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

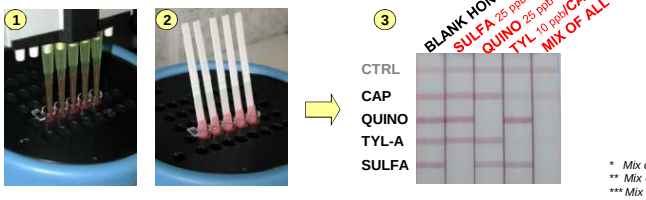
At present the use of antibiotics in apiculture is not permitted in Europe. However, between 2004-2009 approximately 60% of all alerts for drug residues in food of animal origin reported on the European Commission's Rapid Alert System on Food and Feed alerts (RASFFs) related to antibiotics with a high 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-A (TYL) 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 for the "lab-test".

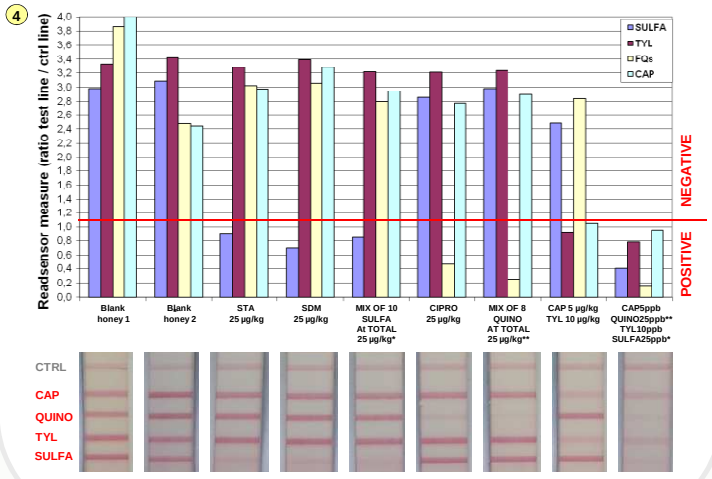
	A) SAMPLE "hydrolized" *(SULFA/QUINO release)*	B) SAMPLE "protected" *(TYL-A/CAP protection)*
HONEY SAMPLE	2,5 gr	2,5 gr
DILUTION	1,2 ml "ACID buffer" → **5 min** at 95°C 1,2 ml "BASE buffer"	2,4 ml H2O → DISSOLUTION
EXTRACTION	10 ml ETHYLACETATE Shake **10 min** Centrifuge **5 min**	
SUPERNATANT	Transfer 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 (CTRL) line. After Readsensor measure, all sample giving a "TEST line/CTRL line" ratio ≤1,1 are considered as positive (see **picture n°4**).



*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 (µg/kg – ppb)

Sulfonamide compounds	LoD LAB	LoD FIELD	CRL**
Sulfapyridine	<10	<50	50
Sulfamethazine	<25	<50	
Sulfamethoxypyridazine	25	50-100	
Sulfamerazine	25	50-100	
Sulfamonomethoxine	25	50-100	
Sulfadiazine	25	50-100	
Sulfadimethoxine	25	50-100	
Sulfathiazole	25	50-100	
Sulfachloropyridazine	25	50-100	
Sulfaquinoxaline	50	<200	

(Fluoro)quinolone compounds	LoD LAB	LoD FIELD	CRL**
Enrofloxacin	<25	5-25	50
Ciprofloxacin	<25	50	
Danofloxacin	25-50	<100	
Difloxacin	250	<500	
Marbofloxacin	50	<100	
Norfloxacin	25	50	
Sarafloxacin	>500	-	
Flumequine	>500	-	

Other compounds	LoD LAB	LoD FIELD	CRL**
Tylosin-A	10	10-50	10
Chloramphenicol	5	<60	0.3

** Single lab validation in progress (FERA) and Inter Lab Validation of the multiplex dipstick assay planned in January 2012.*
*** European limits or recommended concentrations in honey (CRL – AFSSA-LMV France – SANCO /2006/3228).*

"Lab" protocol requires an extraction step (described in paragraph II) and an overall time scale of 90 min for the whole test.
"Field" protocol requires a honey dilution and a overall time scale of 30 min for the whole test.

V. CONCLUSION

We have developed a multiplex dipstick assay able to detect in honey more than 10 sulfonamides (≤50 µg/kg), 5 fluoroquinolones (≤50 µg/kg), tylosin-A (10 µg/kg) below their respective European recommended concentrations together with chloramphenicol (5 µg/kg) in one single analysis. An alternative faster protocol without any extraction step and showing a slightly different sensitivity profile, provides the flexibility to use also the test out of lab for "direct field" testing. An official inter lab validation study of the assay will be conducted in January 2012 under CONffIDENCE project. The kit will be produced and commercialized at UNISENSOR S.A. under the name "BEE4SENSOR". Together with our already commercialized TETRASENSOR® dipstick, this new multiplex dipstick assay is covering the more frequently found and relevant antibiotics in honey.

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