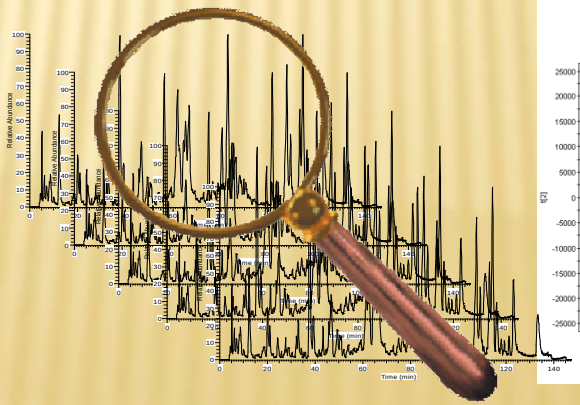
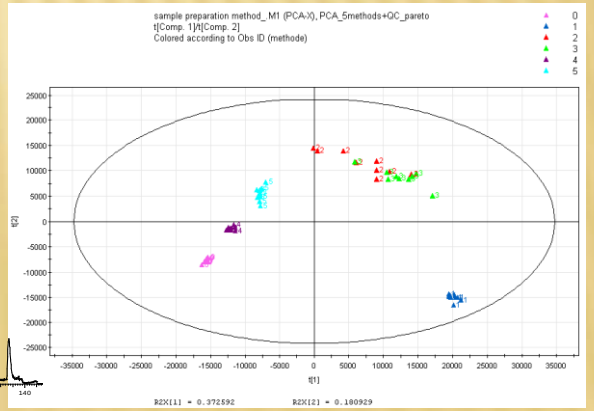
Natali Stojiljkovic
LCH

LC-HRMS based metabolomic approach as a screening tool in horse racing doping control

LC-ESI-HRMS





INTRODUCING LCH

Laboratoire des Courses Hippiques

National and International Reference Laboratory for horse doping control

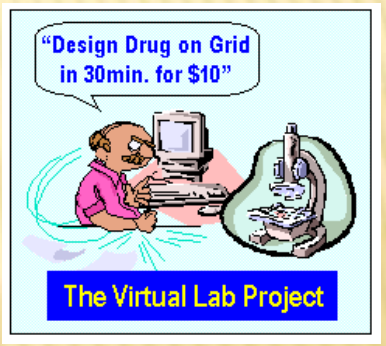
- since **1975**
- **~60** employees
- **35 000** samples per year
- accreditated **ISO CEI 17025** by french accreditation body COFRAC

- Cornerstone methodology: **MASS SPECTROMETRY**

BACKGROUND

Nowdays drug testing difficulties ……

- "endogenous-like" substances
- designer drugs
- low concentration drug cocktail
- gene doping



International Federation of Horseracing Authorities
www.IFHAOnline.org

INTERNATIONAL AGREEMENT ON BREEDING AND RACING

Article 6. - PROHIBITED SUBSTANCES

11. A finding of a prohibited substance means a finding of the substance itself or a metabolite of the substance or an isomer of the substance or an isomer of a metabolite. The finding of any scientific indicator of administration or other exposure to a prohibited substance is also equivalent to the finding of the substance.

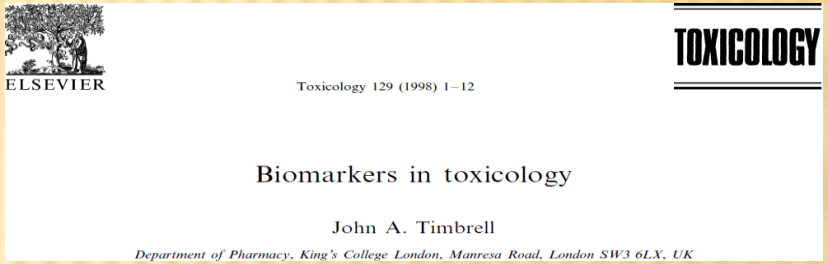
BACKGROUND

Biomarker importance……

The ability or tendency of an organism or cell to maintain internal equilibrium by adjusting its physiological processes.

Biomarkers of exposure

Biomarkers of effect


ELSEVIER

TOXICOLOGY

Toxicology 129 (1998) 1–12

Biomarkers in toxicology

John A. Timbrell

Department of Pharmacy, King's College London, Manresa Road, London SW3 6LX, UK

A **biomarker** is an observable and measurable change revealing the past or present exposure of an organism to toxic compounds or pathologies.

BACKGROUND

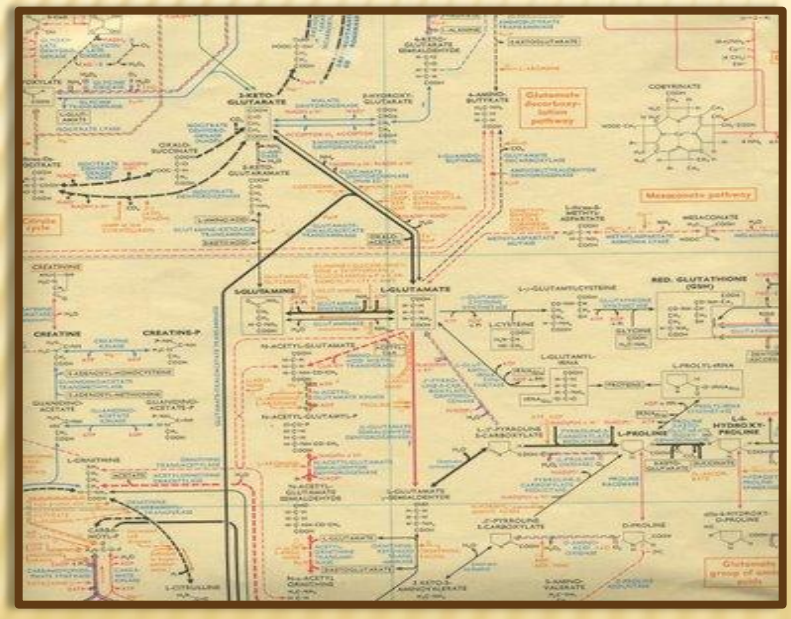
Emergence……

The zebra stripes are simple – I am more concerned of the ***horse*** behind
Alan Turing, mathematician, 1952

Key point in complex systems is not the surface patterns but *deep structures*

from "Emergence", S. Johnson

Biochemical Pathways, by Gerhard Michal, 1974

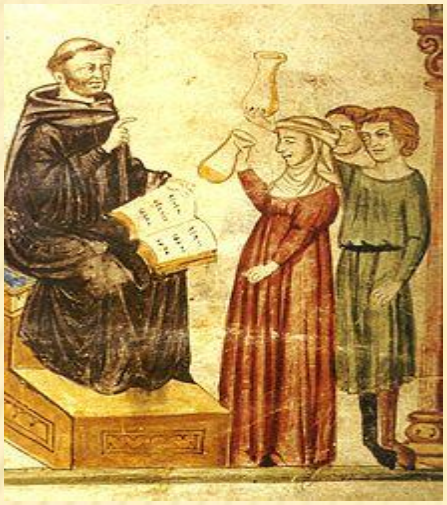


When the many parts of a system interact,
they can generate behaviours in the whole system
that aren't found in the parts themselves.

The whole is more than the sum of its parts. Aristotle, *Metaphysica*

BACKGROUND

Economical aspect……



Constantinus Africanus
11th century

Evolution of Analytical Techniques

Abby from NCIS
21st century

The range of drugs to be screened is wider every year…
Consequence: exponential increase of instruments

- In the 70's: **1 GC/MS**
- In the 80's: **5 GC/MS**
- In the 90's: **10 GC/MS & LC/MS**
- Today: **70 GC/MS & LC/MS**
- In 10 years: **> 100 instruments** ????

Pattern recognition

sad

happy

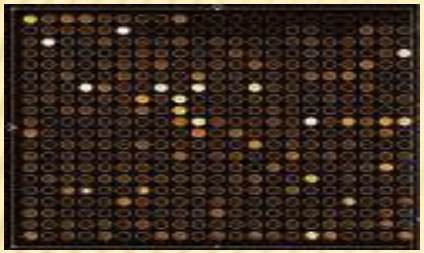
- **A single screening to detect negative samples.**

BACKGROUND

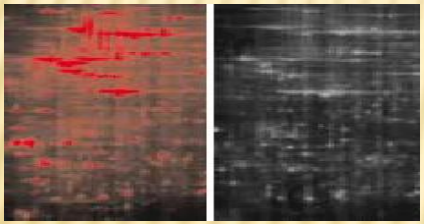
The Wonders of Small Molecules……

Genomics
What can happen

→ **GENOTYPE**

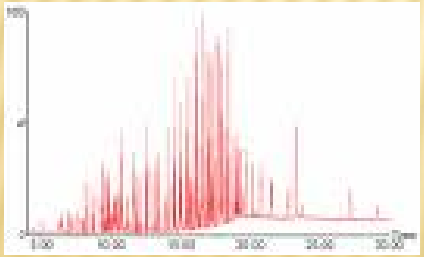


Transcriptomics
What appears
to be happening



Proteomics
What makes
it happen

PHENOTYPE



Metabolomics
What has happened
and is happening

SYSTEMS BIOLOGY

Plant Molecular Biology **48:** 155–171, 2002.
© 2002 *Kluwer Academic Publishers. Printed in the Netherlands.*

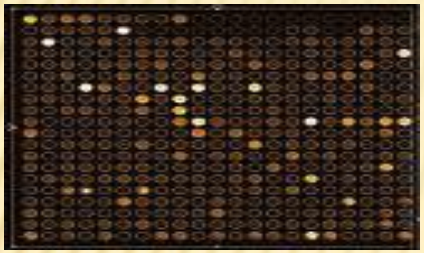
Metabolomics – the link between genotypes and phenotypes

Oliver Fiehn

BACKGROUND

The Wonders of Small Molecules……

Genomics
What can happen



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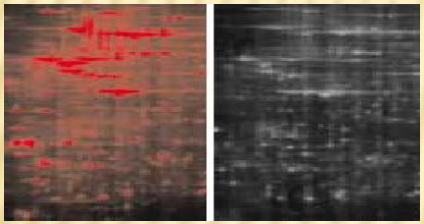

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What makes
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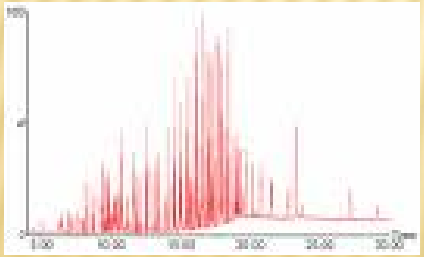


Metabolomics
What has happened
and is happening

Definitions

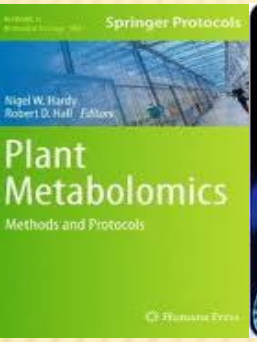
- **Metabolomics**
Comprehensive and simultaneous systematic determination of metabolite levels in the metabolome and their changes over time as a consequence of stimuli.

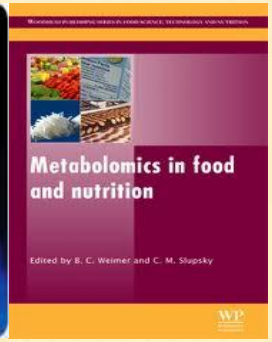
- **Metabolome**
Complete and dynamic set of small molecule metabolites.

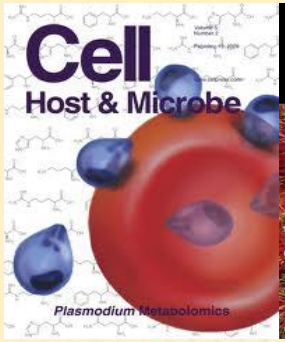
- **Metabolites**
Organic molecule detectable in organisme with MW < 1000 Da.
Intermediates and products of metabolisme.

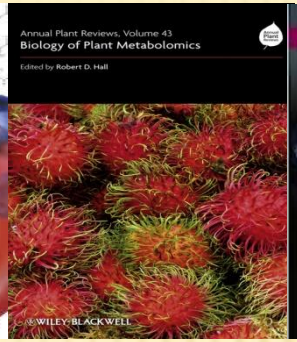
BACKGROUND

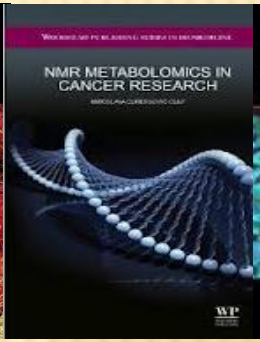
Application……











Brevet (Patent)

« Procédé de discrimination avec repérage et/ou identification de situations de perturbations physiologiques par spectrométrie et reconnaissance de formes »

Alain PARIS, Marc-Emmanuel DUMAS, Joseph VERCAUTEREN et François ANDRE

L'invention vise un procédé de discrimination avec repérage et/ou identification d'une situation de perturbation biologique. Ce procédé est caractérisé en ce qu'on soumet un échantillon biologique à une technique analytique hautement résolutive et qu'on associe cette technique à un traitement statistique ou de classification validé qui prend en compte toutes les variations observées, l'ensemble des variations référençant une situation de perturbation biologique donnée.

perturbation d'un système biologique

utilisation des anabolisants

Application notamment à la caractérisation de l'état physiologique propre à une population, au repérage et/ou à l'identification d'une perturbation d'un système biologique par une substance à activité pharmacologique ou par un microorganisme, à la discrimination indirecte de l'utilisation des anabolisants en élevage et/ou dans le domaine sportif et/ou dans le domaine biomédical et pour constituer un référentiel ou une banque de données.

domaine sportif

ANALYTICAL STRATEGY

Principles……

Routine analysis

Biological interpretation

Biomarker identification

Candidate biomarker selection

? Biological question ?

Data analysis

Data processing

In *vivo* / *vitro* experiments

Sample preparation

Sample fingerprinting

ANALYTICAL STRATEGY

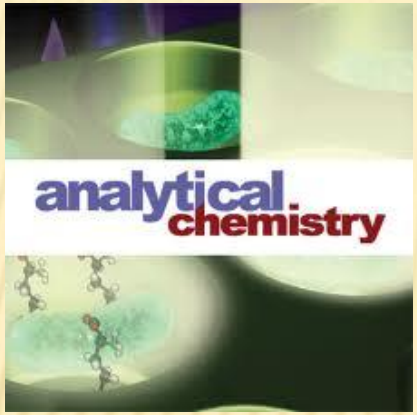
Tools……

Whatfor

Insight into our “material” world

Wherefor

- Experimental design
- Separation
- Detection
- Quantitation
- Identification



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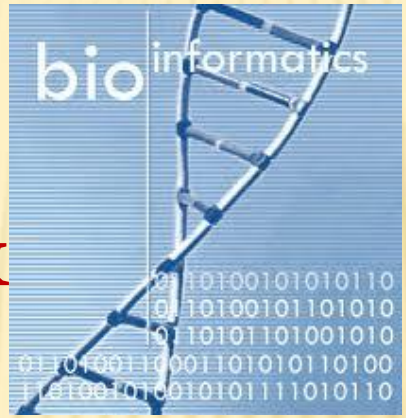


Whatfor

Large scale measurements

Wherefor

- Experimental design
- Processing
- Visualisation
- Identification
- Statistics
- Interpretation

Whyfor

- Inventing and applying the concepts, principles, and strategies for measuring the characteristics of chemical systems and species

Whyfor

- Metabolite and pathway identification
- Quantification of metabolite levels and isotopic composition
- Analysis of dynamic metabolic experiments
- Quantification of metabolic fluxes

ANALYTICAL STRATEGY

Instrumentations……

Separation methods

- **G**as **C**hromatography
  - High resolution
  - Mass spectral libraries
  - Chemical derivatization
- **H**igh **P**erformance **L**iquid **C**hromatography
  - Wide range of components
  - Soft ionization
  - LC depending mass spectra
- **C**apillary **E**lectrophoresis
  - Separation efficiency
  - Easy and predictable selectivity
  - Only charged analytes

Detection methods

- **M**ass **S**pectrometry
  - High sensitivity
  - Fast
  - Sample preparation
- Nuclear **M**agnet **R**esonance spectroscopy
  - Reproducibility
  - No destructive
  - Low sensibility

MATERIAL AND METHOD FOR METABOLOMICS IN LCH

Ultimate 3000 (Dionex, CA, USA)

Uptisphere Strategy (Interchim, Montlucon, France)

Chromatographic conditions

Column	Uptisphere C18 (100 *2.1 mm, 2.2 µm)
Solvent	Water /Acetonitrile (0.1% formic acid)
Flow	0.250 mL/min
Analysis Time	25 minutes

Spectrometric conditions

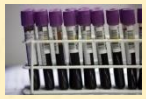
Analyzer	Q-Time Of Flight Rp = 10000
m/z range	Full Scan m/z 50-1000
Ionization / desorption mode	Electrospray Positive / Negative

MicroTOF-Q-II (Bruker, Brem, Germany)

ANALYTICAL STRATEGY

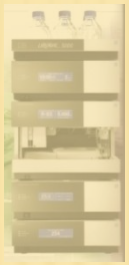
In LCH……

Animal study



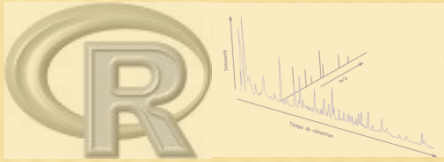
Sample preparation

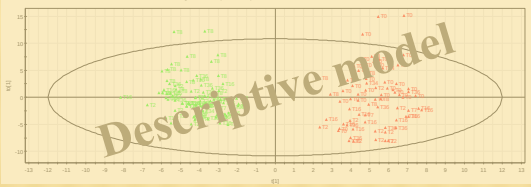
Fingerprinting by LC-HR MS



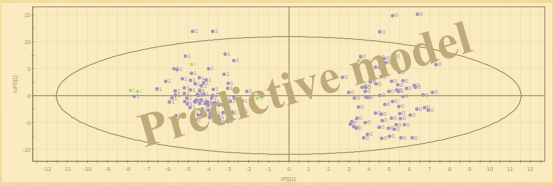
Var	name	mz	RT	riviere_T0_1	riviere_T16_1	riviere_T2_1	riviere_T36_1
2858	M424T436	424.125136	7.3	9.91E+02	2.61E+03	5.31E+03	9.69E+04
686	M221T572	221.127796	9.5	3.15E+04	5.11E+05	3.82E+04	1.47E+02
1782	M316T845	316.035898	14.1	3.80E+02	8.00E+03	5.25E+02	1.51E+04
1193	M267T593	267.158859	9.9	1.48E+04	2.55E+06	0.00E+00	1.55E+04
2560	M389T489	389.089157	8.2	7.29E+03	1.11E+04	8.06E+02	1.34E+05
2629	M395T851	395.329148	14.2	3.03E+01	3.14E+02	4.68E+03	1.41E+04
662	M219T578	219.169063	9.6	1.78E+04	1.72E+06	2.32E+03	2.98E+04
3700	M527T788	527.296711	13.1	6.52E+03	9.00E+02	1.93E+03	1.71E+03
188	M158T78	158.117709	1.3	7.12E+03	6.21E+03	2.11E+04	1.48E+03

Data processing with XCMS





Statistical analysis with SIMCA



Biomarker (selection, identification, bio-interpretation)

ANIMAL STUDY

Principles……

Metabolomics (2007) 3:179–188
DOI 10.1007/s11306-007-0077-z

ORIGINAL ARTICLE

Standard reporting requirements for biological samples in metabolomics experiments: mammalian/in vivo experiments

Julian L. Griffin · Andrew W. Nicholls · Clare A. Daykin · Sarah Heald · Hector C. Keun · Ina Schuppe-Koistinen · John R. Griffiths · Leo L. Cheng · Philippe Rocca-Serra · Denis V. Rubtsov · Donald Robertson

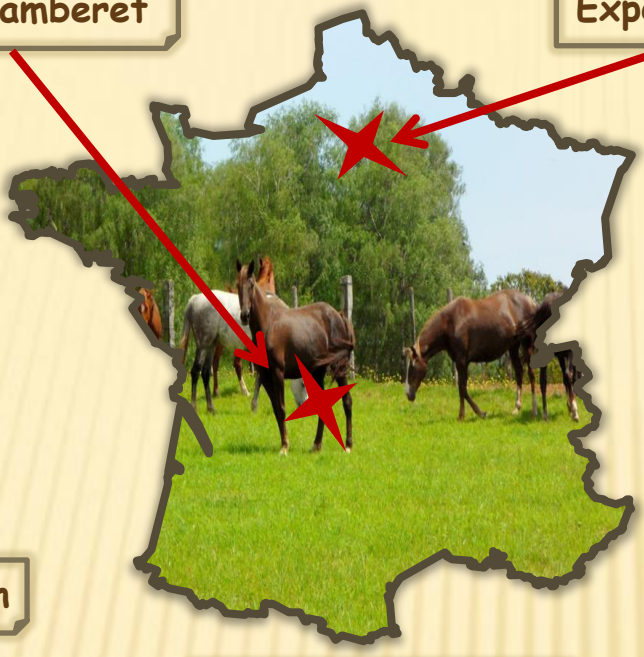
ANIMAL STUDY

In LCH……

Experimental centre of Chamberet

Subject description
- 14 mares
- Anglo-Arab
- 4 years old
- 500-600 kg

Subject description
- 6 pregnante mares
- Anglo-Arab
- 4-17 years old
- 500-600 kg



Experimental centre of Coye la Foret

Subject description
- 2 mares
- Thoroughbred
- 6 years old
- 450-550 kg

Husbandry description

Housing
- padock from mid-April until mid-November
- stable from mid-November until mid-April

Veterinary treatement
- vermifuge (dates)
- vaccin (dates)
- griseofulvin (dates)

Sample description

Urine
- morning collection
- ~ 200mL
- pH 8-9
- freezing at -20°C
- collection before stanozolol treatement: every 15 days during 1 year
- collection after stanozolol treatement:
1) every 4 days during 16 days
2) 1 per week during 6 months

Stanozolol Treatement

Control Animals
- 4 horses
- intramuscular injection
- excipient (oil of sesam)
- chronic: 4 times in 16 days

Treated Animals
- 10 horses
- intramuscular injection
- stanozolol
- chronic: 4 times in 16 days
- 5 horses: total dose of 0.4 mg/kg
- 5 horses: total dose of 1.2 mg/kg

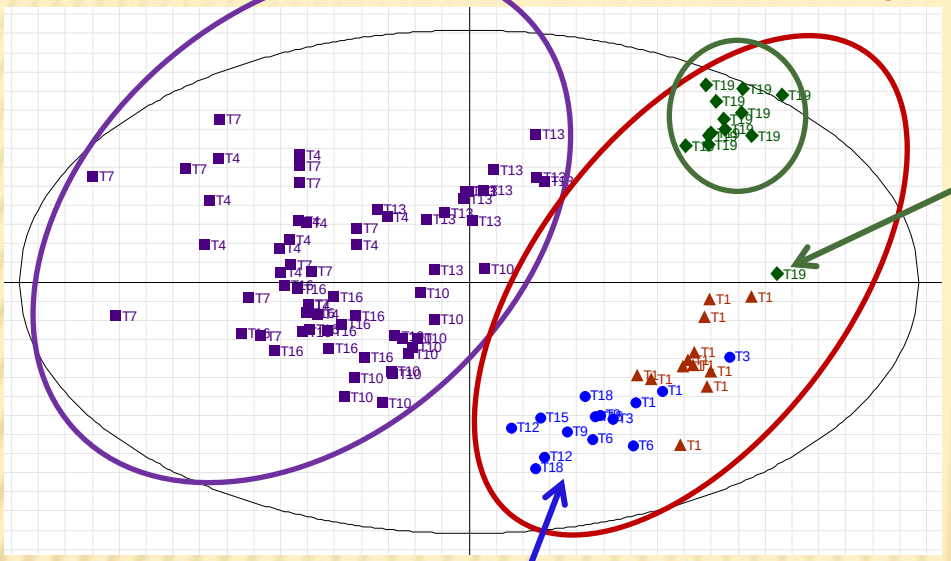
ANIMAL STUDY

Results……

Environmental factor investigation

Pasture

Box



Vermifuge

PCA based on 886 ions

Positive mode of ionization

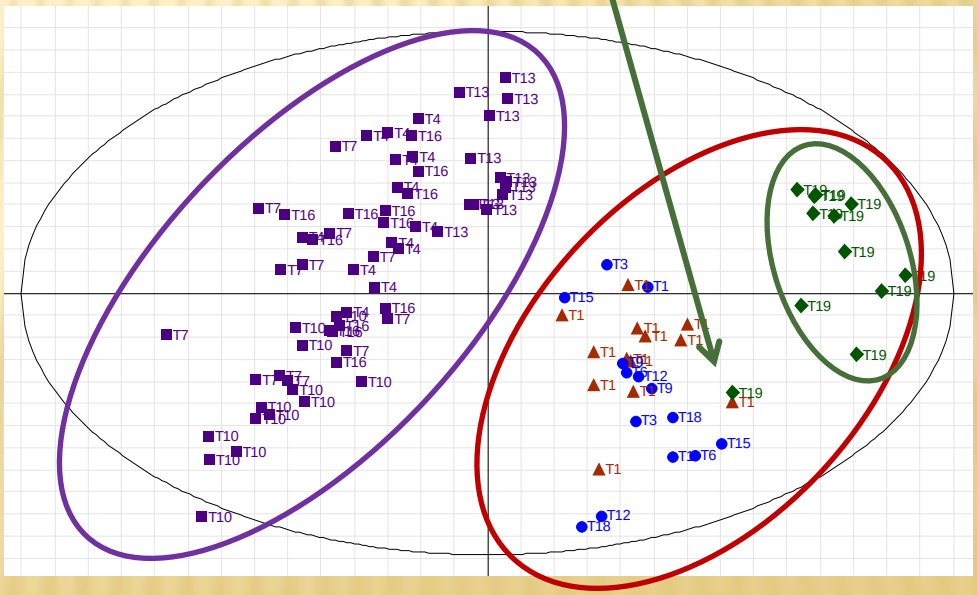
Coye la Foret

PCA based on 1522 ions

Negative mode of ionization

Vermifuge

Box

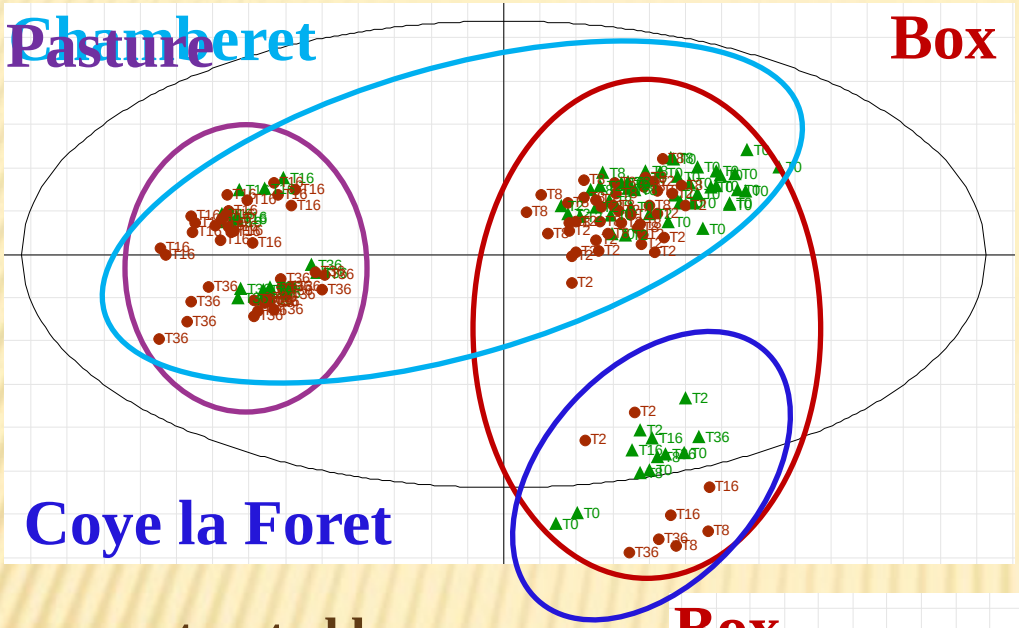


Pasture

ANIMAL STUDY

Results……

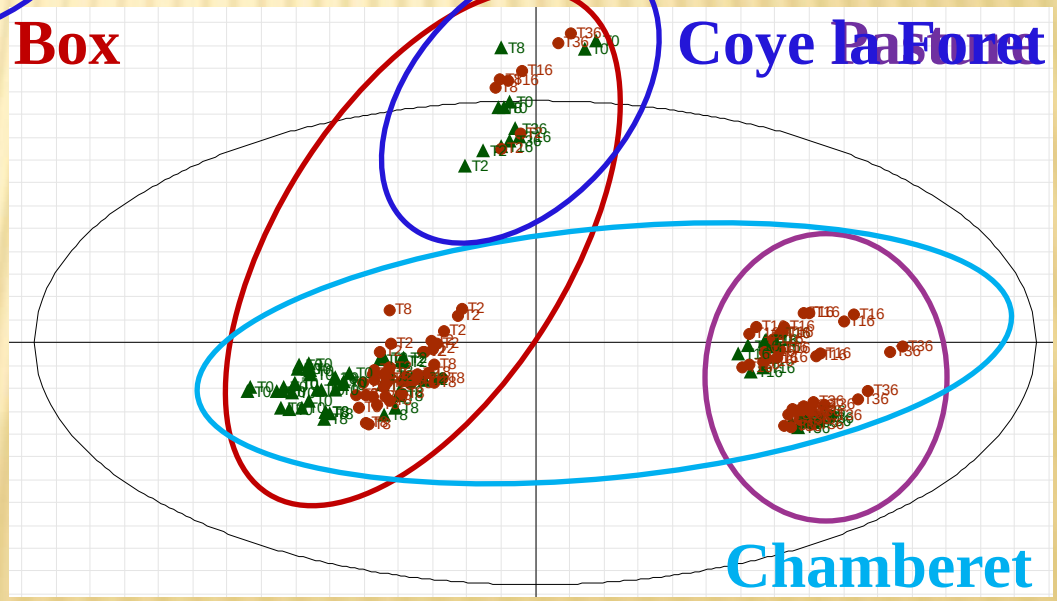
After Stanozolol Treatment



PCA based on 1935 ions
Positive mode of ionization

▲ non-treated horses

● stanozolol-treated horses



PCA based on 1986 ions
Negative mode of ionization

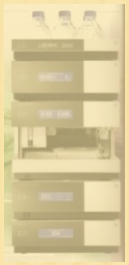
ANALYTICAL STRATEGY

In LCH……

Animal study

Sample preparation

Fingerprinting by LC-HR MS



Var	name	mz	RT	riviere_T0_1	riviere_T16_1	riviere_T2_1	riviere_T36_1
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686	M221T572	221.127796	9.5	3.15E+04	5.11E+05	3.82E+04	1.47E+02
1782	M316T845	316.035898	14.1	3.80E+02	8.00E+03	5.25E+02	1.51E+04
1193	M267T593	267.158859	9.9	1.48E+04	2.55E+06	0.00E+00	1.55E+04
2560	M389T489	389.089157	8.2	7.29E+03	1.11E+04	8.06E+02	1.34E+05
2629	M395T851	395.329148	14.2	3.03E+01	3.14E+02	4.68E+03	1.41E+04
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3700	M527T788	527.296711	13.1	6.52E+03	9.00E+02	1.93E+03	1.71E+03
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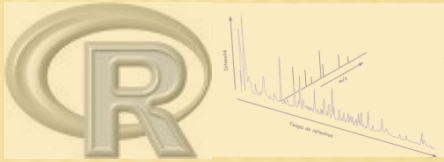
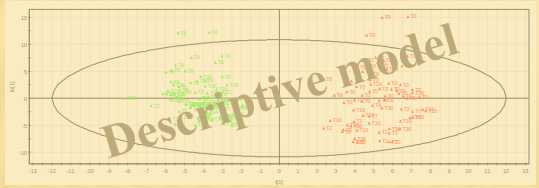
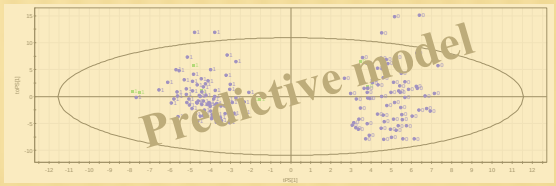




Statistical analysis with SIMCA

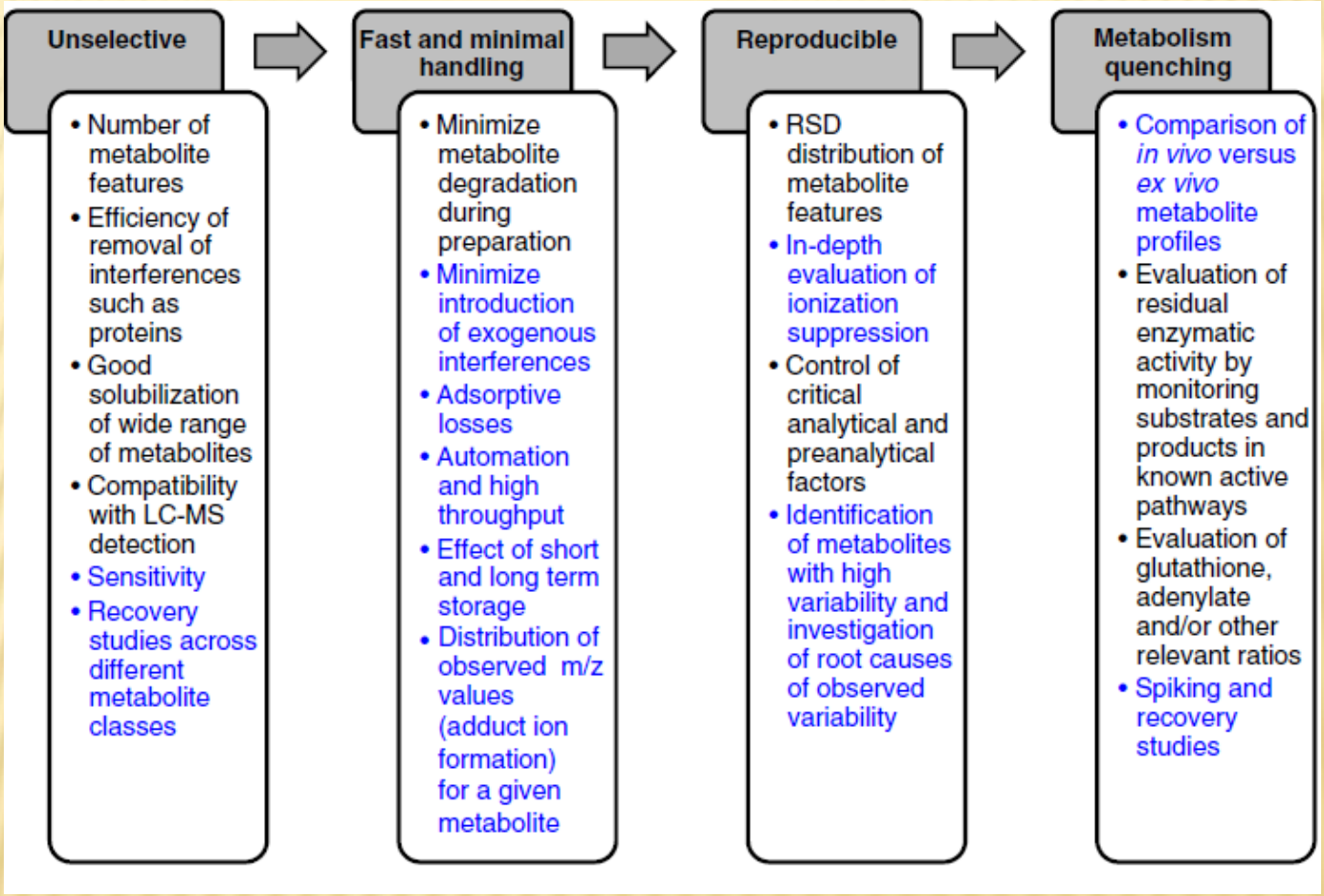


Biomarker (selection, identification, bio-interpretation)

SAMPLE PREPARATION

Principles……

Affects metabolite coverage and biological interpretation of the data!



Vuckovic, D. (**2012**) Current trends and challenges in sample preparation for global metabolomics using liquid chromatography-mass spectrometry. *Anal Bioanal Chem 403*, 1523-1548.

SAMPLE PREPARATION

Principles……

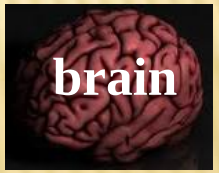
Depends on biological matrix:

BIOFLUIDS

urine

blood
serum plasma

TISSUE



liver

dilute-and-shoot

solvent precipitation

lyophilisation
LLE / SPE extraction

Anal. Chem. 2009, 81, 3285–3296

Investigation of Human Blood Plasma Sample Preparation for Performing Metabolomics Using Ultrahigh Performance Liquid Chromatography/ Mass Spectrometry

Stephen J. Bruce, Isabelle Tavazzi, Véronique Parisod, Serge Rezzi, Sunil Kochhar, and Philippe A. Guy*

Trends in Analytical Chemistry, Vol. 29, No. 2, 2010

Metabolomics analysis II. Preparation of biological samples prior to detection

B. Álvarez-Sánchez, F. Priego-Capote, M.D. Luque de Castro

analytical chemistry

Technical Note

pubs.acs.org/ac

Assessment of Compatibility between Extraction Methods for NMR- and LC/MS-Based Metabolomics

Antoni Beltran,[†,‡,§,*] Manuel Suarez,[⊥] Miguel A. Rodríguez,[†,‡,§] Maria Vinaixa,[†,‡,§] Sara Samino,[†,‡,§] Lluís Arola,[⊥,∥] Xavier Correig,[†,‡,§] and Oscar Yanes[†,‡,§,*]

SAMPLE PREPARATION

Results……

Horse urine specificity:

- **mucus**
- **inorganic salts**
- **wide range of pH**

Method	
1	precipitation with acetonitrile
2	enzymatic hydrolysis (protease K)
3	membrane filtration (10 kDa)
4	dilution at 1/20, direct injection
5	dilution at 1/5, direct injection

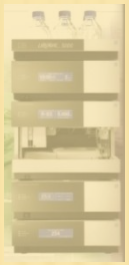
ANALYTICAL STRATEGY

In LCH……

Animal study

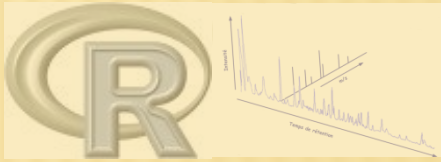
Sample preparation

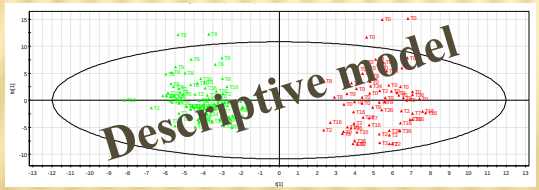
Fingerprinting by LC-HR MS



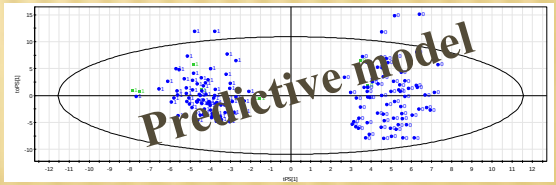
Var	name	mz	RT	riviere_T0_1	riviere_T16_1	riviere_T2_1	riviere_T36_1
2858	M424T436	424.125136	7.3	9.91E+02	2.61E+03	5.31E+03	9.69E+04
686	M221T572	221.127796	9.5	3.15E+04	5.11E+05	3.82E+04	1.47E+02
1782	M316T845	316.035898	14.1	3.80E+02	8.00E+03	5.25E+02	1.51E+04
1193	M267T593	267.158859	9.9	1.48E+04	2.55E+06	0.00E+00	1.55E+04
2560	M389T489	389.089157	8.2	7.29E+03	1.11E+04	8.06E+02	1.34E+05
2629	M395T851	395.329148	14.2	3.03E+01	3.14E+02	4.68E+03	1.41E+04
662	M219T578	219.169063	9.6	1.78E+04	1.72E+06	2.32E+03	2.98E+04
3700	M527T788	527.296711	13.1	6.52E+03	9.00E+02	1.93E+03	1.71E+03
188	M158T78	158.117709	1.3	7.12E+03	6.21E+03	2.11E+04	1.48E+03

Data processing with XCMS





Statistical analysis with SIMCA



Biomarker (selection, identification, bio-interpretation)

STATISTICAL ANALYSIS

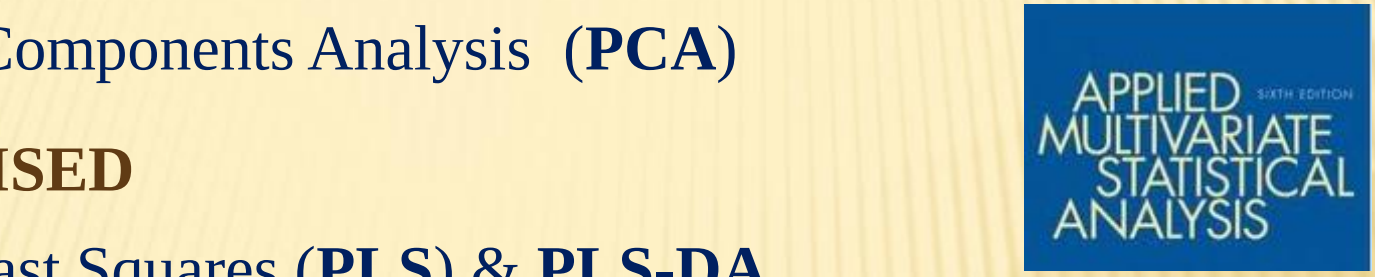
Principles……

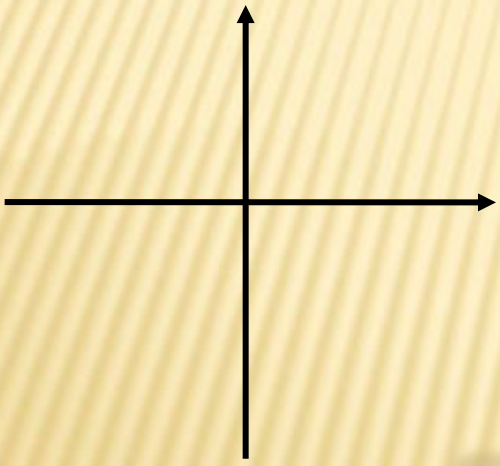
- **UNSUPERVISED**

  Principal Components Analysis (**PCA**)

- **SUPERVISED**

  Partial Least Squares (**PLS**) & **PLS-DA**





Axis 2 → **linear combination** of ions which aims to explain the **total variability**

Axis 1 → **linear combination** of ions which aims to explain the better **separation** between **non-treated** and **treated** populations

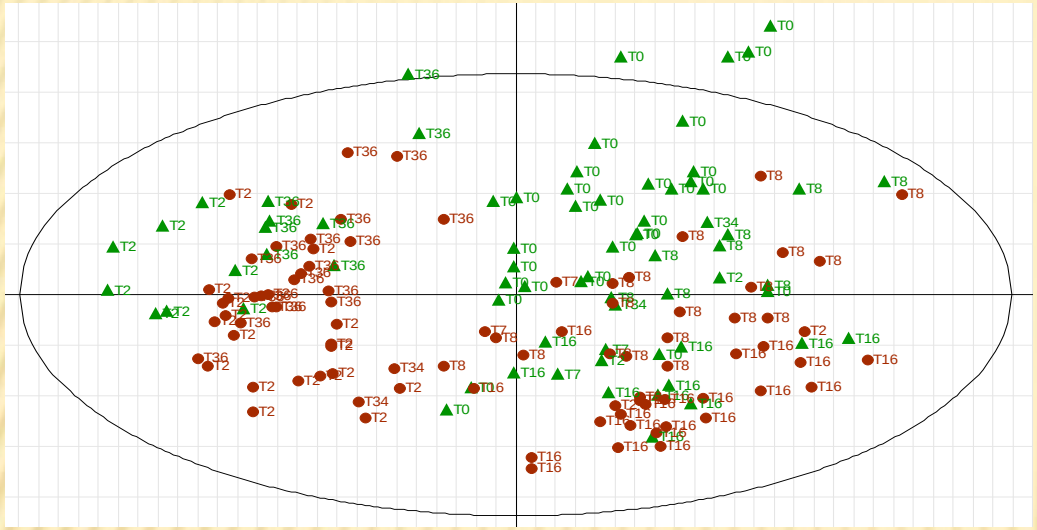
OPLS model

$R^2(X)$: % of the total variance explained by the model
$R^2(Y)$: % of the total variance of Y
Q^2: Predictive ability of the model

STATISTICAL ANALYSIS

Results……

Descriptive model

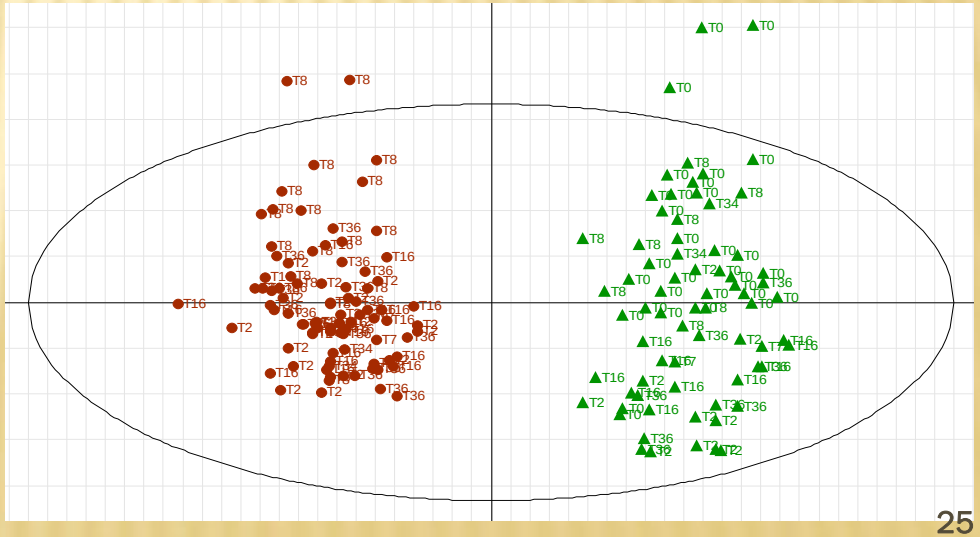


PCA based on 614 ions
(without confounding factors)

▲ **non-treated horses**

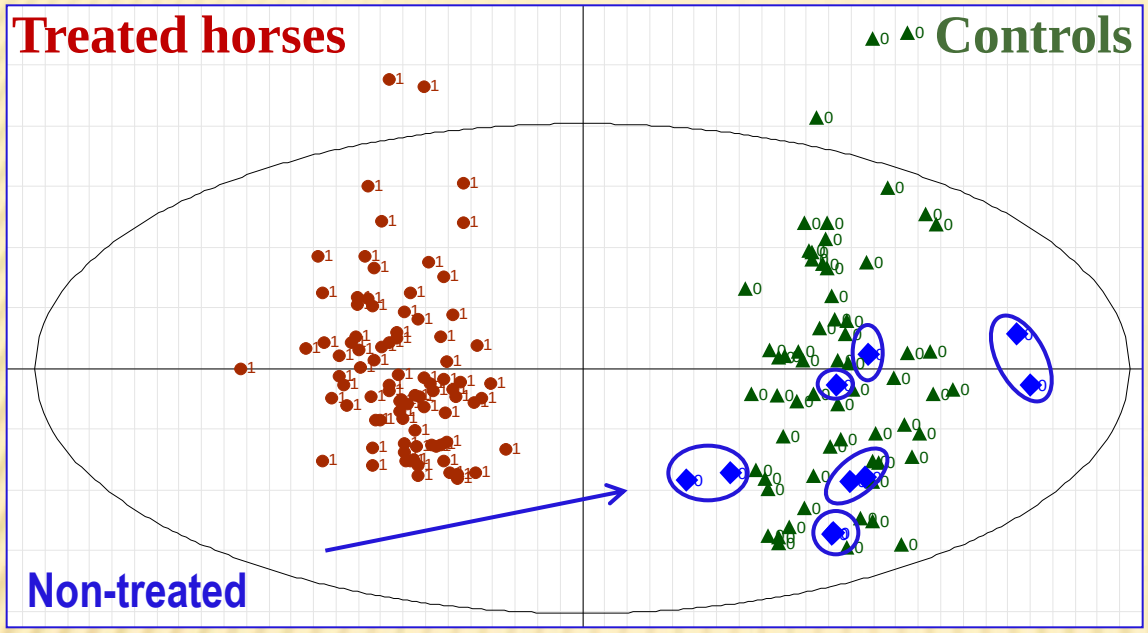
● **stanozolol-treated horses**

OPLS-DA based on 221 ions
(VIP from 614 ions)



STATISTICAL ANALYSIS

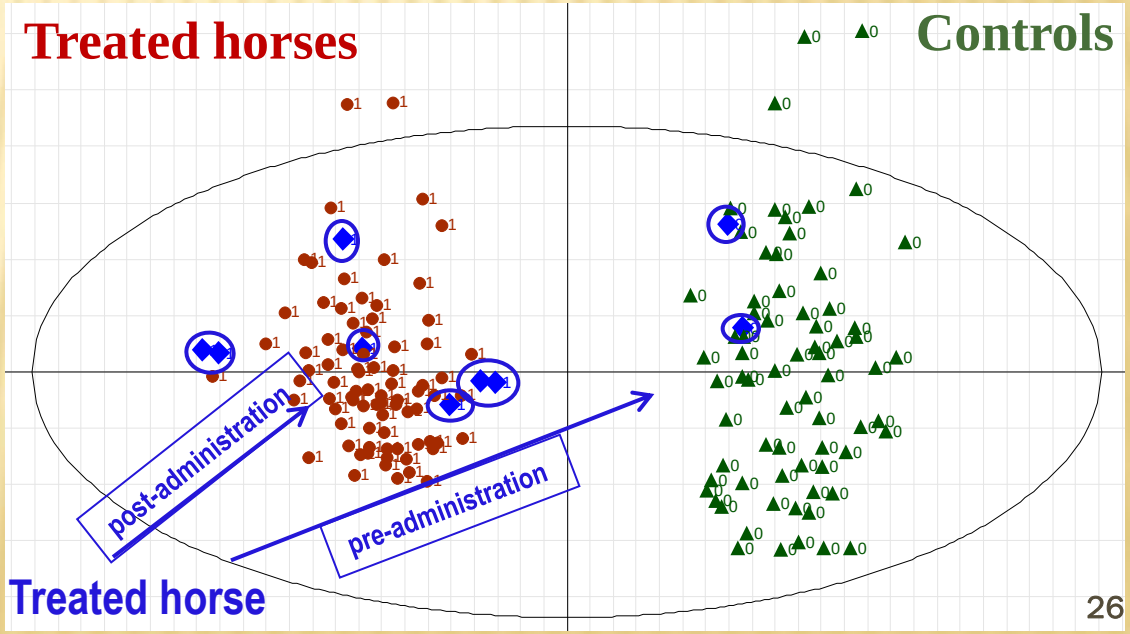
Results……



Predictive model

Prediction of one non-treated horse

Prediction of one stanozolol-treated horse.

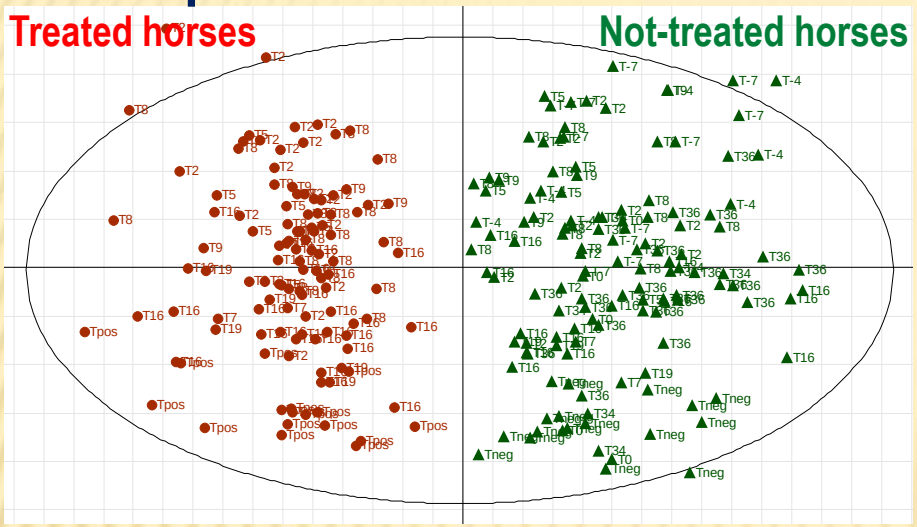


OPLS-DA based on 221 ions

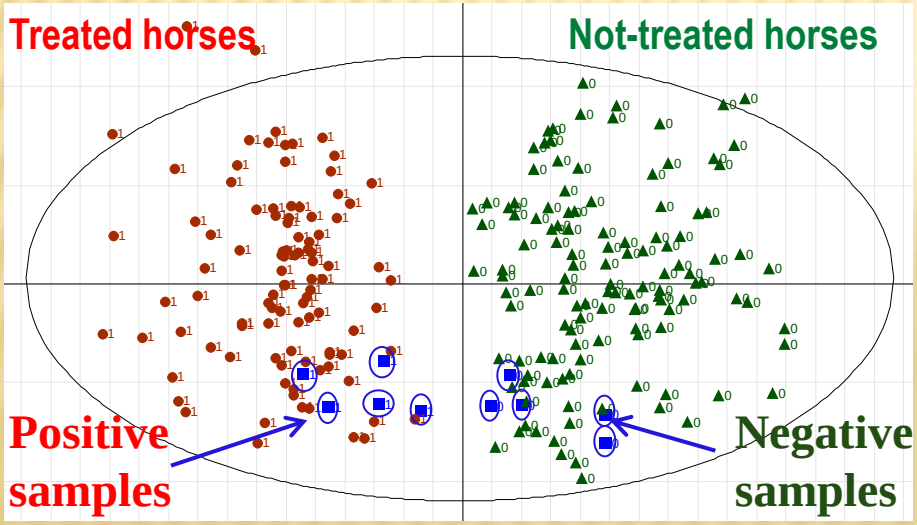
STATISTICAL ANALYSIS

Results……

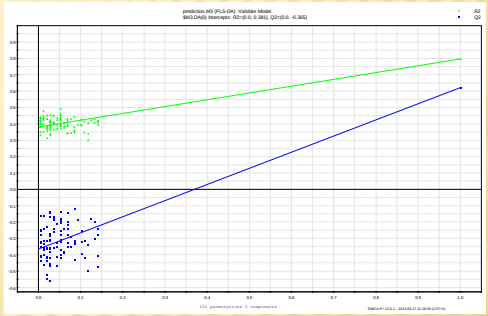
Descriptive model

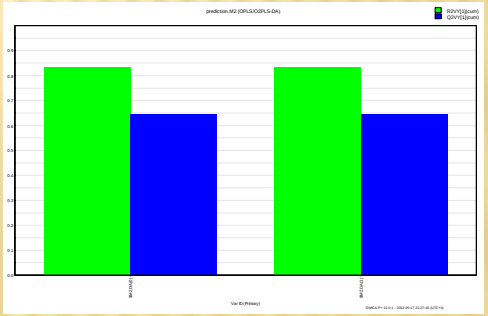


Predictive model



OPLS-DA data analysis





$R^2Y=0.835$

$Q^2Y=0.646$

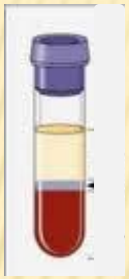
Not OK

LONGITUDINAL PROFILING

Today in LCH……

50 best French Trotters

total blood



urine

Transcriptomics
(EPO, GH)

Proteomics
(anti rGH antibodies)

Biomarkers
(IGF-I ELISA)

Blood count

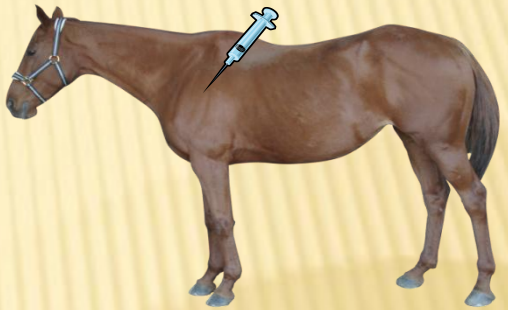
Metabolomics
(GH)

Steroidomics
(AAS)

Equine Biological Passport

LONGITUDINAL PROFILING

Metabolomics GH ……



Sample collection:

before, during and after treatment up to 80 days

GH administration

13 Horses			Week 1	Week 2
Group N 1	7 controls	Physiological Serum	2 mL (S.C.)	
Group N 2	6 treated	**EquiGen-5**	18 µg/kg/day (S.C.)	

Metabolomics (2011) 7:84–93
DOI 10.1007/s11306-010-0233-8

ORIGINAL ARTICLE

Generation and processing of urinary and plasmatic metabolomic fingerprints to reveal an illegal administration of recombinant equine growth hormone from LC-HRMS measurements

Fanny Kieken · Gaud Pinel · Jean-Philippe Antignac · Anne-Christelle Paris · Patrice Garcia · Marie-Agnès Popot · Morgane Grall · Victoria Mercadier · Pierre Louis Toutain · Yves Bonnaire · Bruno Le Bizec

Anal Bioanal Chem (2009) 394:2119–2128
DOI 10.1007/s00216-009-2912-8

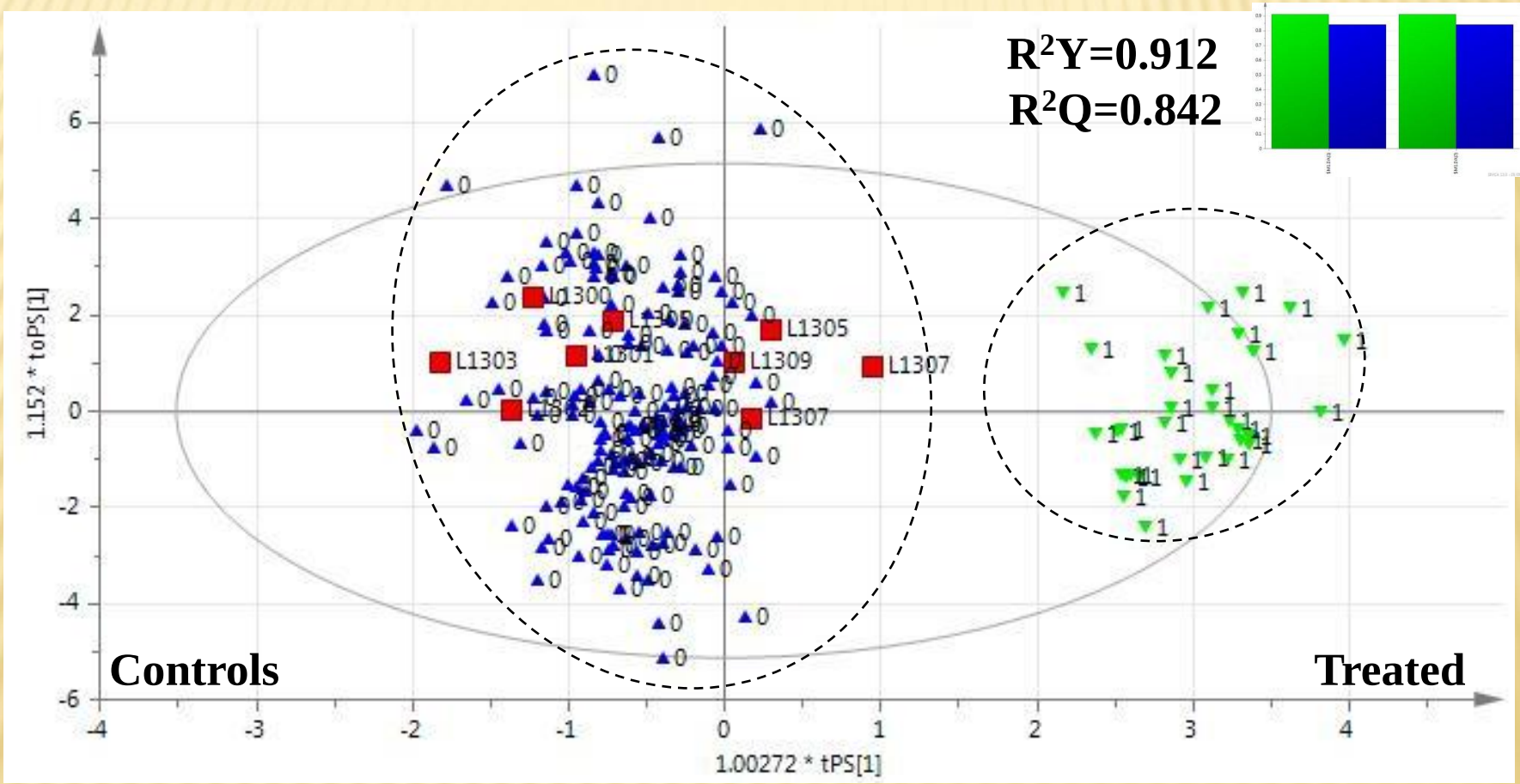
ORIGINAL PAPER

Development of a metabonomic approach based on LC-ESI-HRMS measurements for profiling of metabolic changes induced by recombinant equine growth hormone in horse urine

Fanny Kieken · Gaud Pinel · Jean-Philippe Antignac · Fabrice Monteau · Anne Christelle Paris · Marie-Agnès Popot · Yves Bonnaire · Bruno Le Bizec

LONGITUDINAL PROFILING

- GH predictive model for longitudinal follow-up obtained in August



CONCLUSION

Question of balance……

- **Leader role shift :**

Industrial research → Academic research

Method development

- **Application role shift :**

Descriptive science → Predictive science

- **Status role shift :**

« Nice to have » → « Need to have »

Screening

CONCLUSION

Still a lot of work left….

Improvement and standardization of metabolite detection

High-throughput metabolic profiling

DATA EXCHANGE

Metabolite identification

Data set annotation
Spectral databases
Structural elucidation

Informatics and bioinformatics

Data analysis tools
Data visualization tools
Standard data formats

ACKNOWLEDGEMENTS

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les Haras nationaux

INRA
Institut National de la Recherche Agronomique

Alain Paris

cea

Christophe Junot

anrt
association nationale recherche technologie

THANK YOU FOR YOUR ATTENTION

Natali Stojiljkovic
LCH

LC-HRMS based metabolomic approach as a screening tool in horse racing doping control

LC-ESI-HRMS