Gradient elution in HPLC –

Fundamentals, instrumentation , methodology & and case studies

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OUTLINE

A. Introduction

The general elution problem, challenges of gradient elution

B. Fundamentals

Retention, peak width and resolution, operational parameters: gradient steepness, gradient range, gradient delay

C. Instumentation and gradient generation

Gradient generation: high pressure vs. low pressure gradient, dwell volume, degassing, linear gradient, step gradient

D. Method development

Gradient elution of biopolymers in RPC, IEC, HIC and HILIC, objectives in MD-LC/MS, options and specific features

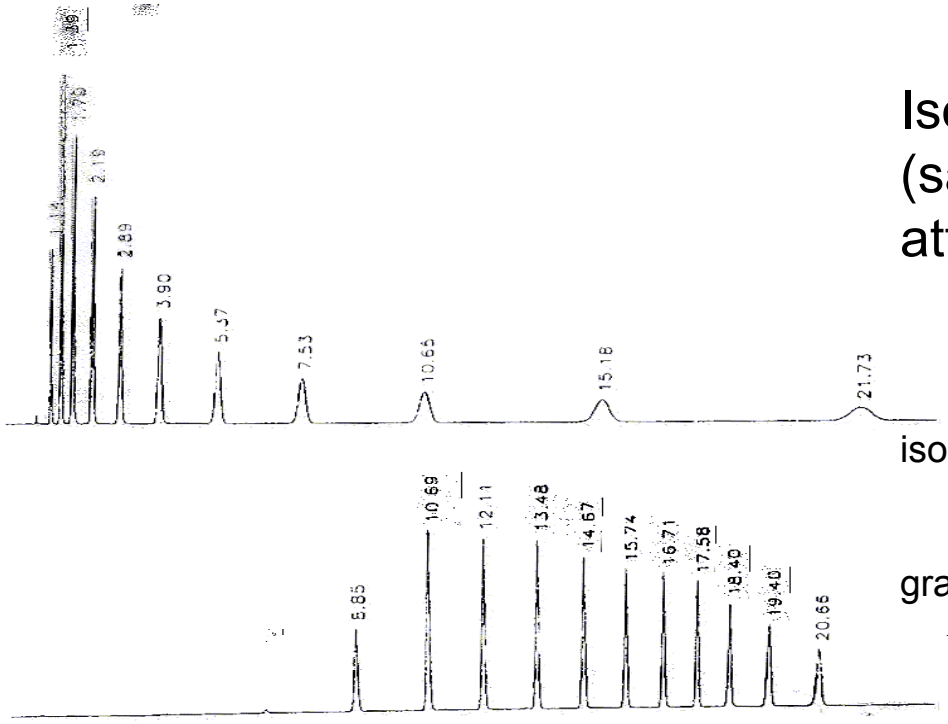
E. Case studies in MD-LC/MS

Elution modes in HPLC

Elution can be achieved by two different modes:

✓Isocratic elution, without changing the mobile phase composition

✓Gradient elution, where the mobile phase composition is changed during the course of the separation



Isocratic elution vs. gradient elution (sample detected at equal attenuation)

isocratic: water/acetonitrile

25/75 (V/V) continuous

gradient: water/acetonitrile

from 90/10 (1min), at 5%/min, to 0/90 (5 min)

A.Introduction

Gradient elution

Continuous or stepwise change of mobile phase during separation in such a way that the elution of late eluting compounds is continuously reduced

Mobile phase has to become steadily stronger as the separation proceeds

The strength of the mobile phase is characterized by the solvent strength which is specific for a given stationary phase,

e.g. Reversed Phase Chromatography :

Water has the lowest solvent strength, acetontrile is a strong solvent

Gradient shape :

linear, concave , convex, segmented, gradient delay, step gradient

Linear solvent strength model

$$\log k = a - b\,(\%\ B)$$

$$\log k = \log k_w - S\,\Phi$$

Where k_w is the retention coefficient of analyte in pure water, Φ is the volume fraction of organic solvent at a RPC separation and S a constant being proportional to the cross-sectional area of analyte

Reasons for using gradient elution

Complex sample mixtures with a wide retention range

Biopolymers whose retention changes markedly for small changes in the mobile phase composition

Samples which contain a variable and/or unknown composition (tool for screening)

Alternatives to gradient elution :

Flow programming

Temperature programming

Column switching

Typical operation conditions for gradient Reversed Phase LC

Eluents

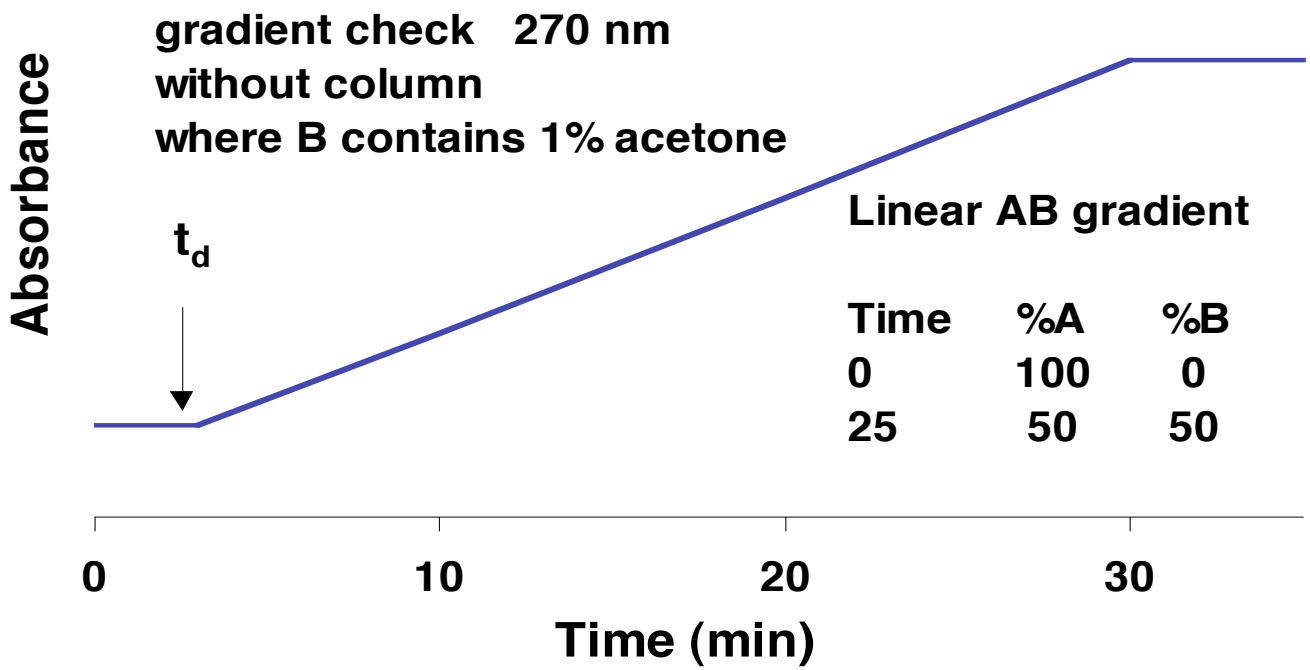
standard conditions:

linear AB gradient
eluent A: 0.1% TFA, eluent B 0.1 %TFA in acetonitrile
gradient rate 1% B / min
flow rate 1 ml / min
room temperature

Operation conditions

flow rate 0.5 - 2.0 ml / min, gradient 0.2 - 5%B / min, low flow rate for proteins
colum dimension (see extra transparency)
type of organic solvent (see extra transparency)
temperature (see extra transparency)
monitoring silanol effects pH 2.0, pH 4.5, pH 7.0
ion pair additives

MONITORING OF GRADIENT LINEARITY



Conditions: linear AB gradient (2% B/min), where eluent A is water and eluent B is 1% aq. acetone; flow rate 1 ml/min; detection at 270 nm. The term t_d denotes gradient delay time. There is no separating column present.

ISOCRATIC CONDITIONS (CONSTANT MOBILE PHASE COMPOSITION)

Retention coefficient k should be in the range between

$$1 < k < 20$$

Elution profiles are expressed as the dependency of k on the concentration of the displacing species

$$\log k = A + B (\log 1 / c)$$

RPC	$\log k = \log k_0 - M \log [\text{solvent}]$
IEC	$\log k = \log k_0 - H \log [\text{salt}]$
HIC	$\log k = \log k_0 - Z \log [\text{salt}]$

GRADIENT ELUTION CONDITIONS (LINEAR CHANGE OF ELUENT OMPOSITION)

k' is replaced by $\bar{k}_1$ $\quad \bar{k}_1 \equiv$ median retention coeffcient, value of the migrating band at the half way point along the columns

c is replaced by $\bar{c}$ $\quad \bar{c} \equiv$ concentration of the displacing component, when the sample band reaches the midpoint of the column

Gradient elution is mandatory in biopolymer separation to match the wide range of molecular weight analytes differing in hydrophobicity, charge etc.

Only size exclusion is performed under isocratic conditions

Resolution in gradient elution mode is proportional to the peak capacity, PC

PC is defined as a number of peaks which are resolved with a chromatographic resolution of Rs = 1

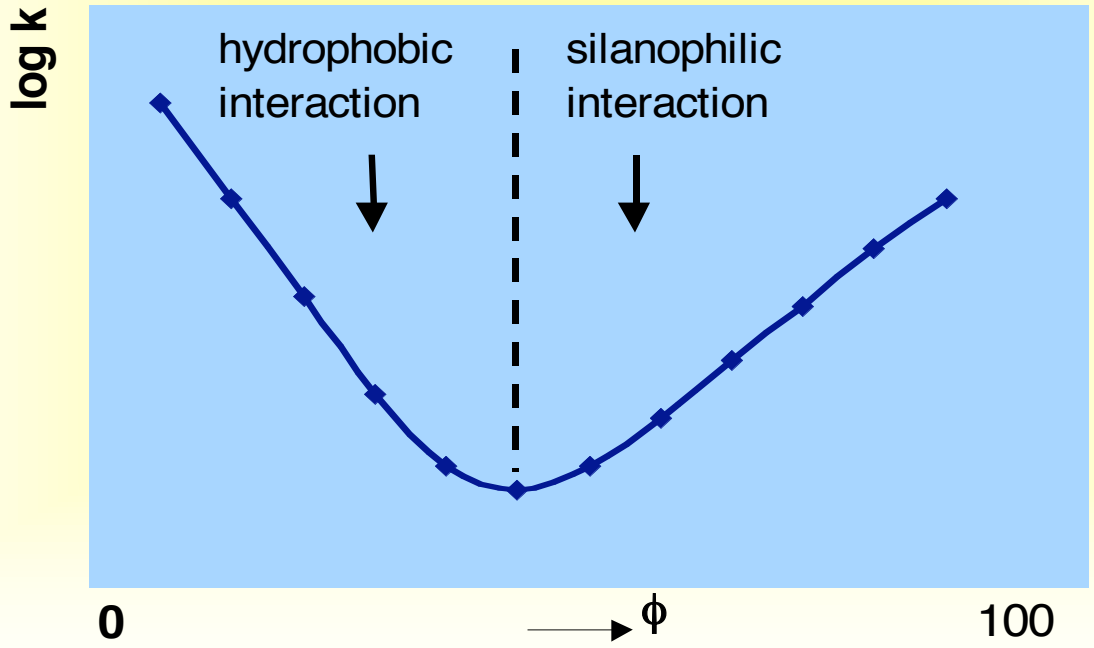
$$PC = \frac{t_g \cdot F}{4\sigma_v}$$

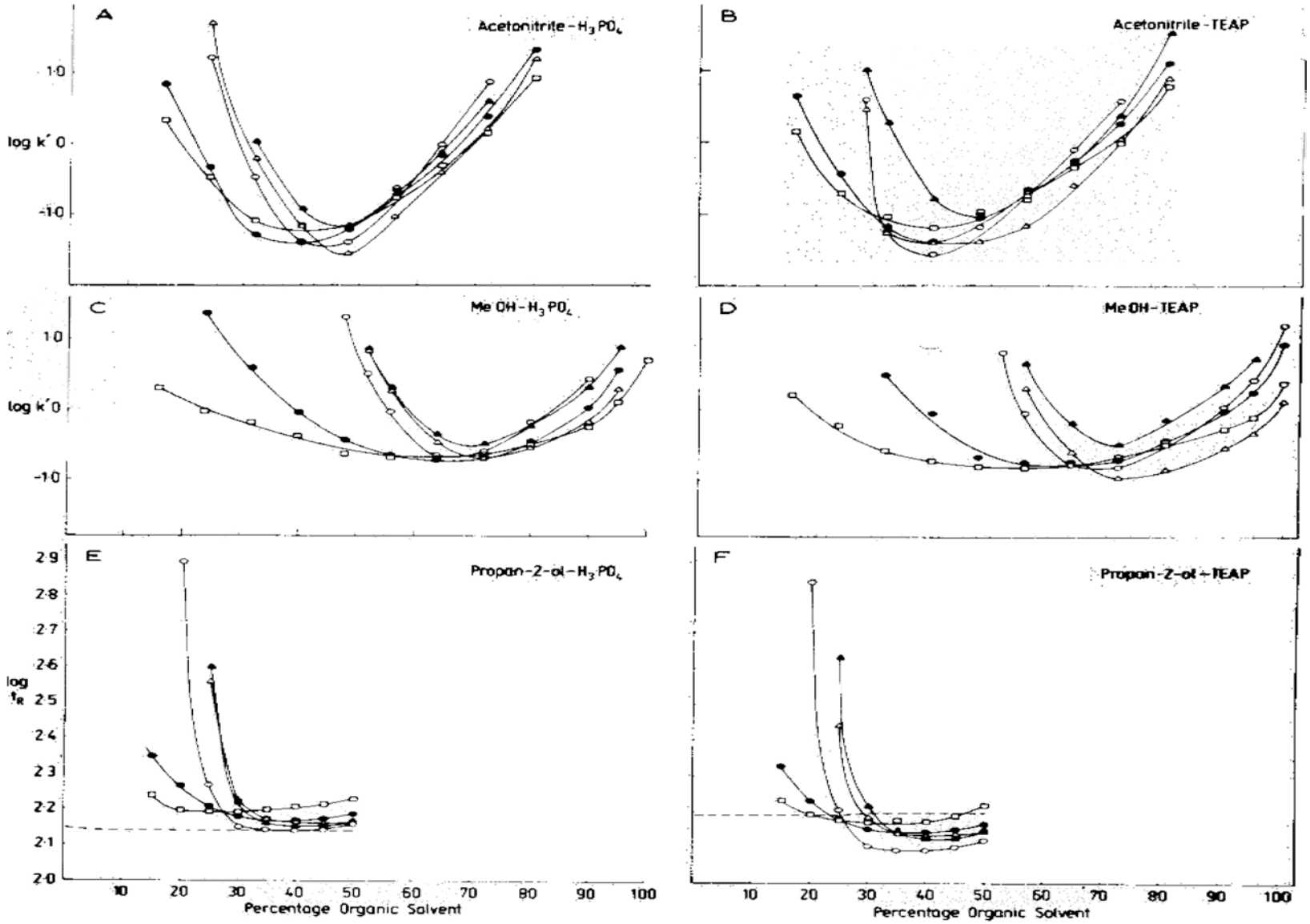
t_g	=	gradient time
F	=	volume flow rate
σ_v	=	standard deviation of peak(s) in volume units

Peak capacity is nearly independent of the column length and can reach values up to 200 in RPC of peptides

Characteristic retention feature of RP columns in gradient elution of peptides and proteins

RP packings have a bifunctional character (hydrophilic, hydrophobic)with is reflected in the bimodal curve: log k' *vs*. Φ




A
Acetonitrite - H_3PO_4
B
Acetonitrile - TEAP
C
MeOH - H_3PO_4
D
MeOH - TEAP
E
Propan-2-ol - H_3PO_4
F
Propan-2-ol + TEAP
log k' 0
1·0
-1·0
log t_R
2·9
2·8
2·7
2·6
2·5
2·4
2·3
2·2
2·1
2·0
10
20
30
40
50
60
70
80
90
100
Percentage Organic Solvent

Effect of gradient conditions on separation (A)

Gradient conditions

Initial and final % B in the gradient

gradient shape

conditions which determine gradient steepness b

gradient time t_g, flow rate F, gradient range, column dead volume

Effect of gradient conditions on separation (B)

Gradient steepness b

Gradient time t_g

Increase in gardient timeresults in better resolution (increased peak capacity), decrease in peak width and longer run times

Column length L

Increase in column length increase the column dead volume ,the column dead time and gradient steepness b. Bcause the peak width increases with column dead volume and column dead time and decreases with gradient steepness, these two effects cancel approximately.

Flow rate F

When F decreases resolution decreases, plate height increases and retention time sligthly increases at otherwise constant conditions

Recommended literature

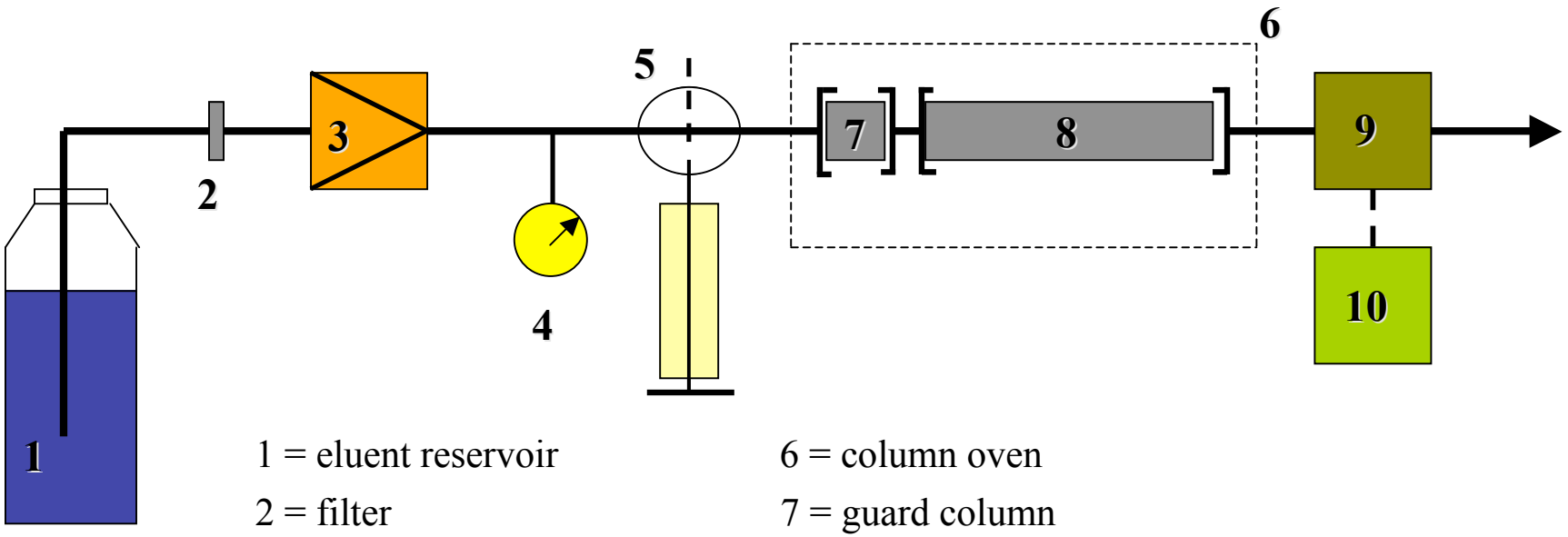
L.R. Snyder and J.W. Dolan, High Performance Gradient Elution, Wiley Interscience, New York, 2007

L.R. Snyder, J.J.Kirkland and J.L Glajch, Practical HPLC Development, Wiley Interscience, New York, 1997

The figure illustrates four case histories for the dependency of the logarithmic capacity factor (log k) on the mole fraction, ς, of the displacing species. As the contact area associated with the solute-ligand interaction increases, the slopes of hte log k vs. ς plots increase, resulting in a narrowing of the elution window over which the solute will desorb. Cases a and b are typically observed for RPC and IEC of polar peptides and small, polar globular proteins, while cases c and d are more representative of the RPC and IEC behavior of highly hydrophobic polypeptides and non-polar globular proteins, including membrane proteins, respectively.

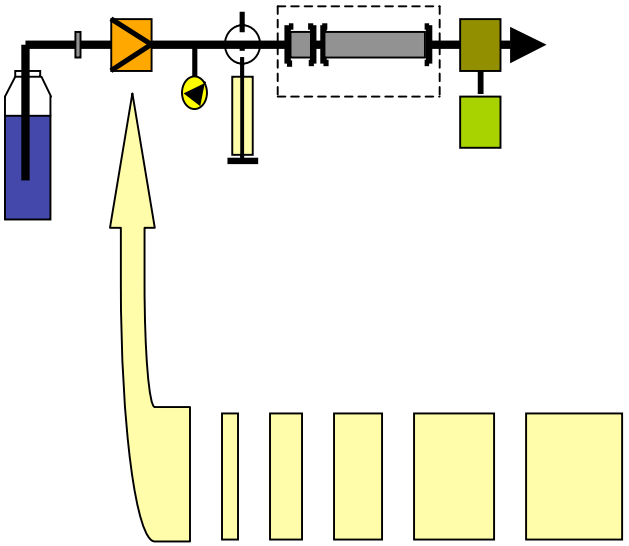
Instrumentation and gradient generation

Schematic diagram of an HPLC system for isocratic operation

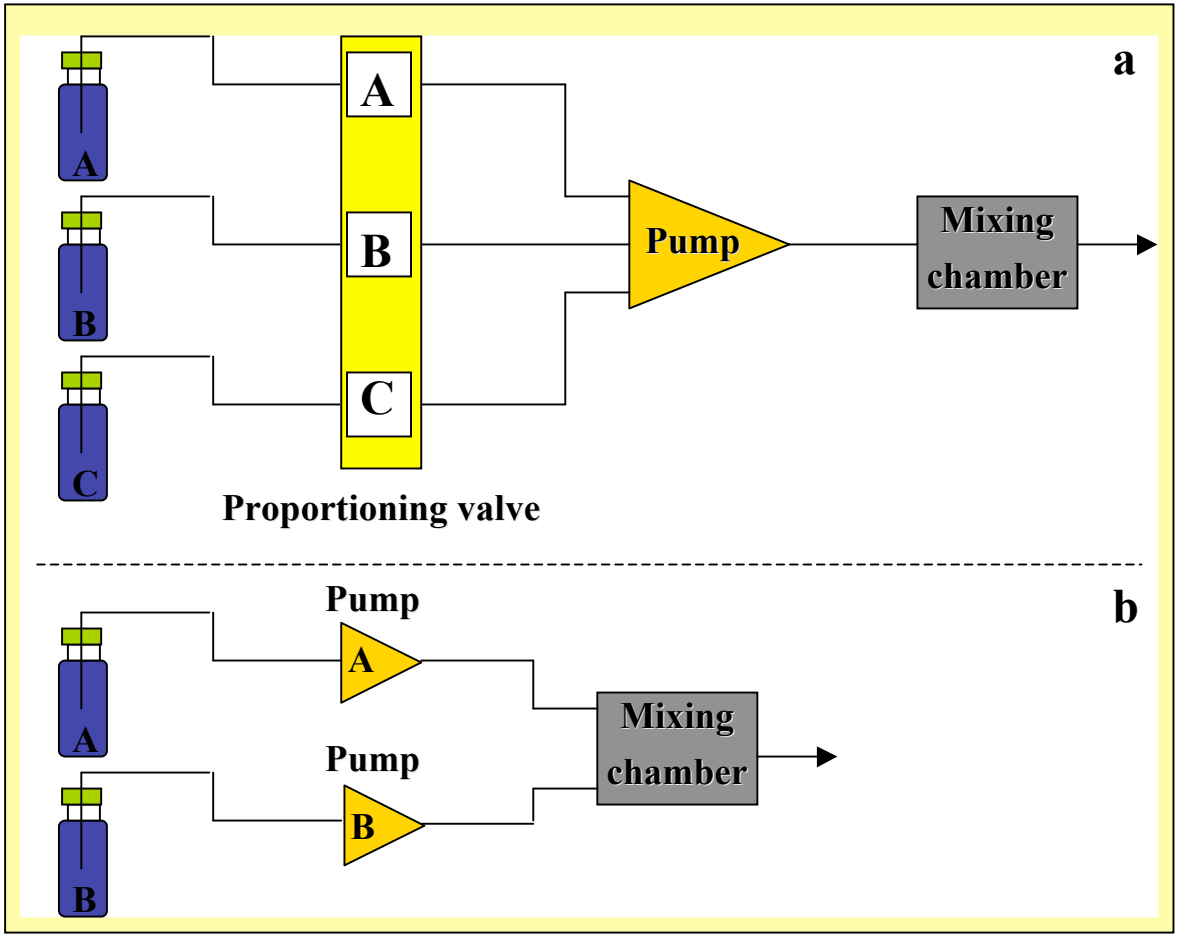


1 = eluent reservoir
2 = filter
3 = high pressure pump with pulse dampener
4 = pressure gauge
5 = sample injection valve with syringe
6 = column oven
7 = guard column
8 = column
9 = detector
10 = recorder (integrator, PC etc.)

Instrumentation and gradient generation



Mixing of mobile phases for HPLC



a) Low-pressure gradient system;

b) High-pressure gradient system

Instrumentation and gradient generation

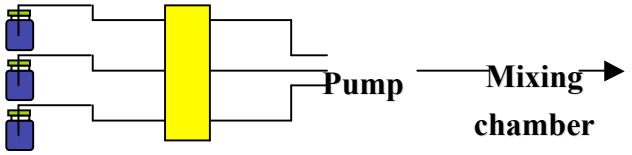
- **The advantages of a low-pressure gradient system are:**

  More than two solvents can be mixed;

  – Only one high-pressure pump is needed;

  – Reproducibility of gradient formation is higher compared to a high pressure gradient system;

  – When the mobile phase composition is changed, it is easier to obtain a constant flow (after changing the mobile phase composition)



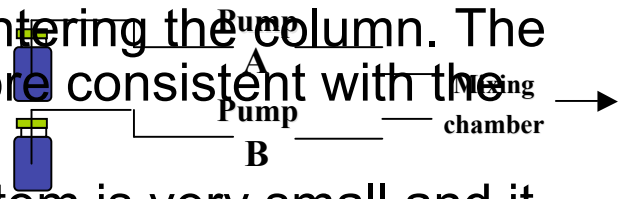
- **Disadvantages of the low-pressure gradient system are:**

  – Proportioning valves are susceptible to contamination and can cause inaccuracies in eluent composition

  – The additional mixing chamber increases the internal volume of the system and causes a delay in the formation of the gradient (the gradient which is actually delivered to the column) compared to the programmed value.

Instrumentation and gradient generation

- **Advantages of a high-pressure gradient system are:**
  - The two solvents are combined just before entering the column. The effective mobile phase composition is therefore consistent with the programmed mobile phase composition
  - The internal volume of the high-pressure system is very small and it is therefore possible to run steep gradient profiles and to perform quick mobile phase changes.
  - Degassing becomes unnecessary when mobile phase is composed at high pressure



- **Disadvantages of a high-pressure gradient system are**:
  - High costs, as two pumps are needed
  - Lower precision of most pumps, when operated at lower flow rates, limits the precision and reproducibility of gradient formation at extreme mobile phase compositions
  - A third pump is needed to create a gradient based on a ternary solvent composition

Instrumentation and gradient generation

- **When using mobile phase gradients it is important to consider the following points:**
  - The solvents must be miscible over the entire gradient composition range.
  - The viscosity of the resulting mobile phase can change considerably when the solvents are mixed resulting in an increased column back pressure.
  - The solvents must be thoroughly degassed otherwise bubbles can be formed during mixing. Water/methanol and water/acetonitrile phases are very susceptible. The solvents can be degassed by vacuum, sonication or helium sparging. Flow-through on-line degassers can also be used.
  - The detector must be able to handle gradients. The baseline should remain stable over the entire mobile phase composition range. UV and fluorescence detectors are suitable for gradient elution whereas a refractive index and an electrochemical detector cannot be used.

Case studies in MD-LC/MS: Objectives

Perform an effective and fully automated sample clean-up

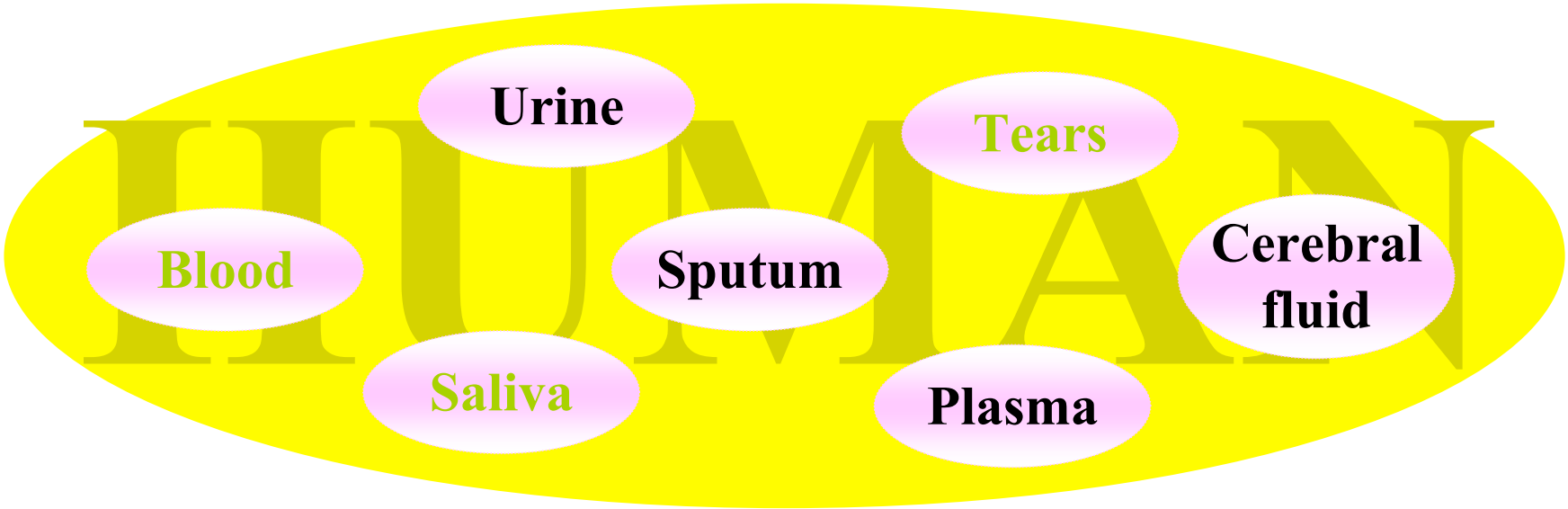
Selective removal of undesired constituents

Enrichment of target compounds

Reduce the complexity of the MD-LC/MS platform

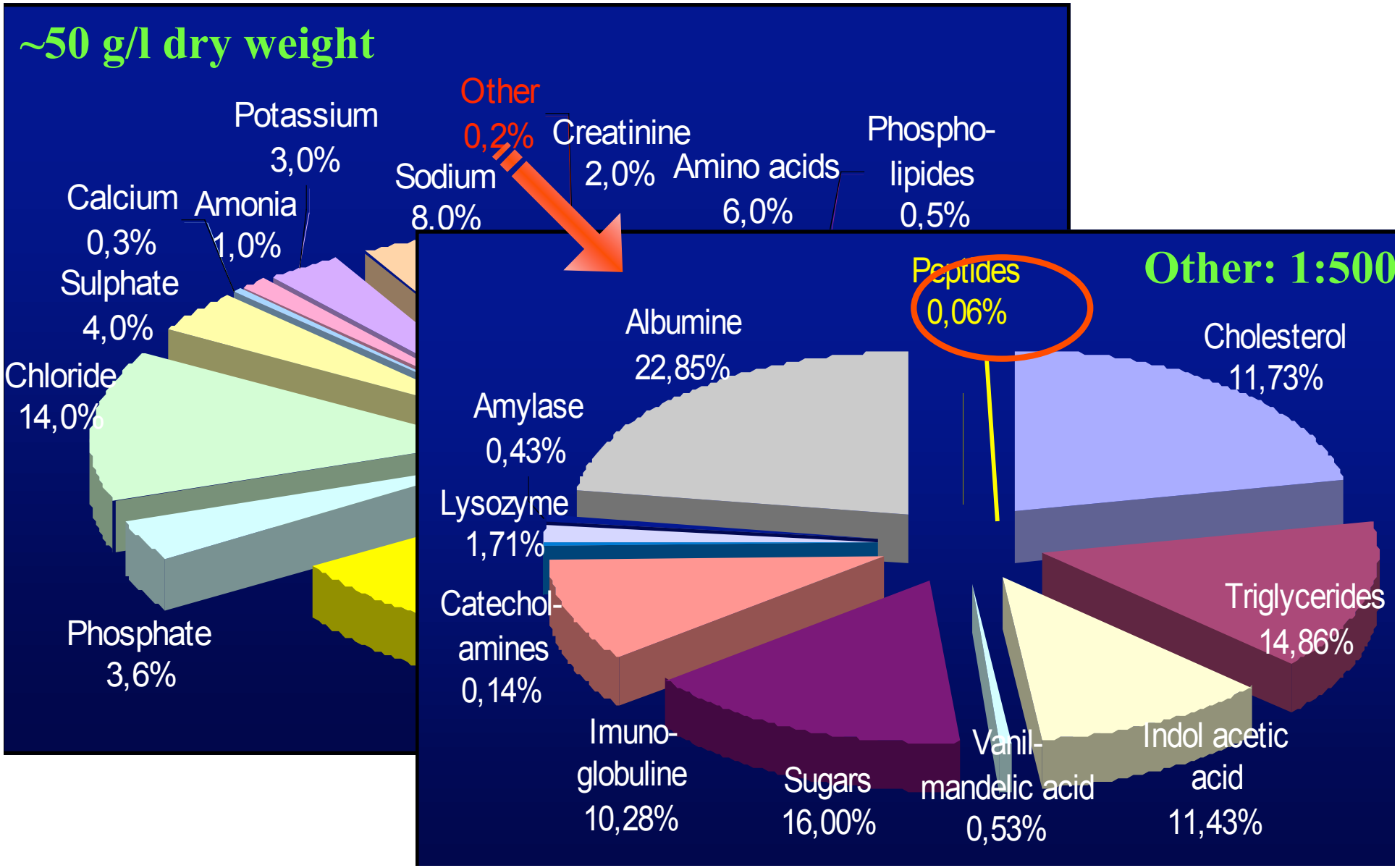
Enhance the reproducibility, robustness and reliability of the system

Types of biofluids studied

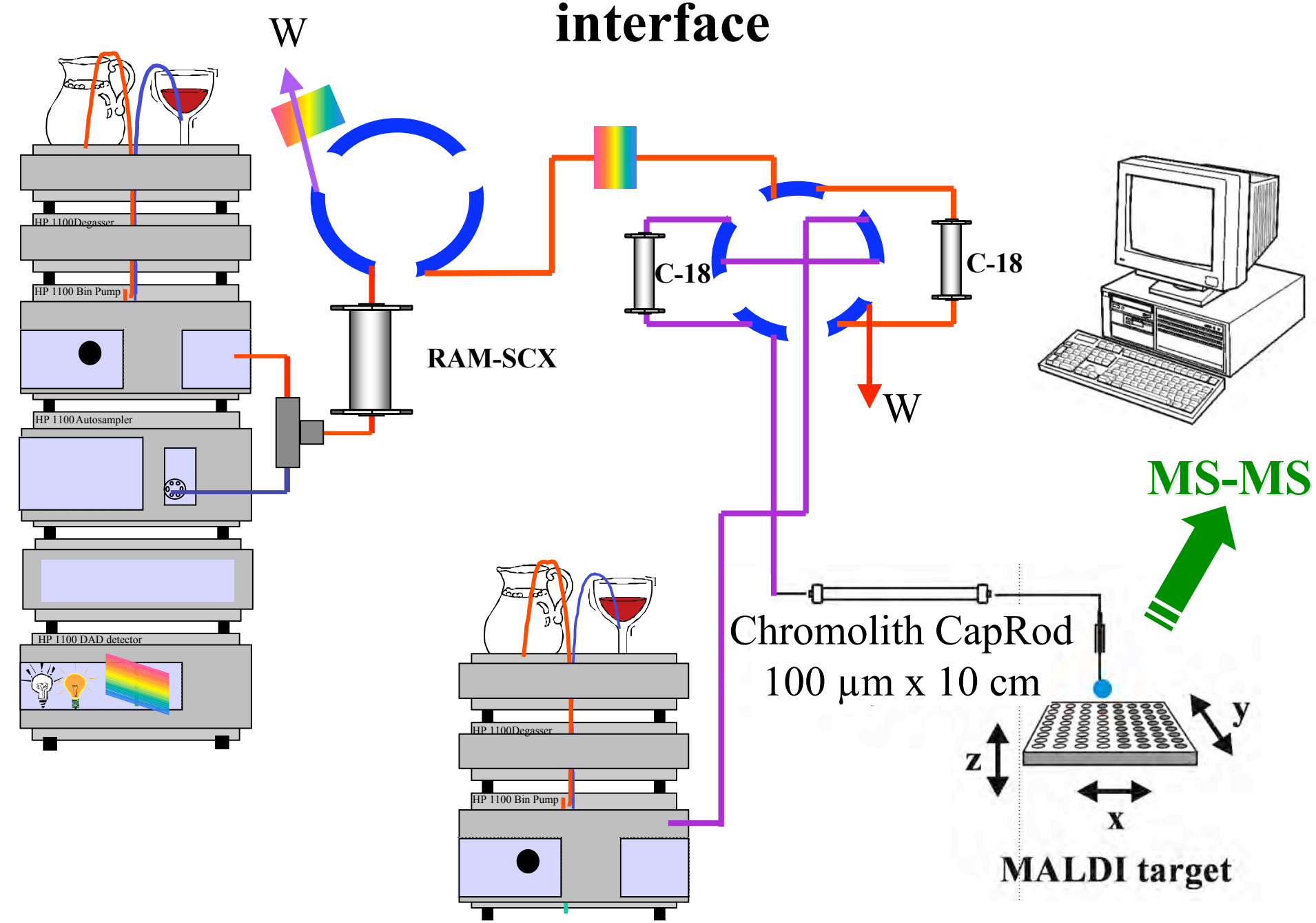


Peptides

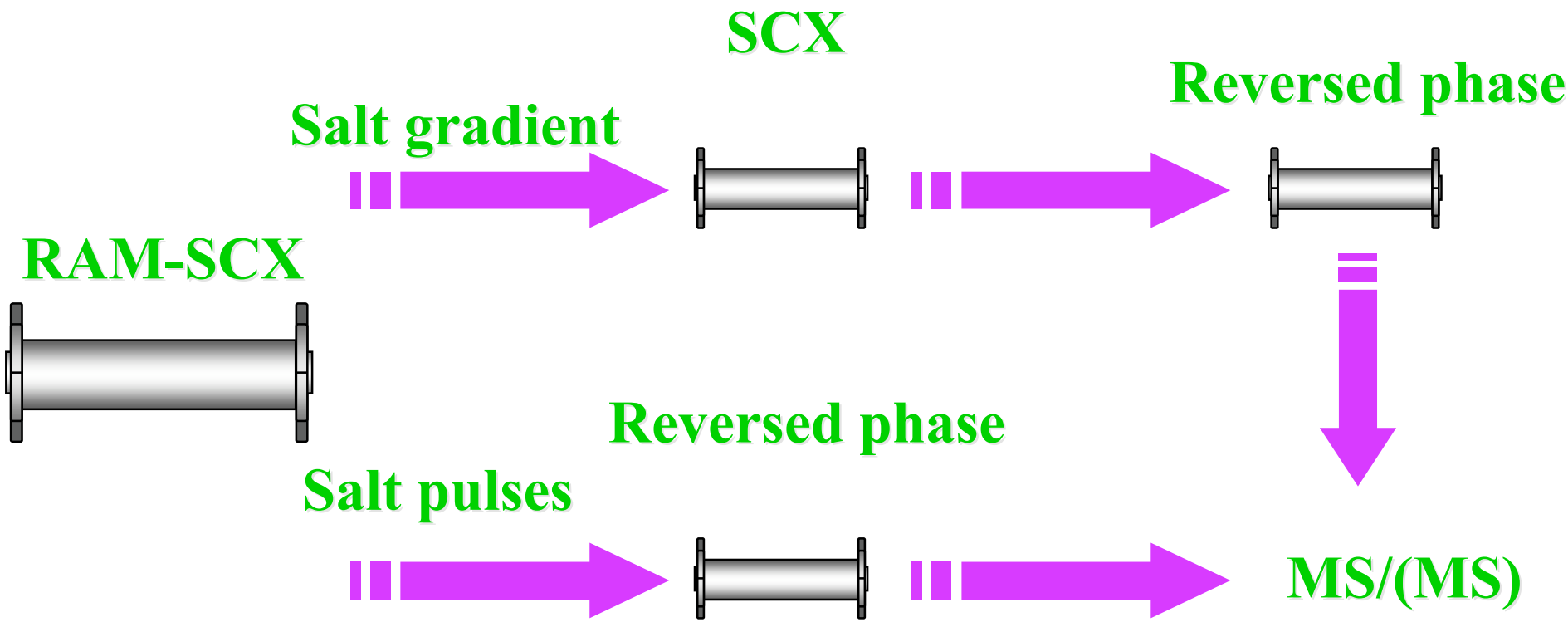
Composition of human urine

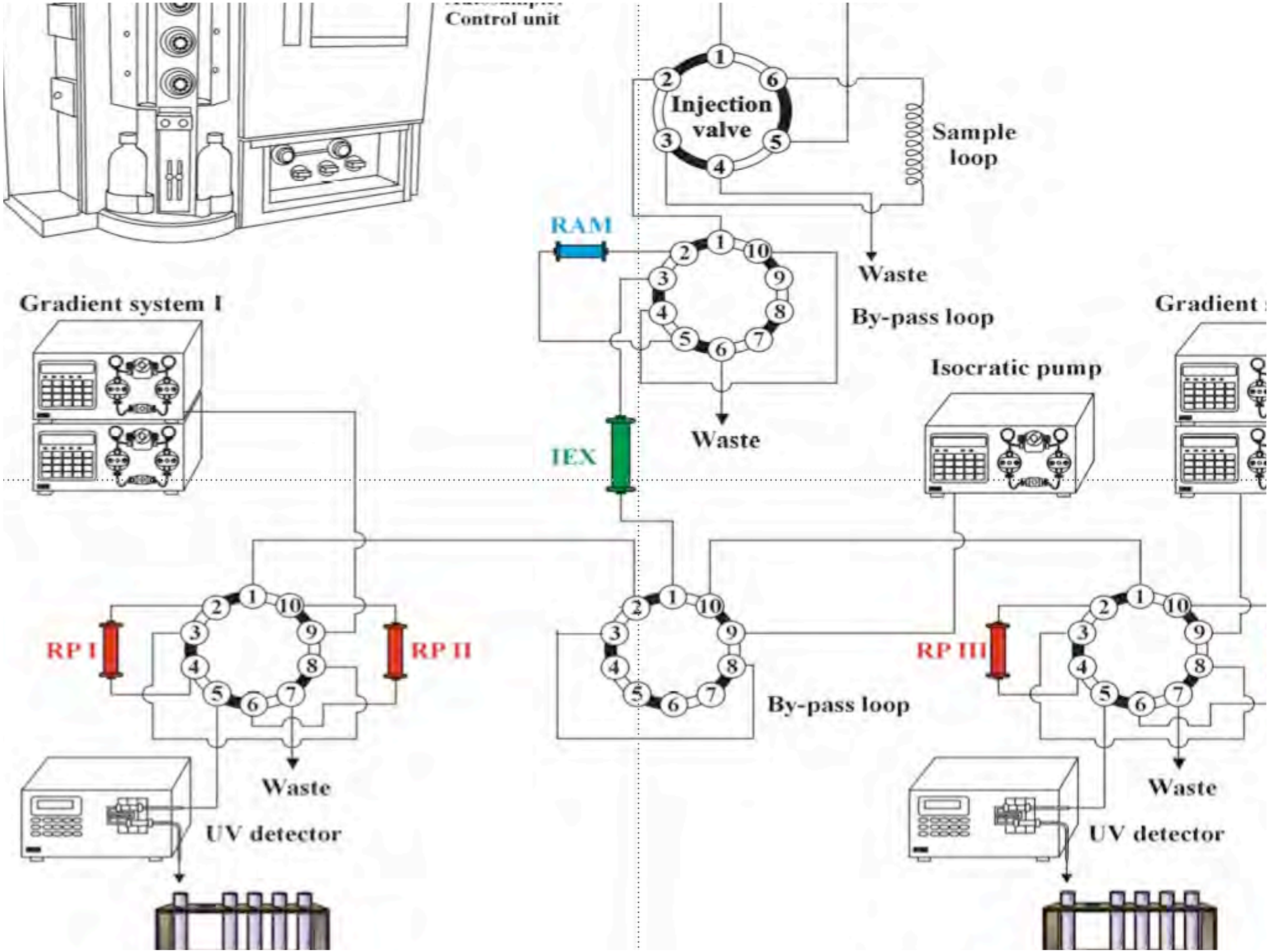


Example of 2D-µLC/MALDI interface



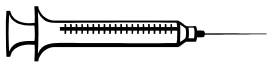
Strategies of the analyses



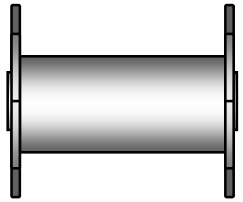

Control unit
Injection valve
Sample loop
RAM
Waste
By-pass loop
Gradient system I
Waste
IEX
Isocratic pump
RP I
RP II
By-pass loop
RP III
Waste
UV detector
Waste
UV detector

Processing strategy

Experimental conditions:

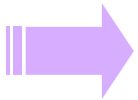


~450 µl

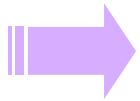


Sample clean-up column - SCX-RAM (Merck)
75 x 10 mm I.D.
0.5 ml/min

5 salt pulses



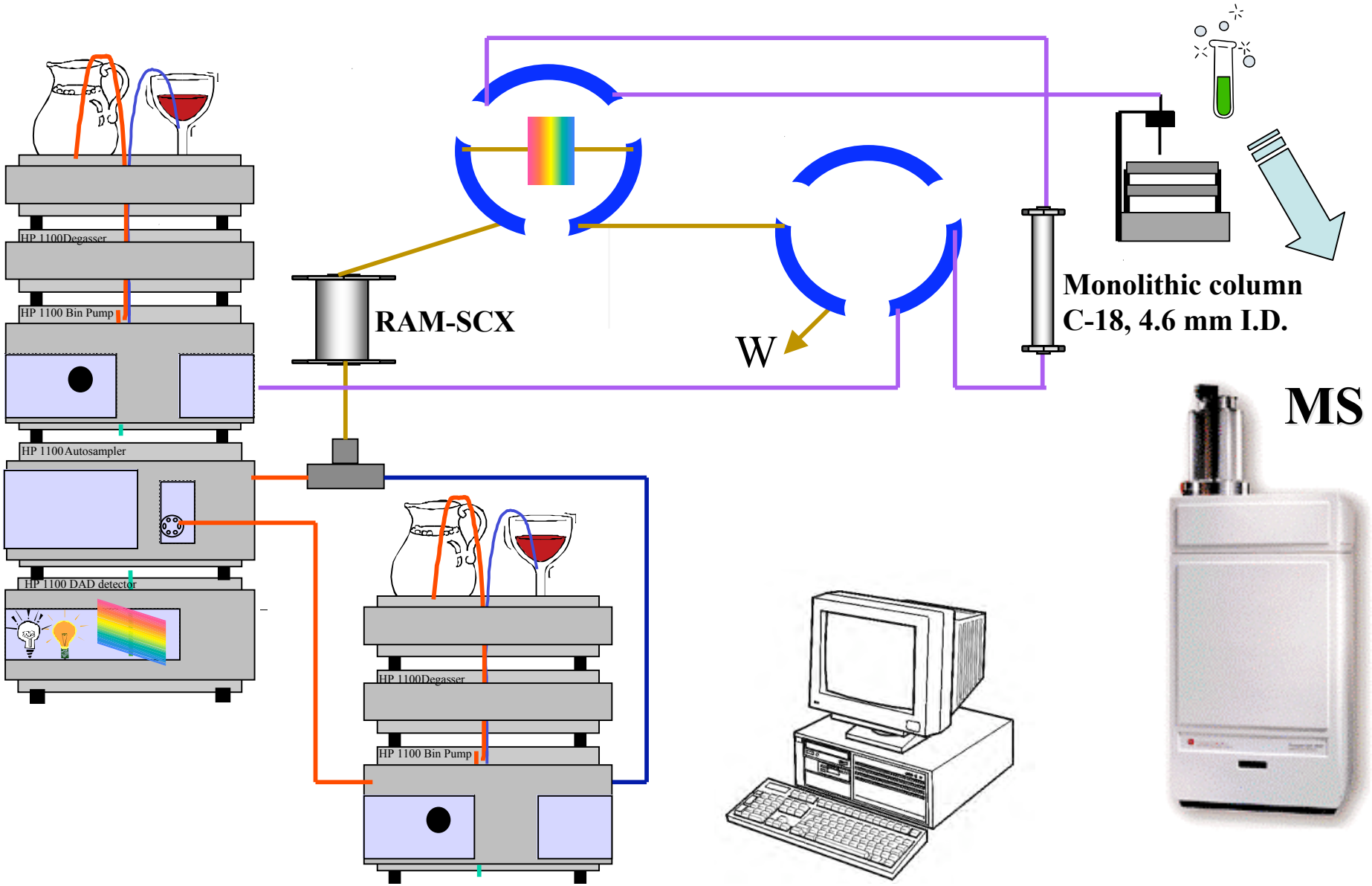
40 min ACN gradient



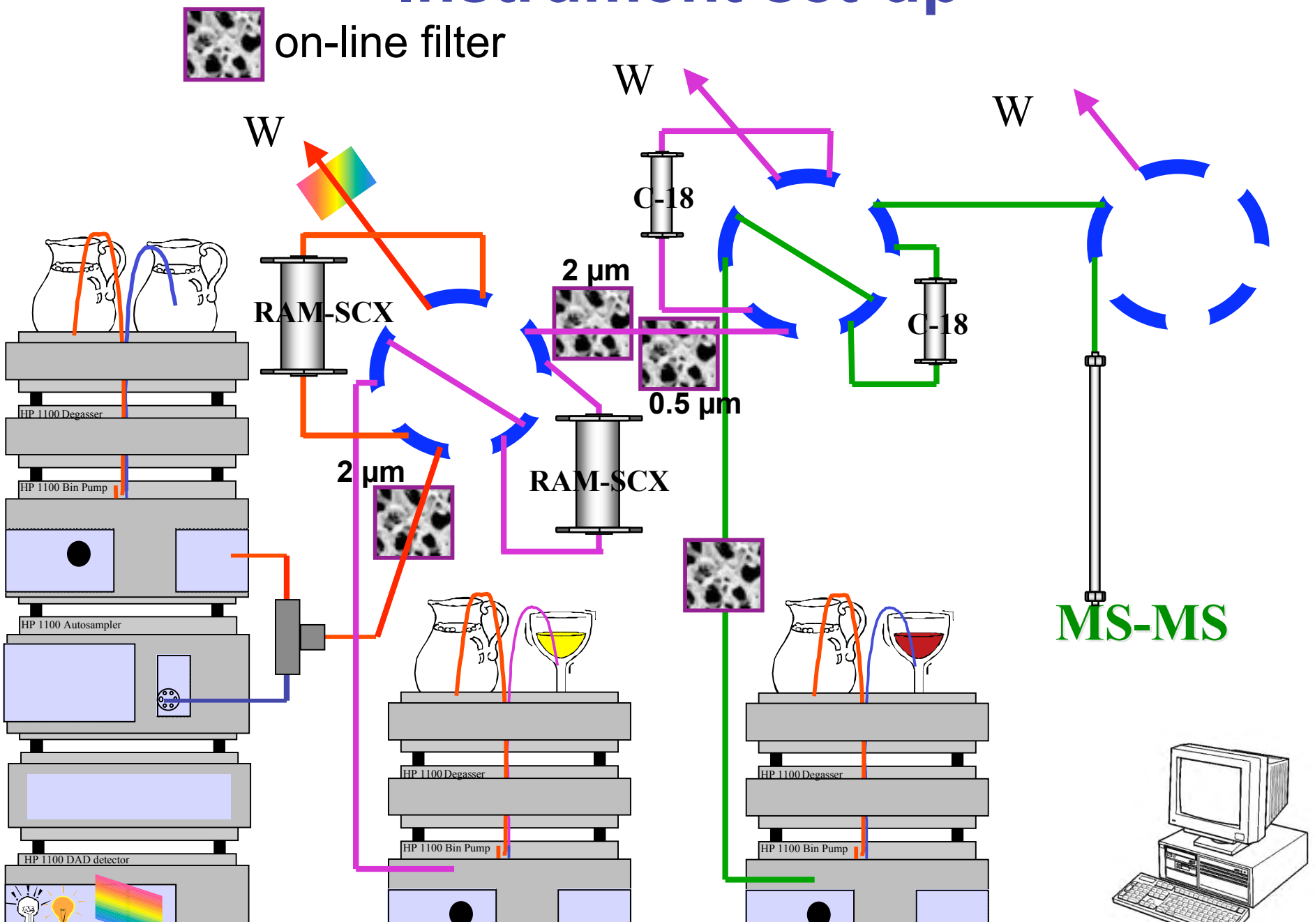
Ion Trap MS

Silica monolith
RP C18 (Merck)
130 x 0.2 mm I.D.
20 µl/min

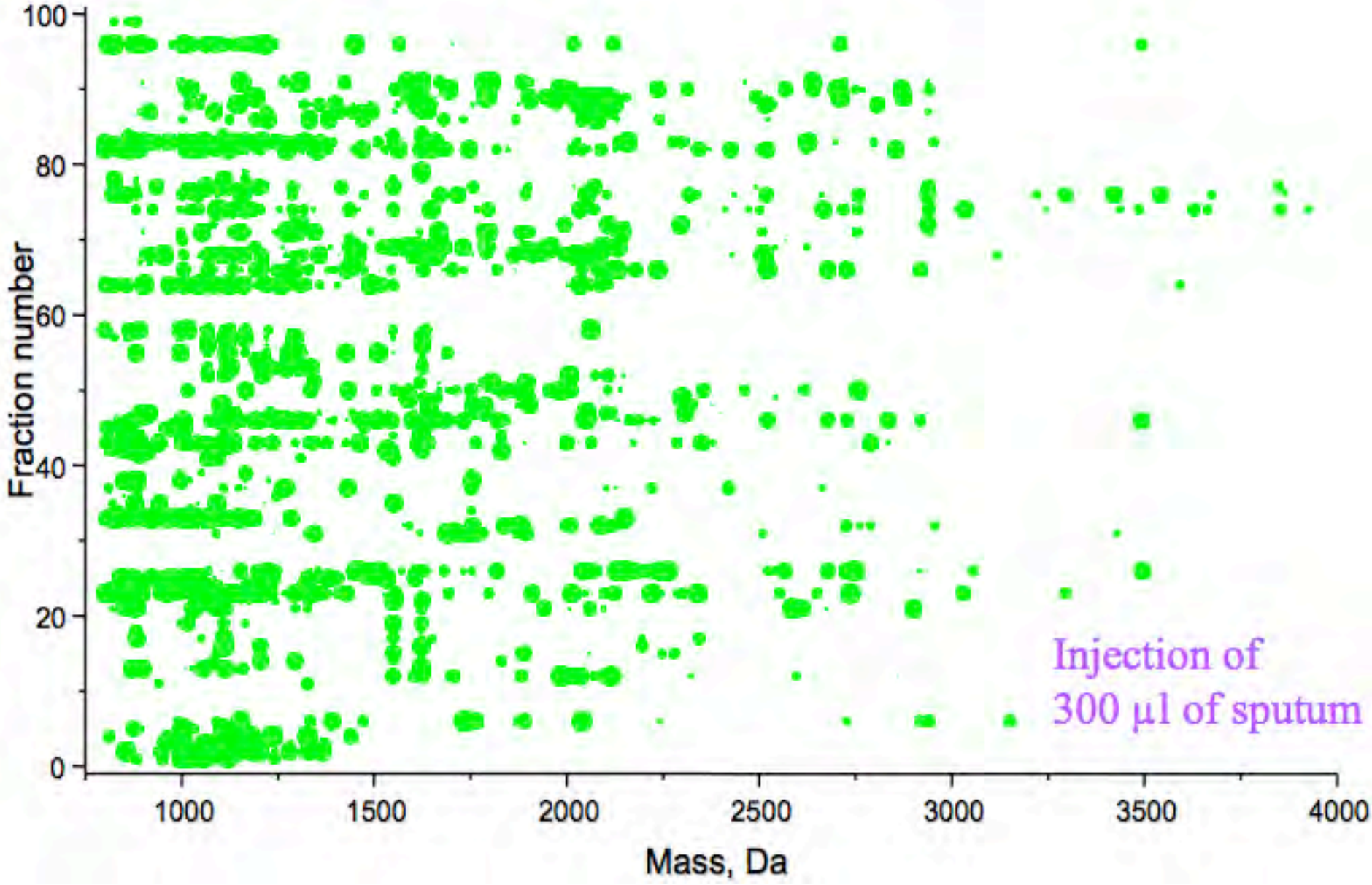
Example for 2D-LC/MALDI interface



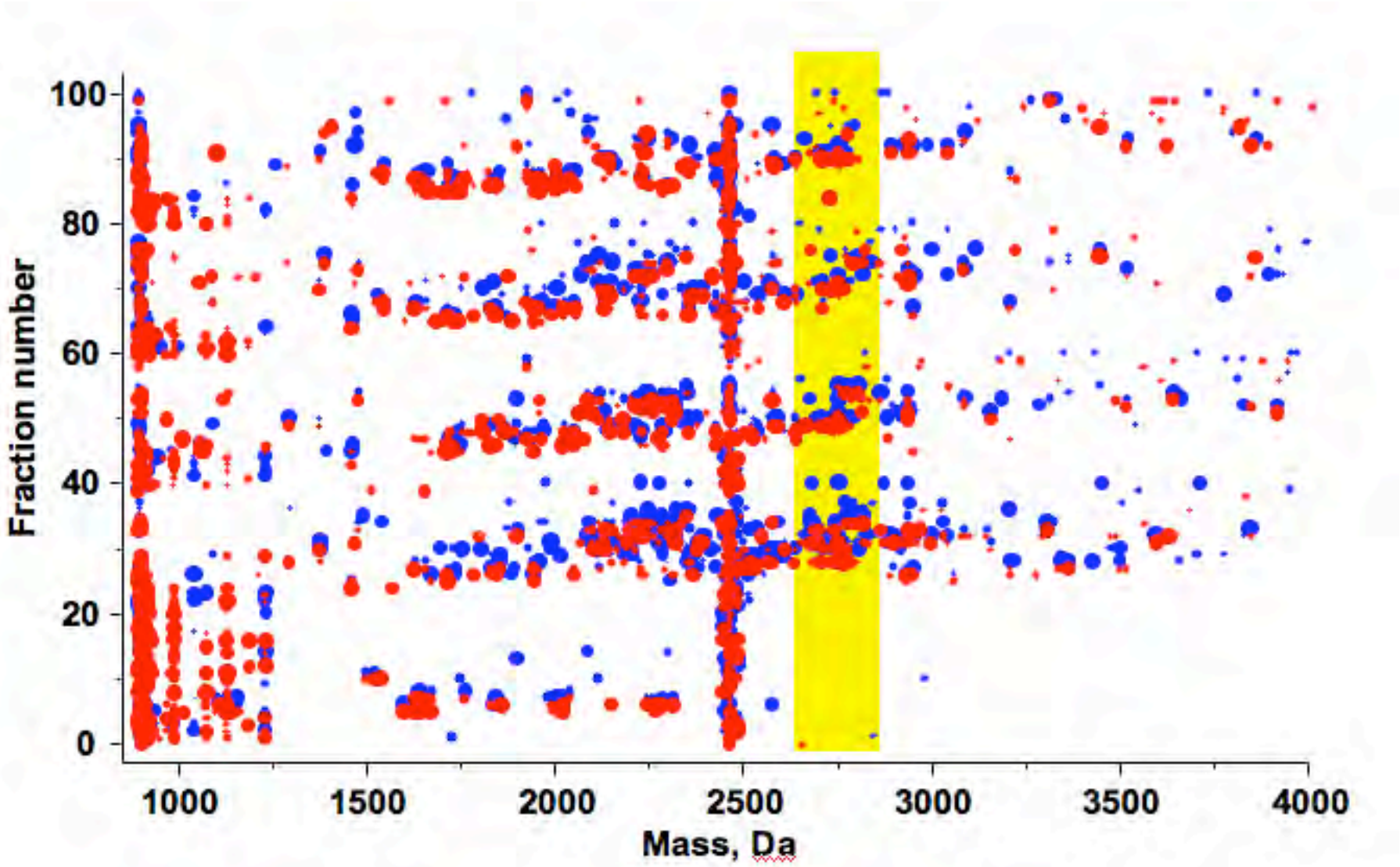
Instrument set-up



Peptide map from Human Sputum



Peptide map (patient plasma)



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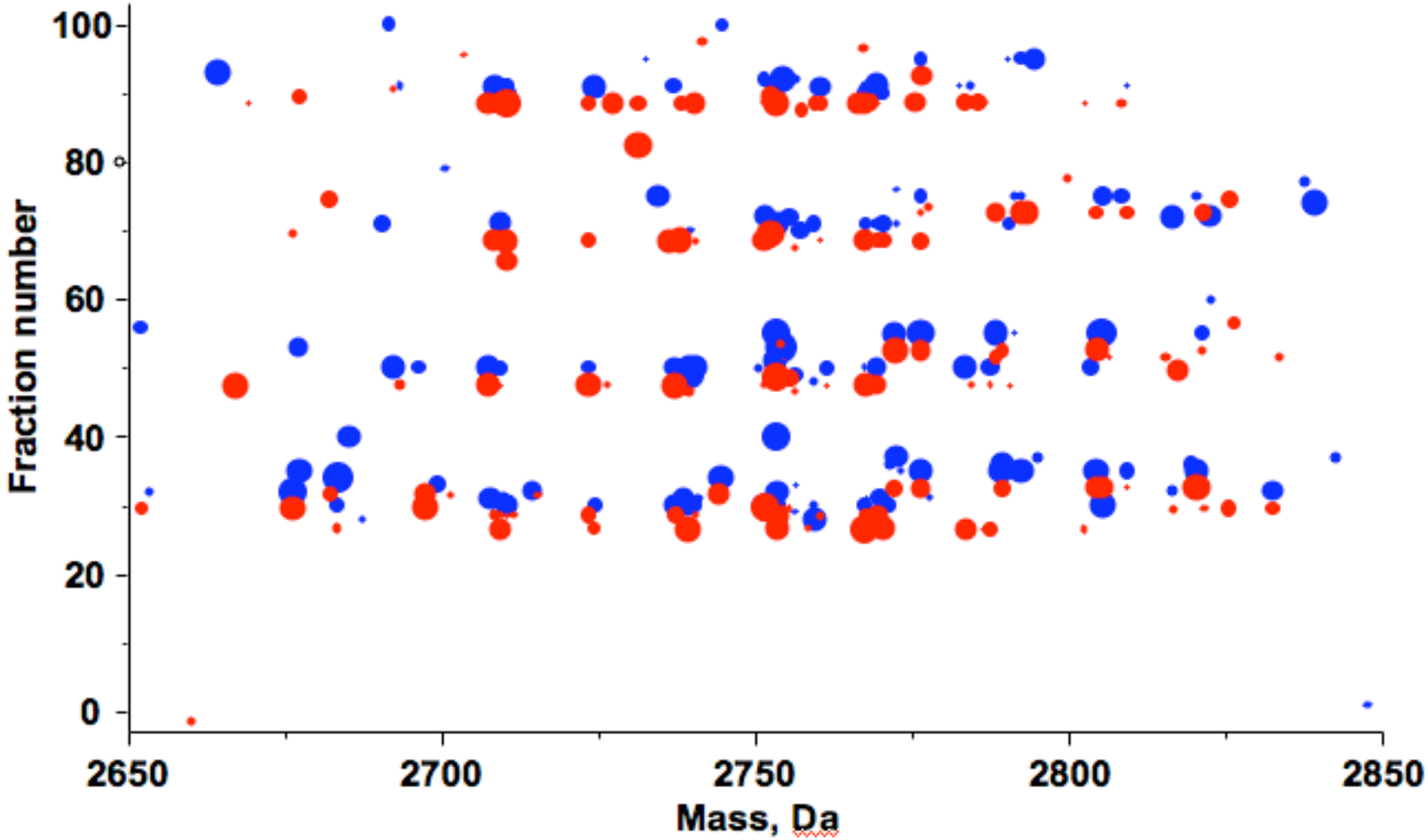

Conclusions

Major achievements:

set-up of fully automated chromatographic systems for biofluid analysis with integrated on-line sample clean-up as 2D-LC system with a RAM-SCX column and the monolithic RP 18 silica column in the second dimension

Added value of monolithic columns are:

- **wide range of flow rates**
- **low back pressures provides the desired flexibility for setting the MD-LC system**
- **Substantial increase of the robustness of the system**

Work to be done:

design and optimisation of monolithic silica columns with adapted pore structure and surface chemistries for protein separations