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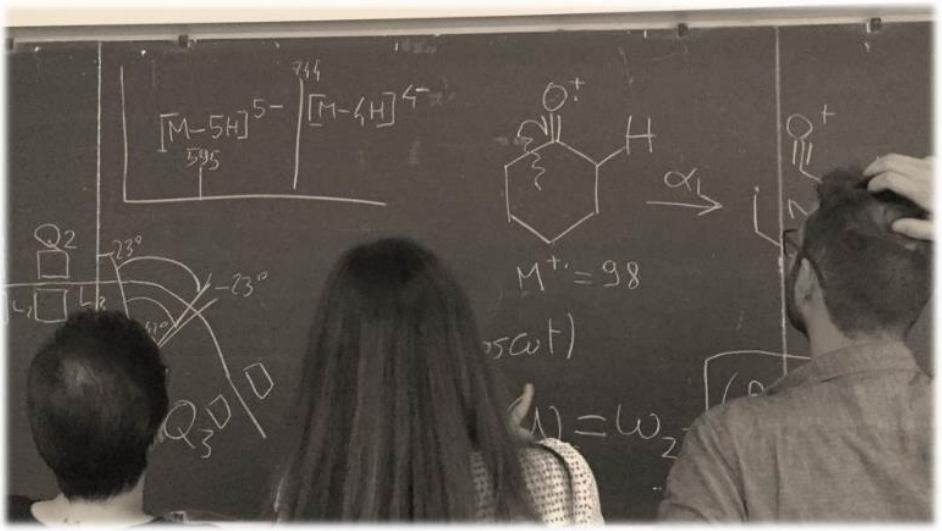

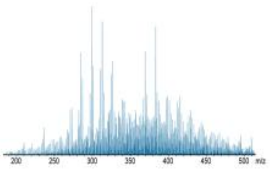

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APPLICAZIONI CHEMIOMETRICHE PER LA VALUTAZIONE DEI PROFILI GASCROMATOGRAFICI NELL'INVESTIGAZIONE DEI REATI INCENDIARI

A COMBINED APPROACH OF GC-MS AND CHEMOMETRIC STRATEGIES FOR FIRE DEBRIS INVESTIGATION PURPOSES

E. Alladio[1,2*], M. Pazzi[1], L. Pacifici[1], F. Malaspina[3], M. Vincenti[1,2]

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In the sphere of fire debris investigations, a largely debated issue deals with the possibility to recognize whether the collected evidences is related to the occurrence of an arson or not. In the practice, data from GC-MS analyses performed on collected fire debris may be compared to the ones from ignitable liquids, e.g. found in possession of a suspect arsonist. With the aim of evaluating the use of gasoline as fire accelerant, several gasolines sampled from different oil stations located within the area of the city of Turin were analysed by SPME-GC-MS methodology.

The use of chemometric strategies allowed to build explorative, classification and likelihood ratio models indicating the probabilities that fire accelerants have been actually employed. Once the chromatograms have been collected, different chemometric approaches have been tested both on raw and semi-quantitative data. In particular, Principal Component Analysis, Self-Organizing Maps (SOM) and Partial Least Squares – Discriminant Analysis, as well as N-way models, were evaluated with the aim of identifying the usage of fire accelerant. Even if in a preliminary stage, this work seems to emphasize the employment of multivariate strategies aimed to potentially help the interpretative process of fire debris investigations.

Keywords Fire debris investigation, GC-MS, Multivariate Data Analysis.

OPTIMIZATION OF A GC-MS METHOD FOR ENDOGENOUS ANABOLIC ANDROGENIC STEROIDS (EAAS) DETECTION AND APPLICATION FOR NEW ANTIDOPING AND CLINICAL SCREENING STRATEGIES

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Abstract

The dosage of endogenous anabolic androgenic steroids (EAAS) founds application in several fields, i.e. doping detection and clinical diagnosis. We selected an extended panel of 18 urinary EAAS that could serve us for many classification purposes. Traditionally, the pre-treatment of the samples provides several step, including solid phase extraction (SPE) of the free and conjugated EAAS, enzymatic deconjugation of phase II metabolites by means of β-glucuronidase, liquid-liquid extraction (LLE) of the free molecules and, finally, the derivatization with trimethylsilyl (TMS) group in order to have a volatility compatible with a gas chromatographic separation. The purpose of this work was the development and optimization of a new strategy cheaper and faster for the treatment of the samples. In particular, we wanted to eliminate the SPE extraction, the most expensive and slowing step of the process.

For this purpose, a full factorial experimental design was arranged, involving three factors (volume of β-glucuronidase, kind of solvent for the LLE and time of derivatization) on two levels with a central point. Then, the chromatographic run was optimized, too. The parameters investigated were the initial and the final temperature of the oven and the speed of the temperature gradient.

Once optimized the analytical strategy, the androgenic profiles of several male subjects coming from the sports and clinical world were investigated. In particular, (i) a marathoner was time monitored in order to investigate the possible anomalous alterations linked to physical stress and (ii) a dataset of individuals affected by benign prostatic hypertrophy (BPH) and prostatic carcinoma (PCa) was assembled, with the purpose to build classification models able to discriminate between the two populations.

Keywords Androgenic profile, GC-MS, design of experiment (DoE), classification tools, exploratory analysis.

Laurea triennale in Biotecnologie

Alberto Asteggiano

Abstract:

Among post-traductional modifications, PTN (protein tyrosine nitration) plays a very important role in determining and modifying the function of a protein such as, for example, an increase or decrease of its functional activity since tyrosine is involved in phosphorylation events, or an increase of susceptibility to proteasome degradation. In this work I will focus on the enrichment of nitrated proteome and it's analysis by LC-MS techniques referring also to a study made on a *Saccharomyces cerevisiae's* signaling pathway and it's nitration events.

Individual and Cyclic Estrogenic Profile in Woman: Structure and Variability of the Data

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Women of childbearing are the ones who still have the period, thus the amount of estrogens, produced by the ovaries, results cyclic with wide fluctuations throughout the menstrual cycle.
To date, the variations of the principal estrogens (estradiol, estrone and estriol) were only evaluated, consequently the aim of the study was to examine in depth the cyclic fluctuations of some metabolites.
A Gas Chromatography coupled to Mass Spectrometry (GC-MS) method capable of detecting estradiol, estrone, estriol and their metabolites in urine was developed, based on an experimental design, and fully validated.
The following validation parameters were investigated according to ISO 17025 requirements: linearity range, selectivity, specificity, limit of detection (LOD), limit of quantitation (LOQ), trueness, intra- and inter-assay precision, repeatability, matrix effect, extraction recovery and carry-over.
A wide panel of estrogens, consisting of 17α-estradiol, 17β-estradiol, estrone, estriol, 4-methoxyestrone, 2-methoxyestrone, 16α-hydroxyestrone, 4-hydroxyestrone, 2-hydroxyestrone, 4-methoxyestradiol, 2-methoxyestradiol, 4-hydroxyestradiol, 2-hydroxyestradiol, 16-epiestriol and 17-epiestriol, was monitored in some young healthy women throughout a complete menstrual cycle.
The resulting data were simultaneously evaluated through the application of the Parallel Factory Analysis (PARAFAC) method, in order to explore the variability of each analyte and each class of estrogens.
The estrogens, furthermore, play a significant role in the development of ovarian and breast tumors. In fact, these carcinomas are closely related to the alteration of the estrogenic production.
Urine samples, collected from healthy post-menopausal women and volunteers affected by breast cancer, were analysed.
Post-menopausal women were chosen because of the higher incidence of these tumors and the greater stability of the urinary estrogenic concentration compared to young women.
The evaluation of the estrogenic profile was performed and the resulting data were analysed by multivariate statistic techniques. Among the most important techniques, Discriminant Analysis (DA), Partial Least Square – Discriminant Analysis (PLS – DA), Principal Component Analysis (PCA), Cluster Analysis and Soft Independent Models of Class Analogy (SIMCA), were tested in order to discriminate between healthy women and the ones suffering from carcinomas.

PROFILO AROMATICO DI VINI PIEMONTESI DOC IN GC-MS CON HS-SPME

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Mediante spettrometria di massa accoppiata alla gascromatografia (Focus GC DSQ Thermo corporation- singolo quadrupolo) siamo stati in grado di ricavare i profili aromatici di alcuni vini della collina piemontese. Le molecole aromatiche presenti nei vini analizzati sono state preventivamente estratte in fase solida in spazio di testa. La HS-SPME è un'alternativa veloce alle tecniche convenzionali di estrazione del campione. Durante la fase di estrazione si stabiliscono gli equilibri tra analita, matrice del campione, spazio di testa sopra il campione e la fibra (nel nostro caso fibra trifasica: CAR/PDMS/DVB 30/50µm) [1]. Questa tecnica di estrazione accoppiata alla spettrometria di massa ha ottenuto un consenso universale, essendo impiegata in molte applicazioni quali appunto il riconoscimento di aromi e di fragranze in ambito alimentare, in tossicologia, in matrici ambientali e biologiche. I risultati preliminari qualitativi costituiscono il punto di partenza per un'analisi sistematica semi-quantitativa mediante la quale, grazie all'utilizzo di moderni approcci chemiometrici, sarà possibile caratterizzare i vini in base al loro profilo di composti aromatici volatili. In un'ottica di ricerca basata su un forte legame con il territorio, lo studio si focalizzerà su alcuni vitigni tipici dell'area della collina torinese. I risultati finali costituiranno una sorta di carta d'identità del vino, utile per la tracciabilità e per la tutela da eventuali frodi alimentari.

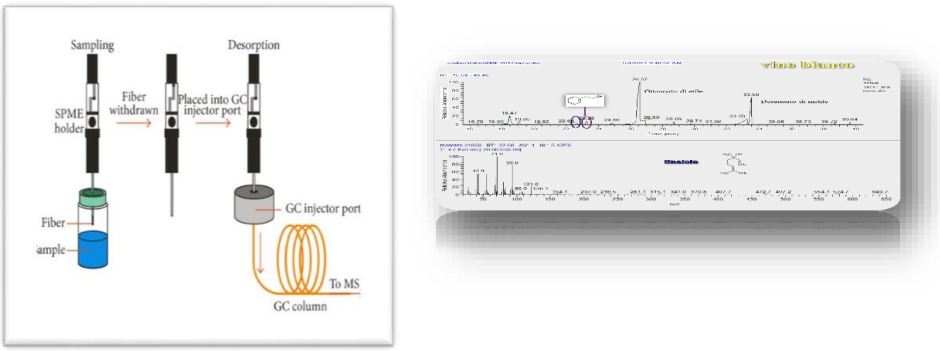

FIG. 1: Tecnica di microestrazione in fase solida accoppiata alla MS

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Individual and Cyclic Estrogenic Profile in Woman: Structure and Variability of the Data

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Women of childbearing are the ones who still have the period, thus the amount of estrogens, produced by the ovaries, results cyclic with wide fluctuations throughout the menstrual cycle.
To date, the variations of the principal estrogens (estradiol, estrone and estriol) were only evaluated, consequently the aim of the study was to examine in depth the cyclic fluctuations of some metabolites.
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A wide panel of estrogens, consisting of 17α-estradiol, 17β-estradiol, estrone, estriol, 4-methoxyestrone, 2-methoxyestrone, 16α-hydroxyestrone, 4-hydroxyestrone, 2-hydroxyestrone, 4-methoxyestradiol, 2-methoxyestradiol, 4-hydroxyestradiol, 2-hydroxyestradiol, 16-epiestriol and 17-epiestriol, was monitored in some young healthy women throughout a complete menstrual cycle.
The resulting data were simultaneously evaluated through the application of the Parallel Factory Analysis (PARAFAC) method, in order to explore the variability of each analyte and each class of estrogens.
The estrogens, furthermore, play a significant role in the development of ovarian and breast tumors. In fact, these carcinomas are closely related to the alteration of the estrogenic production.
Urine samples, collected from healthy post-menopausal women and volunteers affected by breast cancer, were analysed.
Post-menopausal women were chosen because of the higher incidence of these tumors and the greater stability of the urinary estrogenic concentration compared to young women.
The evaluation of the estrogenic profile was performed and the resulting data were analysed by multivariate statistic techniques. Among the most important techniques, Discriminant Analysis (DA), Partial Least Square – Discriminant Analysis (PLS – DA), Principal Component Analysis (PCA), Cluster Analysis and Soft Independent Models of Class Analogy (SIMCA), were tested in order to discriminate between healthy women and the ones suffering from carcinomas.

NanoHPLC-HRMS determination of chicken albumen and pork collagen in red wine

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Abstract

Chicken albumen and pork collagen are used as fining protein agents to clarify red wine. The clarification of wine is useful in order to stabilize its compounds and to remove insoluble (or partially soluble) molecules. In particular natural colloids (polysaccharides and polymerized phenolic compounds), yeast and cell debris cause the turbidity of crude wine after alcoholic and malolactic fermentation. This aspect is not accepted by the consumer and the clarification is mandatory.

Albumen, and its mayor components albumin, are recognized as allergens.

The aim of the project was to verify the presence of albumen and collagen residues in red wine using nanoHPLC-HRMS technology.

Red wine samples were fortified with albumen and collagen, and the proteins were extracted, purified and digested with trypsin.

An Ultimate Dionex 3000 liquid chromatographer coupled with an Orbitrap Fusion mass analyzer (both Thermo Fisher) through a nanoESI source were used to quantify albumen and collagen in red wines. A 50 cm RP column C18 (PepMap™, 50 cm × 0.75 µm, 2 µm, 100 Å) was employed to obtain the chromatography separation of peptides coming from trypsin digestion. Four peptides were chosen to recognized and quantified albumen: the first belonging to ovalbumin protein, the second and the third belonging to lysozyme, and the last belonging to ovotransferrin. Two peptides were chosen for collagen.

Two calibration curves were prepared, one in ammonium bicarbonate buffer (25-5-0.5 ppm) and the other one in red wine proteins pellet (25-10-5-2-1 ppm). Linear regressions and correlation coefficients were excellent. Albumen and collagen were then quantified in fortified red wines.

The developed method was sensitive, selective and robust and it is now ready to be applied to real red wines samples.

Comparative lipidomic analysis of Astrocytes upon graphene and graphene oxide treatment by UPLC-Q-TOF-MS

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Abstract: During the past decade, graphene (GR) and graphene oxide (GO) have been increasingly investigated for their biomedical applications such as neuroprosthetic devices. This possible use of GO/GR make them important to determine their bio-compatibility with neuronal cells. However, the biological interaction of neuronal cells such as astrocytes with GR/GO is largely unknown. Here, we have investigated the effect of GR and GO treatment on astrocyte cells to understand the intracellular metabolic pathways affected. Untargeted lipidome analysis based on high-resolution UPLC-Q-TOF-MS allowed us to observe substantial changes in the lipid composition of astrocyte cells treated with GO and GR flakes (compared to their respective vehicles). Dysregulated lipids indicate that glycerolipid metabolism, glycerophospholipid metabolism, cholesterol biosynthesis,and triglyceride metabolism are perturbed by GO/GR. Interestingly, untargeted Lipidomics data was co-related with untargeted proteomics data. These finding provide further insights into the altered lipid profile across a wide range of biochemical pathways in astrocyte upon GO/GR treatment.

Assessment of photocatalytic transformation of pyridinium-based ionic liquids in water.

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Ionic liquids (ILs) are a subject of active research in the field of alternative solvents, being promoted as "green chemistry" replacements to traditional solvents used in industry. The great interest toward these compounds relies on their attractive properties such as low vapor pressures and flammability, chemical and thermal stability, high ionic conductivity, wide electrochemical potential window and ability to behave as catalysts.
Only a few environmental data for these new "green solvents" are presently available, but the low biodegradability and ecotoxicity of some ILs are a potential risk to aquatic and terrestrial ecosystems.
For these reasons, it is necessary to prevent their leakage and to develop effective means of removal and recovery from wastewater, as well as to minimize their occurrence in such a matrix.
In this work, we studied the behaviour of some ionic liquids belonging to the pyridinium class under heterogeneous photocatalysis treatment by the identification of reaction transformation products (TPs). TPs were separated and analysed by HPLC-HRMS using a hybrid LTQ-Orbitrap instrument on the basis of mass defect filtering. In particular, we analysed how the length of the alkyl chain, the kind of inorganic ion and the type of substituents could influence the disappearance rate, the mineralization extent, the acute toxicity and the transformation mechanism. For such, we selected some pyridinium derivatives with different alkyl chain but the same anion, namely tetrafluoroborate (1-ethylpyridium, 1-butylpyridium, 1-hexylpyridimium), with two alkyl substituents (4-methyl-1-butylpyridium) and with a different substituent (1-cyanopropylpyridinium). Then, on a selected IL, we evaluated the role of different inorganic anions (1-butylpyridium with bromine or chlorine). The whole results permit to conclude that irrespective to the alkyl chain or the number of substituents, the transformation involved an attach to the alkyl chain, proceeded through the formation of armless compounds and the mineralization was easily achieved within 4 h. Nitrogen was mainly released as ammonium ion. When introducing a cyano group, the extent of nitrate ions increased and the number of possible transformation routes increased as well. Conversely, the type of inorganic ion deeply affected the transformation pathways and the extent of mineralization. Actually, in the presence of bromide as anion, IL was only partially mineralized and the formation of highly persistent and hazardous transformation products occurred.

Mass Spectrometry Imaging of Paclitaxel distribution in different tumour models: preprocessing and quantification issues

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Abstract

One of the most important issues of pharmaceutical treatment is to understand how well chemotherapeutic drug can penetrate to the tumour tissues and reach all cells in a sufficient concentration inhibiting tumour growth. An important new tool to better understand and improve drug penetration in tumour tissue is MALDI imaging.
A dedicated automated software has been developed to handle the large number of imaging dataset with the aim of distinguish them basing on the differential intra-tumour drug distribution. In this poster we will describe the application of our pipeline for the assessment of the distribution of paclitaxel (PTX), used for the treatment of different solid tumours, in a model of breast cancer (MDA-MB-231). Six tumour bearing mice were sacrificed 4 h after i.v. treatment with 60 mg/kg PTX, tumours were explanted and three slices for each tumour were analyzed by MALDI 4800 TOF-TOF (AB SCIEX, Old Connecticut Path, Framingham, MA 01701, USA) using TiO_2 nanoparticles as matrix with an imaging raster of 100 x100 µm.
The resulting data were analyzed using our new pipeline developed in Python.
The main functionalities of this pipeline are:
a) Data import and visualization from Analyze 7.5 formats .
b) Image processing (automatic identification of peaks starting from Internal standard, background noise removal, normalization, noise reduction, tissue isolation, filtering, segmentation)
c) Quantification of drug concentration in each pixel using a calibration curve for each plate.
MSI allows to understand which area of tumour was reached of the drug and quantify this amount. For PTX quantification both automatic and manual methods were used and results were compared with HPLC analysis. The mean error obtained comparing the automatic method to HPLC results was -20.63%, while the mean error obtained comparing the manual method to HPLC was 97.03% underling the better performance of the proposed pipeline.
Our pipeline will be implemented with more detailed analysis to describe heterogeneity of drug distribution with texture analysis methods in order to quantify precisely the difference in drug distribution in the different models and after different treatments.

Acknowledgements: this project was supported by CARIPLO grant n. 2013/0692

Gold nanoparticles as MALDI MSI matrix for imatinib distribution and quantitation analysis inside tumor tissues

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MALDI-MS imaging is a useful technique to study drugs distribution inside tumor tissues. Recently, the use of nanoparticles as matrices has opened new opportunities reducing background signals in the low mass range and increasing the spatial resolution. Among several nanomaterials, gold nanoparticles (GNPs) have received attention for their suitability, offering large surface areas, simple sample preparation, flexibility, and selectivity of sample deposition. We used GNPs as matrix to study the distribution inside tumors from different cell lines of the tyrosine-kinase inhibitor Imatinib (IMT). A preliminary drug distribution study in different tumor xenografts is presented. Correlating the IMT ion signal intensities to the concentration values of a calibration curve, it has been possible to develop a drug quantitation method by MSI.

Tumor xenografts were obtained from ovarian, lung, gastric and mesothelioma cancer cells, injected subcutaneously in the flank of nude mice. The animals were treated with IMT 400mg/kg p.o. and sacrificed 1h after treatment.

For drug concentration measurement inside the treated tumors, IMT was spotted at increasing concentrations (2-50pmol) on an untreated tissue slice, to build a calibration curve. GNPs of 20nm diameter were synthesized at 0.4mM and homogenously sprayed with D8-IMT as internal standard. Images were generated with an AB MALDI-TOF/TOF 4800, by plotting the sodium adduct of the molecule at *m/z* 516.3. An automatic signal processing algorithm, developed in Python was used to quantify IMT in each pixel after extraction, noise correction, internal standard calibration and normalization.

The GNPs matrix allows to efficiently ionize IMT inside different tumor tissues harvested from treated mice, with a low background signals from matrix degradation and overcoming the problem of the ion suppression effect of the biological matrix. The internal standard homogenous deposition, over the entire acquired area, compensates for the different tissue specific ion suppression effects, laser variability and other factors that may influence signal intensity, allowing reliable measurement of differences in concentration.

The MSI analysis shows how the drug localizes, highlighting the heterogeneous distribution of this drug that, after 1 hour, poorly penetrates inside these tumors. In particular in the mesothelioma model, characterized by highly fibrotic region, the drug distribution is strongly impaired.

IMT concentration was determined correlating the different normalized ion signal intensity in each pixel to the calibration curve using Python pipeline.

GNPs as matrix allow the visualization and quantification by MALDI-MSI of the tyrosine-kinase inhibitor imatinib inside tumor tissues.

A Deep, Combined Omics Exploration of The FAAH Knockout Brain Lipidome

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Fatty acid amide hydrolase (FAAH) is a serine protease which hydrolyzes bioactive endocannabinoids (ECs). ECs are lipid mediators that bind to and activate cannabinoid receptors CB1 and CB2. Upon binding, ECs initiate a number of signaling pathways which control diverse sets of biological functions including memory, learning, social behavior, appetite and inflammation. Modulating the metabolism of ECs by inhibiting FAAH holds therapeutic promise in a wide range of neurological diseases. Since the discovery of FAAH, it has been a very good target for pharmacological studies: many natural and synthetic analogues have been designed to target this enzyme. Unfortunately, in 2016 a tragedy happened in Phase I clinical trial of a FAAH inhibitor, administered at high dose to healthy volunteers. Several questions remain open on the incident. At present, the fatal outcome seems to be drug-related rather than target-related: 11 potential off-targets were identified for the tested inhibitor and some have been recently confirmed by using ABPP. The aim of this study is to explore the changes occurring in a FAAH knockout mice brain using all the most advanced LC-MS tools, including high-sensitivity targeted investigation of the anandamide biosynthetic pathway, ion mobility mass spectrometry based untargeted lipidomics and shotgun proteomics using isobaric tandem mass tag labeling for expression proteomics.

Our targeted lipidomic analysis of anandamide pathway showed a downregulation of its metabolic precursors, that could act as a possible feedback inhibition to finely tune the endogenous levels of anandamide. Using ion-mobility untargeted mass spectrometry, we also discovered the upregulation of some pro-apoptotic ceramides, besides previously known fatty acid ethanolamides and N-acyl taurines. TMT labelled quantitative proteomics experiment generated relative expression data on 243 mouse proteins, out of which the majority of significantly upregulated proteins are related to cell growth, apoptosis, transport processes, cell to cell adhesion and RNA splicing. Our results indicate that, while a pro-apoptotic signaling appears to be ongoing in the lipidome, a corresponding anti-apoptotic counterbalancing action seems to be carried out at protein level. Overall our study gives a deeper understanding of the lipidome and proteome of FAAH knockout mice brain.

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Set up and application of a hydrophilic interaction chromatography-multiple reaction monitoring (HILIC-MRM) method for the detection and quantification of histidine dipeptides in biological matrices

Ettore Gilardoni, Giancarlo Aldini, Luca Regazzoni

Università di Milano

Histidine dipeptides such as Carnosine and its derivatives have proven antioxidant and buffering properties. Recently it was demonstrated that these compounds improve muscle performance during and after high intensity training [1], for these reasons they are common constituents of nutraceutical products and sport supplements.

Histidine peptides are also powerful scavengers of reactive carbonyl species. They can prevent carbonylation processes of macromolecules like DNA and protein, thus mitigating cell damage in some oxidative-based diseases such as diabetes, obesity and atherosclerosis. [2]

However, the analysis of histidine peptides by reverse phase chromatography is challenging since these compounds are extremely hydrophilic. To overcome this problem different authors developed methods using ion paring agent like perfluorobutirric acid, which improves the retention of these analytes [3], although decreasing the sensibility of mass spectrometry detection through an ion suppression mechanism. Recently, other authors developed chromatographic methods based on hydrophilic interaction column (HILIC) to detect histidine peptides in food by using UV detector [4].

Herein, we show the setup of a new LC-MS method for the detection and quantification of histidine dipeptides in human serum. In detail, a HILIC column was used to achieve a good retention and resolution of the analytes. This approach also permits to simplify sample preparation, which consists in an acetonitrile induced protein precipitation, followed by direct injection without additional sample treatment. Mass detection was performed by a triple quadrupole analyser in MRM mode, obtaining a sensitive, reliable and selective method for the quantification of histidine peptides in complex matrices. The method was successfully applied for the analysis of serum samples, characterizing the distribution of histidine peptides in healthy subjects.

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A Deep, Combined Omics Exploration of The FAAH Knockout Brain Lipidome

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Fatty acid amide hydrolase (FAAH) is a serine protease which hydrolyzes bioactive endocannabinoids (ECs). ECs are lipid mediators that bind to and activate cannabinoid receptors CB1 and CB2. Upon binding, ECs initiate a number of signaling pathways which control diverse sets of biological functions including memory, learning, social behavior, appetite and inflammation. Modulating the metabolism of ECs by inhibiting FAAH holds therapeutic promise in a wide range of neurological diseases. Since the discovery of FAAH, it has been a very good target for pharmacological studies: many natural and synthetic analogues have been designed to target this enzyme. Unfortunately, in 2016 a tragedy happened in Phase I clinical trial of a FAAH inhibitor, administered at high dose to healthy volunteers. Several questions remain open on the incident. At present, the fatal outcome seems to be drug-related rather than target-related: 11 potential off-targets were identified for the tested inhibitor and some have been recently confirmed by using ABPP. The aim of this study is to explore the changes occurring in a FAAH knockout mice brain using all the most advanced LC-MS tools, including high-sensitivity targeted investigation of the anandamide biosynthetic pathway, ion mobility mass spectrometry based untargeted lipidomics and shotgun proteomics using isobaric tandem mass tag labeling for expression proteomics.

Our targeted lipidomic analysis of anandamide pathway showed a downregulation of its metabolic precursors, that could act as a possible feedback inhibition to finely tune the endogenous levels of anandamide. Using ion-mobility untargeted mass spectrometry, we also discovered the upregulation of some pro-apoptotic ceramides, besides previously known fatty acid ethanolamides and N-acyl taurines. TMT labelled quantitative proteomics experiment generated relative expression data on 243 mouse proteins, out of which the majority of significantly upregulated proteins are related to cell growth, apoptosis, transport processes, cell to cell adhesion and RNA splicing. Our results indicate that, while a pro-apoptotic signaling appears to be ongoing in the lipidome, a corresponding anti-apoptotic counterbalancing action seems to be carried out at protein level. Overall our study gives a deeper understanding of the lipidome and proteome of FAAH knockout mice brain.

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SCUOLA DI "SPECIALISTA IN RICERCA BIOMEDICA"

Cristina Matteo

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Dott.ssa Cristina Matteo laureata in biologia con specializzazione in neurobiologia e master in genetica oncologica. Attualmente in formazione presso la scuola di "Specialista in ricerca biomedica" nel dipartimento di Oncologia, laboratorio di Farmacologia Antitumorale, unità di Farmacologia Clinica Antitumorale dell'IRCCS – Istituto di Ricerche Farmacologiche Mario Negri, Milano. Mi occupo della messa a punto di metodi analitici e del dosaggio di farmaci antitumorali in diverse matrici biologiche per studi di farmacocinetica clinici e preclinici mediante HPLC-MS/MS.

DEVELOPMENT OF AN HPLC-MS METHOD FOR STUDIES OF CYP-MEDIATED INTERACTIONS INVOLVING THREE DRUGS

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Multi-therapies are common in clinical practice. It is well known that alterations of CYP-mediated drug metabolism by simultaneous administration of other drugs is one of the most common causes of drug-drug interactions. Using in-silico methods derived from network analysis, and based on available data about the CYP-mediated drug metabolism and the drug effects on CYP activities, complex interactions involving at least three drugs were predicted. The clinical relevance of these triplets was then evaluated by interrogating databases of adverse effects, and the interesting triplet including Amiodarone (AMIO), Paroxetine (PRX) and S-Warfarin (WRF) was identified. In this case, the CYP2C9-mediated metabolism of WRF, which terminates the anti-coagulant activity of the drug, may be affected by co-administration of PRX and AMIO, also acting on CYP2C9, resulting in the reported severe vascular hemorrhagic disorders.

The aim of this study was to obtain an in vitro confirmation of the predicted interactions, to support the interpretation of the clinical data. The effects of PRX and/or AMIO on the metabolism of WRF was thus evaluated by incubating combinations of the three drugs with human microsomal preparations (control CYP-Silensomes™ and CYP2C9-silenced Silensomes™, Biopredic International, France).

Different hydroxylated metabolites of WRF were identified by high-resolution mass spectrometry (HRMS), using an Orbitrap instrument operating in ESI negative ion mode. Subsequently, an analytical method based on liquid chromatography - triple quadrupole mass spectrometry, in Multiple Reaction Monitoring mode, was developed to measure the concentrations of WRF and its main metabolites, using the specific fragment ions identified by HRMS. Since it was not possible to determine the absolute concentrations of the metabolites, because of the lack of reference standards, the data were expressed as absolute peak areas, normalized to the peak area of the used internal standard (Naproxen).

Results showed that both PRX and AMIO decreased WRF metabolism in an additive manner, as indicated by the different amounts of WRF hydroxylated metabolites that were found during in-vitro experiments. These data are consistent and explain the adverse vascular hemorrhagic effects reported in patients receiving combined administration of WRF, AMIO and PRX.

STUDIO DI EQUILIBRI DI COMPLESSAZIONE DI METALLI DELLA 1^{A} SERIE DI TRANSIZIONE MEDIANTE ESI-MS

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Gli equilibri in soluzione dei complessi di coordinazione dei metalli di transizione, con i loro differenti gradi di sostituzione, il grado di solvatazione e la presenza di controioni nella prima sfera di coordinazione, è una tematica che ha sempre avuto un ruolo predominante nella chimica di coordinazione. Le ricerche in questo campo, tuttavia, sono sempre state limitate a prove indirette ed indiziali dell'esistenza di una specie; le spettroscopie in generale diventano di complessa interpretazione in presenza di un gran numero di equilibri simultanei, mentre l'individuazione delle specie tramite le analisi di stato solido come la diffrazione con i Raggi X permette di caratterizzare solamente i complessi che hanno una stabilità tale da avere una esistenza nello stato solido e cristallizzare. Da una ventina di anni, l'avvento delle sorgenti ESI (Electronspray ionization) associate alla spettrometria di massa ha permesso di compiere analisi dettagliate su un gran numero di specie inorganiche ed organometalliche e sulla loro multiforme chimica in soluzione [1]. Tuttavia, le problematiche dell'ESI-MS sono complesse, ed è necessario uno studio accurato per distinguere gli effetti dovuti alla fase gas dai veri equilibri in soluzione. Questi problemi sorgono perché le condizioni della soluzione (temperatura, concentrazione, pH etc) vengono modificate nel processo di ionizzazione e perché gli ioni possono subire varie reazioni in fase gas prima di raggiungere l'analizzatore [2]. Questa perturbazione deve essere presa in considerazione quando si cerca di correlare gli spettri di massa ESI con la soluzione; in casi specifici tuttavia si è potuto persino ottenere le costanti di formazione dei complessi dalle intensità dei picchi relativi [3]. Il lavoro che presentiamo tratta di uno studio compiuto con ESI-MS sulla coordinazione dei metalli della prima serie di transizione dal Mn allo Zn in presenza di due equivalenti di 2,2'-bipiridina, focalizzato allo studio della formazione del complesso di stechiometria $[M(bipy)_2]^{n+}$ per una successiva addizione (o sostituzione) da parte del dicianoaurato $[Au(CN)_2]^-$. L'analisi di differenti solventi e differenti controioni ha permesso di isolare alcuni effetti realmente presenti in soluzione dagli artefatti dovuti all'estrazione in fase gassosa, e perciò dare risposte rivolte all'ottimizzazione della sintesi di aggregati supramolecolari eterobimetallici basati sulle interazioni aurofiliche, materiali di grande interesse per la sensoristica e per l'ottica di alta tecnologia [4]. Si è quindi dimostrato come l'ESI possa essere di grande aiuto nella progettazione di sintesi di materiali complessi formati da frammenti che danno luogo ad equilibri multipli, permettendo di analizzare gli effetti dei vari parametri sperimentali sulle unità di crescita che formano di conseguenza i composti finali.

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Title (120)

Leptin identification and quantification in human milk and infant formulas.

Introduction (120)

Leptin is a small protein (14 KDa) present in plasma and milk. It plays a key role in the regulation of body weight and its concentration is affected by many physio-pathological parameters especially body weight variation and lipodystrophic conditions. Leptin is encoded by the obesity gene on human chromosome 7 and it is mainly secreted by adipocytes. It is possible that serum leptin concentration in breastfed infants is associated to early adipose tissue production and to the leptin levels of milk. In order to understand the role of different newborn feeding (breastmilk, formulas, bovine milk) on infant leptin production and risk of obesity, we aimed to develop a selective LC-MS method to evaluate leptin content in different milk matrices.

Methods (120)

Human milk and infant formulas were provided from local pediatric hospital. In order to detect leptin concentrations <0.1 fM, immunoaffinity extraction was performed. A Thermo Ultimate 3000 nanoLC system Hyphenated to an Orbitrap Fusion high resolution MS and ETD source was utilized. The developed method was compared to a classical SRM quantitation by LC-Qtrap analysis (using a Sciex 5500 Qtrap coupled with Shimadzu Nexera UFLC). C4-RP columns were used with a linear gradient from 5% mobile phase (acetonitrile with 0.1% formic acid) to 60%.

Preliminary data (300)

At first human and bovine leptin were extensively characterized by high resolution mass spectrometry in order to evaluate interferences. Full MS and CID/ETD MS/MS spectra of fifteenth fold charged positive ions of standard proteins were acquired. A top-down approach was used with the aim of reduce to a minimum sample manipulation. Accuracy, precision, LLOQ, linearity, extraction recovery and stability were then assessed. The comparison between nanoLC-Orbitrap HRMS analyzer and UFLC-Qtrap analyzer is at present under evaluation. The method was then applied to investigate about the presence of leptins in about twenty different commercial infant formulas. Finally mean concentration in a small series of plasma and milk samples was determined in order to establish correlations between mother and Infant Body Mass Index. Data acquisition of new samples for statistical purposes is being implemented.

Novel aspects (20)

Development of novel methods to evaluate quality of Infant formulas by top-down protein analysis.

E-cigarettes emissions. Critical aspects in producing and reporting data as from the EC Tobacco Product Directives requests.

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The WHO Tobacco Free Initiative recommends parties to propose guidelines for testing and measuring the contents and emissions of tobacco products and Article 10 recommends to implement effective legislative, executive, administrative measures requiring tobacco companies to disclose contents and emissions of tobacco products. Article 20 of the Tobacco Product Directive (TPD) requires that also e-cigarettes undergo the regulations on ingredients and emissions adopted for tobacco products. Accordingly, the TPD included several Articles to regulate ingredients and emissions of various tobacco products and to obliged tobacco manufacturers to declare all ingredients and additives being used in tobacco product. In particular, Article 7 prohibits the placing on the market of those products containing additives or that have CMR properties in unburnt form.

For these reasons manufacturers must declare e-liquids and emissions components. While direct analysis of liquids does not present particular difficulties, no standardized methods are available to determine emission components. Mass spectrometry probably is the most accurate method but large uncertainties relies in emissions.

We present here the development of a GC/MS method to produce and sample vapours from different e-cigarettes and a critical evaluation of results from a large number of available e-liquids emissions. In our preliminary analysis, we analyzed about 200 emissions by as many samples with 3 different e-cig hardware. Our results confirmed formation of toxic substances like acrolein, formaldehyde, diacetyl and acetaldehyde. Furthermore, our results showed as these molecules ranged depending on the used hardware and the parameters of hardware itself.

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BISPHENOL A AND BISPHENOL S DETERMINATION: AN UPLC/MS-MS APPROCHE

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Plastic objects are very common: bottles, food containers, CD, DVD, medical devices and toys are indispensable in our life, plastic furthermore is often used like coating for her properties. Sometimes is necessary add to the plastic polymers some molecules in order to modify the rheological properties: the more common modifiers are phthalates and bisphenols. Unfortunately the technological requests are in contradiction with the public health. Many studies link an endocrine system's damage to a bisphenols exposition. These molecules simulate the estrogens activity, it produces an alteration to lipogenesis, insulin level and fluids retention control.[1]

Aim of this work is determinate a very sensitive and quick UPLC/MS-MS method in order to quantify bisphenol A and bisphenol S (the more common ones) in urine and breast milk. For the urine we modify a DLLME extraction based on the use of an extractive solvent (chloroform, and a dispersing solvent, acetone, coupled.[2] For the milk samples we use an SPE extraction on C18 stationary phase.

The chromatographic separation consist in an only five minutes gradient on a Phenomenex Luna Omega C18 (2.1*100*1.6) column.

Aid by a triple quadrupole mass spectrometer, Qtrap 5500, we are now able to reach a very low LOQ for both the analytes 10 ng/L for bisphenol S and 100 ng/L for bisphenol A, considering the reconcentration factor we can associate a visible signal respectively to 0.5 ng/L and 5 ng/L of this molecules. The method furthermore is full validated and tested on real sample.

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MORINGA OLEIFERA: THE MIRACLE TREE

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Moringa oleifera Lam is one of the best known and most widely distributed species of the monogeneric family Moringaceae which includes 12 other species belong to the Brassicales order.[1] This plant is native of the western and sub-Himalayan tracts, India, Pakistan, Asia Minor, Africa and Arabia.

Moringa leaves have been reported to be a rich source of β-carotene, fatty acid Ω-3 and Ω-6, protein, vitamin C, calcium, potassium and act as a good source of natural antioxidants due to the presence of antioxidant compounds such as flavonoids, phenolic and carotenoids. So this plant , especially its leaves, can be used as remedy against the malnutrition, by enriching some food supplies. Anyway the nutritional value of Moringa oleifera leaves may vary with cultivar and source. [2,3]

Furthermore a lot of medical properties are ascribed to various parts of the tree: root, bark, leaf, fruit, pods, flowers, seed; which are used for many ailments in the indigenous medicine of South Asia, including the treatment of inflammation, infectious diseases, cardiovascular, gastrointestinal disorders.[4,5]

In the present study seven *Moringa oleifera* leave samples from different geographic areas were analysed. The aim of this work is to compare the different Moringa samples, in term of polyphenolic, glucosinolates content, and in term of nutritional properties taking into account the amount of proteins, starch and sugars. Our goal is to identify the best samples in term of nutritional and therapeutic properties.

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Assessment of the photocatalytic transformation of sweeteners in different water matrices

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Artificial and natural sweeteners are additives often used in food and beverages as sucrose substitutes. However, these sweeteners have recently been recognized as a group of emerging environmental contaminants and their increased global consumption have led to their ubiquitous occurrence in the environment. Their chemical stability leads to a scarce elimination in conventional treatment systems and their subsequent release into water bodies; therefore, they are often detected at levels above 1 μg L^{-1} in river and lake waters. Stevioside and acesulfame K are two examples of respectively natural and artificial sweeteners used in foods. In particular, acesulfame is recognized as one of the most persistent sweeteners, with concentrations in the municipal wastewater treatment plants (WWTPs) effluents reported to be up to 46 μg L^{-1} [1], and average effluent concentration reported up to 76.1 μg L^{-1} in Europe [2]. Several studies proved that acesulfame is resistant to degradation by microorganisms in WWTPs [3] and to hydrolysis under natural environmental conditions [4], so implying a constant accumulation of acesulfame in the aquatic environment.

We investigated the photocatalytic transformation of these two sweeteners by: *i)* following their disappearance kinetics; *ii)* identifying their intermediate compound; *iii)* assessing mineralization and *iv)* evaluating acute toxicity. The photocatalytic transformation of both sweeteners was studied under simulated solar irradiation using different semiconductor oxides. The analyses were carried out by high-performance liquid chromatography (HPLC) coupled with a LTQ-Orbitrap analyzer via an electrospray ionization (ESI) in both positive and negative mode. Stevioside degradation led to the formation of more than one hundred unknown transformation products (TPs), most of them in the form of several isobaric species, while 13 species were identified during acesulfame abatement. By employing accurate mass determination, we were able to attribute an empirical formula to each species and through MS^n analyses we were capable to distinguish several isobaric species. The use of several semiconductors has pointed out differences in terms of both their photocatalytic efficiency and the sweeteners degradation mechanisms: the assessment of the evolution kinetics of each species (TPs, total organic carbon and inorganic ions) has brought to the elaboration of general transformation pathways of acesulfame K and stevioside.

Then we investigated the effect of various aqueous matrices (distilled water, river water and lake water) on the degradation rate of the sweeteners. A fully nested two-level experimental design was employed as a tool to investigate about the effect of various aqueous matrices, as well as the initial sweetener concentration on the variation of the photocatalytic efficiency.

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Stage in Spettrometria di Massa, Università Urbino

Alice Ludovica Scaduto

Sono una studentessa della facoltà di Biotecnologie dell'Università di Urbino Carlo Bo.

Sto svolgendo un tirocinio formativo presso il laboratorio di Spettrometria di massa dell'Istituto di Ricerche Farmacologiche Mario Negri di Milano insieme al Dr. Enrico Davoli e alla Dott.sa Silvia Giordano e mi occupo di imaging con spettrometria di massa. Il periodo dello stage richiesto è di 150 ore durante le quali dovrò effettuare attività di laboratorio, volta alla identificazione di un ambito di ricerca per la mia tesi.

Regional scale modelling of PCBs from a highly contaminated site in Northern Italy

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Brescia is a city located in Northern Italy which suffers the impact of its large scale industrial development on the surrounding environment. This development started at the beginning of 1900 when different industries such as foundry, steel, mechanical, weapon and chemical grew up. Among chemical industries, Caffaro S.p.a. produced PCBs for about 50 years (1930-1984) and its surrounding areas were found to be heavily contaminated with high concentrations in soil at mg/kg levels. For this reason this area was declared National Relevance Site (SIN) for remediation by the Italian authorities. The aim of the present study was to investigate the potential of the contaminated area in driving the PCB contamination at regional scale up to about 100 km from the point source and the current effects on air concentrations. Different sampling campaigns were organized to collect samples of soil and leaves along four 100 km transects (one sampling point every 7 km) that ran in NW, NE, SW and SE directions considering the production plant as starting point. Woods soils were chosen to avoid to collect samples altered by tillage activities. Leaves were sampled above the corresponding soil sample. In each sample the following PCB congeners were determined with GC-ECD and GC-MS: PCB 28, PCB 52, PCB 101, PCB 153, PCB 138, PCB 180 and PCB 209. PCB 209 was included because it can be considered a marker of Caffaro contamination, the only world producer of Fenclor DK, a technical grade decachlorobiphenyl mixture. The results were analyzed to understand the presence of a spatial gradient of decreasing pollutant concentration with distance from the source. Fingerprint data were used to run a number of simulations with a dynamic air-vegetation-litter-soil model in order to 1) predict the order of magnitude of fluxes at each point considering the actual soil and leaf concentrations, 2) understand the source strength in order to predict a temporal emission profile from the site, 3) evaluate the importance of other sources and processes involved in the contamination at a regional scale.

Identification of circulating endothelium-protective factors

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BACKGROUND: In literature many sources report that dog is not a good model for atherosclerosis because it is not susceptible to atherogenesis even under a high-cholesterol diet. Therefore, given the pivotal role of the endothelium in atherogenesis, we have hypothesized the presence of protective factors in the plasma of dog that may reduce the risk of endothelial dysfunction under stress condition. Identification and isolation of the circulating endothelium-protective factors can provide the basis for the development of new drugs against atherosclerosis.
METHODS: Plasma from bovine, pig, dog and human was filtered at 50K and 3K. Human plasma samples were separated in two groups: old human plasma from healthy subjects over 65 years old and young human plasma from healthy subjects under 30 years old. Human aortic endothelial cells (HAEC) were treated with α-tumor necrosis factor (α-TNF 10ng/mL) protein for 24 hours in order to create the experimental conditions that lead to endothelial damage. The different cellular response to the treatment has been evaluated by western blot, through the analysis of VCAM-1 (vascular cell adhesion molecule) expression.
Liquid Chromatography-Mass Spectrometry (LC-MS) was used to identify the endothelium-protective factors in 3K plasma by peptidomic and lipidomic analysis.
HAEC were then treated with stearamide at 0, 0.1 and 1 μM in the presence of 3K bovine and dog plasma. The different cellular response to the treatment has been evaluated by western blot, through the analysis of eNOS (endothelial nitric oxide synthase) expression.
RESULTS: Data showed that the endothelial damage is statistically more effective in cells subjected to the 50K plasma of bovine and old human compared to that one subjected to dog, pig and young human. The endothelial protection is still present in cells subjected to 3K dog, pig and young human plasma.
LC-MS analysis revealed the unexpected presence of amoxicillin in pig plasma. This drug is an exogenous contaminant that was inadvertently administered to the animal. This demonstrated the high sensibility of the method applied.
Lipidomic analysis showed the high levels of stearamide and arachidic amide in bovine plasma. We hypothesized that these molecules may contribute to cause endothelial damage. Indeed cells treated with stearamide in the presence of dog plasma were protected from the endothelial damage compared to that one exposed to bovine plasma.
CONCLUSION: Data confirm the presence of endothelium- protective factors in 3K filtered dog, pig and young human plasma. Proteins should not be involved to exert the endothelium- protection property in HAEC cells, since they were removed by 3K filtration.
High levels of stearamide were found to be a damaging factor for endothelial cells. In 3K bovine plasma stearamide can contribute to its endothelial anti-protective role. Peptidomic and metabolomics analysis merits further evaluation in order to identify the circulating endothelium-protective factors.

Leaching of weathered polychlorinated biphenyls (PCBs) obtained from a contaminated site: role of dissolved organic carbon and saturation conditions in a soil column experiment

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Exposure assessment in contaminated soils is usually performed by the evaluation of the total residual concentrations of target pollutants in soils (e.g. ∑PCBs). This approach does not take into account the actual mobility and bioavailability of the contaminants and may be inappropriate especially for historically contaminated soils where sorption processes may be scarcely reversible due to the presence of bound/weathered residues. Therefore, assuming that equilibrium conditions would occur using standard partitioning coefficients (e.g. Koc/Kd) may lead to misleading results in modelling attempts. Therefore, the estimation of the contaminant fraction available for leaching and transport in different soil conditions is recommended. During the last decades, several authors studied the release of pollutants from soil by using soil column leaching tests but the laboratory experiments were often performed in scarcely realistic conditions (e.g. fresh spiked contaminants). Additionally, often some variables influencing the mobility of contaminants were neglected, as well as the statistical scheme was poor (e.g. lack of replicates). In this context, a soil column leaching experiment was performed to evaluate: 1) the effects of dissolved organic carbon (DOC) content in the leaching solutions, 2) equilibration time, 3) soil saturation conditions on leaching fluxes of selected weathered PCBs (PCB 28, 52, 101, 138, 153, 180, 209) present in an historically contaminated soil. These effects were evaluated collecting leached samples at different contact time (2, 5, 7, 48 days), in flow vs. no flow conditions and in saturated vs. field capacity conditions. The results show that the most influential factors are the DOC content in non-equilibrium conditions (flow condition samples) and the soil saturation conditions.

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Gd-retention in rat's brain: assessment of the amounts of insoluble Gd-containing species and intact complex in rat's brain upon repetitive administrations of GBCAs

Introduction

Gadolinium-based contrast agents (GBCAs) are routinely used in many clinical magnetic resonance (MR) imaging studies. Despite the fact that these chemicals are considered to be extremely safe for human clinical use, recently, a renewed interest on the possibility they may cause adverse effects, came out because a number of studies reported the occurrence of an unexpected hyperintensity in unenhanced T1-weighted MRI of the brain in patients that have been previously administered with multiple doses of GBCAs were published. In the cases where post-mortem histology studies have been carried out, it has been established that the observed hyperintensity correlates well to the presence of gadolinium.

The assessment of the chemical forms Gd is present in the brain tissues is of paramount importance to get more insights into the potential toxic effects as well on the possible interventions to promote its removal.

Methods

A total dose of 13.2 mmoles/Kg of GBCA (Omniscan and ProHance) were administered to healthy rats over a period of 8 weeks. Three days after the last administration, the rats were sacrificed, the brains excised and divided into three portions. Each portion homogenate has been divided in two parts, one for total Gd determination by ICP-MS and one analyzed for the determination of the amounts of intact GBCA and of Gd-containing insoluble species. Separation and quantification of the intact GBCAs was carried out using UPLC-ESI-MS coupled coupled with ACQUITY QDa Mass Detector. An UPLC-BEH-HILIC column (2.1mm×100mm; 1.7 mm particle size) was used by isocratic elution with mobile phase A (ammonium formate 12.5 mM, formic acid 12.5 mM, pH 3.75) set at 76% and mobile phase B (acetonitrile) set at 24% at flow rate of 0.6 mL/min and a column temperature of 40 °C. Injection volume 2 µL or 4 ml.

The total Gd found in the three brain regions of the animals treated with Omniscan was 6.6-12.8 times the amounts found in the corresponding regions of rats treated with ProHance. 100% of Gd found in the case of treatment with ProHance corresponds to intact Gd-HPDO3A. In the case of rats treated with Omniscan, the largest part of Gd-retained in the brain's structures corresponds to insoluble species. In cerebellum, the amount of intact Gd-DTPA-BMA accounts for 18.2±10.6% of the total Gd. The mass balance found for Gd implies the occurrence of other soluble Gd-containing species (ca. 30%). Support to the view that part of the released Gd may be taken up by the polysaccharide component present on the membranes of brain cells parenchima, has been gained by acquiring relaxometric measurements of Gd-DTPA-BMA and $GdCl_3$ in the presence of Poly-syalic acid.

Conclusion

Relevant information on Gd-retained as insoluble/soluble species and as intact GBCA has been gained.

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In the last decades the study about human diseases have evidenced some new key players: the lipids. These latter were recognized not only as the bricks of cellular membrane, but also, and more notably, as the fundamental actors in a broad range of biochemical processes, such as calcium homeostasis and membrane trafficking [1, 2].

It was recently understood that aberration in lipid metabolism caused many human diseases, and the most studied pathologies related to a high fat content diet are obesity and prediabetes syndromes [3, 4]. In particular ceramides and phosphosphingolipids, both belonged to the sphingolipids family, are involved in such disorders [5]. There are four major pathways to synthetize ceramides that involve many enzyme families: from glycosphingolipids, from sphingomyelin, from ceramide-1-phosphate and finally a *de novo* synthesis that involves serine and palmitate. Phosphosphingolipids are metabolized from ceramide, through the alkanolamine sphingosine (2-amino-4-octadecene-1,3-diol). All of the mentioned enzyme-catalyzed reactions are reversible.

Pathological diseases disquiet the enzymatic equilibrium between ceramides, sphingosines and phosphosphingolipids leading to oxidative stress and metabolic cellular damage. Some vitamins, such as pyridoxamine, are able to bring back the disequilibrium.

In our project livers from mice fed with standard (SD), standard plus pyridoxamine (SD+P), high fat (HFAT) and high fat plus pyridoxamine (HFAT+P) diets (Table1) were analyzed using UPLC coupled with a Q trap mass spectrometer (MRM mode). We focused on a peculiar biomarker of hepatic steatosis, the sphingosine-1-phosphate, and monitored its concentration in different diets with the aim to characterize lipidomic of prediabetic disease.

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