The development of a new multiplex dipstick for the simultaneous detection of sulfonamides, (fluoro)quinolones, tylosin and chloramphenicol in honey

Stefan Weigel (RIKILT), Vincent Chabottaux (Unisensor)

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SEVENTH FRAMEWORK PROGRAMME

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CONFFIDENCE

CON*ff*IDENCE project

- **CON**taminants in ***F****ood* and ***F****eed* : **I**nexpensive **DE**tectio**N** for **C**ontrol of **E**xposure…

- **Collaborative Project :** FP7 - European Commission
- **Duration:** 4 years **(**May 2008 – April 2012)
- **Partners:** 16 partners from 10 countries (universities, SME, research institutes,…)
- **Budget:** 7.5 Mio €
- **Coordinator:** RIKILT - Institute of Food Safety (NL)
- **Objective:** Development of innovative, reliable, simple, fast and multiple screening tests for chemical contaminants and residues in food and feed

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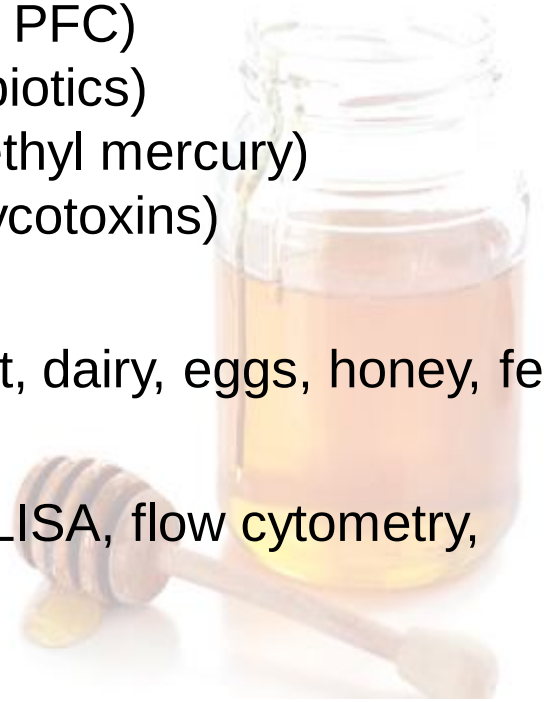
CON*ff*IDENCE project

- **Target analytes:**
  - Organic pollutants (PCB, BFR, PAH, PFC)
  - Veterinary drugs (coccidiostats, antibiotics)
  - Heavy metals (inorganic arsenic, methyl mercury)
  - Biotoxins (alkaloids, phycotoxins, mycotoxins)

- **Commodities**: seafood, cereals, meat, dairy, eggs, honey, feed

- **Techniques**: dipsticks, biosensors, ELISA, flow cytometry, cytosensors, simplified GC/LC-MS



- **Final goals**:
  - Delivering tools to improve food safety
  - Enable more frequent testing
  - Shift testing to the start of the supply chain

CON*ff*IDENCE for honey

- **Antibiotics**
  - ***Electrochemical immunosensor***
    12 sulfonamides < 25 µg/kg

  - ***Multiplex dipstick***
    sulfonamides, tylosin, quinolones, chlorampenicol

- **Pyrrolizidine alkaloids (PA)**
  - original plan: multiplex dipstick for lycopsamine + jacobine
  - major difficulties in dipstick format
  - revision of scope of analytes

➔ currently preparation of new antibodies, shifting to ELISA format

Antibiotics in Honey

- Use of antibiotics by some beekeepers to cure or prevent bacterial infestations of hives (foulbrood)
- 2002/03 alerts relating to chloramphenicol, later nitrofurans
- Continuous usage of tetracyclines, sulfonamides, streptomycin, tylosin, quinolones, lincomycin, erythromycin, ...
- Multiple antibiotics present in (blended) honey

- Concerns about emergence of antibiotic-resistant bacteria strains

- The use of antibiotics in beekeeping is not approved in EU, thus absence is required
- Other countries handle Maximum Residue Limits
- Testing required in both cases!

Multiplex assay concept

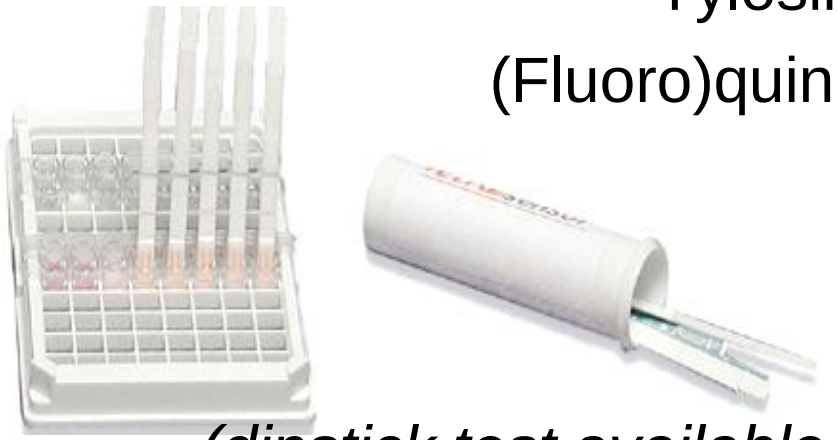
To develop, validate and demonstrate the impact of novel **multiplex dipsticks** for the **rapid, easy** and **cost-effective** detection of the presence of some frequently detected **antibiotics in honey** including…

Sulfonamides

Chloramphenicol

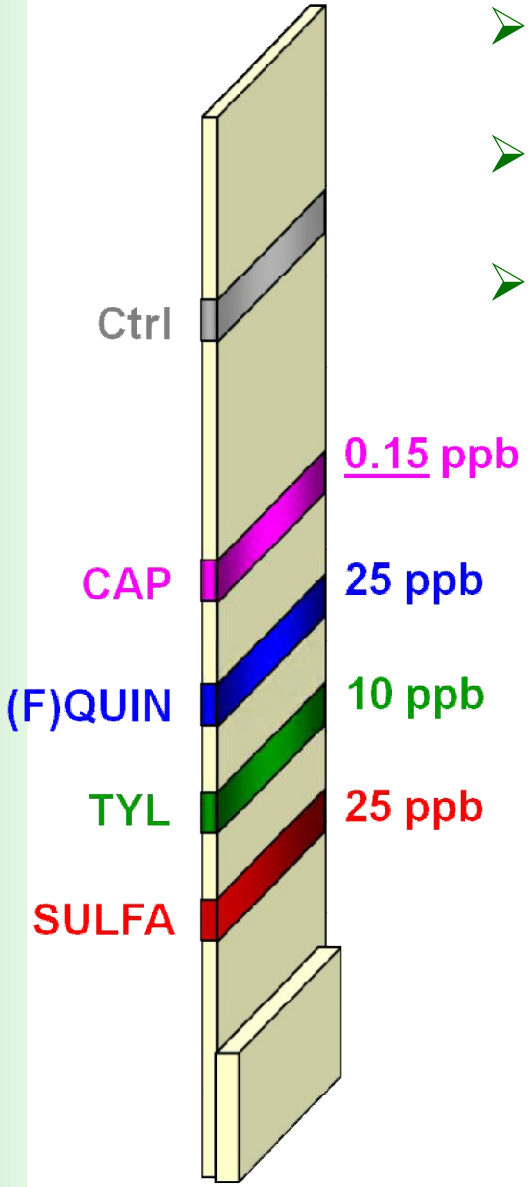
Tylosin

(Fluoro)quinolones



(dipstick test available for tetracyclines)

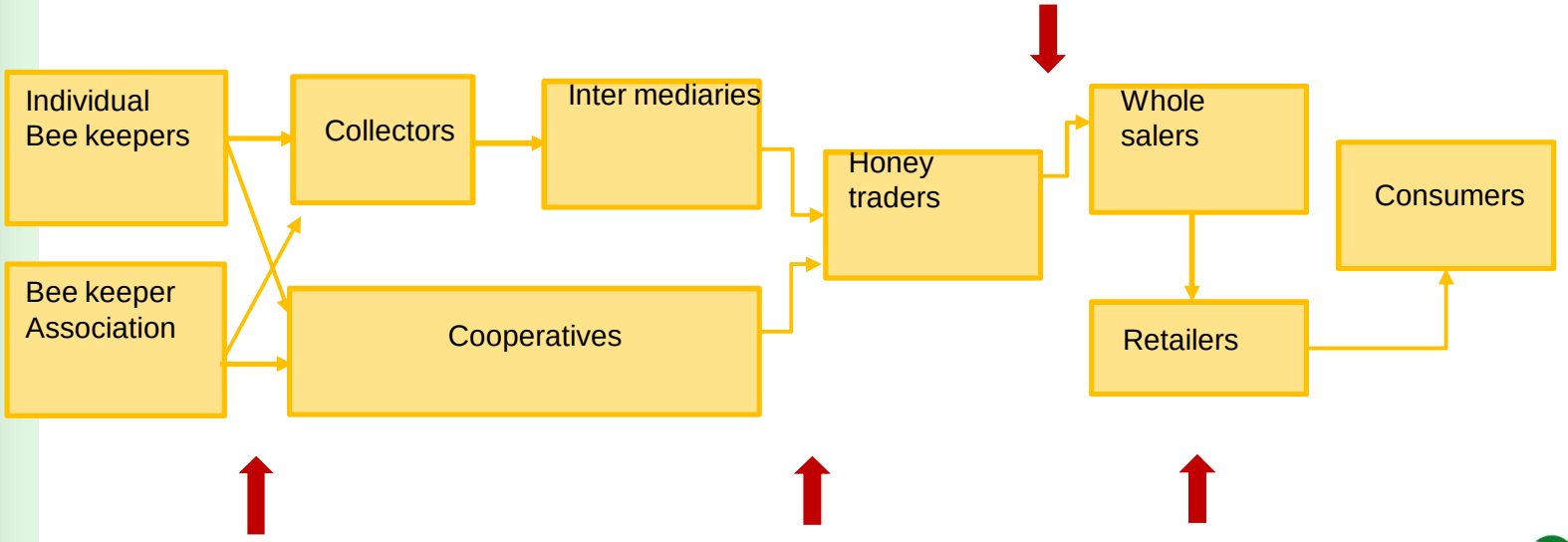
Multiplex assay concept



- Competitive inhibition format (Lateral flow device);
- Incorporating 4 test lines and 1 control line;
- Exploiting matched pairs of antibodies and analyte-protein (OVA) competitors;

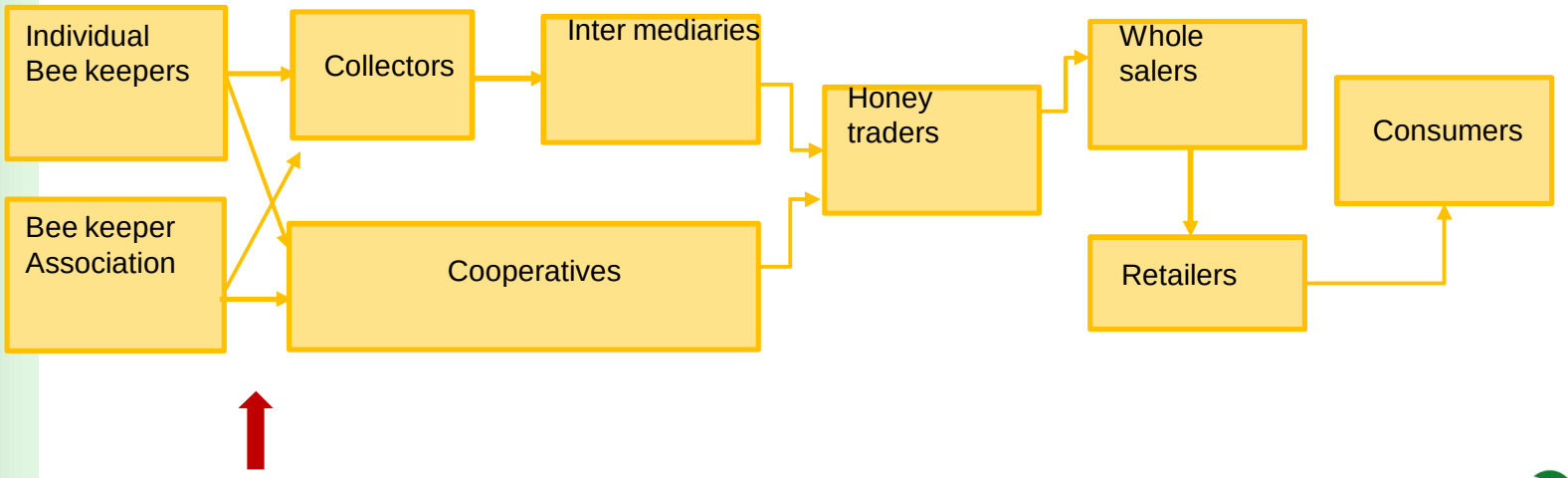
Assay formats

- Lab-based assay
  - simple extraction
  - sensitive, meets recommended reference levels (for sulfonamides, tylosin, quinolones)
  - suited for honey sector QC/QA labs along supply chain as well as external contract labs



Assay formats

- Field assay
  - no lab equipment required, no extraction
  - less sensitive than lab assay, but sufficient to detect contaminated batches from treated hives
  - suited for collectors, cooperatives to test individual lots from beekeepers



Field study

- Evaluation of the applicability and performance of the assay under field conditions
- Testing honey from routine flow (+control samples) with supplied test kit
- Interested cooperatives, collectors, aggregators can register via website or e-mail:
  - www.conffidence.eu
  - dipstick@conffidence.eu

Indirect competitive dipstick principle

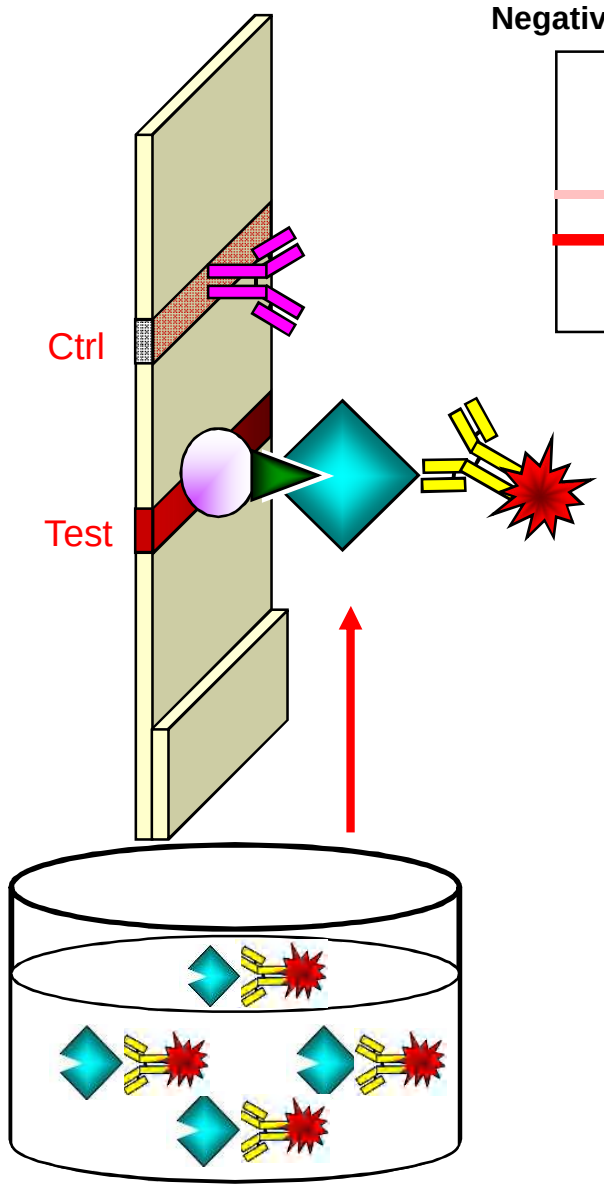
Competitor conjugate (immobilized)

Analyte

Binder anti-Analyte (Antibody, Receptor, …)

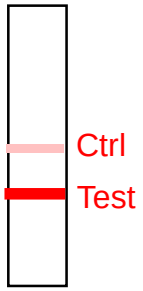
Antibody anti-Binder Labeled (Gold)

Antibody anti-Binder (anti-species)

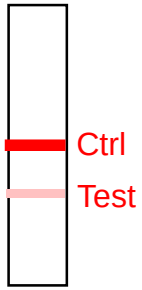


SAMPLE **analyte absent**

Negative test



Positive test



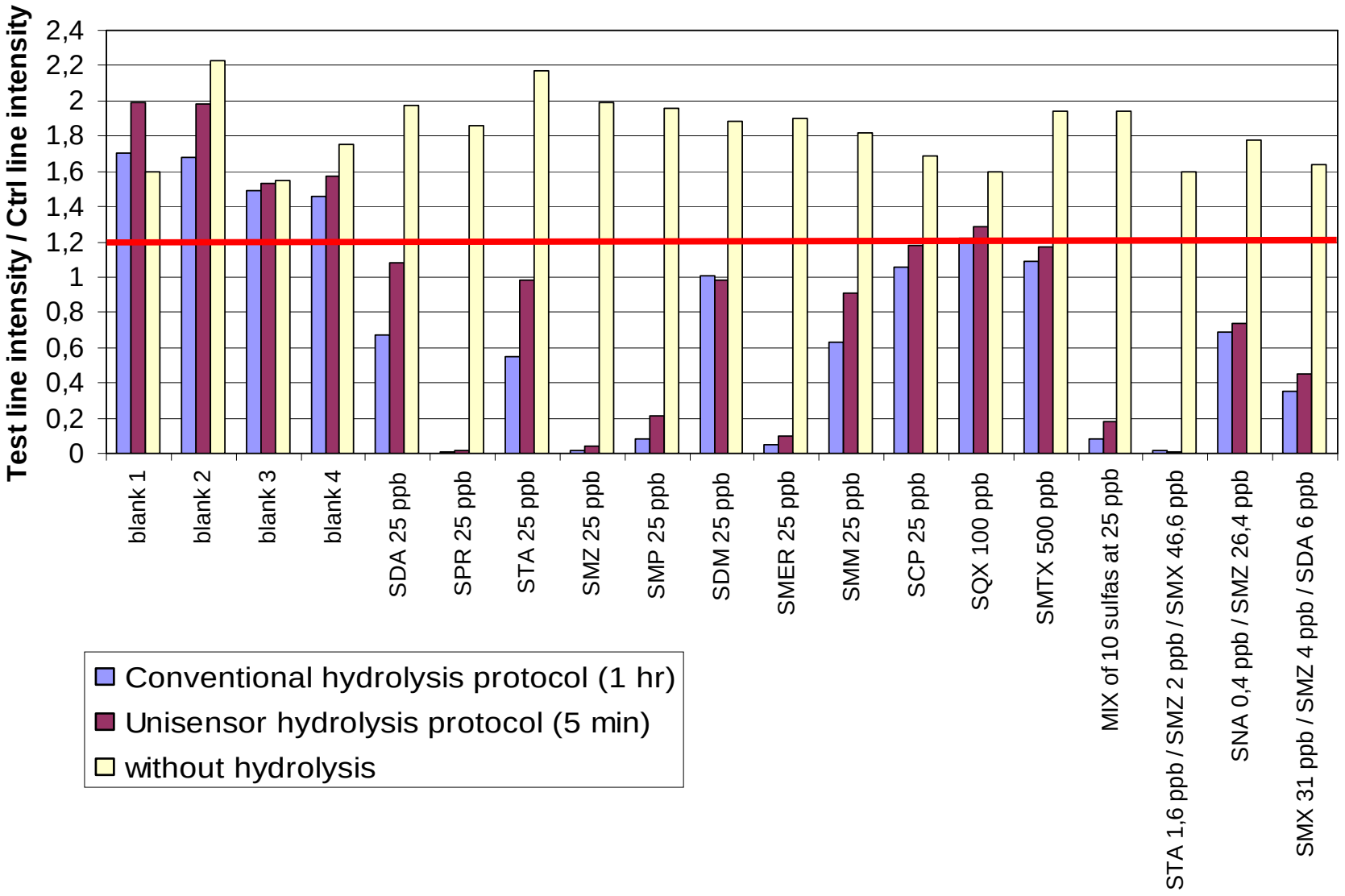
Challenge for a multiple test in honey

- Binding of **SULFAMIDES** to reductive sugars of honey and **QUINOLONES** better soluble in acidic conditions…

  → *NEED of an **acidic hydrolysis** of the sample for drug release/solubilization…*

Challenge of a multiple test for honey

- Development of an easy/rapid hydrolysis for sulfa release…



Challenge for a multiple test in honey

- Binding of **SULFAMIDES** to reductive sugars of honey and **QUINOLONES** better soluble in acidic conditions…

  → *NEED of an **acidic hydrolysis** of the sample for drug release/solubilization…*

BUT…

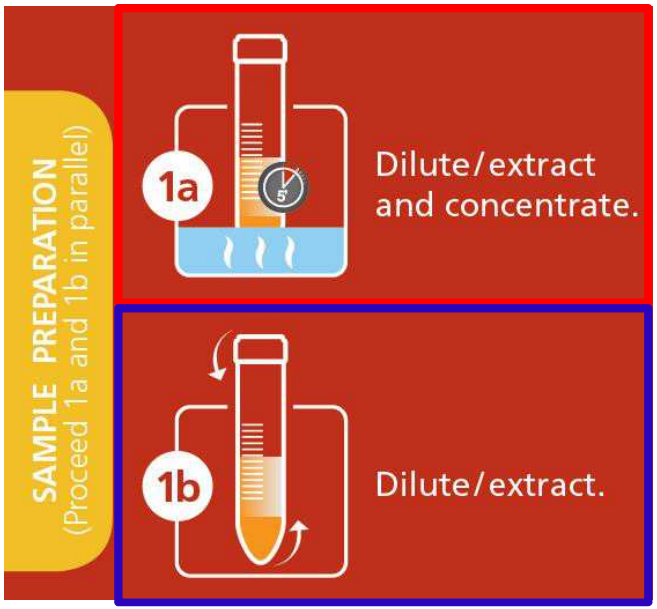
- **TYLOSIN** degrades in acidic condition and **CHLORAMPHENICOL** has a MRPL at 0.3 µg/kg in honey…

  → *NEED to avoid acidic condition and to use of solvent extraction/concentration to reach high sensitivity…*

Challenge of a multiple test for honey

SOLUTION:

- 2 separate honey samples diluted in parallel…
- Pool of the 2 samples just before dipstick analysis

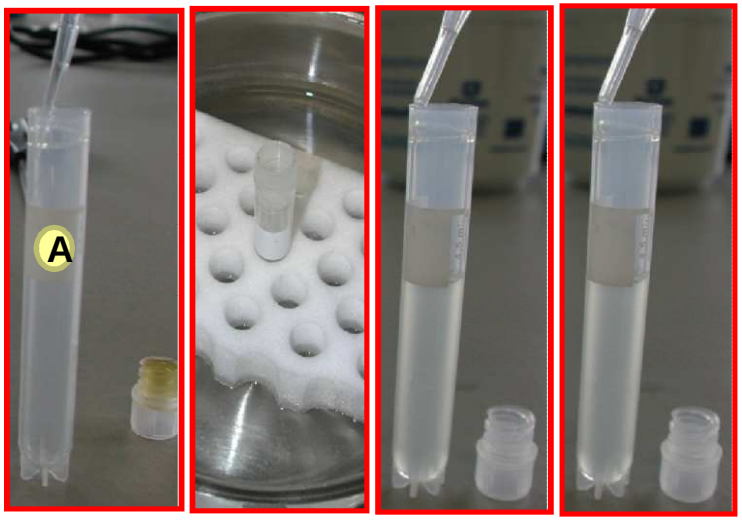


Acidic **hydrolysis** (SULFA / QUINO release)

Buffer **dilution** (TYL / CAP protection)

Field-test test : method schematic

1. DILUTION / HYDROLYSIS

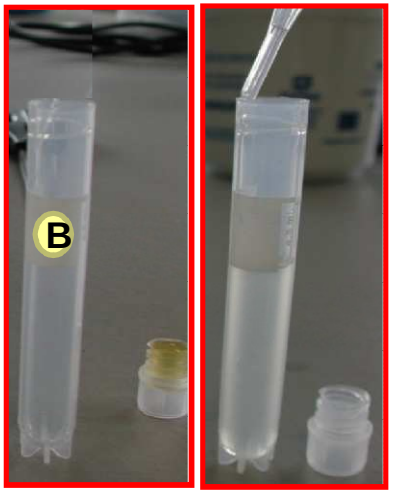


0,65 gr HONEY

300 µl Acid Hydrolysis (5 min 95°C)

300 µl Base Neutralization

2,4 ml buffer Dissolution



0,65 gr HONEY

3 ml buffer Dissolution

2. DIPSTICK

Mix A & B 200µl/200µl

5 min Incubation at 25°C (RT)

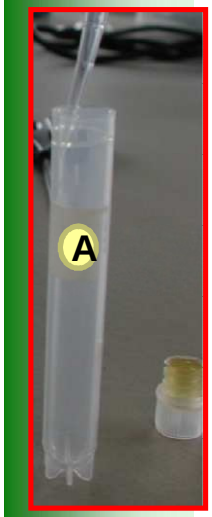
15 min Dipstick At 25°C (RT)

<30 min
TOTAL

All material provided in the kit !

Lab-test format : method schematic

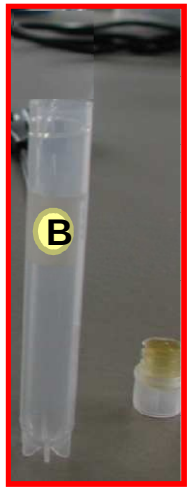
1. DILUTION / HYDROLYSIS



2,5 gr HONEY

1200 µl Acid Hydrolysis (5 min 95°C)

1200 µl Base Neutralization



2,5 gr HONEY

2400 µl warm H20 For dissolution

2. EXTRACTION

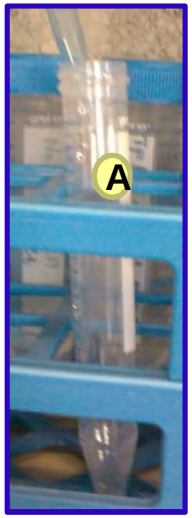
10 ml EA shake (10min)

Centrifuge (5 min)

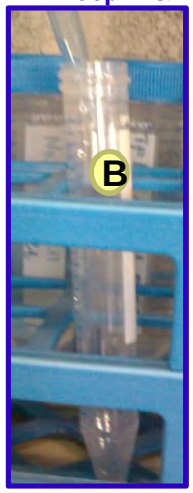
Transfer 8ml Supernatant

Evaporation (40 min 55°C)

3. DIPSTICK



Dilution 250µl Buffer – Mix 200µl A&B



Dilution 250µl Buffer – Mix 200µl A&B

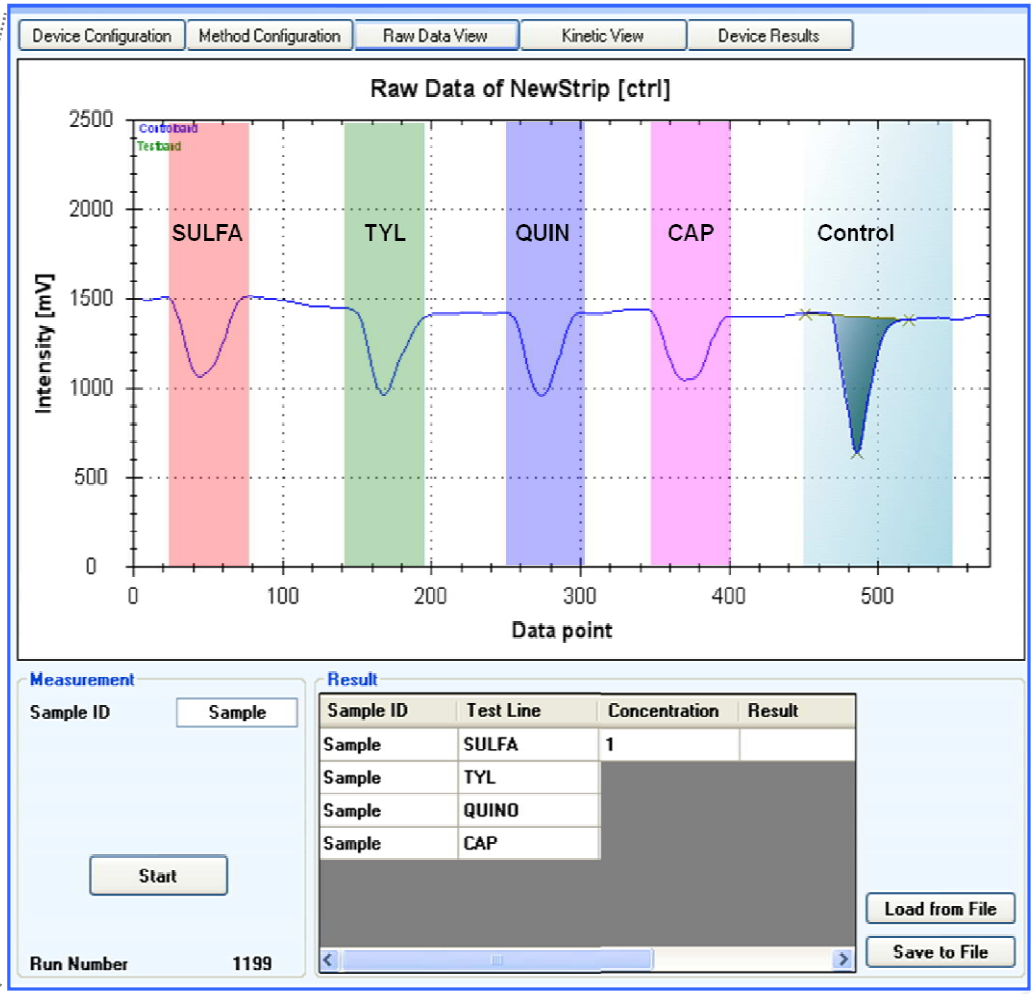
5 min Incubation 40°C

15 min Dipstick 40°C

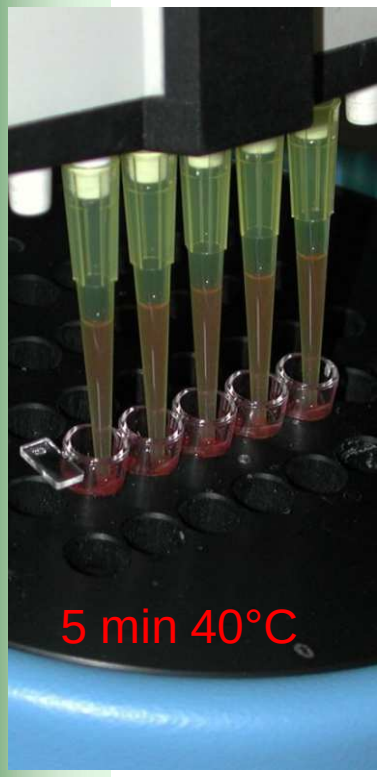
Optional tools for the dipstick analysis

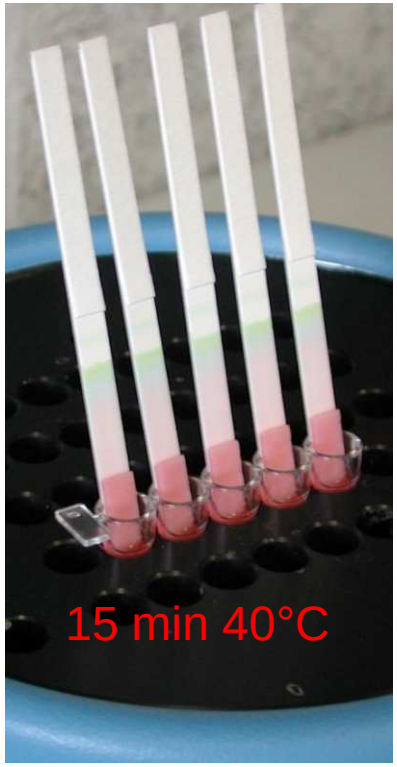
Heatsensor® makes the dipstick analysis automatic in one single step…

Readsensor® makes dipstick measurement more objective, traceable and semi-quantitative



Lab test multiplex dipstick results





BLANK HONEY

SULFA 25 ppb*

QUINO 25 ppb**

TYL 10 ppb/CAP 5 ppb

MIX OF ALL***

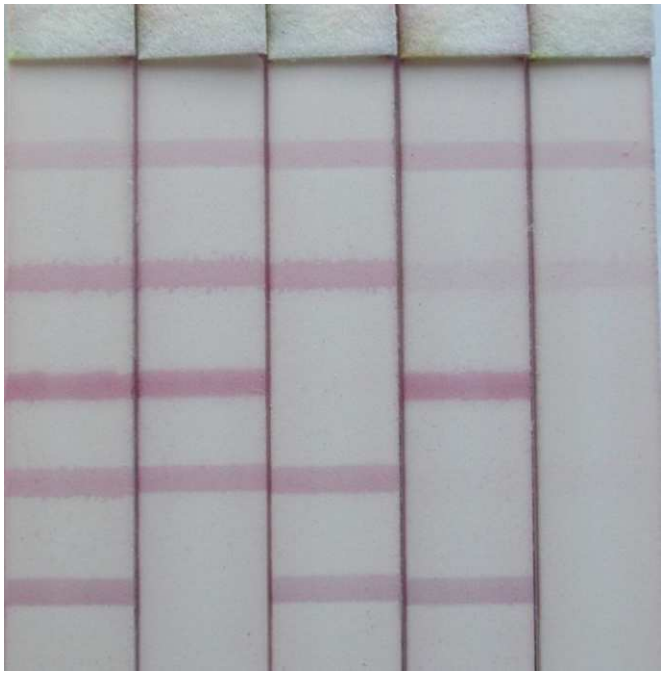
CTRL

CAP

QUINO

TYL-A

SULFA



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

Lab test validation

- **HONEY SAMPLES USED:**

- Liquid, solid, amber, dark, pale, raw, commercial…
- Blank vs Spiked (STA / CIPRO / TYL / CAP at 25 / 25 / 10 / 5 µg/kg)

- **SENSITIVITY:**

- **100%** of **positive** results at 1/2 screening target concentrations for Sulfathiazole, Ciprofloxacin, Chloramphenicol
- **90%** of **positive** result at screening target concentration for Tylosin

- **RUGGEDNESS (n=20):**

- Temperature for extract evaporation = **50°C +/-5°C**
- Time flexibility to read result = **Directly** but OK after **10** & **20 minutes**
- Potential decrease of Tylosin sensitivity for raw honey containing wax

- **SPECIFICITY** (compounds at 50µg/kg) **:**

- No interference on the test with other antibiotics
- Very slight crossreactivity of FQ line with Fumagillin

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	

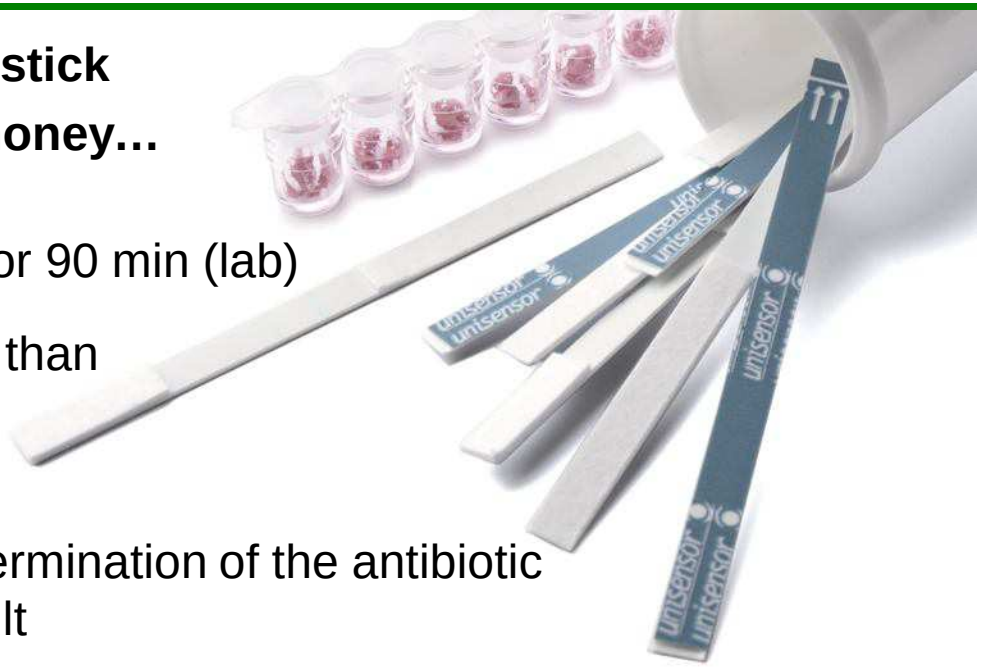
(Fluoro)quinolone compounds	LoD LAB	LoD FIELD	CRL**
Enrofloxacin	<25	5-25	50
Ciprofloxacin	<25	50	
Danofloxacin	25-50	<100	
Difloxacin	250	<500	
Marbofloxacin	50	<100	
Norfloxacin	25	50	
Sarafloxacin	>500	-	
Flumequine	>500	-	

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

*** European limits or recommended concentrations in honey (CRL – AFSSA-LMV France – SANCO /2006/3228).*

Conclusions

- Development of a **multiplex dipstick** assay detecting **antibiotics** in **honey…**



- ✓ **Rapid** - Results in 30 (field) or 90 min (lab)
- ✓ **Multiple** - Detection of more than 18 relevant antibiotics in one single test
- ✓ **Discriminating** – Direct determination of the antibiotic class in case of positive result
- ✓ **Flexible** - Flexibility regarding sensitivity, time and material availability
- ✓ **User-friendly** - Clear visual result or reader interpretation
- ✓ **Convenient** - Performable on site or in the lab
- ✓ **Reliable** and **robust**
- ✓ **Cost-Effective** - Does not need any expensive instrumentation

Availability of the MULTIPLEX

- Extern Lab Validation in progress (FERA, UK) and Inter Lab Validation in January 2012

- Completing the range of existing UNISENSOR's dipstick assay detecting antibiotics in Honey (Tetracyclines, Sulfamides)

- Kit produced and commercialized by unisensor under the name bee4sensor

www.unisensor.be

Thanks to…

- Multiplex dipstick development :
  - **UNISENSOR S.A.** (Belgium)
  - **CER** (Belgium)
  - **CSIC** (Spain)
- Matrix preparation & lab validation :
  - **FERA** (United Kingdom)
  - **NESTLE NRC** (Switzerland)
- Project coordination :
  - **RIKILT** (The Netherlands)
- Funding :
  - **CONffIDENCE** (European Commission FP7 Grant agreement n°211326)