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# How to scale up a method from low throughput to high throughput for the quantification of 16 nucleosides? Lesson from experience.

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## Context : Agriculture, pesticides and cancer

Link between occupational exposures and cancers demonstrated in farmers

- ❑ Molecular epidemiology approach
- ❑ Investigation on biobanks samples
- ❑ Important statistical power needed

Pesticides exposure effects?  
Other determinants?

Agricultural cohort with exposure description and tasks listing

## EPIBIO97 Biobank

Biobank made with

- ❖ 795 individuals at initial sample time in Calvados department area with blood and urine samples
- ❖ 300 follow up after 10 years

Working in

- ❖ Field Crop
- ❖ Bovine breeding



Exposure description by epidemiological questionnaire

## Plausible mechanism of cancerogenicity

### 2 cancerogenicity possible mechanisms studied through analysis of epigenetic and genotoxic alteration

**markers** **Oxidative stress**

Higher oxidative stress in farmers exposed to pesticides

12 Oxidation adducts investigated

Compounds dosed in the previous method Available with internal standards <sup>13</sup>C<sup>15</sup>Nlabelled

New compounds dosed

**Epigenetic**

Augmentation of Methylation within farmers exposed to pesticides

4 Epigenetic alterations investigated

Internal standards available for 5MeC (<sup>13</sup>C<sup>15</sup>N labelled) and 5OHMeC (D labelled)

Adduct	Molecule
5MeC	
5OHMeC	
5fdC	
5CadC	

DNA Demethylation pathway

Development of a HPLC-HRMS/MS method for the simultaneous analysis of 16 nucleosides in human blood

Numerous sample preparation steps

High throughput for the preparation of a high number of samples

## Analytical method

All solution additions are made with an Integra Assist+ pipetting robot

For each 96 well plate, 8 calibration points, 2 blanks, and 10 quality check are prepared, according to ICH M10 recommendation

**Sample preparation**

**Analytical parameters**

**Reduction**

- ❖ NaBH<sub>3</sub>CN, 5 g/L
- ❖ 4 additions of 10 µL
- ❖ Mixing 2 x 30 minutes at 20°C
- ❖ Mixing 2 x 30 minutes at 37°C

❖ NaBH<sub>3</sub>CN, 5 g/L

❖ 1 addition of 10 µL

❖ Mixing 1\*30 minutes

❖ at 37°C

Total reduction obtained with only one NaBH<sub>3</sub>CN addition in 30 minutes  
Operation shortened by 1h30

**Precipitation**

- ❖ Cold ethanol 1 V
- ❖ 50 µL NaCl 0,1mol/L
- ❖ Vortex agitation
- ❖ Centrifugation

❖ Isopropanol 2,5 V

❖ 50 µL NaCl 1mol/L

❖ Plate agitator

❖ Centrifugation

Precipitation adapted to room temperature to be possible with the pipetting robot

**Washing**

- ❖ 2 Washing
- ❖ 1 mL Etoh:H2O (70:30)
- ❖ Vortex agitation
- ❖ Liquid disposal

❖ 1 Washing

❖ 1 mL Etoh:H2O (70:30)

❖ Plate agitator

❖ Liquid disposal

❖ Evaporation

Better yield with 1 washing than 2  
Evaporation allow to clear the samples of the remaining ethanol

**Hydrolysis**

- ❖ 2 additions of enzyme solutions
- ❖ 2 hours agitation at 37°C
- ❖ 10 µL of HCl
- ❖ Centrifugation
- ❖ Transfert into LC vial

❖ 2 additions of enzyme solutions

❖ 2 hours agitation at 37°C

❖ Transfert into 96 deepwell 200µL conic plate

❖ 5 µL of diluted proteinase K

96 plate can be directly inserted in the LC-MS/MS  
Proteinase K prevent adsorption of interest compounds on proteins

**LC-MS/MS analysis**  
Shimadzu UHPLC-MS/MS 8040

Column: Force Biphenyl 1.8 µm, 100\*2,1 mm  
Flow: 0.4 mL/min  
Oven temperature: 40°C  
Mobile phase A: H<sub>2</sub>O + 0.1% Acetic Acid  
Mobile phase B: MeOH + 0.1% Acetic Acid  
5 µL injected

Mobile phase gradient

## Conclusion and current progression

- ❑ Optimisation for the preparation of 380 samples / week
- ❑ Analysis of the 795 agricultural workers from the biobank
- ❑ Association of DNA adduct profiles with occupational exposure to pesticides
- ❑ Already more than 600 samples extracted
- ❑ First analysis currently ongoing

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