TERAMO - 27 MAGGIO 2022

book of abstract

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Scientific Program - Friday, May 27 - Morning Session

Time	Program
10:00	*Registration*
10:30	*Welcome and opening ceremony - Fulvio Magni*
	Plenary Lecture
10:40	*MS-based proteomics: empowering MS-data analysis to address animal infectious diseases* Bruno Tilocca - University "Magna Graecia" of Catanzaro
	Oral Session
11:10	Mass spectrometry-based proteomics to reveal brain signatures of an in vivo model of multiple sclerosis Maurine Fucito - University "G. d'Annunzio" of Chieti-Pescara
11:28	Identification of known and putative allergenic proteins of Tenebrio molitor by liquid chromatography coupled with electrospray ionization and tandem mass spectrometry Mariachiara Bianco - Università degli Studi di Bari Aldo Moro
	Coffee break
12:10	*Application of a GC-qMS method for the determination of atmospheric polycyclic aromatic hydrocarbons (PAHs) deposition flux* Giuseppe Ianiri - University of Molise"
12:28	Use of pure materials to improve background in rare event searches: characterization of plastic samples performed by High Resolution Inductively Coupled Plasma Mass Spectrometry Francesco Ferella - INFN, University of L'Aquila
	Flash Communications
12:46	Proteomics of sorted Leukocyte-derived Extracellular Vesicles in tears as "liquid biopsy" reflecting neuroinflammation in Multiple Sclerosis Maria Concetta Cufaro - University "G. d'Annunzio" of Chieti-Pescara
12:51	*BAG3 in glucose homeostasis: proteomics characterization of BAG3 knockout in pancreatic β-cells* Federica Di Marco - University G. D'Annunzio of Chieti-Pescara
12:56	*Targeting proteomic networks in primary microglia in Alzheimer's disease: new targets for understanding its pathogenesis* Daniel Tortolani - European Center for Brain Research (CERC)/Santa Lucia Foundation IRCCS - Rome
13:01	*Evaluation of plasma metabolomic profile by exploiting the synergy between MS and NMR* Gaia Di Francesco - Sapienza university of Rome
13:06	*Chemical profiles and biological activities of extracts obtained from* Viscum album L. *(Mistletoe)* Lorenza Marinaccio - University G. D'Annunzio of chieti-Pescara
13:11	*Cannabinoids profile in* Cannabis sativa L. *samples by means of LC-MRM/IDA/EPI analysis: a new approach for cultivar classification* Sara Palmieri - University of Teramo
13:16	*Development of a new liquid phase micro-extraction method for the determination of drugs in oral fluids by HPLC-MS / MS analysis* Martina Croce - Sapienza University of Rome
13:21	*Multi-experiment approach analysis for derivatized short chain fatty acids by means LC-MS/MS* Eduardo Viteritti - University of Teramo
	lunch break

Scientific Program - *Friday, May 27- Afternoon Session*

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14:30	LC-MS/MS analysis of phenolic compounds in plant matrices: a multi-experiment approach Fabiola Eugelio - University of Teramo
14:48	Double bond location and geometry assignment in unsaturated fatty acyl chains of drying oils: the interplay between RPLC-ESI-FTMS/MS and m-CPBA epoxidation Davide Coniglio - University of Bari Aldo Moro
15:06	Untargeted metabolic profiling of "Sulmona red garlic" by FT-ICR mass spectrometry Alba Lasalvia - Sapienza University of Rome
	break
15:40	Development of an LC-MS/MS method for the determination of uric acid in bones Chiara Maccari - University of Parma
15:58	IRMPD action spectroscopy of bare deprotonated genistein, a natural antioxidant isoflavone Lucretia Rotari - Sapienza University of Rome
16:13	*Closing remarks*

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Plenary Lecture

MS-based proteomics: empowering MS-data analysis to address animal infectious diseases

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Keywords: *mass spectrometry; data analysis; proteomics; metaproteomics; computational mass spectrometry; animal infectious diseases*

Latest technological advancements in the field of Mass Spectrometry (MS) enabled its massive diffusion under the diverse field of application including the omics sciences with proteomics and metabolomics being the major users of this groundbreaking analytical approach. Since then, several variants of the sample preparation protocol, mass spectrometry and data processing have been applied to elucidate diverse facets of the proteomics, ranging from protein/peptides identification and quantification until elucidating protein-protein interaction and Post-Translational Modification and protein folding assessment.

MS-based proteomics can be broadly divided into Bottom-Up (shotgun) and Top-Down approaches. Shotgun proteomics is particularly suited for the identification and quantification of known and novel biomarkers. On the other hand, Top-Down proteomics, dealing with intact proteins is commonly employed for the thorough elucidation of the protein PTM and 3D-folding, among others.

As of today, the development of powerful separation devices and cutting-edge mass spectrometer technologies warrant analytical performance at unprecedented levels. This generates an impressive amount of data, which appropriate handling is of paramount importance for a deep understanding of the biological systems. Nevertheless, no standardized procedures for the bioinformatic analysis of the MS data are available, opening the research towards the optimized tuning of the MS instrument settings and data processing steps aimed at maximizing the recovery of the biological information held in the MS data. Here, an outline for some of the commonly used setups and the key concepts exploited while improving protein identification and quantification rates are provided along with two illustrative cases of MS-based proteomics in the study of the *Brucella melitensis* immunoproteome [1] and the cheese metaproteomics [2].

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Oral Presentations

Mass spectrometry-based proteomics to reveal brain signatures of an in vivo model of multiple sclerosis

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Keywords: *Brain proteomics, neurodegeneration*

Mass spectrometry-based proteomics, the large-scale study of proteins expression using mass spectrometry, is widely used to investigate molecular pathways and to find biomarkers for complex and heterogeneous neurodegenerative diseases such as multiple sclerosis [1]. As a matter of fact, proteins have several important biological functions and play a crucial role as signaling pathways mediators. Moreover, proteins are regulated by reversible phosphorylation needed for cell life, while abnormal phosphorylation causes or results from diseases [2]. In this context, we used an elegant analytical strategy, highlighted in figure 1, consisting in a high pH (HpH) reversed-phase fractionation approach [3] coupled to tandem mass tag (TMT) mass spectrometry-based proteomics to quantify non-modified and phosphorylated proteins. We performed such approach on the corpus callosum region of extracted brains from a focal experimental autoimmune encephalomyelitis (EAE) in vivo mouse model. Five conditions were chosen namely non-immunized, immunized, immunized with a focal injection of cytokines, immunized with a focal injection of phosphate-buffered saline and treated with minocycline. All experiments were conducted in triplicates. Data allowed to shed light on proteins altered in the different conditions, followed by a functional analysis using Ingenuity Pathway Analysis highlighting molecular pathways involved in the downregulation of the nervous system development and in the upregulation of the inflammatory response.

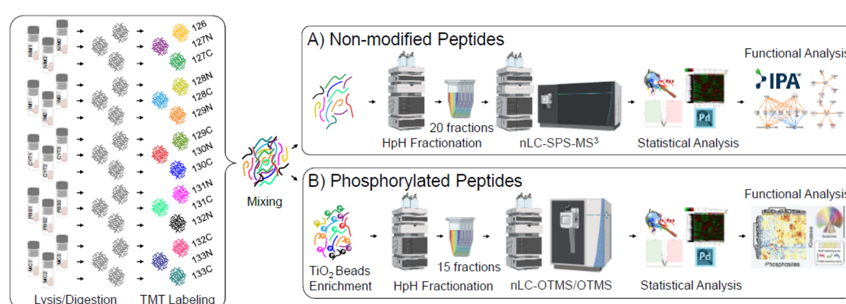

Figure 1: Summary of the mass spectrometry-based proteomics analytical strategy used to separate, identify, and quantify the non-modified and phosphorylated peptides. TMT: Tandem Mass Tag; HpH: High pH; nLC: nano Liquid Chromatography; SPS: Synchronous Precursor Selection; OTMS: Orbitrap Mass Spectrometry.

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Identification of known and putative allergenic proteins of Tenebrio molitor by liquid chromatography coupled with electrospray ionization and tandem mass spectrometry

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Keywords: *allergens, insects, novel food.*

The problem of food allergies is becoming critical in recent years, especially regarding the consumption of novel foods [1], which are mainly represented by imported foods, such as algae, microorganisms, insects, and others. Tenebrio molitor is an insect species largely consumed in Asian countries. Insects are a nutrient-dense food, very similar to meat, rich in proteins and fats, especially polyunsaturated fatty acids such as ω-3, but also contain essential minerals and some vitamins [2]. For this reason, very recently the European Union (EU) authorized the marketing of insects and derived flours in different forms such as dried and processed foods such as snacks. The possible side effects of these foodstuffs should be carefully assessed since insects belong to the same phylum (Arthropoda) of crustaceans, which are among the 14 most allergenic foods declared by the EU. Several cases of food allergy linked to insect species intake are reported in Africa and China where insects have been widely consumed for a long time. Insect-related symptoms are divided into three categories: cutaneous (e.g., hives, itching, angioedema), gastrointestinal (e.g., abdominal pain, nausea), and respiratory (e.g., asthma, breathlessness) [3]. Besides, cases of severe triggering symptoms, such as anaphylaxis, have been reported. [4]. In this communication, we focused on the identification of known allergenic proteins and/or putative ones by liquid chromatography-tandem mass spectrometry with electrospray ionization. The strategy adopted was based on first recognizing the T. molitor proteome using different protein extraction methods, and then proceeding to allergens identification according to FAO/WHO guidelines using an in-silico approach [5]. A great number of putative allergenic proteins of T. molitor, exhibiting an identity percentage higher than 70% with known allergens, mostly belonging to other Arthropoda, species were recognized.

This work was supported by Progetto di Ricerca di Interesse NazionalePRIN 2017YER72K—"Development of novel DNA-based analytical platforms for the rapid, point-of-use quantification of multiple hidden allergens in food samples", financed by the Italian Ministero per l'Istruzione, l'Università e la Ricerca (MIUR).

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Application of a GC-qMS method for the determination of atmospheric polycyclic aromatic hydrocarbons (PAHs) deposition flux

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Keywords: *Polycyclic aromatic hydrocarbons(PAHs); GC-qMS; public health*

La determinazione del flusso di deposizione degli idrocarburi policiclici aromatici diffusi in atmosfera rappresenta uno dei principali indici di monitoraggio della qualità dell'aria di una determinata zona territoriale. Gli idrocarburi policiclici aromatici sono di solito adsorbiti e/o legati chimicamente alle polveri atmosferiche. Quest'ultime, principalmente suddivise in PM_{10} e $PM_{2,5}$, possono depositarsi al suolo e trasportare con esse molteplici molecole contaminanti tra cui gli idrocarburi policiclici aromatici. Come conseguenza, tali molecole possono passare attraverso il suolo, le piante e gli animali nella catena alimentare e giungere all'uomo, causandone un danno per la salute. Il lavoro presentato riassume l'applicazione di un metodo idoneo al campionamento, all'estrazione, all'identificazione ed alla quantificazione dei seguenti idrocarburi policiclici aromatici; Benzo[a]antracene (BaA), Benzo[b]fluorantene (BbFA), Benzo[j]fluorantene (BjFA), Benzo[k]fluorantene (BkFA), Benzo[a]pirene (BaP) e Indeno[1,2,3-cd]pirene (IP). Per campionare le polveri che si depositano al suolo, viene utilizzato un deposimetro, costitutito da una bottiglia in vetro pyrex da 10 L e da un imbuto cilindrico sovrastante, anch'esso in vetro pyrex. Il campionamento dura 30 giorni e le deposizioni secche (DS) ed umide (DU) raccolte rappresentano il campione di partenza. Gli idrocarburi policiclici aromatici contenuti nella DS e nella DU vengono estratti con diclorometano (CH_2Cl_2) rispettivamente in bagno ad ultrasuoni ed in imbuto separatore. I recuperi medi ottenuti da prove di estrazione vanno dall'80 al 120 %. Gli estratti ottenuti vengono combinati e concentrati in evaporatore rotante ad una pressione di 1013 mbar fino ad un volume di 5 mL. Per rimuovere la deposizione umida eventualmente residua, l'estratto viene fatto percolare su una colonnina impaccata con sodio solfato anidro (Na_2SO_4) e successivamente portato ad un volume finale tra 80-100 µL attraverso l'utilizzo di azoto gassoso (N2). L'analisi strumentale viene condotta in GC-qMS. Gli analiti vengono in primis separati in gascromatografia (GC) utilizzando una colonna capillare e successivamente rivelati grazie ad uno spettrometro di massa (MSD) equipaggiato con un analizzatore a singolo quadrupolo (qMS). In conclusione è stato utilizzato il Benzo[a]pirene deuterato (BaP-d12) come standard surrogato e il Benzo[a]antracene deuterato (BaA-d12) come standard interno per quantificare gli analiti presenti nell'estratto.

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Use of pure materials to improve background in rare event searches: characterization of plastic samples performed by High Resolution Inductively Coupled Plasma Mass Spectrometry

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Keywords: *ICP-MS. samples characterization*

In phase of planning and setting-up of an experiment where a low background is required, the choice of materials with high radiopurity appears to be necessary; for each component to be installed it's needed to evaluate his radiopurity to maintain a very weakly radioactive environment. Regarding astroparticle physics experiments, low background can be reached by placed infrastructures where detectors are located in an underground enrivoment (e.g. Gran Sasso National Laboratories), furthermore it also necessary to use of materials with low level of contamination appears to improve low background [1].

Natural radioactivity is present in different elements (Th, U, K, Pb), they have unstable radioactive isotopes that undergo decay processes; their presence in materials used for experiments must be kept under control, in order to determine purity degree and any use. High-Resolution ICP-MS was used to determine amount of impurities regard to natural radioactive elements, lower detection limits were reached improving our analysis. A characterization of different type of plastic material was performed to search impurities inside samples and to monitor contamination level [2]. Search of materials with low level of contamination will be discussed to make a preliminary screening related to materials used for different experiments.

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LC-MS/MS analysis of phenolic compounds in plant matrices: a multi-experiment approach

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Keywords: *Polyphenols, Quantitative analysis, Putative identification*

Polyphenols (PCs) are natural occurring secondary metabolites with significant physiological importance in plants and different biological functions on human health: they are known for their potential in the prevention and treatment of diseases related to oxidative stress. [1].
PCs are generally grouped into two main categories based on their structure and properties: non-flavonoid compounds, such as phenolic acids, stilbenes, lignans, and flavonoids.
Due to their complexity, a complete analysis of this compound is a challenging task, despite numerous analytical approaches are used for the determination of PCs in plants, such as liquid chromatography (LC), generally coupled to different detectors as UV-vis, DAD and MS. With LC-MS it is possible to work either in targeted mode [3], generally coupled to low resolution MS (LRMS), or in untargeted mode, usually with high resolution MS (HRMS) [4], respectively for quantitative and identification analysis.
As PCs are present in fruit and vegetables also in conjugated forms with sugar residues [2], we attempted investigate the conjugated forms of PCs in different food matrices by means of targeted and semi-untargeted approaches. The potential of the quadrupole/linear ion trap hybrid mass spectrometer (LIT-QqQ) was exploited to develop a semi-untargeted method for the identification of polyphenols in different food matrices: green coffee, Crocus sativus L. (saffron) and Humulus lupulus L. (hop). Moreover, thanks to semi-untargeted approaches several conjugate forms of flavonoids and hydroxycinnamic acid were detected using neutral loss (NL) as a survey scan coupled with enhanced product ion (EPI) scan based on information-dependent acquisition (IDA) criteria. Also a reliable and sensitive quantification method was developed and validated, showing good performance on several PCs.
The presented approach is focused on a specific class of molecules and provides comprehensive information on the different conjugation models that are related to specific base molecules, thus allowing a quick and effective identification of all possible combinations, such as mono-, di-, or tri-glycosylation or another type of conjugation such as quinic acid esters.

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Double bond location and geometry assignment in unsaturated fatty acyl chains of drying oils: the interplay between RPLC-ESI-FTMS/MS and m-CPBA epoxidation

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Keywords: *cultural heritage, fatty acidomics, LC-ESI-MS*

As linseed, poppy seed, and walnut oils are chiefly composed of triacylglycerols with high amounts of (poly)unsaturated fatty acyl chains, they have been largely used as paint binders and oil painting became a novel artistical technique [1]. These oils are also named drying oils since upon exposure to oxygen they undergo sequential radical reactions leading to hardening into a densely cross-linked polymer network, which works as a painting medium [2]. Unfortunately, the interaction of such artworks with the environment is a double-edged sword since some aging processes may trigger surface degradation phenomena forming protrusions and efflorescence, called metal soaps [3]. There has been extensive research to assess useable strategies for dealing with these undesired reactions and it emerged that both the drying and degradation processes are strongly affected by the fatty acyl chains profile of the oil; the characterization of these vegetable oils and especially their unsaturated fatty acids (UFA) is thus a key early step for planning suitable restoration and conservation strategies. To date, spectroscopic techniques have led the way in the diagnosis of the metal soap formation shedding light on the formation and crystallization of metal carboxylates, although they often provide preliminary and limited results about the fatty acidomics of metal soaps. Mass spectrometry (MS) represents a valuable tool to investigate cultural heritage specimens [4]. For this purpose, both the free and total fatty acids of mostly used drying oils were investigated by reversed-phase liquid chromatography coupled with high-resolution mass spectrometry by an electrospray ionization source (RPLC-ESI-FTMS) [5]. The epoxidation reaction of the carbon-carbon double bond of UFA by *meta*-chloroperoxybenzoic acid (m-CPBA) was exploited to turn the alkene group into an epoxide ring, which was cleaved upon tandem MS to yield a pair of diagnostic product ions of double bond localization [6]. Moreover, the retention times of derivatized-FA were used to assign the double bond geometry (*i.e., cis/trans*) of UFA [7]. The most relevant outcomes will be discussed in this communication.

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Untargeted metabolic profiling of "Sulmona red garlic" by FT-ICR mass spectrometry

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Keywords: *Sulmona red garlic, Metabolomics, FT-ICR MS*

Garlic (*Allium sativum* L.) belonging to the Amaryllidaceae family represents one of the most diffuse aromatic and food herb. Garlic bulbs have a complex composition in bioactive compounds (sulphur compounds, (poly)phenols, anthocyanins, amino acids) including metabolites with antimicrobial, antiradical and antioxidant activities which gives it a potential involvement in the prevention of chronic and degenerative disorders[1]. Thus, garlic has gained increasing interest as functional foods. 'Rosso di Sulmona' is a variety inserted into the list of the officially recognized commercial varieties of red garlic (DM 296/2009 delivered by Italian Ministry of Agricultural, Food and Forestal Policy). This crop presents a whole bulb covered by a white tunica and a characteristic reddish tunica surrounding each bulbil once the bulb is divided. Its identification is related to morphological and morphometric characteristic factors, while the chemical properties have never been studied. In the present work an untargeted metabolomic approach was conducted on samples of the smaller bulbs (B) and the external white tunica (W) and the inner red tunica (R), which generally represent a waste of the agronomic practices and deserves to be valorized exploring their chemical properties. Hydroalcoholic extracts from samples, collected in two different years, were analysed by direct infusion electrospray ionization (ESI) Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR MS), a fast and powerful method that has been recently demonstrated to fully characterize several matrices[2]. For each extract, more than 400 formulas have been identified in both polarity modes. An overview of the assigned molecular formulas is supported by van Krevelen diagrams (VKd) and relative frequency histograms. VKd offers a qualitative visualization of densities of molecular classes, wherein a relatively higher density of lipids and polyketides, followed by amino acids is displayed for R samples, and a larger number of lipids but less hits for polyketides, amino acids and polyalcohols is revealed for W samples. The averaged relative frequency distribution histograms reveal that all extracts present the major biodiversity of CHO species with organic acids, polyphenols, lipids, and sugars, followed by CHNO compounds, populated by amino acids and alkaloids and, in smaller amount by CHNOS, mostly represented by metabolites responsible for characteristic garlic flavour and bioactivity. Last of all, the application of a statistical method for data visualization has allowed to gain an explorative data analysis by clustering samples based on relevant similarities and differences.

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Development of an LC-MS/MS method for the determination of uric acid in bones

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***Keywords:** Bones, Uric acid, LC-MS/MS*

Gout is a form of inflammatory arthritis caused by monosodium urate monohydrate crystals deposition. In palaeopathology, the diagnosis is almost totally dependent on the morbid appearance and distribution of the bony articular erosions. Gold standard of diagnosis are birefringent urate crystals observed by polarized-light microscopy analysis. However, groundwater usually removes urate crystal from lytic lesions and make difficult to recognize them [1]. Liquid chromatography (LC) coupled with UV detector was applied to aid the diagnosis of gout in detecting an excess content of uric acid in archaeological human bone [2]. The aims of this research were: (a) to develop a new analytical method based on liquid chromatography tandem mass spectrometry to identify and quantify uric acid on paleopathological samples belonging to an individual with a known presumed clinical history of gout; and (b) to validate the same method on different biological matrices, as urine and serum, known to have a natural physiological content of uric acid [3].
As samples we used the bones of Alessandro Farnese (1545-1592), III duke of Parma and Piacenza and his wife, as a positive and negative controls, respectively. In fact, some letters from his personal doctor Ippolito Pennone reported that the duke suffered from gout.
1 Gram of bone dust was collected from the ends of the metatarsals from Alessandro Farnese and his wife. Moreover, to confirm the validity of the analytical method we collected five serum and urine samples from anonymous donors not affected by gout, recruited on a voluntary basis.
Before proceeding with the analysis of bone dust samples of Alessandro Farnese, the LC-MS/MS method was developed using his wife bones, as a negative control. Starting from 200 mg of bone powder in 1 ml of solvent or 1 ml of a uric acid 10 µM solution for the spike sample, different types of solvent (NaOH 1 M and KOH 1 M), extraction conditions (stir, sonication or both), and time of incubation (1 hr, 3, 6 and 18 hrs) were tested to identify the final conditions for sample preparation. The highest recovery was obtained adding 1 ml of NaOH 1 M, stirring the sample for 18 hours followed by 40 minutes of sonication.
Then, we applied the optimized sample preparation to all the other bone samples, but the uric acid concentration was above the detection limit (0.5 µM) in all of them. Only in samples spiked with uric acid was possible to observe a signal. Differently, in serum and urine samples uric acid levels were found to be of the same order of magnitude as the reference values.
This result may suggest that either the uric acid values of the subject have never been so high as to lead to the formation of crystals or that the uric acid present in the terminal metatarsals has deteriorated over time and therefore nowadays is not possible dose it.

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IRMPD action spectroscopy of bare deprotonated genistein, a natural antioxidant isoflavone

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Keywords: *genistein; IRMPD spectroscopy; mass spectrometry*

Flavonoids are widely spread plant secondary metabolites with proven antioxidant, anti-inflammatory, anti-antiangiogenic, and anti-cancer activities [1], [2]. In particular, genistein (4',5,7-trihydroxyisoflavone) belonging to the class of isoflavones, is also a phytoestrogen, naturally occurring estrogen-like compound with a structure resembling 17β-estradiol hormones [3] (Fig 1). Genistein is involved in radical scavenging activity by way of mechanisms based on sequential proton loss electron transfer [4]. In this context, its deprotonated form, $[\text{geni-H}]^-$, is a key intermediate involved in antioxidant mechanisms. For this reason $[\text{geni-H}]^-$ has been generated by electrospray ionization to be assayed by tandem mass spectrometry and infrared multiple photon dissociation (IRMPD) spectroscopy in the 800-1800 cm^{-1} spectral range, aiming to attain a detailed structural picture. Quantum chemical calculations at the B3LYP/6-311+G(d,p) level of theory have been carried out to determine geometries, thermochemical data, and anharmonic vibrational properties of low-lying isomers, allowing to interpret the experimental spectrum. Evidence reveals that the conjugate base of genistein is deprotonated at the most acidic site (7-OH) and in the most stable arrangement it benefits from a strong intramolecular H bond interaction between 5-OH and the adjacent carbonyl oxygen.

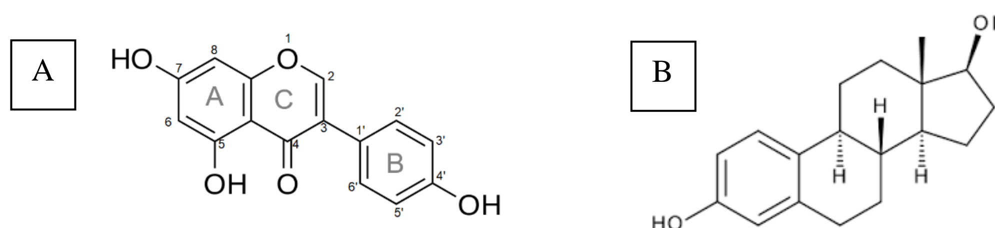

Fig. 1. *Structure of genistein (A) and 17β-estradiol (B).*

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Flash Presentations

Proteomics of sorted Leukocyte-derived Extracellular Vesicles in tears as "liquid biopsy" reflecting neuroinflammation in Multiple Sclerosis

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Keywords: *Proteomics, Leukocyte-Extracellular Vesicles, Multiple Sclerosis, neuroinflammation*

Multiple Sclerosis (MuS) is a multifactorial chronic inflammatory demyelinating disease of the Central Nervous System (CNS). Recent literature has been focusing on Extracellular Vesicles (EVs) as a priceless source for the search of new biomarkers [1]. In fact, our preliminary data highlighted an increased number of neuronal and microglial-derived EVs in tears of MuS patients [2]. In this regard, EVs isolation from whole biofluids is extremely difficult [3-6] especially for the presence of circulating abundant proteins that may impair the quality of proteomics results [1,6]. We have recently optimized an innovative "SORT-omics" workflow for the isolation and subsequent proteomics characterization of EVs directly from untouched biofluids taking advantage of a lipophilic cationic dye (LCD) able to probe intact EVs [7]. Simultaneously, our method offers the great possibility of separating cellular specific EVs by sub-typing them with an appropriate panel of antibodies [7]. Since leukocyte diapedesis trans-blood brain barrier (BBB) is a crucial immune phenomenon that triggers a subsequent cascade of inflammatory events in MuS pathogenesis, here we provided a successful update of "SORT-omics" application by characterizing the proteome of 140,000 Leukocyte-derived EVs (Leuko EVs) from MuS patients compared to healthy controls (HCs). We highlighted that the overlap rate of proteins identified in tears Leuko EVs and Vesiclepedia database was more than 94%. Moreover, functional proteomics analysis revealed that "migration of endothelial cells" function is significantly up-regulated via Leuko EVs proteins by Transforming growth factor beta 1 (TGFB1) as activated upstream regulator in tears MuS versus HCs. Meanwhile, we revealed an activation of "angiogenesis" function in MuS Leuko EVs, according to the complex inflammatory process involving EVs as shuttles in the Immune System machinery in the response to neuroinflammation. In conclusion, our "single-vesicles" proteomics data confirm that Leuko EVs could be considered a useful "liquid biopsy" platform for the assessment of EVs clinical significance also in the tear film where Leuko EVs appear to be a sort of photograph of typical MuS conditions associated with a strong activation of neuroinflammation, oxidative stress and pro-angiogenic processes.

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BAG3 in glucose homeostasis: proteomics characterization of *BAG3* knockout in pancreatic β-cells

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Keywords: *tissues; BAG3; proteomics.*

Adequate glucose homeostasis is fundamental for long term organism health. Impaired glucose homeostatic regulation may result in metabolic disorders, increasing risk of heart disease, stroke and type 2 diabetes. We have previously shown that BAG3, expressed in pancreatic β-cells, regulates insulin secretion. Indeed, silencing BAG3 in β-TC-6 mouse insulinoma cells decreases intracellular insulin content and increases insulin secretion in response to glucose stimulation [1]. β-cells specific BAG3 knockout (BAG3insKO) mice were used as a murine model to study the physio-pathological function of BAG3 in glucose homeostasis. We performed proteomics analysis on livers and muscles (insulin-dependent organs) to identify and quantify the differentially expressed proteins in BAG3insKO and Wild Tipe (WT) mice. The proteomics workflow consists of tryptic digestion with FASP protocol of samples, nanoLC-Orbitrap-MS/MS analysis, label-free quantitative proteomics, evaluation of differential proteins and Functional Gene Ontology term enrichment analysis through Ingenuity Pathway Analysis (IPA software). Proteomics analysis reveals 1030 common proteins in KO and WT livers. In particular, 295 proteins are differentially expressed between two experimental conditions: 96 proteins up-regulated in KO and 199 proteins down-regulated in KO. Specifically, the over expression of cytosolic *HMGCS1* coupled to the down regulation of the mitochondrial isoform *HMGCS2* and *ACAT1*, suggests an increase in the synthesis of cholesterol and a decrease in the formation of ketone bodies. These data are supported by the identification only in KO livers of the following proteins, involved in the synthesis of cholesterol: *Dhcr24, NSDHL, Idi-1*. Functional proteomics analysis also indicated downregulation in fatty acid β-oxidation, ethanol degradation, gluconeogenesis and oxidative phosphorylation in BAG3insKO livers and muscles. Overall, our data demonstrate that BAG3, in pancreatic β-cells, plays an important role in maintaining both the mass and function of β-cells and its depletion increases the tendency to develop insulin resistance.

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Targeting proteomic networks in primary microglia in Alzheimer's disease: new targets for understanding its pathogenesis

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Keywords: *Alzheimer's disease, Microglia, Proteomic profile*

Alzheimer's disease (AD) is the most prevalent age-related neurodegenerative disease worldwide. It includes progressive cognitive deficits, impaired daily activities, and behavioral and psychological symptoms [1]. In addition to beta amyloid (Aβ) and neurofibrillary tangles accumulation in the brain, dysregulated microglia also play key roles in AD pathogenesis. Microglia represent the main innate immune cells in the central nervous system (CNS) and act as guardian by responding to CNS infection or damage [2]. In the initial stages of AD, activated microglia play a role in limiting pathology by removing Aβ through phagocytosis. In contrast, alterations in microglial density, activation, and phenotype, lead to the release of pro-inflammatory cytokines and chemokines that promote neuroinflammation and exacerbate AD neuropathology [3]. In order to highlight microglial proteins and pathways involved in AD pathogenesis, we performed a quantitative label-free proteomic analysis of primary microglia isolated from wild-type (WT) mice and a transgenic mouse model of AD (Tg2576). In addition, we analyzed proteomic profiles of WT and Tg2576 primary microglia after treatment with lipopolysaccharide (LPS), a commonly used neuroinflammation model. Mass spectrometry data have been acquired on a Synapt G2-*Si* Mass spectrometer (Waters Corp.) in $HDMS^E$ mode. Progenesis QI for Proteomics v.4.1 software (Waters Co.) has been used to perform qualitative, quantitative and statistical analysis, allowing the detection of proteins differentially modulated.[4]A deep bioinformatics analysis was further performed, revealing gene ontology and biological pathways enriched by differentially expressed proteins. Interestingly the modulated proteins belong mostly to mRNA processing, proten translation, cell cycle and cell proliferation pathways. Albeit experimental validation will be necessary to confirm candidate molecules associated to the enriched network and possibly identify therapeutic targets, overall, our data identified several regulatory factors and pathways in microglia that could be useful in further understanding AD neuropathology.

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Evaluation of plasma metabolomic profile by exploiting the synergy between MS and NMR

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Keywords: *Metabolomics, Mass Spectrometry, Nuclear Magnetic Resonance*

Metabolomics plays a unique role in the study of biological systems related to health, disease and the environment [1] by studying all metabolites of a biological system [2], or even the end products of the expression of its gene. Due to the complexity of the metabolome, to obtain a comprehensive, sensitive and reliable information, is essential to combine different analytical technique. So, the aim of this project is to integrate NMR and HRMS on plasma samples obtained from patients undergoing treadmill test evaluating the changes occurred in metabolites before and after physical activity. Mass spectra were acquired on an Orbitrap Q-Exactive mass spectrometer equipped with a HESI source operating in positive and negative ionization mode; LC separation was performed with a RP and a HILIC column to detect as many metabolites as possible. Each sample was also acquired in NMR with a Bruker Avance 700 MHz spectrometer equipped with a triple-resonance TXI probe and a SampleXpress Lite autosampler, using a noesypr1d pulse sequence for water suppression. The intensities normalization for all spectra is mandatory, due to the large variability in the analytes total concentration within the different samples. Firstly, PCA was used to obtain an unsupervised evaluation of the results; already from this, a difference emerged between the samples before and after physical activity. Then a reduction in the dimensionality of the LC-MS data was performed, with the application of successive OPLS-DA models; the number of variables was reduced to obtain best Q2 value. So, the reduced dataset was added to the NMR one with an increase in predictivity, compared to the latter. The results obtained from the OPLS-DA, of the NMR/HRMS model, lead us to identify the main metabolic differences in basal samples versus stress samples. The second approach performed was SYNHMET [3] (SYnergic use of NMR and HRMS for METabolomics), the intensities obtained from the MS are linearly correlated with the NMR signals, resulting in standard-free identification of mass hits and conversion of intensities into concentrations. The correlation parameter was the exact mass; a good correlation is essential to ensure that the analyte in question is the same in both techniques. The correlation that does not exist in one sample exists in all of them as a group because, in this case, is the distribution of intensities that determines whether a given chemical shift belongs to a molecule with a specific *m/z*. PCA results demonstrate how all samples can be divided based on the subject gender. The evaluation of two OPLS-DA models showed different significant metabolites for each separation group. So, the applicability of the presented procedure was demonstrated.

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Chemical profiles and biological activities of extracts obtained from *Viscum album* L. (Mistletoe)

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Keywords: *Antioxidant; Phyto-pharmaceutics; HPLC-MS*

Viscum album L., also known as European mistletoe, is a woody shrub sprouting especially on coniferous trees. The extracts obtained from this plant are composed of a huge variety of compounds. The aim of this work is to define the chemical profiles and biological activities of its fruits, leaves and seeds extracts produced using the homogenizer-assisted extraction (HAE) and ultrasound-assisted extraction (UAE). The chemical profile has been identified by high-performance liquid chromatography-mass (HPLC-MS) method. Based on analytical standards and bibliography, thirty-two compounds have been observed. Fruits represent a rich source of a disaccharide (compound **1**) and a dimethylated flavonoid-O-hexoside (compound **32**), while the main compounds located in leaves and seeds are caffeoylquinic acids (compound **3**, **6** and **10**), which represent approximately 30% of the full amount of components, and citric acid (8-11%). Moreover, a caffeoylquinic acid derivative (compound **33**) is abundant in leaves extracts. To determine the antioxidant activity of each extract, we performed different assays including the most common radical scavenging assays, DPPH and ABTS, and reducing power assays (CUPRAC and FRAP). We noticed a strong correlation between the phenolic content and the antioxidant activity, as previously reported in literature [1]. Later, we tested the ability of each extract as cholinesterase inhibitor because of the importance of this type of inhibition in the management of Alzheimer's disease. Fruit-HAE was the most active against AChE, while seed-UAE was the best BChE inhibitor. The inhibition activity is probably due to non-phenolic compounds, e.g. alkaloids and terpenoids, in agreement to previous papers [2]. Additionally, we tested the inhibition activity of each extract against amylase and glucosidase, both involved in the decreasing of blood glucose levels. The regulation of blood glucose level is paramount in order to manage the diabetes mellitus. Leaf-HAE had the best inhibitory activity against amylase and leaf-UAE was the most powerful inhibitor against glucosidase. Finally, we evaluated the tyrosinase inhibitory activity, an enzyme involved in inflammation and melanoma. Leaf-HAE showed a strong inhibitory activity against tyrosinase. The outcomes of the anti-diabetic and anti-tyrosinase assays could be related to the total phenolic content in the leaf extracts. Overall *V. album* extracts could be promising sources of inhibitors useful for the development of new pharmaceutical or nutraceutical drugs.

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Cannabinoids profile in *Cannabis sativa* L. samples by means of LC-MRM/IDA/EPI analysis: a new approach for cultivar classification

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Keywords: *Cannabinoids, HPLC-MS/MS, MRM-IDA-EPI*

Cannabis sativa L. (*C. sativa*, hemp) is a dioceus plant, belonging to Cannabacea family [1]. The importance of this plant is due to the presence of specific compounds, called phytocannabinoids, significant for their biological activity [1,2]. In the last years the presence of new cannabinoids was reported [3]: among these butyl cannabinoid series, as cannabidibutol (CBDB) and Δ9-tetrahydrocannabutol (THCB), and heptyl cannabinoid series, as cannabidiphorol (CBDP) and Δ9-tetrahydrocannabiphorol (THCP), together with the related acid. Moreover, other researchers found a new series of cannabinoids that filled the gap between the pentyl and heptyl homologs of CBD and THC, bearing a n-hexyl side chain on the resorcinyl moiety, called cannabidihexol (CBD-C6) and Δ9-tetrahydrocannabihexol (THC-C6) [4]. Preliminary studies on biological activity of some of these new compounds showed higher activity than the common cannabinoids, [3,5] such as Δ9-tetrahydrocannabinol (THC), cannabidiol (CBD), cannabinol (CBN), cannabichromene (CBC), cannabigerol (CBG), Δ9-tetrahydrocannabinolic acid (THCA), cannabidiolic acid (CBDA), and cannabigerolic acid (CBGA).
In this work, a new multi-target screening procedure for 36 cannabinoids in 12 *Cannabis sativa* L. cultivars (hemp) was developed using multiple reaction monitoring (MRM) coupled with enhanced product ion (EPI) scan in an information-dependent acquisition (IDA) experiment, which can be performed by means HPLC-MS/MS analysis. The MRM-IDA-EPI was used for the analysis of hemp samples and the identification of the compounds of interest. It was performed through the comparison of EPI spectra with literature data and with in-house library. The analytical results, processed by multivariate statistical analysis, showed an accurate classification of the 12 *C. sativa* cultivars, emphasizing the synergic contribution of the new cannabinoids recently discovered, and showing how the traditional classification, based on a common cannabinoid profile, is limiting.

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Development of a new liquid phase micro-extraction method for the determination of drugs in oral fluids by HPLC-MS / MS analysis

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Keywords: *Oral Fluid, PALME, HPLC-MS/MS*

The use of psychoactive substances is increasing worldwide and remains an important social concern. Even if instruments with high sensitivity and precision are available, innovative methodologies to isolate the molecules is essential [1]. The purpose of this work is to develop an analysis method for the simultaneous determination of illicit substances, both natural and synthetic, belonging to different pharmacological classes (synthetic cannabinoids, synthetic cathinones, amphetamines, arylcyclohexylamines, alkaloids, opiates and related metabolites, fentanyl and derivatives and benzodiazepines) in the oral fluid (OF) or saliva (S), an unconventional matrix, alternative to or complementary to plasma and urine in the fields of forensics and therapeutic drug monitoring, as well as for drug-related examination procedures and on-site testing [2]. Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) was used for the identification and quantification of the targeted drugs. Before the analysis with these techniques, a pre-treatment of the sample is necessary to remove any interferences, while a concentration step can be useful to detect parent drugs and/or their metabolites which cn be found at very low concentration in OF. These steps were performed simultaneously, by means of the same technique, the parallel extraction with artificial liquid membrane (PALME), i.e. a type of alternative liquid phase microextraction based on the use of a liquid support membrane which was first described and used in 2013 by Gjelstad et al. for the determination, in human plasma samples, of any basic compounds [3]. This technique uses a pH gradient to facilitate the mass transfer of uncharged analytes across a liquid membrane with organic support and this allows to extract, from an aqueous biological sample, acid, basic or neutral molecules. For this reason it is essential to know the chemical properties of the target analytes [4]. The greatest challenge is the identification of the right compromises, for each parameter (extraction time, extraction pH, the composition of the extracting solvent and acceptor solution, the effect of adding salt and its concentration) which could be adapted to the large number of analytes to be recognized and quantified. The method proved to be suitable for analytes of different classes and since a very small quantities of organic solvent (only 3 µL for each sample) is required, it is an example of green chemistry. The method was subsequently validated following the international guidelines of Scientific Working Group for Forensic Toxicology (SWGTOX) by evaluating a series of parameters, such as precision and accuracy, LOD and LOQ, linear dynamic range, matrix effect and recoveries. Results obtained demonstrate the applicability of the presented method in clinical, toxicological and forensic investigation.

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Multi-experiment approach analysis for derivatized short chain fatty acids by means LC-MS/MS

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Keywords: *Short chain fatty acids, mass spectrometry, Solid phase extraction*

Short-chain fatty acids (SCFAs) are generated as end-products by the degradation and fermentation of indigestible carbohydrates by the gut microbiota, a process called saccharolytic fermentation [1,2]. They can act as signaling molecules as ligands of G-protein-coupled receptors and they are implicated in the increase of anorexic hormone production and energy expenditure [3]. Consequently, SCFA production was linked to preventing the progression of obesity and related complications, [4]. Also gut microbiota-derived acetate is an important precursor for the synthesis of fatty acids and phospholipids in the liver. SCFAs are typically quantified by gas chromatography (GC), liquid chromatography (LC), nuclear magnetic resonance (NMR), and capillary electrophoresis (CE) [5]. These methods require derivatization using, for example, 2-nitrophenylhydrazin, 3-nitrophenylhydrazine, O-benzylhydroxylamine, or aniline. LC coupled with mass spectrometry (MS) with electrospray ionization (ESI) is now the most widely used analytical technique in metabolomics. Ion-exclusion and reversed-phase LC–MS with post-column neutralization, were used for the determination of SCFAs in the pig colon and blood. The complex instrument setup for these two methods, however, makes them unsuitable for routine analysis in most laboratories. On the other hand, LC-MS quantitation without the use of an isotopically-labeled internal standard (IS) for an analyte often makes the analysis questionable because of the notorious matrix effects in ESI, especially when no efficient clean-up is carried out for the quantification of these compounds. The aim of this work was to develop a new analytical method for SCFA based on 3-nitrophenylhydrazone (3NPH) derivatization followed by SPE clean-up to minimize matrix effect and improve extraction selectivity. The LC-MS/MS method was performed both in Multi Reaction Monitoring (MRM) for quantitative analysis and in precursor ion scan analysis (PIS) to achieve semi-targeted analysis for a wide number of SCFA compounds based on derivatization reactions, that could be used for putative identification and screening of SCFA compounds in biological samples.

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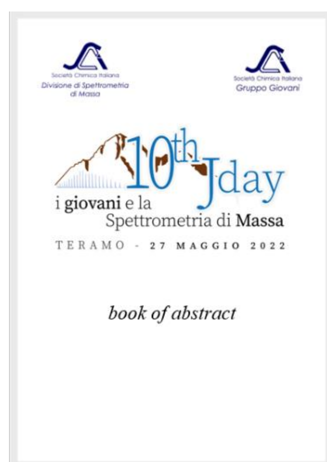

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N. Surname, N. Surname, … and N. Surname; <*abstract title*>, in Proceedings of the 10th MS J day 2022, Ed. M. A. Acquavia, D. Caruso, F. Fanti, G. Giorgi, F. Magni, V. Lazazzara, G. Ventura, F. Vincenti, ISBN: 9788894952094, <*page number*>, 2022, Rome.

***Edited by**: M. A. Acquavia, D. Caruso, F. Fanti, G. Giorgi, F. Magni, V. Lazazzara, G. Ventura, F. Vincenti*

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