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Connaître, évaluer, protéger

Development and optimisation of an amperometric immunosensor for the detection of banned antibiotic residues in honey

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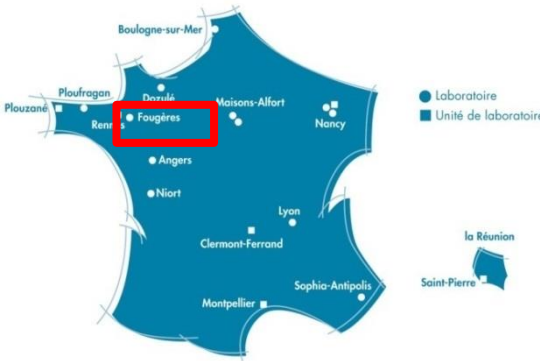
Outline

- Context
- Method
- Optimisation/Validation
- Results
- Conclusions
- Perspectives

Anses

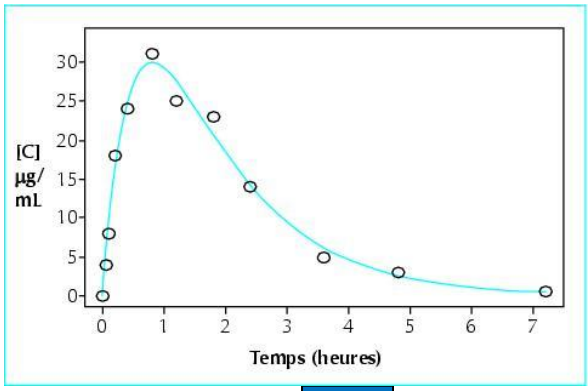
- Missions: contributes to ensuring:
  - Human health and safety in the fields of environment, work and food
  - Protecting animal health and welfare
  - Protecting plant health



- Organisation: Divisions
  - Regulated products and science for expertise
  - Resarch and science division: 11 laboratories
    - Fougères: NRL for vet drugs and EURL for antibiotic residues in food from animal origin

How did antibiotics become part of the food chain?

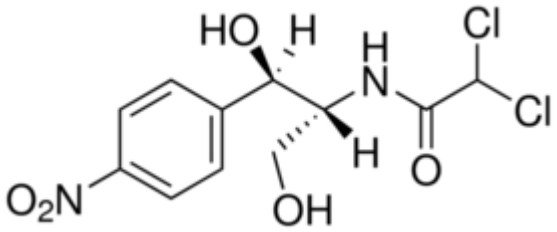
- Animal treatment



- Regulatory limits
  - Authorised substances: MRL
    - Acceptable threshold (toxicity, exposure)
  - Banned substances: RPA/MRPL
    - Detected/identified/quantified (analytical)
- Regulatory control: NRMP

FOOD OF ANIMAL ORIGIN

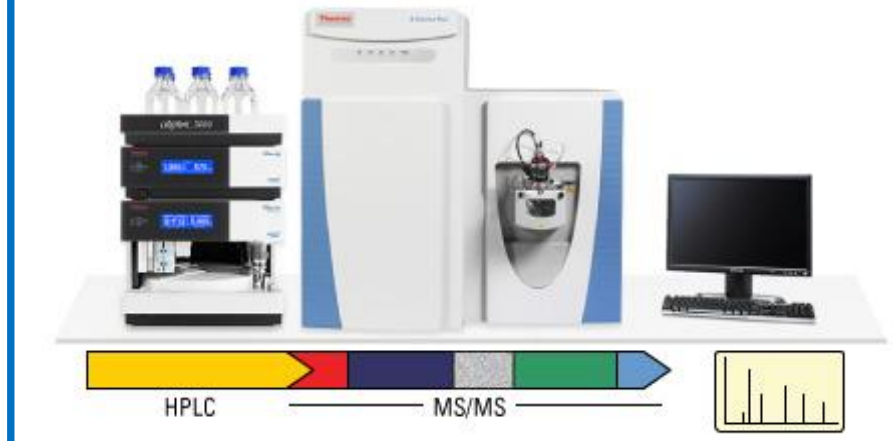
Banned antimicrobials



- Chloramphenicol (CAP):
  - Potent, efficient antibiotic used since years, broad spectrum, cheap
  - Toxicity: Aplastic anemia (AA) and bone marrow suppression
  - Banned from animal food production (1993)
  - MRPL/RPA: the lowest level which can analytically be achieved by the official control laboratories: 0.3 µg/kg

Analytical strategy

SCREENING



CONFIRMATION

MRL
or
MRPL/
RPA

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Screening methods

- Performance characteristics:
  - Cheap
  - Quick
  - Sensitive (< 5 % of false negative results)
  - Specific or with a wide spectrum detection depending on the target analytes
  - High throughput of samples

- Biosensors
  - Optical

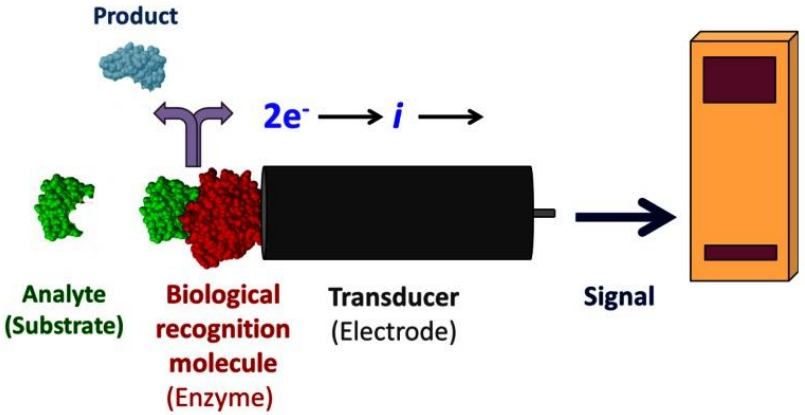
Drawbacks: cost (investment, commercial kits

SPR

FCIA

Chemiluminescence

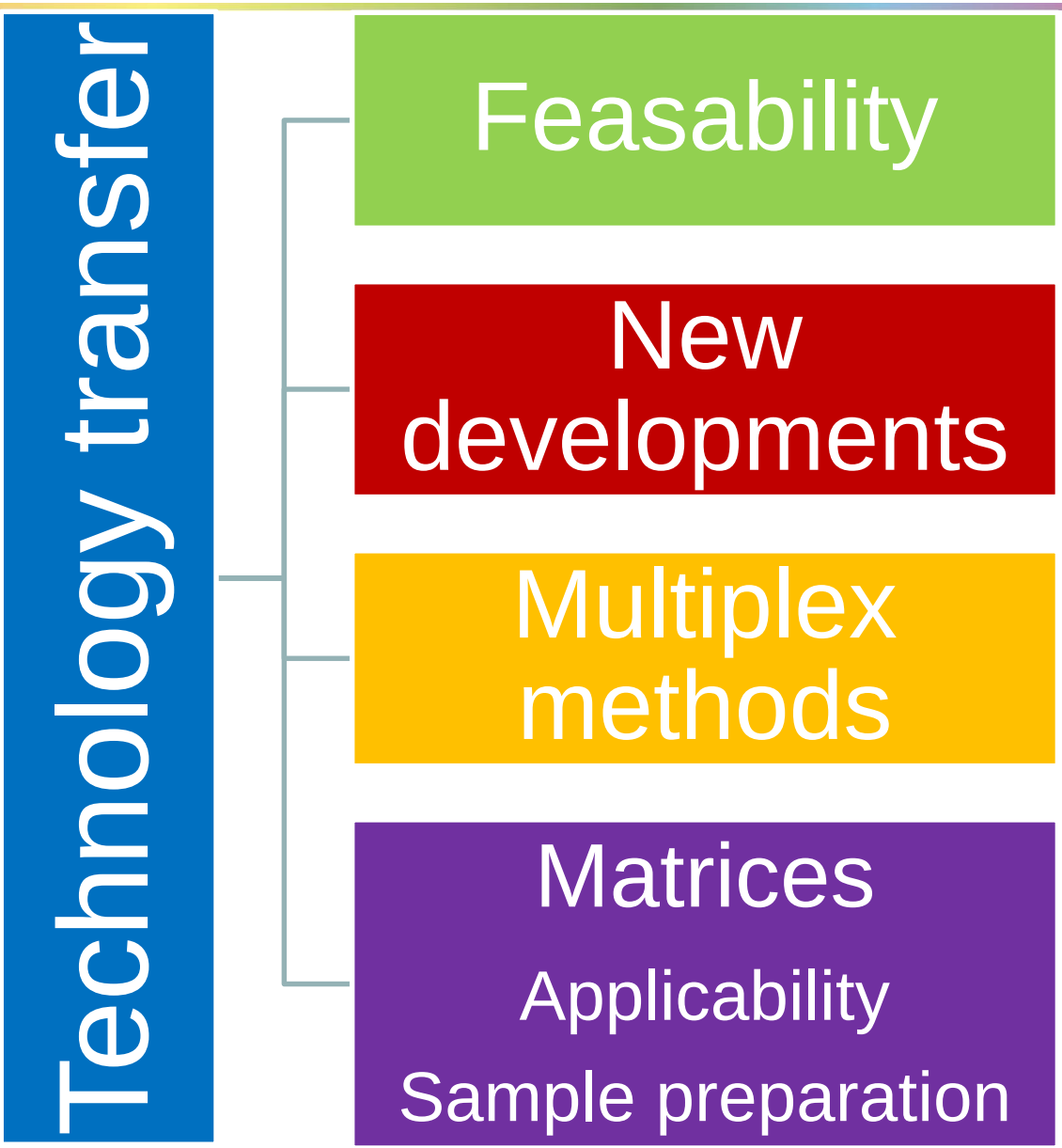
Origin of the project



- Spanish research team
  - Amperometric (electrochemical) biosensor for screening of TCYC, SULF and PEN in milk below MRL
    - Amperometric detection: production of a current when a potential is applied between 2 electrodes
    - Low cost, promising LOD, portable, automatisable
  - On-site training (end 2016)

Conzuelo F, Ruiz-Valdepeñas Montiel V, Campuzano S, Gamella M, Torrente-Rodríguez RM, Reviejo AJ, Pingarrón JM. 2014. Rapid screening of multiple antibiotic residues in milk using disposable amperometric magnetosensors. Anal. Chim. Acta. 820:32-38.

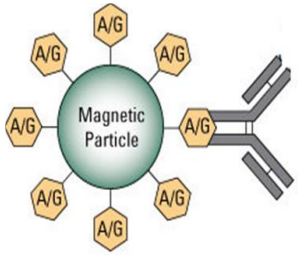
Questions


Technology transfer
Feasability
New developments
Multiplex methods
Matrices
Applicability
Sample preparation

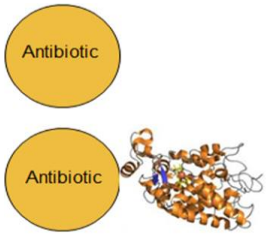
Assay principle and material

METHOD

Competitive IA



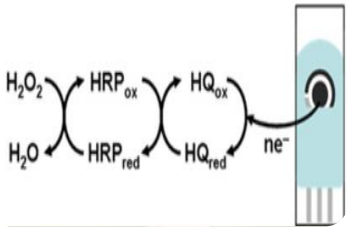
Ab grafting (1st incubation)



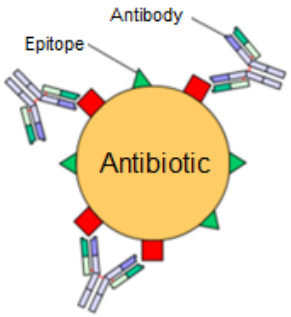
Addition of sample and HRP-conjugate (2nd incubation)

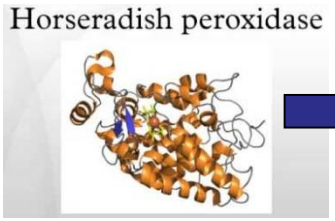
Addition of the mixture onto the electrode

Dipping into buffer + hydroquinone)



Addition of H_2O_2 and measurement



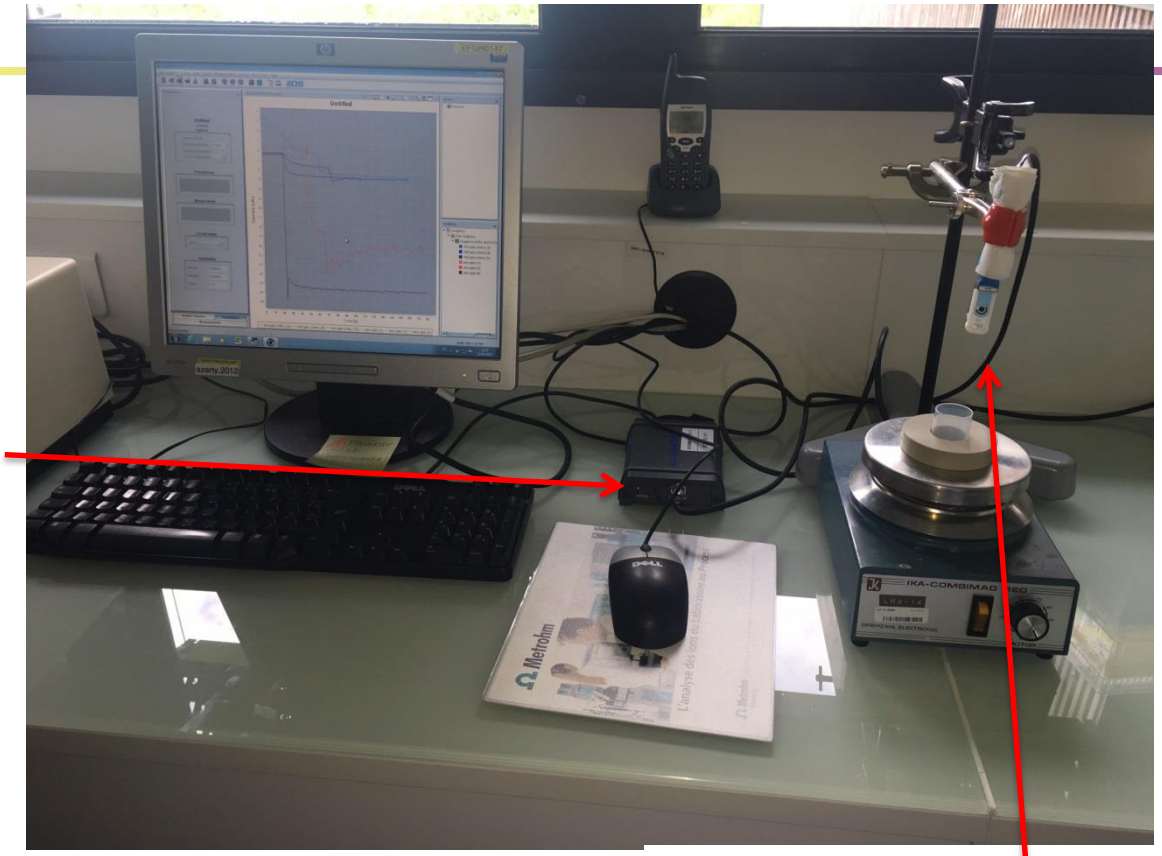


Apply a potential => Read the result

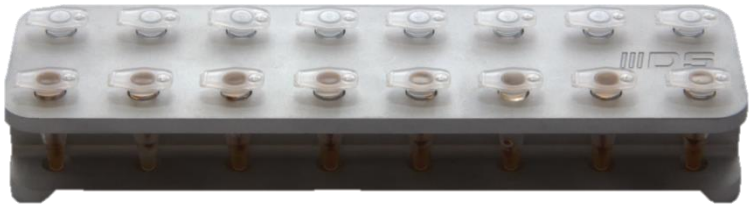
Screen Printed Carbon Electrodes (SPCE), coupled to magnetic beads (MB)

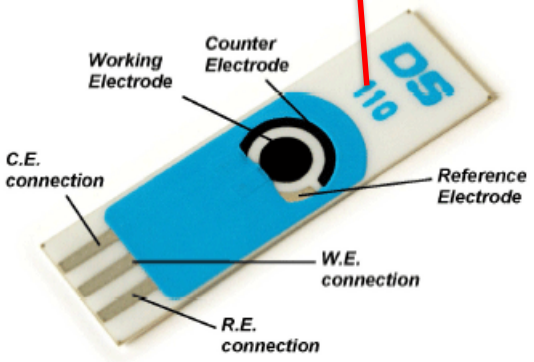
Materials


Bluetooth

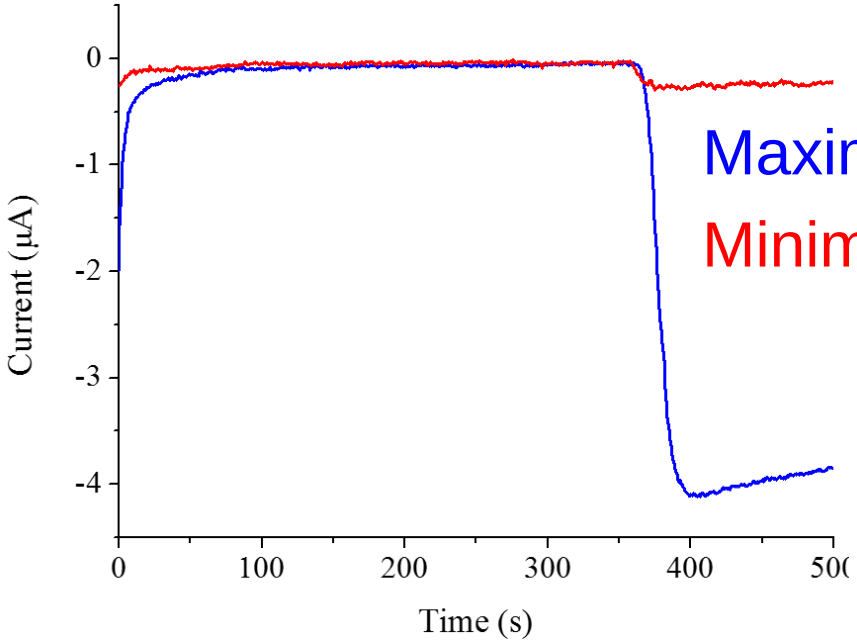

Metrohm


THERMO-SHAKER




Working Electrode
Counter Electrode
C.E. connection
Reference Electrode
W.E. connection
R.E. connection
DS 110

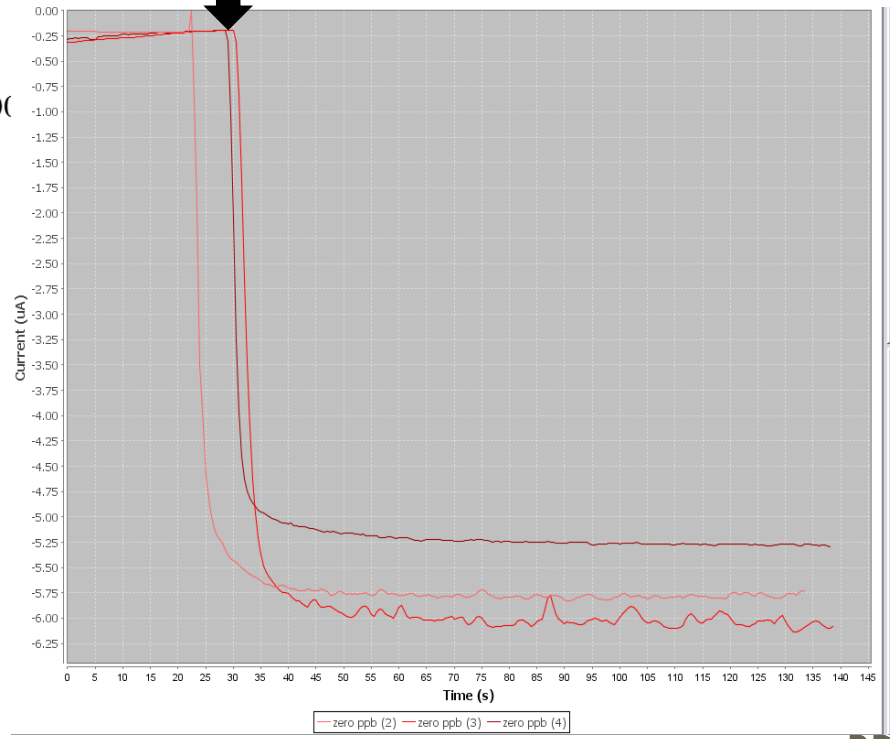
Amperometric signal



Maximum signal (blank sample)

Minimum signal (spiked sample)

H_2O_2



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Protocols

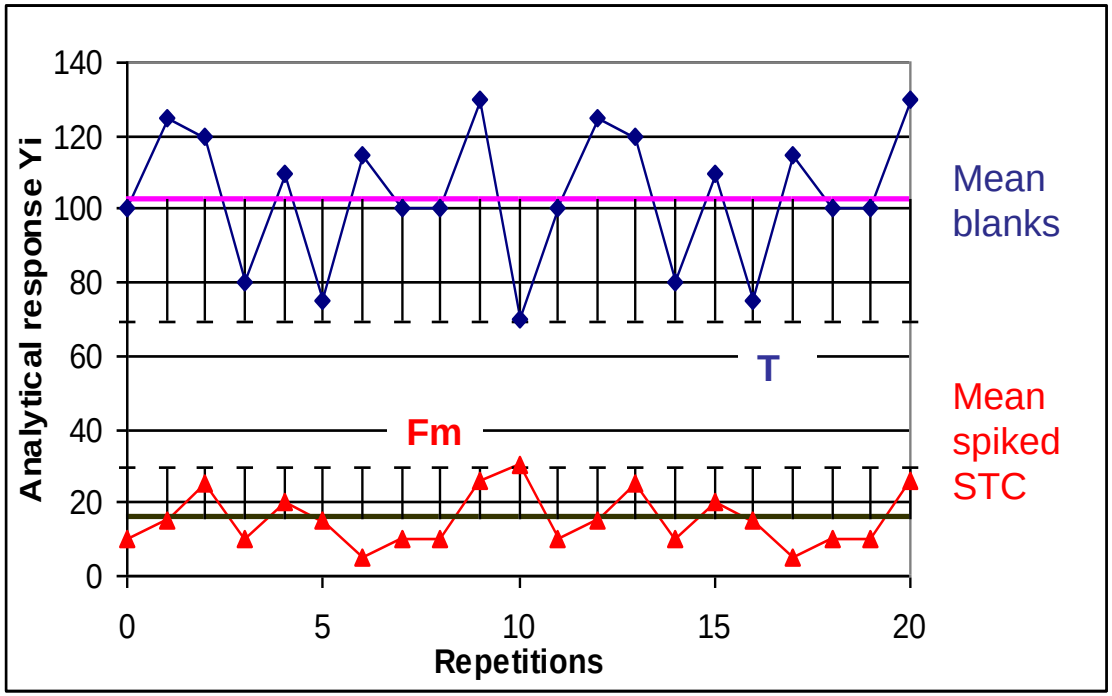
OPTIMISATION/VALIDATION

Optimisation objectives

- Finding the optimal conditions, while considering that:
  - The more dilute the reagents, the less expensive it costs
  - The shorter the manipulation time, the more interesting the protocol

Validation regulations (EC/657/2002, EURL guideline)

- Performance characteristics/criteria:
  - Specificity (N blank samples)
    - Threshold value T:
    - $T = B - 1{,}64 * SD_B$
  - Detection capability CCβ (N spiked samples at STC)
    - Cut-off factor Fm:
    - $Fm = M_{SP} + 1{,}64 * SD_{SP}$
  - βerror ≤ 5%



Competitive IA: response inversely proportional to the concentration => **T > Fm**

⇨ If T>Fm, CCβ = STC

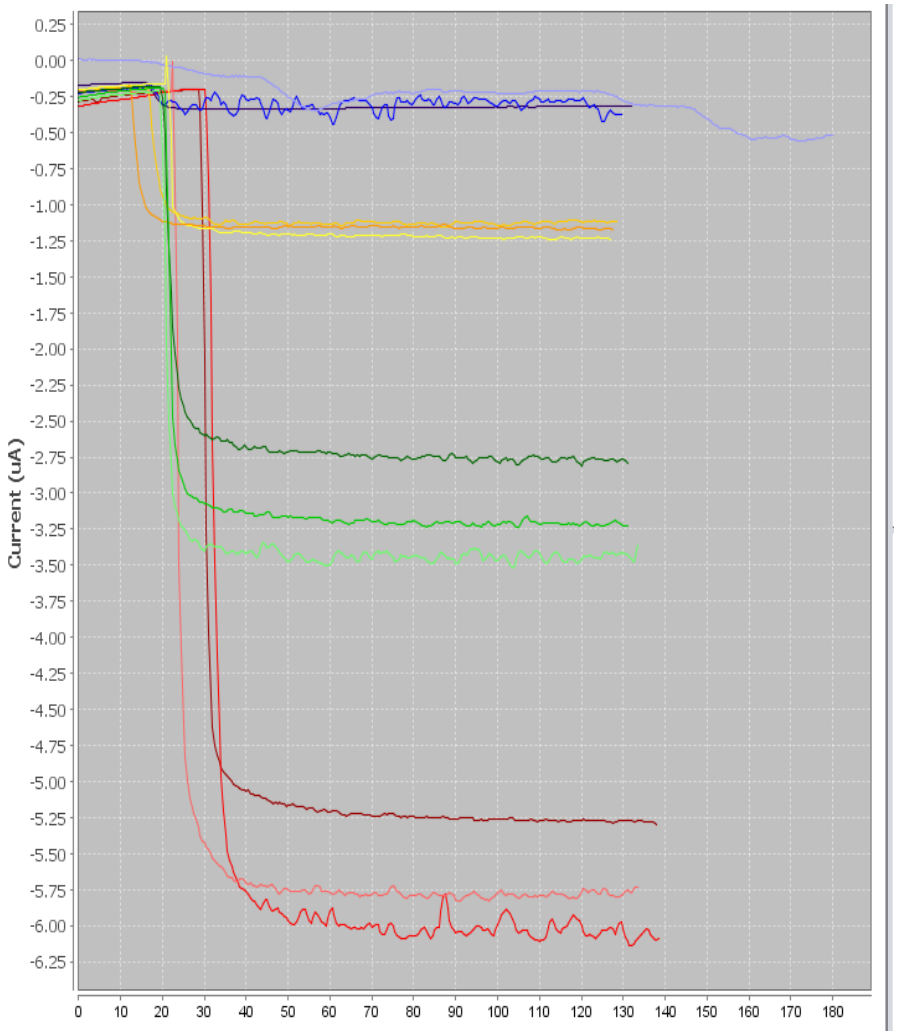
Screening of chloramphenicol in honey at or below regulatory limit

Development and optimisation

RESULTS

Measures in buffer before optimisation

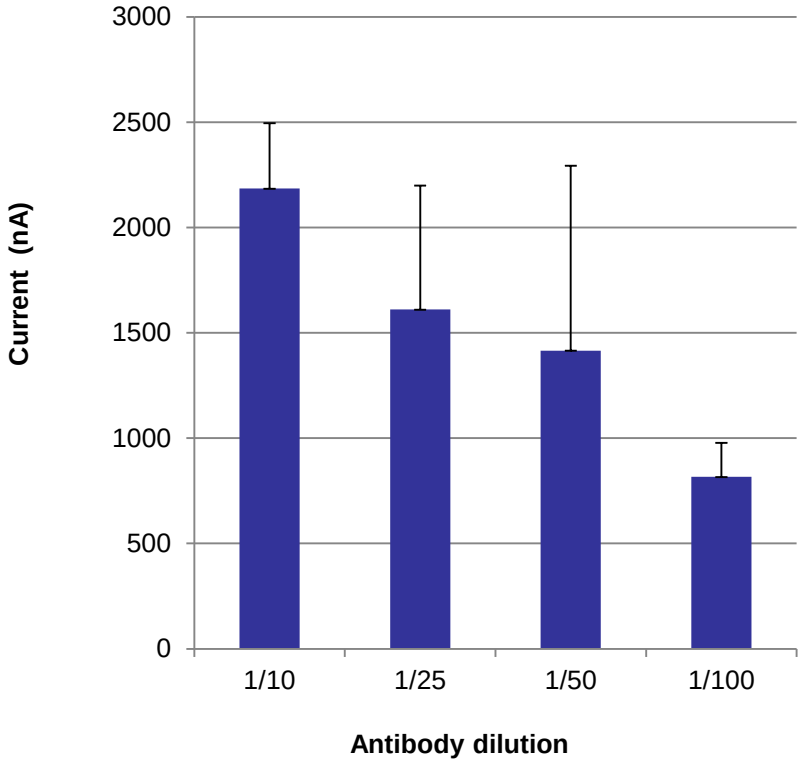
- a series of spiked solutions of CAP in PBS-T.
- The red curves represent blank samples,
- in green the samples spiked to 1ng/ml,
- orange to 10 ng/ml
- and blue to 100ng/ml



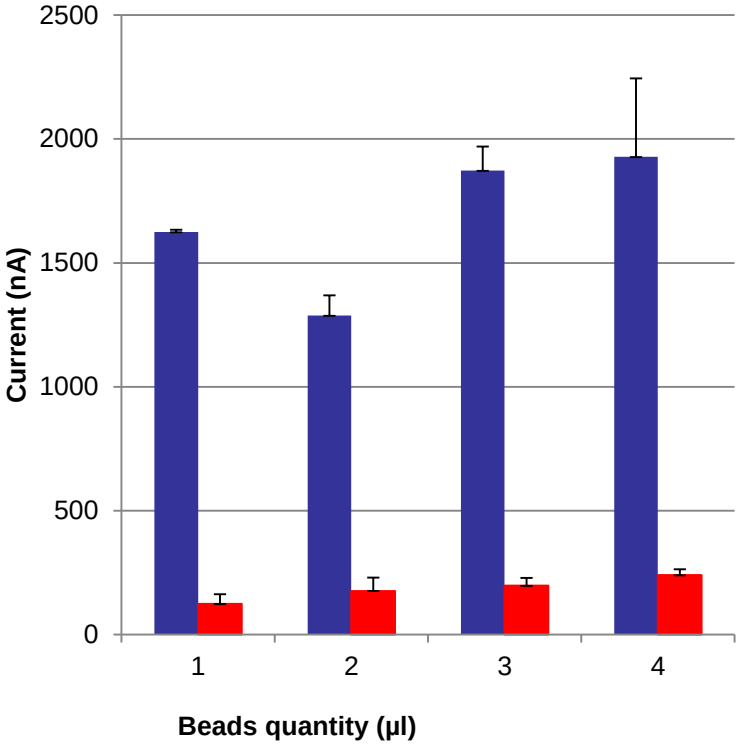
Optimisation results in buffer

Extract of the results: Graph showing the current (nA) as a function of:

the dilution of antibodies used in the blank samples



the quantity of beads used (µL) in the blank (blue) or spiked (red) samples

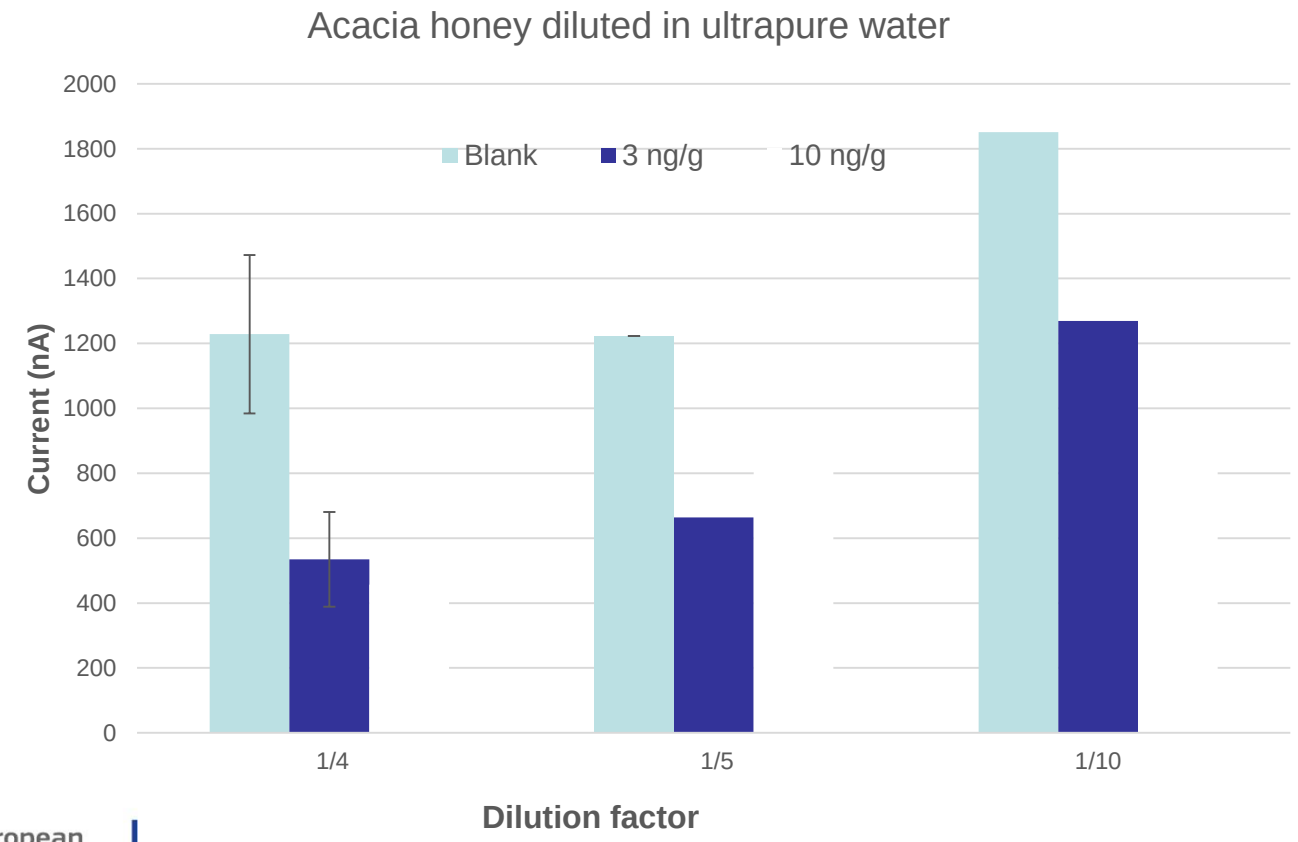


Optimised protocol in buffer

- 3 µL of beads per tube
- Antibodies diluted to 1/50th and incubated for 30 minutes
- CAP-HRP diluted to 1/100th and incubated for 30 minutes with the sample (ie. simultaneous method)
- Hydroquinone concentration of 1 mM and H2O2 of 1 M
- Applied potential of -0.2V

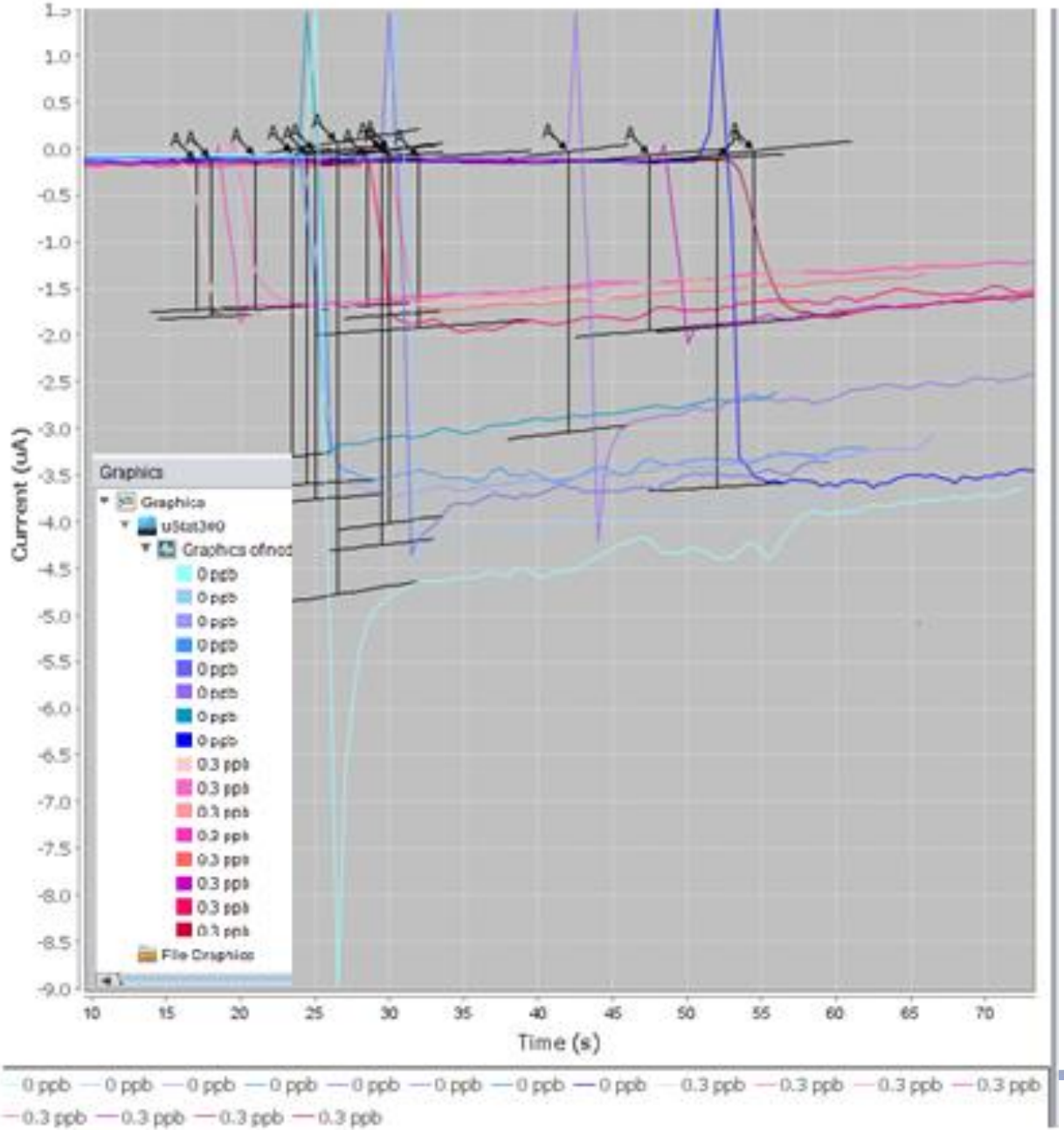
First results in honey

- Honey dilution in water
  - high variability on the measured current (around 20%), taking into account that it is a single acacia honey sample that was analysed several times



- Different extraction protocols tested:
  - Acetonitrile (different volumes)
  - Ethyl acetate (different volumes)
  - Dilution in water or buffer and extraction with ethyl acetate
- Optimised protocol with acacia honey
  - Heating 1.0 g of honey sample at 40° C for 15 min
  - Add 3 ml of ethyl acetate.
  - vortex for 2 min and sonicate for 30 min
  - centrifuge at 5000 rpm for 7 min
  - supernatant evaporated to dryness at 50° C under nitrogen stream
  - Extracts reconstituted with 120 µl of PBS-T

Optimised extraction protocol (1)



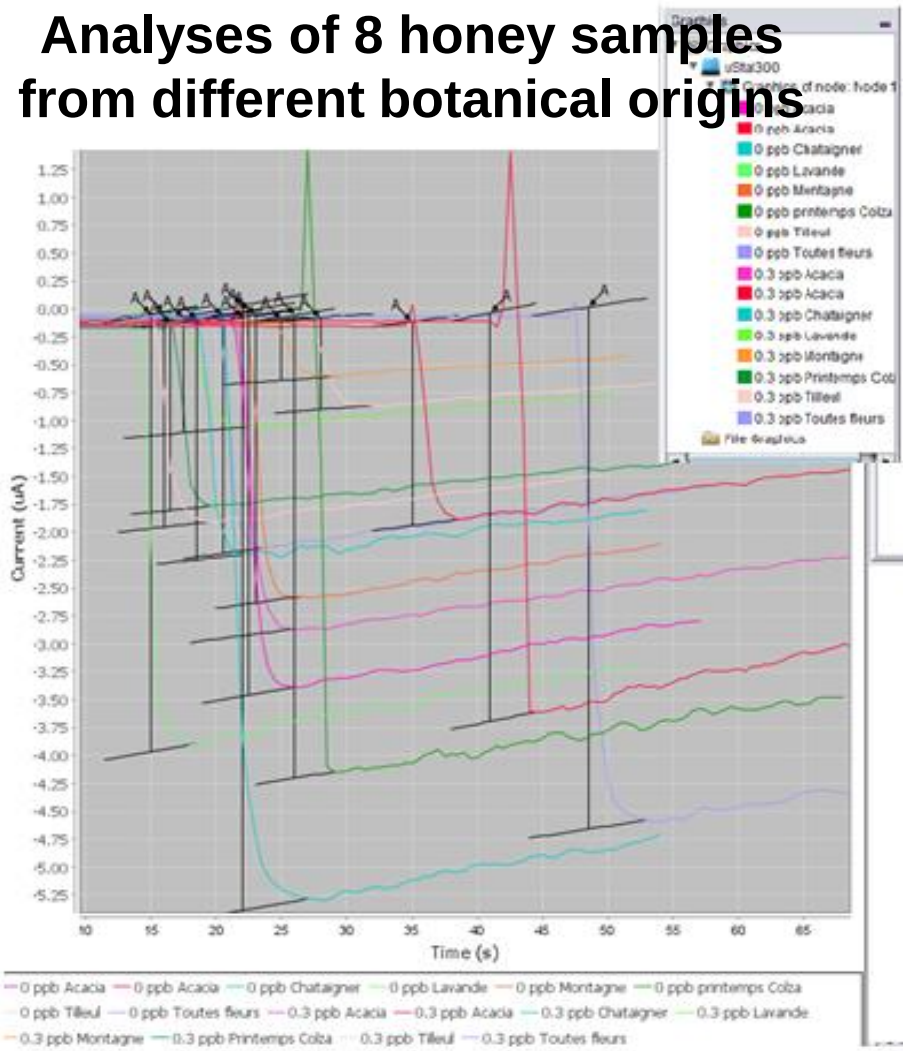
Repeated extractions and analyses of one single acacia honey

- CAP detected at 0.3 ng/g in acacia honey
- T = 2807 > Fm = 1904
- CV on blank samples = 16% and on spiked samples 7%
- Very promising results

Optimised extraction protocol (2)

- T (1902) < Fm (2905)
- CV on blank samples = 30% and on spiked samples 48%
- Clear discrimination of individual results between the signals (current (nA)) from the blank honey and the same sample spiked with CAP to 0.3 ng/g
- **Even with an extraction procedure in ethyl acetate, the honey matrix effect was always strong, impeding and preventing a global analysis for honey from different botanical origins**

Analyses of 8 honey samples from different botanical origins



Acacia (2 samples), chestnut, lavender, mountain, spring, lime tree, multifloral

Complexity of honey matrix

- Not a single honey matrix, wide variety of honeys
- Honey composition and colour varies considerably depending on the botanical origin
  - Sugar (about 76%) (fructose, glucose, sucrose), water (18%) and other ingredients (minerals, proteins (ie. enzymes), Aa, fatty acids, vitamins, essential oils) about 6%, organic and aromatic substances including flavonoids, alcohol, esters, pigments and pollen
    - Eg. dark types of honey are richer in minerals than lighter, higher total phenolic content and consequently a higher antioxidant capacity
- Honey ingredients can interfere with the electrochemical detection, especially substances with antioxidant activities (eg. polyphenols).

PVPP and ethyl acetate extraction

- Poly(vinylpolypyrrolidoe) (PVPP)
  - crosslinked homopolymer that binds with phenolic compounds by hydrogen bonding.
  - used for the removal of phenolics and alkaloids from plant samples.
  - used in wine to absorb some of the phenolic compounds and astringent tannins
  - Two different concentrations of PVPP (0.2 ng/g and 0.8 ng/g of honey) were tested by adding 1 ml and 4 ml of a solution of PVPP 20% in 1 g of honey.

	PVPP 0.2 ng/g		PVPP 0.8 ng/g	
Botanical origin	Blank	CAP 0.3 ng/g	Blank	CAP 0.3 ng/g
Moutain	1440	1090	1808	1347
Forest	2502	1902	1995	1688
Scrubland	2224	1937	1576	1043
Sunflower	2244	1902	1977	1026
Spring	nd	1929	2191	1511
Multifloral	2764	2033	2018	1484

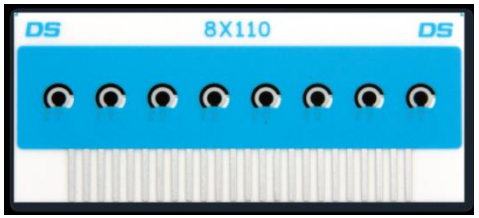
- 0.2 ng/g of PVPP, T = 1422 < Fm = 2374
- 0.8 ng/g of PVPP, T = 1582 <x Fm = 1788
- In both cases, T < Fm, but better when PVPP at the highest concentration (0.8 ng/g)
- Individual honey samples, all the blank samples were discriminated from the samples spiked with CAP to 0.3 ng/g
- Assay to be repated by increasing the PVPP concentration.

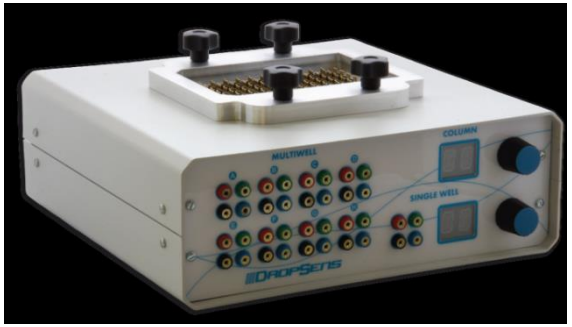
Perspectives

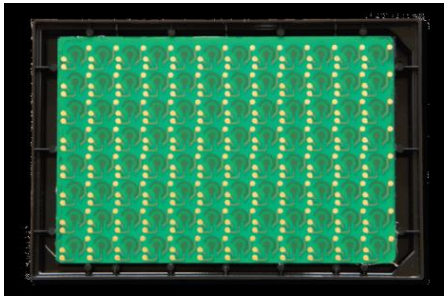
- To develop more efficient extraction protocols, able to remove or at least lower the strong matrix effect of honey
- Positive effect of PVPP on the reduction of the matrix effect to be investigated
- To compare different electrochemical modes of detection: amperometry, voltammetry
- Use of nanomaterials (i.e. Modified SPCE)
- Validation (60 different honey samples)
- Evaluation of an aptamer in replacement of the antibody to detect CAP

Perspectives

- To develop biosensors for other banned antimicrobials (ie. Nitrofuran metabolites) and finally develop a multiplex method for the simultaneous detection of at least all these banned substances.
- Other matrices
- High throughput







Thank you for your attention

Céline Hédou