THE DEVELOPMENT OF A NEW MULTIPLEX DIPSTICK FOR THE SIMULTANEOUS DETECTION OF SULFONAMIDES, FLUORO-QUINOLONES, TYLOSIN AND CHLORAMPHENICOL IN HONEY

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I. ABSTRACT

At present the use of antibiotics in apiculture is not permitted in Europe. However, between 2004-2009 approximately 60% of all alerts for drug residues in food of animal origin reported on the European Commission's Rapid Alert System on Food and Feed alerts (RASFFs) related to antibiotics with a high proportion being in honey. The majority of monitoring for veterinary drug residues is conducted using sophisticated laboratory instrumental equipment e.g., LC-MS/MS. Whilst this type of analysis provides quantitative and confirmatory results the associated turnaround times/costs may be unacceptable for routine screening. This situation underlines the need to develop rapid and inexpensive multiplex screening tests. An indirect competitive multiplex dipstick was developed within the EC funded Conffidence project with the aim of detecting, in one single analysis, some of the most frequently confirmed antibiotics in honey including fluoroquinolones (QUINO), sulfonamides (SULFA), tylosin (TYL-A) and chloramphenicol (CAP).

II. DESCRIPTION OF THE TEST

The dipstick was formulated as follows; freeze-dried antibodies were labelled with gold particles and the competitor conjugates were immobilized on a nitrocellulose membrane. A generic extraction was developed (see **table n°1** on the right) combining an acidic hydrolysis to release the sugar-conjugated residues with an ethyl acetate extraction/concentration step prior to reconstitution in assay buffer. The dipstick is inserted into the liquid sample to initiate the immunochromatographic separation. The dipstick assay requires 5 minutes incubation (see **picture n°1**) and 15 minutes of dipstick migration (see **picture n°2**) at 40°C using Heatsensor and can be analyzed by visual observation (see **picture n°3**) or instrumental reading using a Readsensor.

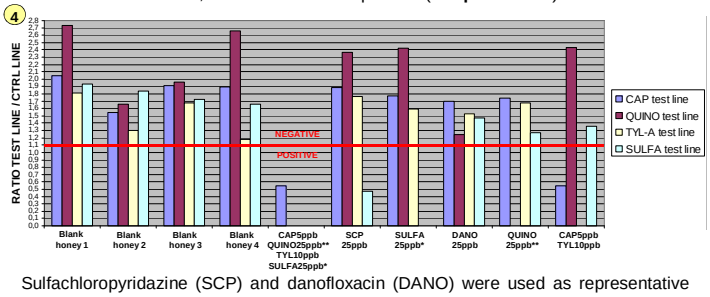
Table n°1 : Sample preparation and dipstick analysis.

	A) SAMPLE "hydrolized" (SULFA/QUINO release)	B) SAMPLE "protected" (TYL-A/CAP protection)
HONEY SAMPLE	2,5 gr	2,5 gr
DILUTION	1,2 ml "ACID buffer" → **5 min** at 95°C 1,2 ml "BASE buffer"	2,4 ml H2O → DISSOLUTION
EXTRACTION	10 ml ETHYLACETATE Shake **10 min** Centrifuge **5 min**	
SUPERNATANT	Transfer 8 ml supernatant	
EVAPORATION	55°C (N2) for **40 min**	
DISSOLUTION	Extract "**A**" dilution in **250µl** BUFFER	Extract "**B**" dilution in **250µl** BUFFER
DIPSTICK ANALYSIS	MIX extracts "**A**" and "**B**" Add **200 µl** of the MIX in the microwell → **5 min** at 40°C Add the **DIPSTICK** in the microwell → **15 min** at 40°C	
TOTAL TIME	**90 min** (up to 8 samples analyzed together)	
LAB MATERIAL	Waterbath, N2 evaporator, Heatsensor®, Readsensor®	

* Mix of 10 SULFA spiked in honey at a TOTAL concentration of 25 µg/kg (ppb).
** Mix of 8 QUINO spiked in honey at a TOTAL concentration of 25 µg/kg (ppb).
*** Mix of SULFA / QUINO / TYL-A / CAP spiked in honey at 25 µg/kg / 25 µg/kg / 10 µg/kg / 5 µg/kg (ppb).

III. RESULTS

In case of contaminated honey sample, the contaminant will prevent the colour to appear on 1 of the 4 test lines corresponding to its antibiotic family. The result of the dipstick can be interpreted by comparing the test line intensity to the threshold intensity of the control (CTL) line. After Readsensor measure, all sample giving a "TEST line/CTRL line" ratio ≤1,1 are considered as positive (see **picture n°4**).



Sulfachloropyridazine (SCP) and danofloxacin (DANO) were used as representative compounds of sulfamides and quinolones families because known among the weakest detected compounds in our assay. In this multiplex prototype, the "TYL-A" test line could be to strengthened in order to decrease probability of "false positive" results and the "QUINO" test line could be lowered for a gain of sensitivity.

**Mix of 10 SULFA spiked in honey at a TOTAL concentration of 25 µg/kg (ppb) : SDA, SPR, STA, SMZ, SMP, SDM, SMER, SMM, SCP, SQX. **Mix of 8 QUINO spiked in honey at a TOTAL concentration of 25 µg/kg (ppb) : CIPRO, DANO, DIFLO, ENRO, FLUM, MARBO, NOR, SARA.*

IV. EXPECTED SENSITIVITY

SULFAMIDES	LoD (µg kg^{-1})	Target* (µg kg^{-1})
Sulfapyridine	<10	25
Sulfamethazine	<25	
Sulfamethoxypyridazine	25	
Sulfamerazine	25	
Sulfamonomethoxine	25	
Sulfadiazine	25	
Sulfadimethoxine	25	
Sulfathiazole	25	
Sulfachloropyridazine	25	
Sulfaquinoxaline	50	

(F)QUINOLONES	LoD (µg kg^{-1})	Target* (µg kg^{-1})
Enrofloxacin	<<25	25
Ciprofloxacin	<25	
Danofloxacin	25-50	
Difloxacin	250	
Marbofloxacin	50	
Norfloxacin	25	
Sarafloxacin	>500	
Flumequine	>500	

OTHERS	LoD (µg kg^{-1})	Target* (µg kg^{-1})
Tylosin-A	<10	10
Chloramphenicol	5	0.15

**Target detection levels are half the official European limits or recommended concentrations as defined in the Conffidence FP7 project for at least 80% of analytes.*

V. CONCLUSION

We have developed a multiplex dipstick assay able to detect in honey more than 7 sulfamides (≤25 µg/kg), 5 quinolones (≤50 µg/kg), tylosin-A (<10 µg/kg) and chloramphenicol (5 µg/kg) in one single analysis. The prototype is in course of optimisation for a gain of sensitivity. Another alternative format of assay without any sample cleaning or extraction is in course of investigation for "direct field" testing. Together with our already commercialized Tetrasensor® dipstick assay, this new multiplex dipstick assay is covering the more frequently found antibiotics in honey.

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