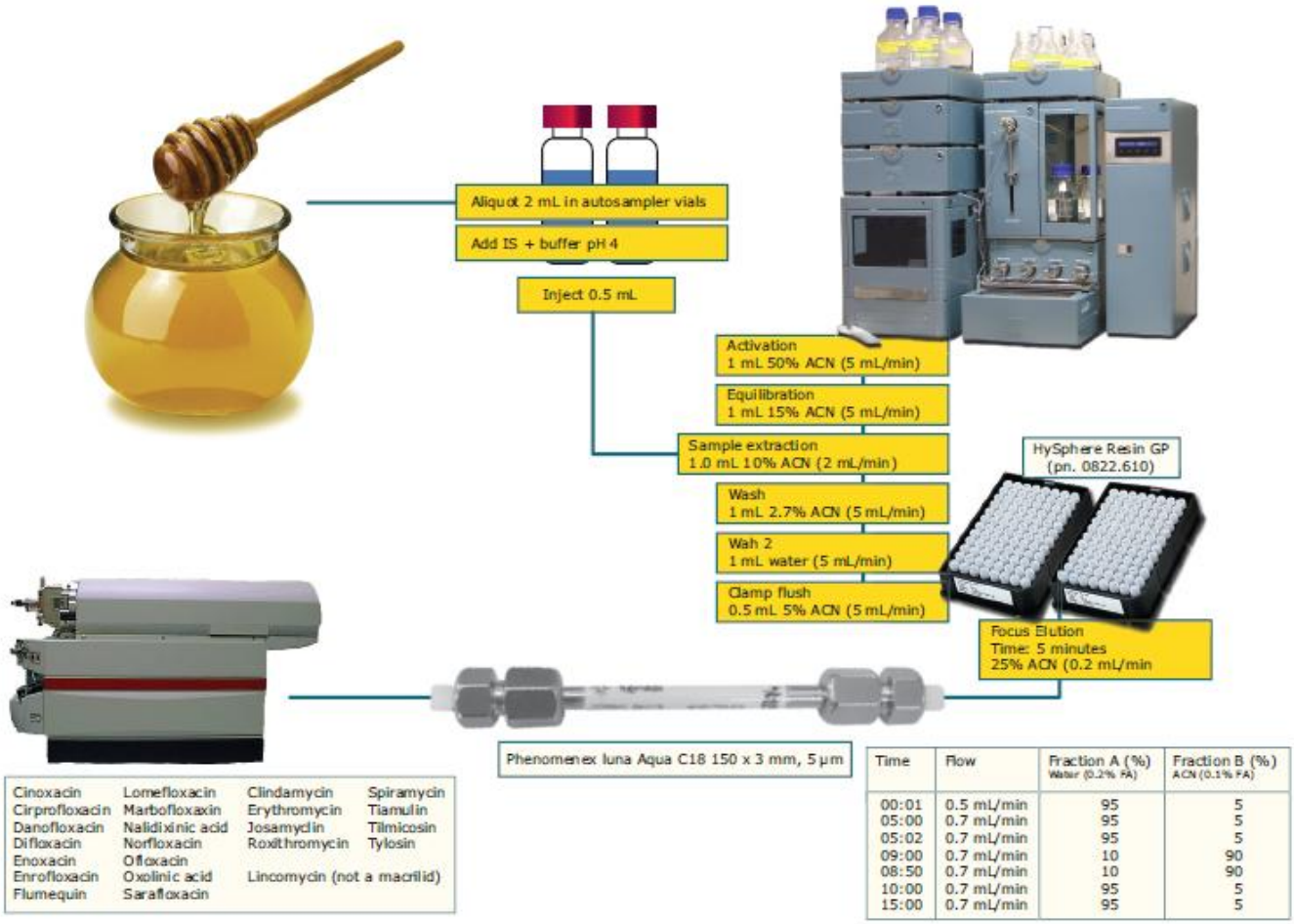
Aliquot 2 mL in autosampler vials
Add IS + buffer pH 4
Inject 0.5 mL
Activation
1 mL 50% ACN (5 mL/min)
Equilibration
1 mL 15% ACN (5 mL/min)
Sample extraction
1.0 mL 10% ACN (2 mL/min)
Wash
1 mL 2.7% ACN (5 mL/min)
Wah 2
1 mL water (5 mL/min)
Clamp flush
0.5 mL 5% ACN (5 mL/min)
HySphere Resin GP
(pn. 0822.610)
Focus Elution
Time: 5 minutes
25% ACN (0.2 mL/min
Phenomenex luna Aqua C18 150 x 3 mm, 5 µm
Cinoxacin
Cirprofloxacin
Danofloxacin
Difloxacin
Enoxacin
Enrofloxacin
Flumequin
Lomefloxacin
Marbofloxacin
Nalidixinic acid
Norfloxacin
Ofloxacin
Oxolinic acid
Sarafloxacin
Clindamycin
Erythromycin
Josamycin
Roxithromycin
Lincomycin (not a macrilid)
Spiramycin
Tiamulin
Tilmicosin
Tylosin
Time
Flow
Fraction A (%)
Water (0.2% FA)
Fraction B (%)
ACN (0.1% FA)
00:01 0.5 mL/min 95 5
05:00 0.7 mL/min 95 5
05:02 0.7 mL/min 95 5
09:00 0.7 mL/min 10 90
08:50 0.7 mL/min 10 90
10:00 0.7 mL/min 95 5
15:00 0.7 mL/min 95 5

图 5 大批量分析流程图

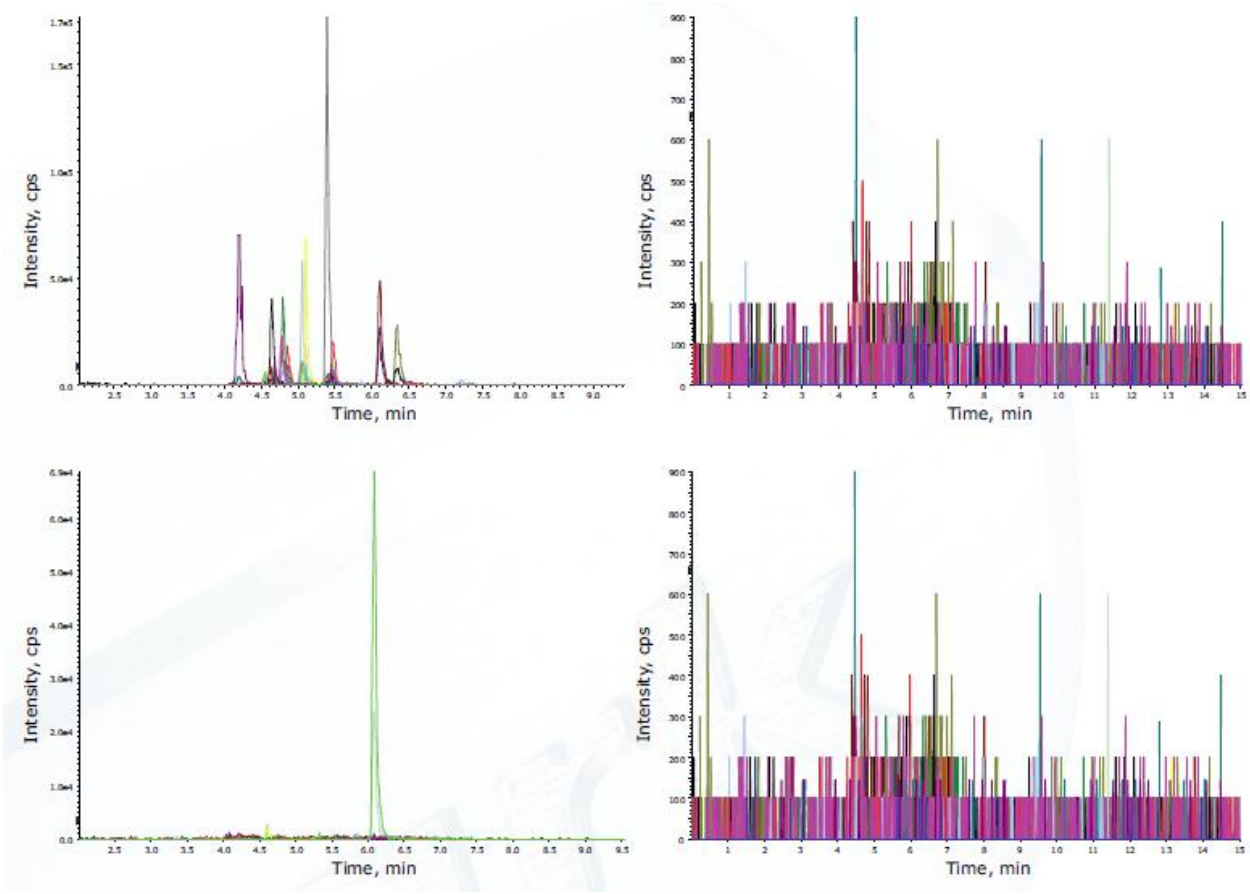

图 6 分析结果

结论

使用在线 SPE-LC-MS/MS 方法进行蜂蜜中的兽残分析，不仅所需溶剂使用量少，SPE 处理时间缩短至 5 分钟，同时可以实现蜂蜜中喹诺酮和大环内酯药物的同时检出，简便的前处理方法更能使检测限低至 10μ g/kg 以下。同时，该方法在其他基质的残留分析检测中也很有很大的推广潜力。

Online-SPE – LC-MS/MS

Determination of 22 Quinolones and Macrolides in Honey

Honey similar to many other food stuffs has to be tested for residues of veterinary drugs. These may come from the entire spectrum of pharmaceutical compounds and their presence is completely forbidden in honey (zero tolerance limit [1, 2]). From an analytical point of view only the lowest determinable content (limit of quantification, LOQ) with a defined accuracy can be defined. To ensure the best possible compliance to the zero tolerance level the quantification limit has to be as low as possible. Therefore the use of very sensitive detection methods such as tandem mass spectrometry (MS/MS) allows the determination of these compounds in the sub ppb-range. For the analysis of honey it is essential to remove sugar containing ingredients to maintain the sensitivity of the analytical system and to avoid its contamination.

Sonja Schittko, project leader and Ute Schwalb, project assistant;
LC-MS Analytics at Eurofins / WEJ.

As a suitable method for this purpose solid phase extraction (SPE) is used to separate the analytes from interfering ingredients (in this case sugars). Usually SPE consists of the conditioning of the SPE-cartridge, the injection of the liquid sample onto the cartridge, the removal of the matrix in a washing step and finally the elution of the analytes from the cartridge. In some cases an additional evaporation step, reconstitution and dilution may be required.

Today automated systems are available for this type of sample preparation procedure, requiring only the insertion of the liquid samples and the solvents as well as the programming of the individual steps. Despite of this automation it is still a quite time consuming process and most often it is executed sequentially, one sample after the other. After this preparation the samples are placed manually into the autosampler and subsequently the analysis starts. This procedure is currently used in routine.

Principle of Online-SPE

The method described here uses online-SPE directly automatically executed prior to analysis. The samples are placed in the autosampler and the XLC-program (XLC = eXtraction Liquid Chromatography) is selected. It consists of two parts: the extraction that finally elutes the analytes from the cartridge onto the analytical column of the directly following LC-MS/MS (liquid chromatography coupled with MS/MS detection).

For the analyses described here a mass spectrometer (API 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 1 demonstrates the principle of online-SPE. In the first step a gripper inserts a cartridge into the left clamp of the XLC-unit. That's where the conditioning of the cartridge, the sample introduction and the wash step to remove the matrix compounds are executed. Afterwards the gripper transfers the SPE-cartridge to the right clamp of the XLC-unit. There the elution directly onto the analytical column and the chromatographic runs take place. This may be done by two different means: either by elution using the LC-gradient or by elution using the high pressure dispenser (HPD) with the so called "focusing". Focusing is a concentration step of the analytes at the top of the analytical column in a small spot. The method described here uses the focusing to achieve an enrichment and a better chromatographic behavior of the very different analytes (22 quinolones and macrolides, see fig. 2) on the analytical column.

One cycle consisting of SPE-purification and LC-MS/MS analysis has a runtime of 20 minutes in total. Immediately after the end of the extrac-

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

Method

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. The automated method development led to the following XLC-method for the analysis of 22 quinolones and macrolides:

XLC: Cartridge: HySphere-resin GP (10 x 2 mm, 10 µm, 13,74 mg, Spark Holland); Conditioning: 1.000 µL acetonitrile (ACN)/water, 15/85; sample injection (2 g honey in 2 mL buffer at pH 4) 500 µL; wash steps: 1.000 µL water/ACN, 97/3, followed by 1.000 µL water; Elution: 1.000 µL ACN/water, 25/75 with 0,1 % formic acid (FA).

LC: Analytical column Aqua 5µ C18 125A (150 x 3 mm, Phenomenex, USA); Eluent A: ACN (0,1 % FA); Eluent B: water (0,2 % FA); Start: 95 % A, 0,5 mL/min, after 2 min 0,7 mL/min, 9 min 10 % A, 10 to 15 min 10 % A, 10 to 15 min 95 % A.

Analysis of Honey Samples

Currently honey samples are purified still using the classical SPE method for routine analysis and the determination of the two veterinary drug compound classes (quinolones and macrolides) is done individually. Applying the online-SPE-method allows the simultaneous determination of both compound groups (except for Lincomycin, requiring a basic buffer).

Figure 3 shows the chromatograms (and several mass traces) of honey samples. Both, the analysis using an API 4000 MS after offline SPE (left side of fig. 3) and the analysis using the API 3200 MS coupled to the Symbiosis Pico online-SPE system, are displayed.

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 quantification limit down to 5 µg/kg for the majority of the analytes.

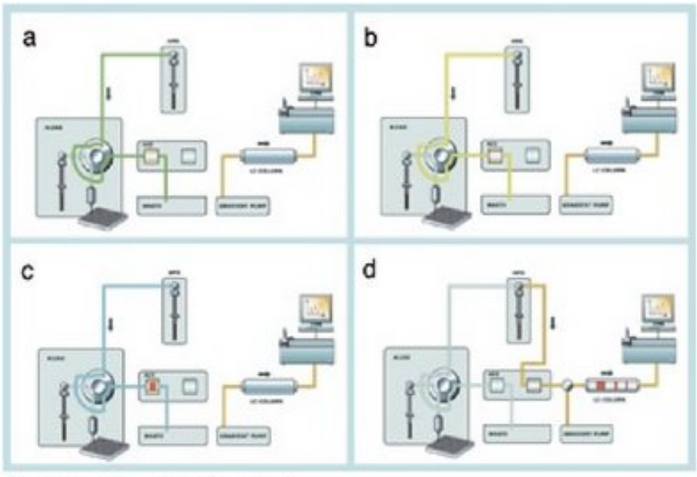

Fig. 1: Function of the Pico System
A: conditioning of the SPE-cartridge
B: Sample application
C: recycling of the SPE-cartridge,
D: Elution and LC-run, sample transfer to the right side

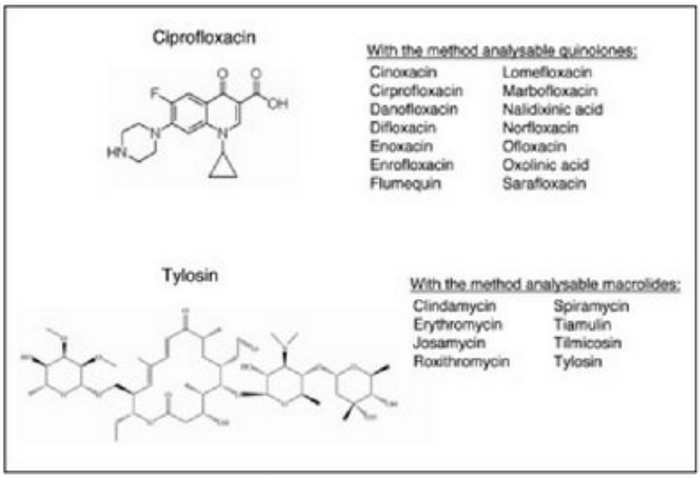

Fig. 2: Chemical structure of Ciprofloxacine and Tylosine and the list of analytes that can be determined with online-SPE and LC-MS/MS

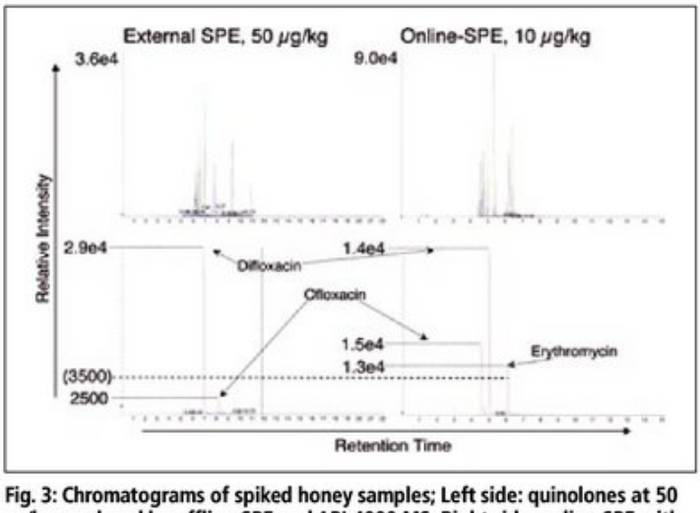

Fig. 3: Chromatograms of spiked honey samples; Left side: quinolones at 50 µg/kg, analyzed by offline-SPE and API 4000 MS; Right side: online-SPE with Symbiosis Pico and API 3200 MS, quinolones and macrolides each at 10 µg/kg

Conclusion

The analysis method described in this article 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.

Having gathered this experience by application of the method shown here it will be possible to substitute classical purification procedures by more comfortable automated online-SPE-methods. Furthermore the transfer of this analysis method to other matrices seems to be very promising.

References

[1] EC-directive 2001/82/EC
[2] EC-regulations 2377/90
[3] GIT Special – Separation 1, 8 (2008)

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Determination of Quinolones and Macrolides in Honey by Online-SPE LC-MS/MS Analysis

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2 - Eurofins Analytik GmbH, Hamburg, Germany

Introduction

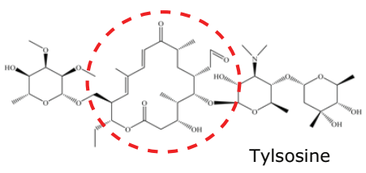
Honey similar to many other food products has to be tested for residues of veterinary drugs. These may come from the entire spectrum of pharmaceutical compounds and their presence is completely forbidden in honey; zero tolerance [1, 2]. From an analytical point of view only the lowest determinable content (quantification limit) with a defined accuracy can be defined. To ensure the best possible compliance to the zero tolerance level the quantification limit has to be as low as possible. Therefore the use of very sensitive detection methods such as tandem mass spectrometry (MS/MS) allows the determination of these compounds in the sub ppb-range. For the analysis of honey it is essential to remove sugar containing ingredients to maintain the sensitivity of the analytical system and to avoid its contamination.

Macrolides

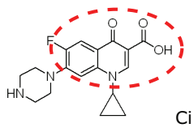
Clindamycin
Erythromycin
Josamycin
Roxithromycin
Spiramycin
Tiamulin
Tilmicosin
Tylosin

Quinolones

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



Tylsosine

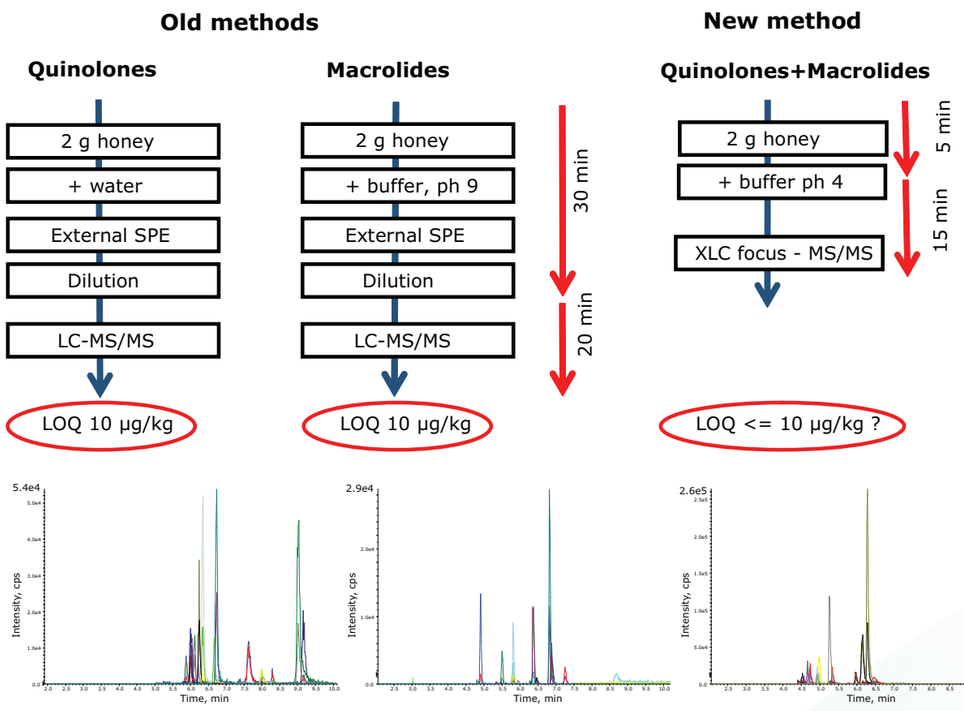


Ciprofloxacin

Figure 1: Chemical structure of both herbicides

Analysis of Honey Samples

As a suitable method for this purpose solid phase extraction (SPE) is used to separate the analytes from interfering ingredients (in this case sugars). Usually SPE consists of the conditioning of the SPE-cartridge, the injection of the liquid sample onto the cartridge, the removal of the matrix in a washing step and finally the elution of the analytes from the cartridge. In some cases an additional evaporation step, reconstitution and dilution may be required.
Today automated systems are available for this type of sample preparation procedure, requiring only the insertion of the liquid samples and the solvents as well as the programming of the individual steps. Despite of this automation it is still a quite time consuming process and most often it is executed sequentially, one sample after the other. After this preparation the samples are introduced manually into the autosampler and subsequently the analysis starts.
Currently honey samples are purified still using this classical SPE method for routine analysis and the determination of the two veterinary drug compound classes (quinolones and macrolides) is done individually. Applying the online-SPE-method allows the simultaneous determination of both compound groups.



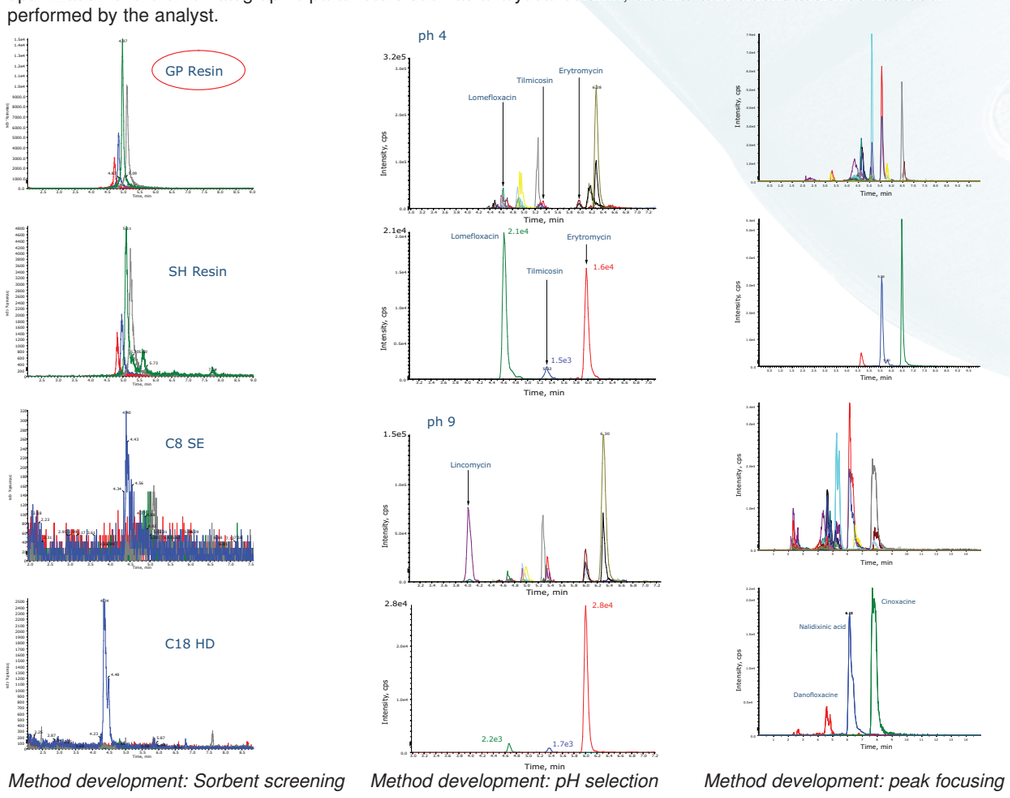
Quinolones (50 µg/kg)
Old method (API4000)

Macrolides (10 µg/kg)
Old method (API4000)

Quinolones + Macrolides (10 µg/kg)
New method (API3200)

Method Development

For the development of the online 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.



Method development: Sorbent screening
Method development: pH selection
Method development: peak focusing

New Online Extraction Method

The method development led to the following XLC-method for the analysis of 21 Quinolones and Macrolides:

Pre-treatment:

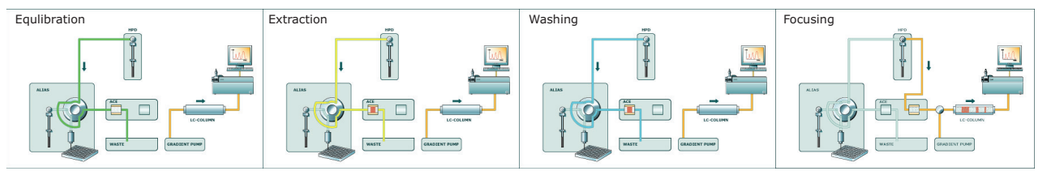
Aliquot 2 grams of honey into a glass vial, add Eritromycine C2 as internal standard and adjust pH to 4 using a buffer

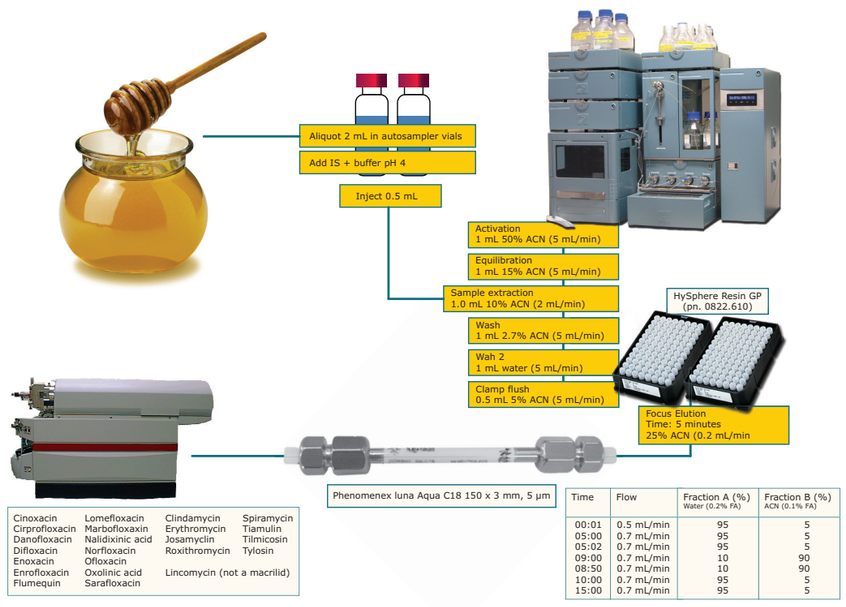
XLC

Cartridge:	HySphere-resin GP (10 x 2 mm, 10 µm, Spark Holland);
Conditioning-I:	1.000 µL acetonitrile/water, 50/50;
Conditioning-II:	1.000 µL acetonitrile/water, 15/85;
Sample transfer	1000 µL acetonitrile/water 10/90;
Wash steps:	1.000 µL acetonitrile/water, 3/97, followed by 1.000 µL water; Focus Elution: 1.000 µL acetonitrile/water, 25/75.

LC

Analytical column	Luna Aqua 5µ C18 125A (150 x 3 mm, Phenomenex, USA);
Eluent A:	0.2% Formic acid in water;
Eluent B:	0.1% Formic acid in Acetonitrile;
Gradient	at Start-5 min. 95% A at 0,5 mL/min; after 5-9 min 10% A at 0,7 mL/min; after 10-15 min 95% A at 0.7 mL/min





Time	Flow	Fraction A (%) Water (0.2% FA)	Fraction B (%) ACN (0.1% FA)
00:01	0.5 mL/min	95	5
05:00	0.7 mL/min	95	5
05:02	0.7 mL/min	95	5
09:00	0.7 mL/min	10	90
08:50	0.7 mL/min	10	90
10:00	0.7 mL/min	95	5
15:00	0.7 mL/min	95	5

Figure 3: Schematic presentation of the method

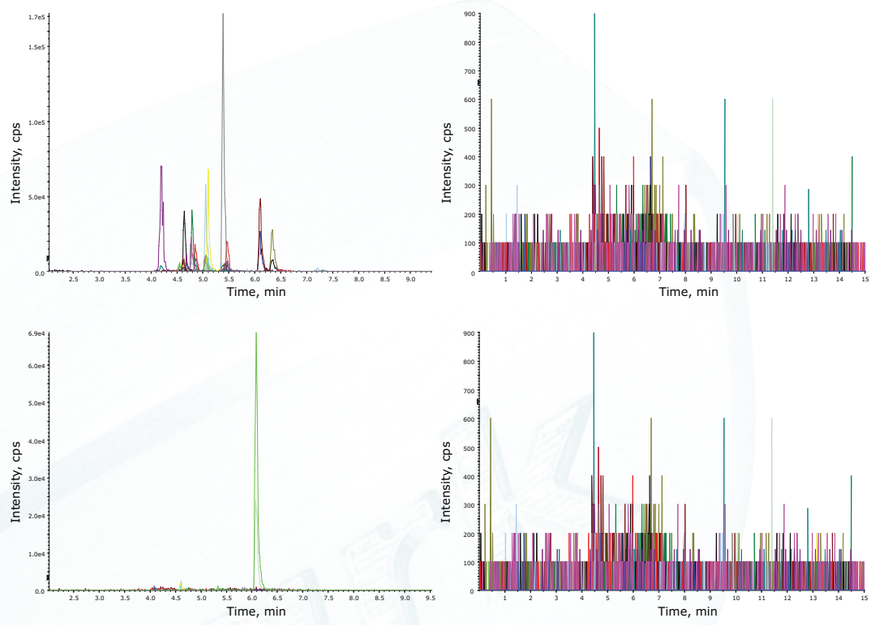


Figure 4: Results

Using the newly developed method several honey samples were analyzed; Figure 4 shows a typical chromatogram of a honey sample and a spiked honey sample and the blank sample immediately after confirms little to none carry over.

Conclusion

The analysis method described in this article using online-SPE followed by LC-MS/MS offers a simple and time saving possibility for the combined analysis of chinolones 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.

Having gathered this experience by application of the method shown here it will be possible to substitute classical purification procedures by more comfortable automated online-SPE-methods. Furthermore the transfer of this analysis method to other matrices seems to be very promising.

Positives

Fast first method developed by software
Method fine-tuning could be done by one person (less sample preparation)
Less time consumptive work in the lab
Time over all for 20 samples 6 h (compared to external SPE 11 h)
Sensitivity of API 4000 with xlc and API 3200

Challenges

For the method fine-tuning an experienced analyst is necessary
First / last analytes in chromatogram react “sensitive” by changes
Adding new substances could be difficult (SPE and LC are coupled)

Literature

[1] EC-directive 2001/82/EC
[2] EC-regulations 2377/90
[3] GIT Special – Separations 1/2008
[4] Spark Holland Method Development Guide 2007

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