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# Development of a high-throughput and high-metabolome coverage UHPLC-HRMS method for the accurate profiling of large clinical cohorts

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## Context

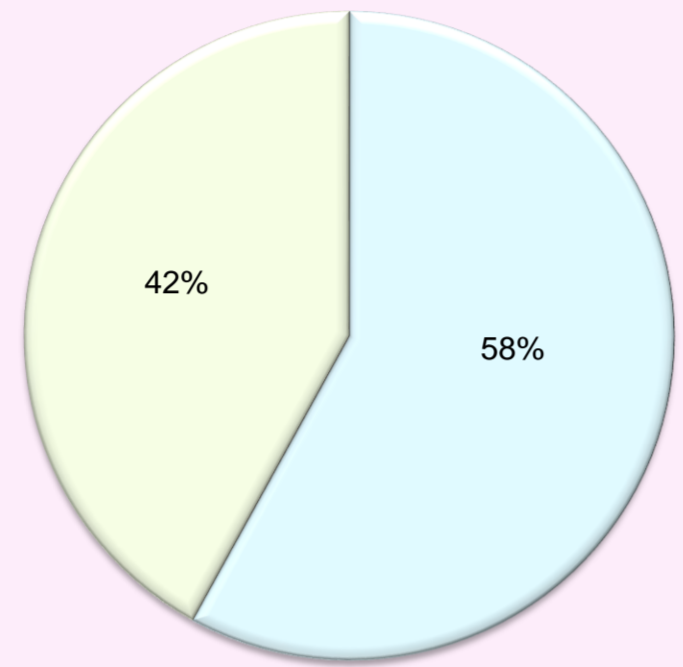
Untargeted metabolomics offers biomedical researchers a powerful tool for assessing human phenotypes through the comparative measurement of metabolome in various biological samples. However, current non-targeted metabolomics methods based on UHPLC-HRMS are often time-consuming and may lack sufficient robustness for successful large-scale cohort analysis (>1000 samples). To address this challenge and analyze a cohort of 6000 human plasma samples (PEPR ProPsy project), we are developing an untargeted metabolomics method with significantly reduced chromatographic separation time.

## Redissolution tests

Test	Phase A	Phase B
1	20	80
2	30	70
3	40	60
4	45	55
5	50	50
6	55	45
7	60	40
8	65	35
9	70	30

Results to be confirmed

Peak broadening

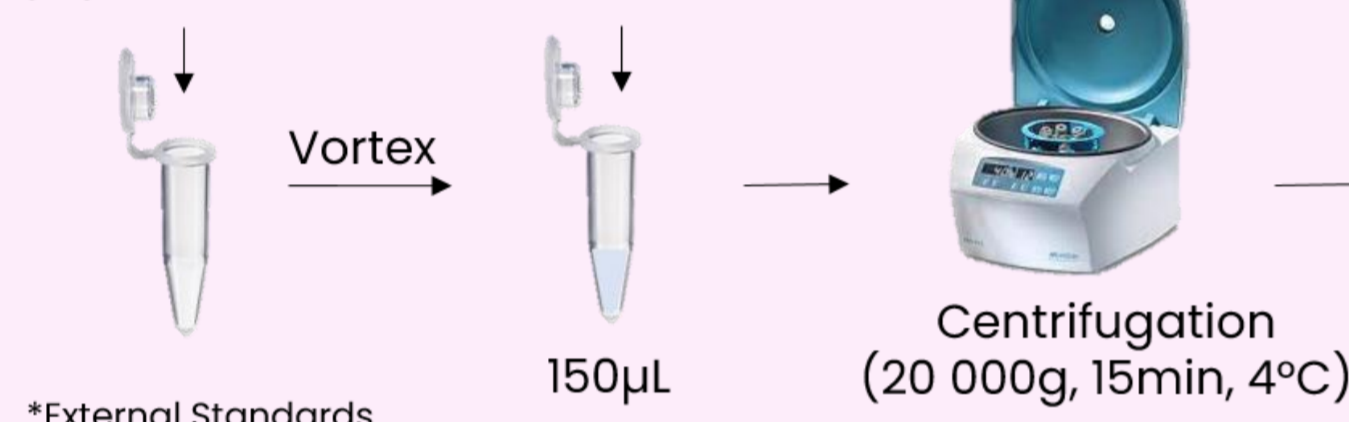


Phase A : H<sub>2</sub>O + 20mM AmFo + 0.1%FA  
Phase B : ACN + 0.1%FA

unaffected affected

The optimization of dry extract redissolution prior to analysis is an important step. Nearly half of all analytes are affected by the ratio between aqueous and organic phases. We observed a broadening of peaks above 45% aqueous phase.

+Xµl phase A + ES\* +Xµl phase B



A two-step redissolution is necessary to avoid precipitation of polar molecules

## Initial Method

42 min 30 min



HILIC-HRMS/MS (neg)  
Polar metabolites  
Merck - SeQuant ZIC-pHILIC  
150 x 2.1mm - 5µm

C18-HRMS/MS (pos)  
Hydrophobic metabolites  
Thermo - Hypersil Gold  
150 x 2.1mm - 2.6µm

One sample → 72 min

Not suitable for large cohorts

## Column selection

Kevin Contrepoix et al., 2015

BEH Amide  
50 x 2.1mm, 1.7µm  
ZIC HILIC  
100 x 2.1mm, 3.5µm  
pH 6.8

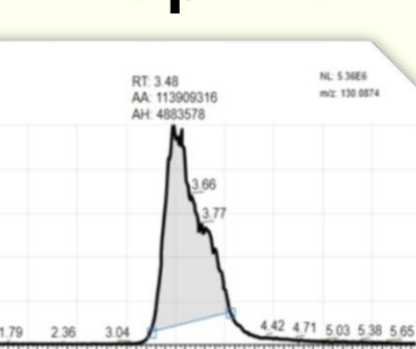
West Coast Metabolomics Center,  
Oliver Fiehn et al., 2022

BEH Amide  
150 x 2.1mm, 1.7µm  
pH 3.3

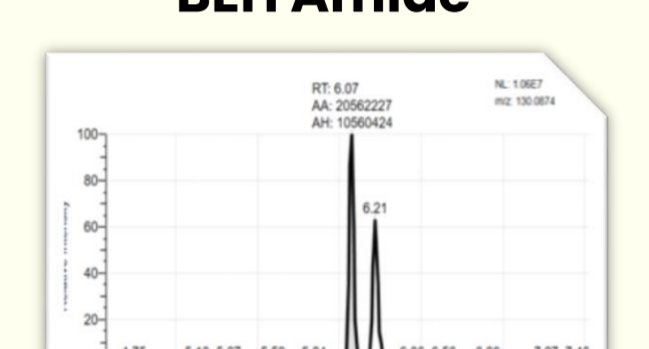
Toxalim, INRAE,  
Carla Orlandi et al., 2023

BEH Amide  
100 x 1mm, 1.7µm  
pH 10.5

According to the literature, the two most widely used columns in current untargeted metabolomics methods are the BEH amide column and the ZIC-HILIC columns.

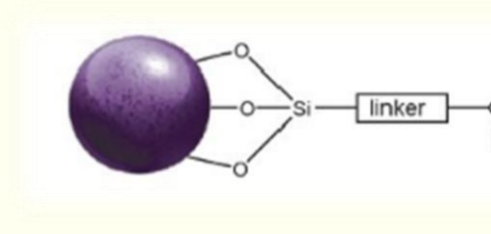


Leucine/Isoleucine



Leucine/Isoleucine

our choice



ACQUITY UPLC BEH Amide Column  
100 x 2.1mm, 1.7µm, 130Å

## Final Method



A fully automated platform is used for high-throughput sample preparation

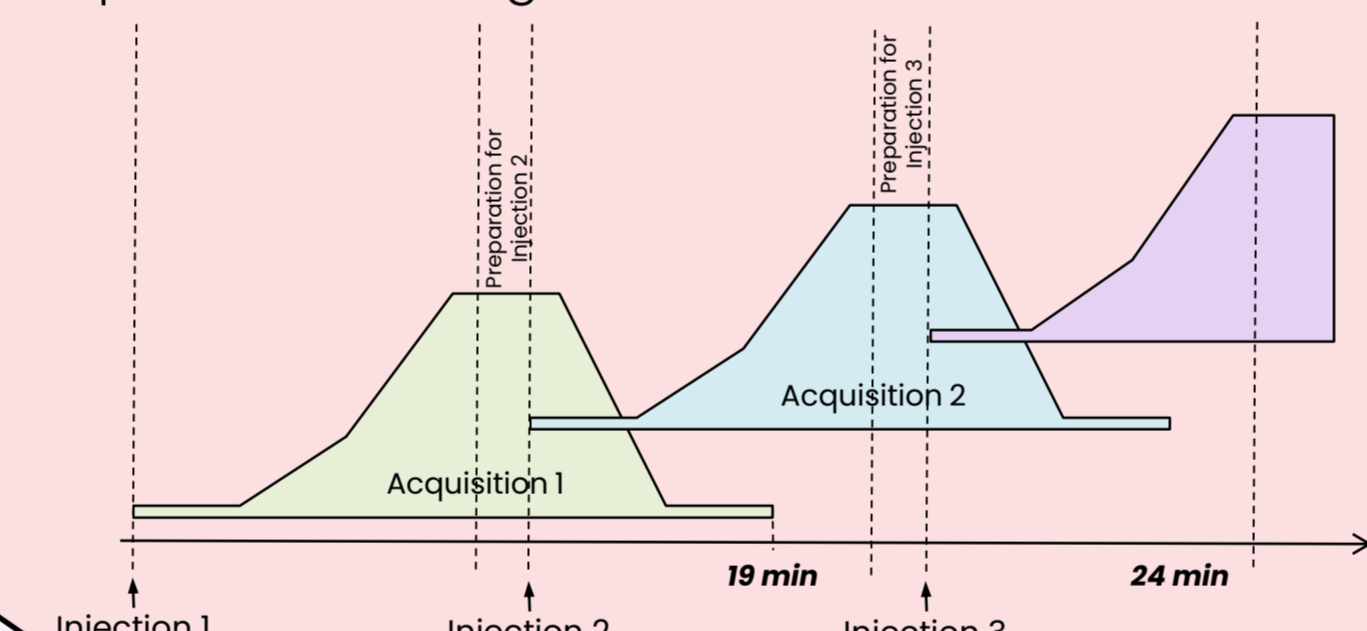
Flow rate: 0.4ml/min  
Injection volume: 5µl  
Temperature: 45°C

ACQUITY UPLC BEH Amide  
100 x 2.1mm, 1.7µm, 130Å



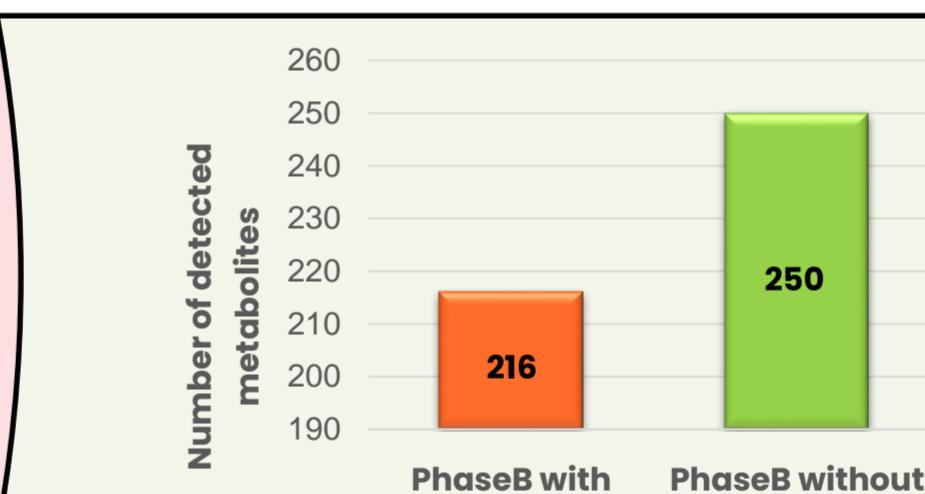
UHPLC Thermo Scientific Vanquish Duo Ref. VQDUO-DUALCMS  
MS Thermo Scientific Orbitrap Exploris™ 240 Ref. BRE72535

A dual HPLC system for parallel sample analysis, enabling us to reduce the analysis time for a sample in positive and negative ionization mode to 24 min.



One sample → 24 min

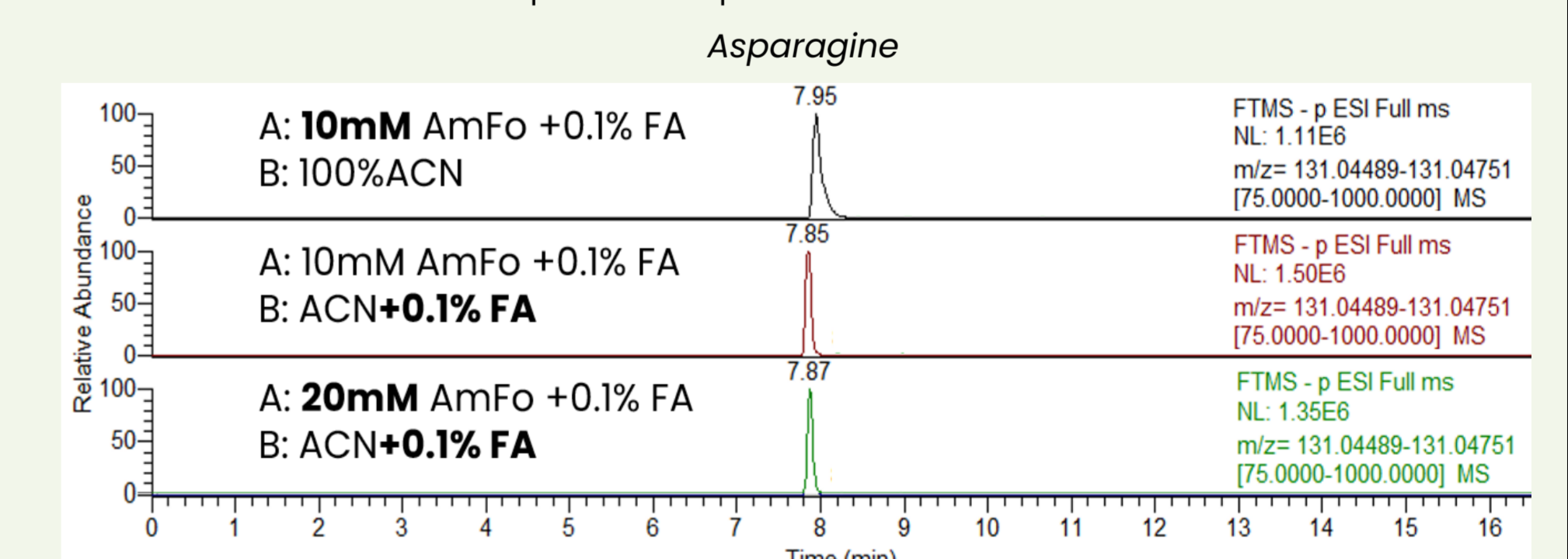
Time (min)	%A	%B
0.00	5	95
2.00	5	95
7.70	35	65
9.50	97	3
13.75	97	3
14.50	5	95
19.00	5	95



The composition of mobile phases has a significant impact on the number of detected metabolites.

Mobile phase	pH	Stability of Retention Time (RT)	Coverage of the metabolites	Background noise	Quality of peaks
A: H <sub>2</sub> O, 10mM AmFo, 0.125% AF B: 100% ACN	10.5	🟢	🟡	🔴	🟡
A: H <sub>2</sub> O, 10mM AmCa B: 100% ACN	10.5	🟢	🟡	🔴	🟡
A: H <sub>2</sub> O, 10mM AmFo, 0.125% AF B: 100% ACN	3.3	🟢	🟡	🔴	🟡
A: H <sub>2</sub> O, 10mM AmFo, 0.125% AF B: ACN, 0.1% AF	3.3	🟢	🟢	🔴	🟡
A: H <sub>2</sub> O, 20mM AmFo, 0.1% AF B: ACN, 0.1% AF	3.3	🟢	🟢	🔴	🟡

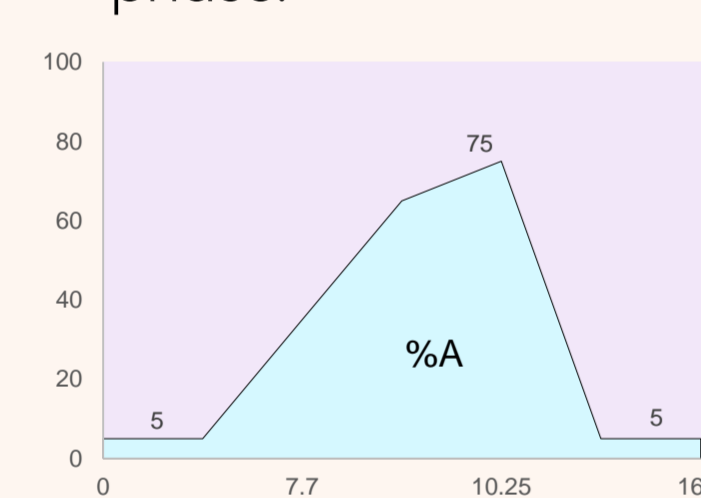
A high concentration of buffer in the mobile phase A (20mM of ammonium formate) with an acidic pH (3.3) has a significant impact on several factors such as peak shape and resolution.



The concentration of ammonium formate in phase A and the percentage of formic acid in phase B have the potential to influence both shape and intensity of the peaks.

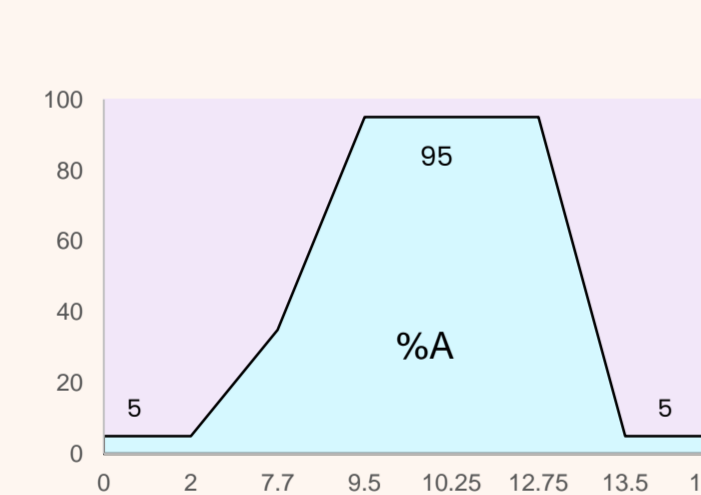
## Mobile phase optimisation

1 According to the bibliography, the initial gradient tested is that of "West Coast Metabolomics Center, Oliver Fiehn et al., 2022". During the elution phase, the phase A percentage increases successfully to 30, 60, and 70%. This step is immediately followed by a 4-minute re-equilibration phase.



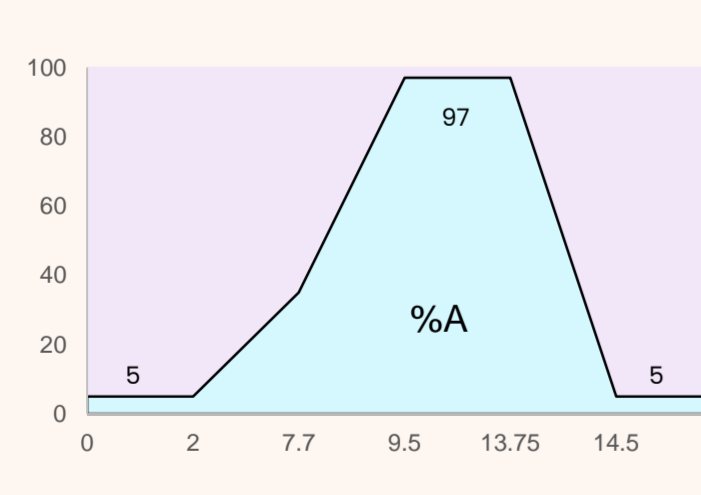
Not all metabolites are eluted

2 The slope of the gradient is almost the same, however the percentage of mobile phase A was increased to 95% instead of 70% from 9.5 to 12.75 minutes.



Improved retention time stability and increased number of compounds eluted

3 The phase A elution plateau is extended by one minute and increased to 97%.



Maximum number of metabolites eluted

One injection → 19 min  
One sample (pos+neg) → 38 min

## Gradient optimisation

## Robustness

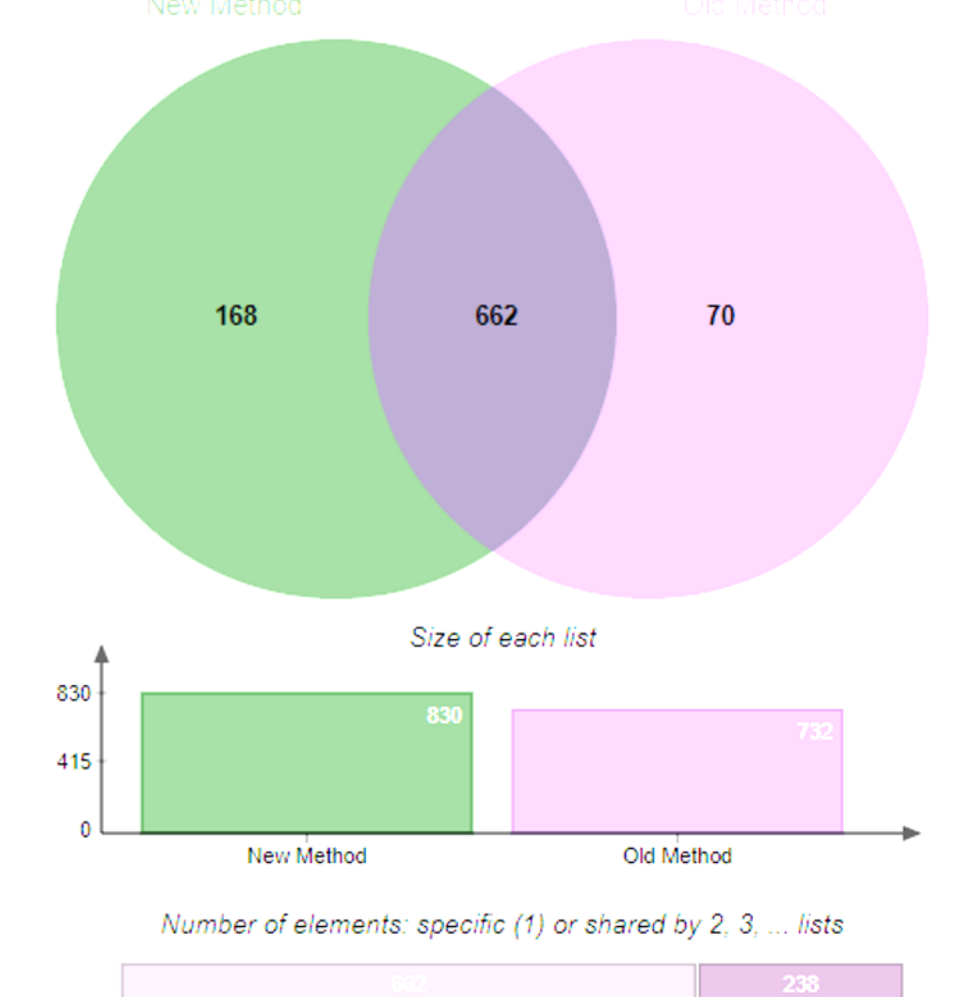
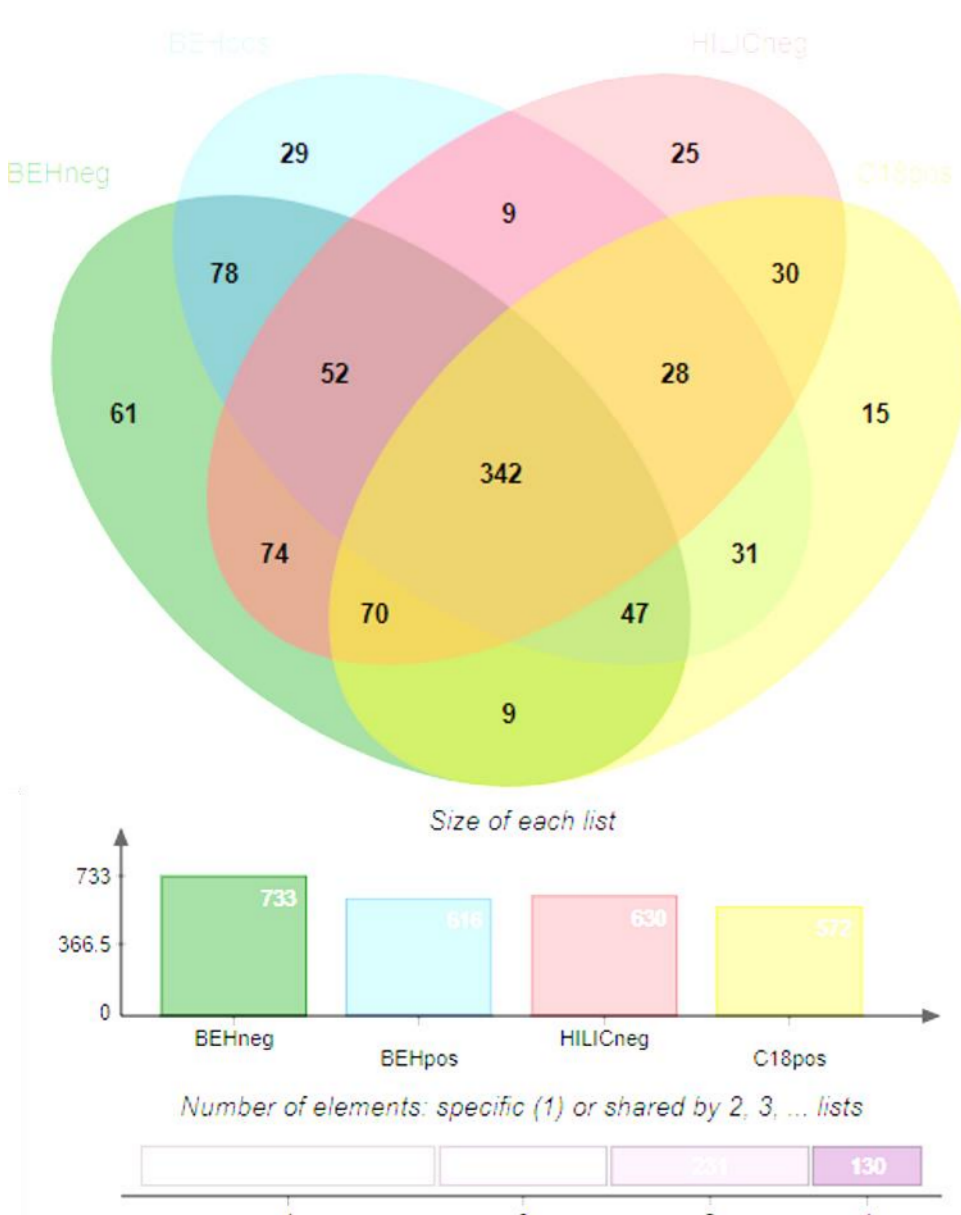
We monitored retention times and peak areas of 260 compounds injected 300 times. The calculated coefficients of variation are quite acceptable.

	Intra-batch	Inter-batch
CV of RT	0.7%	1.8%
CV of peak areas	12%	13%

Evaluation of retention time variations for 3 sequences analyzed during a 4 months period is also very satisfactory.

## Conclusion

Current non-targeted metabolomics methods based on UHPLC-HRMS may not be sufficiently high-throughput or robust enough for successful large-scale metabolomics. This emerging method is capable of detecting **coverage for 90% of our** 1000-compounds library in 38 minutes per sample, thanks to the use of 1 single column type with two injections per sample for ionization detection in positive and negative modes. Additionally, We are currently implementing an analytical method using a dual HPLC system that allows **parallel sample analysis**, thereby theoretically reducing the time to **~24 minutes per sample** - one-third of the time currently required - while ensuring adequate robustness.



The gain of 19% overall for the two acquisitions showed the interest of using this new method.