Uncovering informative content in metabolomics data:

From pre-processing of ^{1}H NMR spectra to biomarkers discovery in multifactorial designs

PhD thesis

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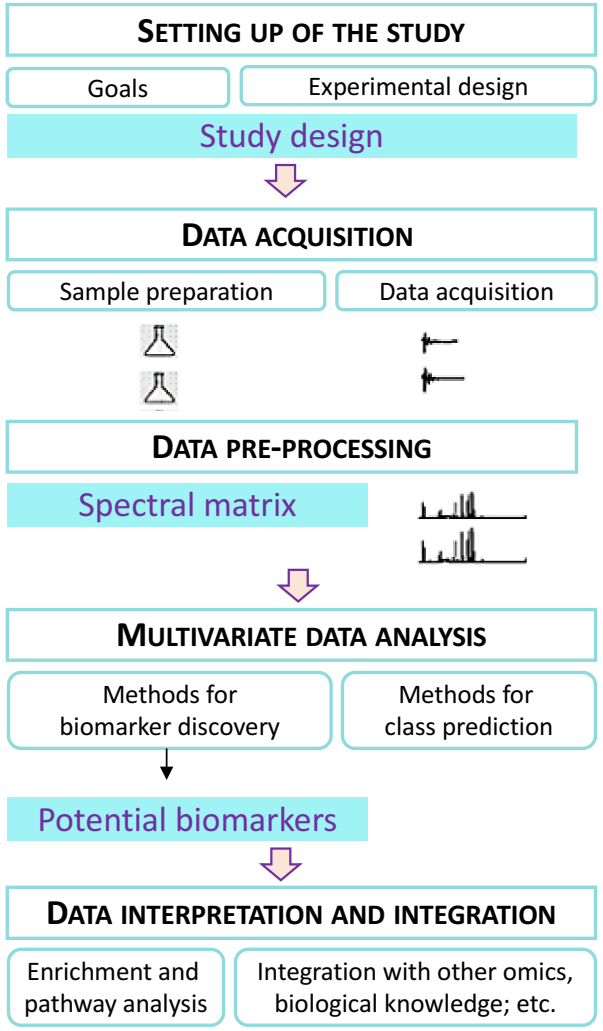
Introduction

[Chapter 1]

Complexity of metabolomics studies

A typical metabolomics study



Challenging data treatment and analysis:

- Expensive data acquisition → reduced Nobs
- Nvar ≫ Nobs: High dimensional but short dataset
- Biological variability and instrumental noise/artifacts
- Need of heavy pre-processing
- Complex correlation structure: 1 metabolite can have ≠ peaks and descriptors, and correlations between metabolites → multicollinearity

Where statisticians do help?

- Everywhere...
- DOE, data pre-processing, biomarker search, predictive modelling, data fusion, etc.

Doctoral project

= 3 different & complementary research axes in metabolomics

Datasets

- Mostly raw and pre-processed metabolomics ^{1}H NMR spectra from blood and urine samples (human, rats)
- Generated from simple or multifactorial experimental designs

End products of this thesis :

Deliver practical solutions to non-statistician researchers

All Chapter's R codes available on GitHub (https://github.com/ManonMartin/thesisMaterial)

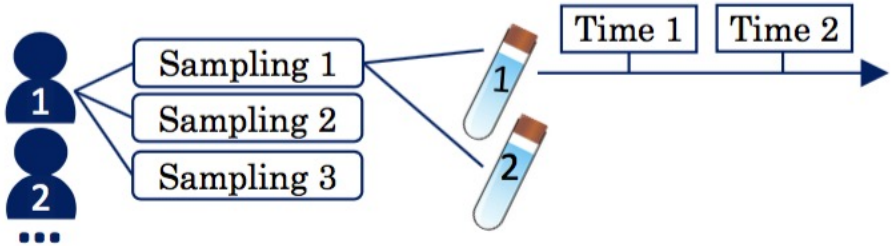
Measure the spectral repeatability

- How to quantify the spectral repeatability/reproducibility and the quality of samples analysed by: ≠ acquisition techniques (1D vs 2D),≠ analytical platforms (MS, NMR), with ≠ pre-processings,≠ sample preparations, etc.?
- Statistical perspective of spectral reproducibility/repeatability and quality control not yet well studied in metabolomics

Applied methodologies:

- Metabolomic Informative Content (MIC) concept from Féraud et al. (2015) with different quantitative quality indices for predictive power and clusters homogeneity applied to different pre-processing strategies [Chapter 2]
- ASCA framework to quantify and compare the sources of variability in the data with fixed and mixed effects models [Chapters 4 and 5]

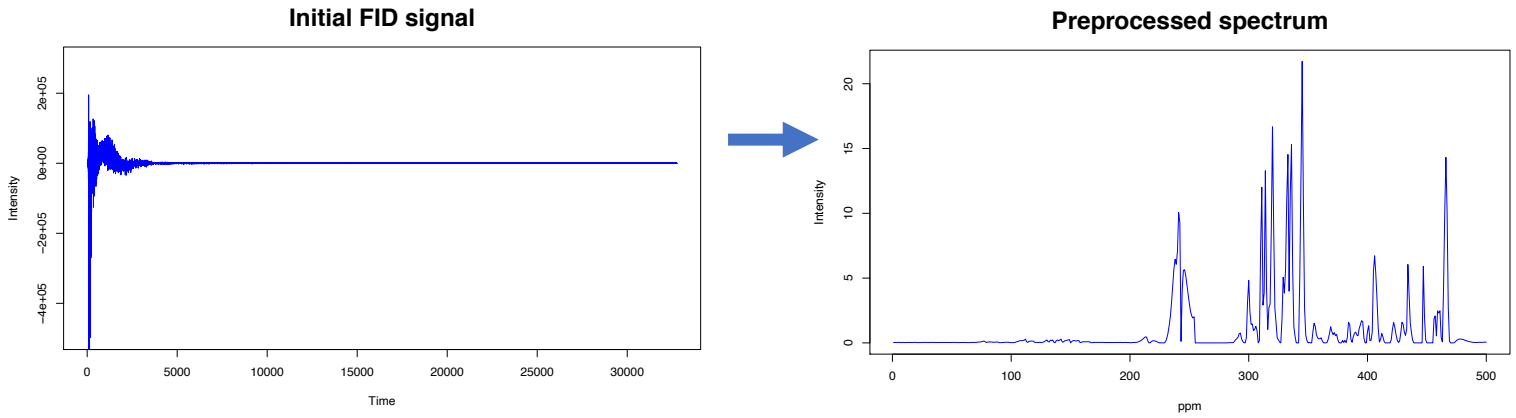
Nested design representation



PepsNMR for ^{1}H NMR data pre-processing

[Chapter 2]

1D ^{1}H NMR data pre-processing



→ These analytical platforms need a dedicated and advanced pre-processing

Current pre-processing (Engel et al., 2013):

- Manual & time-consuming
- Can be incomplete
- Proprietary software and black box
- No clear application strategy: Which method? In which order?

→ Incentive for the package creation

Input: Free Induction Decay (FID) = discretised complex signal in the time domain with > 30.000 points

Output: (n × m) spectral matrix with n observations and m variables (= spectral intensities) in the frequency domain

Pre-processing = mathematical & statistical algorithms designed for:

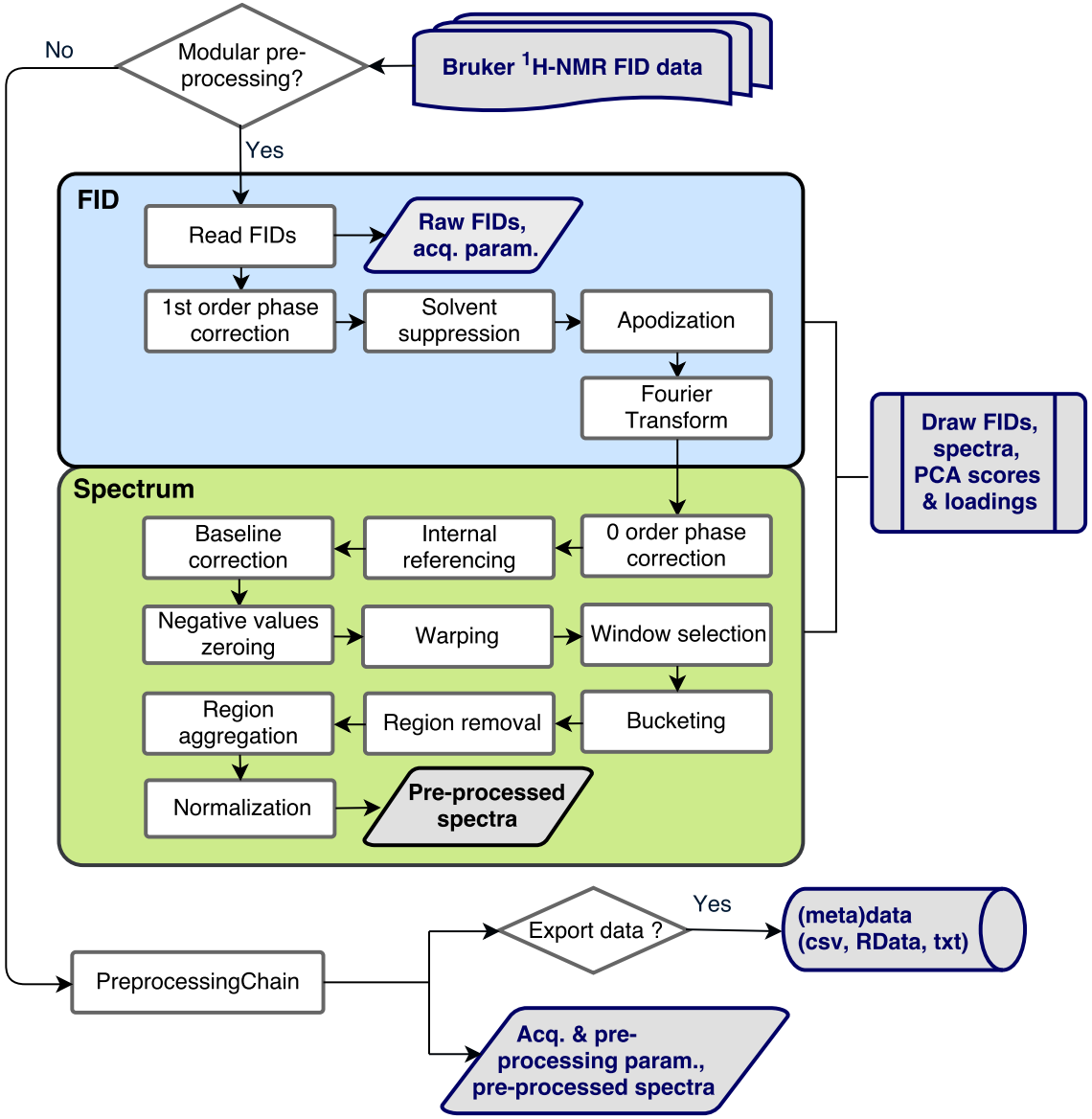
- Instrumental artefacts & biologically-related corrections
- SNR increase
- Data domain or scale transformation
- Data reduction

R package PepsNMR

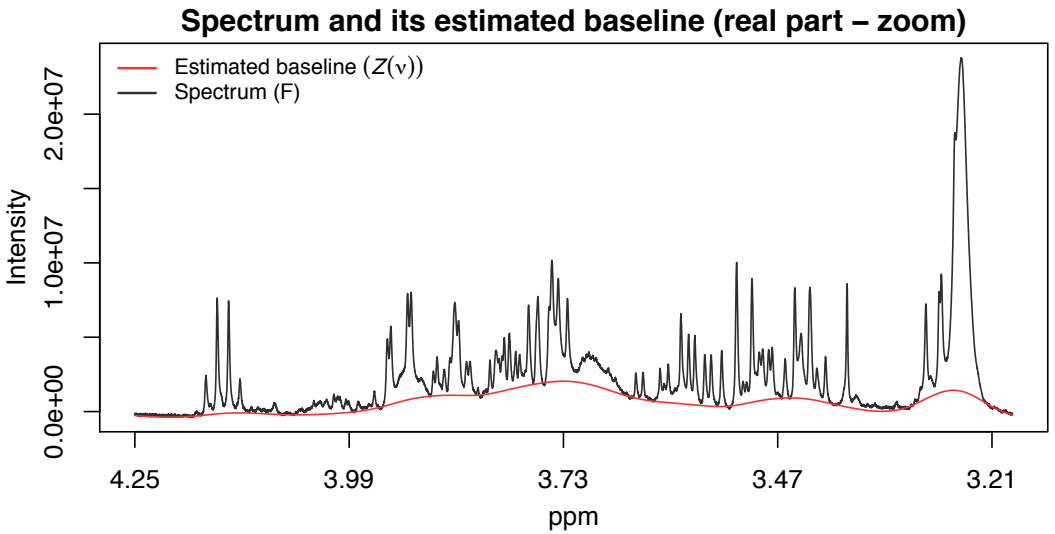
Chapter's objectives

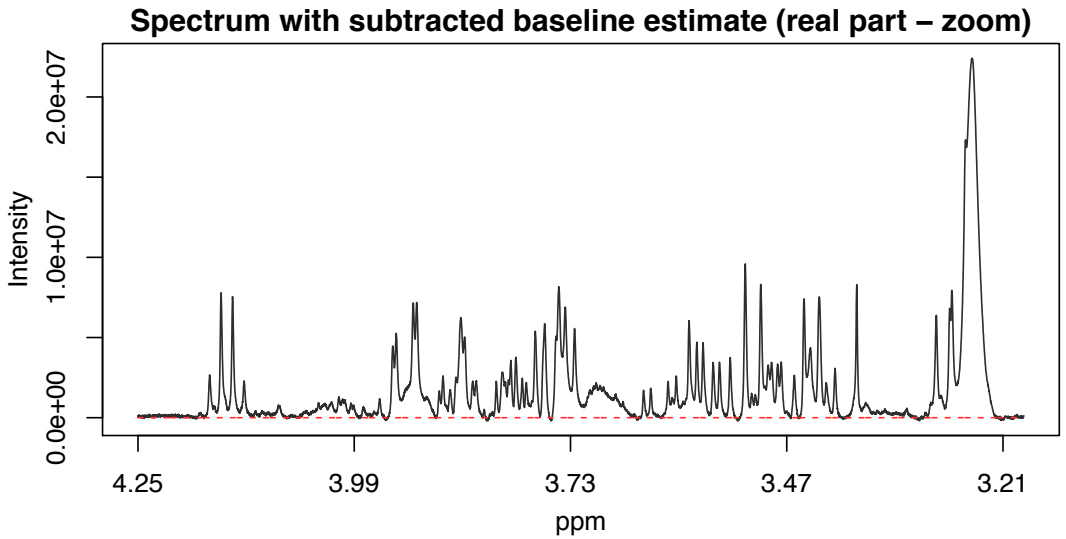
- Publish a complete and flexible software with innovative and well known algorithms for ^{1}H NMR pre-processing
- Package validation and consolidation
- Comparison with standard pre-processing based on quantitative quality criteria

- Former Matlab package (worked with a UI) mainly developed by Eli Lilly, P. H. C. Eilers and J. Vanwinsberghe (Vanwinsberghe, 2005)
- Includes gold-standard but also non- and semi-parametric methods



Baseline Correction





Issue: Presence of baseline artifacts (should be flat) in the spectrum *f*

Example of a non-parametric smoothing method:

- Asymmetric Least Squares smoothing (Eilers and Boelens, 2005) to estimate & remove the baseline *z*

$$\arg\min_{z} \; Q = \sum_{j=1}^{m} \psi_j (f_j - z_j)^2 + \lambda \sum_{j=3}^{m} (\Delta^2 z_j)^2$$

with weight ψ_j for the j^{th} frequency: if $f_j > z_j$, $\psi_j = p$,
else $\psi_j = (1-p)$ with $p \in [0, 1]$ close to 0.

Spectra realignments

Issue: Variation in experimental conditions (*e.g.* pH, temperature or concentration) induce misalignments between identical features from different spectra → intensities cannot be compared

Example of a semi-parametric method: Semi-parametric Time Warping (SPTW)
SPTW is inspired by (Eilers, 2004) and (Van Nederkassel et al., 2006)

Principle: Build a warping function $w(\nu)$ such that the distance between a warped spectrum $F(w(\nu))$ and a reference spectrum G is minimised by Penalized Least Squares:

$$\underset{\beta_k,\alpha_l}{\arg\min}\ Q = \sum_{j=1}^{m}(g_j - f_j(w(\nu)))^2 + \lambda\sum_{l=3}^{L}(\Delta^2\alpha_l)^2 + \kappa\sum_{l=1}^{L}\alpha_l^2$$

$$\text{with } w(\nu) = \sum_{k=0}^{K}\beta_k\nu^k + \sum_{l=1}^{L}\alpha_l B_l(\nu)$$

Soft Bucketing Used for dimension reduction & soft realignment (either trapezoidal or rectangular integration)

Comparison with classic pre-processing

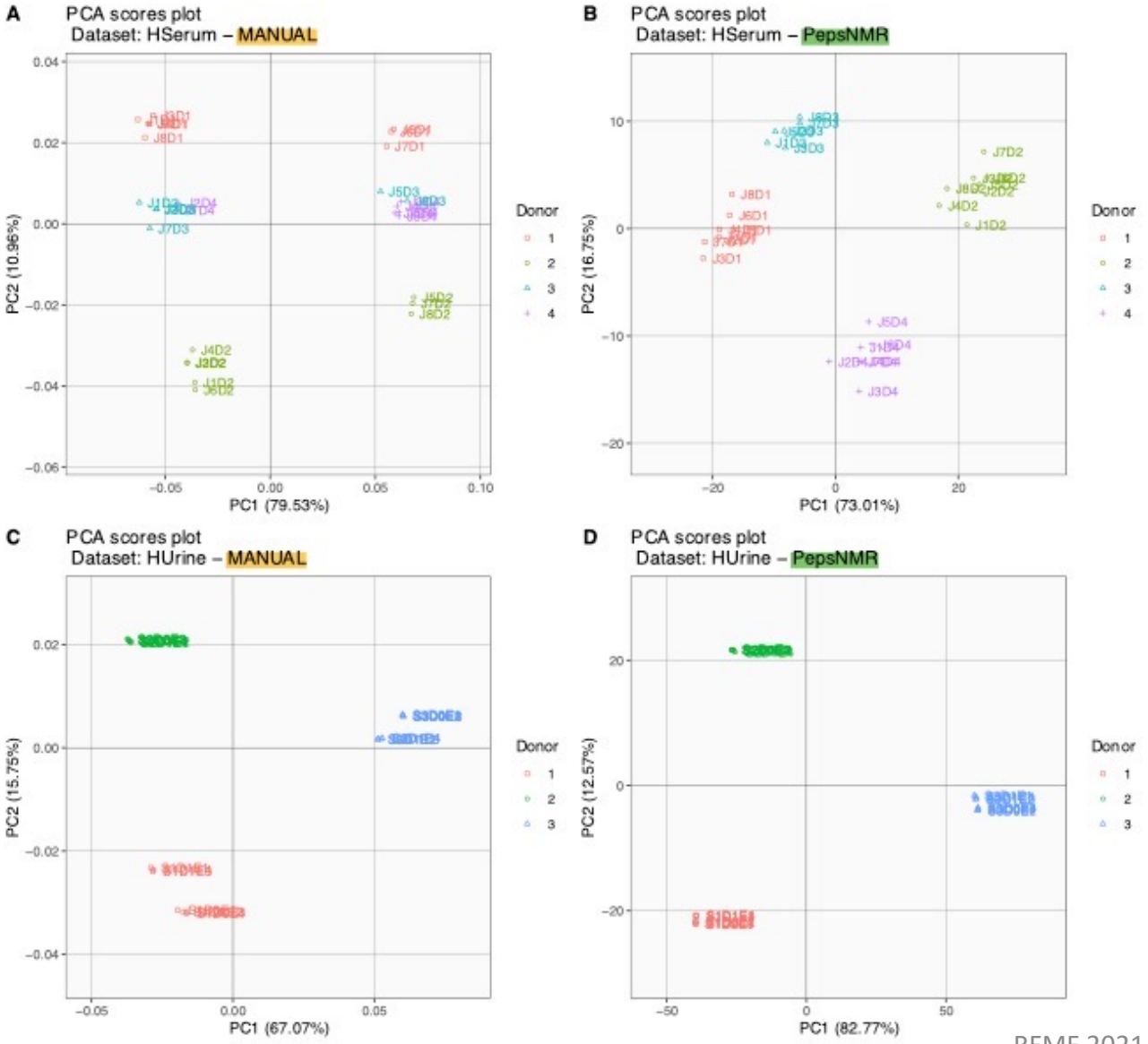
- Compare PepsNMR to “classic” pre-processing (Topspin+AMIX)
- 2 experimental datasets (HUrine & HSerum): ≠ donors (class), noisy factors (dilution, day, etc.)
- **MIC Quality criteria** measured from: inertia, PCA, clustering & PLS-DA indices

Max
Min

Dataset	Pre-processing	Inertia (%)		Ward hierarchical clustering				PLS-DA
		Between	Within	Rand	Adj. Rand	Dunn	D-B	Q2
HSerum	manual	21.44	78.56	0.64	0.14	0.62	0.56	-0.53
HSerum	PepsNMR	88.86	11.14	0.88	0.72	0.87	0.68	0.79
HUrine	manual	82.14	17.86	1	1	0.83	0.69	0.98
HUrine	PepsNMR	95.32	4.68	1	1	1.38	0.32	1

Table: Repeatability is partly assessed by variance decomposition methods: inertia decomposition (% of **between** and **within** inertia), Clustering/classification results for repeatability assessment: indexes derived from unsupervised Hierarchical clustering ((**Adjusted**) **Rand**, **Dunn** and Davies-Bouldin (**D-B**) indexes) and a supervised PLS-DA validation criterion (the cross-validated coefficient of determination **Q2**

Comparison with classic pre-processing



Main results for the comparison study

- Increased spectral repeatability
- Overall better information recovery
- Enhanced predictive power in a classification context

Built-in assets

- Modularity: can be combined/compared to other processing tools & software
- Open-source, semi-automated, structured documentation, reproducible results and reporting

End products

Publication This chapter has been published as a **journal article**:

M. Martin, B. Legat, J. Leenders, J. Vanwinsberghe, R. Rousseau, B. Boulanger, P. H.C. Eilers, P. De Tullio, and B. Govaerts. PepsNMR for 1H NMR metabolomic data pre-processing. *Analytica Chimica Acta*, 1019:1 - 13, 2018

R package The development version of **PepsNMR** is available on **GitHub** (https://github.com/ManonMartin/PepsNMR). The release version is published on **Bioconductor**. The package has *help* pages for functions and datasets, as well as a *vignette*, a long format case study. The *ad hoc* package, **PepsNMRData**, is also available on Github and Bioconductor.

The 9 first pre-processing steps of PepsNMR are implemented in **Workflow4Metabolomics** (Guitton et al., 2017) for NMR pre-processing on the Galaxy web platform.

Biomarker search in simple designs

[Chapter 3]

Feature selection with multivariate projection methods

Discrimination problem: dummy-coded response **y**

Tested methods:

- t-tests
- PLS (Varmuza and Filzmoser, 2016),
- OPLS (Trygg and Wold, 2002): PLS with an OSC filter and 1 predictive latent variable
- SPLS (Chun and Keleş, 2010): Addition of a L1 sparsity constraint (lasso) to the SIMPLS maximisation problem
- LSOPLS (Féraud et al., 2017): Take advantage of OPLS and SPLS for biomarkers discovery: sparsity and orthogonal variation filtering

Chapter's objectives

- Evaluate and compare different common biomarker selection techniques derived from multivariate projection methods in metabolomics based on a dataset with known true biomarkers' location.
- Compare several evaluation criteria

Meta-parameters:

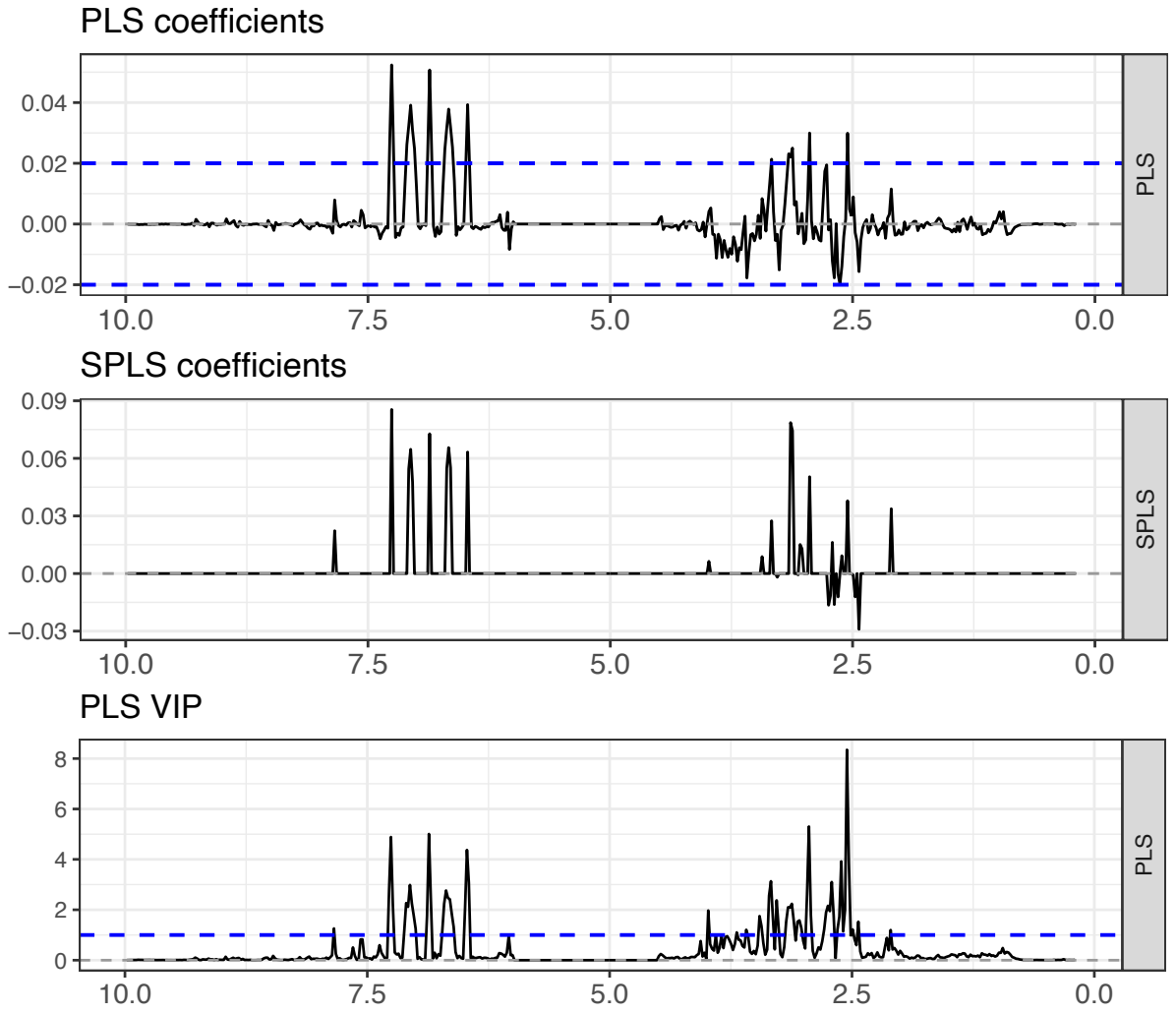
	PrC	OC	η
PLS	?	-	-
OPLS	1	?	-
SPLS	?	-	?
LSOPLS	1	?	?

Incentives for sparse methods

- Consistency of PLS estimators breaks down when m >> n
- Embedded: FS is integrated to the classifier training
- Sparse models are easier to interpret by clinicians

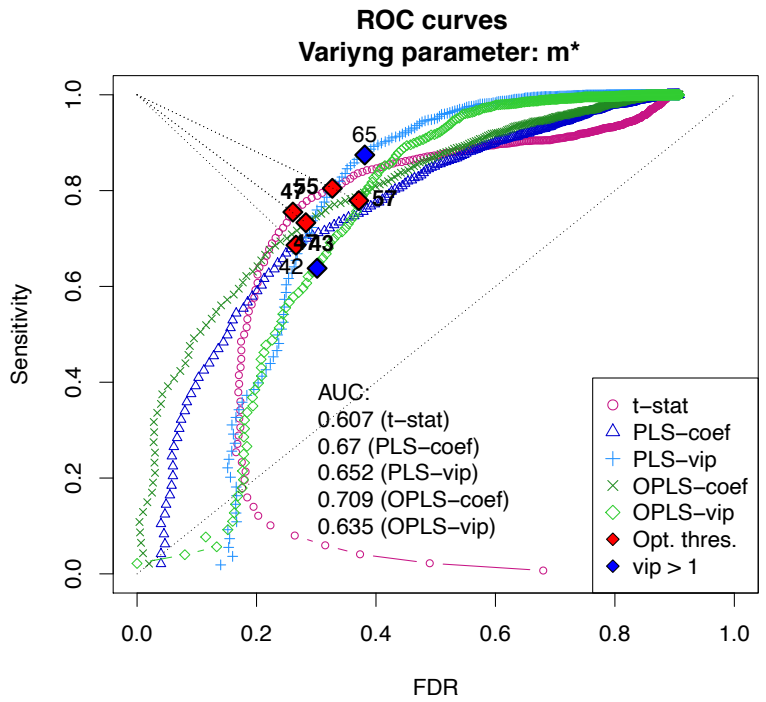
Comparison study

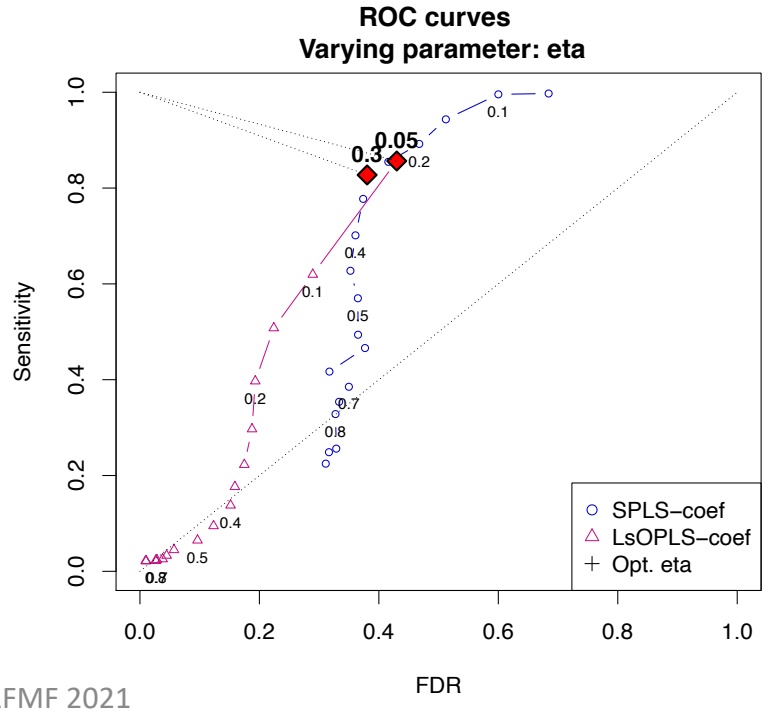
- **Semi-artificial NMR spectra**
  Rat urine spectra, half are artificially altered with artificial biomarkers
  ≠ Sample sizes (n = 60, 200)
  ≠ Signal-to-Noise-Ratio
  ≠ Biomarker intensities
- **Feature** *ranking or selection* based on **r**egression coefficients or VIP values
- **Comparison study:** double CV loop based on the AUC criterion
- **Evaluation criteria** of the final models:
  - ROC curves
  - Discriminant power
  - Stability
  - Graphical outputs



Main results

- Success of biomarker discovery heavily dependent on the multivariate method(s) used to extract the features but also on the data characteristics (different sample sizes, different levels of noise, etc.)
- Importance of the MP choice, especially for sparse embedded methods
- T-test: lots of false discoveries, sensitive to a smaller sample size, recovers correlated true features
- VIP ≥ 1 threshold: adapted for PLS but too high for OPLS
- Orthogonal filter: better for recovery in noisy area but not for lower intensities (and inversely without)
- Sparse classifiers: still a lot of FP, do not detect all correlated features
- PLS-VIP1: best overall performances on this dataset





End products

Publication This chapter has been published as a **Discussion Paper** at ISBA, UCLouvain:

> Martin, M. and Govaerts, B. (2019). Feature selection in metabolomics with PLS-derived methods. Discussion Paper DP 2019/20, Institute of Statistics, Biostatistics and Actuarial Sciences, UCLouvain, Belgium

The implemented (LS)OPLS methods were also employed in Féraud et al. (2017).

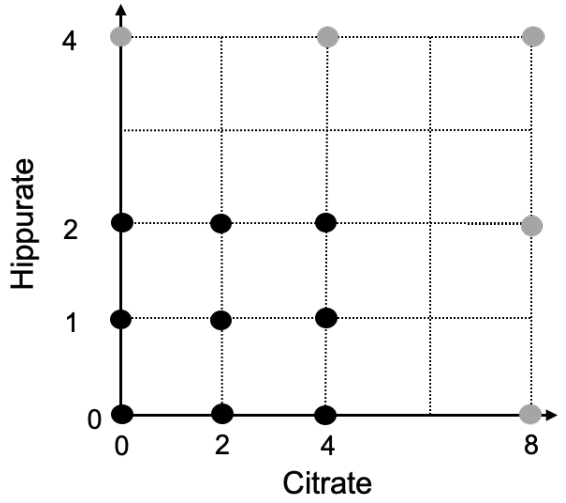
R package The R package **MBXUCL**, on GitHub (https://github.com/ManonMartin/MBXUCL) also contains all the applied methods, in addition to other common multivariate methods (PCA, clustering).

Extending ASCA^{+}

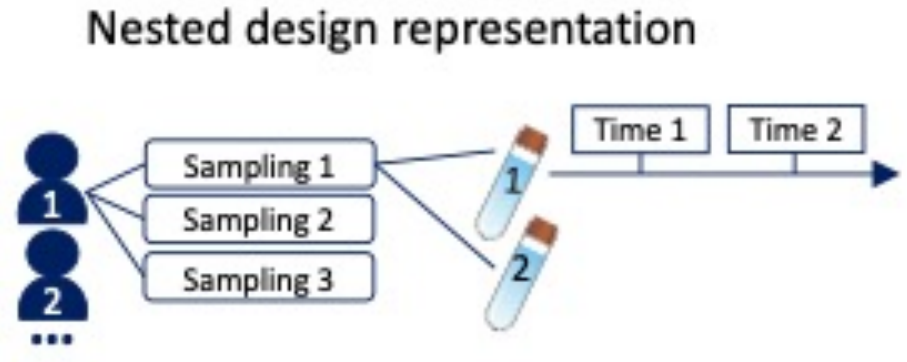
[Chapters 4 and 5]

Examples of multifactorial designs

- **Citrate-Hippurate Urine** dataset [Chapter 4]
  Urine spectra spiked with ≠ amounts of citrate and hippurate



- **Repeatability/reproducibility** dataset [Chapter 5]
  Human serum spectra affected by ≠ factors (nested design):
- biological (volunteer, sampling), analytical (tube, time replication) factors



Data frequency table

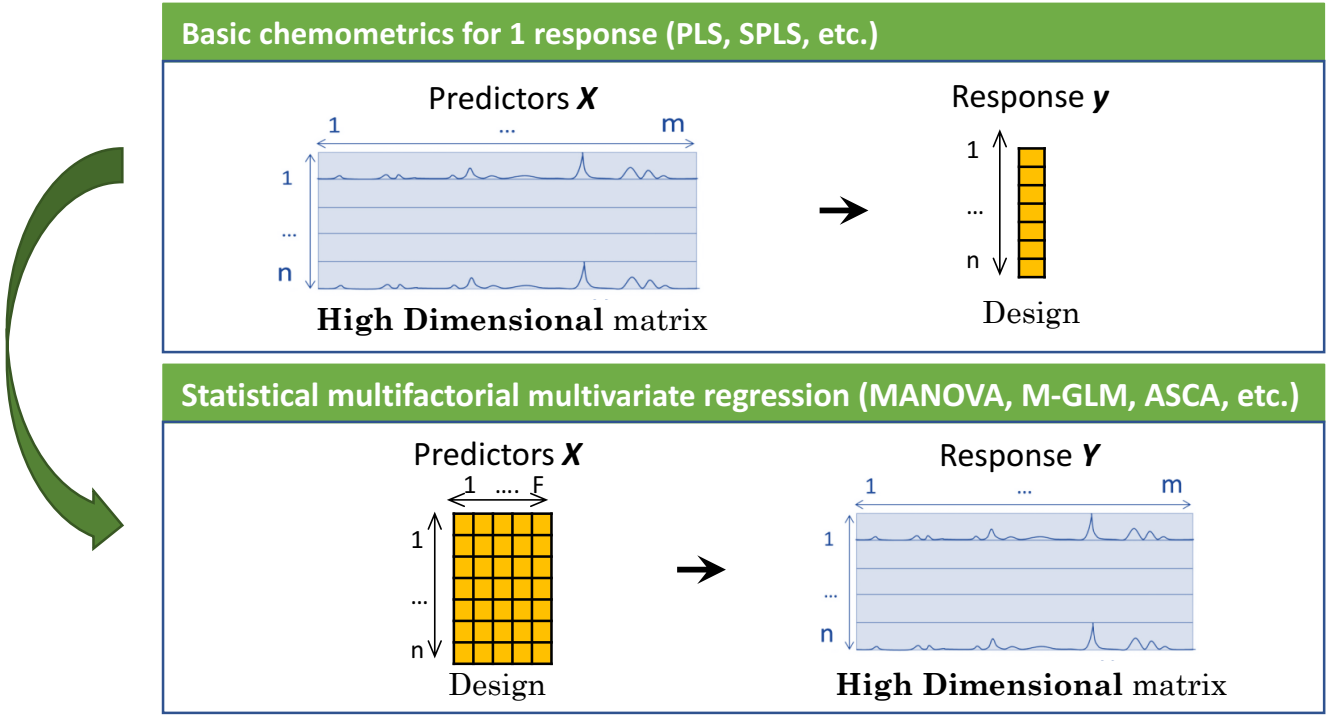
Tube	Time	Sampling		
		1	2	3
1	1	12	12	12
1	2	12	12	12
2	1	11	11	12
2	2	11	11	12

Multivariate projection methods

- **Disadvantages of multivariate projection methods (e.g. PLS):**
  - Rely on simple experimental designs (often only the class of the observation), without considering the full info. from the design
  - Few statistical tests
- **Classic statistical methods:**

Dealing with the dependent variable(s) dimension

- Linear or logistic regression, ANOVA, mixed models: **y** (1×m)
- MANOVA or multivariate-GLM: **Y** (n×m) with n < m
- **But** m ≫ n for metabolomic data !



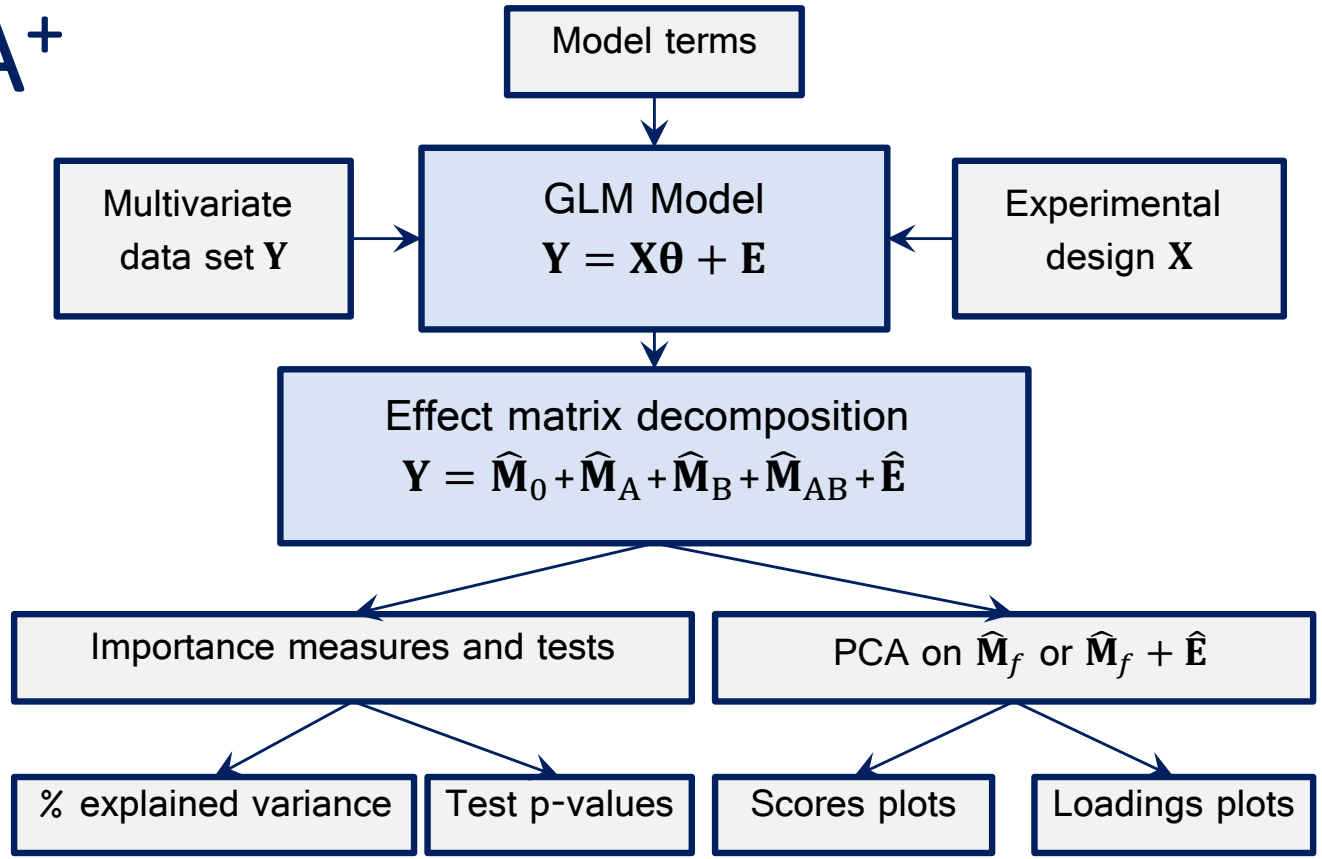
→ **One solution**: combine dimension reduction techniques and statistical modeling

State of the art: ASCA+

2 main steps:

1. **Parallel GLM** on each column of the response matrix **Y** and effect matrix decomposition with LS estimators according to the experimental design.
2. **Effect matrix interpretation**: Importance measure and effects test, PCA on each pure ($\hat{\boldsymbol{M}}_f = \boldsymbol{T}_f \boldsymbol{P}'$) or augmented ($\hat{\boldsymbol{T}}_f^a = (\hat{\boldsymbol{M}}_f + \boldsymbol{E}) \times \hat{\boldsymbol{P}}_f$) effect matrix.

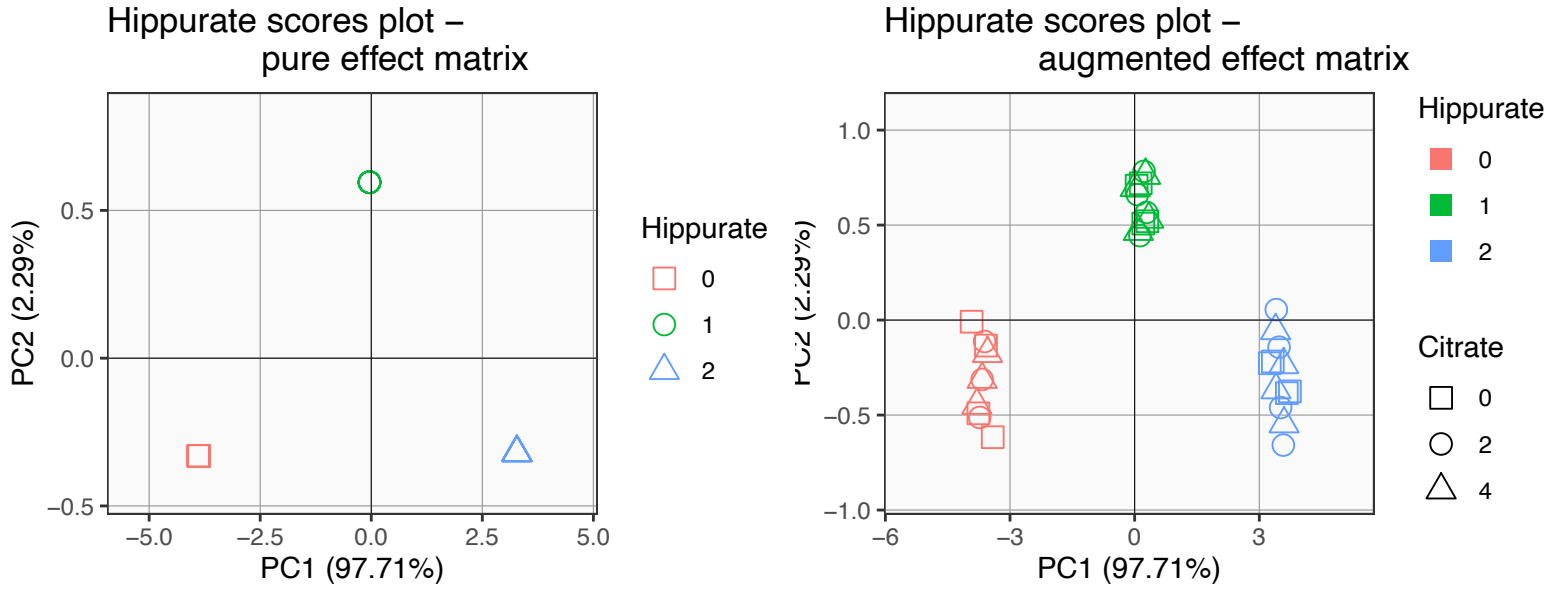
- = General Linear Model (GLM) version of ASCA
- For (un)balanced designs with fixed categorical variables
- Factors *sum* coding in the model matrix



Effect matrix augmentation

Table: Two way fixed ANOVA table with the $E(MS)$ and the F-test values

Effect	SS	df	MS	$E(MS)$	F-test
A	SSA	$a-1$	MSA	$\sigma^2 + \frac{nb\sum \alpha_i^2}{(a-1)}$	$\frac{MSA}{MSE}$
B	SSB	$b-1$	MSB	$\sigma^2 + \frac{na\sum \beta_i^2}{(b-1)}$	$\frac{MSB}{MSE}$
AB	$SSAB$	$(a-1)(b-1)$	$MSAB$	$\sigma^2 + \frac{n\sum\sum (\alpha\beta)_{ij}^2}{(a-1)(b-1)}$	$\frac{MSAB}{MSE}$
$Error$	SSE	$ab(n-1)$	MSE	σ^2	



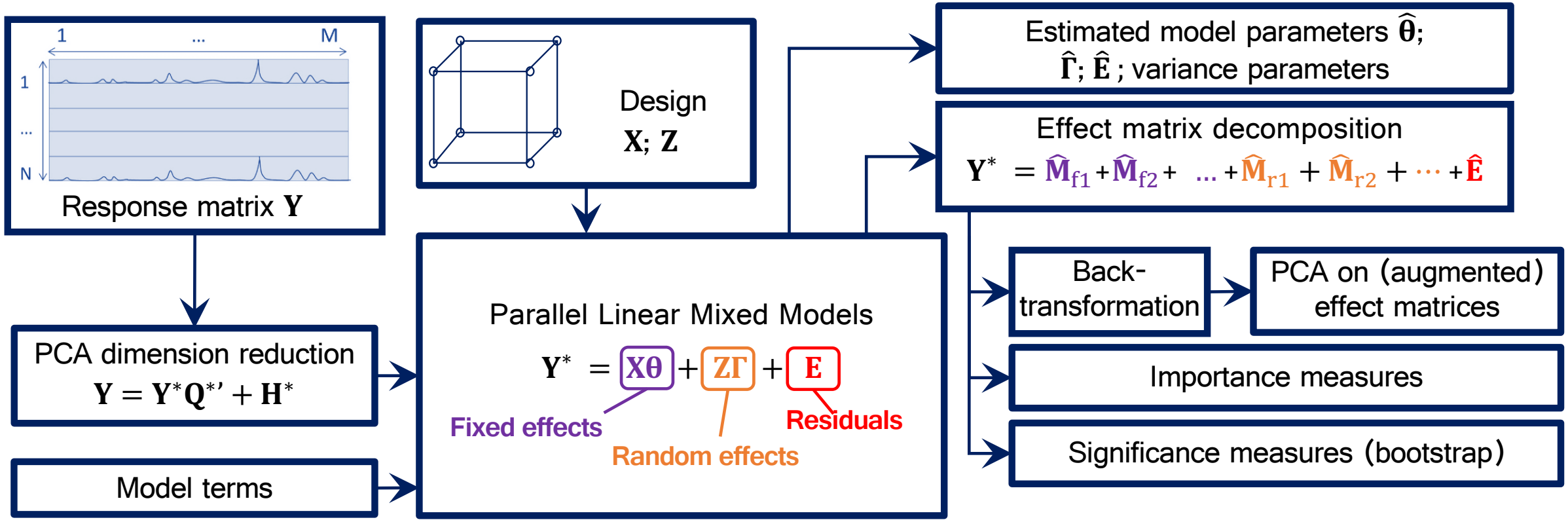
New developments

- **A. Modification of step 2** [Chapter 4]: Extension to other dimension reduction methods
  - **Incentive**: Applying other dimension reduction methods than PCA could provide supplementary information or other visualisation tools
- **B. Modification of step 1** [Chapter 5]: Extension of GLM to Linear Mixed Model (LMM) and bootstrap tests
  - **Incentives**:
    - ASCA+ not adapted to model random effects
    - Permutation tests implementation is challenging for advanced DOE [Anderson and Braak, 2003]

Chapters' objectives

- **Chapter 4**
  - Present and compare within a unified framework with common notations ASCA$^+$ and three global techniques (PARAFASCA, AComDim and AMOPLS) and extend them with the ASCA$^+$ GLM approach
  - advanced graphical results and permutations test to test the effects significance
- **Chapter 5**
  - Suggest a generic framework, LiMM-PCA, to extend the ASCA$^+$ methodology to deal with random effects and allow more flexible modelling to analyse more advanced designs, identify, quantify and compare the mixed (fixed or random) sources of variability
  - Adapt the workflow (effect matrix augmentation, effect importance, bootstrap test)
- Application of the methodologies to experimental datasets

LiMM-PCA methodology description



LiMM-PCA methodology description

- **Effect matrix augmentation**
  - Based on the E(MS) and F-tests in ANOVA tables for mixed effects + correction factor (involving the effective model dimension and the corresponding F-distribution quantile)
- **Quantification of effects importance**
  - Compare the fixed and random sources of variation
  - Nakagawa and Schielzeth (2013): General definition of marginal and conditional R2 in the univariate LMM for independent random effects.
  - Random effects: variance components $\widehat{var(\boldsymbol{y}_j^*|\boldsymbol{X})} = \sum_{r=1}^{R} \hat{\sigma}_{rj}^2 + \hat{\sigma}_{\epsilon j}^2$
  - Fixed effects: population variance of the estimated effect matrices $\hat{\sigma}_{fj}^2 = var(\hat{\boldsymbol{M}}_{fj})$
  - Global variance $\widehat{var(\boldsymbol{Y}^*)} = \sum_{j=1}^{m^*} \widehat{var(\boldsymbol{y}_j^*)} = \sum_{j=1}^{m^*} \left(\sum_{f=1}^{F} \hat{\sigma}_{fl}^2 + \sum_{r=1}^{R} \hat{\sigma}_{rl}^2 + \hat{\sigma}_{\epsilon j}^2 \right)$
- **Parametric bootstrap test**
  - Multivariate generalisation of the LLR test under the hypothesis of near independence between the individual transformed responses
  - Global Log Likelihood Ratio test statistics for (a group of) effect(s):
    $\Lambda_{\tilde{g}}^{obs} = 2[\sum_{j=1}^{m^*}(\log(L_{H_1,j\tilde{g}}) - \log(L_{H_0,j\tilde{g}}))]$
  - Parametric random effects and residuals bootstrap: simulate $\boldsymbol{Y}^*$ under H_0, measure $\Lambda_{\tilde{g}}^{b}$
  - $p_{\tilde{g}}^{\text{boot}} = \frac{\sum_{b=1}^{B} I(\Lambda_{\tilde{g}}^{b} \geq \Lambda_{\tilde{g}}^{\text{obs}}) + 1}{B+1}$

Results on the repeatability/reproducibility dataset

Unbalanced multifactorial design with 4 effects:

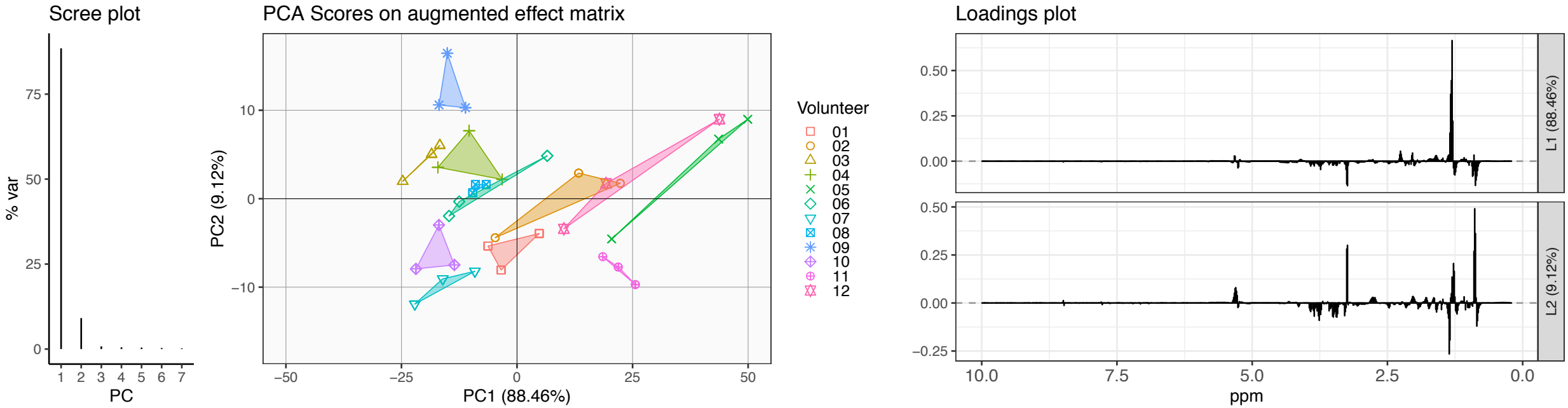
- 2 fixed (Tube, Time)
- 2 random (Volunteer, Sampling)

Main research questions:

- Compare the groups
- Quantify the variability of the repetitions and the patients
- Test the significance of these random/fixed effects

D

Volunteer effect matrix



Results on the repeatability/reproducibility dataset

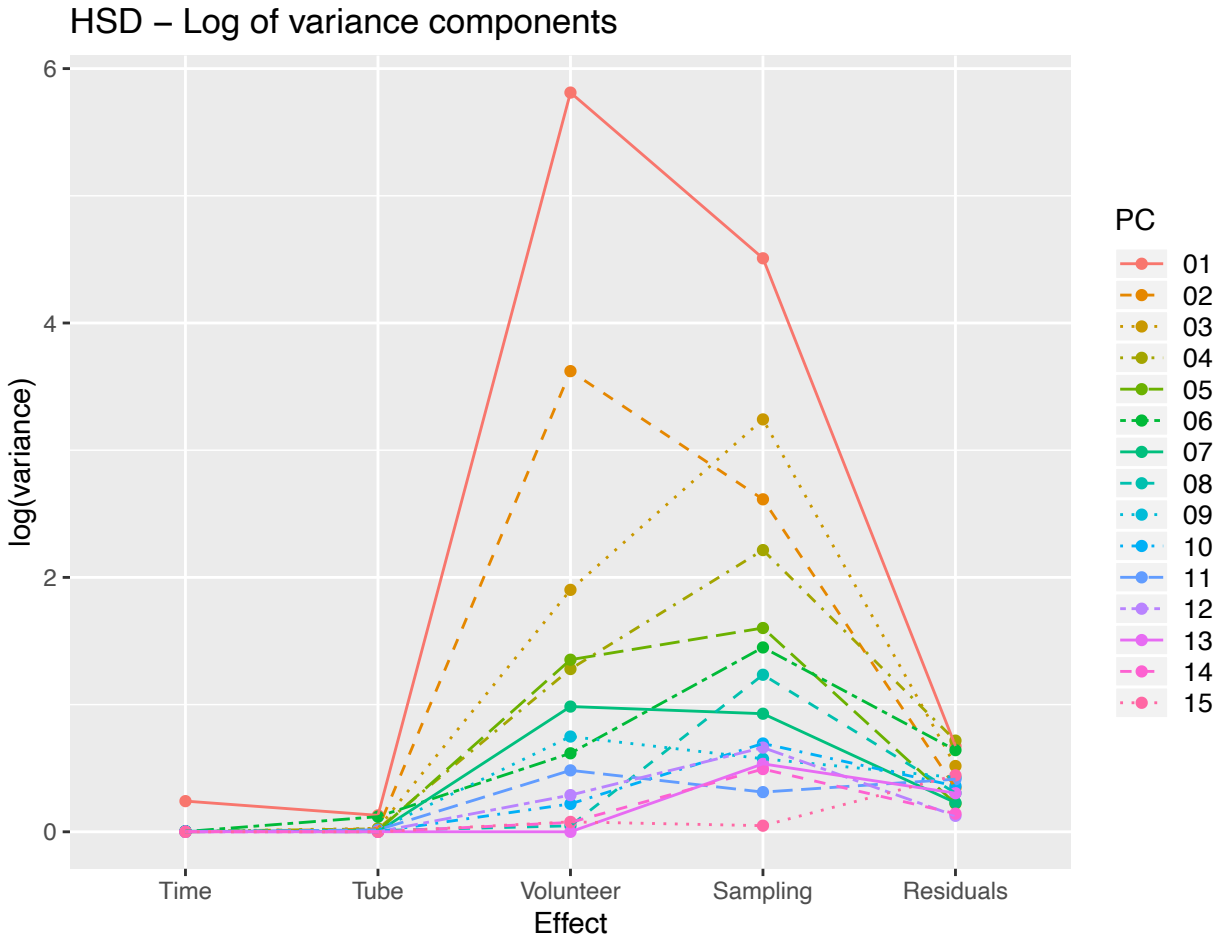
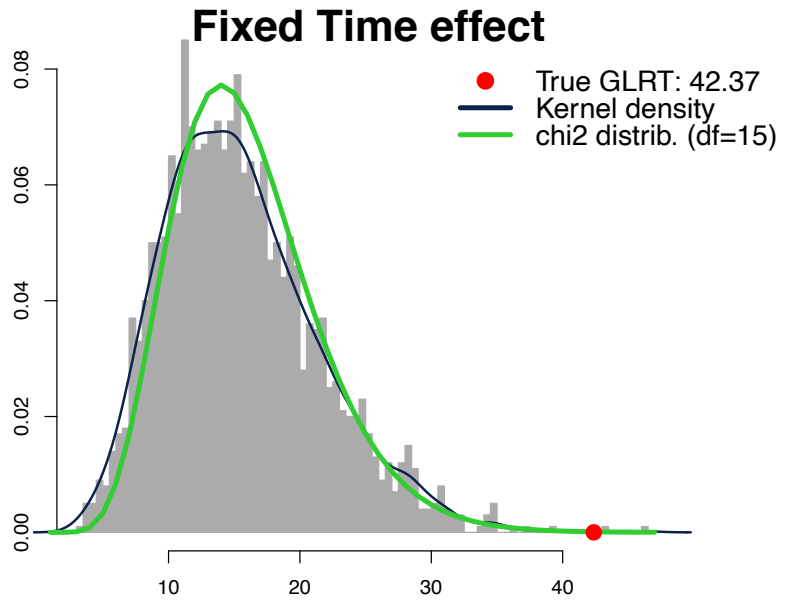


Table: Global effect importance and tests of effect significance.

Effect	**Global variance** (%)	**Bootstrapped p-value**	χ^2 **test**
Time	0.05	0.0015	$<$ 5e-04
Tube	0.07	$<$ 5e-04	$<$ 5e-04
Volunteer	70.80	$<$ 5e-04	-
Sampling	27.69	$<$ 5e-04	-
Residuals	1.38	-	-



Main results

LiMM-PCA

- Innovative extension and generalisation of the ASCA^{+} framework
- Enable to model unbalanced designs with random factors
- Global test of effect significance
- Quantification and comparison of the mixed variability sources
- Targeted applications: repeatability/reproducibility study & longitudinal data

End products

Publication These chapters have been published as **journal articles**:

Chapter 4

Guisset, S., Martin, M., and Govaerts, B. (2019). Comparison of PARAFASCA, AComDim, and AMOPLS approaches in the multivariate GLM modelling of multi-factorial designs. *Chemometrics and Intelligent Laboratory Systems*, 184:44 - 63

Chapter 5

Martin, M., and Govaerts, B. (2020). LiMM-PCA: Combining ASCA$^+$ and linear mixed models to analyse high-dimensional designed data. *Journal of Chemometrics*, *34*(6), e3232.

R packages This work contributed to the creation of the R package **LMWiRe** for ASCA$^+$-related methodology and graphical outputs that is currently under active development on GitHub: https://github.com/bgovaerts/LMWiRe

General conclusions and perspectives

- **Field of metabolomics**
  - Recent research field
    Very informative generated data but heavy data (pre-)processing
  - Technology- and data-driven science → statistical and bioinformatics tools have to adapt
- **Doctoral research** = 4 different contributions to the field of metabolomics
  - Pre-processing: R package for ^{1}H NMR pre-processing published on Bioconductor
    Data analysis (can be generalised to datasets from other research fields)
  - Comparison of classic PLS approaches for biomarker discovery
  - Extension of ASCA^{+} to other global methods and to LMM
- **Perspectives**
  - **PepsNMR R package:** Continuous improvement of the package
    - Fix bugs, update codes, etc. ; Test and include alternative methods Read other raw data formats; Develop a strategy based on DOE methodology to assess the quality of the pre-processing steps for methods and parameters selection
  - **Feature selection**
    - Combine different FS methods
    - Take into account the correlation structure of the features eg: use sparse Group LASSO
    - Bagging principle applied in the features space
  - **ASCA^{+} and LiMM-PCA**
    - Theoretical extensions to include continuous coefficients in the model
    - Deal with data from longitudinal studies

Thank you for your attention!

- Thanks to the RFMF thesis prize and conference organising committees and to all the collaborators of my PhD thesis.
- Link to the slides of this presentation and the thesis codes: https://github.com/ManonMartin/thesisMaterial
- Any questions?

Manon Martin

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