

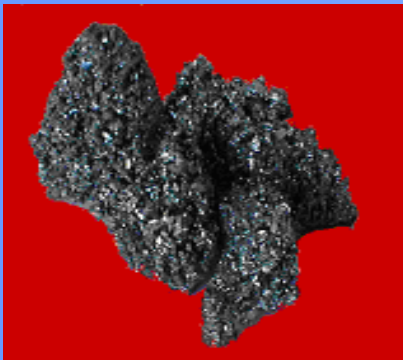
HEAVY METAL DETECTION USING CELL BASED AND SENSOR BASED ASSAYS

**Danila Moscone
University of Rome Tor Vergata,
Italy**

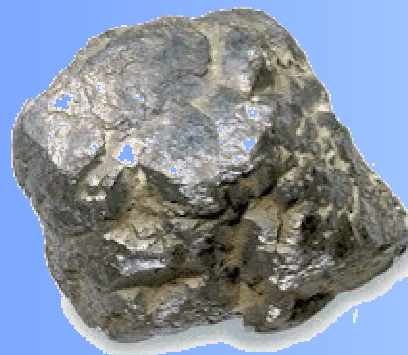
**Matti Karp
Tampere University of Technology,
Finland**

Heavy metals

The term **heavy metal** refers to any metallic chemical element that has a relatively high density (*greater than 5 g/cm³*) and is **toxic or poisonous at low concentrations**.



Arsenic



Lead



Mercury



Cadmium

Heavy metals

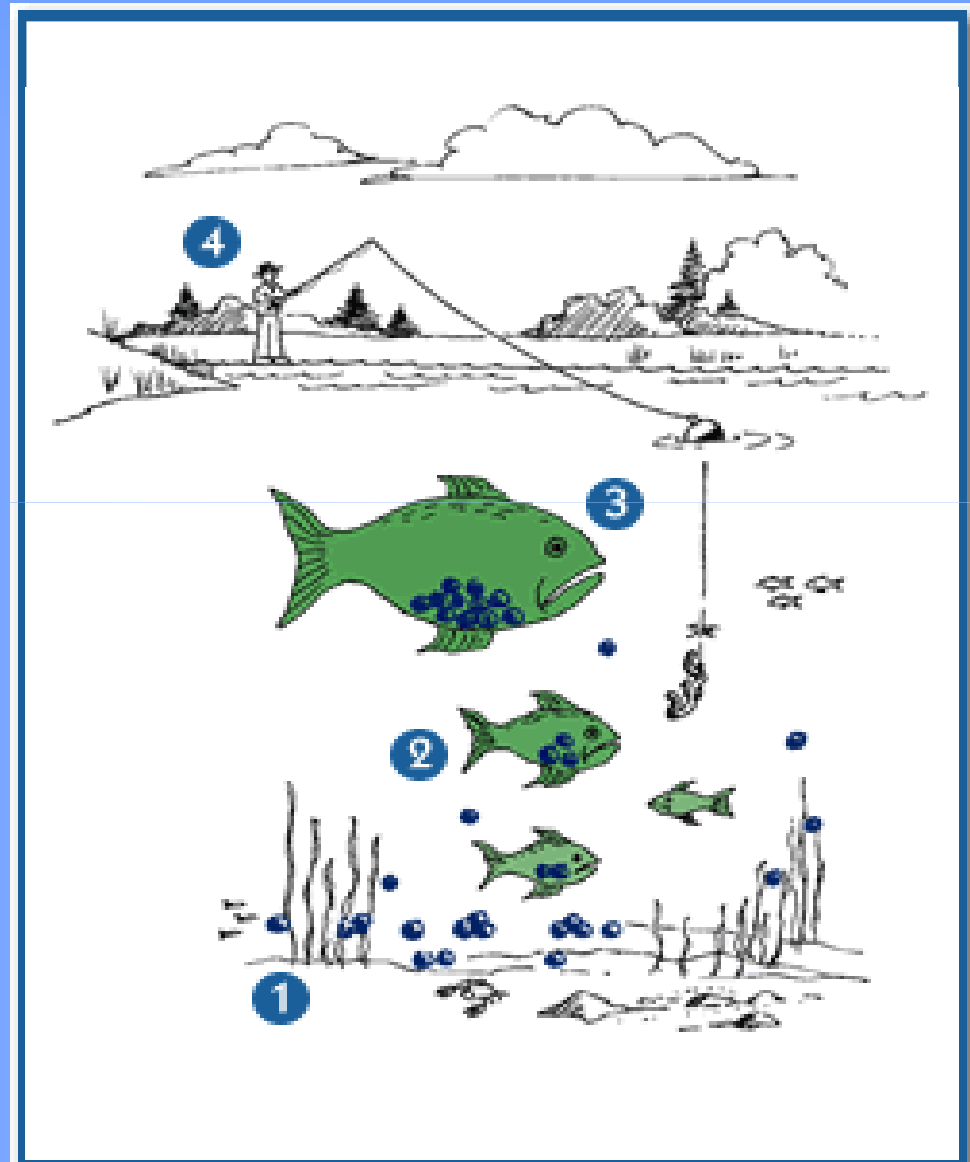
- Are natural components of the earth's crust
- They cannot be degraded or destroyed
- To a small extent they enter our bodies via food, drinking water and air
- As trace elements, some heavy metals (e.g. Copper, Selenium, Zinc) are essential to maintain the metabolism of the human body
- **However, at higher concentrations they can lead to poisoning**
- Heavy metal poisoning could result, for instance, from drinking-water contamination (e.g. Lead pipes), high ambient air concentrations near emission sources, or intake via the food chain

Bioaccumulation

Most plants and animals can regulate their metal content to a certain point.

Metals that can't be excreted, build up in an organism over its lifetime

Biomagnification



Heavy Metals Analysis

- **Quantitative and qualitative analysis**

Atomic Absorption (AA)

- Flame, gas furnace

Inductively Coupled Plasma (ICP)

- Atomic emission spectrometry, mass spectroscopy

- **Experience level of operators**

- **Collection technique**

Proper sample container

Preservation and storage

ATOMIC ABSORPTION



ICP-MS



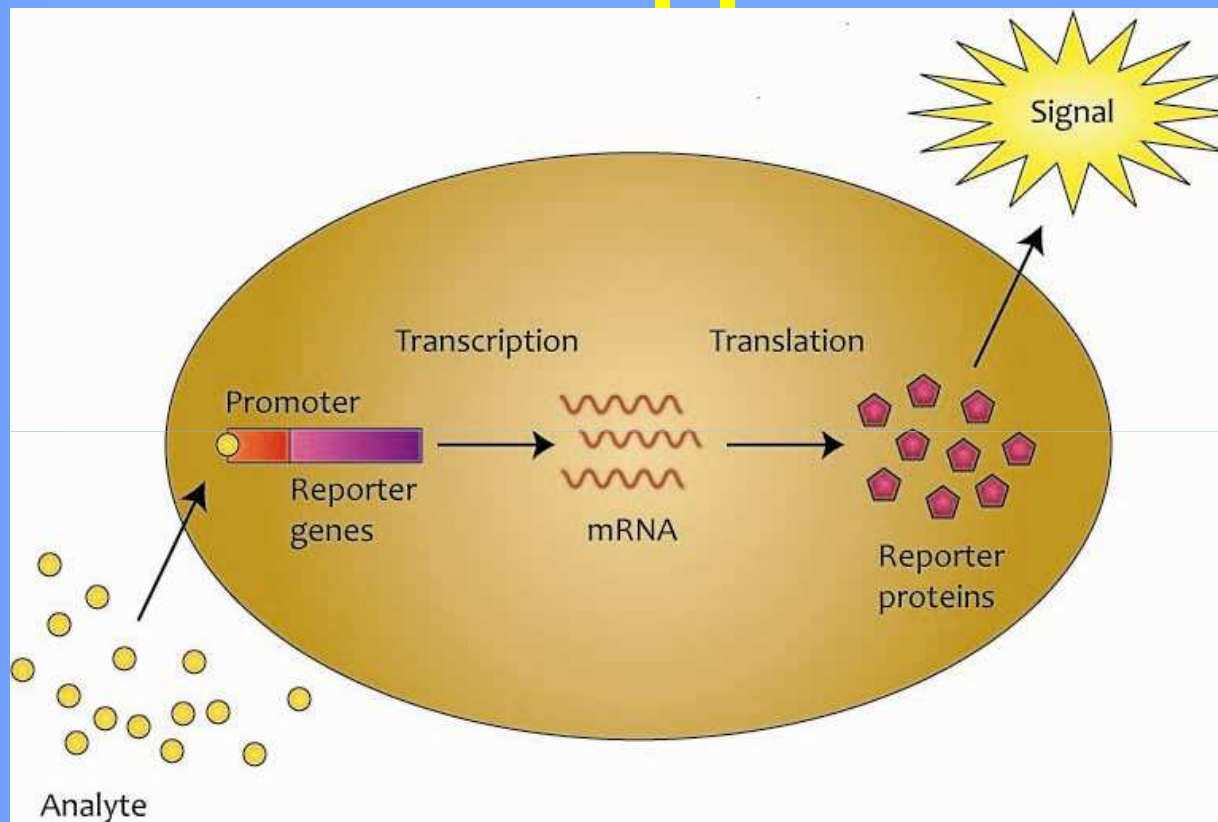
BIOLUMINESCENT SENSORS

- Naturally non-bioluminescent organisms can be converted to bioluminescent by means of genetic engineering.
- A bacterial cell can function as a **microbial biosensor** if contains two linked genetic elements: a **sensing element** and a **reporter**.
- The former senses the presence of the target molecule(s), and turns on the latter, which emits a detectable bioluminescent signal.

Principle of Bioluminescent Sensor Approach

- **Promoters** (sensing elements) are generally found in bacteria that are able to survive in environments contaminated by heavy metals or other toxic compounds. This ability is usually based on a genetically encoded resistance system, the expression of which is regulated very precisely.
- **Luciferase** genes are widely used **reporter genes** because they provide sensitive and simple detection of gene expression and regulation.

Principle of Bioluminescent Sensor Approach



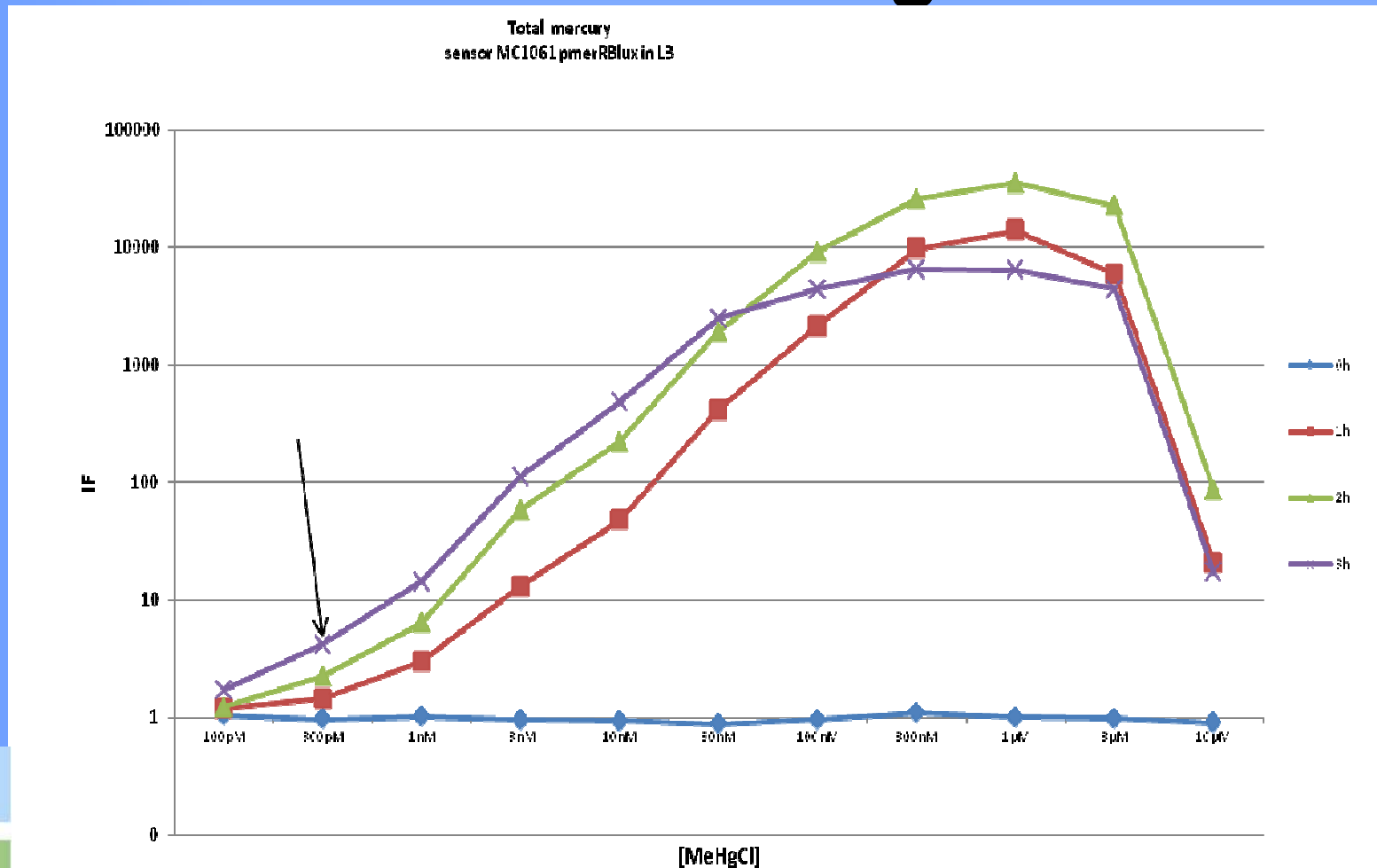
The presence of an analyte (**inorganic arsenic** or **methyl mercury**) triggers a reaction that results in the emission of visible light.





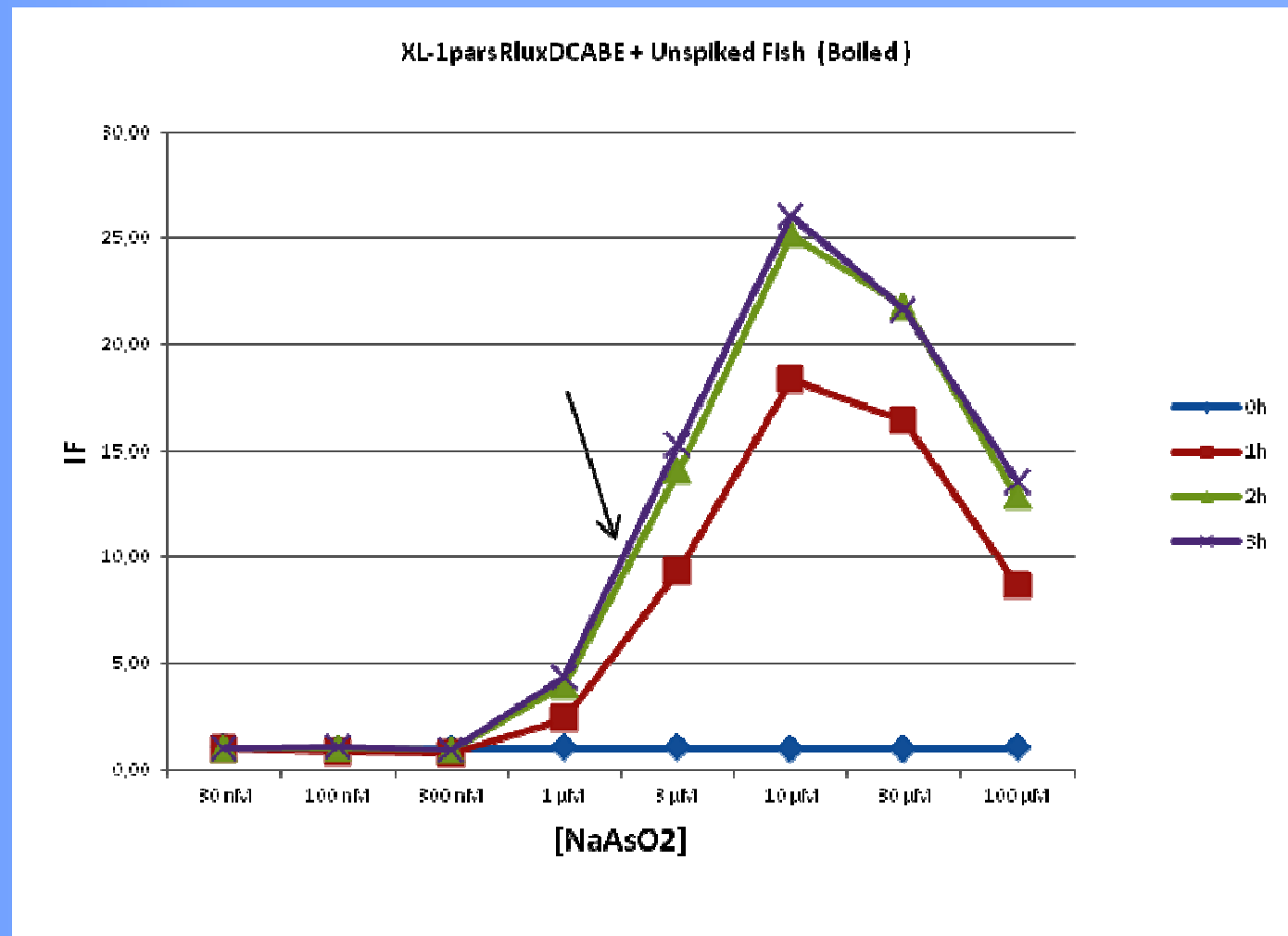
- ✓ ***E.coli*** bacterial bioluminescent proteins are encoded by the ***luxCDABE*** genes
 - The activity of the genes is controlled by a promoter element
 - The **promoter** is specific to a certain **analyte** (**inorganic arsenic or methyl mercury**, Ars-operon, Mer-operon)
 - **Mercuric sensor contains an extra gene coding for organic mercury degradation to ionic form**
 - The presence of the metal triggers the activation of the bioluminescent genes
 - Production of **bioluminescent proteins**
 - Emission of **visible light at 490 nm**

Total mercury sensor, standard curve for Methylmercury, pMolar sensitivity, wide dynamic measurement range



Arsenic sensor as tested by spiking fish and extracting the metalloid by boiling

The arrow shows concentration found from a arsenic containing sample (contains ~1,88 mg/kg iAs, target 3,2 mg/kg)





Work Package 9 (H'METALS)

www.biocop.org

BioCop

New Technologies to Screen Multiple Chemical Contaminants in Foods



**University of Tor Vergata,
Rome, Italy**



**Inst. of Biochemistry,
Vilnius, Lithuania**



Nestlé Research Center



Palm Instruments BV



www.biocop.org





Pb maximum admissible levels

www.biocop.org

BioCop

New Technologies to Screen Multiple Chemical Contaminants in Foods

Drinking water

10 $\mu\text{g/l}$

Air

0.5 $\mu\text{g/m}^3$

Foods

0,02-1 mg/Kg

milk, baby foods, meat,
fish, cereals, vegetables,
fruits and fruit juices,
oils and fats, wines

Milk = 20 ppb



www.biocop.org





RESEARCH LINES

www.biocop.org

New Technologies to Screen Multiple Chemical Contaminants in Foods

**- ELECTROCHEMICAL MEASUREMENT
(ASV)**

- SAMPLE TREATMENT



www.biocop.org

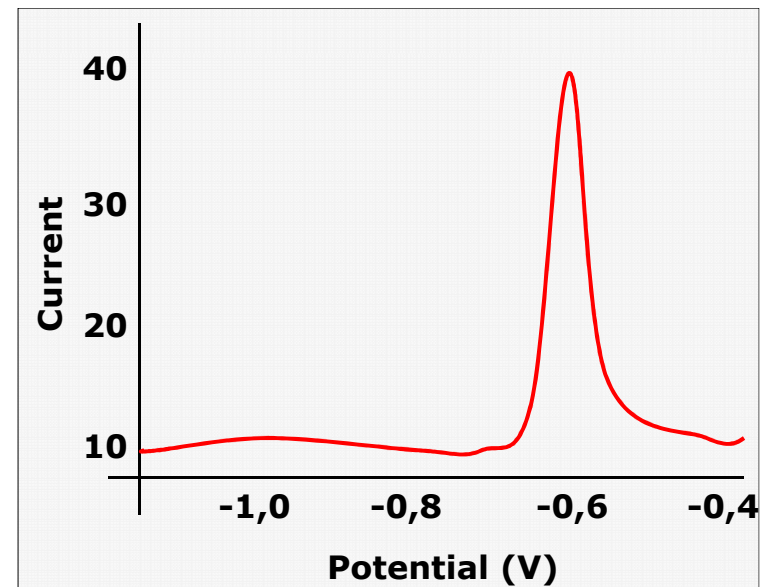
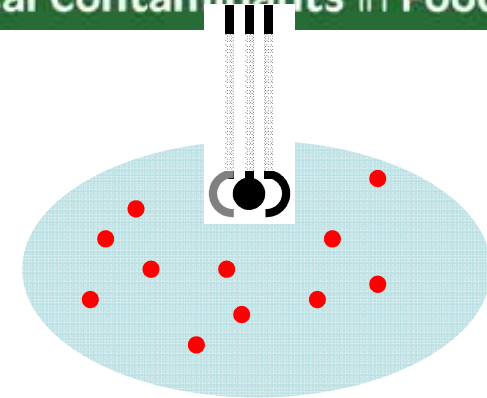


1 – PRE-CONCENTRATION STEP

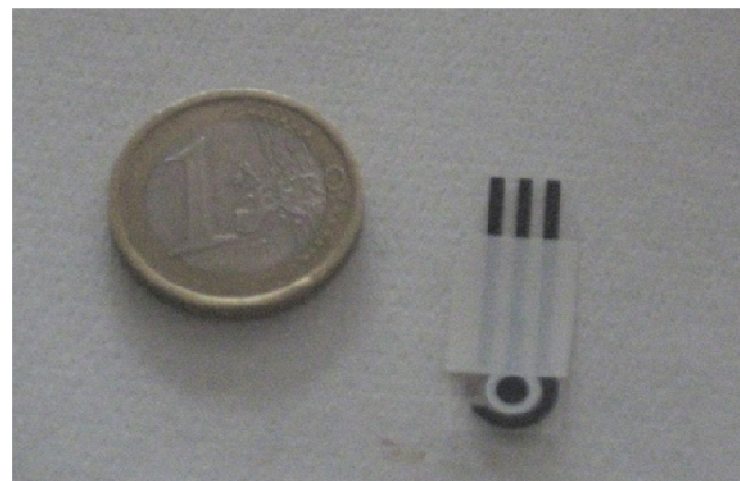
The lead Pb^{2+} is reduced and accumulates on the surface of the sensor on which a film of Hg (or other metals, e.g. **Bi**) has been deposited

2 – STRIPPING STEP

The lead is stripped away and a peak is recorded



SPE and Anodic Stripping Voltammetry carried out with the portable PalmSens Instrument



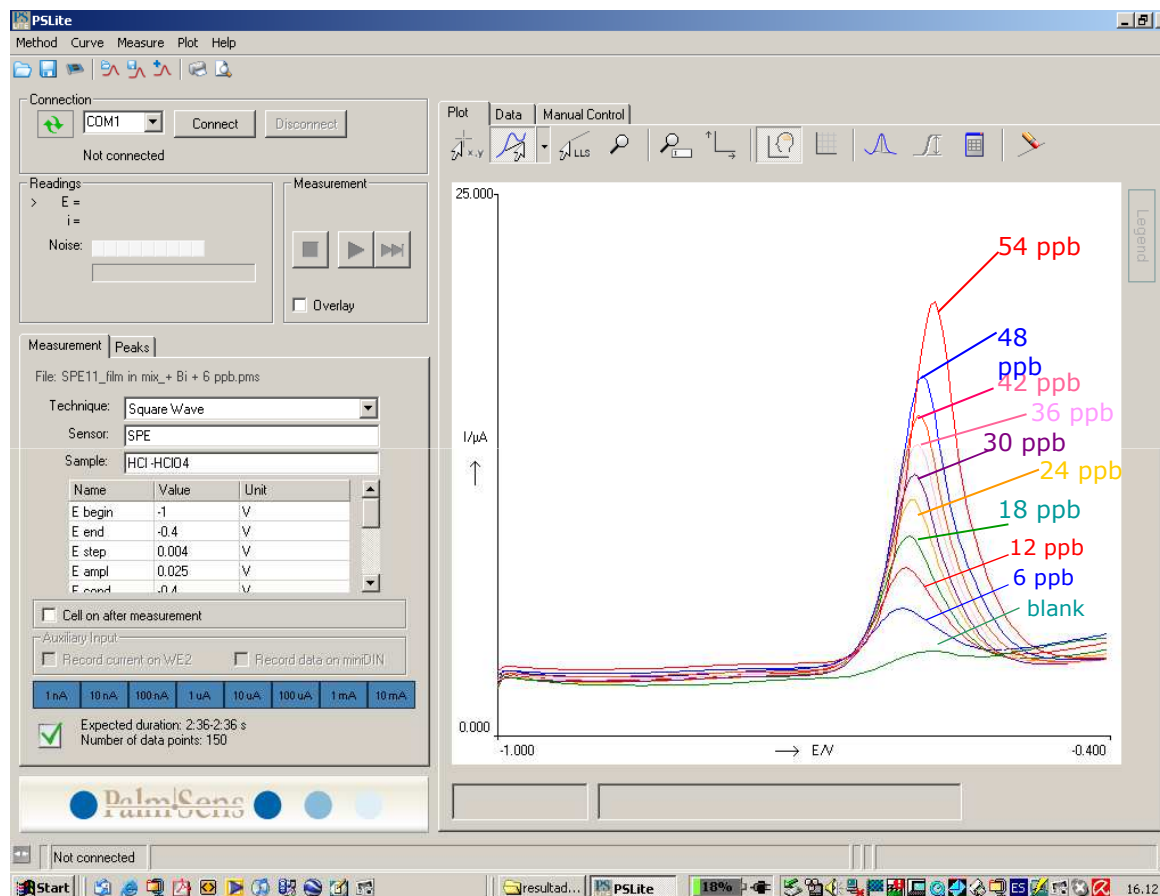
STUDIES CARRIED OUT:

- Type of Electrode (GC, CP, **SPE**)
- SPE (Bi, Hg, Hg+Bi, Bulk-modification with Bi_2O_3 , **Bi+Nafion**)
- Type of electrochemical techniques (DPV, **SWV**)
- Bi deposition (*in situ*; *ex situ*)
- Study of effect of buffer, pH and Nafion % (AcOH, HCl, HNO_3 , HClO_4 , **HCl+ HClO_4 , pH=2, Nf 0.5%**)

**Optimized SPEs +
Nafion + Bi**

SW Anodic Stripping Technique

E dep: -1,0 V
E end: -0,3 V
E beg: -1,0 V
E step: 4 mV
E ampl: 25 mV
E cond: -0,4 V
t dep: 120 sec
t cond: 30 sec
t eq: 5 sec
Freq: 100 Hz



All measurement were performed without removing oxygen from HCl/HClO₄, pH 2 solution

1. Dry samples overnight at **120°C**
2. Place sample in furnace at **250°C** and slowly raise to **350°C** until smoking ceases
3. Increase temperature slowly to **500°C**
4. Ash overnight at **500°C**
5. If ash is grey rather white: HNO_3 + 3 h of furnace

PROBLEMS:

- **LONG PROCEDURE (2 days)**
- **HIGH TEMPERATURES**

Problems....

- **Low recovery in spiked milk samples!!**

In literature: treatments with strong oxidizing agents such as ~~HNO₃~~, H₂O₂

Solutions....

⇒ **Introduction of HClO₄ and H₂O₂ in addition to HCl**

⇒ **Introduction of sonication steps**

Milk sample

- **Addition of H₂O₂ and Sonication for 30 min**
- **Addition of HClO₄ and Sonication for 15 min**
- **Addition of HCl and Centrifugation at 48000g for 10 min**
- **Filtration, dilution and adjustment to pH 2**

TOTAL TIME: 1 h



Validation carried out at ISS

www.biocop.org

New Technologies to Screen Multiple Chemical Contaminants in Foods



Istituto Superiore di Sanità
299 Viale Regina Elena
00161 - Roma (I)
Phone: +39 06 4990 1
Fax: +39 06 49 38 71 18

The Istituto Superiore di Sanità (ISS) is the leading technical and scientific public body of the Italian National Health Service. Its activities include research, control, training and consultation in the interest of public health protection and **Inspection, Monitoring and Certification**

The validation has been carried out at the FOOD LABORATORY (Heavy Metals section) of ISS

In accordance with the provisions of Regulation (EC) No 882/2004 of April, 29, 2004 it was decided to consider for evaluation (validation) of the method suitability the parameters given in **Regulation (EC) No 333/2007 OF THE COMMISSION of March. 28, 2007**



www.biocop.org



Performance criteria for methods of analysis for lead, cadmium, mercury and inorganic tin

Parameter	Value/Comment
Applicability	Foods specified in Regulation (EC) No 1881/2006
LOD	For inorganic tin less than 5 mg/kg. For other elements less than one tenth of the maximum level in Regulation (EC) No 1881/2006, except if the maximum level for lead is less than 100 µg/kg. For the latter, <u>less than one fifth of the maximum level</u>
LOQ	For inorganic tin less than 10 mg/kg. For other elements less than one fifth of the maximum level in Regulation (EC) No 1881/2006, except if the maximum level for lead is less than 100 µg/kg. For the latter, <u>less than two fifth of the maximum level</u>
Precision	HORRAT _T or HORRAT _R values of <u>less than 2</u>
Recovery	The provisions of point D.1.2. apply
Specificity	Free from matrix or spectral interferences



Milk validation: LOD and LOQ

www.biocop.org

BioCop

New Technologies to Screen Multiple Chemical Contaminants in Foods

blank	ppb
1	2,7
2	3,28
3	5,3
4	2,06
5	2,13
6	2,08
7	1,18
8	1,15
9	0,44
10	4,56
11	3,61
12	0,92
13	3,55
14	2,53
15	3,96
16	2,06
17	0,89
18	2,11
19	1,76
20	2,7

Xm_b (ppb)	2,4
SD	1.3
LOD = 3xSD	3.9
LOQ = 6xSD	7.8

'LOD' = Limit of detection, smallest measured content, from which it is possible to deduce the presence of the analyte with reasonable statistical certainty. The limit of detection is numerically equal to three times the standard deviation of the mean of blank determinations ($n > 20$).

'LOQ' = Limit of quantification, lowest content of the analyte which can be measured with reasonable statistical certainty. If both accuracy and precision are constant over a concentration range around the limit of detection, then the limit of quantification is numerically equal to six or 10 times the standard deviation of the mean of blank determinations ($n > 20$).

COMMISSION REGULATION (EC) No 333/2007



www.biocop.org



Statistical parameters in milk	
Meas. No.	20
Level of Pb in samples (ppb)	20
Mean (\bar{X}_m)	16.8
Sr	1.96
CV	11.7%

CVs for quantitative methods at a range of element mass fractions

Mass fraction	CV (%)
$\geq 10 \mu\text{g/kg}$ to $100 \mu\text{g/kg}$	20
$> 100 \mu\text{g/kg}$ to $1\,000 \mu\text{g/kg}$	15
$\geq 1\,000 \mu\text{g/kg}$	10

COMMISSION DECISION of 12 August 2002 (2002/657/EC)

$$\text{HORRAT}_r = \frac{\text{RSD}_r \text{ observed}}{\text{RSD}_r \text{ Horwitz (Thompson)}}$$

Where RSD_r is the the RSD calculated by a set of 20 independent measurements on the same milk sample, and RSD_r Horwitz is:

$$\text{RSD}_H = 0,67 \times 2^{(1-0,5 \log C)}$$

Horrat_r	
RSD_r	11,7%
C	16,8
adim C	1,67859E-08
log	-7,7750547
CV%	29,6
RSD_H%	19,8
Horrat_r	0,59
Limit Value	2



Milk validation: TRUENESS

www.biocop.org

BioCop

New Technologies to Screen Multiple Chemical Contaminants in Foods

Statistical parameters	
Reference material BCR 150	1000 ppb
Meas. No.	11
Mean (Xm)	871.6
Recovery	87.2 %
Sr	142.2
CV	16,3%
Bias	128.4
Bias %	12.8%

COMMISSION DIRECTIVE 2001/22/EC

Recovery

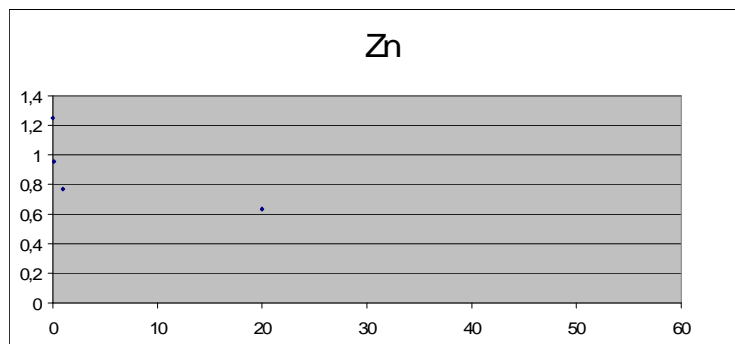
80-120 % (as indicated in the collaborative trial)



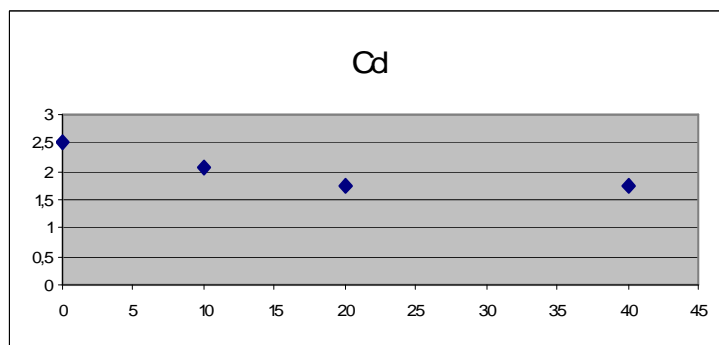
www.biocop.org



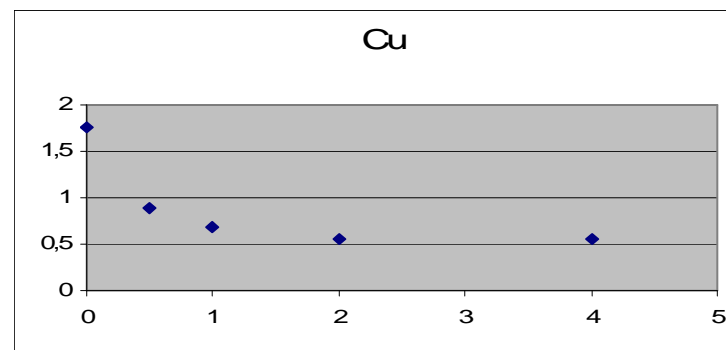
- 10 ppb Pb + increasing concentrations of interferents: Zn (ppm), Cd, Hg, Cu (ppb)



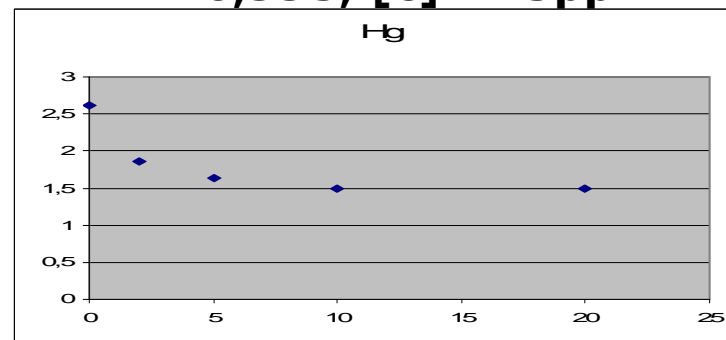
$R^2 = 0,965$, [c]=50ppm



$R^2 = 0,956$, [c]=22ppb



$R^2 = 0,995$, [c]=2.3ppm



$R^2 = 0,985$, [c]=9.4ppb

pH	variation
2,0	100%
1,8	93,5%
2,2	94,4%

variation < 10% were found
in the range of pH 1,8-2,2



Milk validation: UNCERTAINTY

www.biocop.org

New Technologies to Screen Multiple Chemical Contaminants in Foods

$$U_f = \sqrt{(LOD/2)^2 + (\alpha C)^2}$$

$$C = 0.020 \text{ mg/kg};$$

$$LOD = 0.004 \text{ mg/kg};$$

$$\alpha = 0.20$$

$$U_f = 0.005 \text{ mg/kg}$$

where:

U_f is the maximum standard measurement uncertainty ($\mu\text{g/kg}$);

LOD is the limit of detection of the method ($\mu\text{g/kg}$);

C is the concentration of interest ($\mu\text{g/kg}$);

α is a numeric factor to be used depending on the value of C. The values to be used are given in Table 8.

The uncertainty $U(X)$ has been calculated following the **Horwitz approach**, and resulted **equal to 5 ppb**

The measurement of blank milk samples fortified with 20 ppb can be expressed as **17 ± 5 ppb**



www.biocop.org



Procedure for PalmSens program

- Pre-treatment of screen printed (SPE) working electrode surface
 - Deposition of Nafion film (30 min drying)
 - Measurement of Pb 6 ppb (+Bi) in HCl+HClO₄+Ferrocyanide
 - Measurement of Pb 12 ppb (+Bi) in HCl+HClO₄+Ferrocyanide
 - Measurement in pre-treated milk (+Bi+Ferrocyanide)
 - Measurement in pre-treated milk + Pb 6 ppb (+Bi+Ferrocyanide)
 - Measurement in pre-treated milk + Pb 12 ppb (+Bi+Ferrocyanide)
- These 2 steps can be carried out in advance

PALMSENS DEDICATED PROGRAM

BioCop - Pb detection 2

Analysis info | Pretreat sensor | Precalibration | Analysis

Operator: X
 Sample name: SPE1
 Sample ID: 1
 Dilution factor: 1
 Started:

Solution	Color	Concentration (ppb)
Solution 1	Red	0
Solution 2	Green	10
Solution 3	Yellow	20
Solution 4	Purple	30

Number of calibration solutions: 4

Result: Recalculate Show Result

BioCop - Pb detection 2

Analysis info | Pretreat sensor | Precalibration | Analysis

Start

or put a drop of 100 µl on the sensor.

E = 1800 mV - 120 s
 E = 1800 mV - 60 s

- Remove electrode.
 - Wash with water and dry with filter paper.
 - Put 5 µl nafion solution (0.5%) just on the surface of the working
 - Let dry for 30 minutes at 37 °C.

Result: Recalculate Show Result

Underloads: 0 Overloads: 0

BioCop - Pb detection 2

Analysis info | Pretreat sensor | Precalibration | Analysis

Start

- Immerse sensor in buffer solution (0.05 M acetate buffer pH 4.6) or put a drop of 100 µl on the sensor.

E = 1800 mV - 120 s
 E = 1800 mV - 60 s

- Remove electrode.
 - Wash with water and dry with filter paper.
 - Put 5 µl nafion solution (0.5%) just on the surface of the working

Result: Recalculate Show Result

BioCop - Pb detection 2

Analysis info | Pretreat sensor | Precalibration | Analysis

Start

- Immerse the pre-treated sensor in 1 ppm Bi solution (in 5 or 10 ml of a solution 0.44 M HCl and 0.44 M HClO₄, pH 2)

Square wave voltammetry

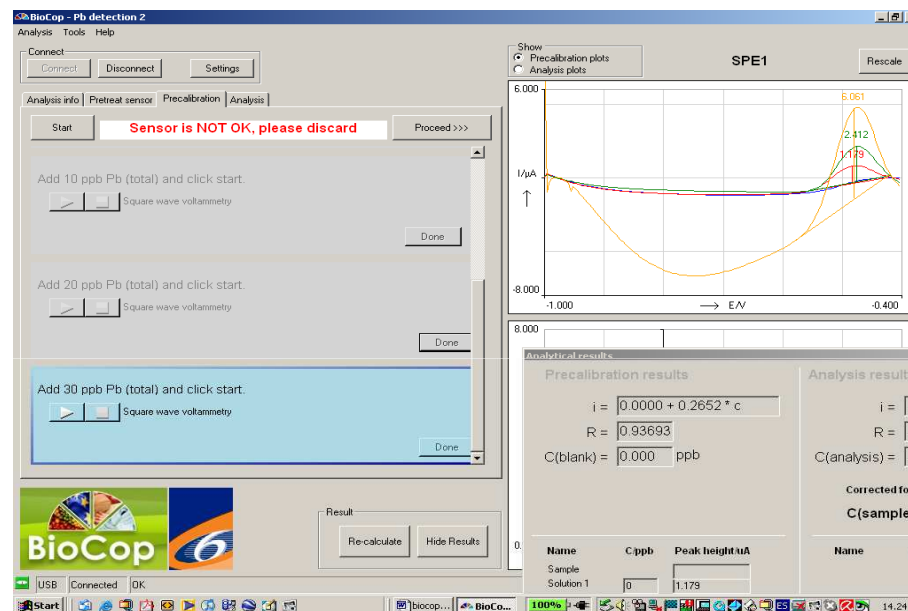
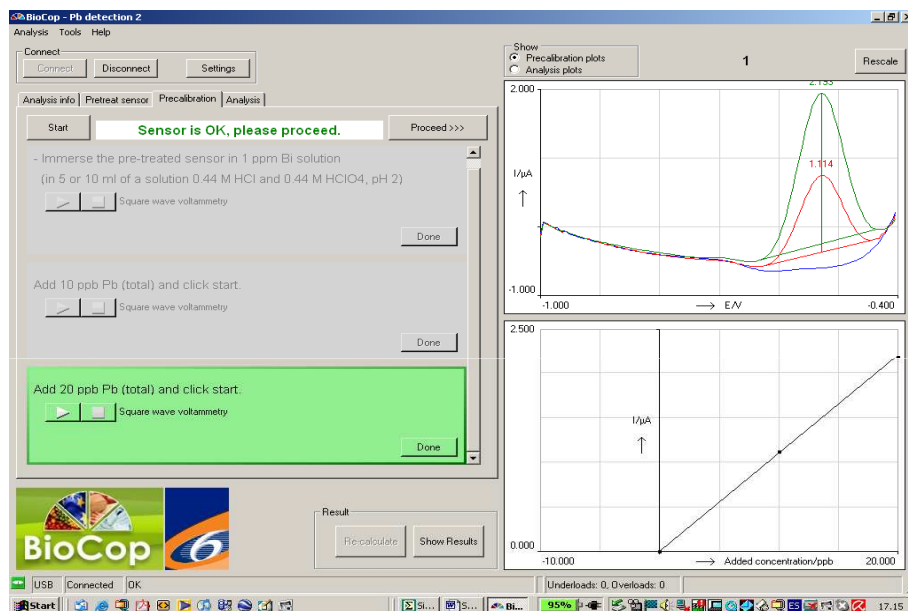
Add 0 ppb Pb (total) and click start.
 Square wave voltammetry

Add 10 ppb Pb (total) and click start.
 Square wave voltammetry

Result: Recalculate Show Results

Underloads: 0 Overloads: 0

PALMSENS DEDICATED PROGRAM



BioCop - Pb detection 2

Analysis Tools Help

Connect:

Analysis info | Pretreat sensor | Precalibration | Analysis

Start

Analytical results

Precalibration results

$i = 0.0000 + 0.1097 * c$

R = 0.99991

C(blank) = 0.000 ppb

Analysis results

$i = 1.6587 + 0.5620 * c$

R = 0.97276

C(analysis) = 2.951 ppb

Corrected for blank and dilution:

C(sample) = 7.378 ppb

Name	C/ppb	Peak height/uA
Sample		
Solution 1	10	1.114
Solution 2	20	2.193

Name	C/ppb	Peak height/uA
Sample		1.659
Solution 1	10	5.65
Solution 2	20	12.899

Result:

USB Connected OK

Underloads: 0, Overloads: 0

1

→ E/V -0.400

→ Added concentration/ppb 20.000



Thanks

for the attention