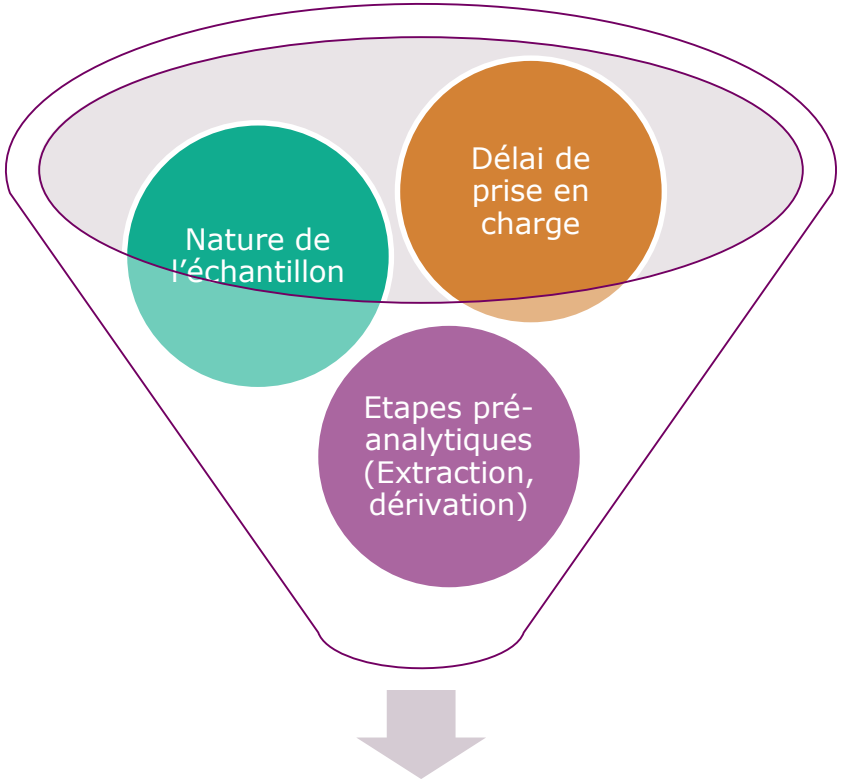
Université de Lille

CM2
Sample preparation

Les points clés de la préparation d'échantillon



Facteurs à risque

Facteurs confondants susceptibles de masquer le phénomène biologique que l'on souhaite étudier :

- *Facteurs d'origine biologique : âge, sexe, effets chronobiologiques, facteurs environnementaux*
- *Très grande diversité des métabolites (propriétés physico-chimiques, concentrations)*
- *Des métabolites de masse ou de structures voisines*
- *Facteurs analytiques (Type d'appareillage)*
- *Qualité de la préparation de l'échantillon*
  - *élimination partielle de la matrice biologique*
  - *Métabolomique globale => traitement simple pour éviter des pertes d'informations*
  - *éliminer les sels et les macromolécules*
- *Normalisation des résultats selon les milieux : urines, tissus…*

Sample preparation

Wide physicochemical properties diversity of metabolites
- *Hydrophobycity/hydrophilicity*
- *Volatility*
- *Chemical reactivity/stability*

No single method of sample collection
- *Sampling*
- *Quenching*
- *Storage*

No single method of sample preparation
- *Extraction*
- *Dilution*
- *Clean-up*

Conditions that stabilize one type affect other types
- *Appropriate methods for the majority of metabolites*

Sample preparation

Metabolism is always changing => concentration variation

Ideal sample prep. method for global metabolomics requires:

i. *Quenching step*
ii. *Nonselective strategy*
iii. *Simple & fast*
iv. *Reproducibility*

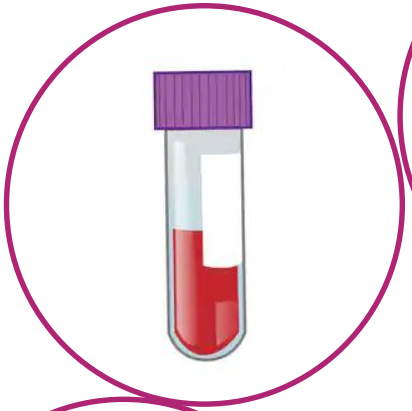
Le délai de prise en charge

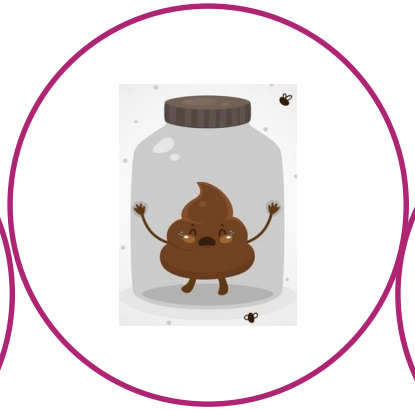
- Point critique majeur
- Corrolaire = « bloquer les voies métaboliques le plus vite possible» = « quenching »
- Labilité des métabolites
- Risque d'interconversion ou de dégradation

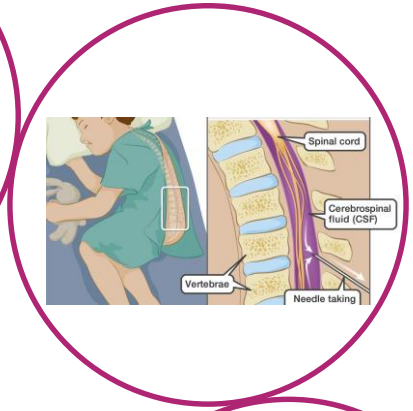
Métabolome à l'instant T ≠ T+1

- La manière de le réduire au max doit être envisagée avant toute mise en œuvre pratique (« snap-freezing »?)

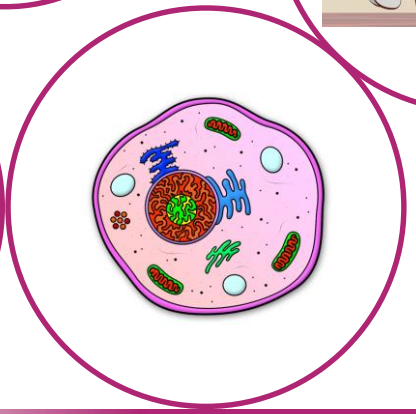
La nature de l'échantillon







Diverse range of sample type



La nature de l'échantillon

- Influence indirectement le temps de prise en charge

- Effet matrice : influence le rendement des étapes ultérieures
  - ✓ *Sang: peu de problème*
  - ✓ *Urine : nécessité de standardisation initiale (pH, sel)*

- Nécessité de normaliser
  - ✓ *Urines : créatininurie*
  - ✓ *Tissus : pesée de tissu préalable*
  - ✓ *Selles : pesée de selles préalable*
  - ✓ *Cellules : numération cellulaire ou dosage de protéines ?*

La nature de l'échantillon

- Influence indirectement le temps de prise en charge :
  - ✓ *LCR : congeler*
  - ✓ *Selles : congeler (penser à peser le tube vide avant…)*
  - ✓ *Urine : centrifuger, décanter, aliquoter, congeler*
  - ✓ *Sang : centrifuger (10 min 2500 ×g), décanter, aliquoter, congeler*
  - ✓ *Tissus : broyage dans azote liquide, homogénéisation dans tampon*
  - ✓ *Cellules en culture : adhérentes ou tapis cellulaire ?*
    - laver ou pas ?
    - trypsine ou pas ?

Urine & sang

- Blood and urine => the most frequently studied samples
- Sample collection is noninvasive (urine) or minimally invasive (blood)
- Blood and urine are integrative biofluids that incorporate the functions and phenotypes of many different parts of the body in a single sample,

  'metabolic footprint' of tissue metabolism
- These biofluids also contain many hundreds or thousands of metabolites
  - related to gut microflora
  - lipid and drug metabolism

Etape d'extraction

- Dépend du milieu biologique (extraits cellulaires ou fluides biologiques)
- Dépend de la structure des composés (polarité, lipides…)
- Dépend du mode d'analyse choisi (ciblé ou non ciblé) et donc du nombre de métabolites à rechercher
- Ajout de standards internes pour quantification
  - Au cours de l'étape
  - Préalablement à l'étape
- Différentes approches
  - Précipitation par un acide fort (ex : acide sulfosalicylique pour CAA)
  - Extraction sur phase solide
  - Extraction liquide-liquide
  - Concentration (lyophilisation, évaporation)

Quenching strategy

Quenching step

(i) ensure a fast and complete blockage of any intracellular metabolic reaction, as most intermediates have high conversion rates

(ii) avoid contamination of the sample with extracellular metabolites present in the supernatant

(iii) lose none of the intracellular sub-stances through leaky membranes

Inactivation of the metabolism should be faster than metabolic changes

*Turnover rates of many primary metabolites are in the range of 1mM/s (*1.5 mM/s for ATP, 2.0 mM/s for ADP, 1.0 mM/s for cytosolic glucose)

Sample integrety should be carefully preserved during the process

No significant variations in chemical and physical properties or in the concentration of metabolites

Resulting quenched sample should be amenable to subsequent steps of the analytical process

Quenching strategy

Quenching methods are based on rapid modification of sample conditions

pH variation
Quenching quenching is achieved by instantly changing to extreme pH, either to high alkali (e.g., by adding KOH or NaOH) or to high acid pH (e.g.,by adding perchloric, hydrochloric or trichloroaceticacid)

Temperature variation
Quenching is mainly carried out by cooling at values usually lower than -20°C

No significant variations in chemical and physical properties or in the concentration of metabolites

Quenching strategy

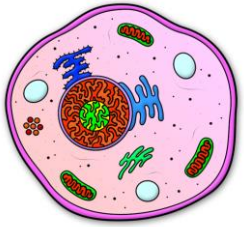
Biofluids

- Cold organic solvents (-40°c)
- pH variation
- I variation

Tissues

- Liquid N_2 (-196°c)

Cells, yeast…



- Cold organic solvents (<-20°c)

Quenching strategy

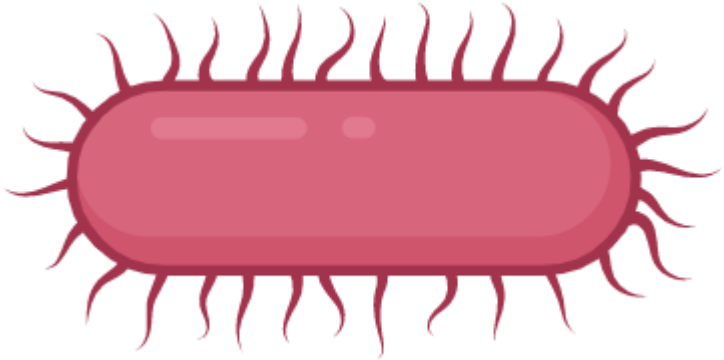
Cold Methanol
Most popular quenching method; allows a rapid interruption of the metabolism in the sub-second time scale

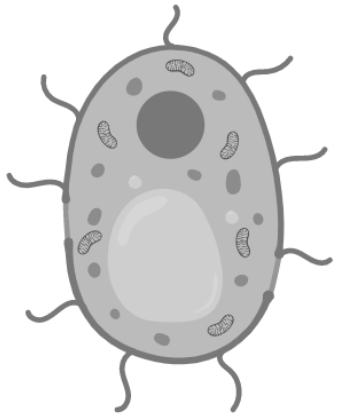
Can be implemented in protocols to discriminate between intracellular and extra cellular metabolites

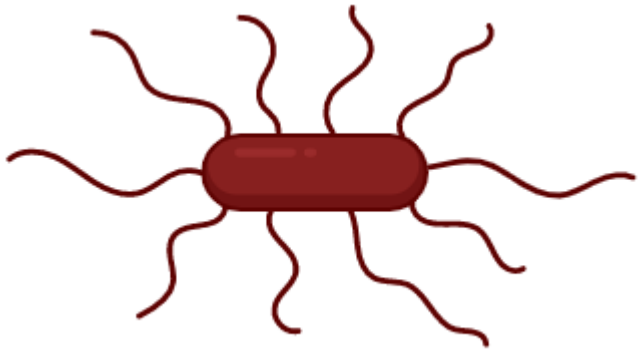
For culture cell

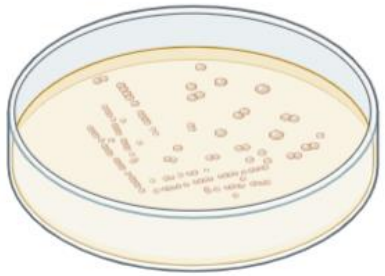
1. Isolation of the cells from media by fast filtration
2. Adherent cultures => aspirate the media
3. Add quenching solution (MeOH)
4. Maintaining the sample temperature below -20°C
5. Cells collection by a centrifugation step prior to extraction

Bacteria & yeast









Quenching strategy

Loctabacillus plantarum cells

Quenching

Table 1: ATP leakage (%) of different samples from different cultures.

Samples from same culture				Samples from different cultures	
Samples	**MeOH/HEPES**	**Samples**	**MeOH**	**Samples**	**MeOH/HEPES**
1	2.3	5	13.0	7	2.5
2	1.6	6	12.0	8	6.4
3	3.7	-	-	9	4.3
Average	2.5 ± 0.9	Average	12.5 ± 0.5	Average	4.4 ± 1.5

- Percentage of ATP that was measured in the supernatant of L. plantarum cells after quenching.
- Samples 1, 2 and 3 come from the same batch culture and were quenched with MeOH/HEPES (70mM, pH 5.5)
- Samples 7, 8 and 9 come from different batch cultures and were also quenched with MeOH/HEPES solution
- Samples 5 and 6 come from the same batch culture but were quenched with 60% aqueous methanol solution

Microbial Cell Factories 2007, 6:27

Quenching strategy

Loctabacillus plantarum cells

Quench + wash

Table 2: ATP leakage (%) with four different quenching procedures.

	Batch culture			Chemostat culture (D = 0.06 h^{-1})		
Supernatants	**MeOH**	**MeOH/HEPES**	**MeOH/NaCl**	**MeOH/HEPES**	**MeOH/NaCl**	**MeOH/AC**
Quenched	12.0	6.4	9.5	4.3	15.0	8.1
1st washed	14.7	1.2	4.8	0.5	5.4	0.8
Total	26.7	7.6	14.3	4.8	20.4	8.9

- Percentage of ATP that was measured in the supernatant after quenching and washing with different quenching solutions:
  - ✓ *60% methanol (MeOH)*
  - ✓ *60% methanol and 70 mM of HEPES (pH 5.5) (MeOH/HEPES)*
  - ✓ *60% MeOH and 0.85% NaCl (MeOH/NaCl)*
  - ✓ *60% methanol and 0.85% ammonium carbonate (pH 5.5) (MeOH/AC)*

Microbial Cell Factories 2007, 6:27

Quenching strategy

Loctabacillus plantarum cells

Quench + wash + extraction

Table 3: Adenine nucleotide concentrations and energy charges in cell extracts of a chemostat grown culture of *Lactobacillus plantarum*.

Quenching solution	ATP (mM)	ADP (mM)	AMP (mM)	Total (mM)	EC
MeOH/HEPES	5.88	2.04	0.44	8.36	0.83
MeOH/AC	4.75	2.11	1.53	8.39	0.70

- Intracellular concentrations of ATP, ADP and AMP in cell extracts derived from a pH-controlled continuous culture.
- Two samples were quenched with:
  - ✓ *60% MeOH and 70 mM HEPES (pH 5.5) (MeOH/HEPES)*
  - ✓ *60% MeOH and 0.85% ammonium carbonate (pH 5.5) (MeOH/AC)*
- Cell pellets washed the same quenching solution
- Extraction solution: cold methanol and freeze-dried

The energy charge (EC) of the cell extract was calculated by $EC = \frac{[ATP] + 0.5[ADP]}{[ATP] + [ADP] + [AMP]}$

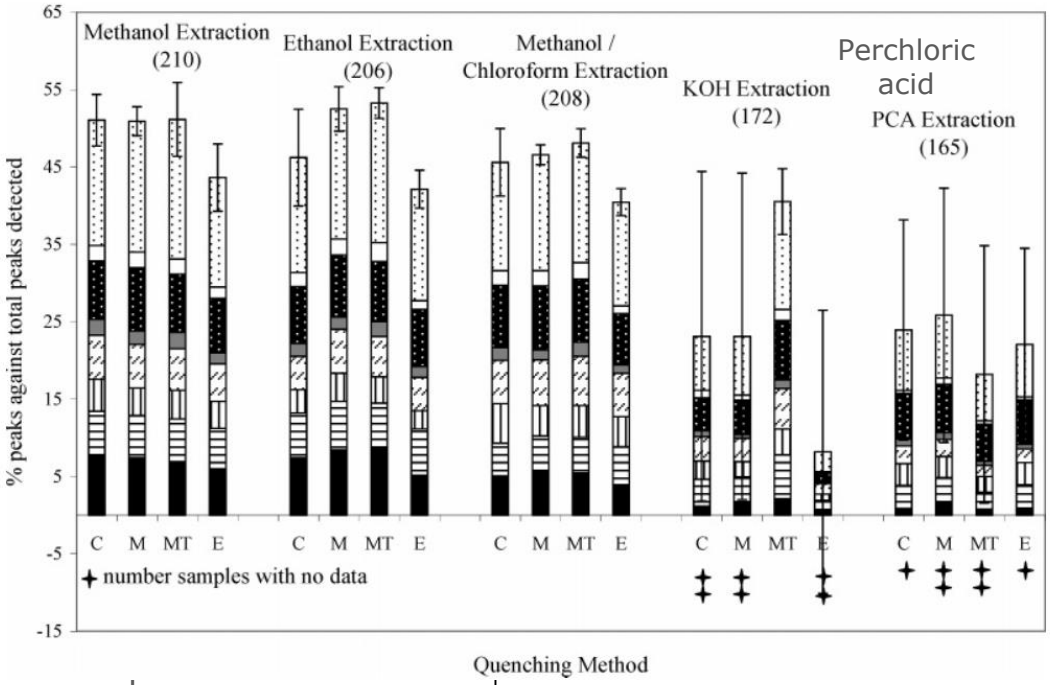
Microbial Cell Factories 2007, 6:27

Quenching strategy

Evaluation on *Escherichia coli* cultures

Effect of quenching methods

- **C**: control, chilliing at -48°c
- **M**: 60% MeOH (-48°c)
- **MT**: 60% MeOH + 0.5mM tricine (-48°c)
- **E**: Boiling absolute ethanol (90° C)



- **Black spots**: unknown
- **White**: sugar phosphates
- **White spots**: sugars and sugar alcohols
- **Gray**: phosphate derivatives
- **Diagonal lines**: organic acids,
- **Vertical lines**: others
- **Horizontal lines**: fatty acids
- **Black**: amino acids and nitrogen-containing compounds

- Number of peaks for each method, represented as a percentage of the total number of peaks detected
- The largest number of peaks was observed with either M & MT solutions
- Hot ethanol as a quenching solution had a detrimental effect => degradation of thermolabile metabolites, increase in the cell permeability and leakage of metabolites

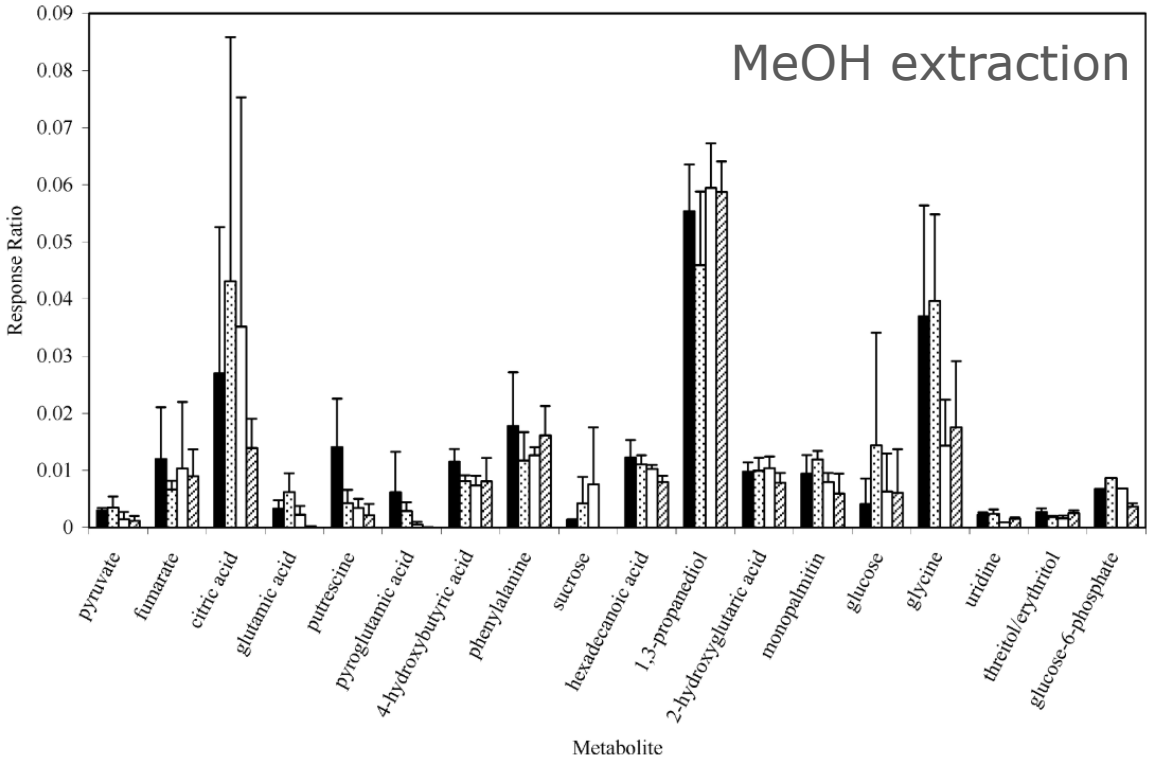
Anal. Chem. 2008, 80, 2939-2948

Quenching strategy

Evaluation on *Escherichia coli* cultures

Effect of quenching methods

- Black, control
- Black dots, 60% methanol
- White, 60% methanol and 0.5 mM tricine
- Diagonal lines, boiling ethanol



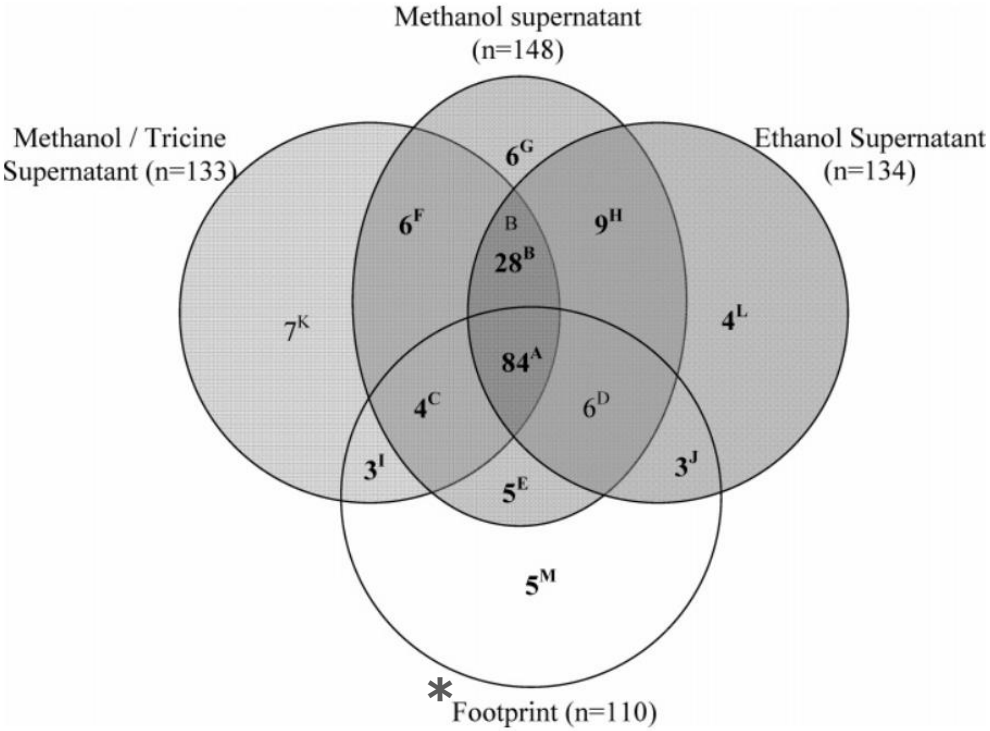
- Contact with solvents during the quenching step decreased the relative recovery of some metabolites (e.g. putrescine and glutamic acid/pyroglutamic acid)
  - ✓ *Increase in the amount of these metabolites released into the quenching solutions*
- 60% methanol produced the greatest recovery of the majority of the selected metabolites, whereas boiling ethanol quenching decreased the recovery

Quenching strategy

Evaluation on *Escherichia coli* cultures

Leakage evaluation during quenching

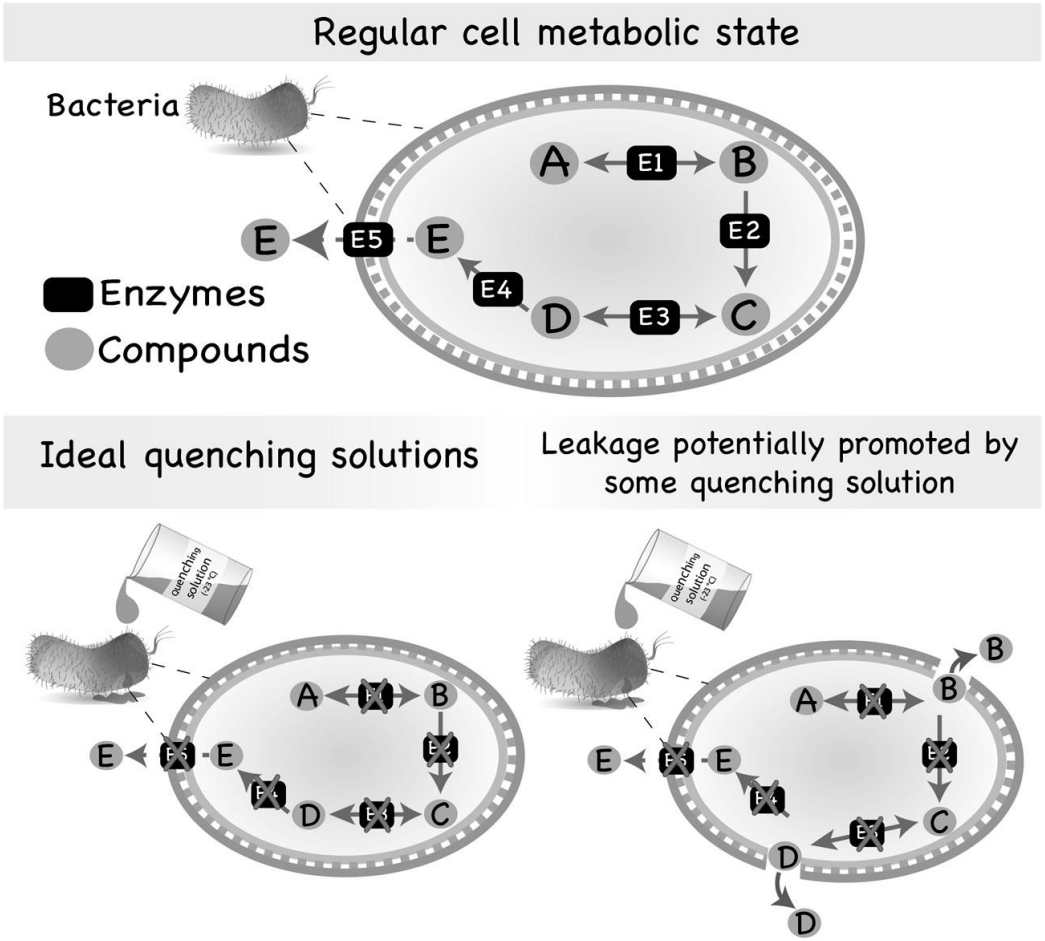
65 metabolites were present in the quenching supernatants but not the footprint samples



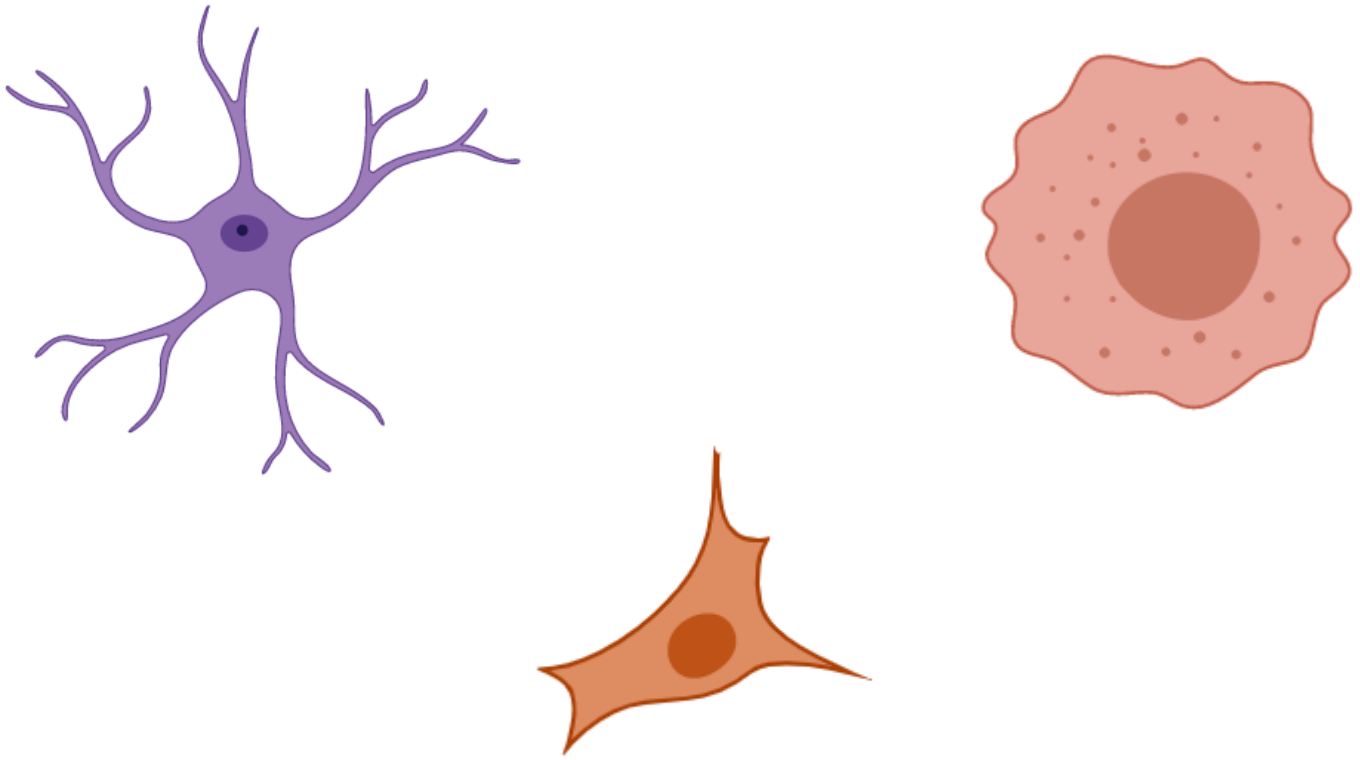
*Metabolic footprint = exometabolome

Anal. Chem. 2008, 80, 2939-2948

Quenching strategy
Metabolites leakage

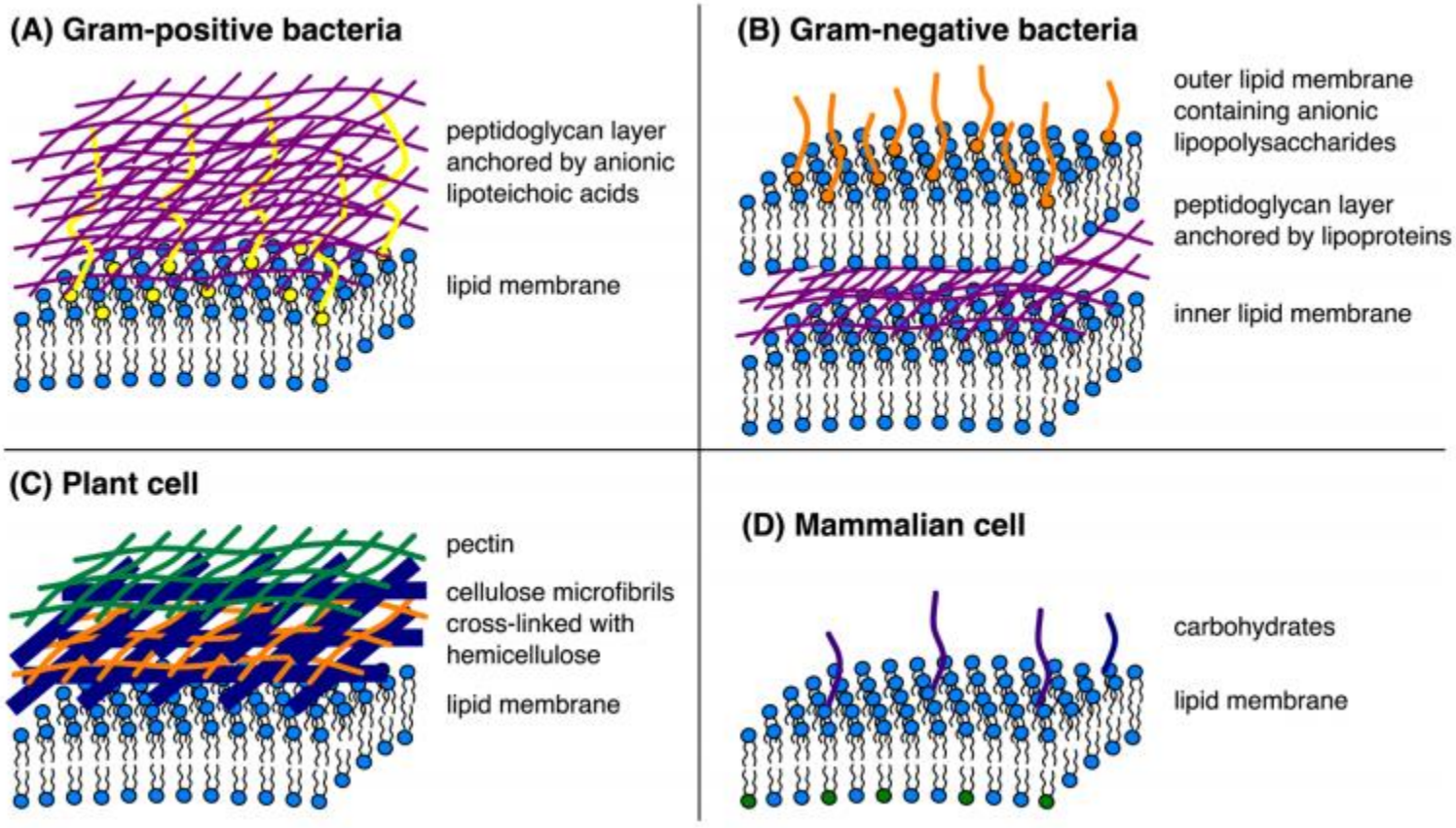


Mammalian cells



Quenching strategy

Metabolites leakage

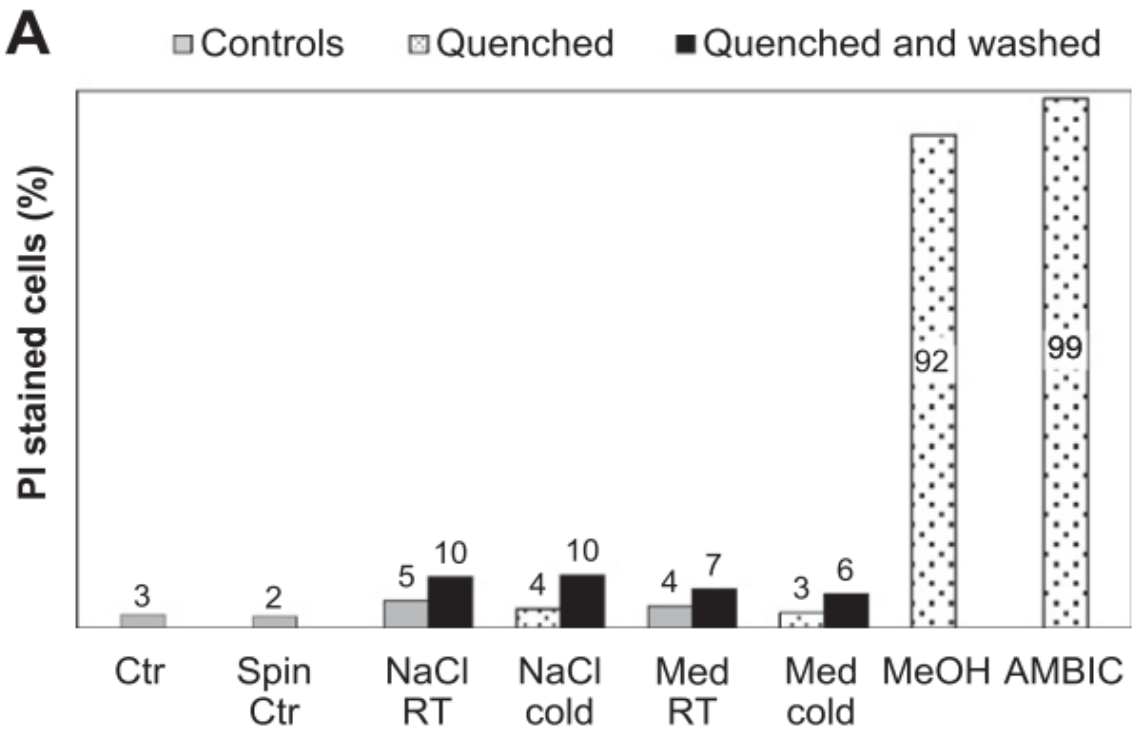


Quenching strategy
Metabolites leakage

Cold organic solvent => successfully implemented for yeast and bacteria
Direct application to mammalian cells => high leakage of intracellular metabolites as mammalian cells lack a cell wall.

A less aggressive quenching method : ice-cold PBS, prevents leakage while at diluting extracellular contaminants
Do not achieve temperatures < 0 ◦C

Analysis of CHO *(Chinese hamster ovary)*

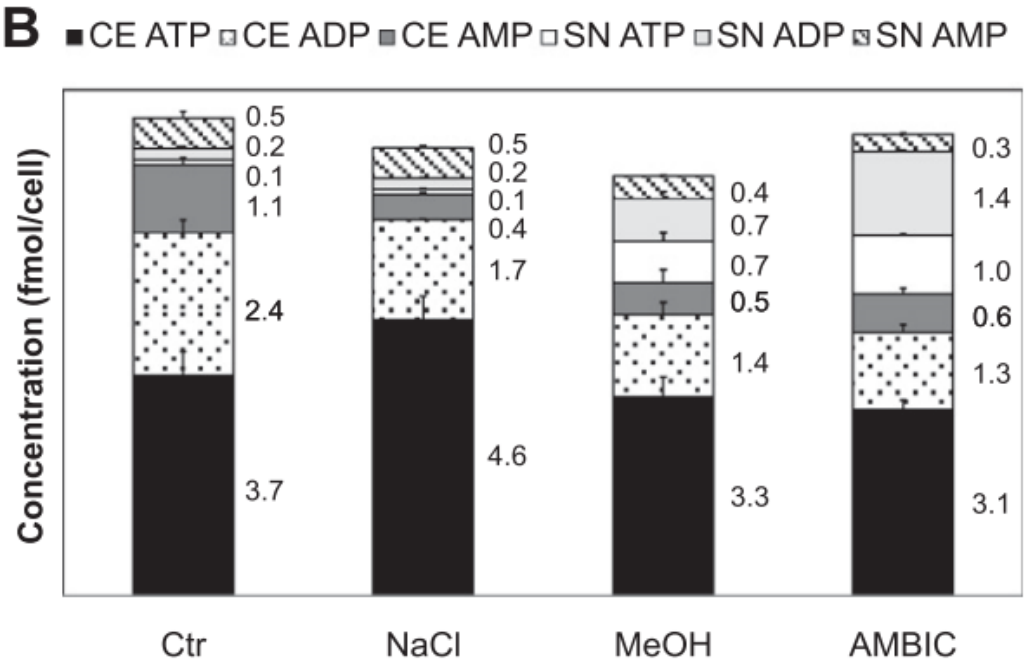


PI (Iodure de propidium)
= viability marker
PI is a fluorescent
molecule that binds to DN

Quenching with ice-cold NaCl increased the percentage of PI stained cells only slightly compared with the untreated control. In contrast, more than 90% of cells quenched with MeOH and MeOH/AMBIC were stained with PI. This indicates that even brief contact with MeOH damages the cell membrane, possibly allowing intracellular metabolites to leak from the cells

Anal. Biochem. 404 (2010) 155–164

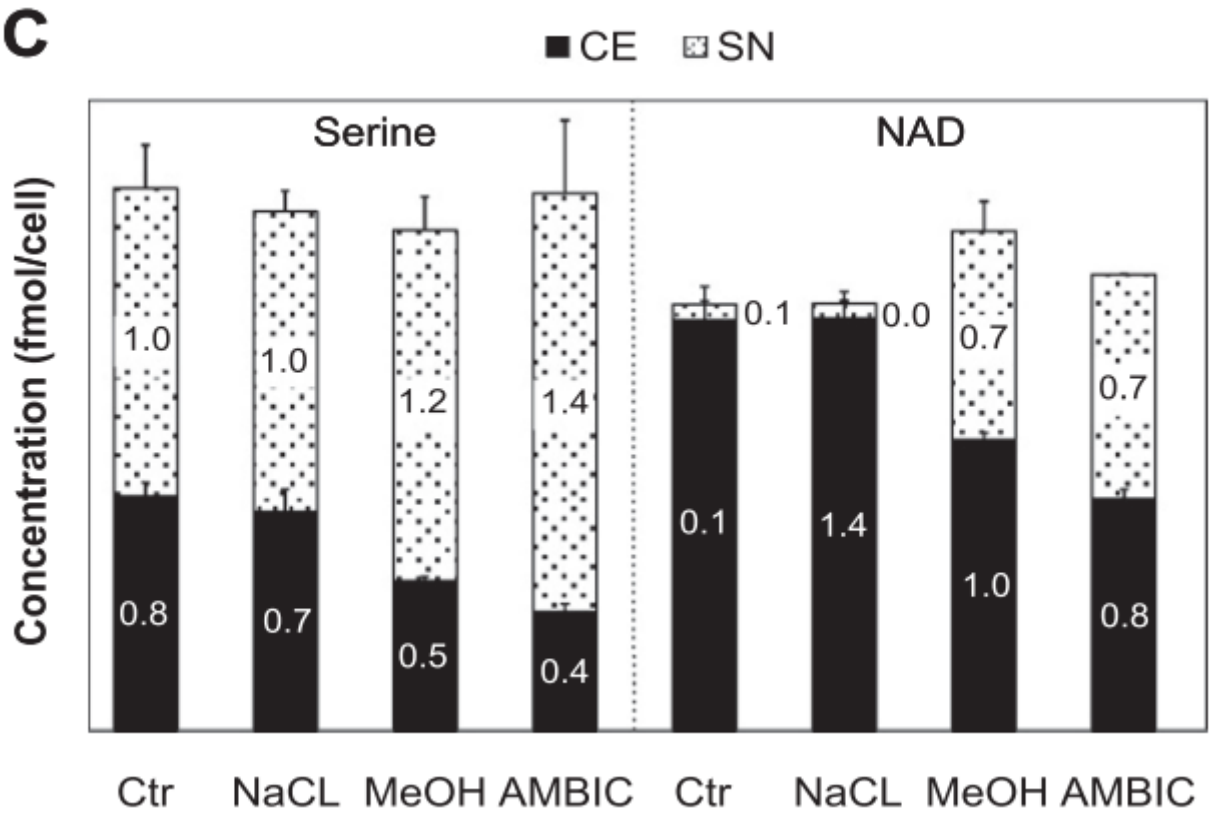
Analysis of CHO



- Concentrations of ANPs (ATP, ADP, AMP), NAD, and serine in the cell extracts (CE) and supernatants (SN) of quenched and unquenched cells
- The concentration of intracellular ATP in cell extracts from NaCl quenched cultures is significantly higher than in unquenched control cultures???
- Cold NaCl slows down the conversion of ATP to ADP

Anal. Biochem. 404 (2010) 155–164

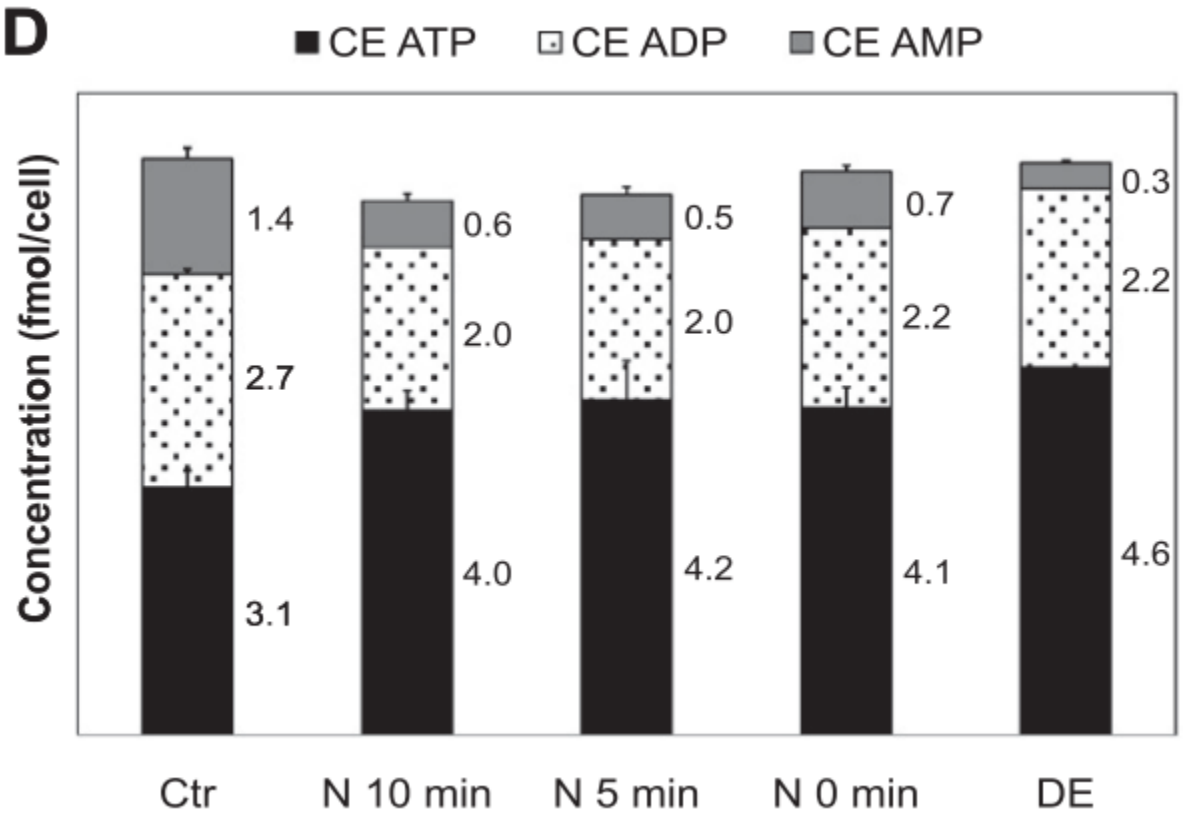
Analysis of CHO



Concentrations of NAD, and serine in the cell extracts and supernatants of quenched and unquenched cells

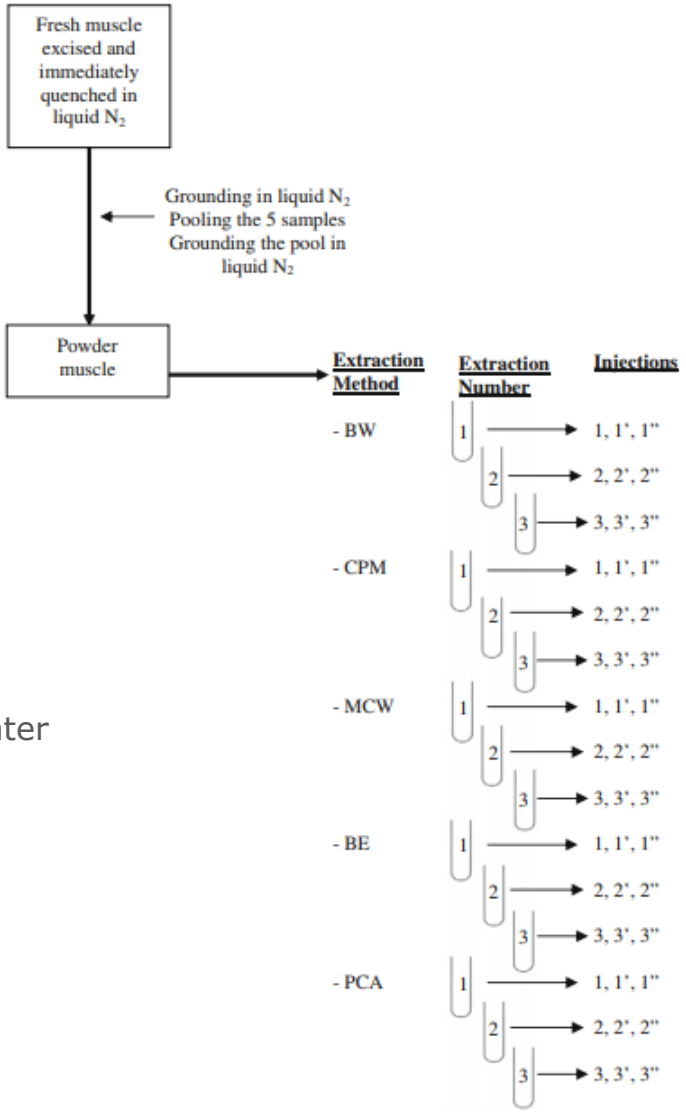
Anal. Biochem. 404 (2010) 155–164

Analysis of CHO



Intracellular ATP concentration in NaCl quenched cells was not significantly different from the ATP concentrations in directly extracted cultures but was significantly higher than in unquenched control samples

Analysis of skeletal muscle metabolome



- **BW**: Boiling water
- **CPM**: Cold pure methanol
- **MCW**: Methanol/chloroform/water
- **BE**: Boiling ethanol
- **PCA**: Perchloric acid

Analysis of skeletal muscle metabolome

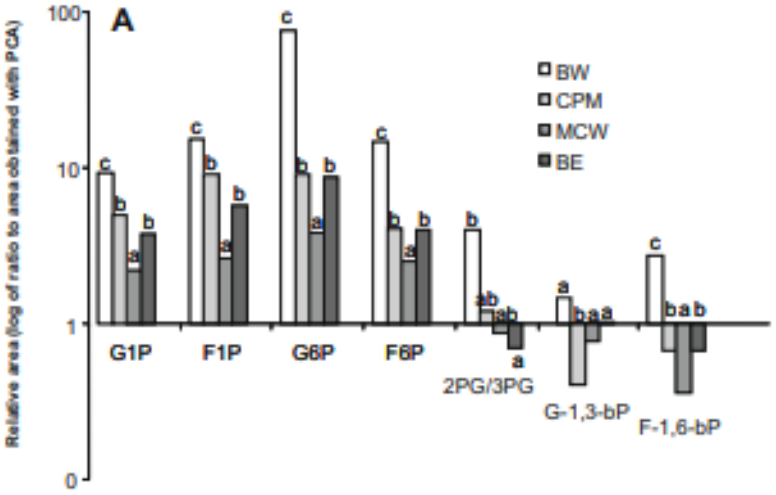
Retention times and transitions of selected metabolites

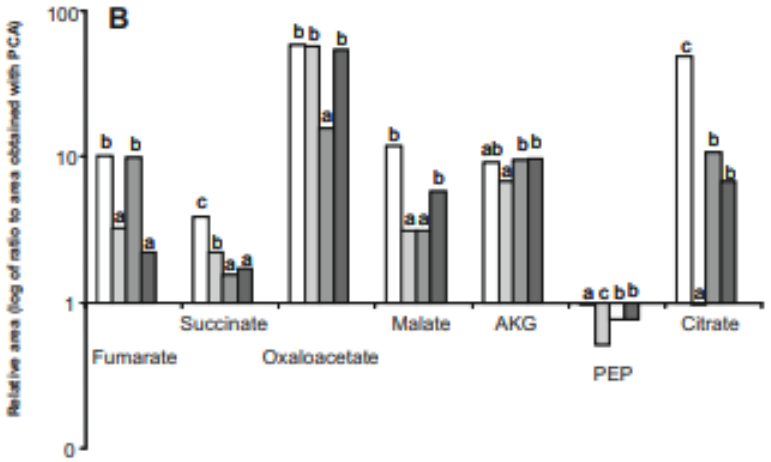
	Retention time (min)	Transition (MRM)
Sugar phosphates		
G1P	5.70	259/97
F1P	14.19	259/97
G6P	15.79	259/97
F6P	17.60	259/97
F-1,6-bP	44.20	339/97
Phosphorated organic acids		
2PG/3PG	29.40	185/97
G-1,3-bP	33.30	265/79
Other phosphorated compound		
Creatine-P	4.00	210/79
Nucleotides		
AMP	18.63	346/79
ADP	39.00	426/79
ATP	50.30	506/159
IMP	50.70	347/79
IDP	52.80	427/79
ITP	61.00	507/159
Organic acids		
Fumarate	11.40	115/71
Succinate	7.79	117/73
Oxaloacetic acid	7.38	131/87
Malate	7.84	133/71
AKG	7.35	145/101
PEP	33.40	167/79
Citrate	31.30	191/111

Note. 2PG/3PG, 2- and 3-phosphoglycerate; AKG, α-ketoglutarate.

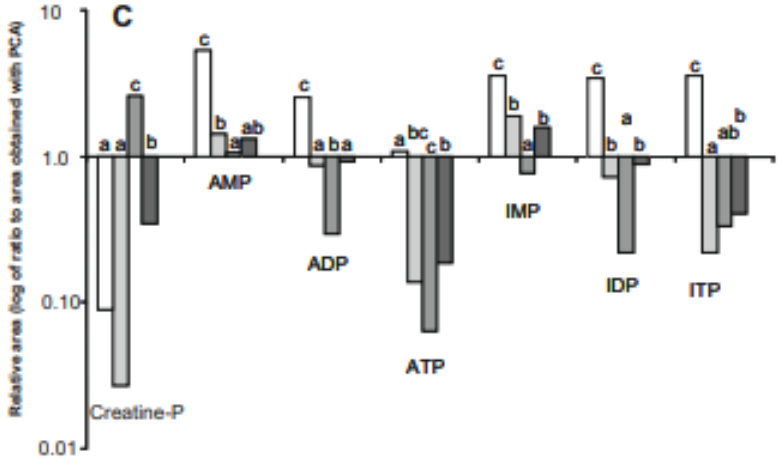
Analysis of skeletal muscle metabolome

Recovery of metabolites from different extraction methods relative to the PCA extraction





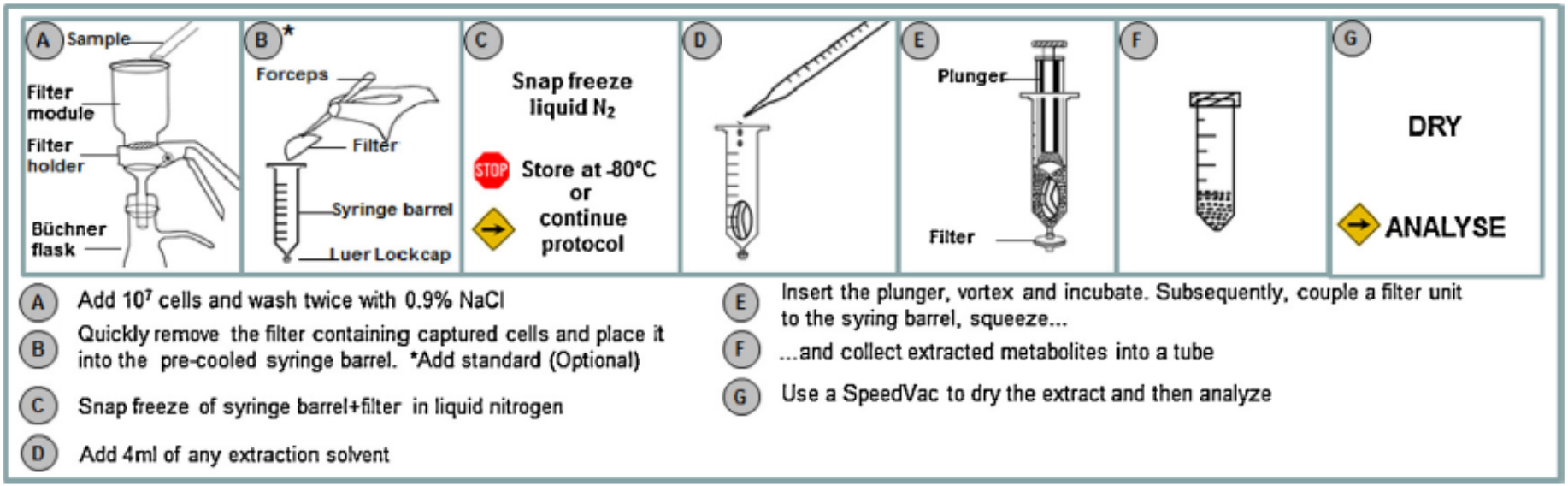
- The PCA extraction is the usual extraction method
- Used as reference



BW > MCW > CPM > PCA > BE

Filtration and syringe extraction

Reduced quenching and extraction time for mammalian cells

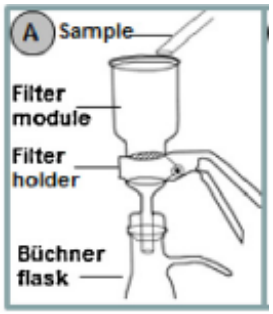


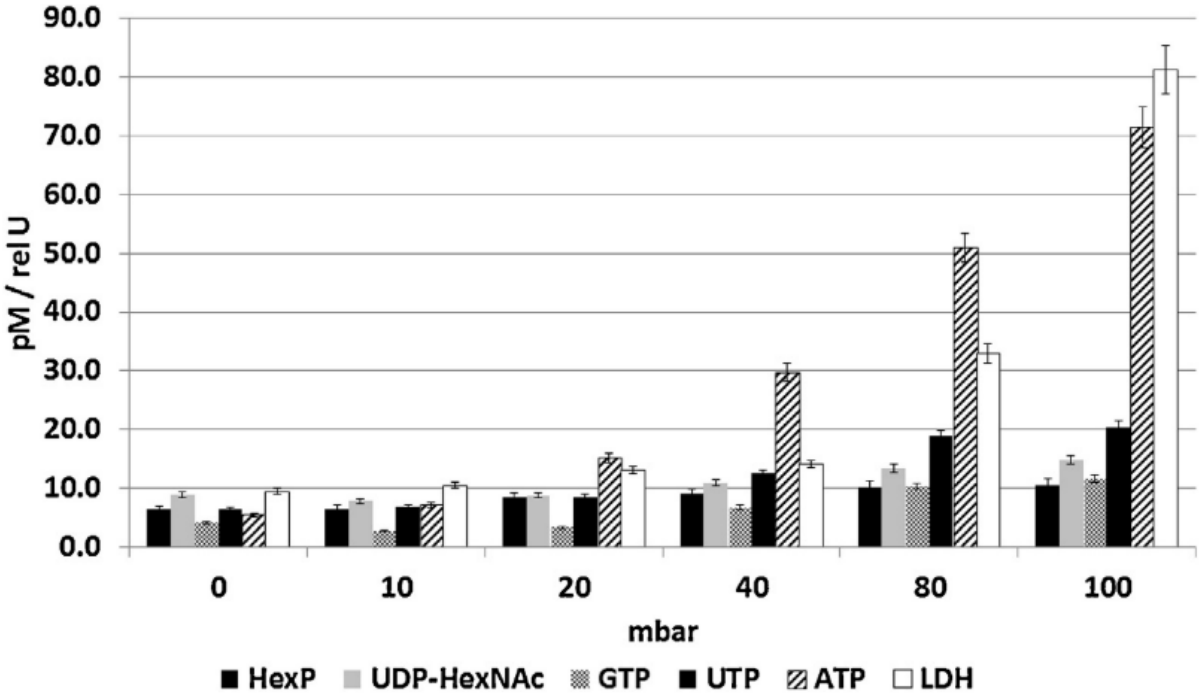
To avoid metabolite leakage, fast filtration was proposed as a washing step prior to quenching with liquid N2

Journal of Biotechnology 182–183 (2014) 97–103 Contents

Filtration and syringe extraction

Influence of vacuum on leakage of intracellular molecules





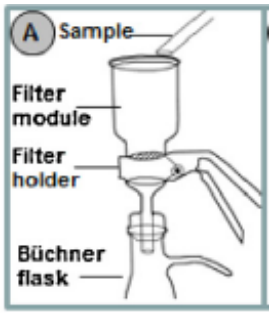
Low molecular weight substances and an intracellular protein (LDH) were analyzed in the filtered wash solution

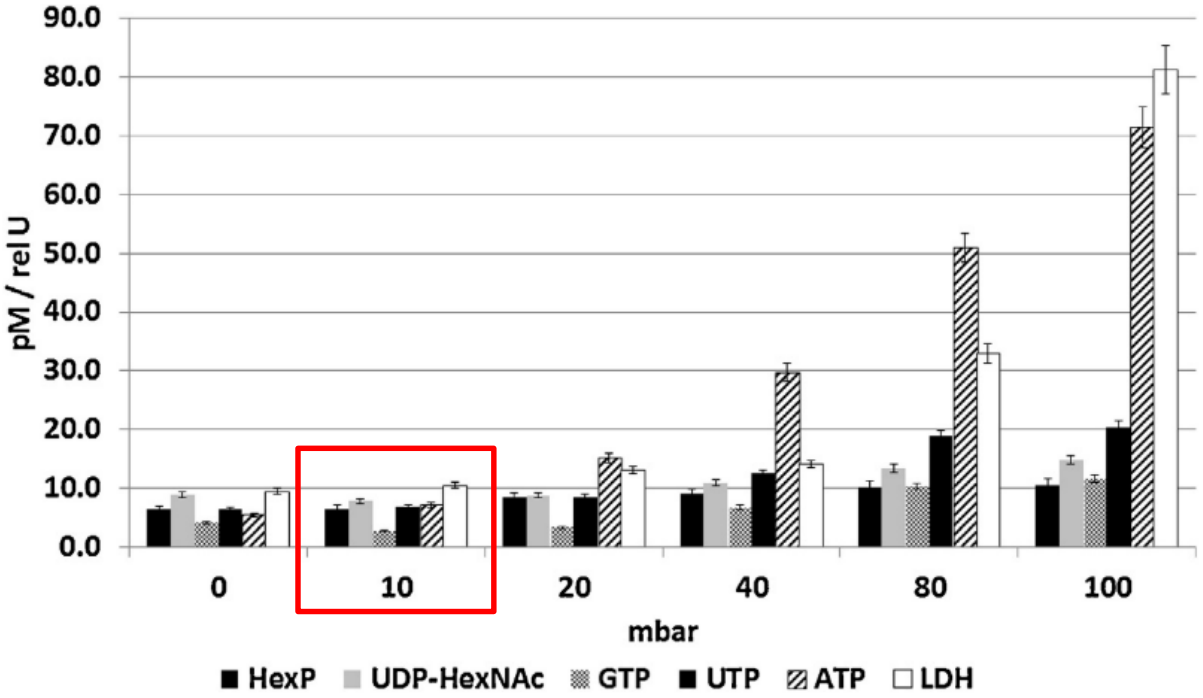
CHO (Chinese hamster ovary) cell line

Journal of Biotechnology 182–183 (2014) 97–103 Contents

Filtration and syringe extraction

Influence of vacuum on leakage of intracellular molecules





- Concentrations of released small molecules start to increase at 20 mbar vacuum
- 10mbar vacuum has been chosen

CHO (Chinese hamster ovary) cell line

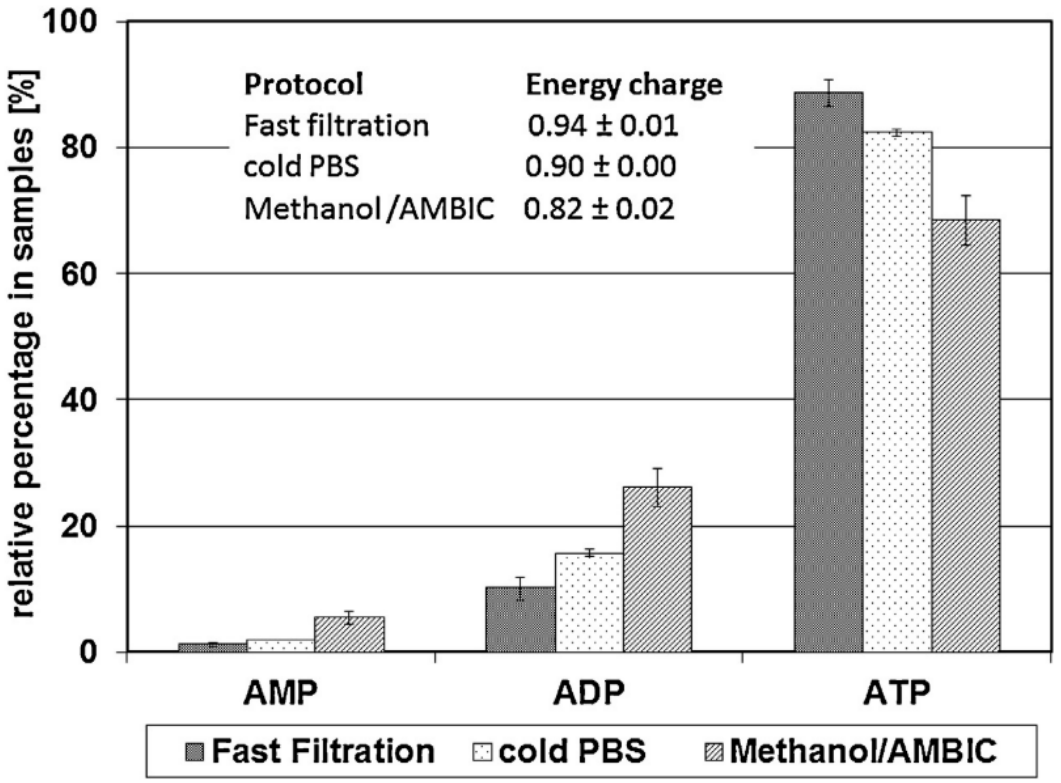
Journal of Biotechnology 182–183 (2014) 97–103 Contents

Filtration and syringe extraction

Comparison of quenching efficiency

- Cells quenched by:
  - ✓ fast filtration (=N_2)
  - ✓ cold PBS (pH 7.4, 0.5 ◦C)
  - ✓ cold 60% MeOH + 0.85% (w/v) AMBIC (pH 7.4; −20 ◦C)
- Relative distribution of AMP/ADP/ATP
- Associated calculated energy charge

$$\text{Energy charge} = \frac{\text{ATP} + 0.5\text{ADP}}{\text{ATP} + \text{ADP} + \text{AMP}}$$



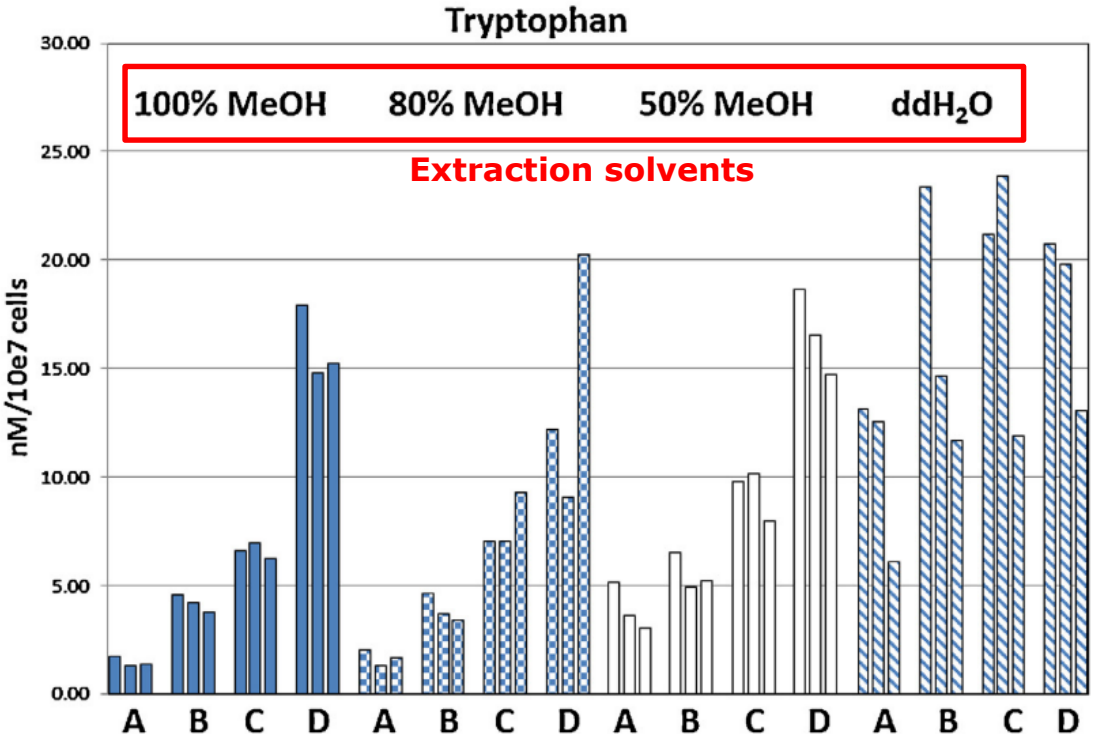
CHO (Chinese hamster ovary) cell line

Journal of Biotechnology 182–183 (2014) 97–103 Contents

Filtration and syringe extraction

Effects of extraction volume, drying and extraction solvents

- Metabolite extraction from 10^7 cells using:
  - ✓ *8 ml (left bar within each group)*
  - ✓ *6 ml (middle bar)*
  - ✓ *4 ml (right bar)*
- Different volume of extract were dried and reconstituted in 250µl.
  Volumes dried:
  - ✓ *1ml (A)*
  - ✓ *500 µl (B)*
  - ✓ *250 µl (C)*
  - ✓ *No drying (D)*
- 100% MeOH provide better reproducible results
- Other solvents showed higher variability
  - ✓ *less efficient cell disruption*



CHO (Chinese hamster ovary) cell line

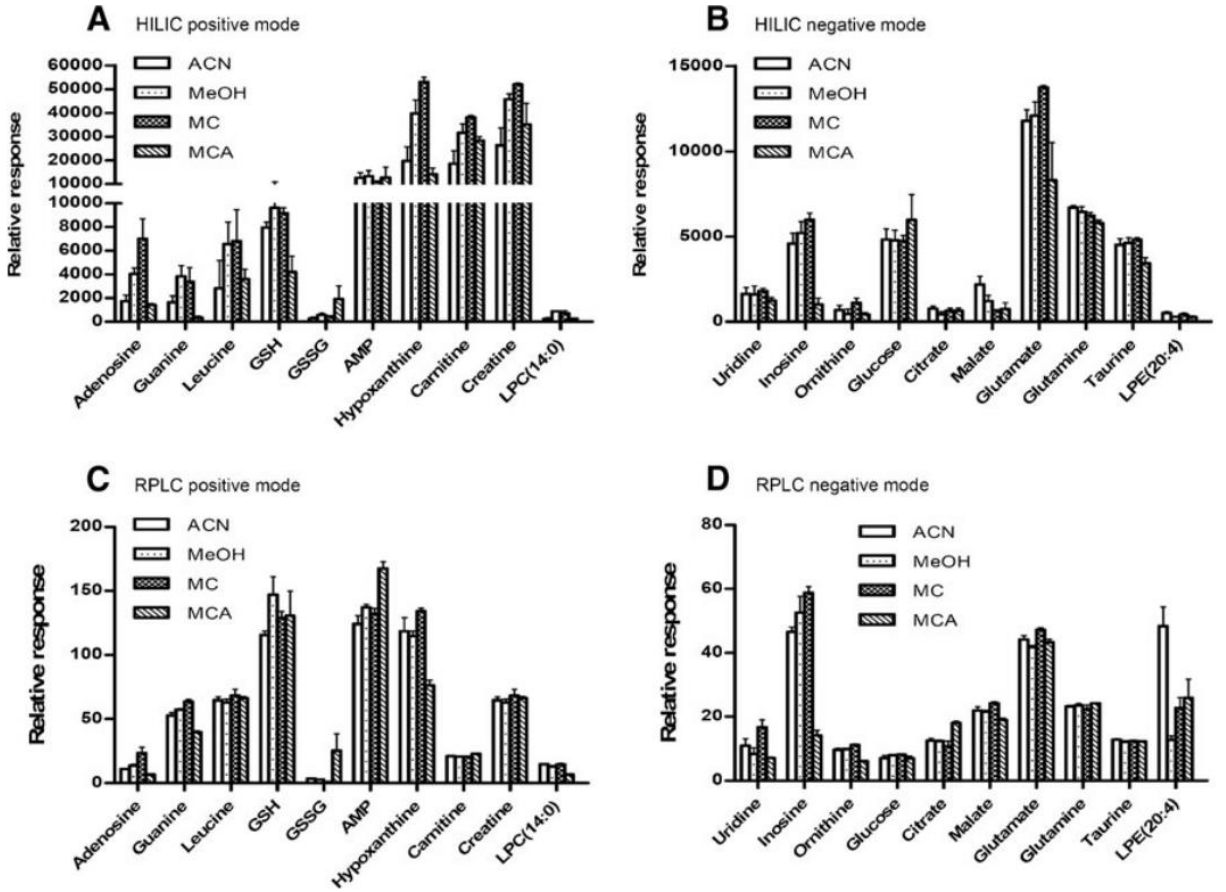
Journal of Biotechnology 182–183 (2014) 97–103 Contents

Human pancreatic cancer cell line Panc-1

Adherent cells

Extraction from pooled cell suspensions harvested using trypsinization or scraping

- Pooled Human pancreatic cancer (Panc-1) cell line suspensions harvested using trypsin or scraping
- Relative abundance of 20 different representative metabolite classes and metabolic pathways
- Extraction solvents:
  - ✓ *ACN*
  - ✓ *MeOH*
  - ✓ *MeOH/Chloroform (MC)*
  - ✓ *MeOH/Chloroform/AcN (MCA)*
- Analytical methods:
  - ✓ *HILIC-MS, positive mode (A)*
  - ✓ *HILIC-MS, negative mode (B)*
  - ✓ *RPLC-MS, positive mode (C)*
  - ✓ *RPLC-MS, negative mode (D)*



Relative responses showed that MeOH and MC extractions were most effective for most of the metabolites MCA method the lowest

Adherent cells

Extraction from pooled cell suspensions harvested using trypsinization or scraping

Metabolites	HILIC mode				RPLC mode			
	ACN	MeOH	MC	MCA	ACN	MeOH	MC	MCA
Positive mode								
Adenosine	100.0±21.3	231.1±21.0	*400.8±71.0*	80.9±5.1	100.0±11.1	125.5±23.0	*217.1±91.3*	60.7±10.4
Guanine	100.0±24.9	*234.1±39.9*	205.8±52.5	20.7±5.6	100.0±9.4	108.8±2.6	*120.6±6.6*	75.1±5.4
Leucine	100.0±61.5	231.2±49.8	*240.8±69.8*	127.1±21.7	100.0±8.4	97.2±8.4	*105.3±17.4*	102.2±4.9
GSH	100.0±4.3	115.1±4.4	*120.9±11.6*	52.6±12.5	100.0±6.3	111.6±9.8	*127.1±27.5*	113.0±37.3
GSSG	100.0±33.2	171.1±8.9	211.7±41.8	*412.7±303.9*	100.0±10.7	76.9±33.3	84.3±28.5	*312.2±345.2*
AMP	100.0±12.9	87.2±2.1	*105.4±13.2*	100.9±25.6	100.0±11.5	106.4±7.6	110.5±3.9	*134.8±9.6*
Hypoxanthine	100.0±23.0	*269.6±7.5*	202.1±21.5	72.2±9.4	100.0±19.6	*113.1±4.6*	96.8±7.4	64.2±8.1
Carnitine	100.0±22.4	171.0±14.9	*205.4±3.1*	152.5±7.0	100.0±7.9	97.8±4.7	98.5±3.6	*108.6±4.6*
Creatine	100.0±20.8	*197.9±1.5*	174.3±6.1	133.9±24.8	100.0±8.4	*105.3±17.4*	97.2±8.4	102.2±4.9
LPC (14:0)	100.0±33.1	315.0±61.4	*380.5±17.6*	106.1±13.6	*100.0±4.4*	99.1±14.1	87.3±10.4	45.0±6.5
Negative mode								
Uridine	100.0±17.6	*110.6±7.3*	98.2±23.2	76.7±8.0	100.0±45.4	*153.6±45.1*	77.3±37.4	65.2±5.2
Inosine	100.0±10.2	113.9±10.8	*130.7±6.6*	72.1±6.2	100.0±7.1	112.9±24.1	*126.1±9.5*	60.3±7.9
Ornithine	100.0±31.3	78.4±30.9	*163.2±30.4*	61.5±12.4	100.0±9.8	102.5±6.2	*116.0±6.4*	63.4±5.6
Glucose	100.0±9.6	98.4±5.0	99.1±9.2	*123.9±23.0*	100.0±27.0	116.4±6.6	*119.4±3.6*	99.2±23.0
Citrate	*100.0±13.9*	82.8±14.6	75.2±14.7	83.3±31.6	100.0±12.9	85.4±29.9	98.9±4.6	*144.3±9.6*
Malate	100.0±22.1	96.0±19.5	*172.4±5.6*	75.5±14.1	100.0±11.3	98.0±5.1	*110.1±5.1*	86.3±6.3
Glutamate	100.0±4.2	102.6±5.1	*116.7±7.0*	70.5±14.0	100.0±6.1	94.4±2.7	*107.1±3.2*	98.1±4.8
Glutamine	*100.0±1.2*	93.0±2.1	96.3±3.4	86.7±1.7	100.0±3.1	96.7±11.8	*105.1±5.8*	104.4±1.6
Taurine	100.0±5.9	*106.9±1.7*	102.3±5.1	76.2±5.3	*100.0±4.5*	96.5±4.6	94.8±6.3	95.6±3.9
LPE (20:4)	*100.0±10.2*	83.5±11.8	64.7±4.7	61.8±9.2	*100.0±63.5*	63.3±10.2	58.7±3.9	67.8±19.5
Average	100.0	149.5	168.3	102.4	100.0	103.1	107.9	100.1
Minimum	100.0	78.4	64.7	20.7	100.0	63.3	58.7	45.0
Maximum	100.0	315.0	400.8	412.7	100.0	153.6	217.1	312.2

Relative response of each metabolite normalized by the internal standard and protein concentration (n=5
Extraction efficiency relative to pure ACN set at 100 %

Adherent cells

Extraction from pooled cell suspensions harvested using trypsinization or scraping

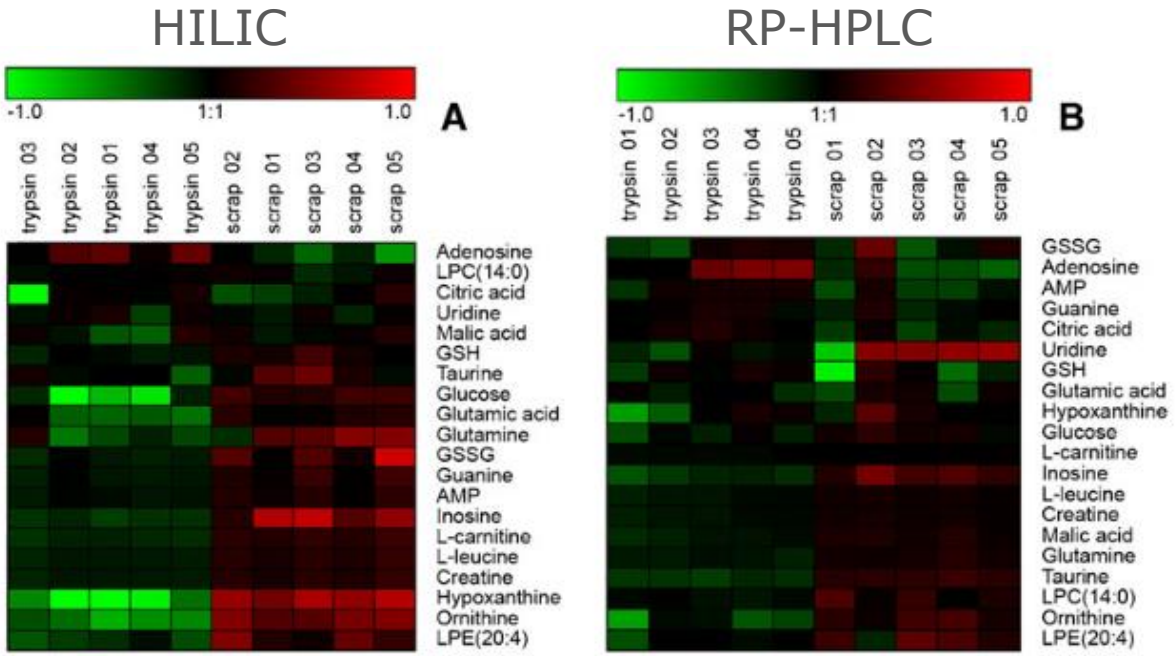
- Comparison of extraction methods showed advantages and disadvantages
  - ✓ *Metabolites with Very different chemical and physical properties such as structure and polarity.*
- Thus, the overall intracellular metabolite profiling may be limited by the extraction method
- A desirable extraction method would enable recovery of the broadest range of metabolites with highest possible extraction efficiency.
  - ✓ *The results in this study indicate that the MC extraction is the most suitable extraction method for extraction of broadest range of metabolites*

Adherent cells

Trypsin versus scrapping collection

Heatmap analysis of targeted metabolites with trypsinization versus scrapping collection analyzed by:

- HILIC (A)
- RPLC (B)

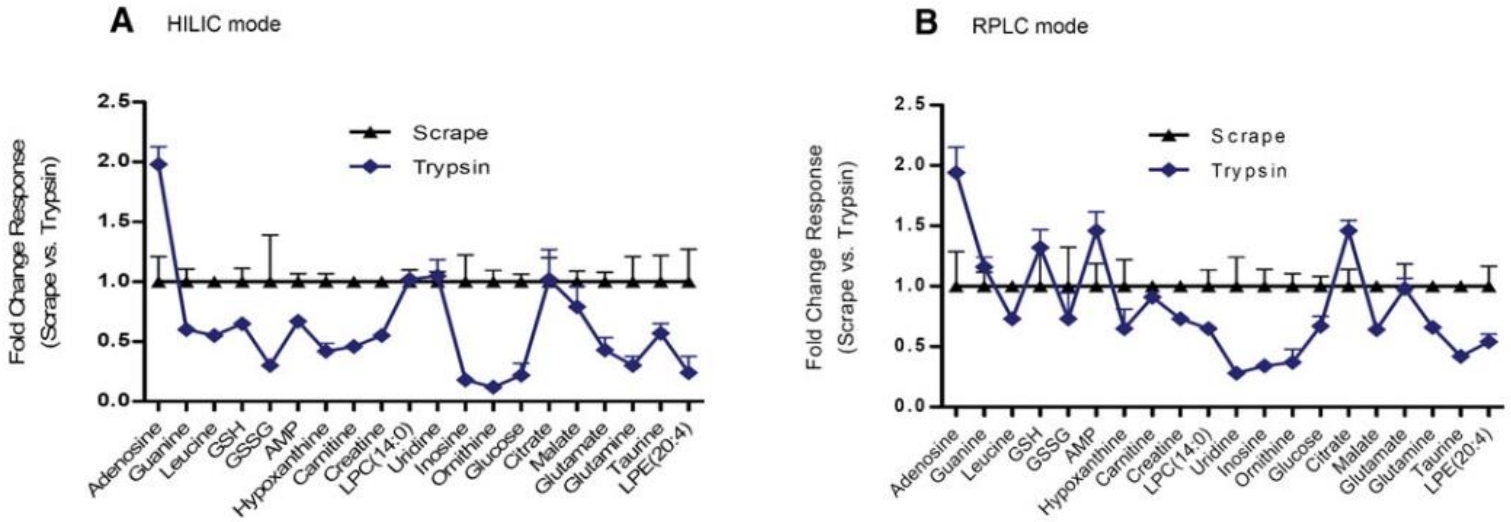


Metabolites concentration were lower in the cells harvested by trypsinization thus indicating substantial metabolite leakage during trypsin treatment

Anal Bioanal Chem (2013) 405:5279–5289

Adherent cells

Metabolites with trypsin versus scrapping collection



Fold change decrease of Panc-1 metabolite peak area with trypsinization versus scrapping collection in both HILIC and RPLC modes

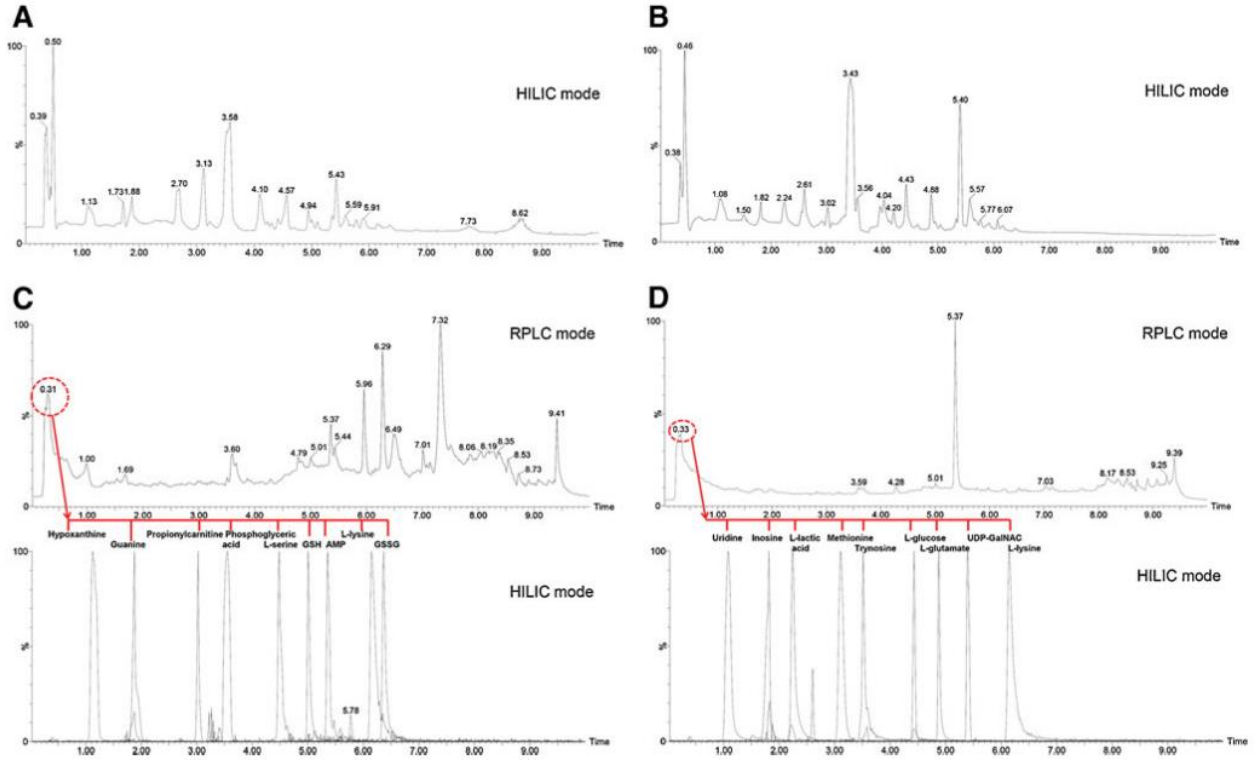
The response of the scrapping collection was set at 100 % as a reference.

In the HILIC mode, the relative response of most of the target metabolites was significantly decreased by 2- to 8-fold, except for adenosine that showed a 2-fold increase with the trypsinization protocol.

Anal Bioanal Chem (2013) 405:5279–5289

Adherent cells

Metabolites with trypsin versus scrapping collection



HILIC had significantly larger number of molecular features than RPLC irrespective of the harvesting and extraction
It can be seen that response under HILIC mode was stronger than that of RPLC mode.
Volatile nature of HILIC mobile phases => greater response than the RPLC in the ESI-MS
More detected peaks appeared under positive and negative HILIC modes, which indicates a better separation of metabolites

Anal Bioanal Chem (2013) 405:5279–5289

Adherent cells

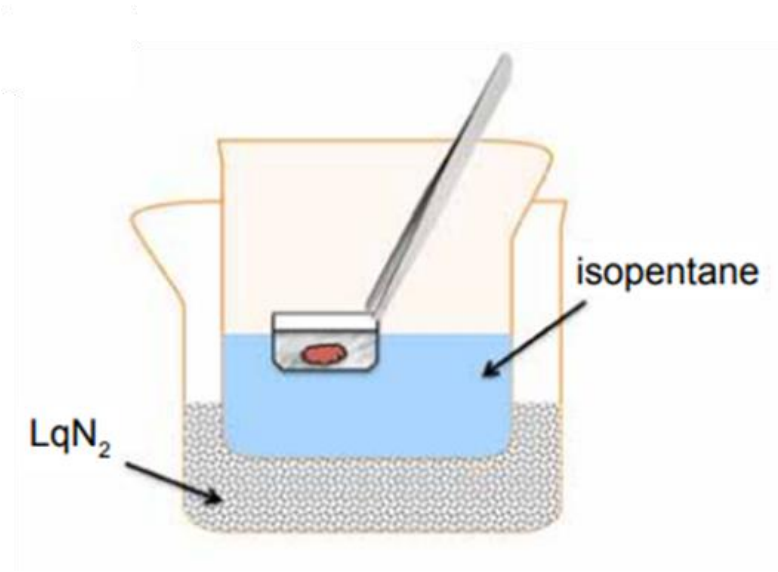
Metabolites with trypsin versus scrapping collection

Compounds	Experimental ion mass (*m*/*z*)	RT in HILIC$^+$ (min)	RT in HILIC$^-$ (min)	RT in RP$^+$ (min)	RT in RP$^-$ (min)
Adenosine	268.1041+	1.06		0.33	
Hypoxanthine	137.0459+	1.13		0.33	
Guanine	152.0568+	1.88		0.32	
L-leucine	132.1021+	2.70		0.28	
Propionylcarnitine	218.1387+	3.02		0.34	
Acetylcarnitine	204.1232+	3.44		0.30	
L-carnitine	162.1126+	3.96		0.29	
Phosphoglyceric acid	186.9955+	3.55		0.32	
L-serine	106.0503+	4.48		0.29	
L-glutathione reduced (GSH)	308.0916+	5.01		0.32	
AMP	348.0709+	5.36		0.32	
L-lysine	147.1134+	6.14		0.30	
L-glutathione oxidized (GSSG)	613.1598+	6.37		0.32	
Creatine	132.0773+	3.96		0.28	
LPC (14:0)	468.3077+	0.52		6.45	
Uridine	243.0618−		1.08		0.31
Inosine	267.0725−		1.81		0.31
L-lactic acid	89.0239−		2.24		0.27
Methionine	148.0431−		3.11		0.30
Trynosine	180.0667−		3.51		0.33
L-glutamine	145.0610−		4.37		0.30
L-glucose	179.0556−		4.43		0.28
L-glutamic acid	146.0453−		4.88		0.31
UDP-GalNAC	606.0736−		5.40		0.46
L-lysine	145.0976−		6.14		0.26
Malic acid	133.0136−		4.94		0.31
Citric acid	191.0192−		6.02		0.33
Ornithine	131.0821−		4.52		0.31
Taurine	124.0070−		3.29		0.39
LPE (20:4)	500.2789−		0.49		6.65

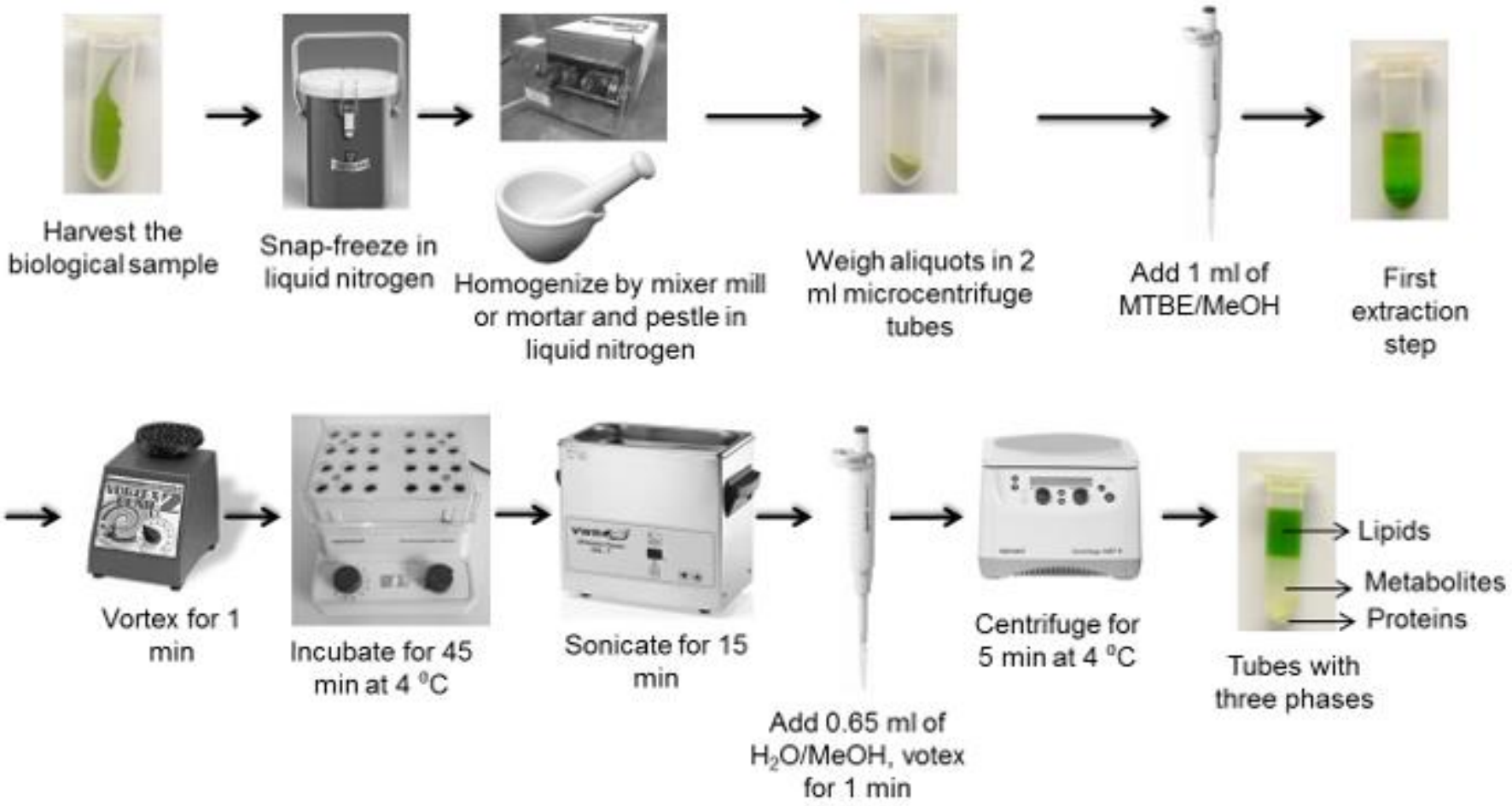
- Metabolites that were not retained by RPLC and eluted in the void volume with very early and close retention time at 0.3–0.4min were well separated by HILIC under both positive and negative modes.
- Polar molecules showed a much better separation in HILIC mode
- Hydrophobic molecules such as LPC and LPE were better separated by RPLC

Tissue preparation

Snap freeze



Tissue preparation



MTBE: methyl tert-butyl ether

Tissue homogenization and metabolite extraction

- Exemple of extraction buffer
  - *10mM phosphate buffer (pH 7.5)*
  - *Methanol*
  - *Cold Methanol*
  - *Methanol/ phosphate buffer (85/15 v/v),*
  - *Ethanol*
  - *Ethanol/phosphate buffer (85/15 v/v)*
  - *Ethanol/dichloromethane (1/ 1 v/v)*

Tissue homogenization and metabolite extraction

example

High-throughput extraction and quantification method for targeted metabolomics in murine tissues

Sven Zukunft[1,2] · Cornelia Prehn[1] · Cornelia Röhring[3] · Gabriele Möller[1] · Martin Hrabě de Angelis[1,2,4] · Jerzy Adamski[1,2,4] · Janina Tokarz[1]

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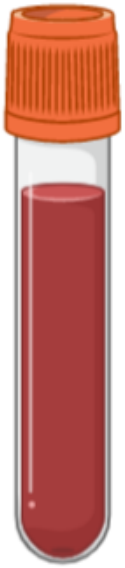
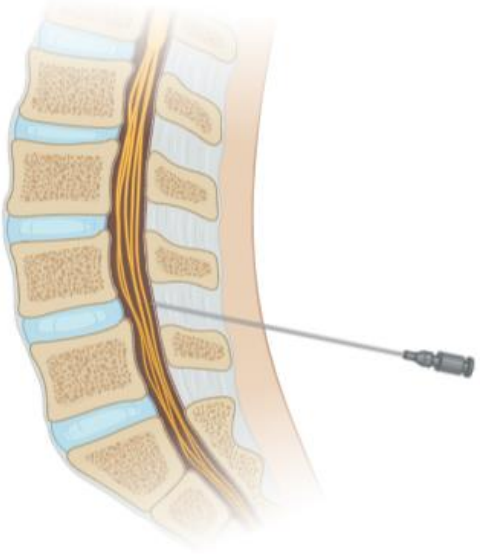
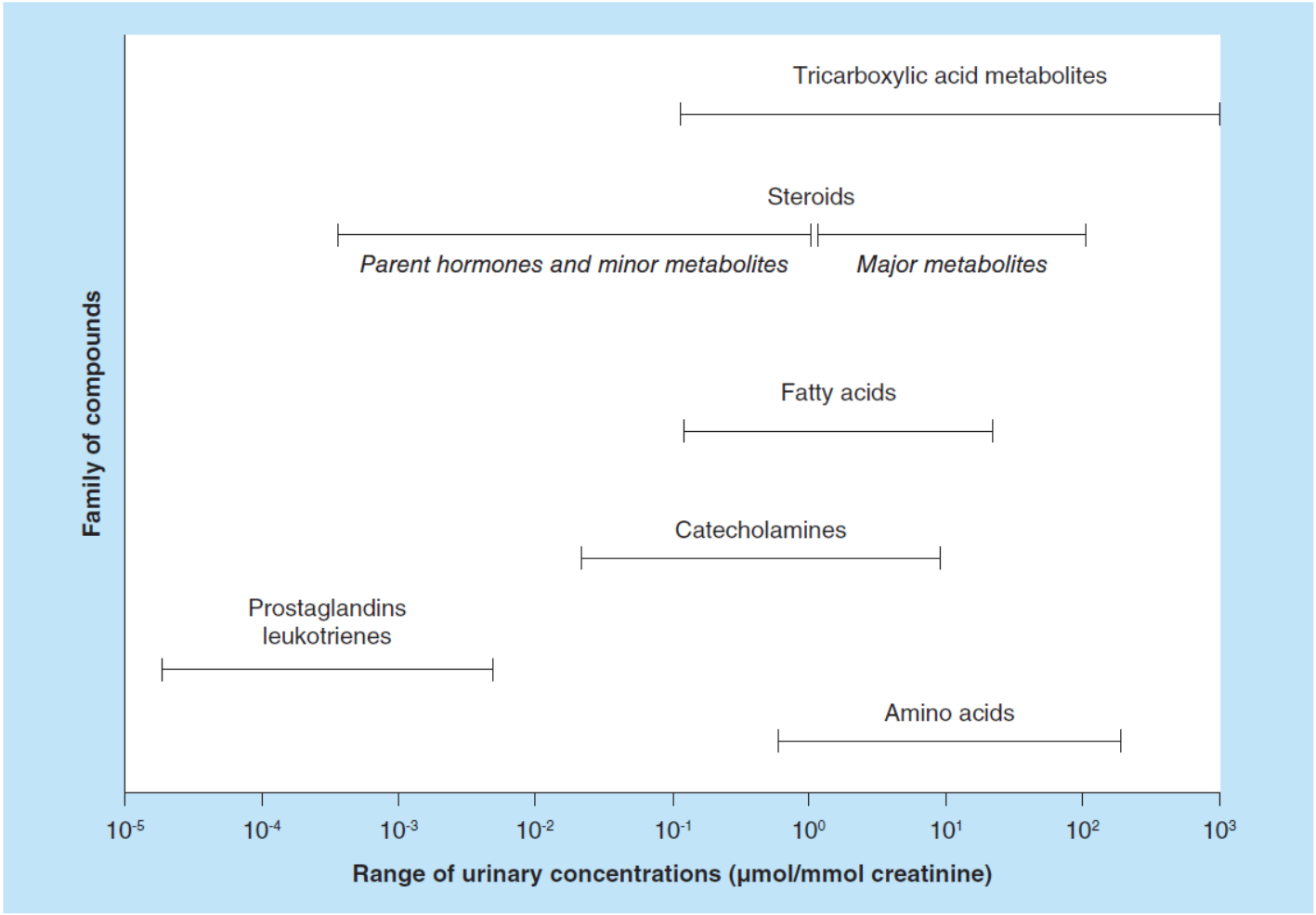
Body fluids





Urine

Broad range of concentrations



Cas de l'urine

Préconcentrations & dilution

High [C]
µg/mL

Low [C]
ng/mL

- Quantification issues
  - ✓ *poor linear behavior LC–MS/MS methods*
  - ✓ *dilution factors up to 100-fold*

- Required to evaluate metabolic pathways
- Development of adequate targeted LC–MS/MS approaches
- SPE and liquid–liquid extraction (LLE)
  - ✓ *0–50-fold preconcentration*
- Clean-up of the sample
  - ✓ *eliminates interfering compounds*
  - ✓ *favors analyte ionization*

- Urine samples are processed prior to storage or analysis to remove cellular debris and to minimize the effect of any contaminating bacteria