Terminology

• **Metabolite: substance produced or used during metabolism such as lipids, sugars and amino acids**

A metabolite may be described as a compound which is internalized, chemically converted or secreted by an organism, but is not synthetized by DNA replication, transcription, or translation

• **Metabolome: the quantitative complement of all the low molecular weight molecules present in cells or biofluids in a particular physiological or developmental state**

• **Metabolomics: a comprehensive analysis of the whole metabolome under a given set of conditions**

Metabo*omics

• **Metabonomics:**

The quantitative measurement of the dynamic multiparametric response of living systems to pathophysiological stimuli or genetic modification (*Nicholson et al., Xenobiotica 1999*)

holistic analysis of biofluids and tissues in order to determine metabolic composition

deals with integrated, multicellular, biological systems including communicating extracellular environments in animal and human biochemistry

• **Metabolomics:**

Measurement of metabolite concentrations and fluxes in isolated cell systems (*Nicholson et al., Xenobiotica 1999*)

deals with simple cell systems and mainly intracellular metabolite concentrations in microbial and plant biochemistry

Metabolomics vs genomics and proteomics

Genomics and proteomics tell you what might happen, but metabonomics tells you what actually did happen
(*Bill Lasley, UC Davis*)

Although changes in the quantities of individual enzymes might be expected to have little effect on metabolic fluxes, they can and do have significant effects on the concentrations of numerous individual metabolites.

The metabolome is further down the line from gene to function and so reflects more closely the activities of the cell at a functional level. Thus, as the 'downstream' result of gene expression, changes in the metabolome are expected to be amplified relative to changes in the transcriptome and the proteome.

Metabolic fluxes (at least as exemplified by glycolysis in trypanosomes) are not regulated by gene expression alone.

mRNA levels do not always correlate well with protein levels
Once translated a protein may or may not be active, as determined by post-translational modifications, protein sorting, protein-protein interactions, proteolysis

There is still large uncertainty in accuracy of annotations of the genome, and annotation is limited
Metabolomics does not rely on annotated databases and can provide more direct functional info

General aplications

- **Assessing gene function and relationships to phenotypes**
- **Understanding metabolism and predicting novel pathways**
- **To increase metabolite fluxes into valuable biochemical pathways using metabolic engineering**
- **To compare genetically modified organisms**
- **To assess the effect of environmental/stress/temperature changes that lead to changes in gene expression, flux pathways, and extent of carbon and electron flow through them**

Who believes in metabolomics?

NIH Roadmap

http://nihroadmap.nih.gov/initiatives.asp

Building Blocks, Pathways, and Networks Implementation Group

***Metabolomics Technology Development*. This initiative will promote development of novel technologies to study cellular metabolites, such as lipids, carbohydrates, and amino acids. Knowledge gained from these studies will be used to understand more precisely the role of metabolites in the context of cellular pathways and networks.**

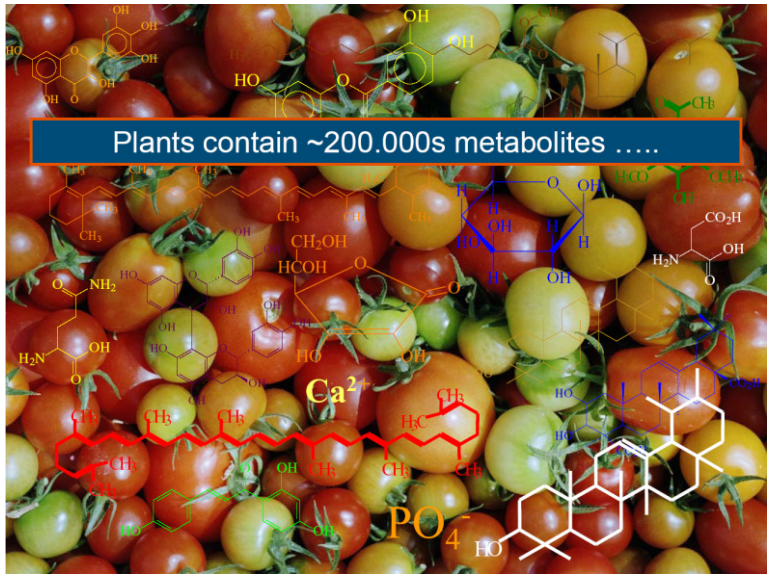
RFA for "Metabolomics Technology Development"

http://grants.nih.gov/grants/guide/rfa-files/RFA-RM-04-002.html

Characteristics of the metabolomes

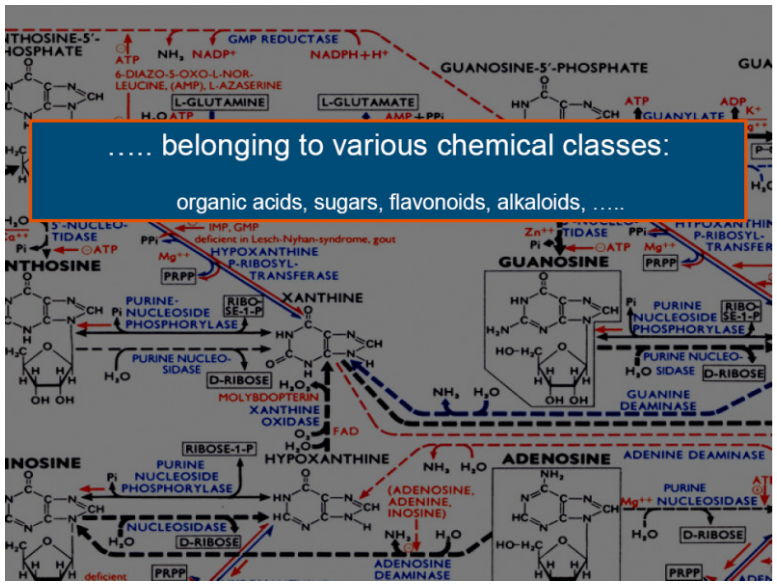
Metabolome size:

- **S. cerevisiae: about 600 metabolites**
- **Plants: estimated 200,000 primary and secondary metabolites**
- **Mammalians: ?**

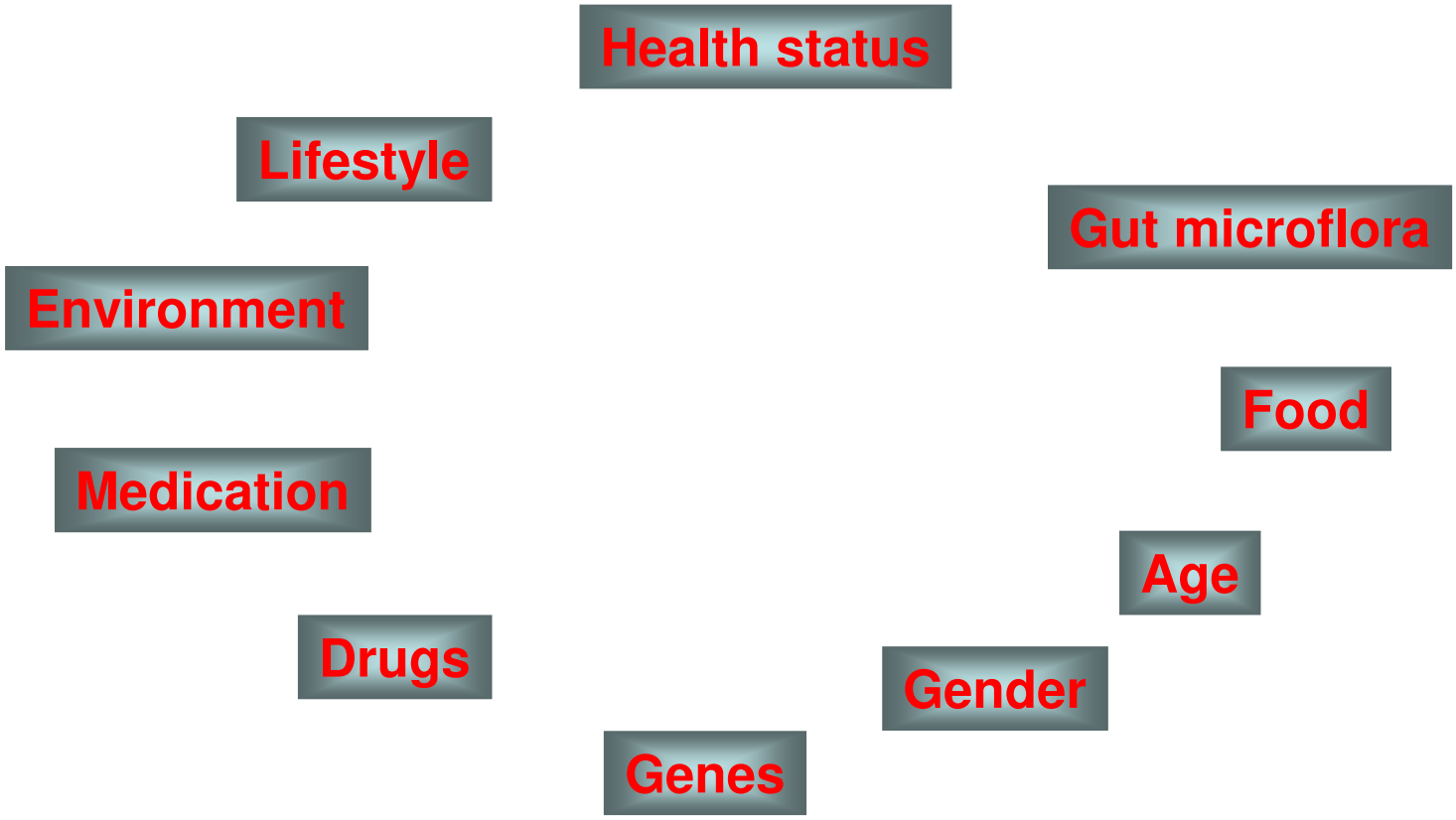


Metabolite chemical diversity:

- **the metabolome extends over an estimated 7–9 magnitudes of concentration (pmol–mmol)**
- **wide variations in chemical (molecular weight, polarity, solubility) and physical (volatility) properties**



Factors affecting the human metabolome



Dietary contributions to the human metabolome

Macronutrient energy Sources

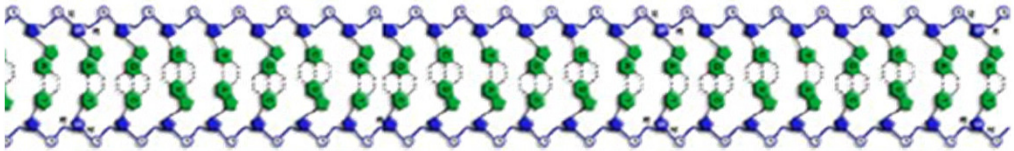
Essential micronutrients

Non-essential, beneficial dietary components

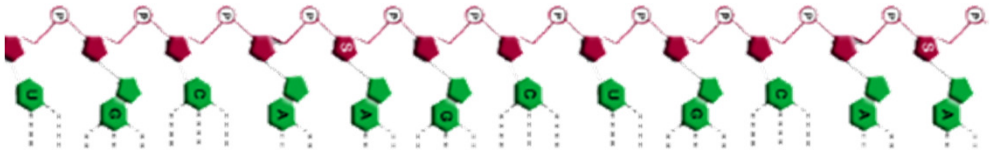
Metabolically neutral dietary components

Dietary toxins and toxicants

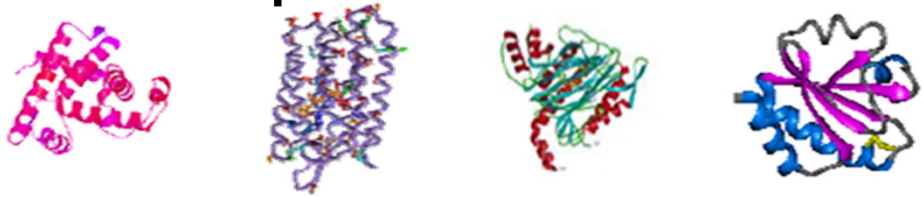
genome



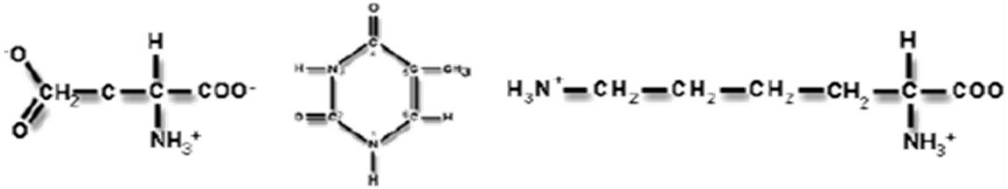
transcriptome



proteome



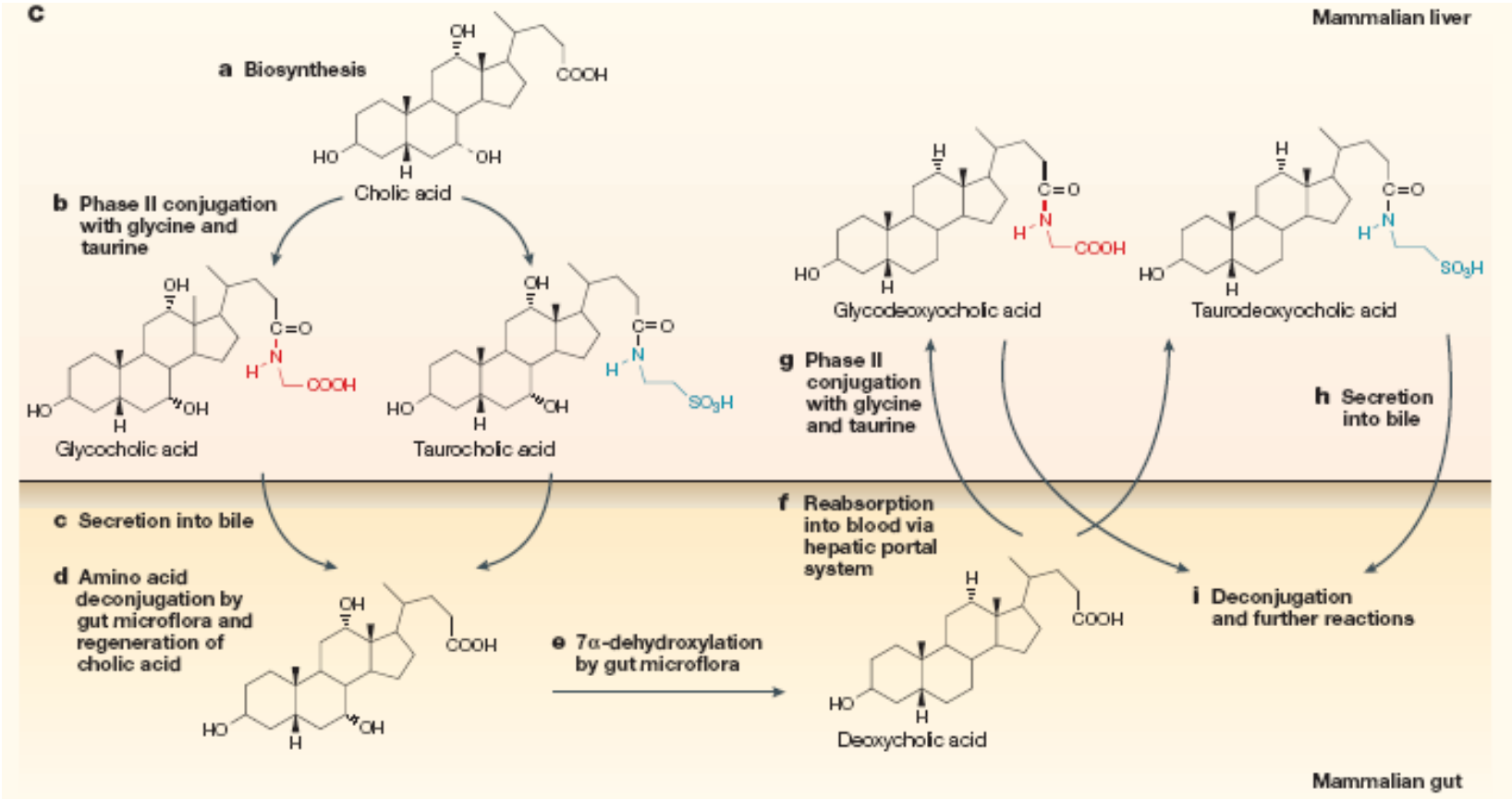
metabolome



Biological function/ health

interacting metabolomes

The origin of a metabolite is not exclusively dependent on the biosynthetic capacity of an organism or delimited by the genomic inventory. Metabolites may readily be exchanged between organisms, for example in plant microbe interactions, and – like drugs or pesticieds – can today be of anthropogenic/xenobiotic origin



Example of sym-xenobiotic metabolism occurring in mammals

Cholic acid and other bile acids biosynthesized in the liver undergo a series of conversions in both the host liver and inside the gut microflora; these compartments are connected by the entero-hepatic circulation

Classification of metabolomics approaches

Metabolomics is the study of metabolic changes. It encompasses metabolomics, metabolite target analysis, metabolite profiling, metabolic fingerprinting, metabolic profiling, and metabonomics – *the Metabolomics Society*

Metabolite target analysis: analysis restricted to metabolites of, for example, a particular enzyme that would be directly affected by abiotic or biotic perturbation

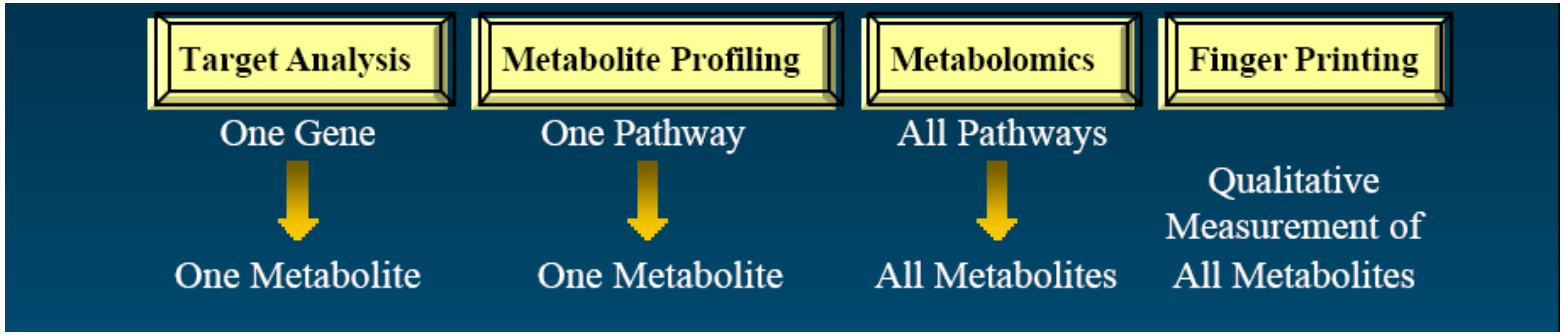
Metabolite profiling: analysis focused on a group of metabolites, for example, a class of compounds such as carbohydrates, amino acids or those associated with a specific pathway

Metabolomics: comprehensive analysis of the whole metabolome under a given set of conditions

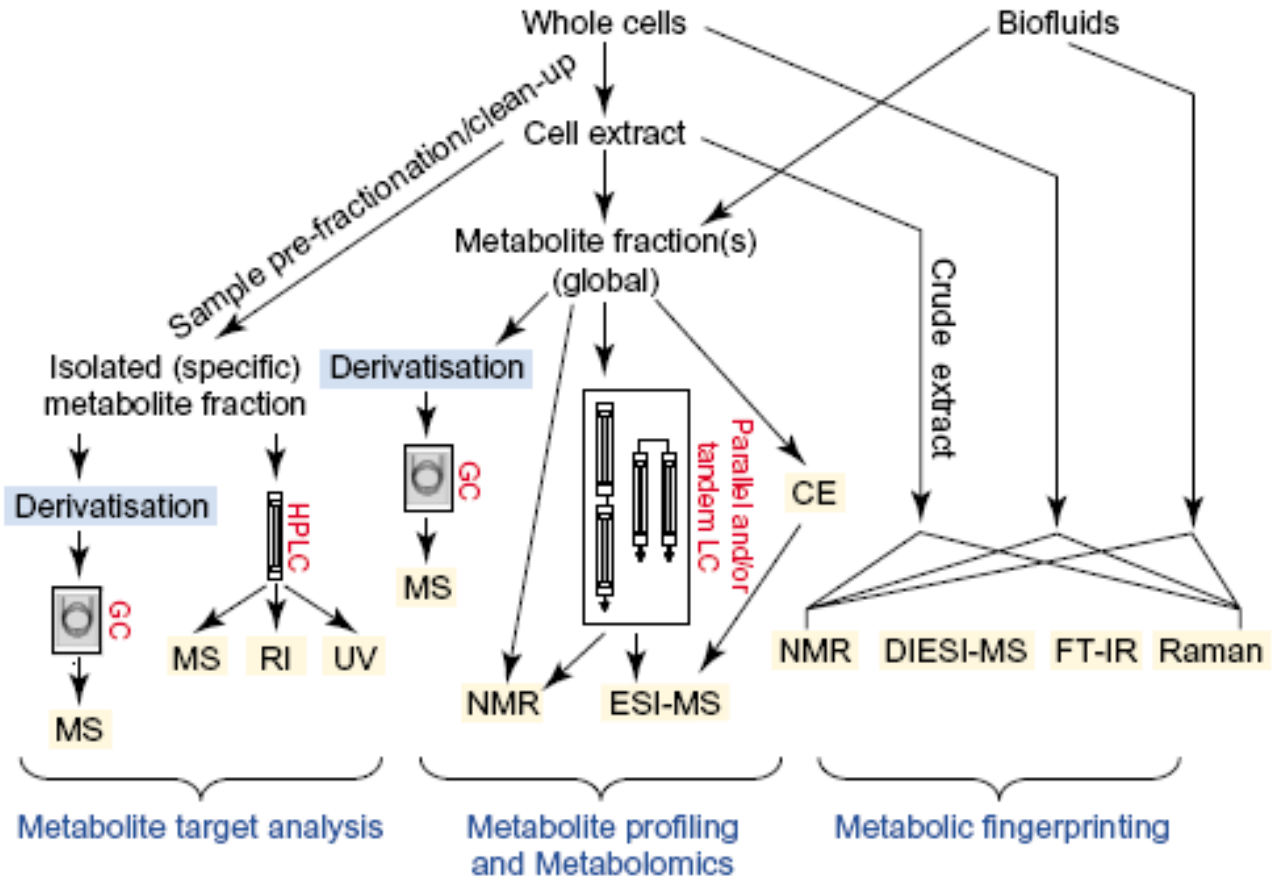
Metabolic fingerprinting: classification of samples on the basis of provenance of either their biological relevance or origin

Metabolic profiling: often used interchangeably with 'metabolite profiling'; m.p. is commonly used in clinical and pharmaceutical analysis to trace the fate of a drug or metabolite

Metabonomics: measure the fingerprint of biochemical perturbations caused by disease, drugs and toxins



Technologies for metabolome analysis



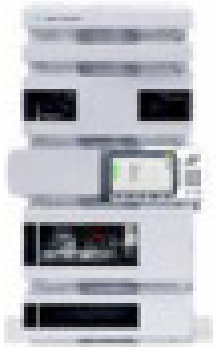
General strategies for metabolome analysis.

CE, capillary electrophoresis; DIESI, direct-infusion ESI, which can be linked to Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR-MS); NMR, nuclear magnetic resonance; RI, refractive index detection; UV, ultraviolet detection

Differenti tecniche separative

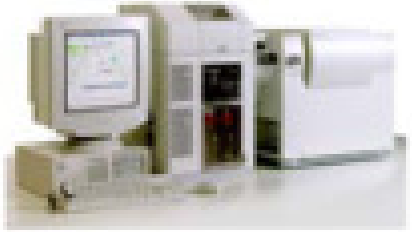
Basate su...

Solubilità



Liquid chromatography (HPLC)

Polarità

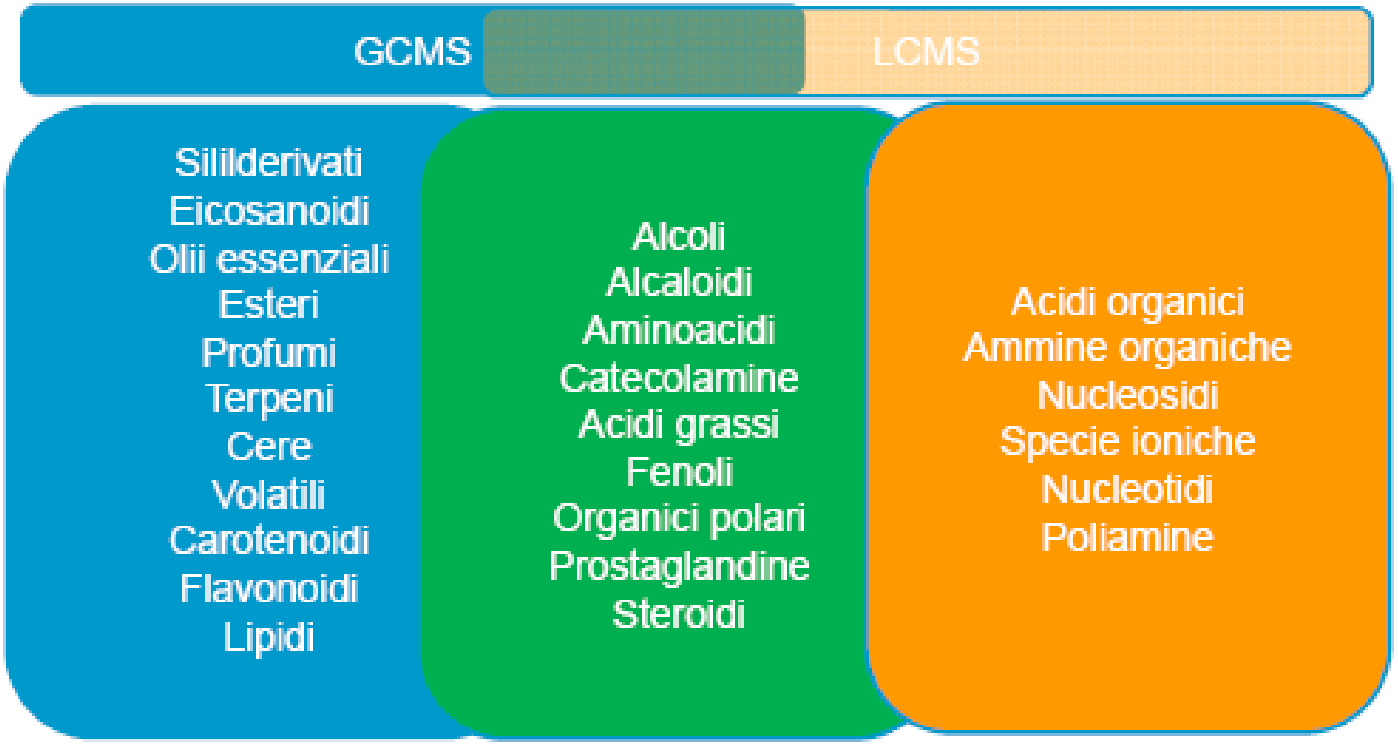


Capillary electrophoresis (CE)

Volatilità

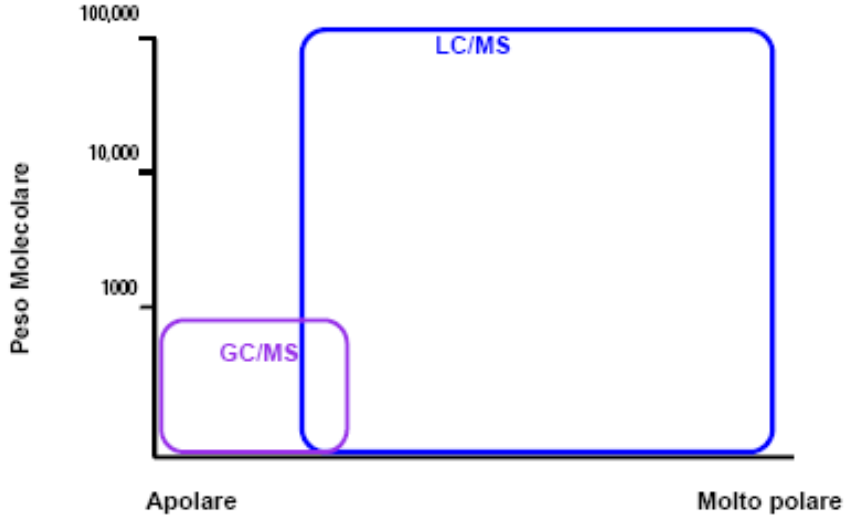
Gas chromatography (GC)

Quale tecnica...

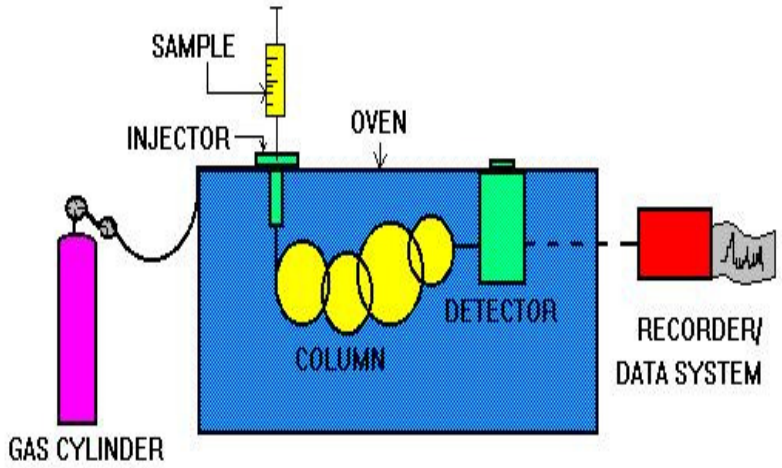


Meno polari

Più polari

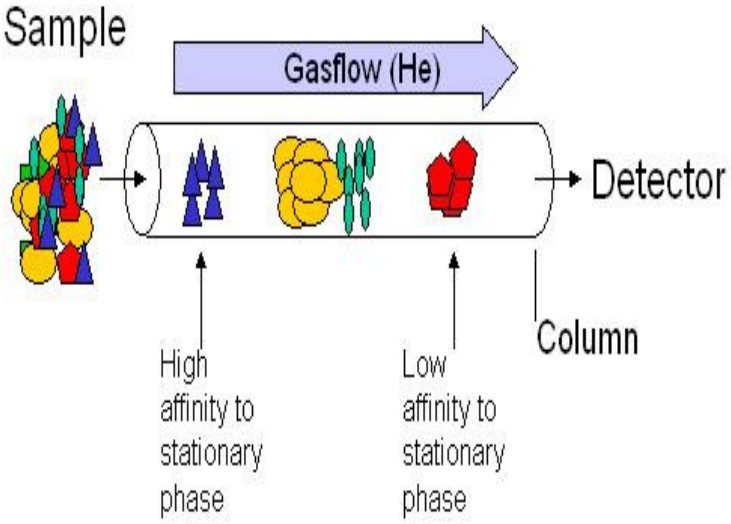


GAS CHROMATOGRAPHY

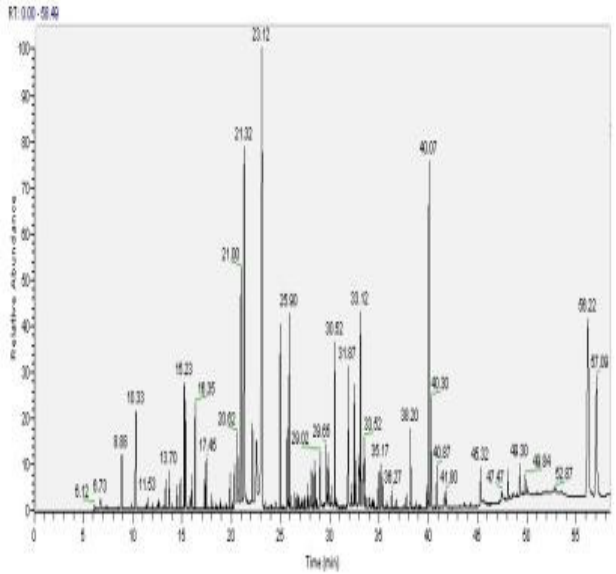


programmed temperature gradient

A



B



Gas chromatography-mass spectrometry (GCMS)

Ionization of the molecules in GCMS can be done in different ways:
- Electron ionization (positive and negative)
- Chemical ionization (positive and negative)

The fragment ions are detected by time-of-flight (TOF) or by quadruple mass spectrometry (MS). Often derivatisation methods are used to make metabolites more volatile.

GCMS mainly separates metabolites that are smaller than 500 Dalton. Separation is based on boiling point and binding to the column. Many metabolites can be identified in the GCMS, such as sugars, fatty acids, organic acids and amino acids. GC-MS is poor for the analysis of substances, which are non-volatile due to their high molecular weight and/or polarity. GCMS is suitable as a broad metabolic profiling technique.

Some biofluids (e.g. plasma) require removal of interfering proteins for example by precipitation in acetonitrile
Evaporate supernatant to dryness, derivatize (e.g. first methoxylamine hydrochloride in pyridine at 28 ℃ 90', then MSTFA: N-methyl-N-(trimethylsilyl)-trifluoroacetamide 37 ℃ 30')

Derivatization requires extraction for use of pyridine
Solid phase extraction (SPE) cartridges can be used

GC/MS

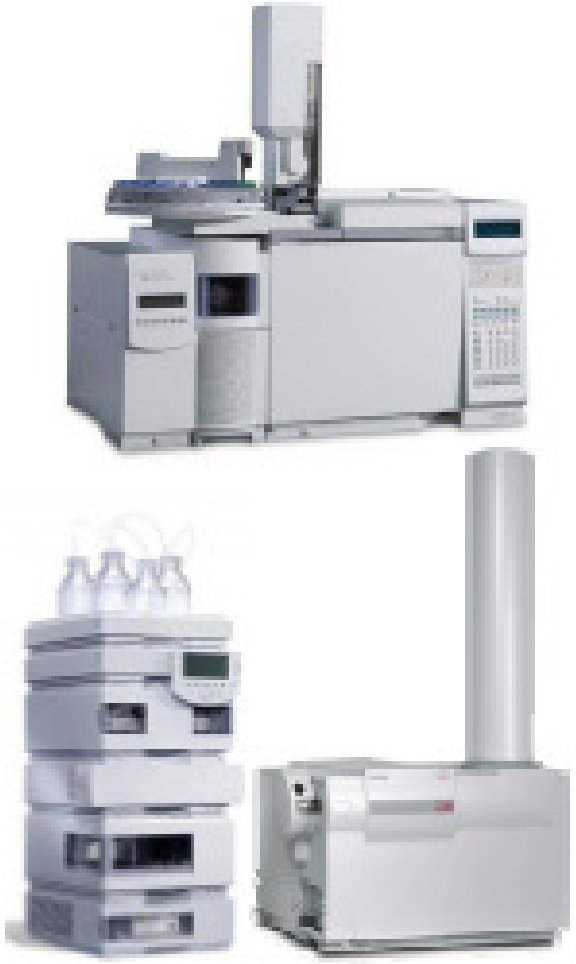
Vantaggi

- Separazione ad alta efficienza
- Alta sensibilità
- Librerie EI
- No soppressione ionica
- Economico

Svantaggi

- Volatilità analiti
  - Derivatizzazione
- No ione molecolare

Derivatizzazione + tempi di acquisizione lunghi = Low throughput



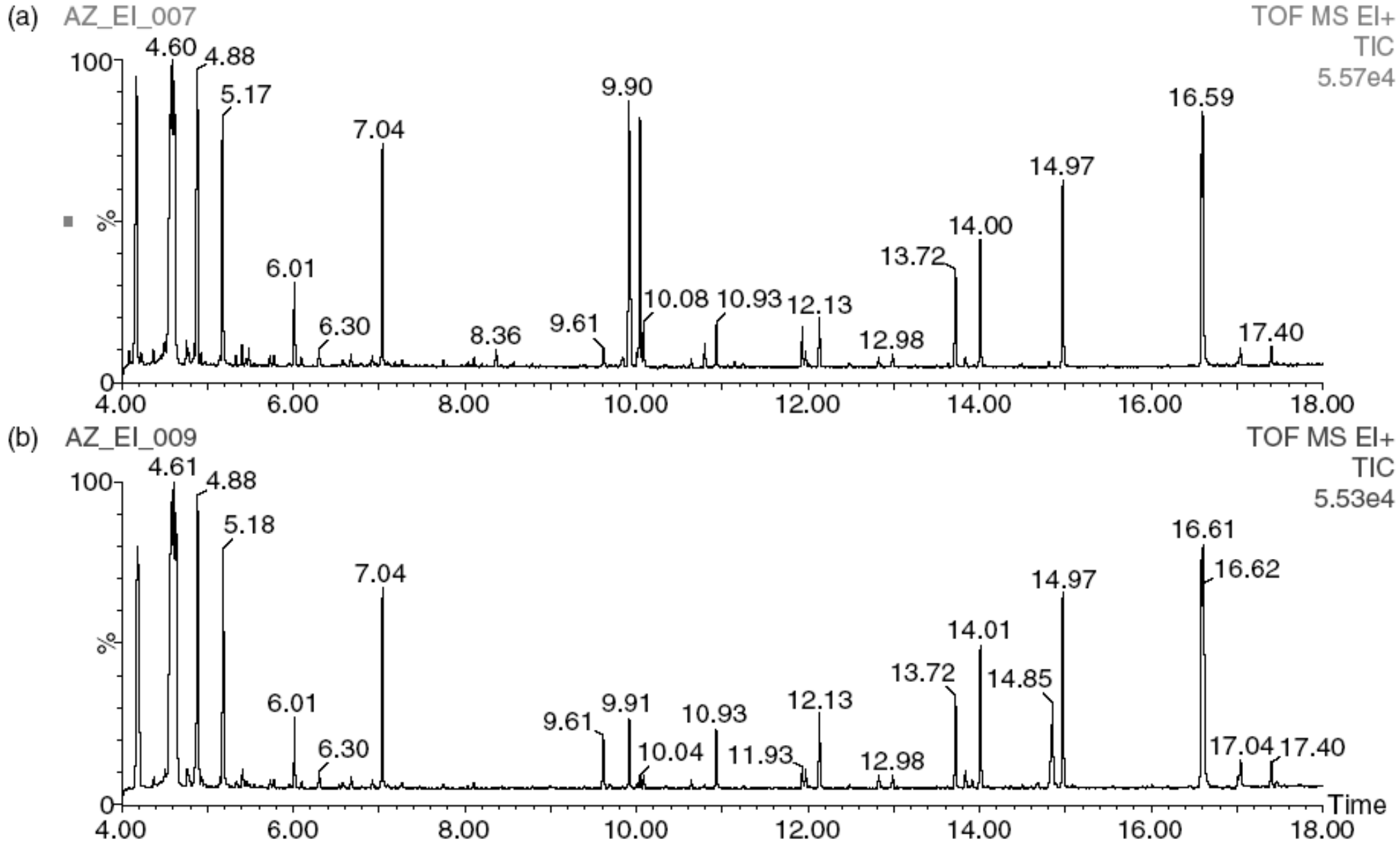


Figure 5.1. Typical TIC traces obtained from GC-EI-MS analysis of plasma obtained from (a) Wistar-derived and (b) Zucker (fa/fa) obese rats.

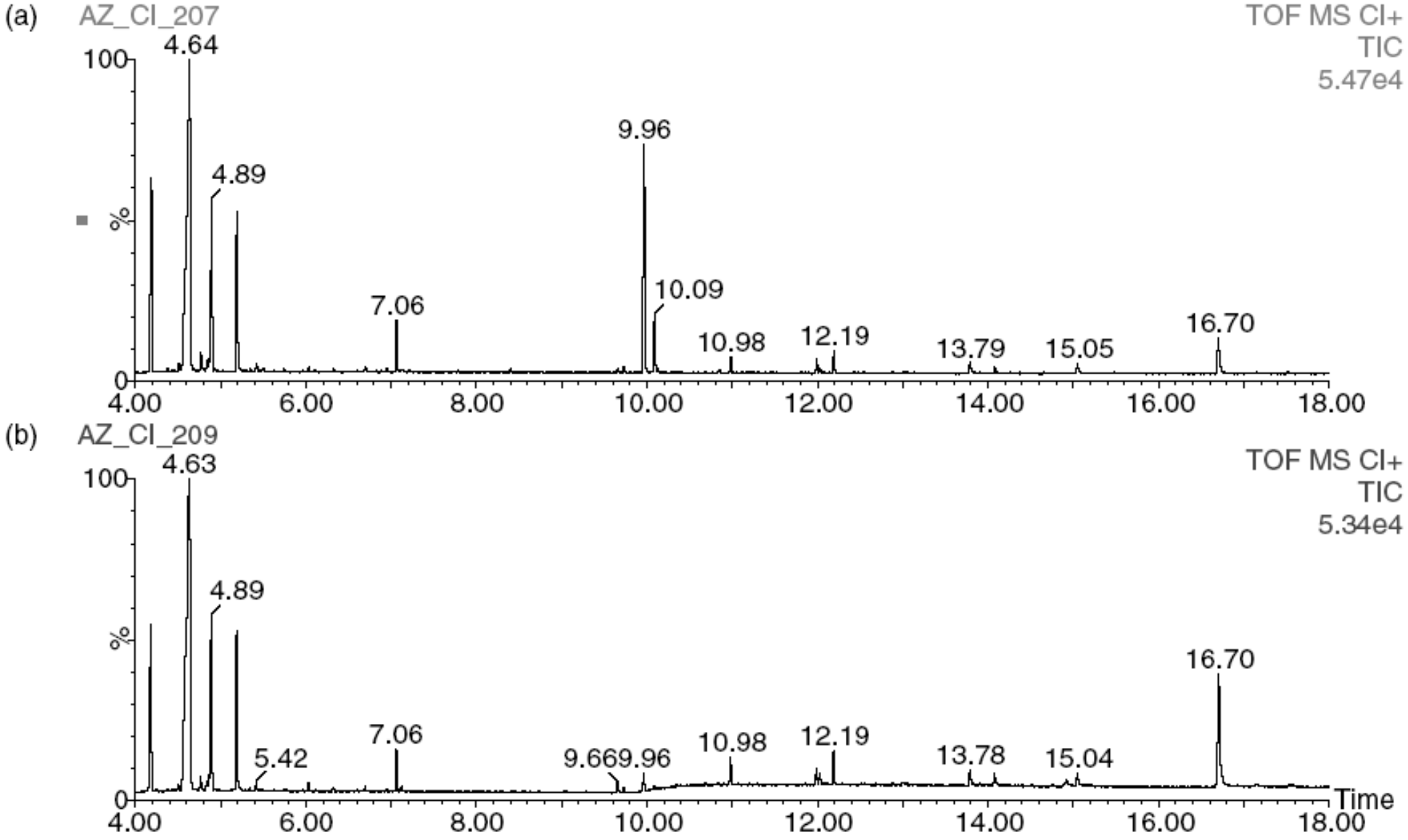


Figure 5.2. Typical TICs obtained from GC-CI-MS analysis of plasma obtained from (a) Wistar-derived and (b) Zucker (fa/fa) obese rats.

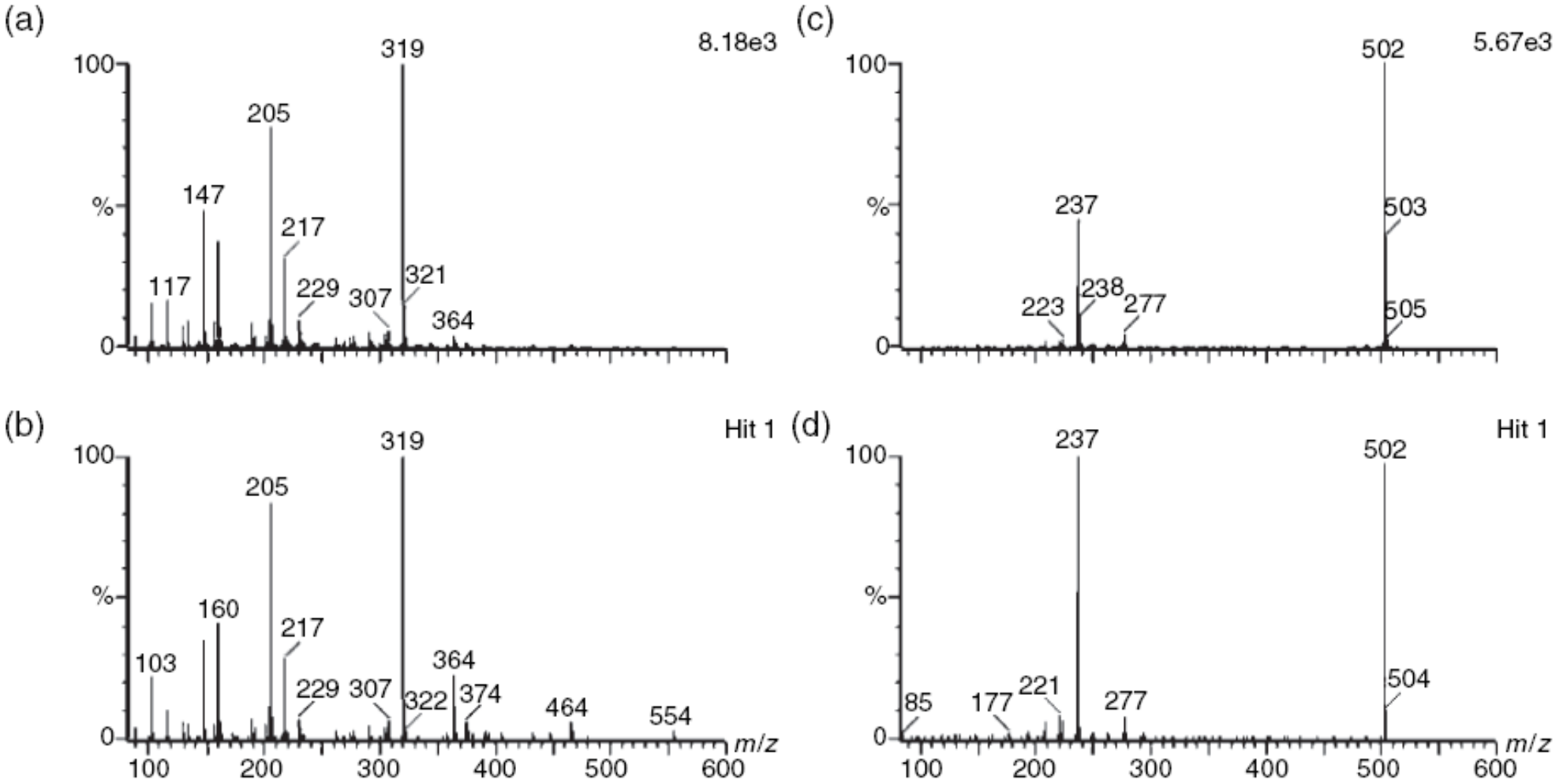


Figure 5.4. Identification of perturbed metabolites from GC-EI-MS analysis: (a) EI-MS for peak eluting at 9.91 min and (b) its library match D-glucose 2,3,4,5,6-pentakis-*O*-(trimethylsilyl-,*O*-methyloxime, (c) EI-MS for peak eluting at 16.58 min and (d) its library match tocopherol (vitamin E), trimethylsilyl derivative.

LC-MS

Hyphenation is technically difficult

Generally used as reversed-phase gradient chromatography (hydrophobic stationary phase, compatible with aqueous samples with no pre-processing)

Detection commonly made by ESI (both positive and negative modes), but also CI

Not all molecules ionize equally well -> differences in sensitivity

Co-eluting substances adversely affect the ionization of the analyte

Column degradation and contamination of the ion source are common problems

Liquid chromatography-mass spectrometry (LCMS)

Different types of LCMS approaches:
- lipid LCMS
- ion pair LCMS
- polar (derivatised) LCMS

LCMS is the better choice for (semi) polar and non-volatile compounds. It can also be applied to profiling of polar compounds, but special (ion pair) agents need to be used or derivatisation in order to retain polar compounds on the column. In LCMS, more combinations of LC (Normal Phase, Reversed Phase, Ion Pair, Hilic,..) and MS (TOF, Ion trap, Quadrupole, FTMS instruments…) parts are available for different applications However, the identification of metabolites is more difficult than with GCMS. Often derivatisation methods are used to make metabolites better solvable. LCMS polar is a suitable technique when you would like to apply a fingerprinting procedure specifically on polar compounds. Lipid LCMS techniques are suitable as metabolic profiling techniques when you are specifically interested in lipid metabolism.

Analisi dei campioni – GC/MS o LC/MS

LC/MS

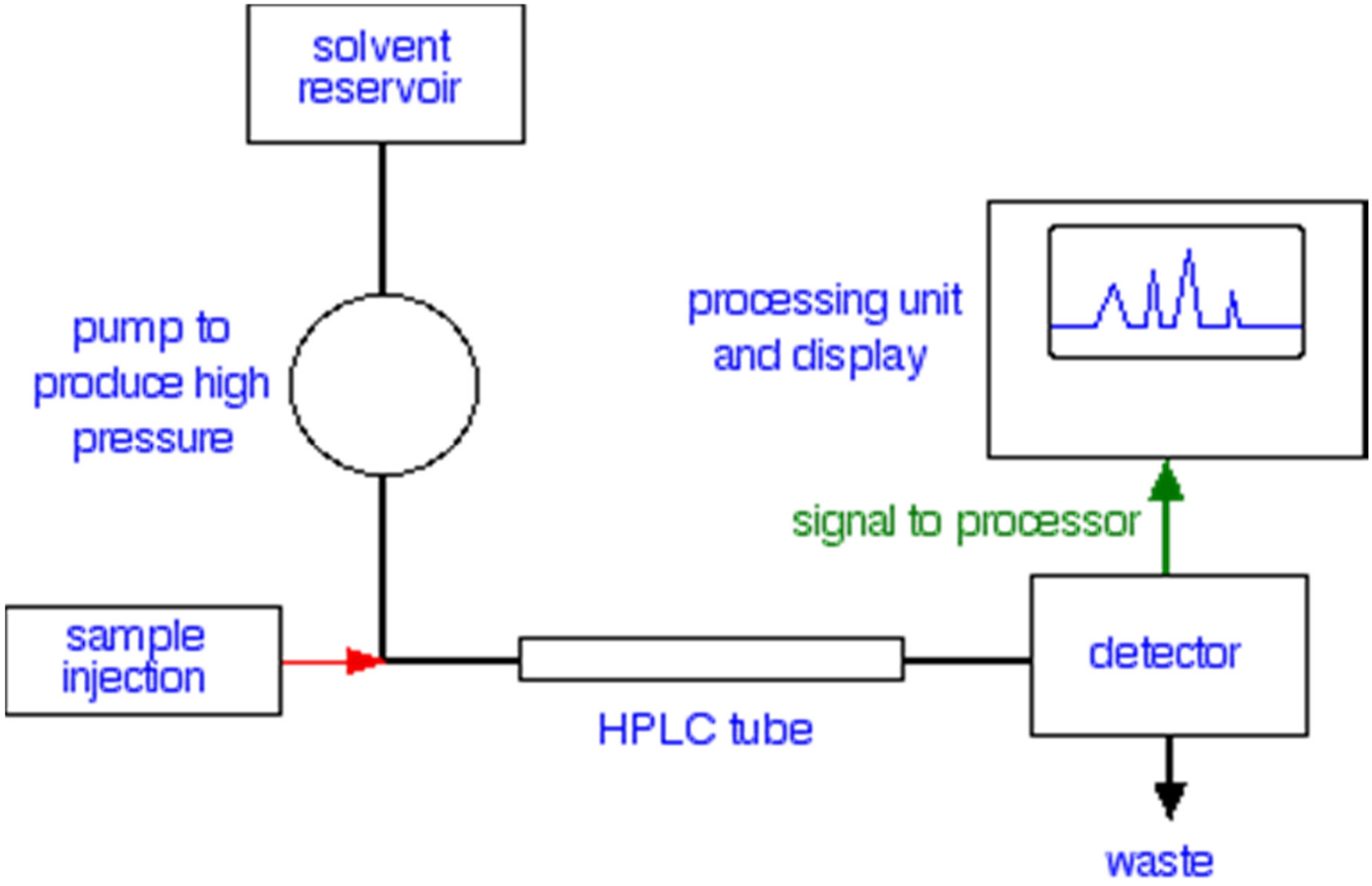
Vantaggi

- Più ampio spettro di analiti
  - Non richiede derivatizzazione
- Alta sensibilità
- Ione pseudomolecolare

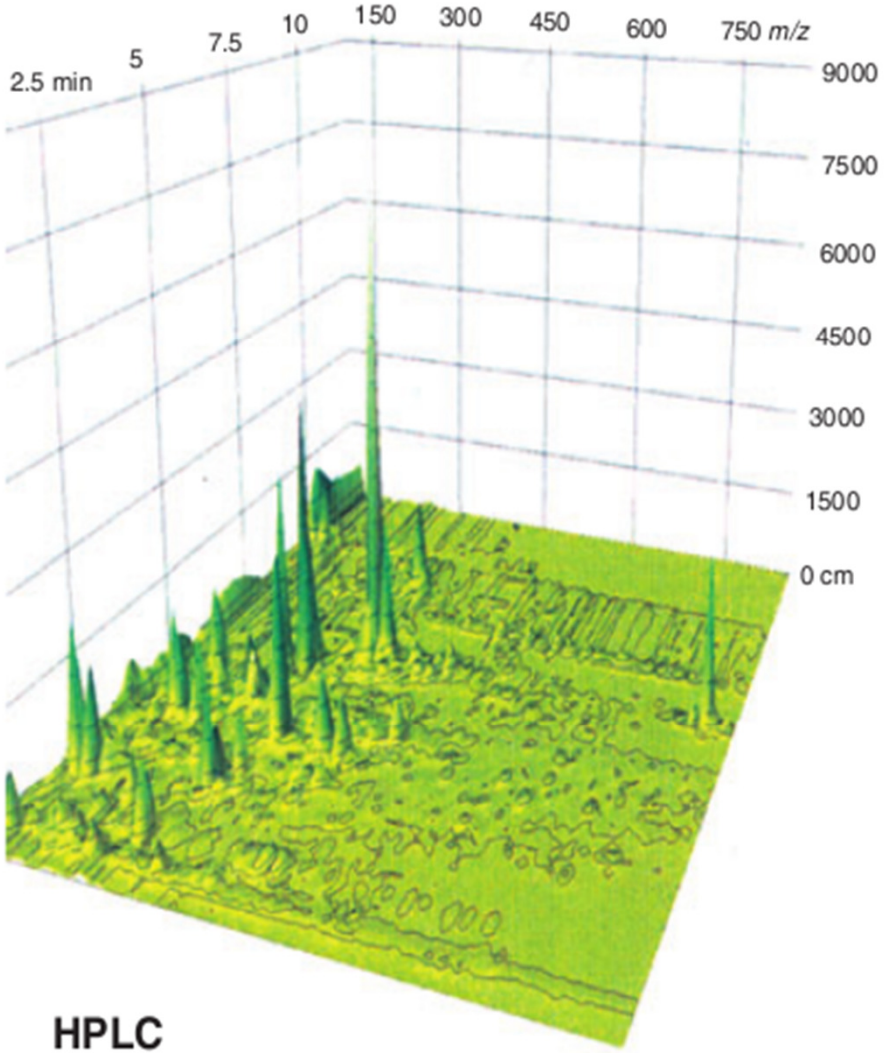
Svantaggi

- Risoluzione media
- Soppressione ionica
  - ESI o APCI
- Non ci sono librerie di spettri
- Investimento maggiore

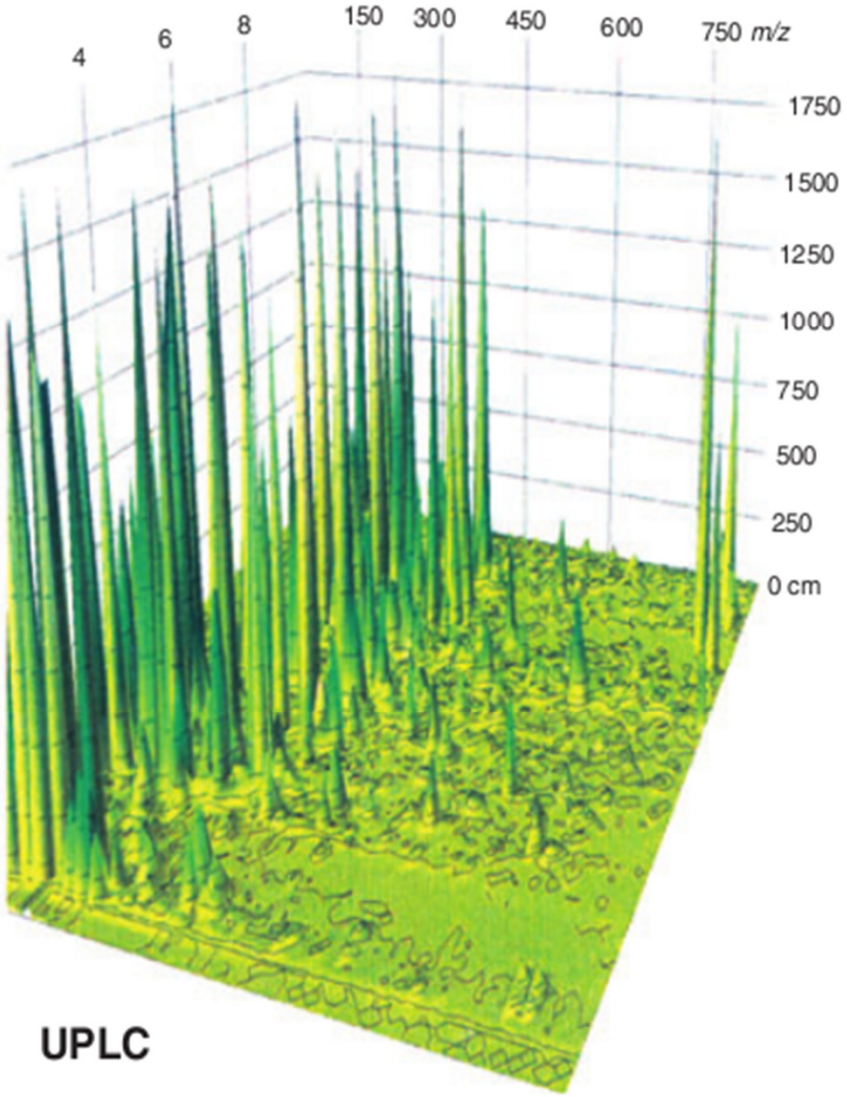
HPLC



HPLC (conventional 3.0-5.0 mm i.d., narrow bore 2mm, micro bore 0.5-1.0mm, capillary)
UPLC (1.7 um stationary phases, better resolution, reduced run-time)


2.5 min
5
7.5
10
150
300
450
600
750 m/z
9000
7500
6000
4500
3000
1500
0 cm

HPLC


4
6
8
150
300
450
600
750 m/z
1750
1500
1250
1000
750
500
250
0 cm

UPLC

Resolution

$R \propto \sqrt{N}$

N: theoretical plate number = L/d_P (length/particle size)

2× increase in resolution requires to square column length e.g. 250mm -> 625cm
this increases enormously operating pressure, so requires reduction of mobile phase viscosity by increasing temperature

Peak capacity

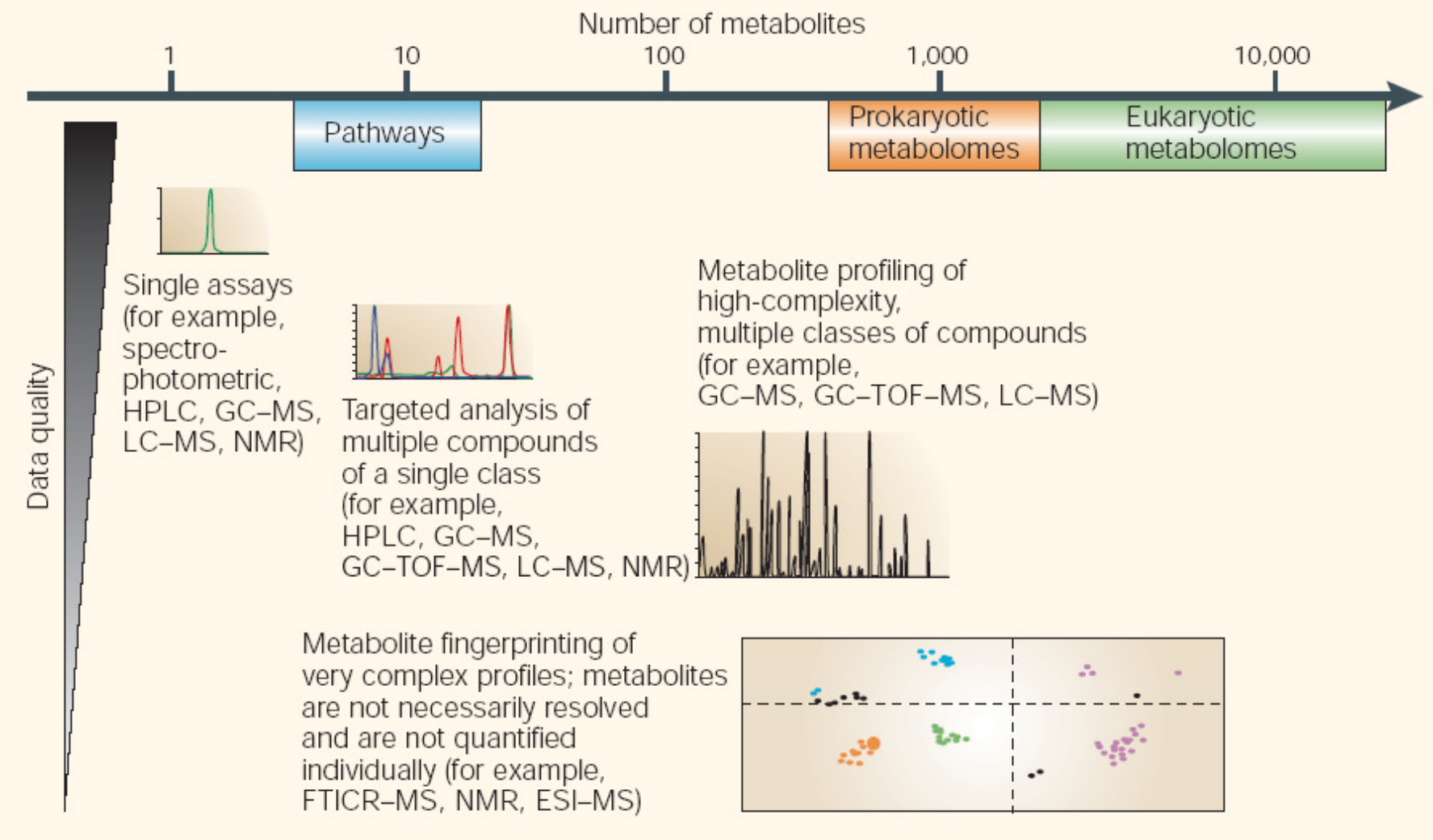
$n = \frac{\sqrt{N}}{4} \cdot \frac{t_n}{t_1} + 1$ measures the maximum number of theoretical peaks resolvable by the system

ideally should approach or exceed the no. of compounds that need to be separated

t_1, t_n: retention times of first and last peak

Metabolomics samples require n > 10000
Analytical scale HPLC (4.6×250mm) has a maximum peak capacity of ~300
Best solution is multidimensional chromatography: $n_{tot}=n_1 \times n_2$ (e.g. 150×50 = 7500)
LC×LC, GC×GC, LC×GC

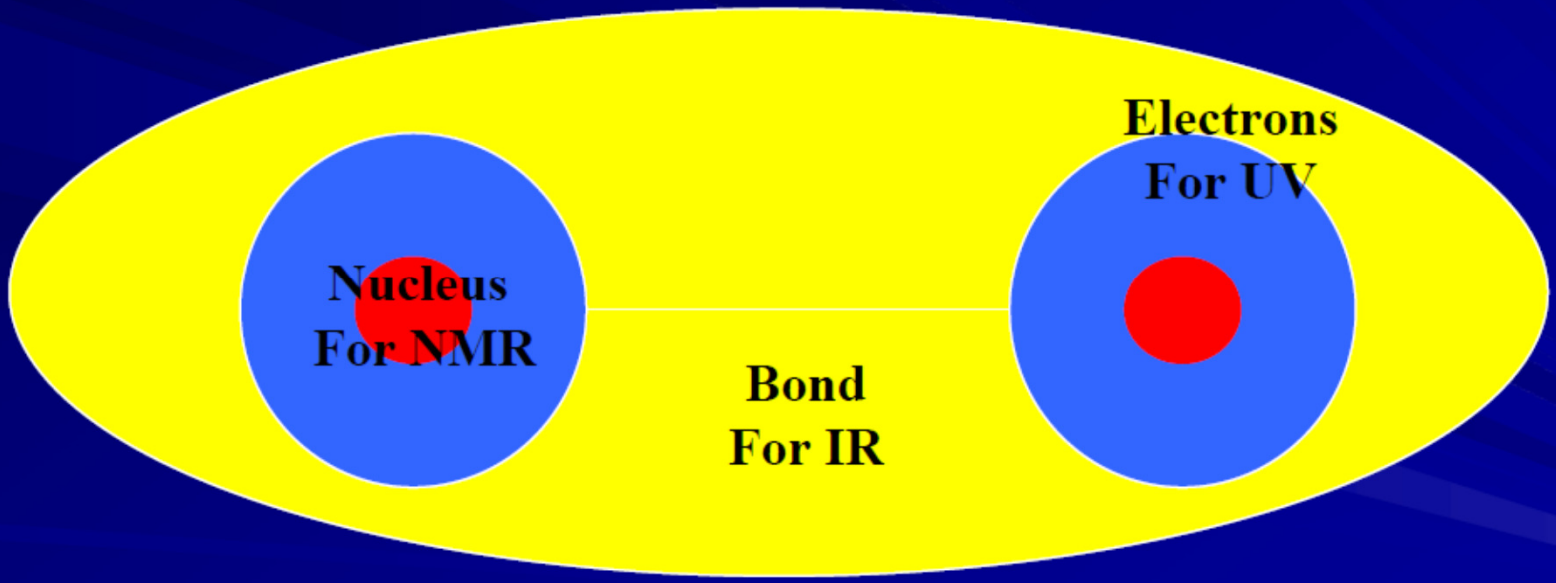
Quality vs metabolic coverage



The trade-off between metabolic coverage and the quality of metabolic analysis

Non-chromatographic based methods for metabolomics

Available spectroscopy methods



Molecules for MS with ionization is necessary

NMR, an ideal method as a pattern recognition technique?

NMR

UV or IR

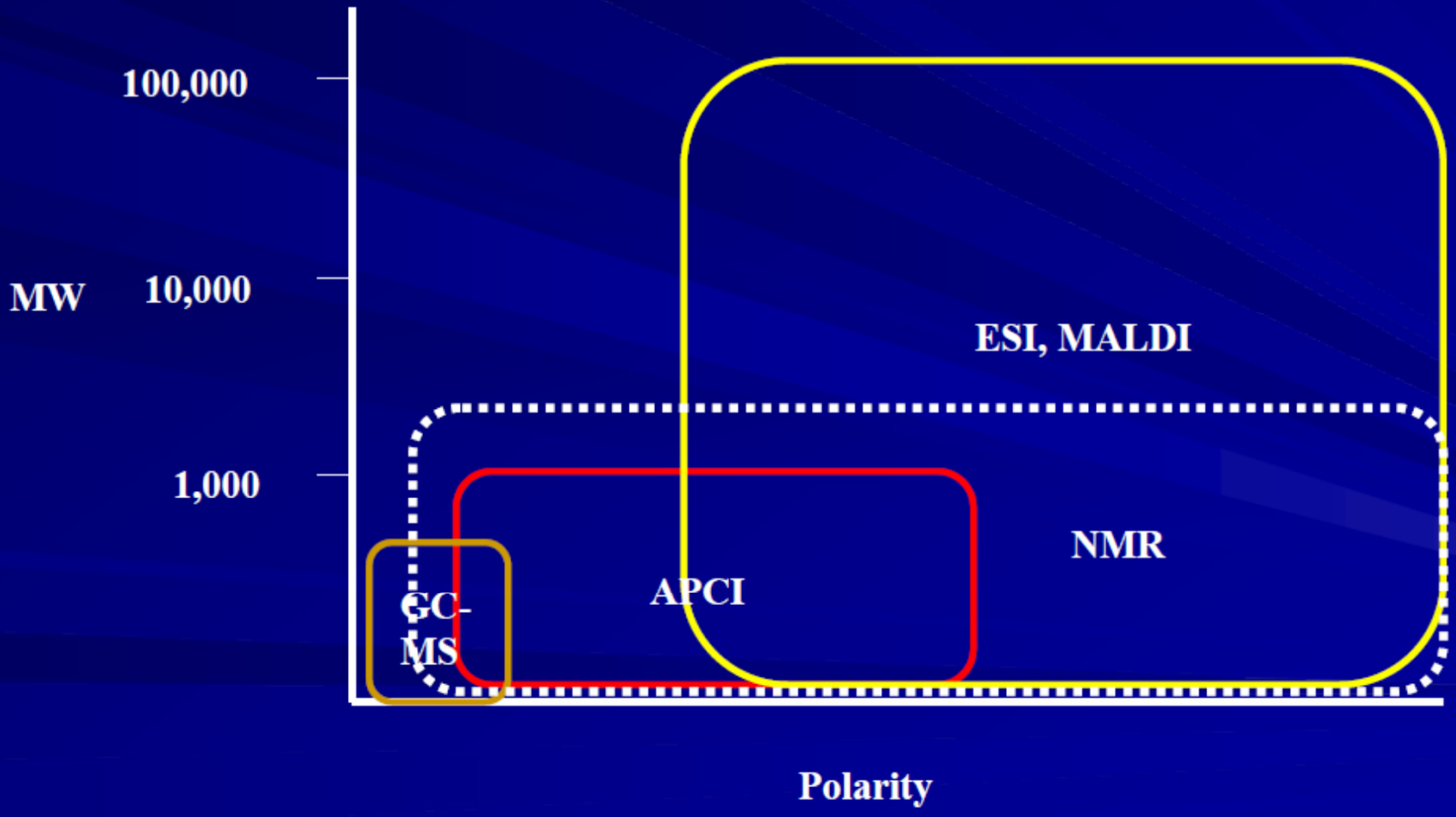
MS

- **Macroscopic approach** : to cover broad range of metabolites

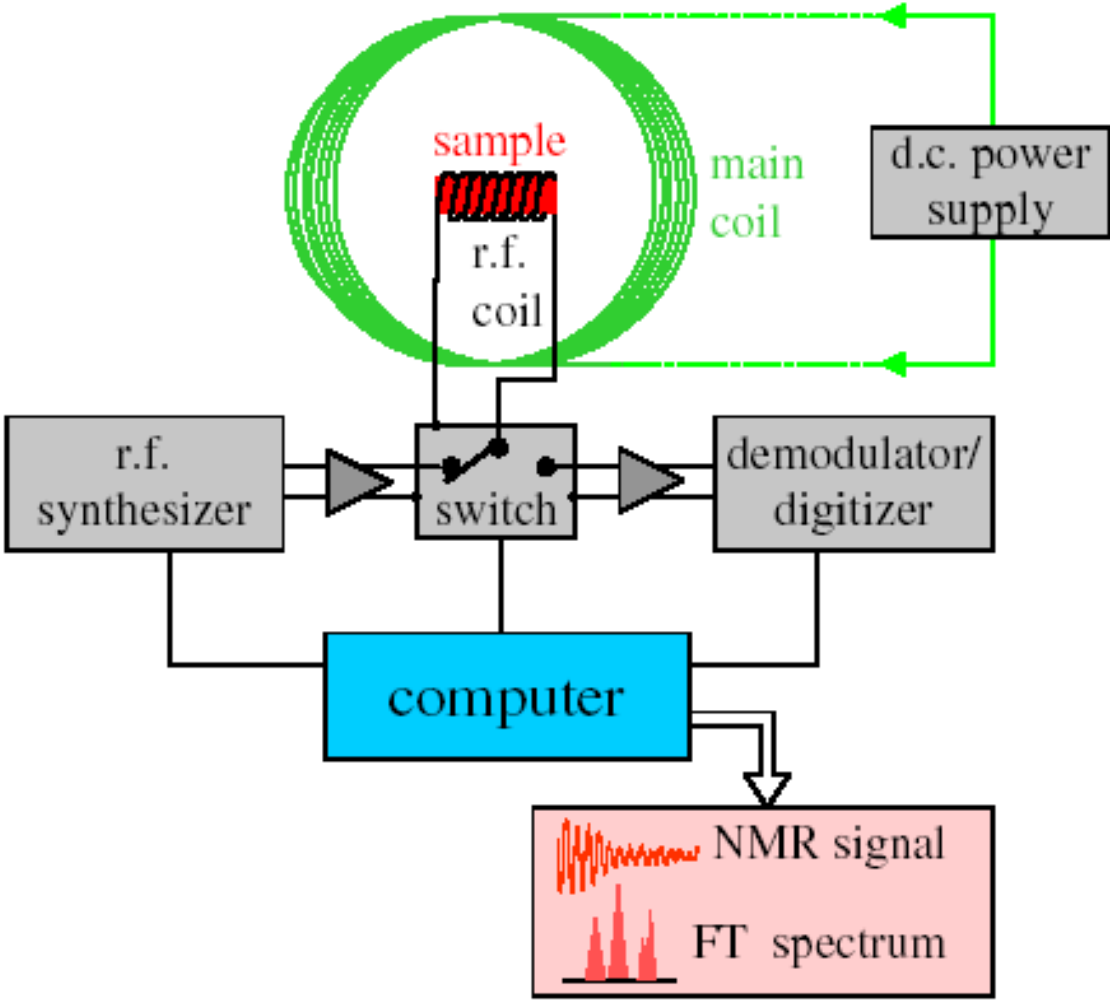
  e. g. amino acids, alkaloids carbohydrates, flavonoids, terpenoids, etc

- **Unbiased** or non-targeted investigation

Range of Metabolites Detected by NMR



NMR


sample
r.f.
coil
main
coil
d.c. power
supply
r.f.
synthesizer
switch
demodulator/
digitizer
computer
NMR signal
FT spectrum

NMR-based metabolomics

What kind of samples can be analysed by NMR?

•All type of biological liquids (urine, plasma, cerebrospinal fluid, amniotic fluid, sperm, synovial fluid, saliva) or cellular or organ extracts

•All kind of biological samples such as biopsies of organs and cell cultures

Why is NMR competitive?

• NMR offers a direct biochemical window into a living system in a holistic way (no a priori selection)

• NMR is fully quantitative

• There is no need for special sample preparation (fractionation, derivatization, …)

• NMR is non-destructive and allows to completely recover the samples

• NMR has emerged into a high throughput analysis system with minimal sample preparation (cost effective)

• Nearly all metabolic intermediates have unique NMR signatures

Factors underlying the enormous challenges of metabolomics

Radius of a typical eukaryotic cell (meter)	5×10^{-6}
Volume of one cell (liter)	5×10^{-13}
Maximum number of cells in 10 g tissue	2×10^{10}
Maximum quantity (mole) of a metabolite with one copy/cell recoverable from 10 g cells/tissue	3×10^{-15}
Detection limit for MS (mole)	1×10^{-18}
Dynamic range limit for MS (factor)	1×10^{6}
Detection limit for ^{1}H NMR (mole)	1×10^{-9}
Dynamic range limit for NMR (factor)	1×10^{6}

NMR vs MS

NMR and MS dominate metabolomics research. "There is no one magic tool that can capture the diversity of composition and concentration which is present in a single sample," says Aram Adourian, senior director of advanced technologies at Beyond Genomics. "It really depends on the question you are asking. You need to have an array of tools available."

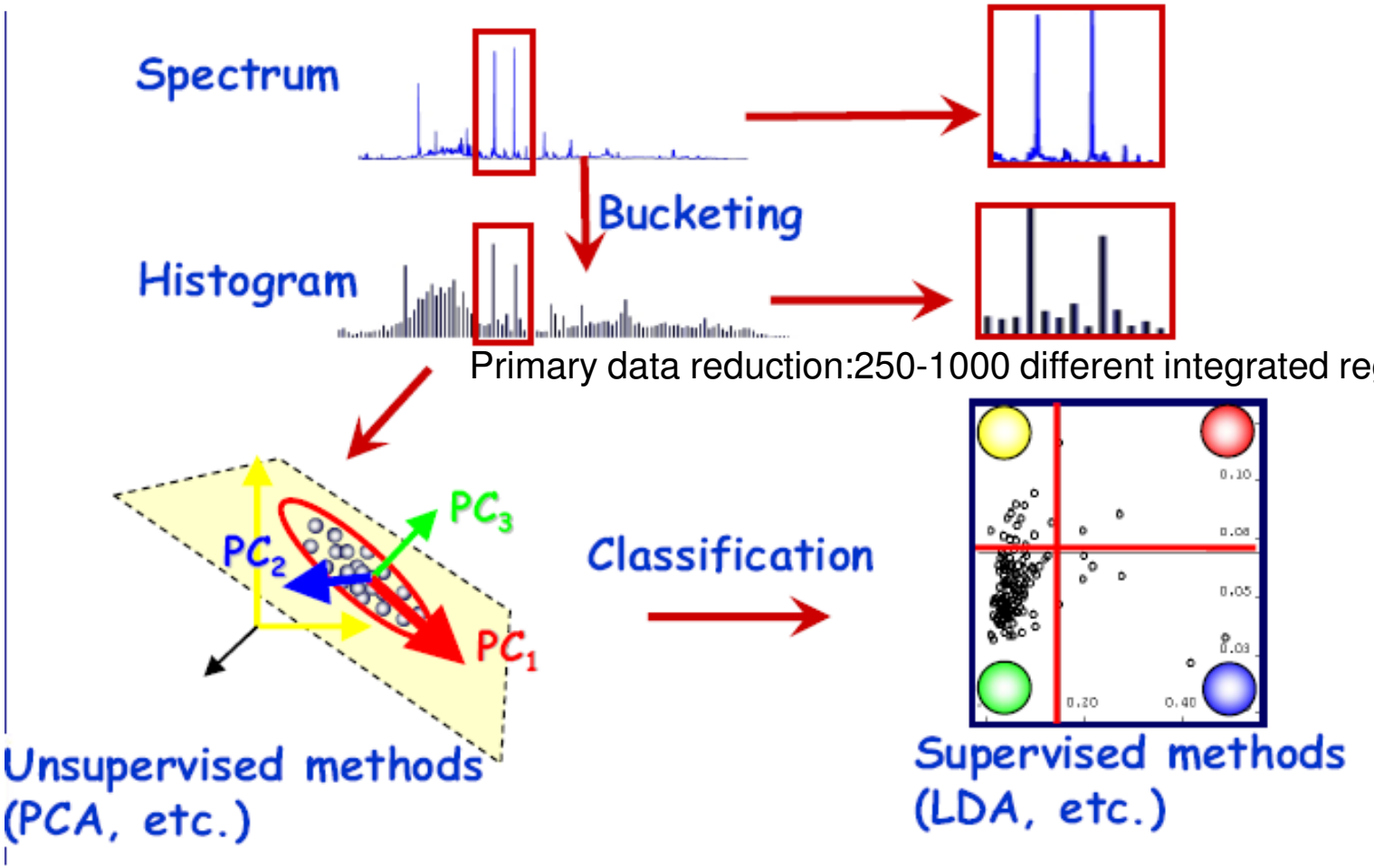
www.the-scientist.com
Amy Adams, “Metabolomics”,
Volume 17 | Issue 8 | 38 | Apr. 21, 2003

Applications and examples of NMR-based metabonomics

APPLICATION	EXAMPLES
Classification of toxicity	Nephrotoxicity Hepatotoxicity Phospholipidosis Testicular toxicity Mitochondrial
Classification of disease	Inborn errors of metabolism Cancer (prostatic, brain, renal etc) Renal disease Diabetes Muscular Dystrophy
Investigation of physiological status	Diurnal variation Hormonal variation
Monitoring efficacy of therapeutic intervention	Renal transplantation (cyclosporin)
Functional genomics	Assessment of strain differences in animal models Evaluation of transgenic models
Characterisation of natural products	Batch to batch variation in commercial Feverfew

Antti et al.
http://www.acc.umu.se/~tnkjtg/Chemometrics/Editorial

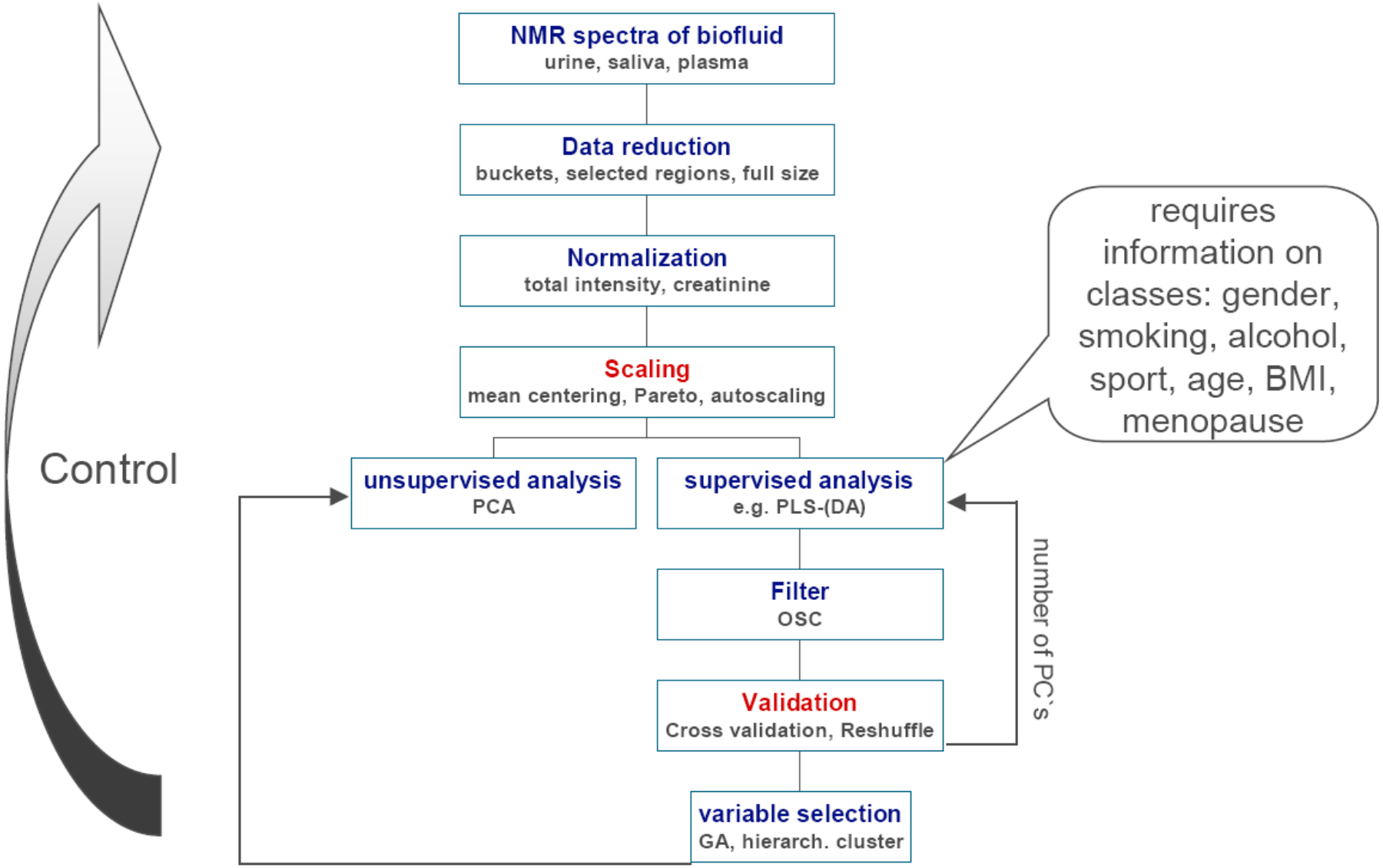
NMR-based metabolomics: the concept



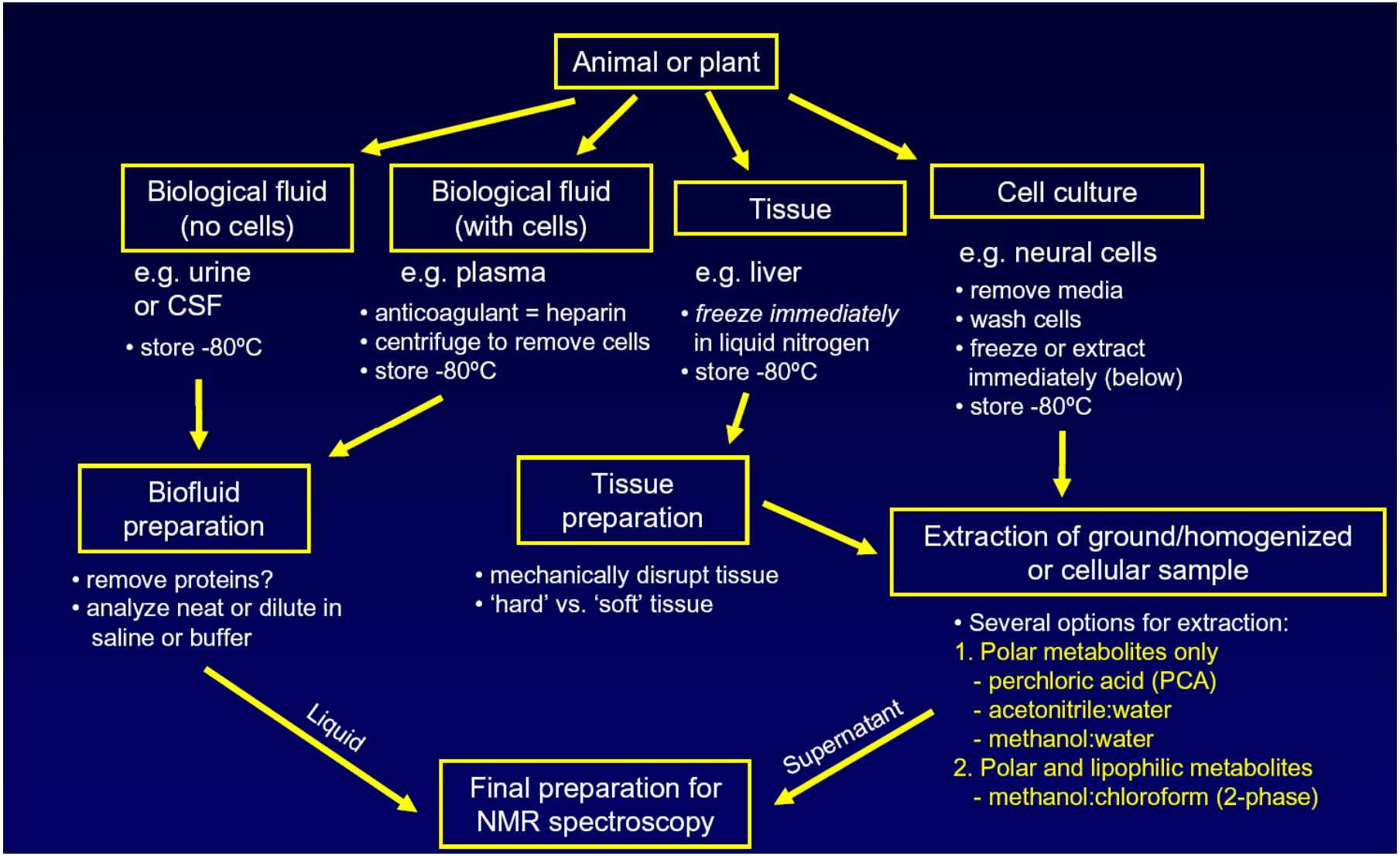
No *a priori* knowledge of the class of samples

Model for the prediction of independent data
Use class information to maximise separation among classes

NMR-based metabolomics (…)

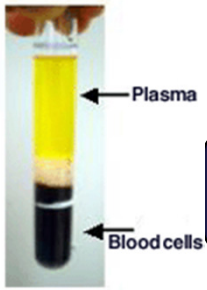


Sample preparation for NMR-based metabolomics



Plasma sample preparation

Blood collection



Plasma separation

Filtration

Sample volume and pH adjustment

Ready for NMR spectroscopy