Pražské analytické centrum inovací

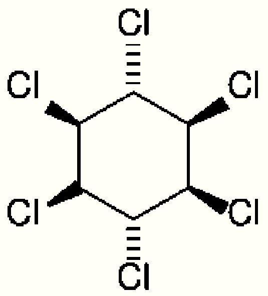
Projekt CZ.04.3.07/4.2.01.1/0002 spolufinancovaný ESF a Státním rozpočtem ČR

Trends in sample preparation: innovative strategies from trace elements to metalloproteins

Marco Aurélio Zezzi Arruda
Associate Professor
GEPAM - http://gepam.iqm.unicamp.br
e-mail: zezzi@iqm.unicamp.br

Grupo de Espectrometria, Preparo de Amostras e Mecanização

Sample preparation: why?



Lindane determination in vegetable samples

1,2,3,4,5,6-hexachlorocyclohexane

Homogenization

Can the sample be directly injected ???

Water: 90 – 96%

Fibers: 0.4 – 1.8%

Starch: 0 – 0.6%

Salts: 0.4 – 0.7%

Fat: 0 – 1.3%

Proteins: 0.7 – 1.2%

NON VOLATILE MATERIALS PRESENT IN SAMPLE MATRIX

Incompatible with GC!!!

(columns/injectors occlusion)
(contaminant introduction)
(artifacts formation)

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OUTLINE

from trace elements to metalloproteins

CPE
→Cd
→Proteins

US/MAWE
→Inorganic
→Organic

MIP
→Catechol

Miscellaneous
→Metalloproteins

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Unicamp: Institute of Chemistry

87 teachers → 4 areas
ca. 400 under-graduation students
ca. 300 MSc/PhD students

Unicamp: Institute of Chemistry

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Unicamp: GEPAM

4 main research areas
-Sample preparation
-Bio-analytical
-Spectrometry
-Mechanization

4 under-graduation students
7 MSc/PhD students
2 Post-doc collaborators

Unicamp: GEPAM

Adilson
Alessandra
Aline Lopes
Ana Cristi
Cristiana

Eduardo
Eraldo
Fabiana
Herbert
Marcelo
Márcia
Pedro
Renata

OUTLINE

CPE

→Cd

→Proteins

from trace elements to metalloproteins

US/MAWE

→Inorganic

→Organic

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SURFACTANT

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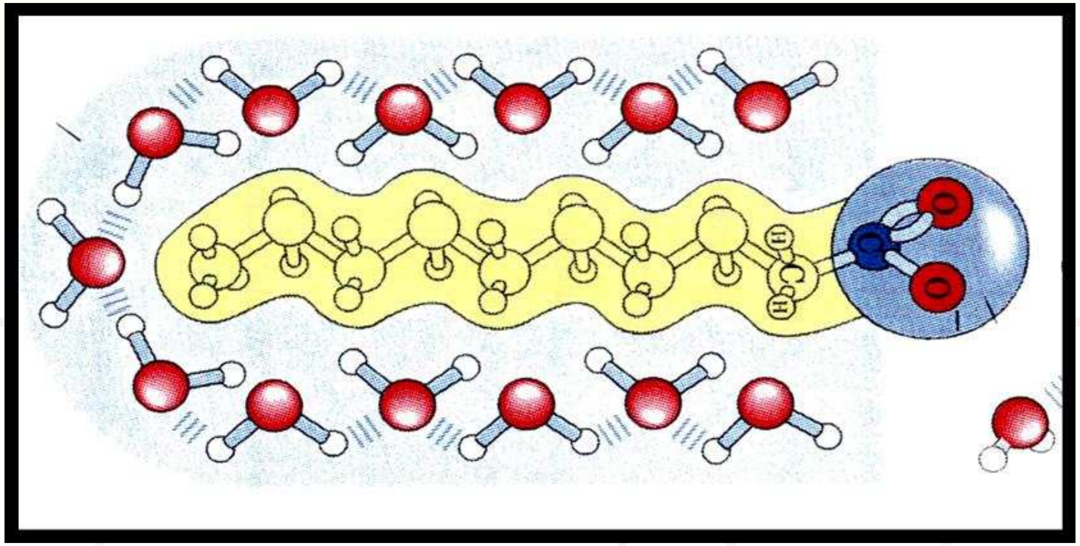
(*Surface active agent*)



R-X structure

R: hydrocarbon chain (8-18 atoms)

X: polar or ionic head group

hydrophilic + hydrophobic groups → dissolution in water or other solvents

SURFACTANT

•Non-ionic

Brij 35, Polyoxyethylene(23); $CH_3(CH_2)_{11}(OCH_2CH_2)_{23}OH$

•Anionic

SDS, Sodium dodecil sulfate; $CH_3(CH_2)_{11}OSO_3^{-}Na$

•Cationic

CTAB, Cetyl trimetyl ammonium bromide; $CH_3(CH_2)_{15}N^{+}(CH_3)_3Br$

•Amphoteric (Zwitterionic)

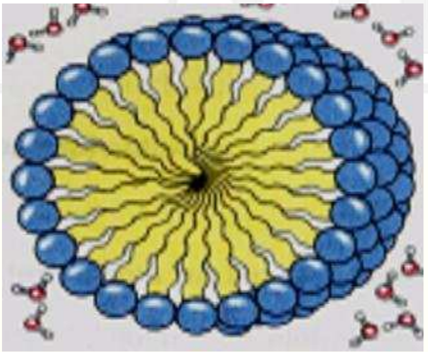
DAB, 4-(Dodecyldimetyl ammonium) butirate

$CH_3(CH_2)_{11}N^{+}(CH_3)_2(CH_2)_3COO^{-}$

The aggregate micellar formation

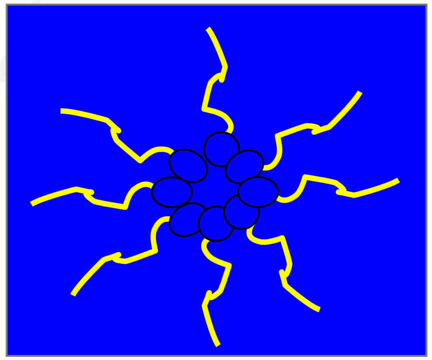
Amphiphilic molecules → self association in several solvents

organized medium (microscopically ordered molecular aggregates)



normal micelle

bi-layers
vesicles
micro emulsions



reversed micelle

final structure: monomer, solvent, surrounding ions

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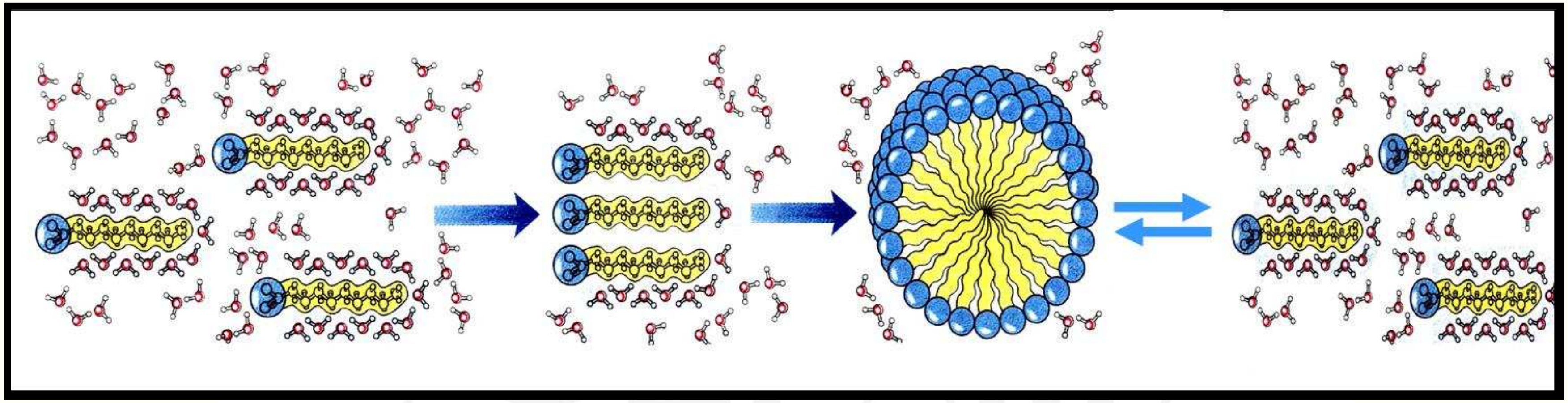
The aggregate micellar formation



monomers
(below the CMC)

micelles and monomers in dynamic equilibrium
(above the CMC)

Micellar medium in analytical chemistry

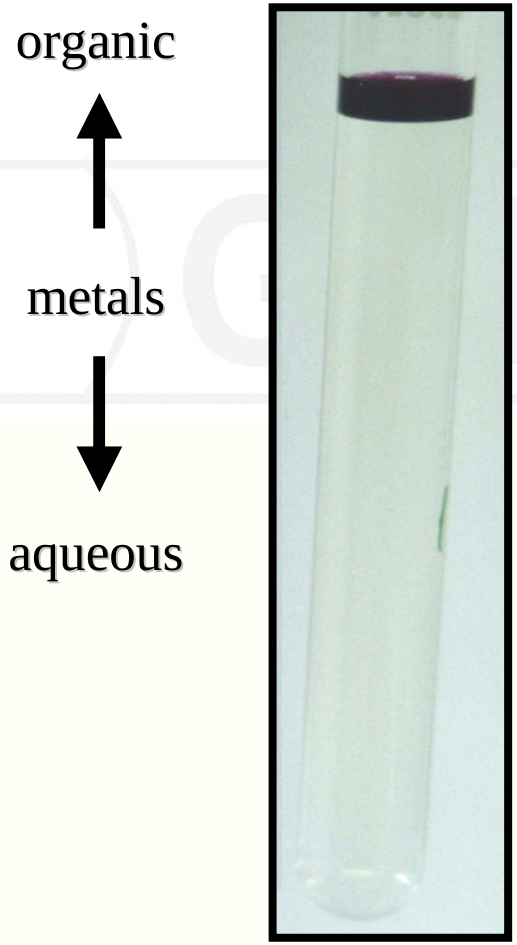
-water solubilization of hydrophobic substances
-enhancing detection (spectrophotometric methods)
-improvement on transport and nebulization (AAS methods)
-catalytic reactions
-extraction
-preconcentration

} **CPE**

Cloud point extraction – CPE
Extraction mechanism

Bezerra et al., *App. Spectrosc. Review* 40(2005)269

surfactant molecules act as organic solvent

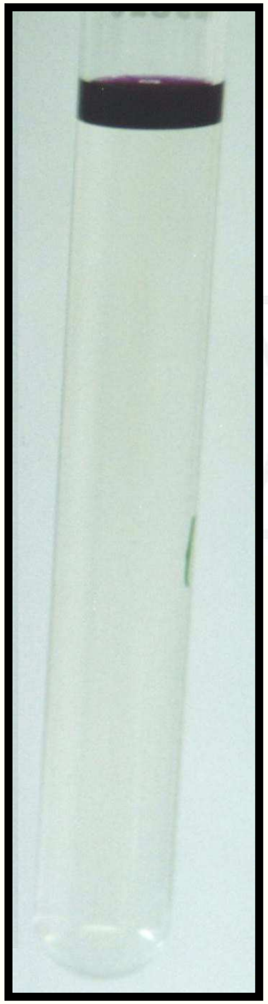


CPE efficiency:

1) formation constant of the metallic complexes;
2) kinetics of complexation reaction;
3) phase transference of metallic species or chelate into micelle medium

Cloud point extraction – CPE
Extraction mechanism

Bezerra et al., *App. Spectrosc. Review* 40(2005)269



- hydrated nature of the surfactant phase → distribution coefficient (D) lower than conventional LLE

- LLE: > ionic strength does not seriously modify extraction efficiency; CPE: salt addition → increase on phase separation

Cloud point extraction – CPE

Extraction mechanism

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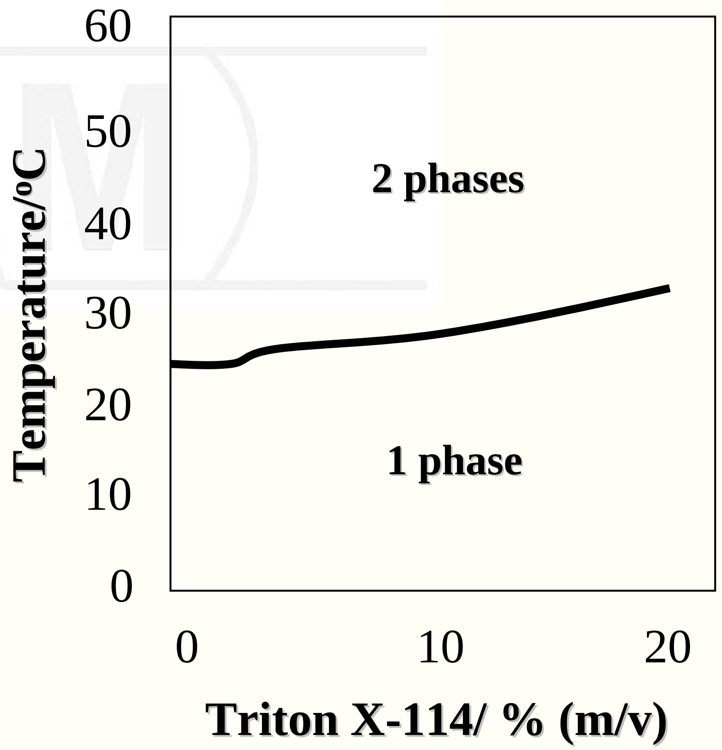
Gu & Sjöblom, *Colloids Surf.* 64(1992)39

Surfactant	T/°C
C_6E_3	40.5
$C_{10}E_4$	19.7
$C_{12}E_5$	28.9
$C_{14}E_8$	70.5
Triton X-114	22.0
Triton X-100	*ca.* 64

cmc/mmol L^{-1}

Triton X-100: 0.17 – 0.30

Triton X-114: 0.20 – 0.35



$$C(^oC) = 220\log N_E - 5.5N_C - 55$$

N_E = number of ethylene oxide

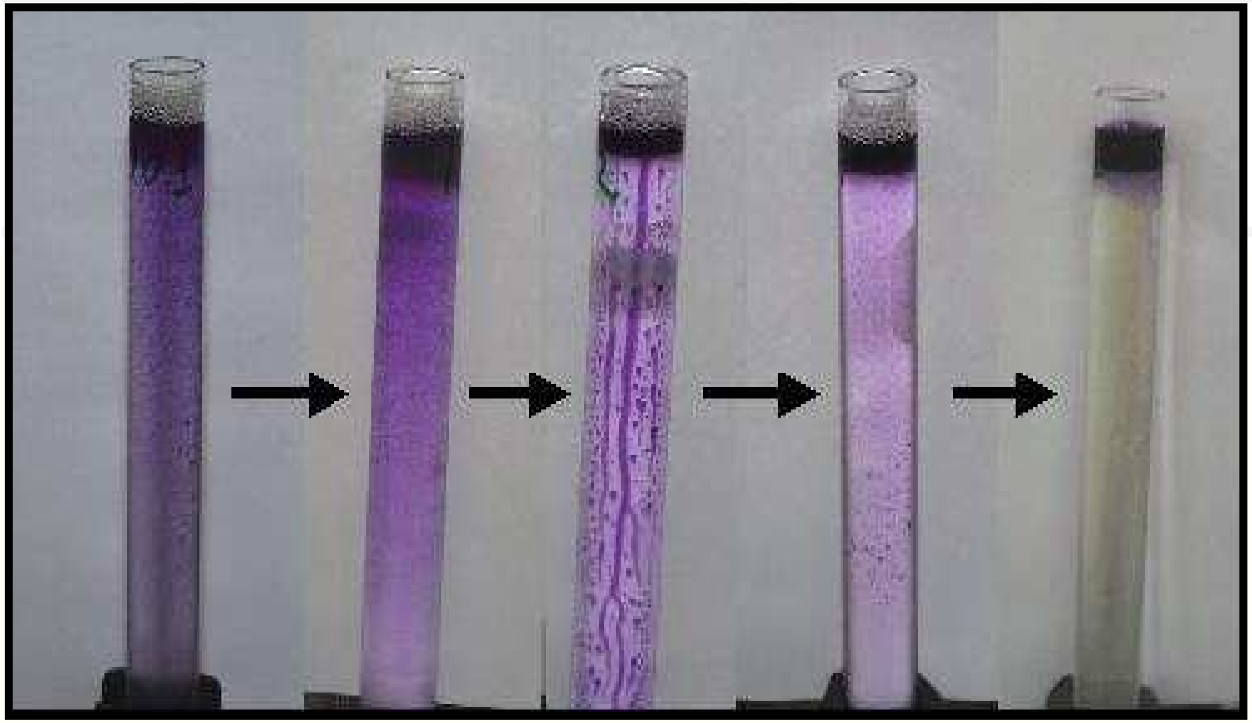
N_C = number of alquil carbons

Cloud point extraction – CPE
Temperature modification

- ✓**Ionic surfactants**
- ✓**Inorganic salts**
- ✓**Mineral acids**
- ✓**Pressure**

Cloud point extraction – CPE

Co
+ 5-Br-PADAP
+ SDS + Triton X-100
+ NaCl
pH = 9



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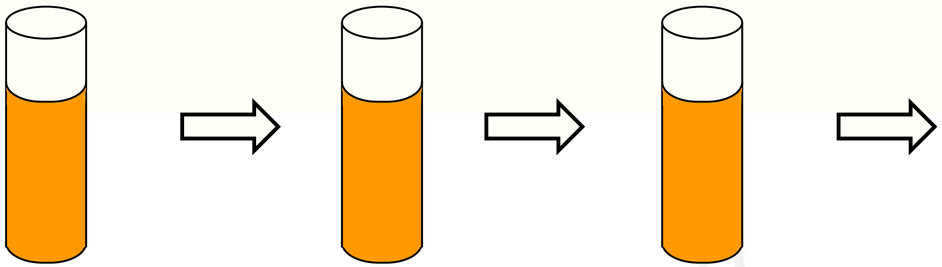
Cloud point extraction – CPE: Cd

Coelho, <u>et al</u>. *Talanta* 71(2007)353

DDTP



Cd (8.0 mL)
0.32 mol/L HCl,
(0.5 mL)

DDTP (1.5 mL)
complexing
time
→ 0 - 20 min

TX-114
(1.5 mL)

NaCl
(0.5 – 5 % m/v)
(200 μL)

2500 rpm (10 min)

rich phase
diluted (0.4 mL) with 9:1 (v/v)
ethanol and HNO_3

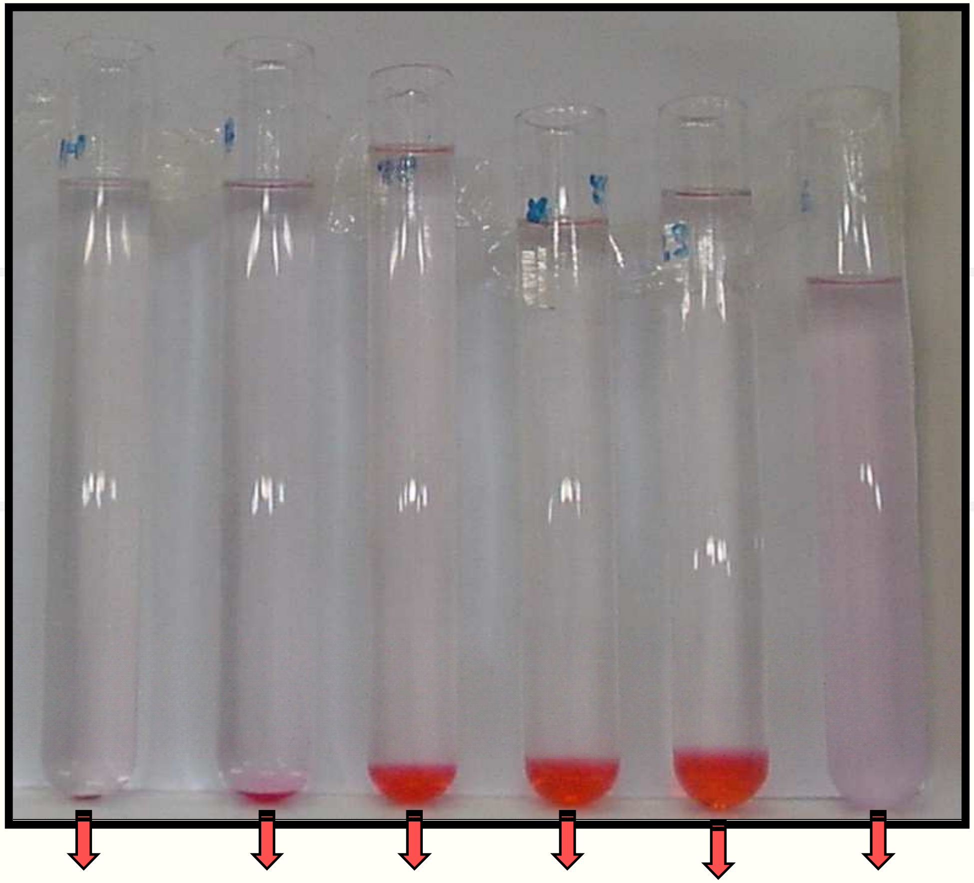
Cloud point extraction – CPE: Cd

Studied variables :

→complexing time
→nature of complexing agent
→complexing agent concentration
→surfactant concentration
→volume ratio between Cd and surfactant
→electrolyte concentration
→ethanol volume

Cloud point extraction – CPE: Cd

rich phase: Triton X-114 (m/v)



0.04% **0.08%** **0.13%** **0.44%** **0.86%** **0.44% - before centrifuging**

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Cloud point extraction – CPE: Cd

Analytical performance

Linear equation	Y = 0.0026 + 0.0018X
r	0.997
Linear range (µg/L)	3 - 400
Limit of detection (µg/L)	0.9
Limit of quantification (µg/L)	2.9
Precision (100 µg/L)	4% (n=11)
CF	19

Cloud point extraction – CPE: Cd

Cd determination (µg/g) in tobacco samples by FAAS and ETAAS (n=4)

Sample	FAAS	ETAAS
1	0.546 ± 0.050	0.479 ± 0.038
2	0.437 ± 0.036	0.437 ± 0.013
3	0.353 ± 0.022	0.364 ± 0.019
4	0.229 ± 0.022	0.250 ± 0.025

Cloud point extraction – CPE: Cd

Sample	Cd added (µg L^{-1})	Cd found (µg L^{-1})	Recovery[a] (%)
Mineral water	0	---[b]	-
	10	10.7 ± 1.8	107
	20	20.7 ± 4.8	104
Lake water	0	---[b]	-
	10	11.4 ± 1.0	96
	20	22.0 ± 1.8	101
Physiological serum	0	---[b]	-
	10	10.5 ± 1.5	105
	20	18.6 ± 0.2	93
Tobacco 1	0	10.9 ±1.0	-
	4	15.3 ± 1.4	103
Tobacco 2	0	8.7 ±0.7	-
	4	13.4 ± 1.6	106
Tobacco 3	0	7.1 ±0.5	-
	4	11.5 ± 0.5	104
Tobacco 4	0	4.3 ±0.4	-
	4	8.6 ± 0.6	103

[a] recovery in fortified samples; [b] < LOQ

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Cloud point extraction – CPE: Organics

Quina & Hinze, *Ind. Eng. Chem. Res.* 38(1999)4150

PAHs – river water
$C_{12}E_4$, 40 ºC, HPLC-F (fluorescence)

Fungicides (captan, captafol) – river water
Triton X-114, 40 ºC, HPLC-EC (electrochemical detector)

Fulvic acid – river water
Triton X-100, 90 ºC, HPLC-UV (ionic pair)

Phenol – aqueous solution
PONPE-10, 70 ºC, GC-FID

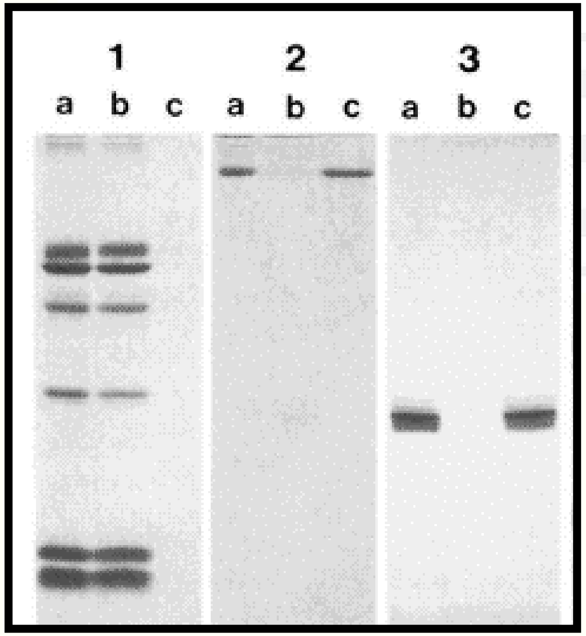
Cloud point extraction – CPE: Proteins

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 256, No. 4, Issue of February 25, pp. 1604-1607, 1981
Printed in U.S.A.

Phase Separation of Integral Membrane Proteins in Triton X-114 Solution*

(Received for publication, March 31, 1980, and in revised form, October 6, 1980)

Clément Bordier‡
From the Biozentrum der Universität Basel, Klingelbergstr. 70, CH-4056 Basel, Switzerland



a = before phases separation

b = aqueous phase after separation

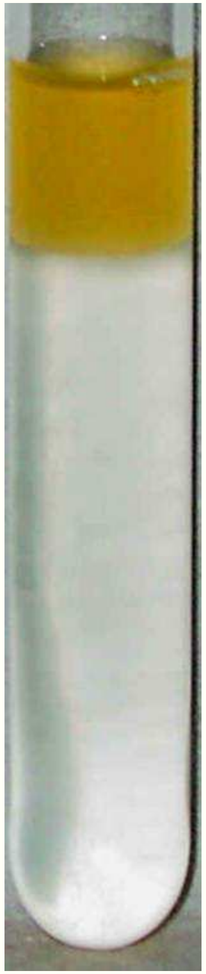
c = surfactant phase after separation

1. serum albumin, catalase, ovalbumin, concanavalin A, myoglobin and cytochrome c;

2. human erythrocyte acetylcholin-esterase; **3.** bacteriorhodopsin

Cloud point extraction – CPE: Proteins
Extraction mechanisms

Nikas et al., *Macromolecules* 25(1992)4797



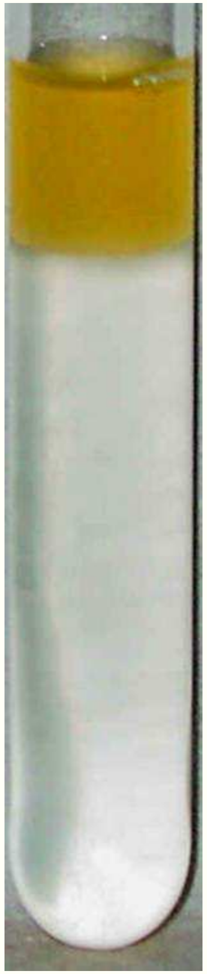
$$K_p = \exp\left\{-(\phi_t - \phi_b)(1 + R_p / R_0)^2\right\}$$

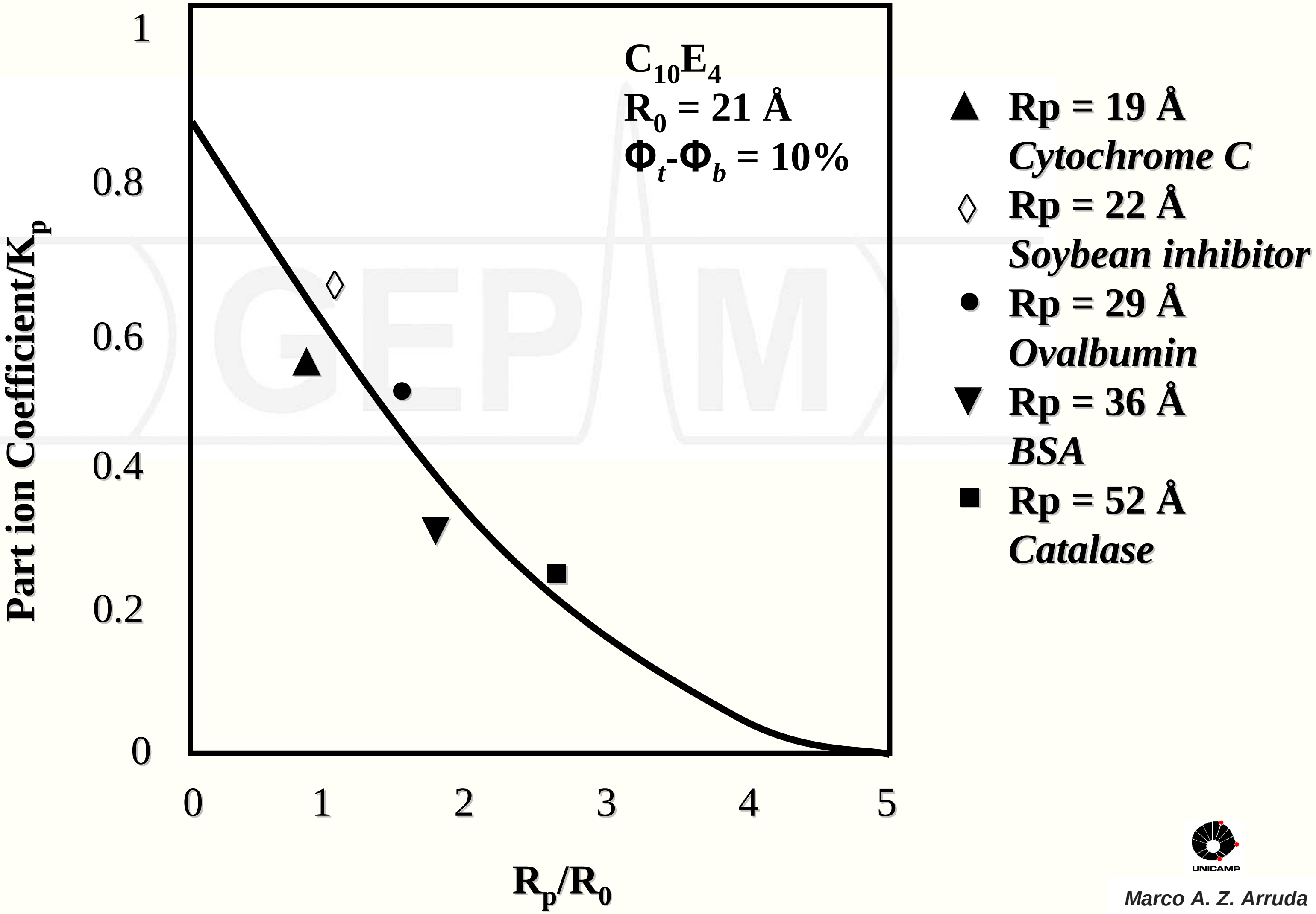
$$K_p = \exp\left\{-(\phi_t - \phi_b)(1 + R_p / R_0)^3\right\}$$

Φ_t = top fraction volume
Φ_b = bottom fraction volume
R_p = protein hydrodynamic radius
R_0 = transversal section radius of the cylindrical micelle (or spherical)

Cloud point extraction – CPE: Proteins
Extraction mechanisms

Tani et al., *Anal. Sci.* *14*(1998)875





Cloud point extraction – CPE: Proteins

Tani et al., *J. Cromatogr. A* 780(1997)229

Bacteriorodopsin, hemoglobin in serum
Triton X-114, 0.15 mol/L NaCl (pH 7.4), 30 ºC

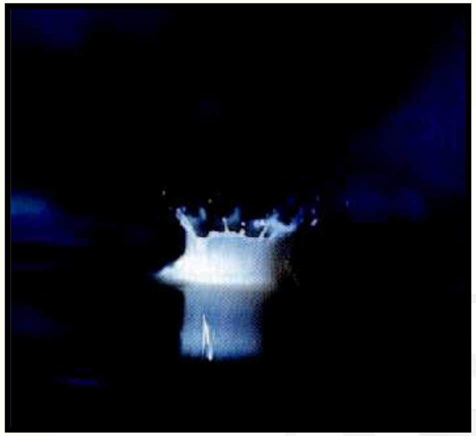
Cytochrome b_5, cytochrome c_1, Fe-S proteins
in bacteria
Triton X-114, 0.05-0.15 mol/L NaCl (pH 7.5), 30 ºC

Pyruvate oxidase in *Escherichia Coli*
Triton X-114, 0.15 mol/L NaCl, (pH 6.0), 30 ºC

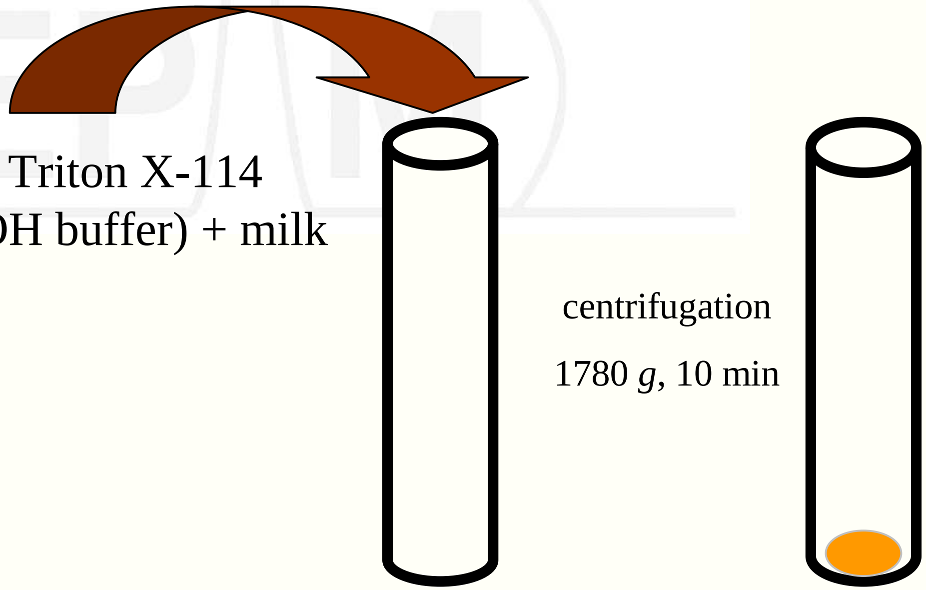
Tyrosinase in mushroom
Triton X-114 (pH 7.3), 37 ºC

Cloud point extraction – CPE
Proteins in milk

Lopes et al., *Anal. Chim. Acta* 590(2007)166



$NaCl_{(s)}$, 8 mL Triton X-114
(in KH_2PO_4/NaOH buffer) + milk



Optimized conditions:

• 1% (w/v) Triton X-114;

•50 µL – sample volume

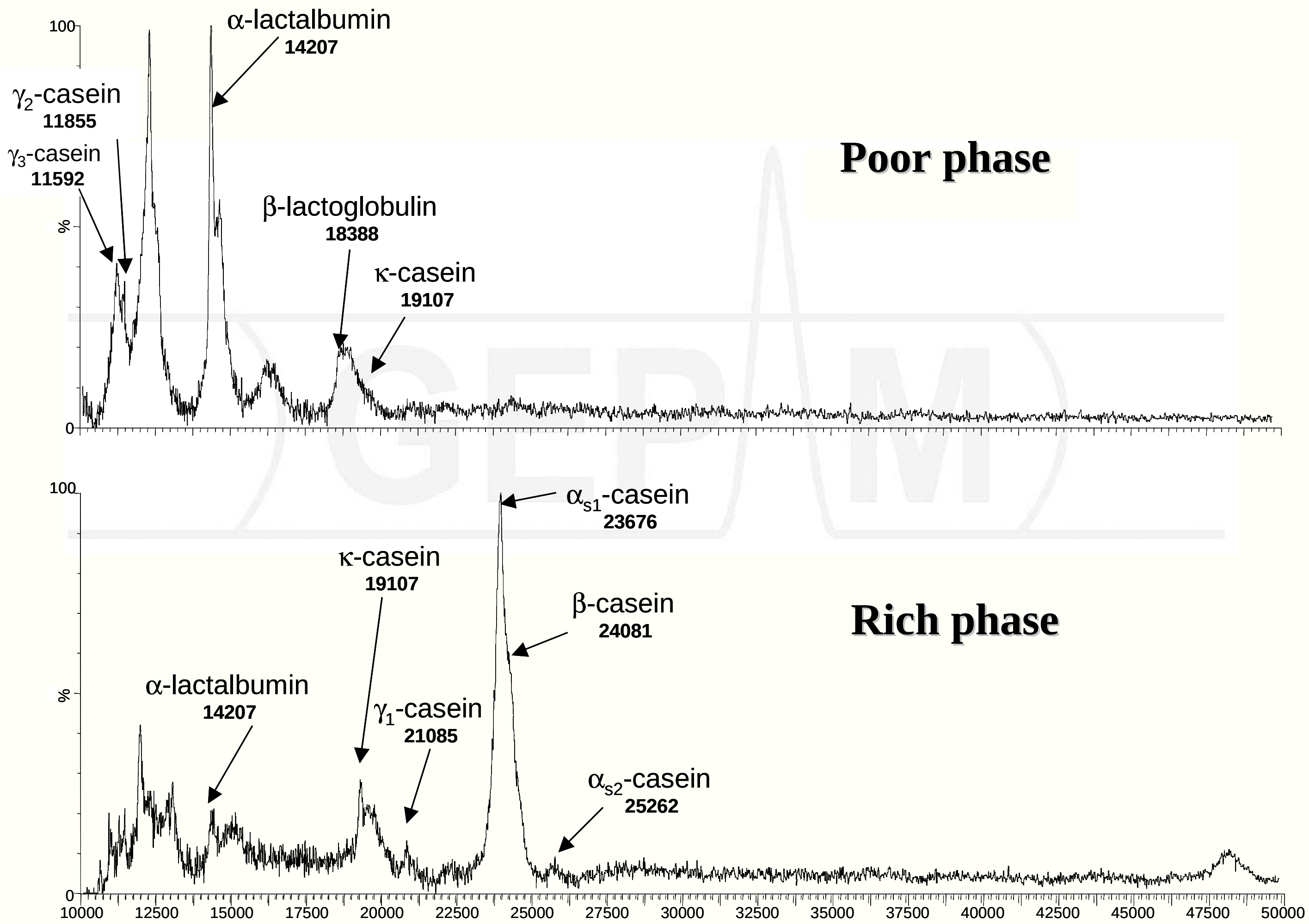
•6% (w/v) NaCl,

•pH = 7.0

✓ Rich phase: surfactant removed using acetone

✓ Protein quantification: Bradford method

Cloud point extraction – CPE
Proteins in milk



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Cloud point extraction – CPE
Proteins in milk

Phase	Protein	MM (Da)		Deviation (%)
		Literature	MALDI-MS	
Poor	γ_3 -Casein	11500	11592	0.8
	γ_2 -Casein	11800	11855	0.5
	α- Lactalbumin	14200	14207	0.05
	β-Lactoglobulin	18360	18388	0.2
	κ-Casein	19000	19107	0.6
Rich	α- Lactalbumin	14200	14207	0.05
	κ-Casein	19000	19107	0.6
	γ_1-Casein	21000	21085	0.4
	α_{s1}-Casein	23600	23676	0.3
	β-Casein	24000	24081	0.3
	α_{s2}-Casein	25250	25262	0.05

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Cloud point extraction – CPE
Some references

C. Bordier, *J. Biol. Chem.*, 236(1981)1604

K. Selber et al., *Process Biochem.*, 39(2004)889

T. Minuth et al., *Biotechnol. Bioeng.*, 55(1997)339

G. L. McIntire, *Critical Reviews in Anal. Chem.*, 21(1990)257

M. A. Bezerra et al., *Appl. Spectrosc. Reviews*, 40(2005)269

Cloud point extraction – CPE
Conclusions

good alternative
for extracting metals
and bio-molecules

biocompatible
system

CPE

good CF

safe, fast and low cost procedure

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OUTLINE

CPE

→Cd

→Proteins

US/MAWE

→Inorganic

→Organic

from trace elements to metalloproteins

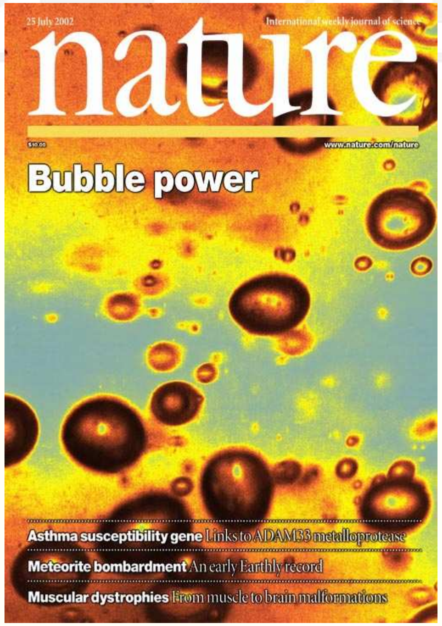
MIP

→Catechol

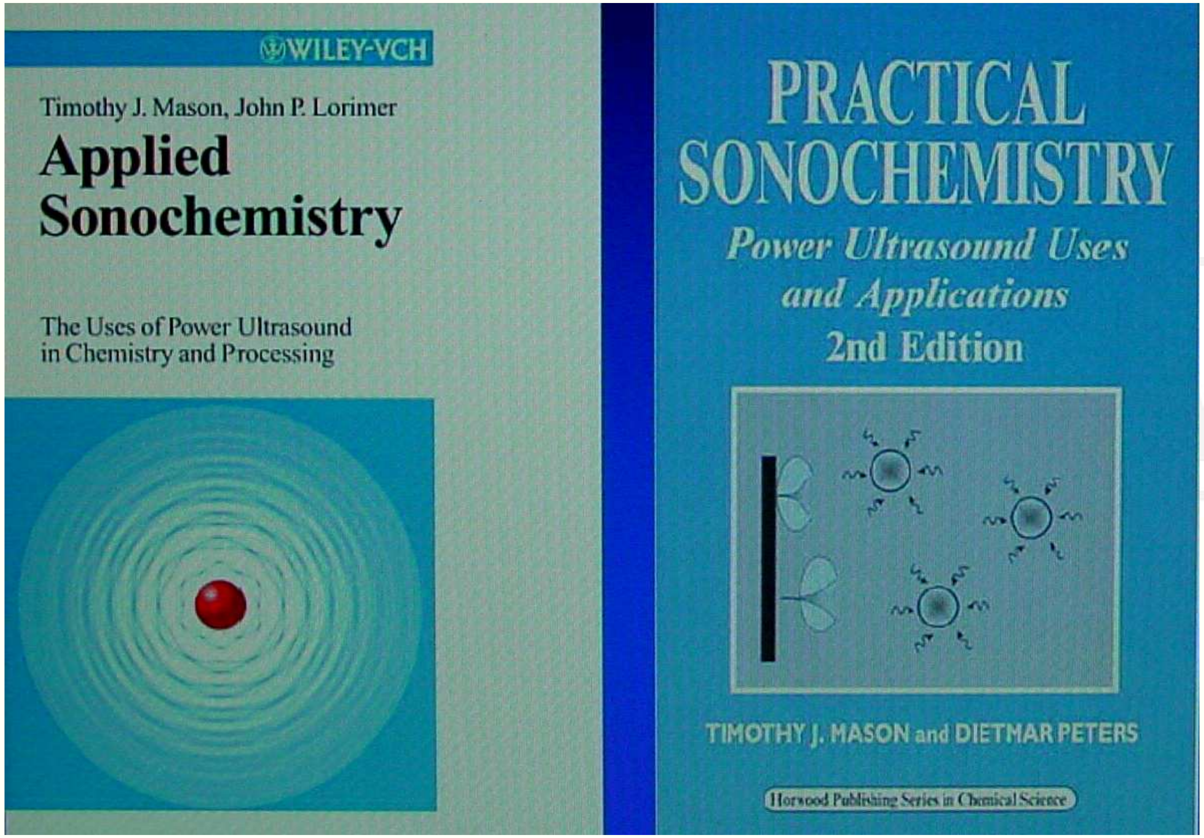
Miscellaneous

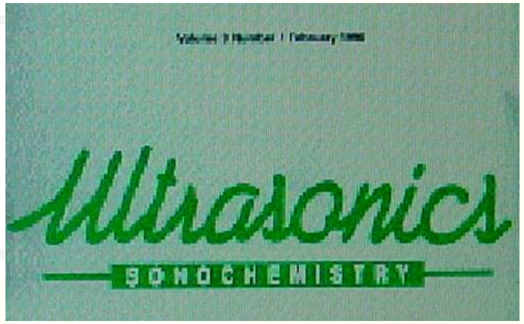
→Metalloproteins

- Sound: propagation of mechanical waves through successive compression and expansion cycles in the medium
- Ultrasound: sound presenting frequency higher than 16 kHz
  - Sonochemistry: study of ultrasonic waves influence on chemical systems



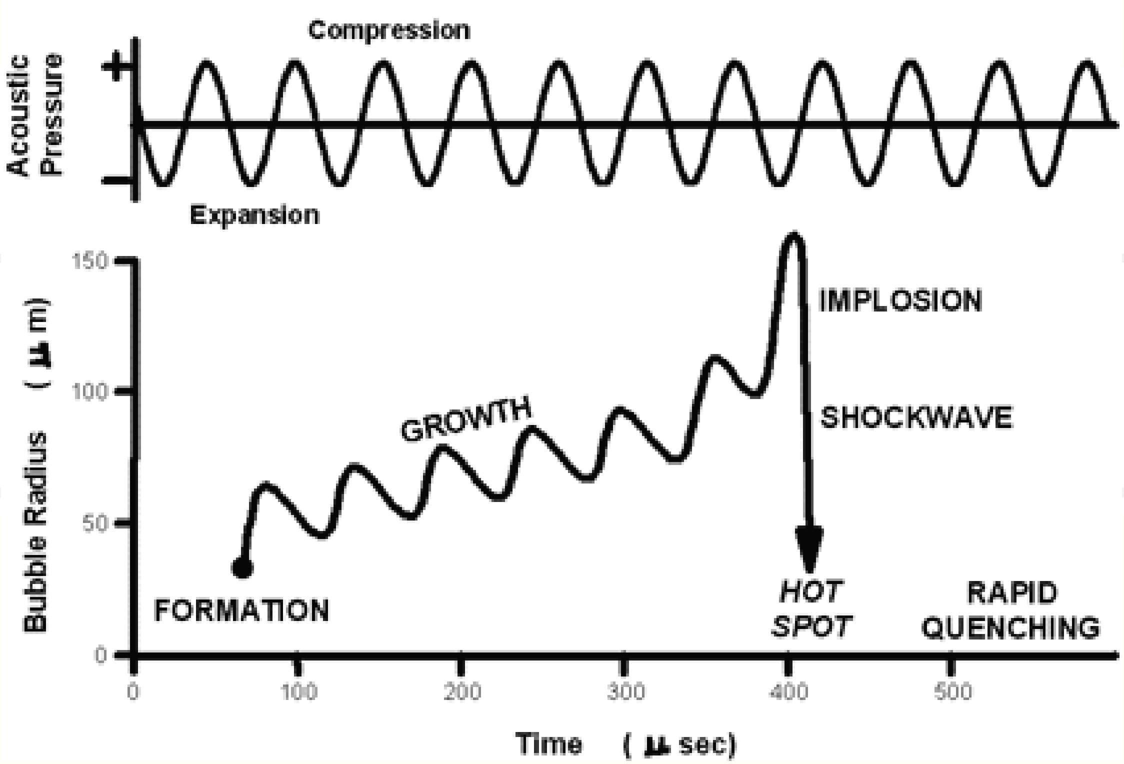
SONOCHEMISTRY





CAVITATION

Process of formation, growth and collapse of micro bubbles in liquids



…a way to concentrates the ultrasound energy to a chemically useful form

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CAVITATION

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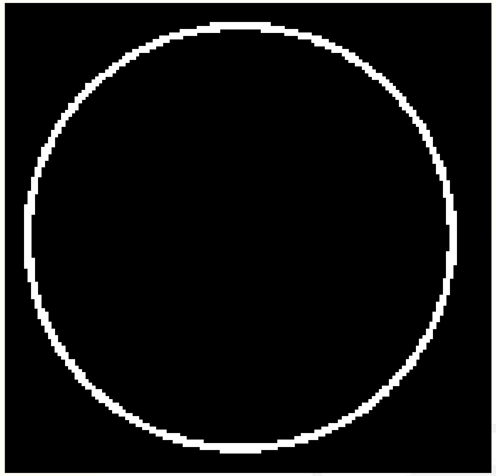
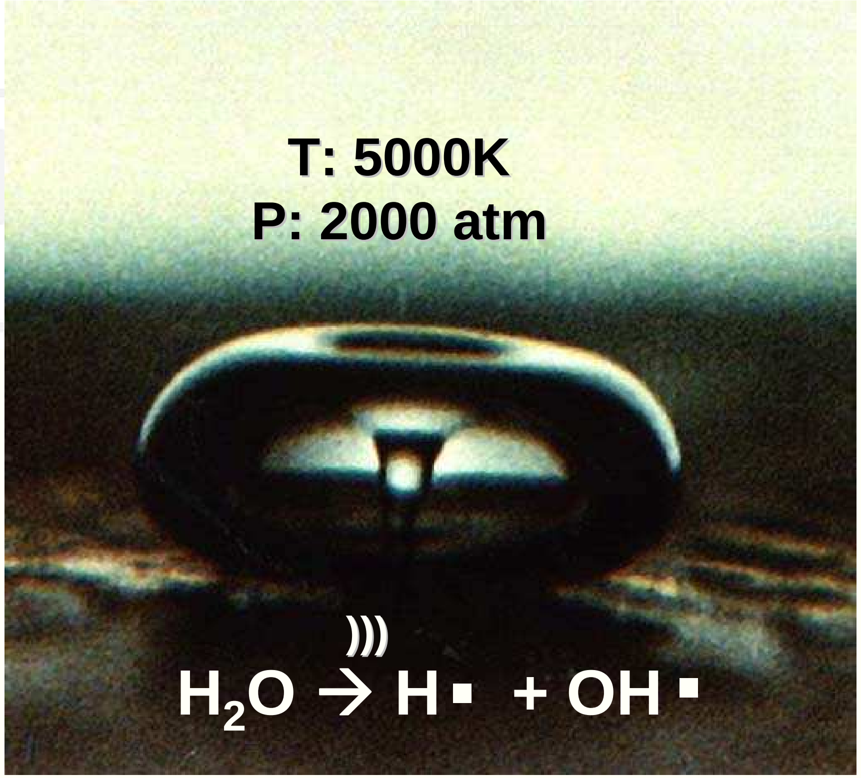
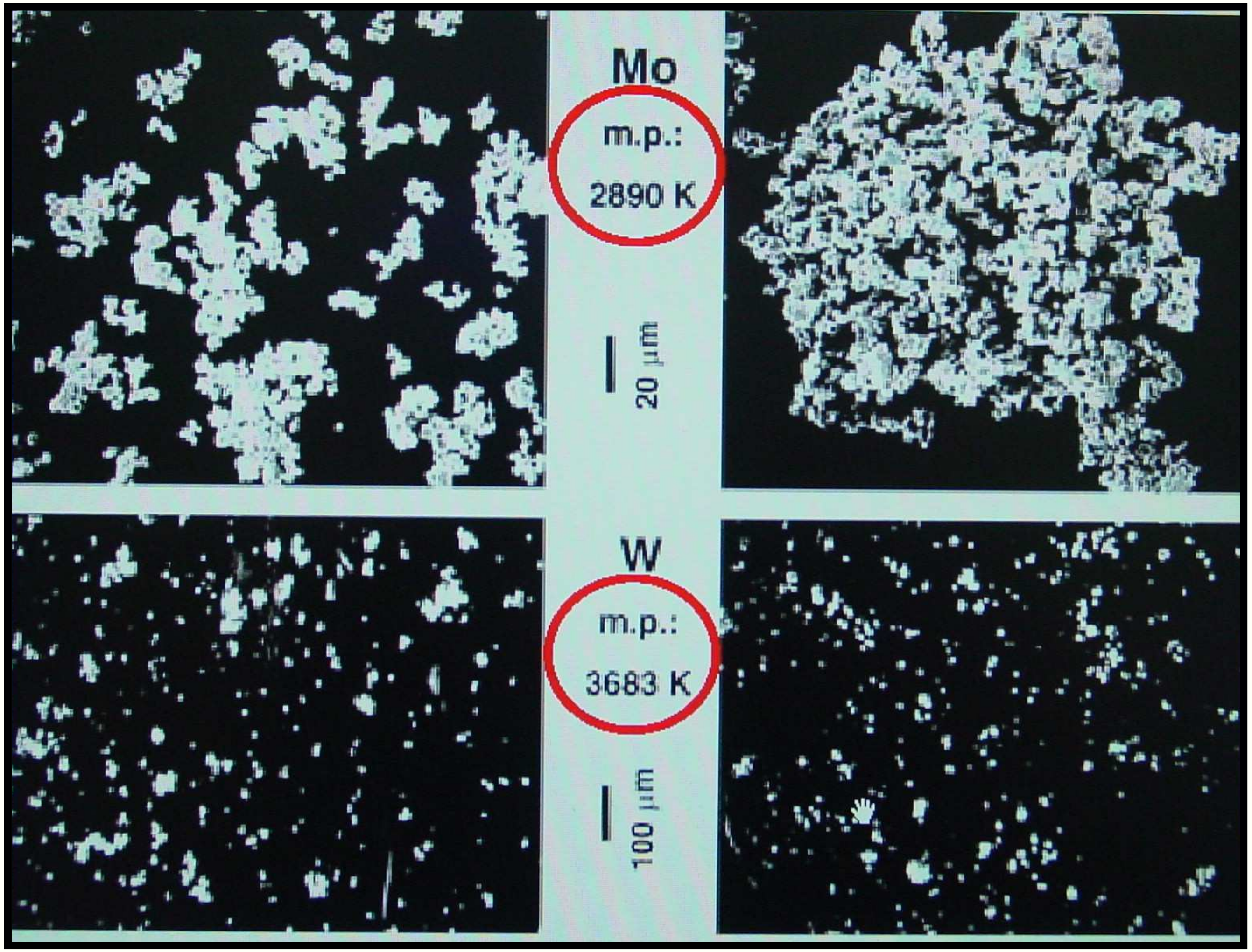


adapted: www.scs.uiuc.edu/~suslick/britannica.html



CAVITATION

Cavitation effect and micro-jet effect:
fusion of Mo particles after sonication



without ultrasound **with 30 min ultrasound**

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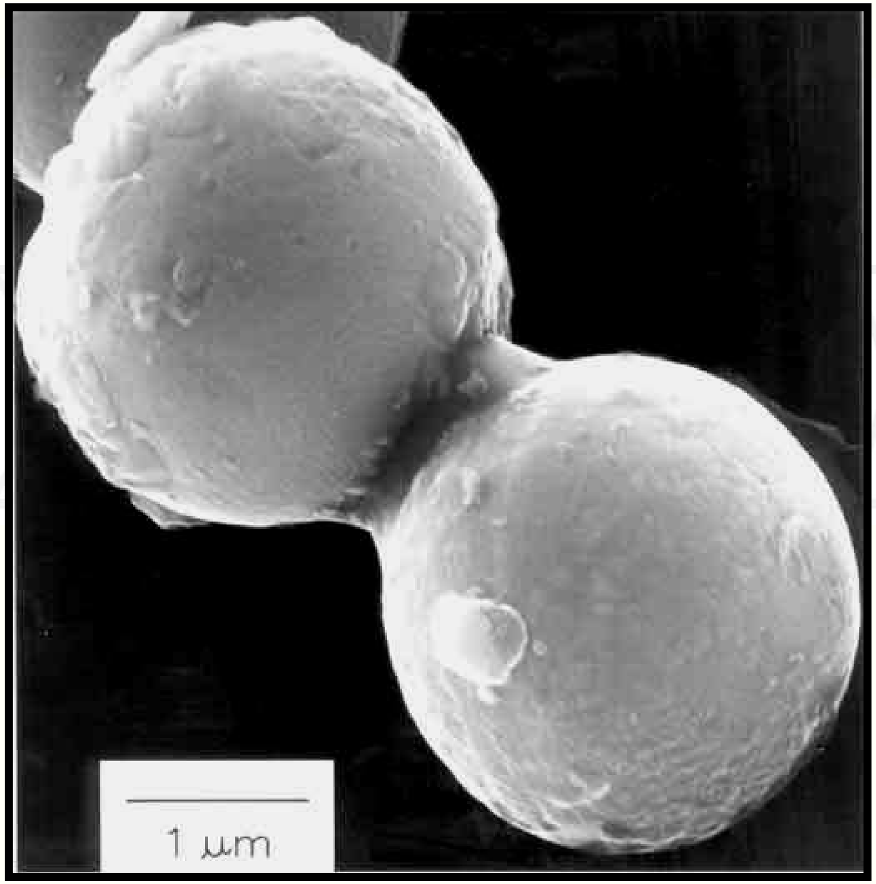
CAVITATION

Cavitation effect and micro-jet effect:
fusion of Zn particles after sonication

20 kHz
50 W cm^{-2}



m.p. 419.5 ^{o}C

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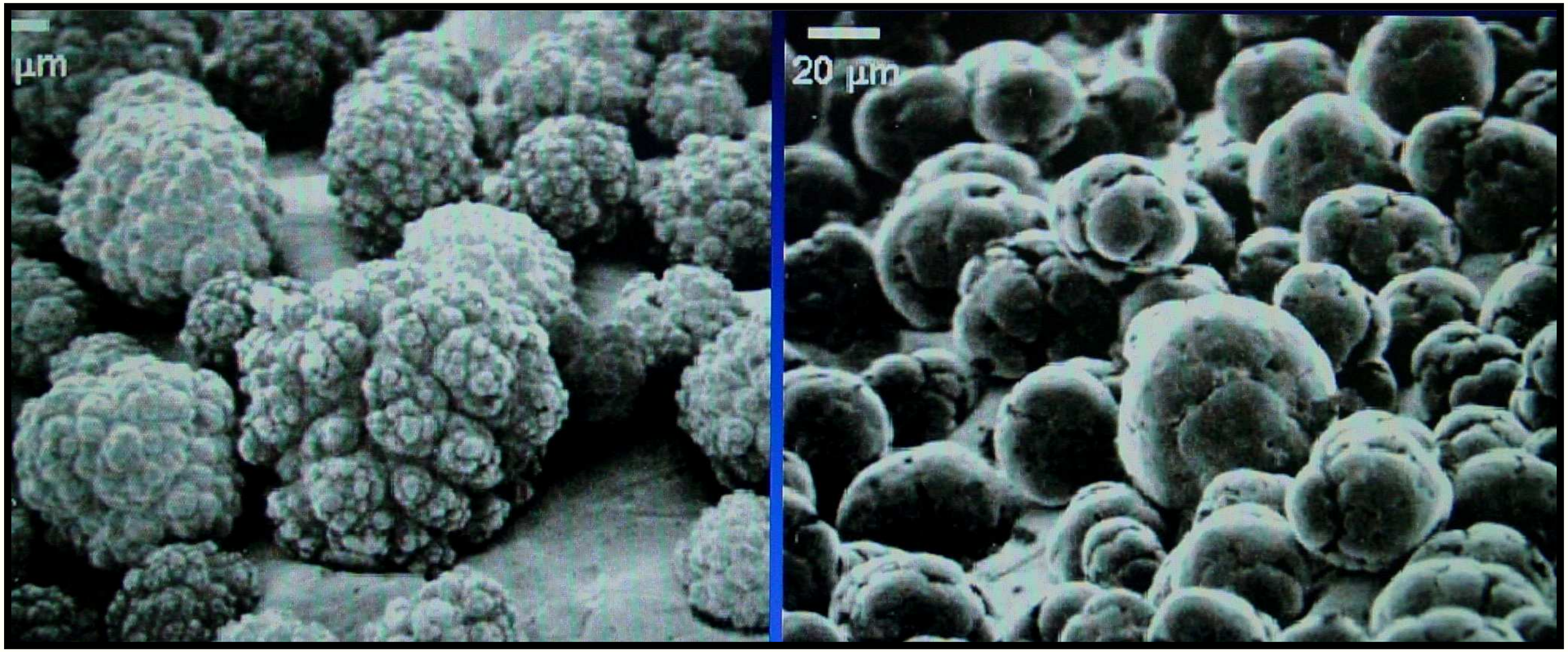
CAVITATION

Cavitation effect and micro-jet effect:
morphology and particle sizes changes → nickel oxide



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CAVITATION

Factors affecting

- Dissolved gas
- Irradiation frequency
- Temperature
- Viscosity and surface tension
- External pressure
- Presence of particles in solution

CAVITATION - Sonochemistry

- Organic synthesis
- Polymers degradation
- Polymerization
- Sonoluminescence
- Sonolysis (free-radicals formation)
- Sonogels formation
- Catalysts preparation
- Sample preparation

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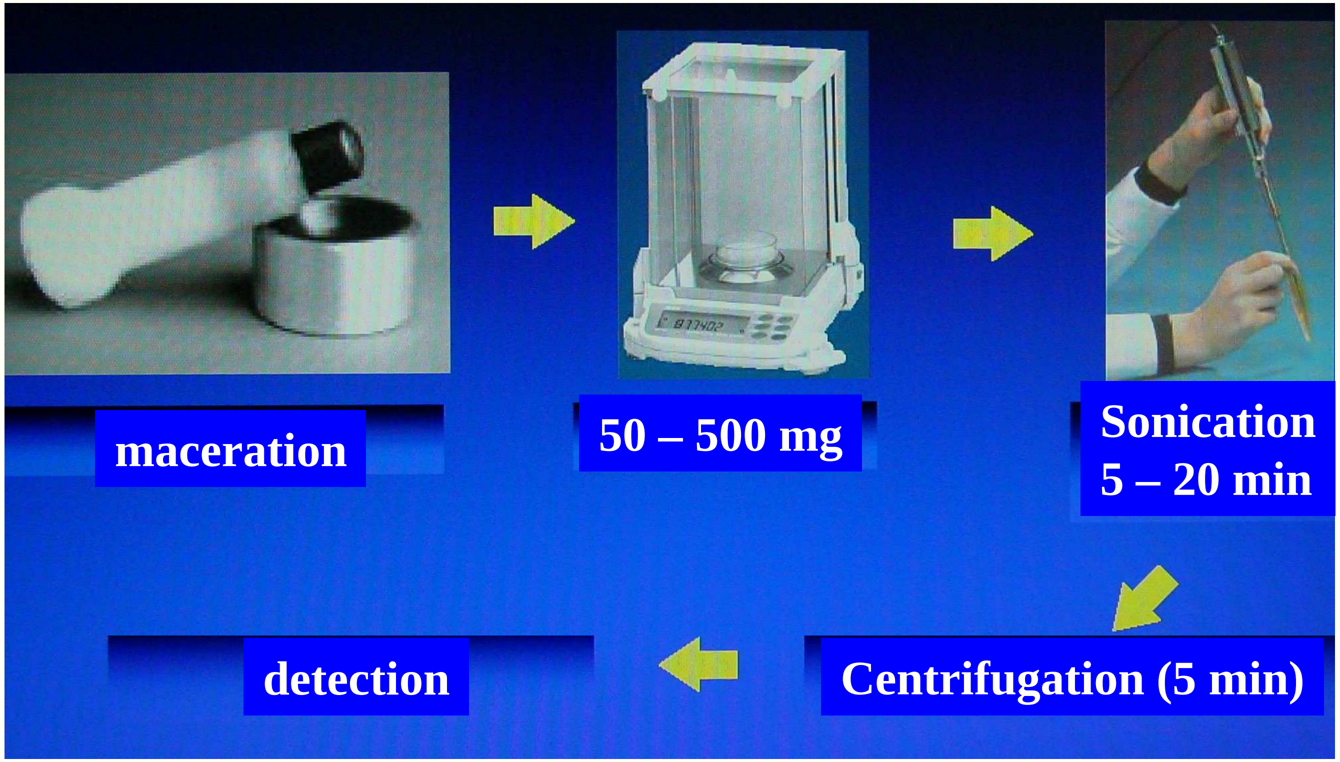
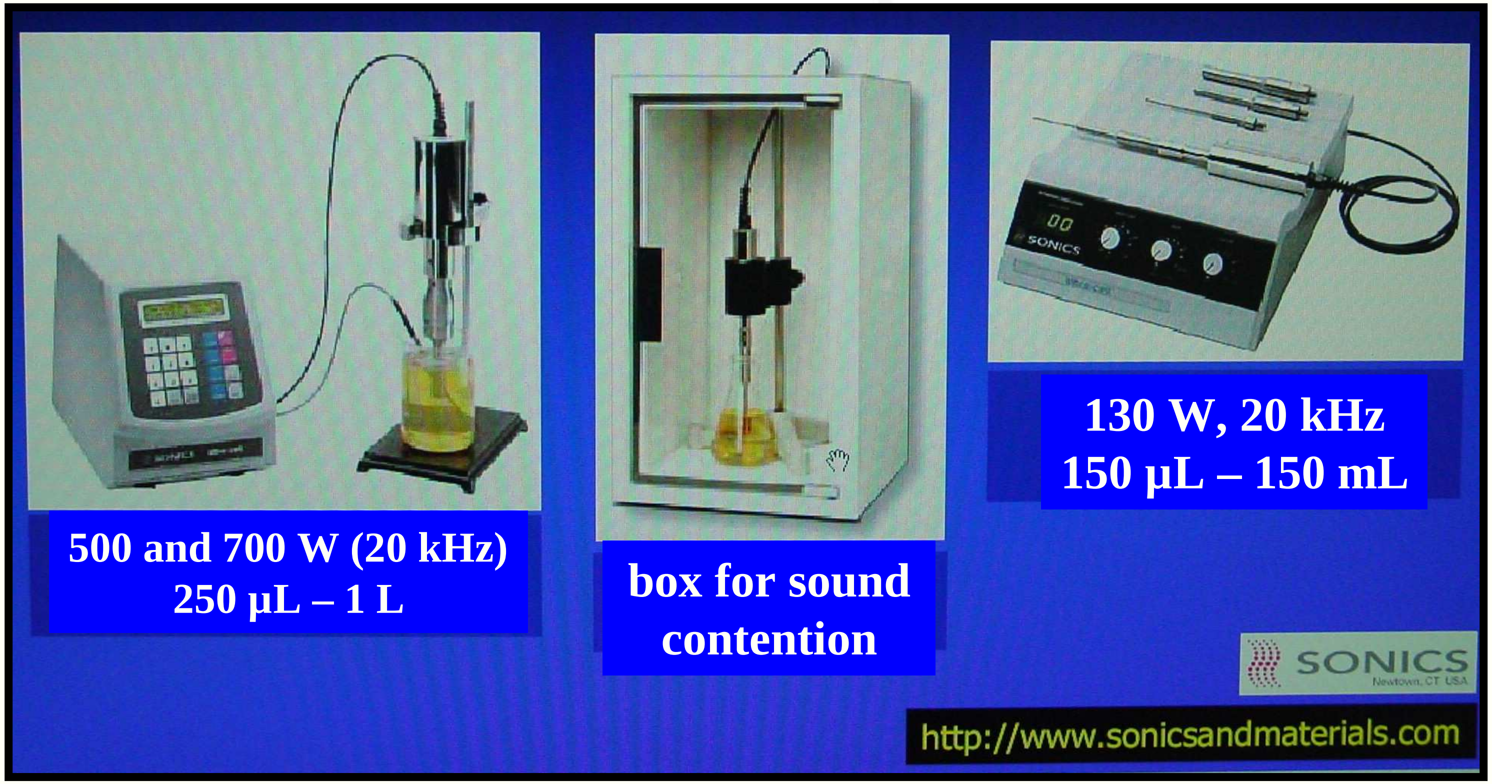
CAVITATION - Sonochemistry



CAVITATION - Sonochemistry

Commercial available instruments
ultrasonic probes



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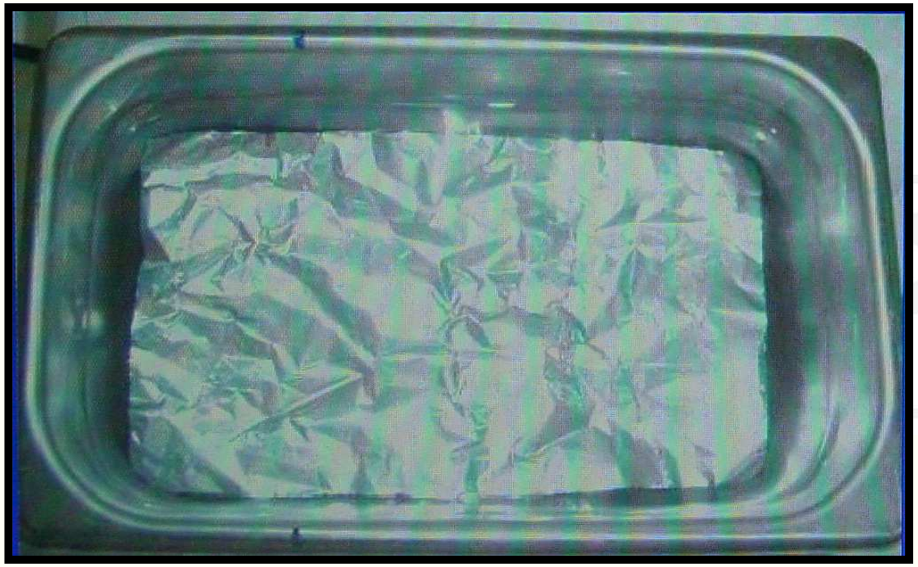
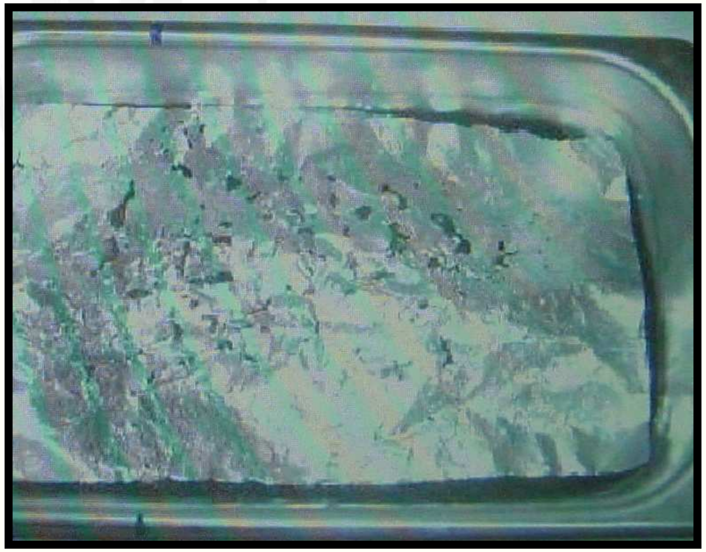
CAVITATION - Sonochemistry

Commercial available instruments
ultrasonic baths

CAVITATION - Sonochemistry

Energy distribution
ultrasonic baths





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CAVITATION - Sonochemistry

Energy distribution – ultrasonic baths

Nascentes et al., *J. Braz. Chem. Soc.*, 12(2001)57

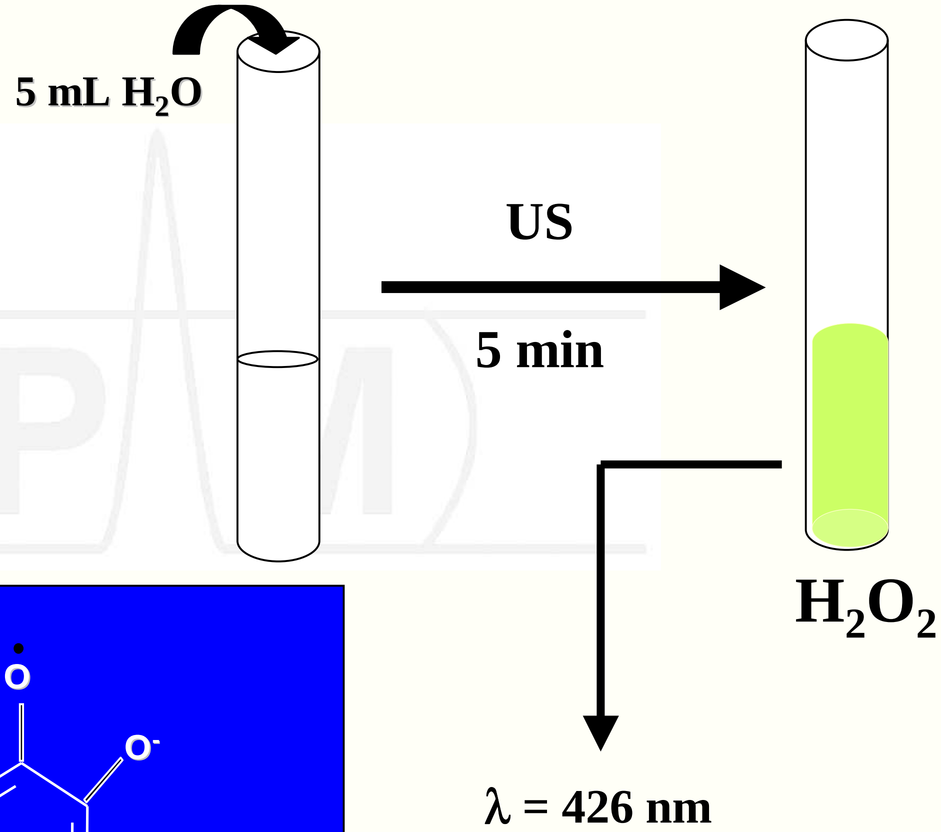
- **H_2O_2 method**

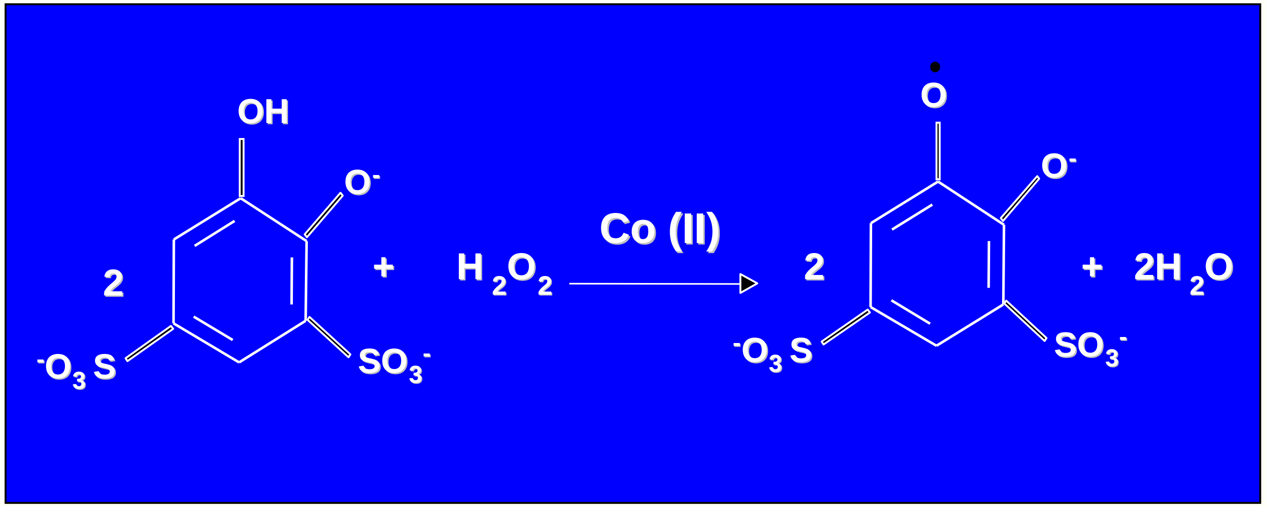
➢ **H_2O_2 formation**

$$H_2O \rightarrow H\cdot + \cdot OH$$

$$HO\cdot + \cdot OH \rightarrow H_2O_2$$

➢ **Tiron oxidation by H_2O_2**





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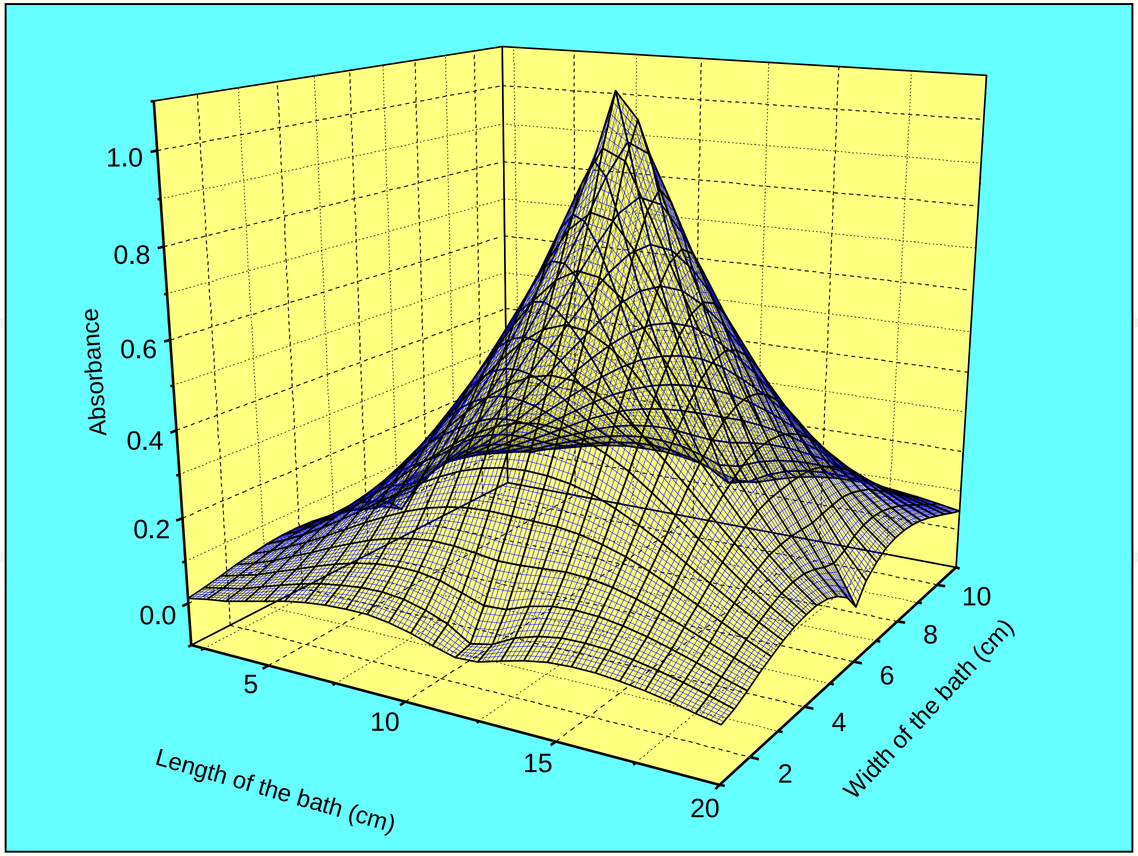
Energy distribution – ultrasonic baths



Point 5 → highest H_2O_2 concentration
Others: < LOD

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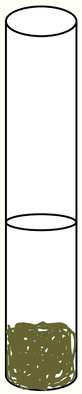
CAVITATION - Sonochemistry

Sample preparation

Nascentes et al., *Microchem. J*. 69(2001)37

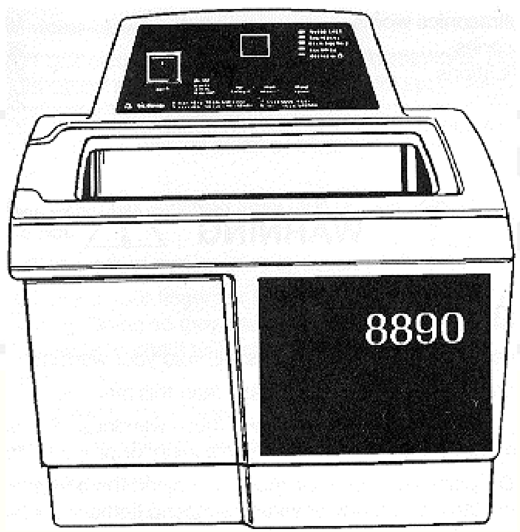


0.3 g sample

+

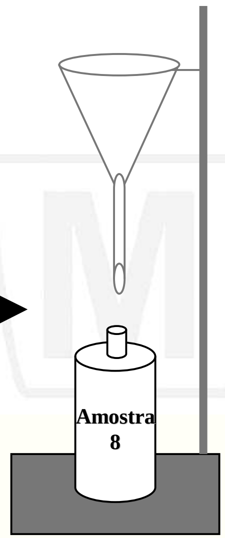
15 mL extracting solution

→



sonication

→



filtration

→

Ca, Fe, Mg, Mn and Zn determination by FAAS

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CAVITATION - Sonochemistry

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Sample preparation

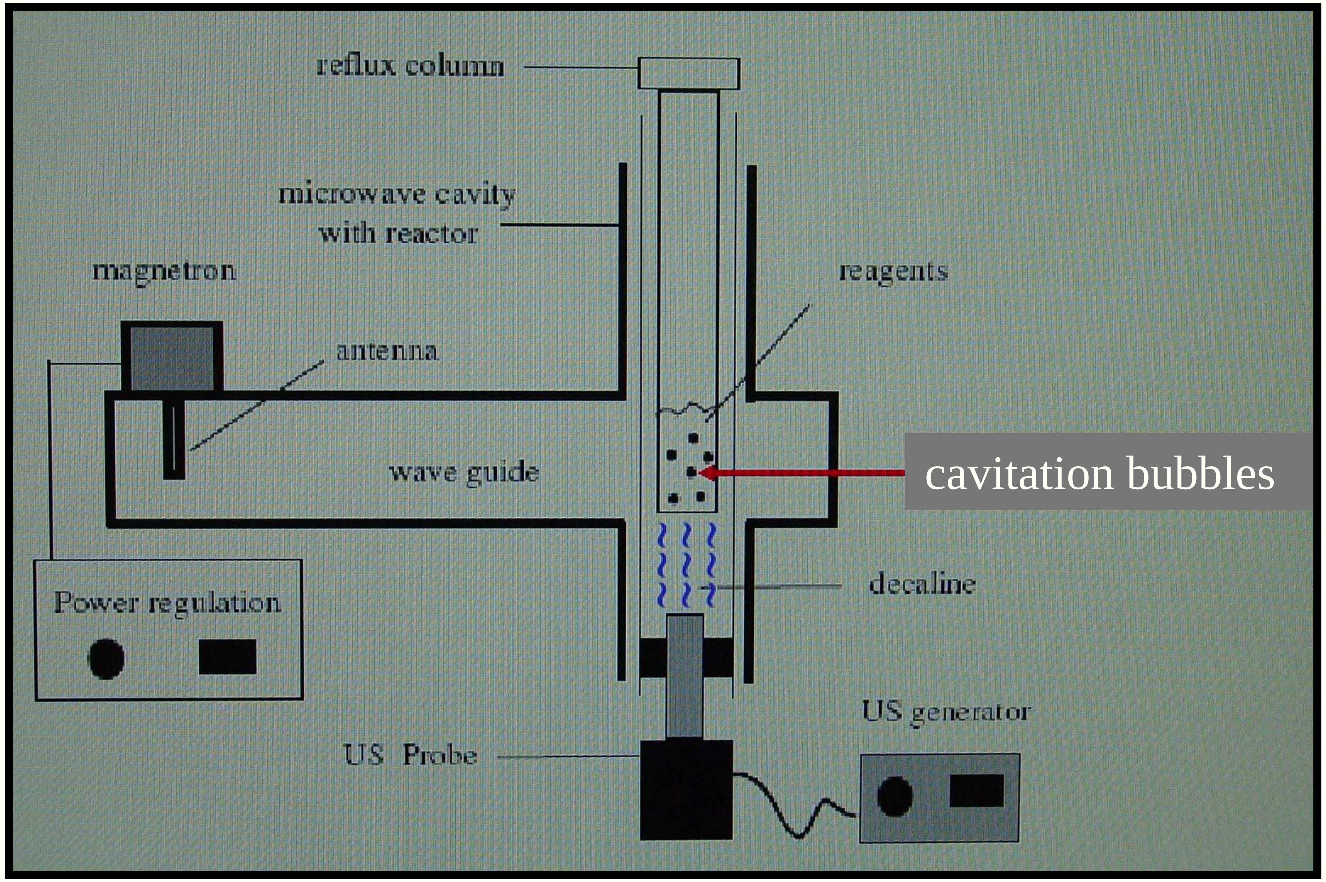
Samples	Wet digestion (μg g^{-1})			
	Ca	Mg	Mn	Zn
Lettuce	1.69±0.06	0.280±0.009	177±7	119±5
Cabbage	0.88±0.05	0.180±0.008	31±1	26±1
Cavalinha	2.64±0.07	0.200±0.005	80±1	36±1
Spinach[a]	---	---	---	---
Apple leaves[b]	---	---	---	---
	Ultrasound extraction (μg g^{-1})			
	Ca	Mg	Mn	Zn
Lettuce	1.68±0.03	0.280±0.012	166±2	112±2
Cabbage	0.89±0.04	0.180±0.010	32±1	26±1
Cavalinha	2.68±0.02	0.190±0.008	75±3	33±1
Spinach[a]	1.10±0.05[c]	0.850±0.022	72±3	74±4
Apple leaves[b]	1.60±0.14	0.250±0.007	53±1	11±1

[a]NIST 1570a: 1.53±0.05; 0.9; 79±2; 82±8 μg g^{-1} for Ca, Mg, Mn and Zn
[b]NIST 1515: 1.51±0.02; 0.270±0.012; 54±2; 12.5±0.4 μg g^{-1} for Ca, Mg, Mn and Zn
[c]no statistical differences at 99.5% confidence level

CAVITATION - Sonochemistry

Sample preparation

Chemat et al., *Ultrasonics Sonochem*. 11(2004)5-8



CAVITATION - Sonochemistry

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Sample preparation

Chemat et al., *Ultrasonics Sonochem.* 11(2004)5-8

Efficiency of simultaneous MW-US irradiation on the digestion time

Heating system	Digestion time of sunflower oil (min)	Digestion time of sesame oil (min)
(1) Microwave	40	50
(2) Classical	50	70
(3) Microwave + US	**24**	**35**

Comparative Kjeldahl nitrogen determination in food and agricultural products between MW and classical standards methods

Food products	Classical Kjeldahl		MW Kjeldahl		MW-US Kjeldahl	
	%N	t (min)	%N	t (min)	%N	t (min)
Cow's milk	0.5	180	0.48	30	0.45	**10**
Rice	1.04	180	1.18	30	1.1	**10**
Corn	1.12	180	1.09	30	1.19	**10**
Flour	1.57	180	1.72	30	1.62	**10**
Beef	2.67	180	2.81	30	2.91	**10**
Corned beef	3.69	180	3.61	30	3.53	**10**
Chick pea	3.20	180	3.35	30	3.55	**10**
Powdered milk	4.75	180	4.82	30	4.63	**10**

Ultrasound – US
Conclusions

Simple and robust

Multi-elemental (?)

US

Low costs

Reduced residual acidity

Low sample throughput

Metrological trustable (?)

Extraction success → interactions between analyte-sample

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Microwave assisted water extraction-MAWE

- Advantages:
  - No cost and abundant substance
  - Changes in its polarity by changing T
  - Clean extraction methods

- Limitations:
  - Low significance as extracting agent when not assisted by auxiliary energies

- Alternative extraction when assisted by:
  - High temperature – high pressure
  - Microwave radiation
  - Ultrasound

Microwave assisted water extraction-MAWE

water←→microwaves interaction

- ✓ High dielectric constant compound necessary for producing heating when MW is used (presence of water!!)
- ✓ >>>T → < viscosity. MW penetrates into sample destroying macrostructure of the matrix
- ✓ T and P produce a decrease on polarity with increases on analytes solubility. Process completely different to conventional extraction → solvent diffusion through the sample and analytes are removed from matrix by solubilization

Microwave assisted water extraction-MAWE

water properties

- ✓ Polarity modified by temperature

- ✓ P and room temperature:
  - ✓ Dielectric constant (80) – extremely polar solvent, favoring the solubility of high polarity compounds

- ✓ High T and high P (water in liquid phase)
  - ✓ Lower dielectric constant, favoring the solubility of compounds presenting low polarity
    - ✓ Example: water (at 250^{o}C)/ε = 27
    - ✓ ethanol/ε=24; methanol/ε=33 (at room T)
  - ✓ Water at high T contributes to the non-polar compounds solubility increases: benzo(e)pyrene solubility increases 25 million times (water → from room T to 350^{o}C)
  - ✓ Extraction varying from ionic analytes to non-polar compounds

Microwave assisted water extraction-MAWE

5092 J. Agric. Food Chem. 2001, 49, 5092–5097

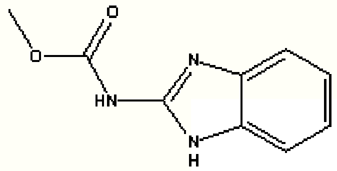
Focused Microwave Assistance for Extracting Some Pesticide Residues from Strawberries into Water before Their Determination by SPME/HPLC/DAD

Clara Falqui-Cao, Zhi Wang, Louise Urruty, Jean-Jacques Pommier,[†] and Michel Montury*

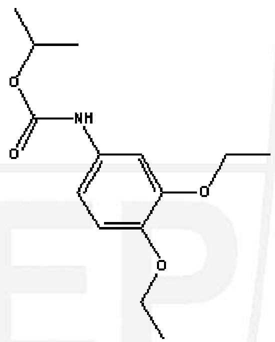
Equipe Périgourdine de Chimie Appliquée, Laboratoire de Physico et Toxico Chimie des Systèmes Naturels UMR 5472, Université Bordeaux 1/CNRS, BP 1043, 24001 Périgueux Cedex, France and Centre Interrégional de Recherche et d'Expérimentation de la Fraise, 24130 Prigonrieux, France

A novel and simple method for the determination of some pesticide residues in strawberries using both focused microwave-assisted extraction (FMAE) and solid-phase micro extraction (SPME), coupled with high-performance liquid chromatography (HPLC), has been developed. The pesticides were first extracted from strawberries with water and the assistance of focused microwaves at 30 W for 7 min. Then, an aliquot of the resulting aqueous extract was subjected to SPME with a 60-μm thick poly(dimethylsiloxane)/divinylbenzene (PDMS/DVB) fiber for 45 min at room temperature, with the solution being stirred at 1000 rpm. The extracted pesticides on the SPME fiber were desorbed into the SPME/HPLC interface for quantitative analysis with a diode array detector (DAD). The whole sample pretreatment procedure before chromatographic analysis did not use any organic solvents or involve any blending or centrifugation steps. The five compounds (carbendazim, diethofencarb, azoxystrobine, napropamide, and bupirimate) were chosen because they cannot be analyzed easily by GC. The efficiency of this relatively fast procedure was comparable to that of previously reported methods, with detection limits at low μg/kg levels and linear responses in the range from 0.05 to 1 mg/kg of pesticide in strawberries, with RSDs between 3 and 7.3%, depending on the analyte. In all but one case results obtained by this method for field-incurred samples were comparable to those obtained with traditional methods.

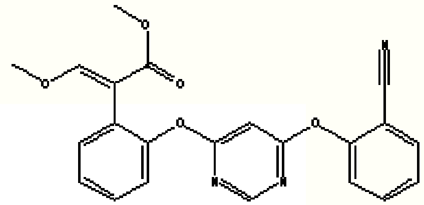
Microwave assisted water extraction-MAWE



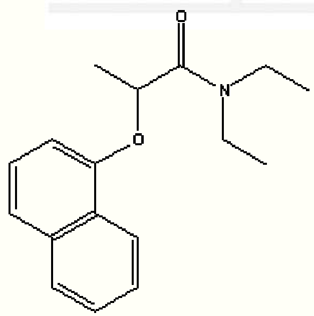
Carbendazim
[C9H9N3O2]



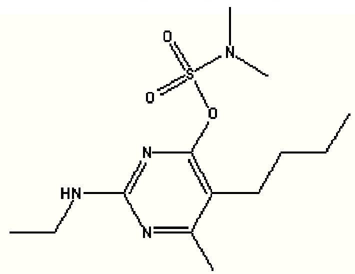
Diethofencarb
[C14H21NO4]



Azoxystrobine
[C22H17N3O5]



Napropamide
[C17H21NO2]



Bupirimate
[C13H24N4O3S]

Microwave assisted water extraction-MAWE

- ✓ Extraction:
  - ✓ FMAE
  - ✓ SPME – direct analytes transference to the PDMS/DVB fiber *via* pure water

  Matrix processing and centrifugation are avoided

- ✓ Quantification: HPLC

Microwave assisted water extraction-MAWE

- ✓ Samples:
  - ✓ synthetic (contamination by adding the analytes)
  - ✓ real
- ✓ Optimization:
  - ✓ SPME – through literature and manufacturer information
  - ✓ FMAE – time of fiber exposition (at a fixed power)

Microwave assisted water extraction-MAWE

Extraction efficiency

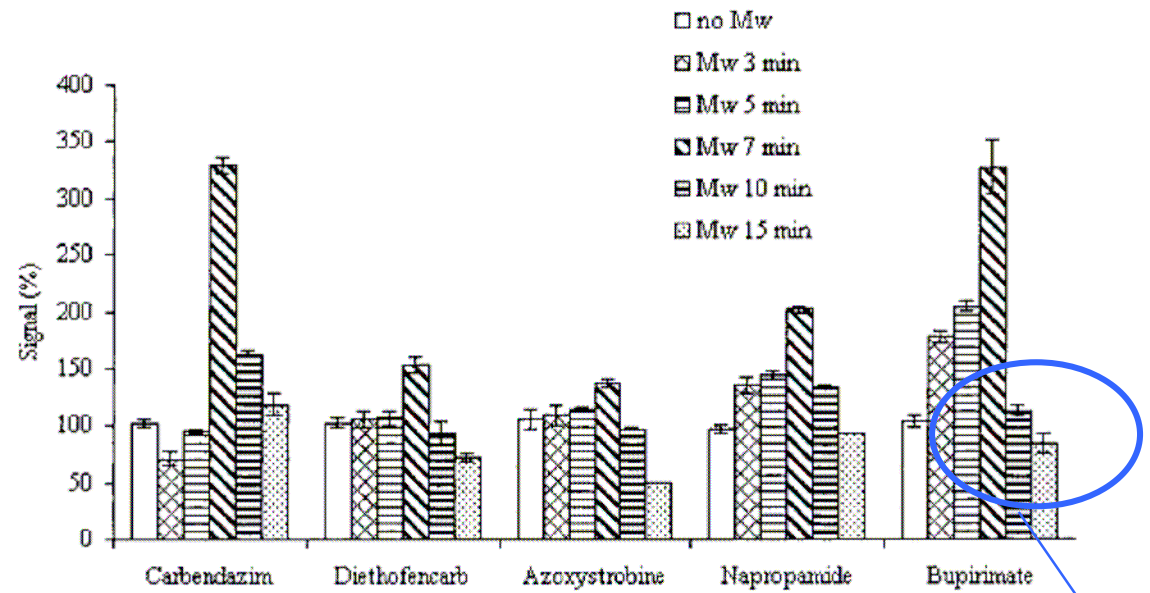


Figure 1. Effect of microwave irradiation time at 30 W on the signal obtained by SPME/HPLC/DAD from strawberries spiked at the concentration of 1 mg/kg (signals normalized to those obtained with no microwave).

↑ Power
↑ T
→ analytes degradation

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Microwave assisted water extraction-MAWE

Recoveries

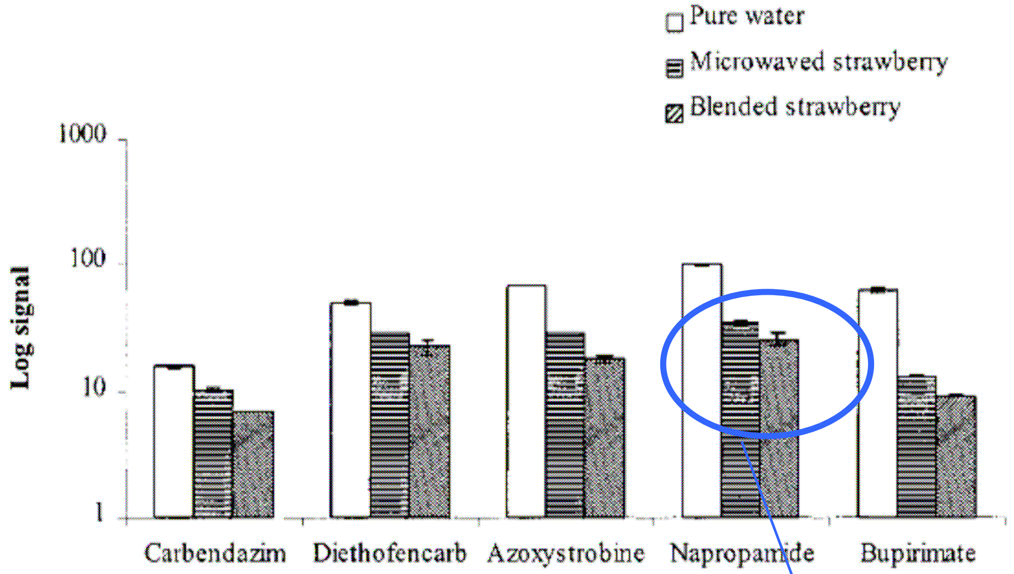

Figure 2. Comparison of signals obtained from pure water, microwaved strawberries, and blended strawberry solutions spiked at the concentration of 0.5 mg/kg.

Matrix effect:
- lower recoveries

Extraction efficiency:
- FMAE ↑

Microwave assisted water extraction-MAWE

Results

Table 2. Comparison of Concentrations Obtained by Using SPME and Traditional Methods from Field-Incurred Samples Coming from Experimental Assays and Commercial Production (n.a., not analyzed; LOD, limit of detection)

	experimental assay		commercial sample	
pesticide	SPME	traditional method	SPME	traditional method
Carbendazim	3.7	3.5		
	1.9	1.7		
	0.04	0.04		
	0.09	0.06		
Diethofencarb	1.8	2.07	0.66	0.73
	0.99	1.22	0.03	0.03
			0.03	0.27
Azoxystrobine	2.9	n.a.		
	0.38	n.a.		
Napropamide	< LOD	< LOD		
Bupirimate	< LOD	< LOD	0.09	0.09
			0.09	0.07
			0.19	0.21
			< LOD	0.03
			0.13	0.15

Table 1. Calibration Curve, Relative Standard Deviation (RSD), Limit of Detection (LOD), Limit of Quantification (LOQ), and French Maximum Residue Limit (MRL) Corresponding to the 5 Pesticides Analyzed

pesticide	λ	regression equation	r	RDS	LOD (mg/kg)	LOQ	MRL (mg/kg)
Carbendazim	205	$y = 1093x$	0.9994	5.8	0.022	0.074	0.100
Diethofencarb	205	$y = 3069x$	0.9977	7.3	0.018	0.060	0.500
Azoxystrobine	205	$y = 3149x$	0.9981	5.8	0.016	0.053	-[a]
Napropamide	205	$y = 4639x$	0.9964	3.0	0.013	0.067	0.100
Bupirimate	240	$y = 2331x$	0.9977	4.1	0.017	0.044	0.500

[a] Registration process for strawberry in progress.

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Microwave assisted water extraction-MAWE
Conclusions

ε similar to organic solvents

No organic solvent for extraction

→Water
low cost,
abundant,
high purity material

MAWE

Non-polar compounds extraction

(almost) no matrix modification

Speciation studies??

Microwave assisted water extraction-MAWE

Recommended literature

Morales-Muñoz et al., *Anal. Chim. Acta* 557(2006)278

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Analytica Chimica Acta 557 (2006) 278–286

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Pure and modified water assisted by auxiliary energies: An environmental friendly extractant for sample preparation

S. Morales-Muñoz, J.L. Luque-García, M.D. Luque de Castro*

Department of Analytical Chemistry, Marie Curie Building, Campus of Rabanales, University of Córdoba, E-14071 Córdoba, Spain

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Abstract

The growing trend both to avoid the use of organic solvents and reduce the time needed to extract pollutants from environmental solid samples has led to the use of water assisted by auxiliary energies for leaching. The most common of the auxiliary energies used are high pressure–high temperature, microwaves and ultrasounds. One of the most interesting aspects of the use of water in combination with auxiliary energies is the possibility of coupling extraction with other steps of the analytical process, thus enabling partial or total automation of the analytical process, so it is expedited. The addition of reagents (such as surfactants, acids, etc.) to water enlarges its field of application and provides an additional way of shortening the leaching time; thus allowing the establishment of environmental friendly methods. A review about the potential of water as extractant and its main applications so far is here presented.

Keywords: Water; Auxiliary energies; Sample preparation

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OUTLINE

CPE
→Cd
→Proteins

US/MAWE
→Inorganic
→Organic

from trace elements to metalloproteins

MIP
→Catechol

Miscellaneous
→Metalloproteins

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Biomimetic Receptors
Molecularly Imprinted Polymers (MIP)

Synthetic polymers presenting selectivity to a target molecule

Science tries to imitate those natural recognition sites

Antigen/Antibody

Enzyme/Substrate

Drug/Receptor

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MIPs: Application to the Analytical Chemistry

Samples

Drugs, Enzymes,

Hormones, Proteins,

Amino acids, Sugars,

Agro-toxics, etc.

Techniques

Chromatography

(as stationary phase),

Capillary electrophoresis,

SPE, SPME

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MIP – Synthesis

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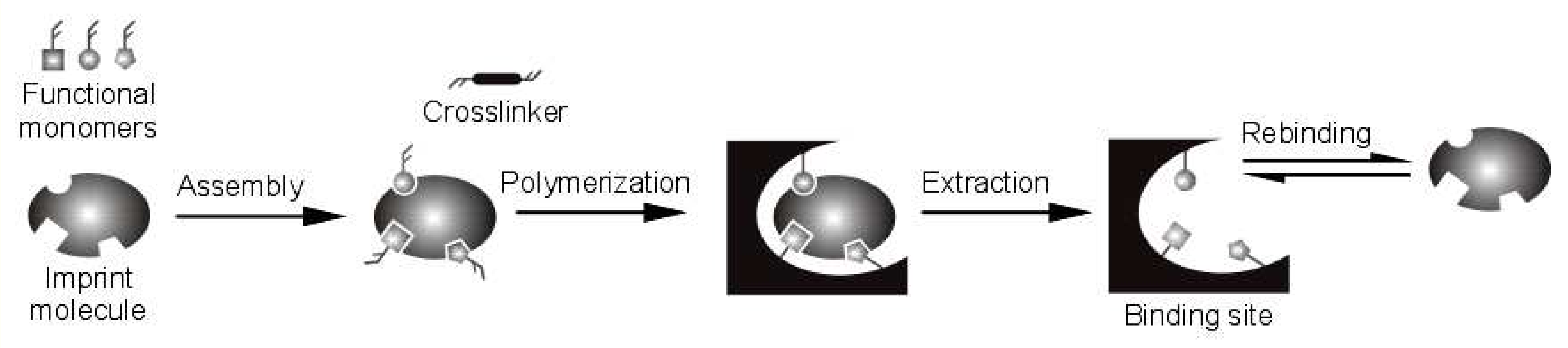
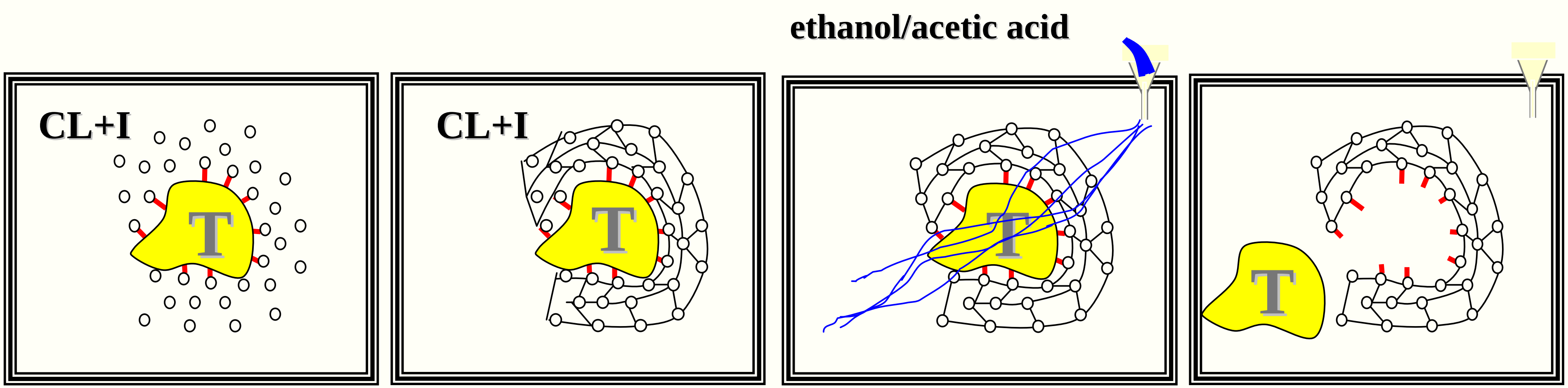
Medium (solvent, water); Template (IM); Functional monomer (FM); Cross-linker (CL); Initiator (I)





MIP – Reagents

Imprinted molecule – IM

-has specific groups to promote the bond to the FM
-no presence of polymerizable groups (insertion to the polymeric net)
-no groups that increase or decrease the polymerization reaction (ex. tyol groups)
-stability (T *ca*. 60^{o}C)

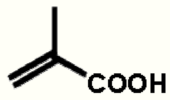
MIP – Reagents

Functional monomer – FM

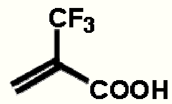
- Complementarities between FM and IM.
- if FM is proton donor (acidic character) → IM must be proton acceptor (basic character) and *vice-versa*
- Commonly used: methacrylic acid (for basic IM) and 4-vinylpyridine (for acidic IM)
- Concentration: interaction between FM and IM is based on equilibrium processes. >> [FM] than [IM] - *ca*. 4:1 [FM]:[IM] → formation of a great number of specific recognizing sites
- Possibility of combining different FM → polymerization cocktail

MIP – Reagents

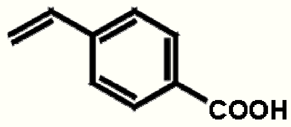
acrylic acid



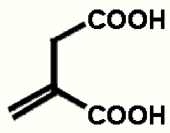
methacrylic acid



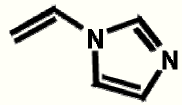
trifluoro-methacrylic acid



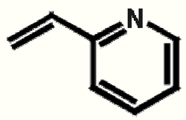
4-vinylbenzoic acid



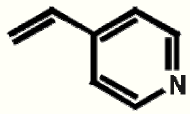
itaconic acid



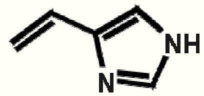
1-vinylimidazole



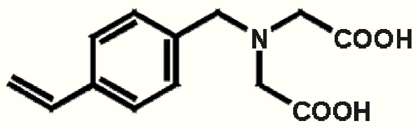
2-vinylpyridine



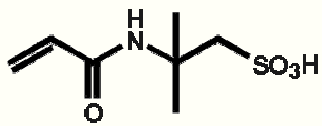
4-vinylpyridine



4(5)-vinylimidazole



4-vinylbenzyl-iminodiacetic acid



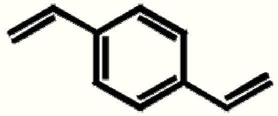
2-acrylamido-2-methyl-
1-propane sulphonic acid

MIP – Reagents

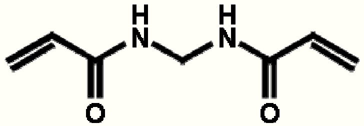
Cross-linker – CL

-Function:
*morphology control of the polymeric matrix
*stabilizes the bond sites
*promote mechanical stability to the polymer
-necessary at higher proportions in the MIP synthesis → to access the porous of the polymer, guarantying mechanical stability
-Ethylene glycol dimethacrylate → commonly used

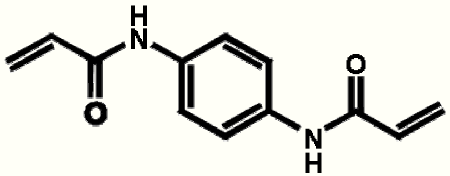
MIP – Reagents



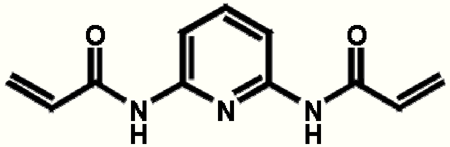
4-divinylbenzene



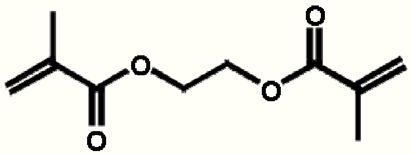
N,N'-methylene-bisacrylamide



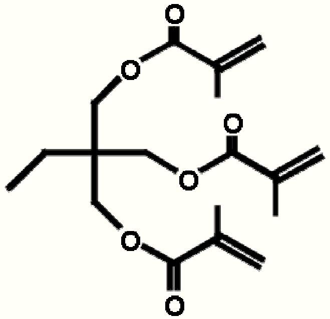
N,N'-phenylene-bisacrylamide



2,6-bisacrylamidopyridine



ethylene glycol dimethacrylate



trimethylolpropane trimethacrylate

Cross-linker

MIP – Reagents

Solvent

-Function:
* reagents solubilization in the synthesis
*contribute to best porous formation
*contribute to macro-porous formation in the polymer → large surface area
-does not interfere in the complex FM-IM formation (probability to form low selective sites and in less quantities)
-majority of the interactions between FM-IM is due to the electrostatic forces and hydrogen bounds → solvents should present non polar characteristics and have lower dielectric constant (*e.g*. chloroform and toluene)
-solubility problems: uses acetonitrile. MIP presenting lower selectivity can be formed

MIP – Reagents

Initiator - I

- Function: creates free radicals for polymerization
- external stimulus: T, UV radiation
- check its influence on others reagents (T increase without its degradation?, etc.)
- commonly used: 2,2'- azobisisobutyronitrile

Caution:

- synthesis processed at 60^{o}C as maximum T (azobisisobutyronitrile)
- it must be carried out in an oxygen-free atmosphere

MIP – Synthesis: Reagents

Medium	Chloroform/acetronitrile
Functional monomer	Methacrylic acid (MA) used for basic molecules
	4 vinylpyridine (4VP) used for acidic molecules
Cross-linker	Ethylene glycol dimethacrylate (EGDA).
Initiator	Azobisisobutyronitrile

MIP – Applications

On-line molecularly imprinted solid-phase extraction for the selective spectrophotometric determination of catechol

Figueiredo et al., *Microchem. J.* 85(2007)290

antifungal

antiseptic

antioxidant

Catechol

vaseconstrictor

damage in renal tubes

decreased liver function

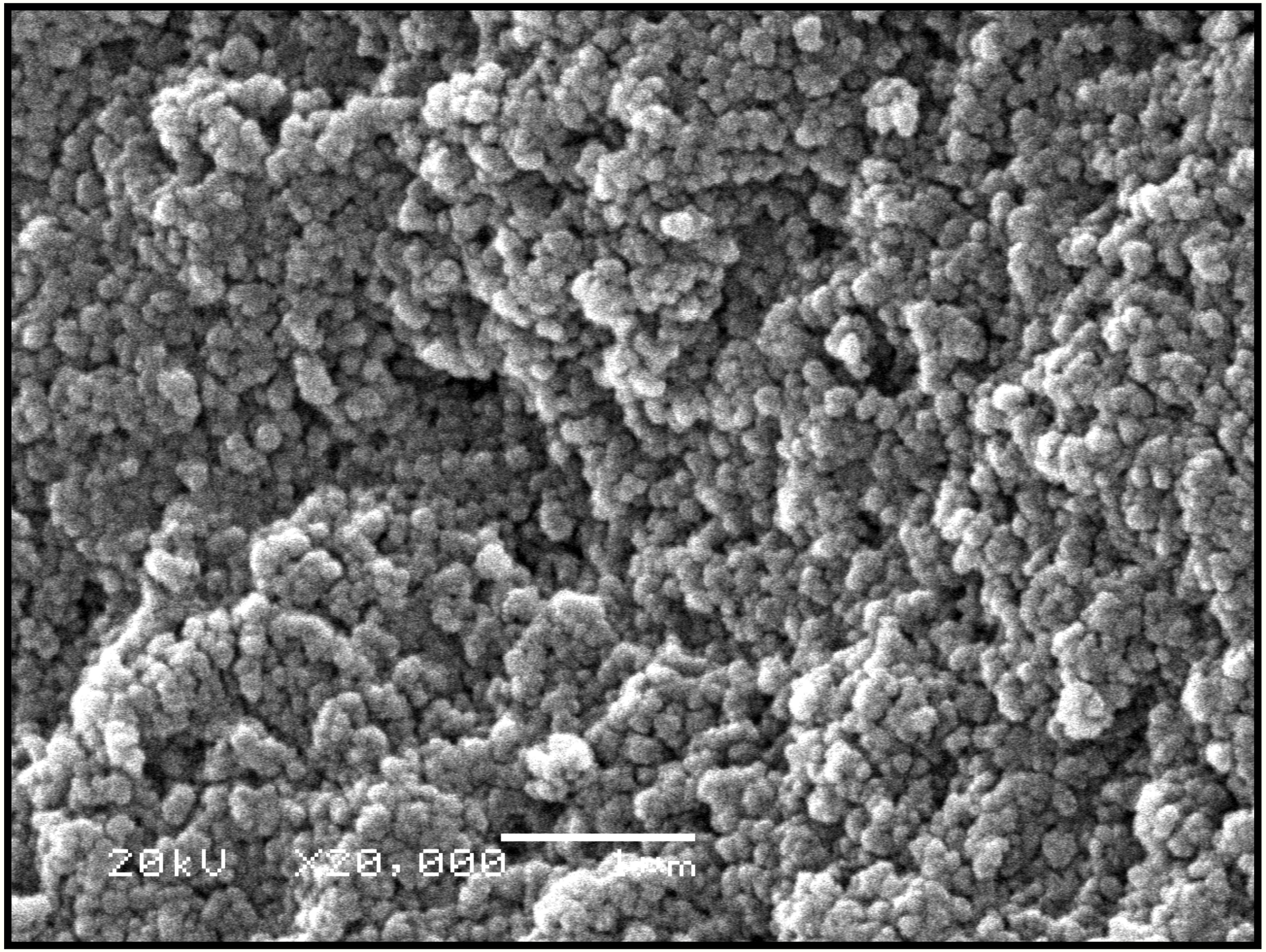
Mn(VII) → Mn(II)

selectivity

MIP

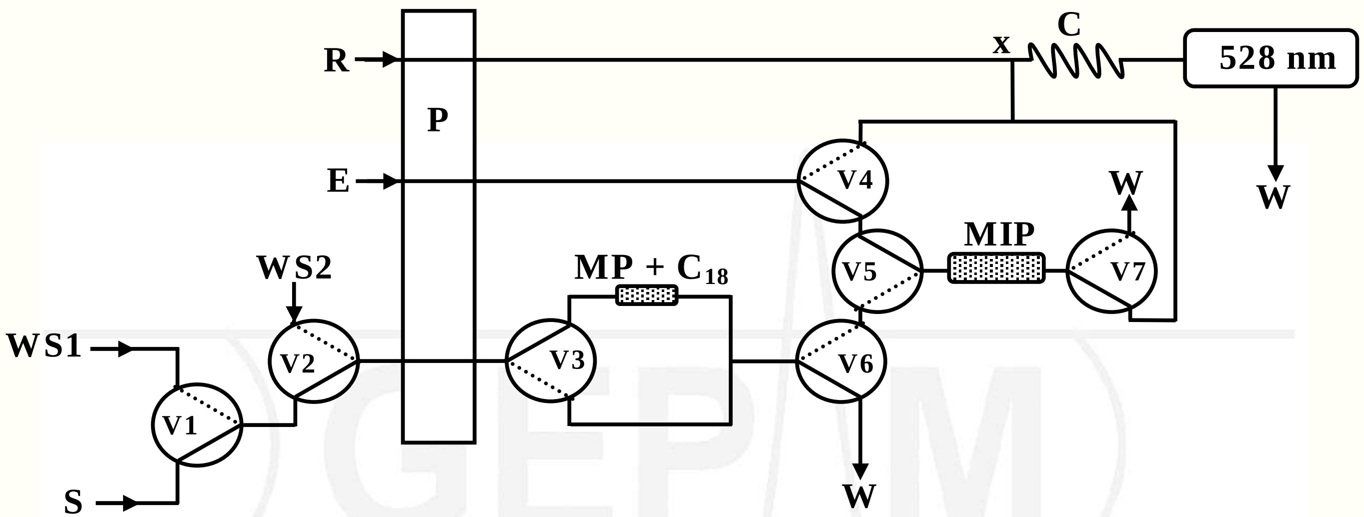
MIP – characterization

Tarley et al., *Talanta* 69(2006)259



MIP – Applications

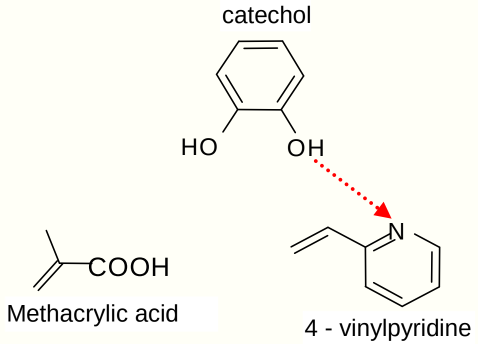
Flow system

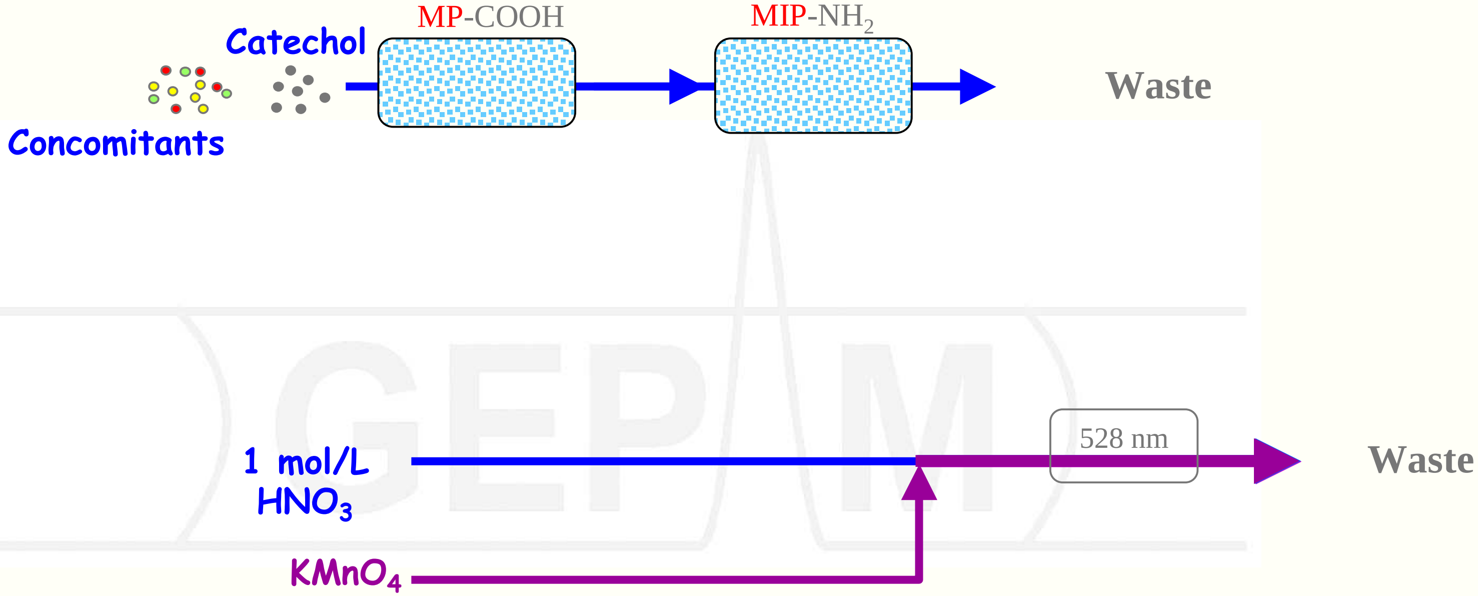


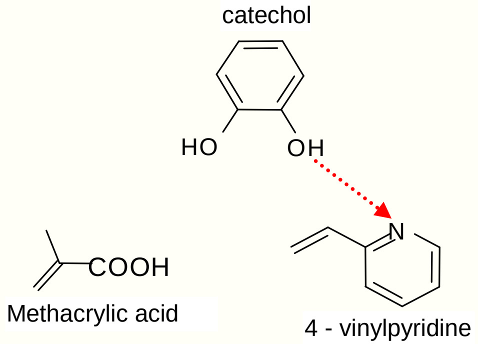
R: $KMnO_4$ 0.010% (m/v)
E: HNO_3 1 mol/L
WS1: washing solution (0.01 mol/L HNO_3 + 2 % v/v ACN)
WS2: washing solution (ACN 50% v/v)
MP+C_{18}: 70 mg (70% polymer + 30% C_{18})
MIP: 70 mg
C: coil 20 cm

MIP – Applications

Flow system

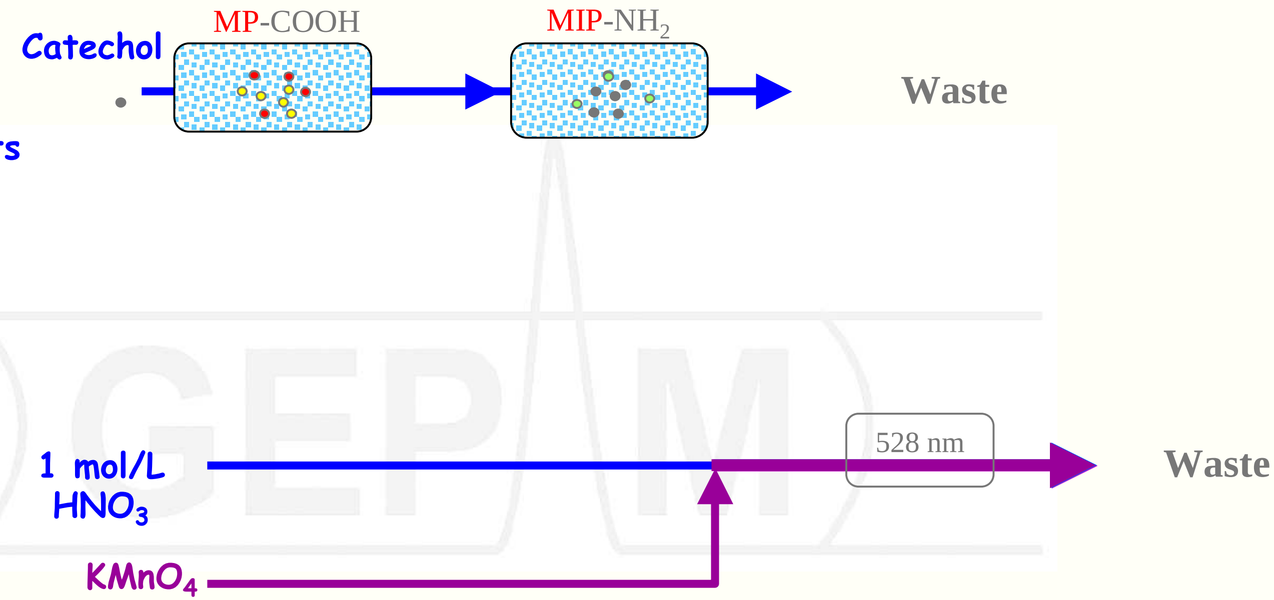






MIP – Applications

Flow system



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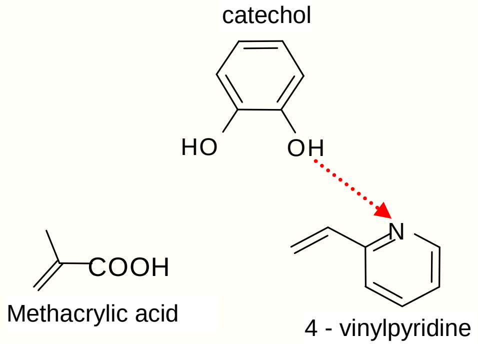
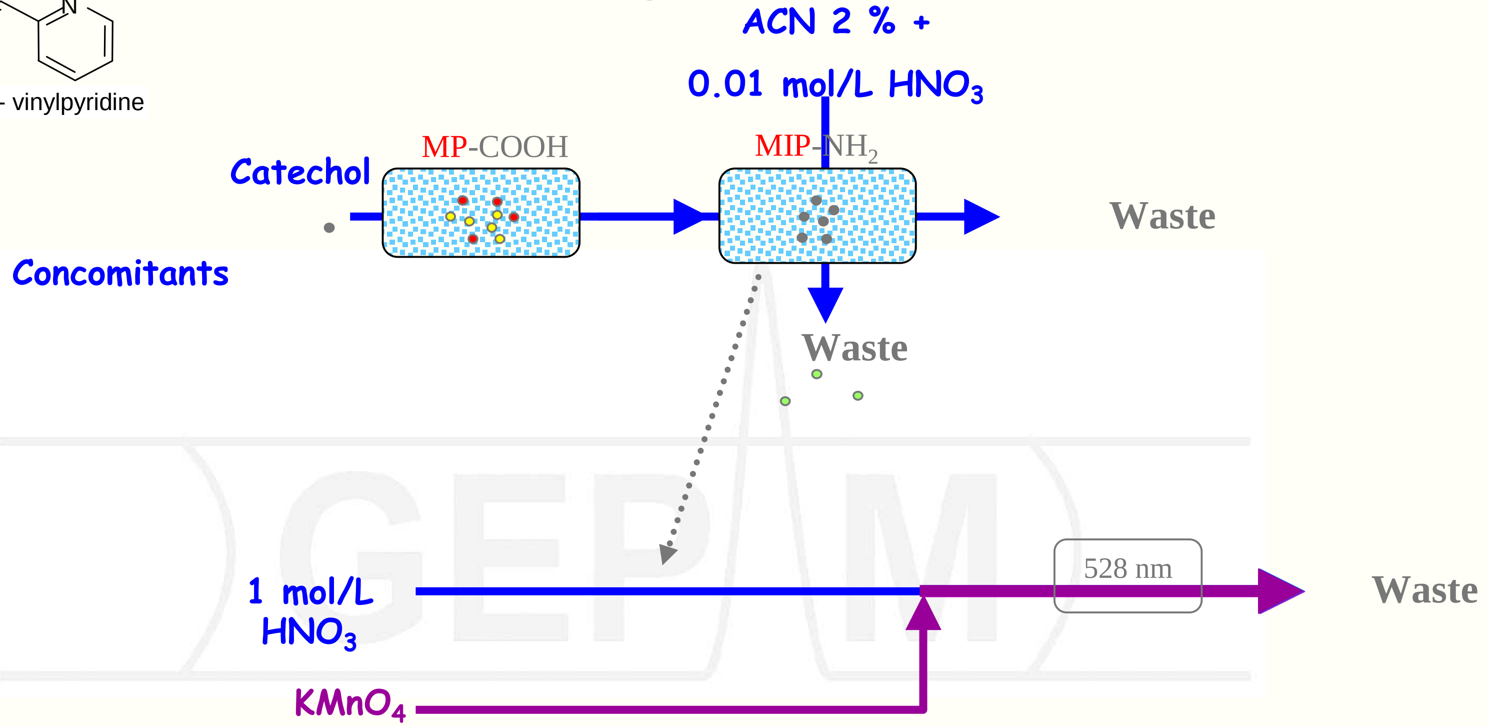
MIP – Applications

Flow system





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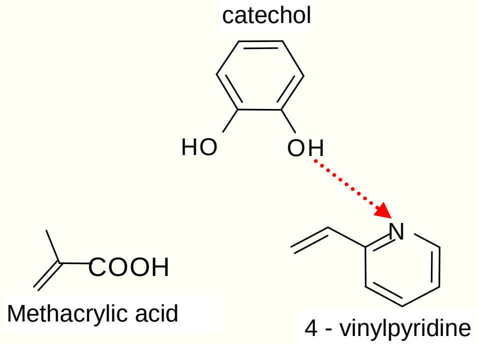
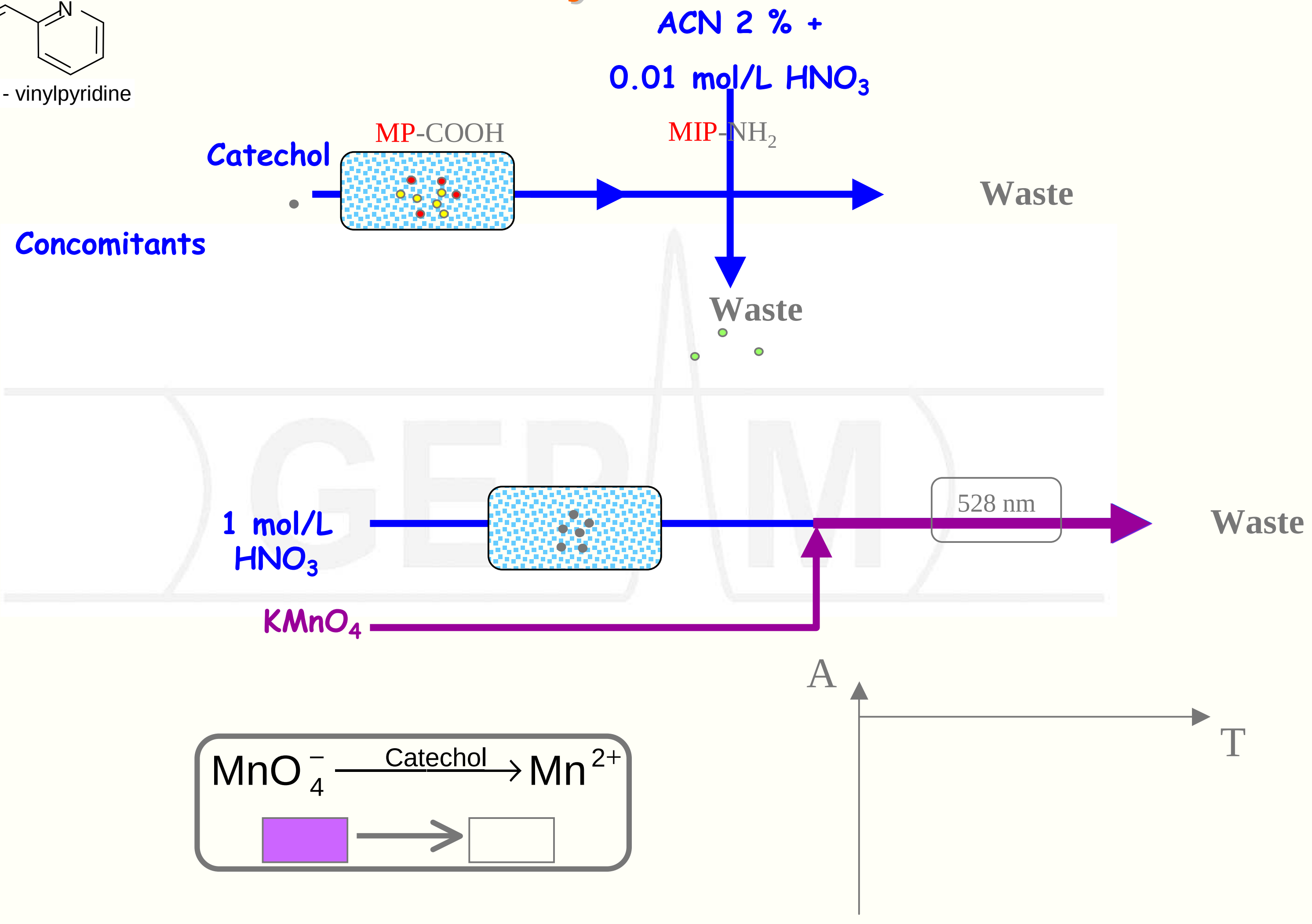


MIP – Applications

Flow system



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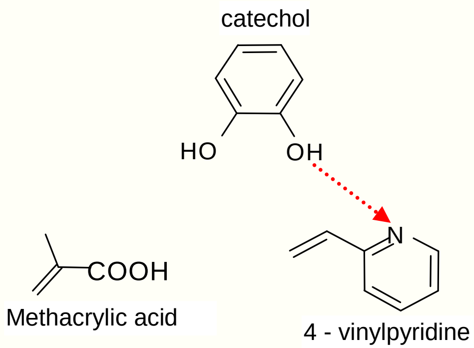
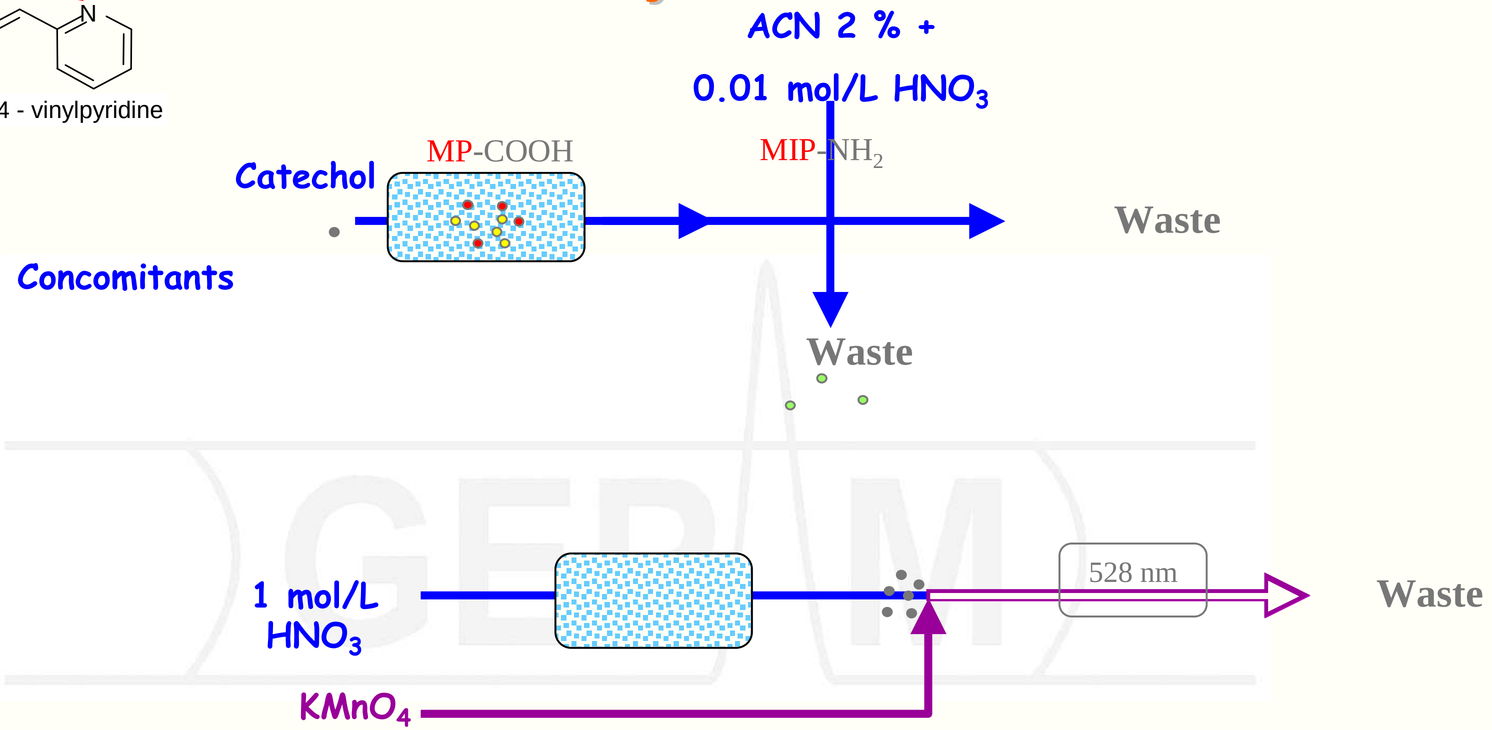
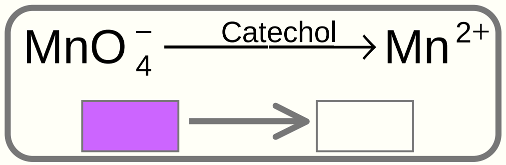
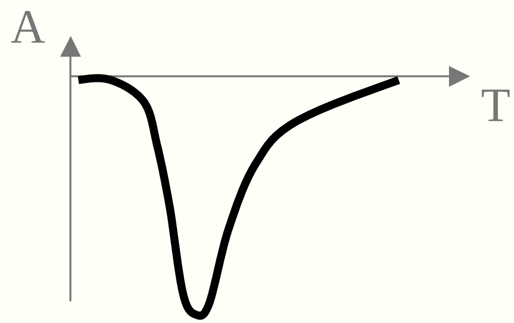
MIP – Applications

Flow system









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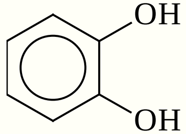
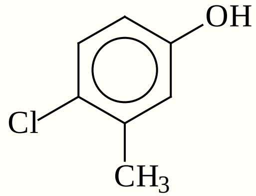
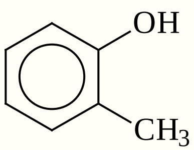
Concomitant evaluation



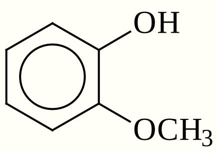
Catechol



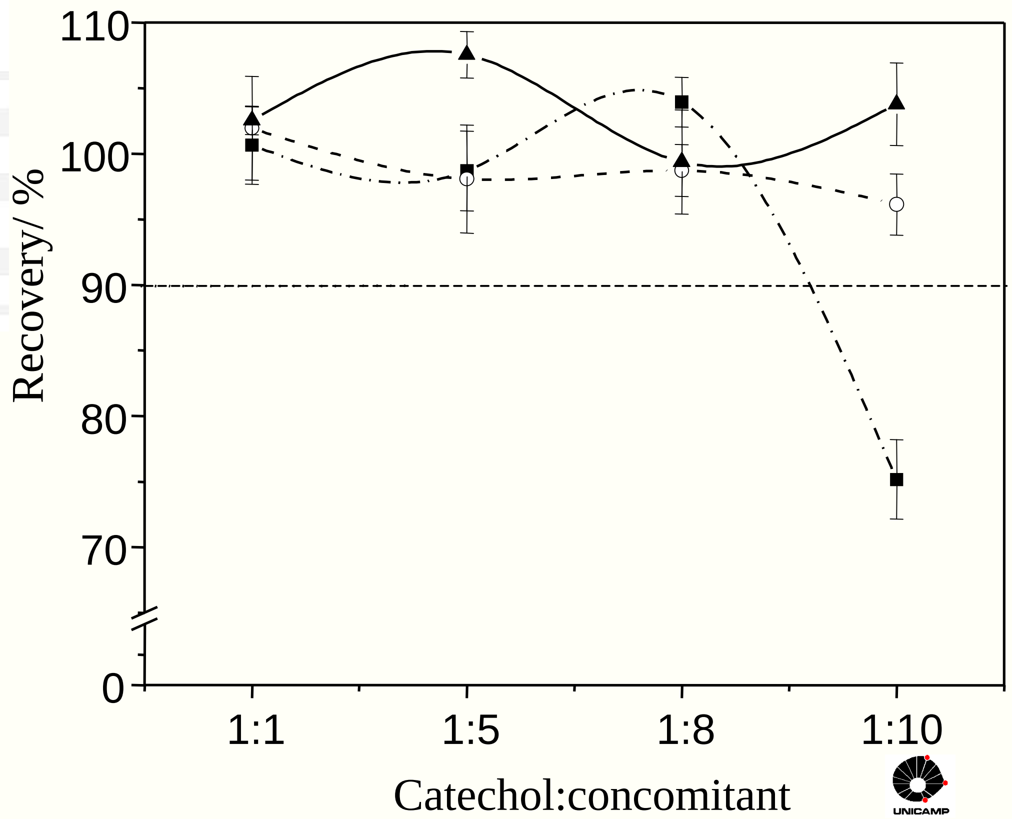
4-chloro-3-methylphenol



2-cresol

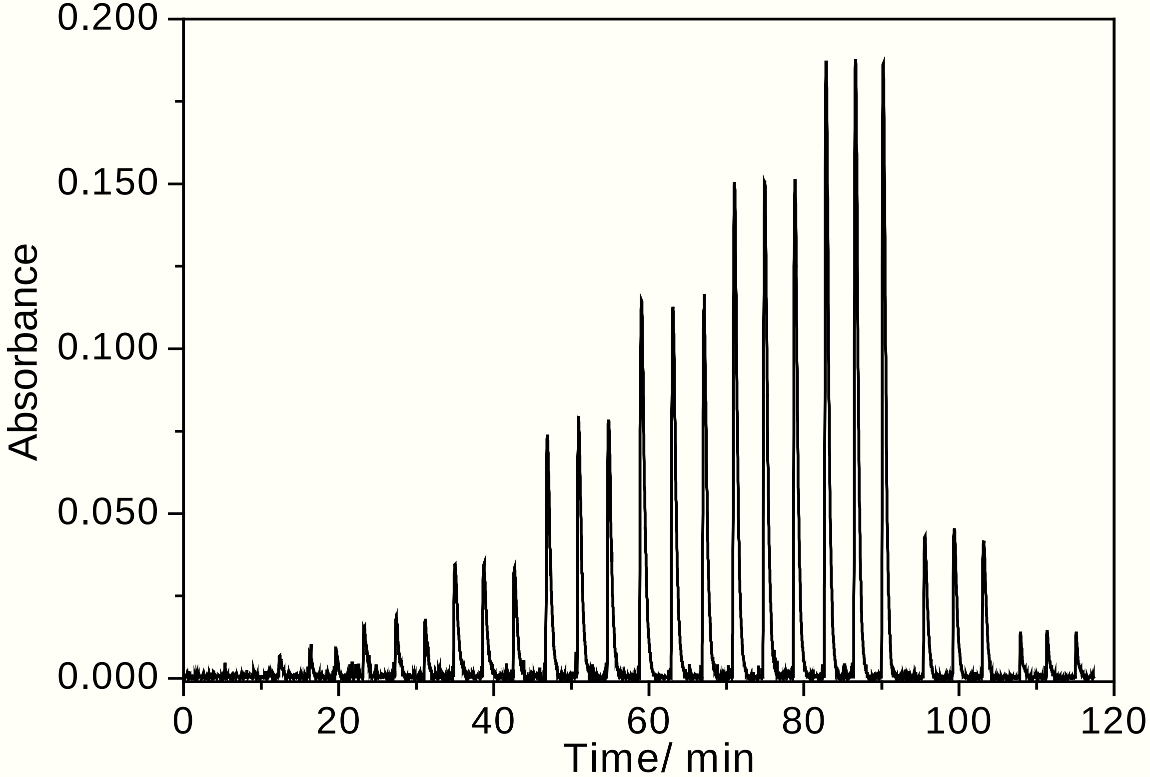


2-methoxiphenol



Figures of merit

Linear range: 3.00 – 100 μmol/L
(r > 0.999; n=7)
Precision: 3% RSD (n=10)
LOQ: 2.7 μmol/L
LOD: 0.8 μmol/L



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Results

Sample		Addition (μmol L^{-1})	FI-MISPE	FI-MISPE recovery/%	HPLC
Guaraná	A	0	< LOQ[1]	-	< LOQ[2]
		10	10.5 ± 0.5	105	10.4 ± 0.5
		20	20.6 ± 0.6	103	21.3 ± 0.2
	B	0	2.8 ± 0.4	-	< LOQ
		10	11.9 ± 0.2	90	12.0 ± 0.1
		20	23.2 ± 0.4	102	24.0 ± 0.3
Mate tea		0	7.7 ± 0.5	-	7.4 ± 0.2
		10	18.9 ± 0.2	112	17.0 ± 0.1
		20	29.5 ± 0.4	109	27.4 ± 0.2
Tap water		0	< LOQ	-	< LOQ
		10	9.5 ± 0.4	95	10.3 ± 0.8
		20	19.6 ± 0.2	98	20.0 ± 0.6

[1]LOQ = 2.7 μmol L^{-1} y [2]LOQ = 4.2 μmol L^{-1}

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Molecularly imprinted polymers – MIP
Conclusions

Alternative for separating those compounds presenting similar structure

MIP

Good analytical performance

Use of non-selective spectrophotometric reactions

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OUTLINE

CPE

→Cd

→Proteins

US/MAWE

→Inorganic

→Organic

MIP

→Catechol

from trace elements to metalloproteins

Miscellaneous

→Metalloproteins

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Some definitions

METALLOME: Set of metals or metalloids inside a cell or tissue – *ionome* and *metalloproteome*

Ionome: Metal (free) contained in a cell

Metalloproteome: Set of complexed metals with proteins in a cell

METALLOMICS: Study (quali and quantitative) of the metallome

HARAGUCHI, 2002

Examples of metalloenzymes (and metalloproteins)

Element	Enzymes/Proteins
Selenium	*Glutathione peroxidase*. An enzyme that catalyzes the reduction of peroxides and protects the cells from oxidative damage
Chromium	*Transferrin* (plasma protein). Transports Cr(III) throughout the body in the blood cells
Copper	*Ceruloplasmin* (human serum protein), *ascorbate oxidase* (plants and bacteria), *plastocyanin* (higher plants and cyanobacteria), *superoxide dismutase*, *tyrosinase*, *cytocrome oxidase* and *hemocuprein* (animals).
Lead	Around 95% of total Lead in human blood is bound to erythrocytes. In most erythrocytes, the lead is bound within the cell in *haemoglobin*. In extracellular fluids lead is bound to *albumin* and some high molecular weight proteins (*globulins*).
Zinc	Zinc is a constituent in more than 200 enzymes and proteins. The principal examples are *insulin* and *carboxypeptidase* A.
Manganese	This metal is found in a variety of enzymes such as *pyruvate carboxylase* and *oxalacetate decarboxylase*. It can be found in proteins such as *glutamine synthetase*, *β-globulin* and *albumin*.
Iron	Proteins containing iron are classified into two categories. The first is haeme, where this metal is chelated by *porphyrin* (a water-insoluble ligand). This class is constituted by *haemoglobin*, *myoglobin* and *cythochromes*. The second is formed by non-haeme iron. The principal examples are *transferrin*, *ferritin*, *ovotrasferrin*, *casein*, *hemosiderin* and *albumin*.

DECIPHERING METALLOMICS…

I) How the element (metal or metalloid) is distributed in cellular compartments of a cell

II) Its coordination environment; in which biomolecule is complexed or what is the bioligand involved in its complexation

III) Individual concentrations of the metallic species

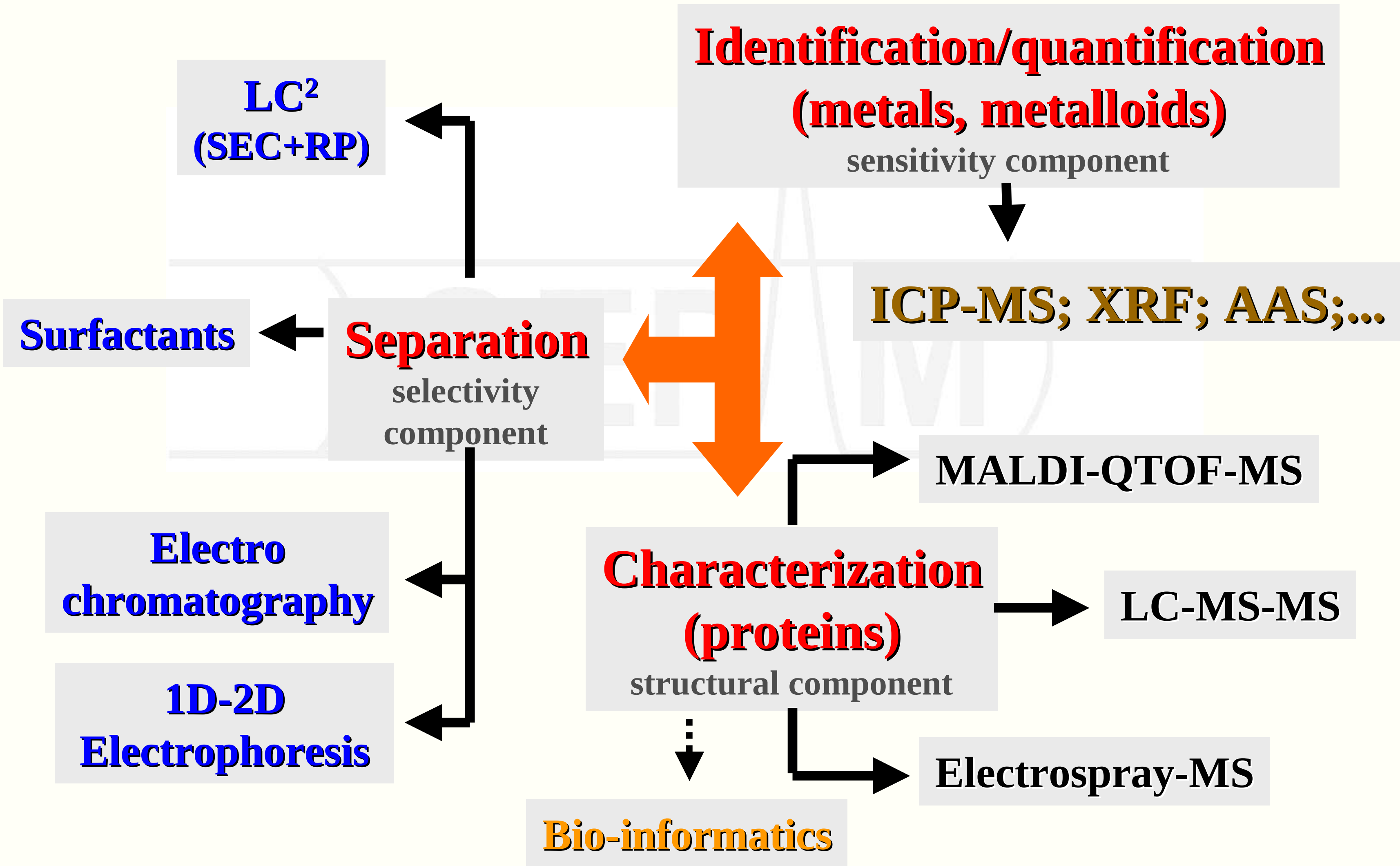
Classification on the metallomic information

Qualitative metallomics: individual species identification

Quantitative metallomics: concentration determination of the species

Comparative metallomics: monitoring of metallome alterations related to an organism, under influence of external stimulus.

Strategies on metalloproteomics studies



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Strategies on metalloproteomics studies

Should the sample preparation be the same one employed to proteomics studies?

To be or not to be?...it is the question

Evaluation of metalloprotein extraction procedures in phytotherapic medicines

Aesculus hippocastanum I. – Horse Chestnut

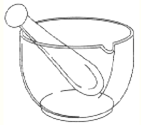
Magalhães & Arruda, *Talanta* 71(2007)1958

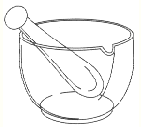
Protein extraction procedures

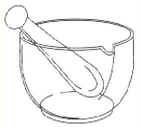
1 shaken (water) → centrifugation → 25°C

2 shaken (buffer) → centrifugation → 25°C

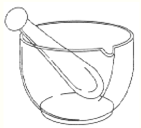
3 shaken → dialysis (42h) → centrifugation → 25°C

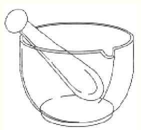
4  (water) → centrifugation → 25°C

5  (buffer) → centrifugation → 25°C

6  (water) → dialysis → centrifugation → 25°C

7 shaken (water) →  → centrifugation → 25°C

8  (water) →  → centrifugation → 25°C

9  (buffer) →  → centrifugation → 25°C

10 as in procedure 8 → 40°C

11 as in procedure 9 → 40°C

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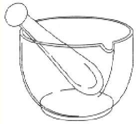
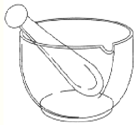
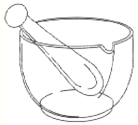
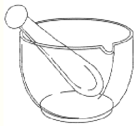
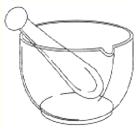
	Procedure	mg/g
1	shaken (water) → centrifugation → 25^{o}C	1.81±0.06
2	shaken (buffer) → centrifugation → 25^{o}C	1.51±0.06
3	shaken → dialysis (42h) → centrifugation → 25^{o}C	1.22±0.04
4	 (water) → centrifugation → 25^{o}C	3.03±0.08
5	 (buffer) → centrifugation → 25^{o}C	3.6±0.2
6	 (water) → dialysis → centrifugation → 25^{o}C	1.74±0.08
7	shaken (water) →  → centrifugation → 25^{o}C	2.51±0.04
8	 (water) →  → centrifugation → 25^{o}C	3.38±0.09
9	 (buffer) →  → centrifugation → 25^{o}C	**5.5±0.1**
10	as in procedure 8 → 40^{o}C	4.8±0.2
11	as in procedure 9 → 40^{o}C	3.8±0.2

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Mapping the metals… (qualitative metalloproteomics)

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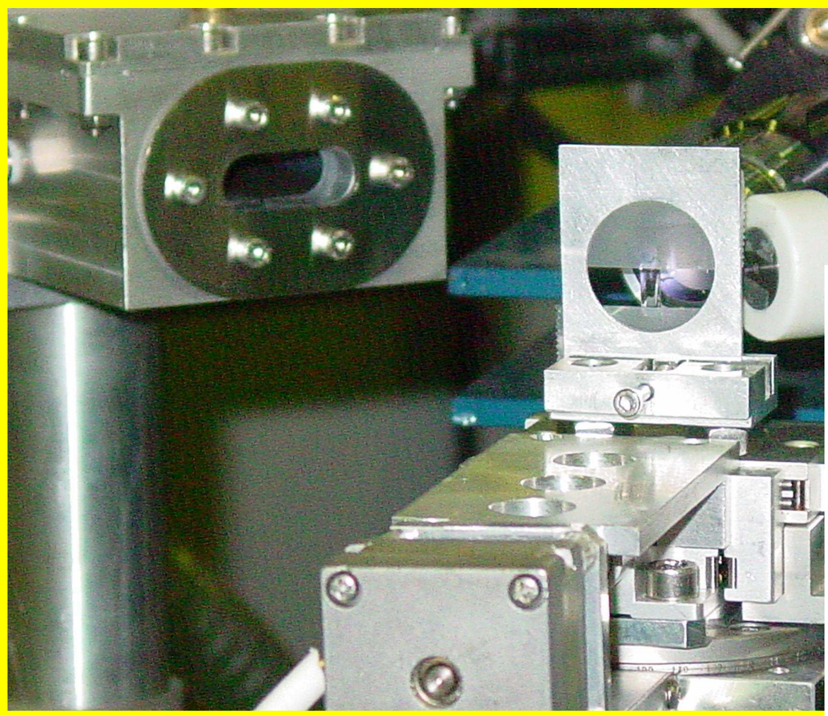
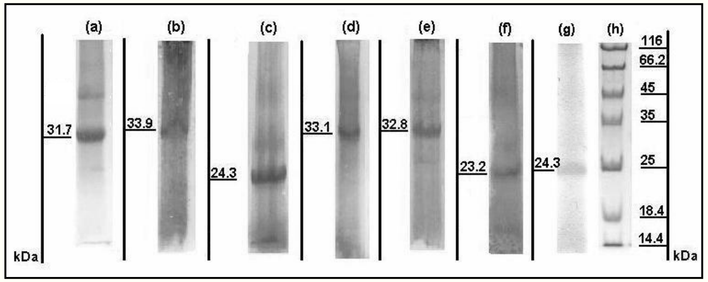
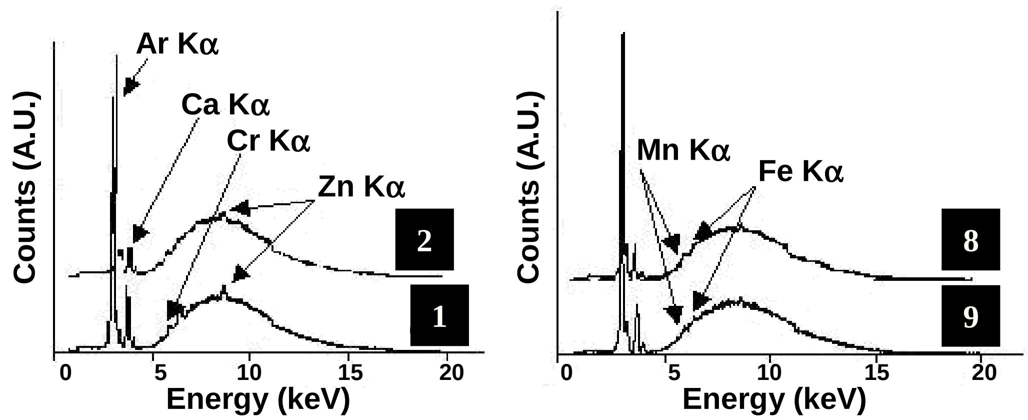
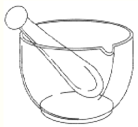
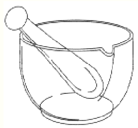




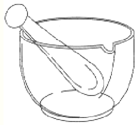


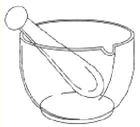
1	shaken (water)	⇒	centrifugation	⇒	25^{o}C		**1.81±0.06**
2	shaken (buffer)	⇒	centrifugation	⇒	25^{o}C		**1.51±0.06**

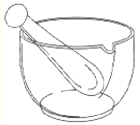
8  (water) ⇒  ⇒ centrifugation ⇒ 25^{o}C **3.38±0.09**

9  (buffer) ⇒  ⇒ centrifugation ⇒ 25^{o}C **5.5±0.1**

Determining the metals… (quantitative metalloproteomics)

4  (water) → centrifugation → 25^{o}C **3.03±0.08**

5  (buffer) → centrifugation → 25^{o}C **3.6±0.2**

9  (buffer) →  → centrifugation → 25^{o}C **5.5±0.1**

Procedure	Ca (µg g^{-1})	Cr (µg g^{-1})	Fe (µg g^{-1})	Mn (µg g^{-1})
1	12.5±0.5	4.98 ±0.05	35 ±6	2.7 ±0.4
2	9.8 ±0.7	1.6 ±0.7	38 ±2	1.9 ±0.2
4	6.4 ±0.3	4.00 ±0.02	40 ±7	15 ±1
5	11.4 ±0.8	1.5 ±0.4	37 ±7	12 ±2
8	0.74 ±1	< LOD	23 ±9	< LOD
9	< LOD	1.3 ±0.1	38 ±3	1.1 ±0.4

LOD: 0.05 µg g^{-1} Ca; 0.054 µg g^{-1} Cr; 0.99 µg g^{-1} Fe; 0.06 µg g^{-1} Mn

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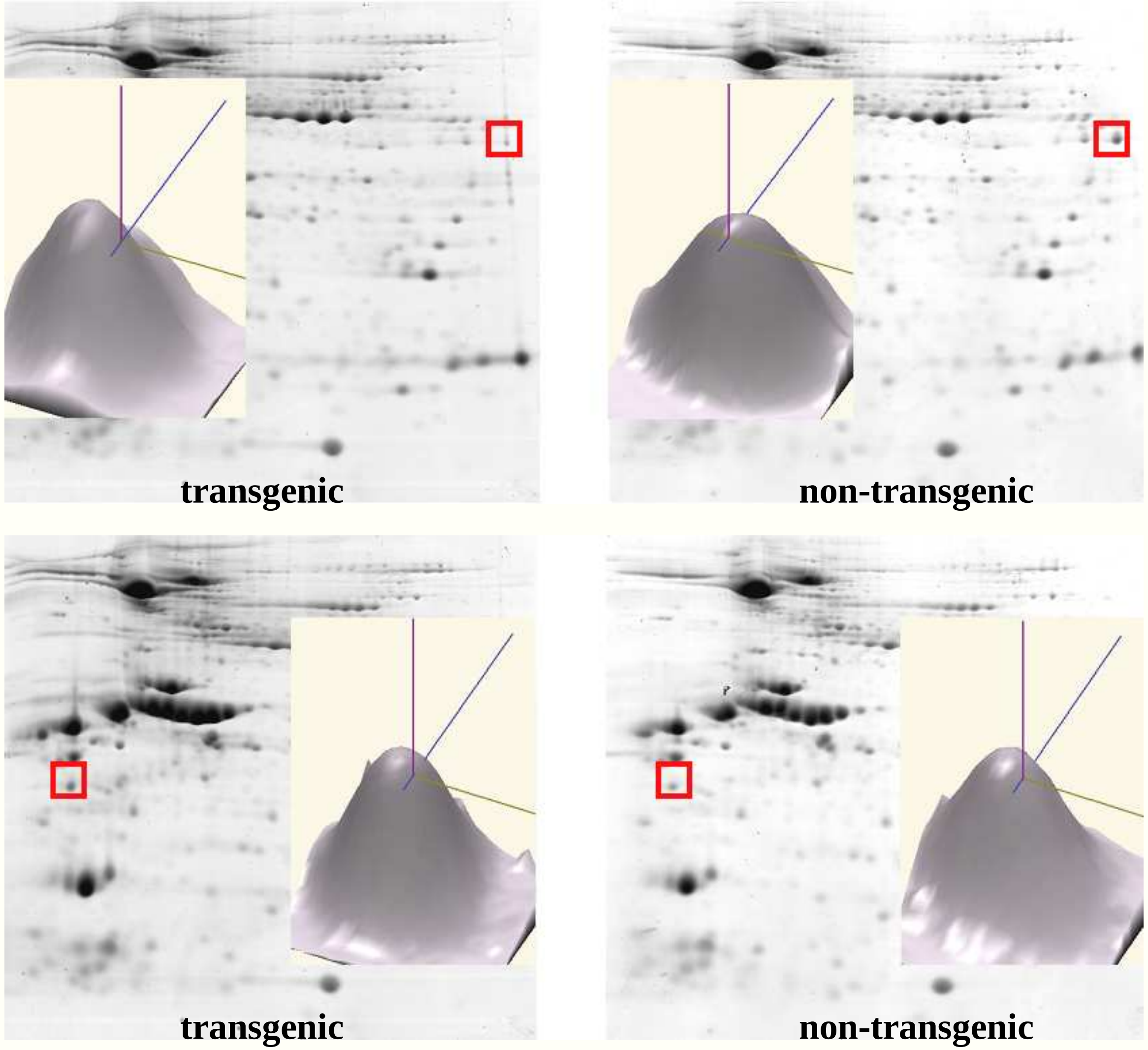
Comparative metallomics

Sussulini et al., *J. Anal. At. Spectrom*, 2007, **DOI**: 10.1039/b706684h

Transgenic organisms: changes in the proteome!!



54.50 kDa
pI: 6.83

27.03 kDa
pI: 4.37

Hypothesis: also metallome changes??

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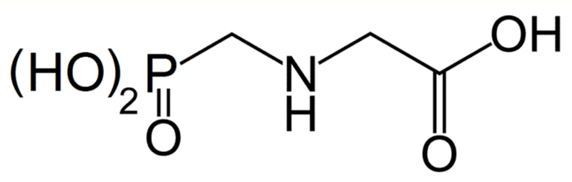
Comparative metallomics

Transgenic soybean [*Glycine max* (L.) Merril]: *Roundup Ready*®

Action of glyphosate: inhibits the EPSP synthase enzyme, which participates on aromatic amino acids synthesis in plants

Soybean genetic modification: based on inserting the CP4 EPSPS gene from *Agrobacterium* sp. strain CP4 that provides CP4 EPSPS (EC 2.5.1.19) protein (tolerant to glyphosate)



Non-selective herbicide: non-transgenic plants are also exterminated

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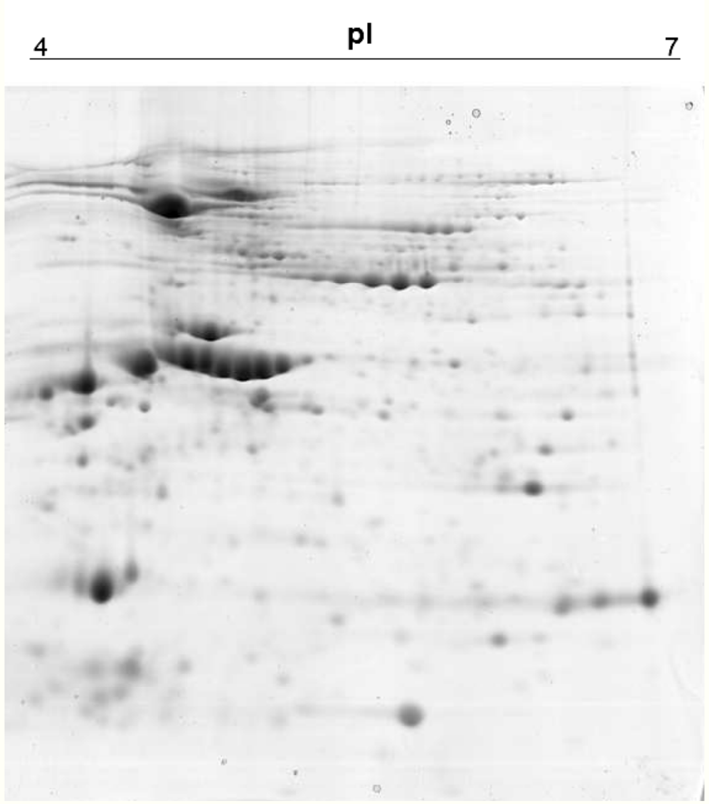
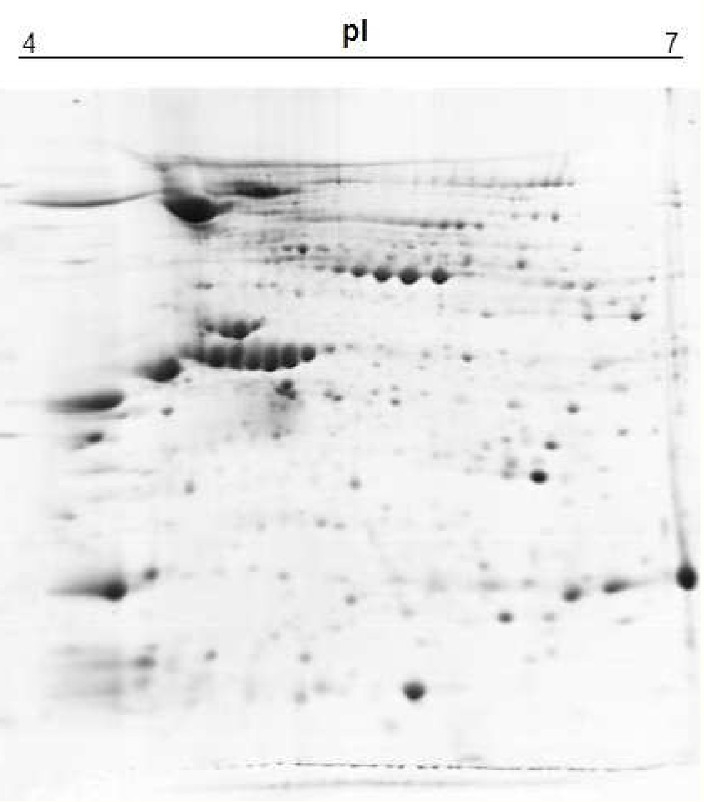


transgenic



non-transgenic

Transgenic soybean: 408±27
Non-transgenic soybean: 397±26
***match*: *ca*. 70% (n=3) and *ca*. 40%**

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Comparative metallomics - Strategy

Identification/quantification (metals, metalloids)
Sensitivity component

SR-XRF and AAS

2D electrophoresis

Separation
Selectivity component

Characterization (proteins)
Structural component

MALDI-QTOF-MS

Bio-informatics

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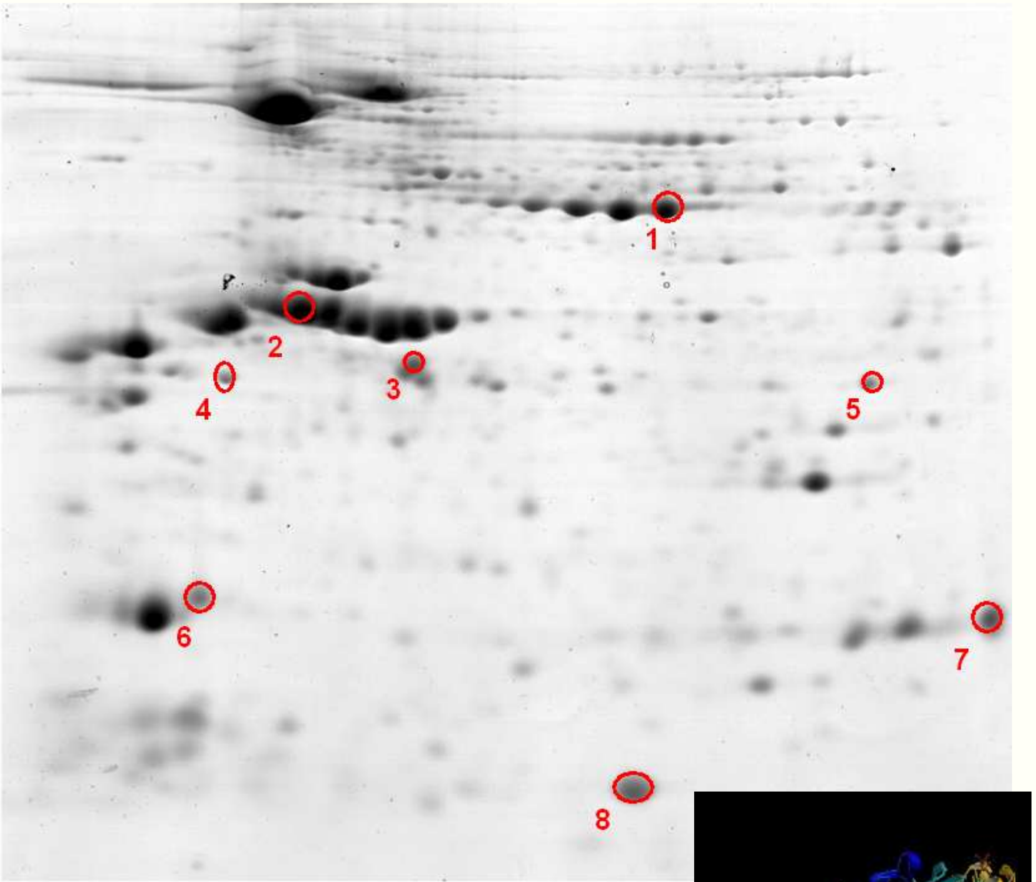
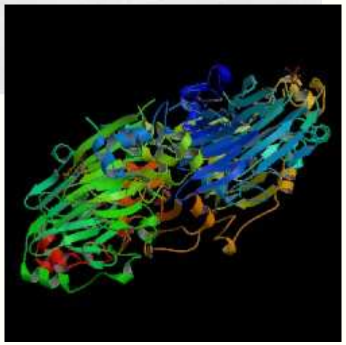
Comparative metallomics
Selectivity and Structural components

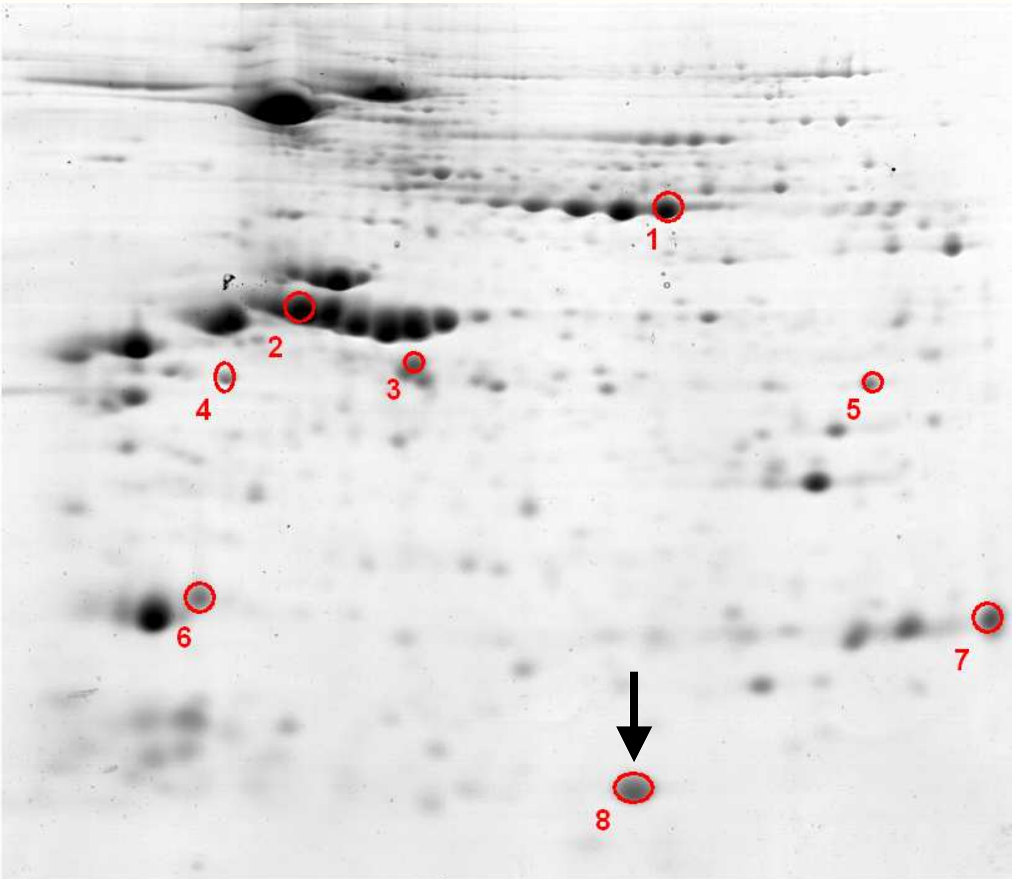


transgenic



1: β-conglycinin β-chain precursor
2: Glycinin G2
3: Soybean agglutinin
4: Seed maturation protein PM 25
5: not identified
6: not identified
7: Glycinin chain A2B1a precursor
8: Glycinin G4 (precursor)
→ **MALDI-QTOF MS**

Comparative metallomics
Sensitivity component



LA-ICP-MS

Extracted from Agilent CD

Binet et al., *Anal. Biochem.* 318(2003)30
Escherichia coli (Cd/Zn) → >> [metalloproteins] under stress conditions

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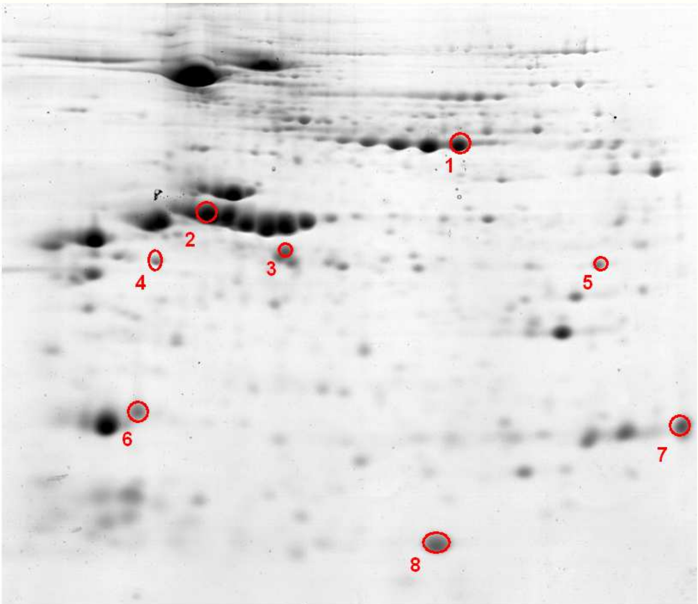
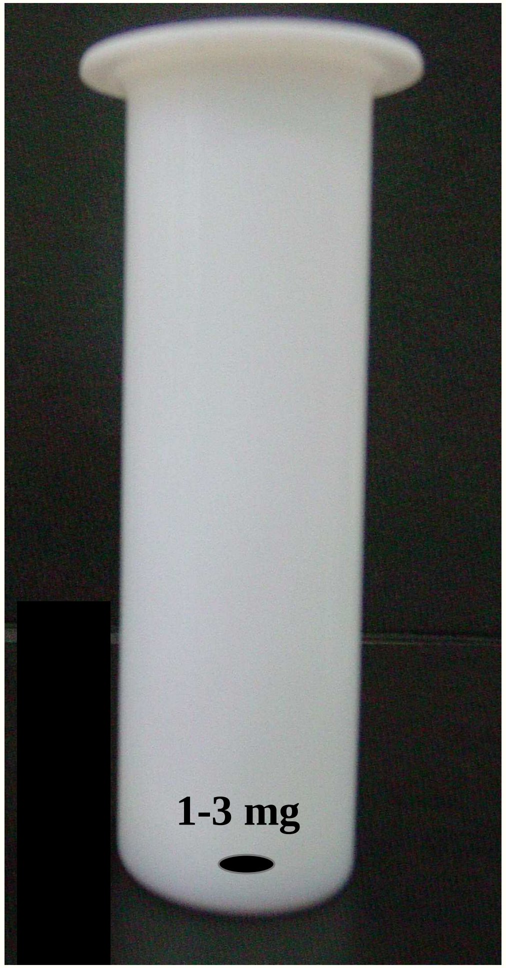
Comparative metallomics
Sensitivity component





gel decomposition

mini-vials

Sussulini <u>et al</u>., *Anal. Biochem.* 361(2007)146

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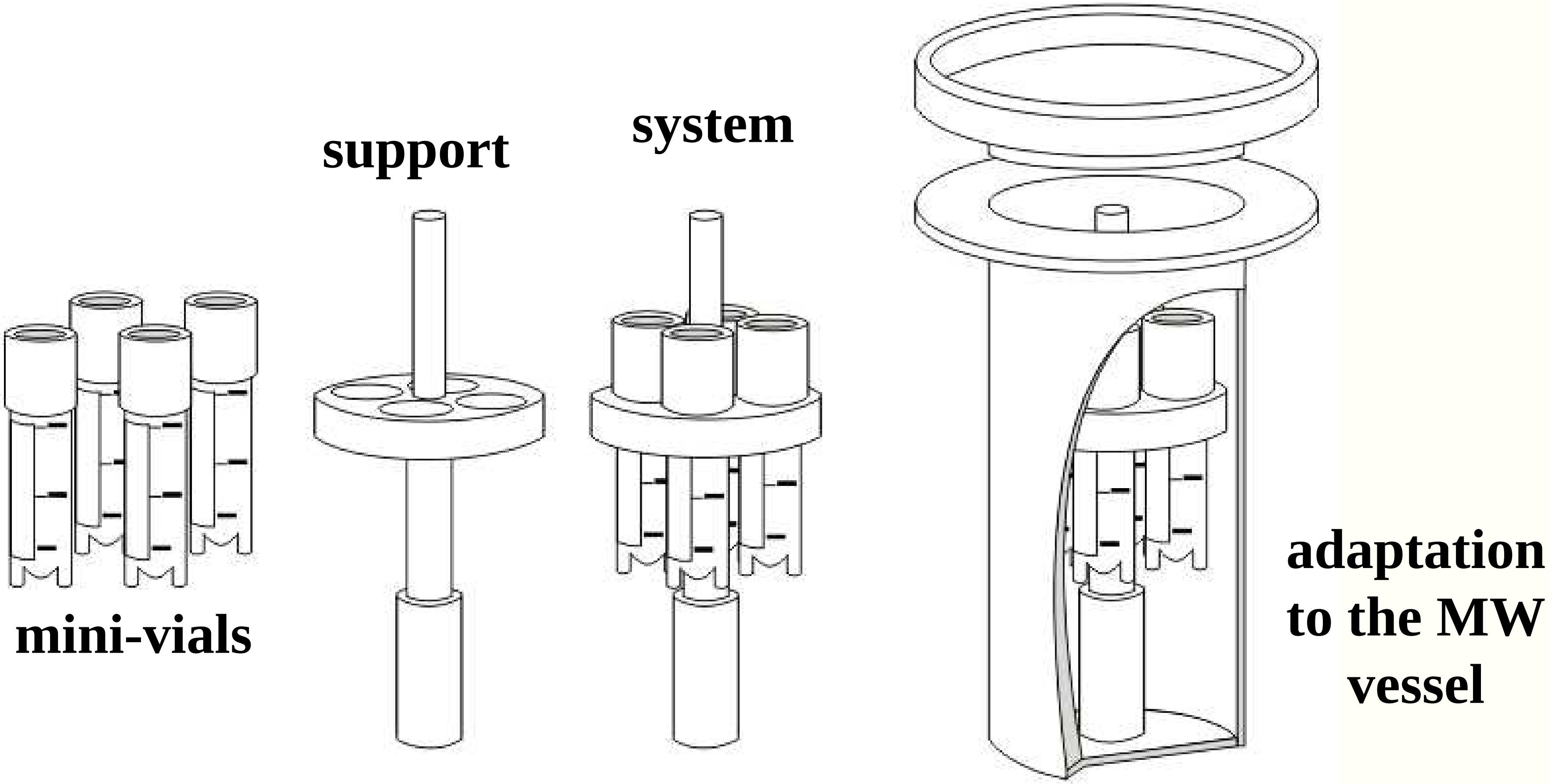
Comparative metallomics
Sensitivity component

Sussulini et al., *Anal. Biochem.* 361(2007)146



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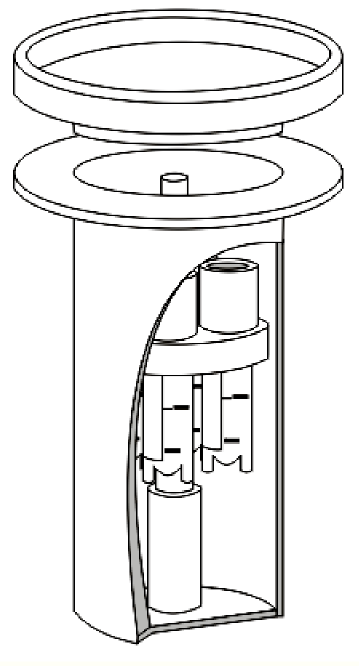
Comparative metallomics
Sensitivity component

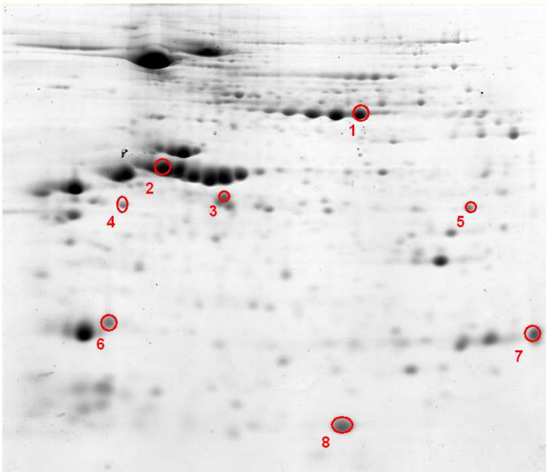


1ª step	300W - 1 min
2ª step	0W - 1 min
3ª step	500W - 30 s
4ª step	0W - 2 min
5ª step	500W - 1 min
6ª step	0W - 2 min
7ª step	800W - 30 s
8ª step	0W - 2 min
9ª step	800W - 30 s
10ª step	0W - 2 min
11ª step	800W - 30 s
12ª step	0W - 2 min
13ª step	500W - 1 min

Time: 16 min (2x)
Reagents: 200 µL HNO_3 + 150 µL H_2O_2
Mass: 1 – 2.5 mg
residual C : < 1%

Comparative metallomics
Sensitivity component – SR-XRF

Spot	TS	N-TS
1	Fe(+)	Fe
2	ND	Ca, Fe
3	Ca(+), Cu(+)	Ca, Cu
4	Ca	ND
5	Ca(+), Cu(+), Fe(+), Zn(+)	Ca, Cu, Fe, Zn
6	Ca, Fe	Ca(+), Fe(+), Mn, Ni
7	Ca	Ca(+), Fe
8	ND	Fe



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Comparative metallomics

Sensitivity component – AAS

	Ca[a] (mg g^{-1})		Cu[b] (µg g^{-1})		Fe[b] (µg g^{-1})	
Spot	TS	N-TS	TS	N-TS	TS	N-TS
1	ND[c]	ND	ND	ND	< LOQ[d]	< LOQ
2	< LOQ	< LOQ	ND	ND	212 ± 31	348 ± 49
3	2.6 ± 0.2	2.2 ± 0.4	1.5 ± 0.2	2.8 ± 0.2	ND	ND
4	< LOQ	< LOQ	ND	ND	ND	ND
5	17 ± 2	3.6 ± 0.5	1.6 ± 0.1	< LQ	691 ± 78	447 ± 54
6	3.5 ± 0.2	15 ± 2	ND	ND	663 ± 79	869 ± 93
7	< LOQ	< LOQ	ND	ND	< LOQ	< LOQ
8	ND	ND	ND	ND	< LOQ	< LOQ

a: FAAS; b: ETAAS; c: not determined; d: limit of quantification

Proteins of spots 1,2,7,8 → do not present metal in their structures;
Spot 2: Fe(II) micronutrient → better nutrient preservation??
Protein of spot 3 → involved in the metallic ions coordination from a bond site with carbohydrate [Ca(II), Mn(II) and others transition metallic ions]

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Conclusions

Decisive to preserve metals
in the protein structure

Different results in terms of
protein and/or metal extraction

Extraction procedure
Extraction medium

Must be carefully chosen:
proteomics and/or metalloproteomics

References

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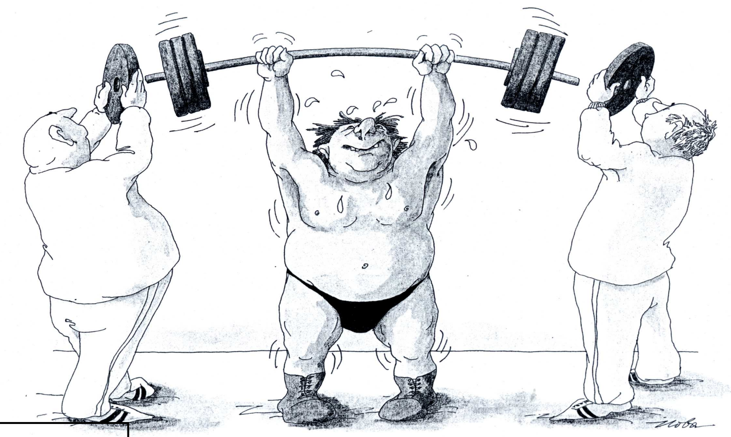
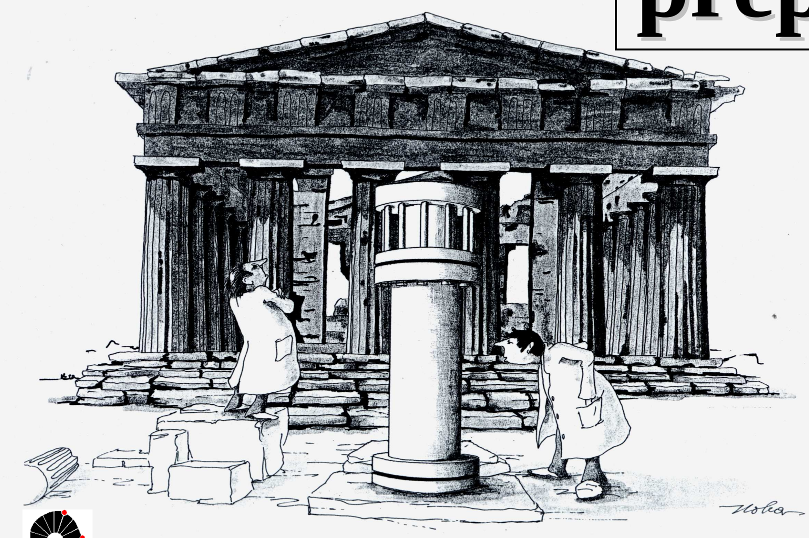
General Conclusions

in-deep view

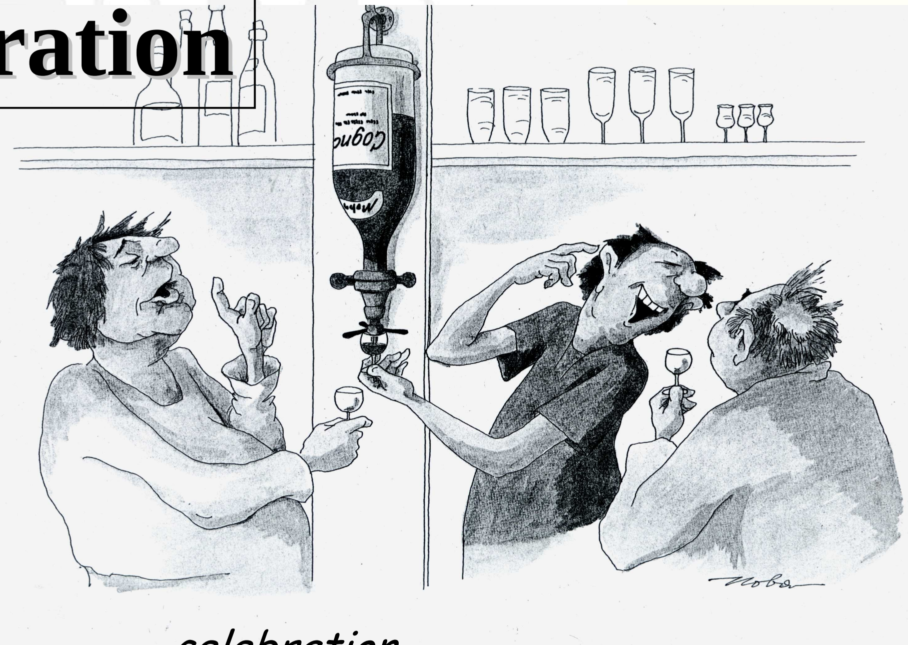


ability *knowledge*

Sample preparation



first stages



celebration...

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Special thanks…

FAPESP

CNPq
50 ANOS

CAPES

LNLS

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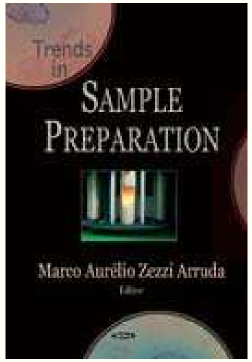
…and even more

Trends in Sample Preparation



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"That's all Folks!"

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