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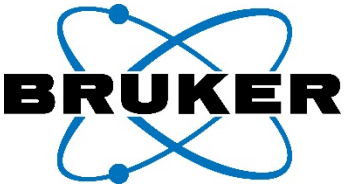

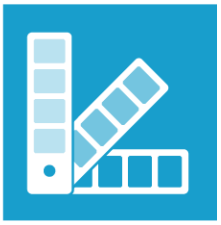

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Scientific Program

THURSDAT, JUNE 9	
9.30	Registration
10.00	Welcome and opening ceremony *Fulvio Magni*
Plenary Lecture	
Chairperson: Valentina Lazazzara	
10.15	**Applications of high-resolution mass spectrometry in food quality and authenticity research** *Dr. Ksenia Morozova, Free University of Bozen-Bolzano*
Oral Session	
Chairperson: Federico Fanti, Giovanni Ventura, Flaminia Vincenti	
10.45	**Online solid-phase extraction with High Performance Liquid Chromatography–Tandem Mass Spectrometry: a Green Analytical Method for the determination of perfluorinated compounds at sub ng· L− 1 Level in water** *S. Barreca,* Agenzia Regionale per la Protezione dell'Ambiente della Lombardia (ARPA Lombardia)
11.05	**Identification and quantitation of hidden food allergens by mass spectrometry techniques** *M. Bianco,* Università degli Studi di Bari Aldo Moro
11.25	**Inositolphosphoceramides in spirulina microalgae and their characterization by LC-ESI tandem MS** *D. Coniglio,*Dipartimento di Chimica, Università degli Studi di Bari Aldo Moro
11.45	**Proteomics characterization of Extracellular Vesicles FACS-sorted from untouched biofluids: an application on Multiple Sclerosis** *M. C. Cufaro,* Analytical Biochemistry and Proteomics and Cytomorphology Laboratory, Center for Advanced Studies and Technology (CAST), University "G. d'Annunzio" of Chieti-Pescara
12.05	**The applicability of strontium isotope ratio analysis to investigate the traceability and authenticity of agricultural products** *H. Puntscher,* Laimburg Research Centre
12.25	**Interactome analysis of bioactive molecules: optimization of a label-free functional proteomics platform.** *E. Morretta,* Department of Pharmacy, University of Salerno
12.45	**Untargeted profiling of different wild and crop Allium species** *A. Maccelli,* Dipartimento di Chimica e Tecnologie del Farmaco, Università di Roma "La Sapienza"
13.05	Lunch and Poster session
14.15	Award ceremony and closing remarks *Fulvio Magni, Donatella Caruso*

Summary

Plenary Lecture

Applications of high-resolution mass spectrometry in food quality and authenticity research

Dr. Ksenia Morozova[1]

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Keywords: *high-resolution mass spectrometry, food chemistry, food by-products, food technology*

Liquid chromatography coupled to mass spectrometry is largely applied in food analysis due to high accuracy and selectivity. High-resolution mass spectrometry alongside UV-Vis spectrophotometry consents the detection, identification and quantification of a wide range of food constituents, such as antioxidants, vitamins, fats, proteins etc. However, the amount of data provided by the mass spectrometer detectors often is huge and needs complex data treatment for selection of the compounds of interest. Moreover, UV-VIS detectors do not provide information of the antioxidant activity of the analyzed compounds. This can be resolved by the combination of LC-MS with electrochemical methods in a continuous system, which allows to select the compounds of interest according to their antioxidant capacity and identify them using the accurate mass and fragmentation pattern. In this lecture most common approaches (targeted and untargeted) to food analysis with high-resolution mass spectrometry will be discussed.

The examples of studies to detect valuable compounds and antioxidants in natural extracts and food products and by-products will be used to illustrate different applications of high-resolution mass spectrometry in food quality and authenticity research [1, 2, 3].

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Oral Presentations

Online solid-phase extraction with High Performance Liquid Chromatography–Tandem Mass Spectrometry: a Green Analytical Method for the determination of perfluorinated compounds at sub ng· L^{-1} Level in water

S. Barreca [1], M. Busetto [1], L. Colzani [1], L. Clerici [1], D. Daverio [1] and P. Dellavedova [1]

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Keywords: *PFAS determination; Green Analytical Chemistry; Hyphenated Techniques*

In this presentation, we report a hyphenated technique based on ultra-high performance liquid chromatography–tandem mass spectrometry for the determination of twelve Perfluorinated Alkylated Substances in surface and groundwater samples. A green analytic method was developed by using an online Solid Phase Extraction coupled with UHPLC-MS/MS [1], and applied for 4992 determinations conducted on 416 samples from 109 different sampling stations located in Lombardia Region. Through the use of an online SPE system integrated with a UHPLC/MS-MS, it was possible to automize procedure, achieve low LOQ values (0.2 ng/L for PFOS) and reduce the amount of mobile phases compared to offline SPE, in compliance with Green Analytical Chemistry principles.
Concerning to analytes, PFOS, PFOA, PFBA, PFBS, PFPeA and PFHxA were identified as the most abundant compounds. PFAS concentrations, in most cases, were below the limits of quantification and, in the cases where the limits of quantification have been exceeded, the values found were lower than Italy directive. PFOS is an exception and in fact this compound was detected in 76% of analysed samples (surface and ground waters). Solid phase extraction with high performance liquid chromatography–tandem Mass Spectrometry has proved to be a very good Hyphenated techniques able to detect low concentrations of pollutants in surface and groundwater samples.

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Identification and quantitation of hidden food allergens by mass spectrometry techniques

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Keywords: *food allergens, mass spectrometry, milk proteins*

Food allergens are proteins or peptides responsible of food allergenicity that can trigger immune-mediated reactions in predisposed subjects [1]. Food allergy is recognized as a serious health concern that currently affects about 3% of the European population but its incidence is continuously growing [2] [3]. The only effective treatment for allergy sufferers remains the total avoidance of food at risk. The most allergenic foods define the family known as "the big 8", namely eggs, milk, fish, peanuts, crustaceans, soybeans, wheat and tree nuts [4]. Recently, the number of sensitive subjects to other foods has increased in Europe and the list has been extended to 14 foods, including lupin, molluscs, celery, mustard, sesame and sulphur dioxide [3]. However, often it could be not enough to avoid the mentioned foodstuffs but also all the processed foods that derive from allergenic ingredients even though their occurrence (declared or not) has significantly increased. With the aim of protecting the consumer health, advanced mass spectrometry (MS) techniques also coupled to liquid chromatography should be employed to identify and quantify the most common allergens of milk and egg in different food matrices. After optimizing the extraction and digestion protocols, the samples were analysed using two platforms: Q-Exactive Orbitrap Fourier-transform MS (FTMS) coupled to reversed-phase liquid chromatography through an ESI source and MALDI ToF MS. Standard allergen solutions (casein, lactoglobulin, ovalbumin) were initially investigated in order to recognize the qualitative and quantitative marker peptides [5]. Subsequently, complex and processed food matrices were examined; "spiked" and "incurred" cookies were prepared in laboratory to determine the matrix and processing effect of these foodstuffs [6]. The inspection was thus carried out on commercial biscuits made with bovine milk and eggs, and alternative derivative soy products (drink and biscuits) with the aim of identifying any trace of hidden bovine milk and egg allergens. Marker peptides were identified in food samples by database search of proteolytic digests and consecutive tandem MS analysis. In this way it was possible to determine and quantify the ensuing presence of food allergens in the examined samples.

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Inositolphosphoceramides in spirulina microalgae and their characterization by LC-ESI tandem MS

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Keywords: *spirulina algae, inositolphosphoceramides, LC-MS*

Spirulina microalga (*Arthrospira platensis*) is an interesting phototrophic organism that acclaims a remarkable content of nutrients [1]; the high amount of proteins, vitamins, fatty acids, sterols, among others, makes its production and application by the food industry very interesting [2]. Here we discuss the characterization of a minor lipid class, namely inositolphosphoceramides (IPC) (Fig. 1) [3,4] never reported in spirulina.

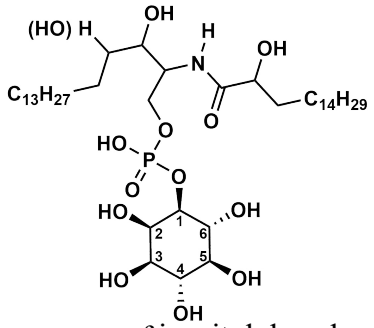

Figure 1. Generic structure of inositolphosphoceramides (IPC).

The occurrence of IPC in lipid extracts of spirulina samples was carried out by hydrophilic interaction liquid chromatography (HILIC) via electrospray ionization (ESI) coupled to Orbitrap Fourier-transform mass spectrometry (FTMS) for high-resolution/accuracy measurements or to a linear ion trap (LIT) MS to exploit the multistage (MS^n, with n=2, 3) collision-induced dissociation (CID) of deprotonated molecules, $[M-H]^-$. Four different commercial samples of spirulina were examined: powder, capsules, noodles, and tablets. Each specimen was independently ground and rehydrated, and the lipid fraction was extracted by a conventional Bligh and Dyer protocol [5]. While HILIC-ESI-FTMS allowed us to identify the chemical formula of all IPC species, their chemical structure was established by tandem MS experiments. Specifically, the identification of the sphingoid base and the fatty acyl chain required a further fragmentation stage (*i.e.*, MS^3) on the product ion arising from the neutral loss of a dehydrated sugar moiety $[M-162-H]^-$. Strikingly, the four commercial samples revealed the occurrence of the same IPC species, but in different relative abundance. Results are reported and discussed in this contribute.

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Proteomics characterization of Extracellular Vesicles FACS-sorted from untouched biofluids: an application on Multiple Sclerosis

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Keywords: *Proteomics, Extracellular Vesicles, Multiple Sclerosis*

Extracellular Vesicles (EVs) are small membrane-enclosed particles released by cells able to vehiculate information between them [1]. In the last decade, their emerging role in many pathophysiological processes is demonstrated by the exponentially increase of published papers on this topic. Recent evidences have shown that distinct types of brain cells release EVs for shipping biological information to different target cells [2,3]. In this context, an increased concentration of EVs was identified in various biological fluids of Multiple Sclerosis (MuS) patients [4-7]. The importance of their biological role prompted researchers to adopt and standardize purification methods able to isolate EVs for proteomics purposes[1,3,8]. Their isolation from biofluids is very difficult for many reasons, especially linked to the presence of abundant proteins that may influence the recovery of isolated EVs, impairing quality of proteomics results [9, 10]. In this work we propose an innovative integrated FACS (Fluorescent-Activated Cell Sorting)-proteomics approach for the isolation and characterization of the EVs proteome cargo from untouched biofluids (Patent Code: 102018000003981) [7,9]. The FACS-proteomics workflow demonstrated a significant decrease of contaminant soluble proteins in the purified EVs from cerebrospinal fluid (CSF) compared to traditional purification methods like ultracentrifugation, showing that the EVs represent a circulating magnifying glass for protein biomarkers search [9]. This innovative experimental scheme was used to characterize EVs proteins from CSF and tears of MuS patients. Their specific protein cargo highlighted, for the first time, the ability of EVs to deliver information from Central Nervous System (CNS) into a peripheral biofluid, bringing the photo print of CNS inflammation and immune status [8]. In conclusion, our approach allows to study protein cargo in high purified EVs with a significant decrease of protein dynamic range in the investigated biofluids. Moreover, the first application demonstrated an important disease-specific molecular cross-talk between central and peripheral biofluids carried by EVs [9].

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The applicability of strontium isotope ratio analysis to investigate the traceability and authenticity of agricultural products

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Keywords: *food traceability, MC-ICP-MS, geographical tracer*

In the last years, the increasing appreciation of consumers for locally produced agricultural crops has also given rise to profitable mislabelling of food products with respect of their origins. To prevent food fraud and to assure food authenticity, suitable routine analysis is required to determine a products' provenance.

Strontium isotope ratio ($^{87}Sr/^{86}Sr$) analysis allows for the correlation of agricultural products to their respective growing areas. In fact, the relative abundance of these isotopes in soil is characteristic to the geological features of the surroundings and allows therefore geographical allocations. The plants' strontium uptake reflects the isotope ratio in the respective soil of the growing area, which makes it a possible geographical tracer for agricultural products. However, for the development of robust analytical tools and the generation of reliable data, it is necessary to investigate possibly interfering factors.

Soil represents the main Sr source for plants. However, an alteration of the isotope ratio over long periods of time caused by agricultural measures including irrigation, the use of fertilizers or the addition of different soils cannot be excluded [1]. Due to the slow absorption, perennial plants may take some time after planting or relocation to adapt to the local conditions. Moreover, geologically complex regions demonstrate variabilities of Sr isotope ratios even within small areas or in soil depth, which makes representative sampling and unambiguous correlations difficult. In case of large, geologically homogeneous areas on the other hand, discrimination of products with very similar Sr isotope ratios is limited [2]. The analysis of different plant parts (wood, leaves, fruits) of the same plant confirmed a high intra-plant homogeneity of the Sr isotope ratio [3]. Therefore, any plant part can be used for representative sampling. Moreover, no significant differences were found for various cereal and vegetable species or apple varieties grown on the same soil.

In conclusion, our studies demonstrate Sr isotope ratio analysis to be a promising and broadly applicable technology for the investigation of food authenticity. Therefore, we aim for further analyses of international samples covering the most important agricultural regions worldwide and to investigate post-harvest measures like storage or food-processing to estimate their influence on the Sr isotope ratio in the respective products.

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Interactome analysis of bioactive molecules: optimization of a *label-free* functional proteomics platform.

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Keywords: *Functional proteomics, limited proteolysis, targeted & untargeted mass spectrometry.*

Natural products (NPs) have long been explored as invaluable sources for drug discovery, thus the identification of their target proteins is a crucial step. In this scenario, affinity chromatography coupled to mass spectrometry (AP-MS) has been a top-choice technique in the last 15 years. Nevertheless, due to the NPs chemical features for the mandatory *on-beads* immobilization step, this strategy is not universally applicable. Furthermore, the covalent modification of a NP functional group could alter its original bioactivity [1].

Thus, to provide a universally applicable and more comprehensive target identification strategy, I started to develop and optimize a *label-free* functional proteomics platform based on two complementary strategies, Drug Affinity Responsive Target Stability (DARTS) [2] and targeted Limited Proteolysis coupled to Multiple Reaction Monitoring MS (t-LiP-MRM) [3]. This platform does not require any chemical modification of the molecule for both the characterization of its protein partner(s) and of their interaction features. Indeed, in a first step DARTS gives the identification of NPs most reliable interacting proteins and then t-LiP-MRM is used to pinpoint the proteins regions directly or distally involved in the NPs/targets interactions. The results are then confirmed by additional techniques, such as Western Blotting and molecular docking**.**

At first, this *label-free* platform *proof of concept* was achieved through the well-known radicicol/Hsp90s system; subsequently the optimized method was exploited for the *interactome* analysis of several NPs. As an example, the *targetome* characterization of the *Mycale rotalis* poly-brominated acetogenin BrACG [4] will be presented.

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Untargeted profiling of different wild and crop Allium species

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Keywords: *Metabolomics, FT-ICR MS, Food Analysis, Allium species*

The health-enhancing properties of *Allium* species are well recognized in scientific literature and in everyday life [1]. In particular, *Allium sativum* L. represents the most economically significant species in the Amaryllidaceae family, due to its antioxidant, anti-inflammatory and anti-hypertensive activity, thus justifying the world-wide spread consumption. Most of the data available from scientific repositories are focused on the characterization of the volatile fraction, including sulfur-containing compounds like alliin, allicin and essential oil. More recently, an increasing attention has been also devoted to the study of the polar fraction of garlic metabolome, characterized by the presence of less volatile nutraceutical components as (poly)phenols, anthocyanins and amino acids. In this context, we have undertaken a comparative study on the phytochemical composition of the microwave assisted extracts from eight different crop and wild *Allium* species, including *Allium sativum* L., *Allium triquetrum* L., *Allium roseum* L. and *Allium ampeloprasum* L., all originating from the Mediterranean basin, an area rich in biodiversity. In order to evaluate their nutritional and potential therapeutic value, and contribute to preserve and valorize natural resources. In order to cover a widest set of metabolites without the employment of any time-consuming preliminary chromatographic technique, an untargeted metabolomics approach has been applied by using high-resolution electrospray ionization FT-ICR mass spectrometry. By this fast and sensitive method, more than 900 compounds, belonging to different classes of metabolites, like lipids, flavonoids, organic and fatty acids, amino acids and saponins, have been identified. In a complementary way, a targeted assay has been carried out on the pigment and polyphenol profile by a spectrophotometric and chromatographic (HPLC-PDA) method and CIEL*a*b* colorimetric assay. Catechin, gallic acid and carvacrol have been revealed as the most abundant phenols, broadly distributed among all the samples, while the red and the yellow components reflect a different admixture of anthocyanins and flavonoids typical of each Allium extract.

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Poster Presentations

Insight the statistical workflow for MALDI-MSI data analysis

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Keywords: *pre-processing, classification model, MALDI-MSI*

MALDI-MSI represents an ideal tool to explore the spatial distribution of proteins directly in-situ, integrating molecular and cytomorphological information and enabling the discovery of potential diagnostic markers. Given the amount of data generated from MALDI-MSI analysis, it is of paramount importance to use proper statistical methods in order to correctly interpret data.
An overview of the main statistical approaches to deal with high dimensional data is presented, from the construction of an adequate pre-processing step to the choice of the statistical models able to answer the clinical question. Various statistical methods can be applied in the omics field, but two are the main approaches usually considered in the workflow of a omics' analysis. Firstly, unsupervised methods can be applied in order to explore the data structure; then, supervised methods are used to construct either diagnostic, prognostic, or predictive models, based on the specific clinical question. None of these algorithms works best for every problem, because there are different factors, such as the size, structure of data and clinical question, which play an important role on the choice of the approach to be used. Advantages and weaknesses of the different algorithms are discussed. Moreover, no attention is given in the correct choice of the pre-processing process, leading to biased results if incorrectly used. Given the great possibility of different situations, an example of an adequate workflow is reported to find discriminant features in a thyroid nodules classification problem. The actual gold standard to exclude the malignant nature of thyroid nodules in the clinical routine is represented by thyroid Fine Needle Aspirations (FNAs) biopsies [1]. Approximately the 20-30% of cases have an indeterminate for malignancy final report, but after surgery the 80% of these thyroid nodules are benign. This overtreatment has of course important consequences in the quality of life of the patients and high healthcare costs.
This preliminary study involved data from 18 subjects with benign and malignant thyroid nodules and, an additional sample of 11 patients with different type of lesions (i.e. benign, indeterminate and malignant) was used for validation. Results are very promising and highlight the possibility to introduce MALDI-MSI as a complementary tool for the diagnostic characterization of thyroid lesions [2].

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Carbamoyl-Phosphate Synthase 1 as a Novel Target of Phomoxanthone A, a Bioactive Fungal Metabolite

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Keywords: *drug affinity responsive target stability; targeted-limited proteolysis; bioactive xanthone*

Phomoxanthone A (PXA) is a tetrahydroxanthone dimer isolated from the endophytic fungus *Phomopsis longicolla*. Recent studies revealed that it disturbs the form and the function of inner mitochondrial membrane (IMM) in several ways. Indeed, PXA causes the rapid inhibition of the electron transport chain, the loss of the membrane potential ($\Delta\Psi m$), the release of mitochondrial Ca2+, the cristae disruption and fragmentation of the IMM [1]. However, the identification of the mitochondrial interactome of PXA in an unbiased way has not been performed so far. To determinate the targets of this natural compound, the chemoproteomic approach of Drug Affinity Responsive Target Stability (DARTS) has been performed on mitochondrial lysates. Obtained results have been validated by t-LiP-MRM, a method that couples limited proteolysis (LiP) with multiple reaction monitoring mass spectrometry (MRM-MS). DARTS relies on the proteolysis protection conferred on a protein by the interaction with a ligand: under physiological conditions, a protein fluctuates between multiple alternative conformations ("breathing"), but, upon saturation with a specific ligand, the equilibrium shifts to favor the thermodynamically more stable bound-ligand state, decreasing protein susceptibility to proteases degradation [2]. T-LiP-MRM enables to validate the small molecule interactome, shedding light on the protein regions protected by PXA through the quantitation of its proteolytic peptides by means of MRM based strategies [3]. DARTS revealed Carbamoyl-phosphate synthase 1 (CPS1) as a novel biological target of PXA, confirmed by Western Blotting and t-LiP-MRM [4]. CPS1 plays an important role in removing excess ammonia from the cell, catalyzing the first reaction of the urea cycle: the ATP-dependent synthesis of carbamoyl phosphate from ammonia and bicarbonate. Activity assays in presence of PXA suggest a positive modulation of CPS1 activity by this compound. Thus, PXA can be considered as a potential new lead compound for the treatment of CPS1 deficiency, a recessively inherited urea cycle error due to CPS1 gene mutations, which leads to life-threatening hyperammonemia [5].

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Phenolic compounds quantification in raw and steamed Brassica vegetables from Trentino-Alto Adige (Italy) and Italian retailers

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Keywords: *Brassica, UHPLC-MS/MS, secondary plant metabolites*

Brassica (Cruciferae) vegetables include a large group of horticultural crops and they are the world's most commonly cultivated vegetables [1]. Trentino-Alto Adige (Italy) is an emerging producer of Brassica vegetables such as cauliflower and cabbage [2]. Besides the well-studied class of glucosinolates [3], another major group of secondary plant metabolites (SPM) in Brassica vegetables gathers phenolic compounds which have been linked to their health-promoting properties [4, 5]. Most Brassicaceae species are cooked before consumption and the cooking process influences the composition of SPM [6]. The work aims to characterize the phenolic composition of nine Brassica vegetables and to investigate the effect of cooking processes have on these bioactive compounds [7]. Fresh Brassica vegetables are collected from Val Venosta (BZ), Trento, San Genesio (BZ) as well as six retailers from Bolzano. Steaming treatments are carried out using a commercial steamer according to Ferracane et al. [6] and the extraction method, which involves methanolic extracts of lyophilized samples, is adapted from Li et. al [5]. The phenolic compounds are analyzed by UHPLC coupled to a triple quadrupole mass spectrometer (MS/MS) with an electrospray ionization (ESI) in negative ionization mode, except for kaempferol and (±)-catechin-2,3,4-13C3. The mass spectra were acquired in multiple reaction monitoring (MRM) including at least 2 MRM transitions for each analyte [5]. The quantification of single polyphenols is carried out using external calibration curves and they are normalized to catechin-2,3,4-13C3 added to each sample as internal standard. This method is able to identify and quantify 46 phenolic compounds including 15 hydroxy-cinnamic acids derivatives, 16 unsubstituted kaempferol and acylated kaempferol glycosides, 9 unsubstituted quercetin and acylated quercetin glycosides and 5 isorhamnetin glycosides [5]. Recently, the general interest in sustainable foods has significantly increased, in opposition to vegetables grown and processed with intensive practices. This study draws attention towards raw and steamed Brassica vegetables which are cultivated in Trentino-Alto Adige (Italy) or purchased in retailers, as well as a source of nutraceutical compounds.

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Distinguishing geographical origin of various vegetable species grown in South Tyrol applying strontium isotope ratio analysis

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Keywords: *food traceability, isotope ratio analysis, ICP-MS*

The geographical origin of food and agricultural products is becoming increasingly important for consumers [1]. However, mislabelling is the most common type of food fraud in the EU and results in illegitimate economic competition [2]. For this purpose, innovative analytical methods are required to confirm the geographical origin of agricultural crops and to ensure the correct labelling of the respective products. One promising methodology is the analysis of the $^{87}Sr/^{86}Sr$ isotope ratio. In plants, this ratio reflects the soil characteristics of the growing area [3]. In recent studies, this parameter has already been successfully applied as a geographical tracer for North-Italian apples [4, 5]. However, detailed information and case studies about its application as a geographical indicator for different species of vegetables are still lacking. In the present work, six different vegetables (carrot, potato, cauliflower, beetroot, radicchio, pumpkin) and soil samples of their corresponding growing areas were sampled from two selected locations in South Tyrol. The $^{87}Sr/^{86}Sr$ isotope ratios were determined using an established and validated mc-ICP-MS method. The obtained results demonstrated significant differences in the isotope ratios between the two locations of provenience, while within samples of the same location there were no significant differences between the soil and vegetable products. Moreover, no significant differences between crops grown above (cauliflower, radicchio, pumpkin) or under the ground (potato, carrot, beetroot) were found. Therefore, we conclude that the Sr isotope ratio in the vegetable samples was mainly dependent on the Sr characteristics of the respective soils, but it was not significantly affected by the vegetable species. The results allowed to assign the vegetables to their respective place of origin. Further investigations are required to confirm the robustness of the Sr isotope ratio analysis as a geographical indicator for different agricultural products. Therefore, we will extend our studies to additional crops and international samples to generate a more comprehensive database.

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MALDI-MS imaging of gelatin hybrid hydrogel based tumor spheroids

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Keywords: *MALDI-MSI imaging, tumor spheroids, proteomic*

Monolayers cell culture systems have classically been used for pharmacological evaluation of anticancer drugs. However, solid tumors grow naturally in a 3D conformation, and consist of a heterogeneous microenvironment. Many types of in vitro 3D culture methods have been developed and are now widely used as tumour surrogates. Among these systems tumor spheroids are a powerful biological model which closely mimics the structure of primary avascularized tumors.

MALDI-MSI can be considered an ideal tool that can be used to obtain spatially defined molecular information from these structures, improving our understanding of tumour development and for monitoring the in vitro efficacy of antitumor agents. In this work, we aimed to establish the feasibility of performing high spatial resolution MALDI-MSI of tumour spheroids embedded in a novel, highly biocompatible, hybrid hydrogel [1].

MALDI mass spectrometry imaging was performed on tumor spheroids that were created starting from the human glioma U87-MG cell line and later included in gelatine (GE) and chitosan (CH) hybrid hydrogel prior to formalin-fixation and paraffin embedding. All mass spectra were then obtained in the *m/z* 700 to 3000 range using a rapifleX MALDI Tissuetyper™ and the images were acquired using a beam scan of 6 µm and a raster setting of 10 µm in both x and y dimensions.

Briefly, distinct protein profiles could be generated from the tumour spheroids as well as the histologically diverse regions within them, indicating the feasibility to analyse these types of tumour spheroids by MALDI-MS imaging. In particular, those regions containing necrotic cells could be distinguished from those regions containing primarily proliferative cells. This was initially highlighted by using unsupervised principal component analysis (PCA), with the spectra of the corresponding regions distributed in distinct locations within the three-dimensional space. This was also highlighted by the spatial distribution of individual m/z signals, with m/z 1325.6 being particularly expressed in proliferative regions while both *m/z* 1045.462 and 1011.541 were co-localized within the necrotic regions.

Here we present the feasibility of performing spatially resolved proteomics analysis of tumour spheroids prepared using an innovative hydrogel formulation. The possibility to combine MALDI-MS imaging with this specific type of biocompatible tumor spheroid represents a highly promising approach to investigate, at the proteomic level, the efficacy of new antitumor drugs designed to target specific cells implicated in tumour development and progression, thus providing new outlooks in this field of research.

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Tri-modal MALDI-MS imaging of lipids, N-glycans, and proteins on a single tissue section

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Keywords: *MALDI-MS imaging; multimodal; FFPE*

Introduction: Matrix-assisted laser desorption/ionization (MALDI) mass spectrometry imaging (MSI) is a powerful technology that allows the in-situ detection of a broad range of biomolecules, including lipids, N-glycans, and proteins [1]. Importantly, the combination of multilevel molecular data can possibly enhance the detection of novel disease signatures and improve the stratification of patients [2]. However, the detection of multiple class of molecules on formalin-fixed paraffin embedded (FFPE) tissue samples, the primary source of clinical tissue, is so far challenging. Here, we illustrate a workflow that highlights the feasibility to detect lipids, N-glycans, and proteins from the FFPE tissue section.

Methods: For each section of FFPE clinical specimens, MALDI-MSI was performed in three modalities using a Bruker rapiflexTM MALDI tissuetyperTM. Initially, deparaffinization, tissue rehydration and antigen retrieval were performed. Hence, lipids were analysed in positive ion mode within the m/z 420 to 820 range. Subsequently, N-glycans, released following digestion with PNGase-F, were analysed within the m/z 1000 to 3000 range. Finally, tryptic peptides, obtained following enzymatic digestion were analysed within the m/z 700 to 3000 range. All image acquisitions were performed using a laser beam scan of 44 μm and a raster setting of 50 μm in both x and y co-ordinates.

Results: Initially, we detected a limited number of lipids within FFPE tissue sections, as expected [3]. Furthermore, we noticed that lateral diffusion of the N-glycans and tryptic peptides in the subsequent analysis was limited, regardless of the numerous tissue washes and matrix applications. Finally, comparing the spatial distribution of key signals detected at these multi-molecular levels, complementary information could be obtained and regions of tissue with altered molecular profiles could be highlighted.

Conclusions: The tri-modal approach proposed here would be the first of its kind to consecutively detect lipids, N-Glycans and tryptic peptides on the same FFPE tissue section. Concluding, the workflow described in this work may be useful for a deeper understanding of complex diseases, where both lipids, and proteins pathways are involved, and for supporting the pathologist in the diagnosis and stratification of patients, that may be unlikely when considering a single group of analytes.

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Identifying Natural Products Targetome: the case of Artemetin.

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Keywords: DARTS, targeted-LiP-MRM, proteomics

5-hydroxy-3,6,7,3',4'-pentamethoxyflavone, simply known as Artemetin, is a flavonoid isolated from *Achillea millefolium L*. (Asteraceae). In addition to exerting anti-inflammatory action, Artemetin has been shown to significantly counteract peroxidation in CCl (4)-intoxicated livers of rats and to reduce the elevated serum levels of transaminases and bilirubin. In addition, intravenous infusion of Artemetin in rats has been reported to reduce mean arterial blood pressure and to inhibit the hypertensive response to angiotensin I. Such data suggest that Artemetin would play a beneficial role against the onset of cardiovascular diseases. The regulation of arterial blood pressure and the antioxidant properties could make Artemetin a very interesting agent potentially useful for the prevention/treatment of ischemic myocardial disease. This natural product (NP) presents beneficial effects in the cardiovascular system, including vasodilatation, antioxidant and antihypertensive activities [1]. The identification of natural products target proteins is crucial to understand the mechanism of action for the development of molecular probes and/or potential drugs. In this scenario, Drug Affinity Responsive Target Stability (DARTS) coupled to mass spectrometry has emerged as a valid tool for NPs *targetome* characterization. DARTS exploits the thermodynamic stabilization conferred by a molecule to its protein target(s), whose conformational fluctuations (i.e. *breathing*) are dramatically decreased due to the interaction with the compound: the NPs targets showing an altered proteolytic pattern when submitted to unspecific protease exposures can be identified by mass spectrometry and/or immunoblotting analysis [2]. Moreover, a DARTS-like *gel-free* approach has been recently developed to probe proteins structural transitions in complex biological environments. This strategy, termed targeted-LiP-MRM [3], couples limited proteolysis (LiP) with the targeted Multiple Reaction Monitoring approach (MRM-MS) and tracks the ligand-induced targets proteins proteolytic resistance measuring changes in abundance of their tryptic peptides. Targeted-LiP-MRM is able to pin-point proteins region directly or distally involved in the interaction with a small molecule, being complementary to DARTS in the characterization of NPs target profile.

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Untargeted analyses for the characterization of New Psychoactive Substances

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Keywords: *GC-MS, LC-HRMS/MS, Fenanyls*

Over the years, the diffusion of New Psychoactive Substances (NPS) has undergone a slight but constant growth. Nowadays, an additional issue is related to the easy and quick availability of these substances on the internet, in particular due to the restriction policies aimed at reducing their sale as street-drug [1]. Constantly, new drugs are introduced on the market and recently their number has risen to 730 [2].

Synthetic opioids are one of the most important threat and fentanyl, a widespread narcotic agent, act as a μ-receptor agonist binding 80 to 100 times more strongly than morphine and some fentanyl derivatives even higher [3].

In this work we reported a case study describing the comprehensive characterization of an online purchased product: it was shown how a new fentanyl derivative, 4-Fluoro-Furanylfentanyl (4F-FuF), was identified and characterized by exploiting different analytical techniques, namely IR, Raman, GC-MS, LC-HRMS/MS and NMR.

Two different synthetic opioids, belonging to the family of fentanyl derivatives were identified in the same seizure occurred in Italy. A comprehensive elucidation of 4F-FuF structure is provided in this work.

Through IR analysis we were able to identify the class to which the compound belongs, and the presence of a particular type of diluent. Raman spectroscopy gaves a deeper information regarding the presence of two different fentanyl derivatives in the same seizure, and in particular it highlighted the presence of a FuF. With GC-MS the presence of two different compound in the same powder was confirmed and the characteristic EI fragmentation was observed.

Structure elucidation procedure was facilitated by HPLC-HRMS and the related HRMS/MS spectra were also added to a public library, HighResNPS [30]. Finally, NMR analysis provided the isomeric structure for the compound: 4F-FuF.

In Italy, as a result of this study, a formal notification of these substances to National Early Warning Sistem was sent, notification to the EMCDDA followed [4].

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High-resolution mass spectrometry as a powerful tool for determination of chlorophyll catabolites in apple peel

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Keywords: *Phyllobilins, high resolution mass spectrometry*

Chlorophyll breakdown is an important catabolic process of fruit ripening. The yellowing of fruits seems to be connected with an increasing concentration of a group of catabolites called phyllobilins (PBs) in apple peels [1]. Thus, a possible indicator of fruit ripening could be the degradation of chlorophyll to PBs, including the most common compounds in this degradation pathway, such as nonfluorescent chlorophyll catabolites (NCCs), nonfluorescent dioxobilane chlorophyll catabolites (NDCCs) and yellow chlorophyll catabolites (YCCs). Moreover, PBs have been demonstrated to show antioxidant activity, enriching the list of compounds with possible beneficial effect on human health found in apple fruits [1].

Thus, a deep understanding of the characterization of PBs is fundamental to shed light on the composition of chlorophyll catabolites in apple peels.

Two clones of cv Royal Gala apples (Schniga and Buckeye) and two belonging to cv Golden Delicious apples (Parsi and Reinders) were used in this study. The protocol for the PBs analysis was adapted from Mittelberger et al. [2] and peel extracts were analyzed using an ultra-high pressure liquid chromatography-high resolution quadrupole-time of flight-mass spectrometry (UHPLC-Q-TOF-MS) instrument. Data were acquired using auto MS-MS mode based on an inclusion list containing the pseudo-molecular ions of all the known catabolites. The annotation of PBs relied on mass accuracy, adducts formation and fragmentation spectra interpretation. Compounds were confirmed by comparing experimental retention times, MS and MS/MS spectra with those available in literature [2].

While in literature only two catabolites in apple peel are known [1], more than twenty-five chlorophyll catabolites were annotated in this study, including five NCCs, one YCC, and three DNCCs. All of them seem to be present in at least two different isomeric forms that elute at different retention times. Interestingly, similar results have been found in apple leaves [2], confirming a possible common chlorophyll degradation pathway in both fruits and leaves. Further analyses are required to confirm the uncertain isomers.

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MALDI-MS Imaging, a new resource for the triage of thyroid nodules

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Keywords: *thyroid carcinoma, MALDI-MSI, Fine Needle Aspiration*

Thyroid nodules diagnosis is performed by fine needle aspiration (FNA) biopsy and approximately only 4-8% are diagnosed as malignant. However, 15-30% of FNABs are indeterminate nodules (THY3), showing insufficient cytological alterations to determine their benignity or malignancy [1]. Today, the debate about the management of these THY3 cases is still open and surgery represents the only possible therapeutic approach. Nevertheless, the post-operative histological evaluation shows that about 80% of THY3 nodules were benign and the surgery could have been avoided [2]. For this reason, the aim of this work was to apply the MALDI-MSI technique as a new possible diagnostic tool to support the pathologist to improve the triage of thyroid nodules FNAs diagnosis.
Cytological thyroid samples were collected from patients (San Gerardo Hospital, Monza, Italy), transferred onto conductive slides by cyto-centrifugation [3], and analysed by MALDI-MSI within the *m/z* 3,000–20,000 range. Data analysis (PCA and ROC analysis) was performed comparing two different approaches: focusing on the overall MALDI-MSI area and thyrocytes regions of interest. Finally, a diagnostic model was built through a pixel-by-pixel classification approach using 9 subjects with a THY2/hyperplastic nodules diagnosis and 9 patients with a THY5/papillary thyroid carcinoma diagnosis. The data analysis of the overall MALDI-MSI area of benign and malignant patients showed an area of overlap, probably due to the heterogeneous cellular background present in both classes, and five statistically significant *m/z* signals (AUC≥0.7) with higher absolute intensity in HP than PTC samples. However, when only data from ROIs were investigated, differences between benign and malignant samples were more evident and two additional peaks were found as statistically significant (AUC≥0.7) with higher absolute intensity in PTC than HP samples. For all these reasons we decided to build up a classification training model using only spectra from ROIs. This model was able to successfully validate, with a pixel-by pixel classification performed on all the sample area, a second set of samples when the cellularity of FNA was adequate. Even though the FNAs material is challenging, MALDI-MSI shows potential as a new complementary tool in the clinical routine, for the classification of indeterminate thyroid nodules.

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Analysis of Polyphenols in food samples by UHPLC-MS/MS in target and semi-untarget acquisition mode

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Keywords: *Polyphenols, Mass Spectrometry, Precursor ion scan.*

Phenolic compounds (PCs) are natural-occurring secondary metabolites with considerable physiological and morphological importance in plants and different biological functions. PCs have been widely studied for their potentiality in prevention and treatment of oxidative stress-related diseases; several in vitro and in vivo studies exposed their involvement in the prevention of several diseases, such as cardiovascular and some neurodegenerative illness, cancers, osteoporosis, and diabetes mellitus [1]. Antioxidant capacity of polyphenols is one of the key issues to understand their role in health and food systems; this can be rapidly assessed using classical spectrophotometric assays or newly proposed colorimetric or sensor-based approaches. Different analytical methods have been presented for the PCs determination in food samples, usually in target mode [2] or in untarget mode by using mostly Q-TOF or Orbitrap MS intruments [3].

In this work, a targeted and semi-targeted analysis procedure was developed by high performance tandem liquid chromatographic mass spectrometry, which allowed the identification and quantification of PC and glycosidic derivatives of flavonoids in different matrices food.

A tailored SPE clean-up was also developed allowing the use of a limited quantity of sample, and its subsequent enrichment; the use of a small solvent consumption marks the convenience of this technique for the extraction/isolation of compounds of interest, including both the most polar compounds, such as Gallic acid, and apolar flavonoids, such as Quercetin. Furthermore, the use of an Excel 2 C18-PFP column allowed to obtain a good retention both from phenolic acids and flavonoids, thus obtaining a good separation of the analytes of interest, in a fast and effective chromatographic run. Working with the MRM acquisition mode and the precursor ion scan for each analysis, allowed a reliable quantitative analysis of the target compounds (>30) and furthermore to characterize the glycosidic derivatives of the flavonoids present in the food samples.

The validation parameters showed good results of the method with adequate sensitivity (low ppt for most analytes) and specificity, with recoveries between 55 and 100%, reproducibility ≤11%. The UHPLC-MS/MS method was applied to different food matrices in order to evaluate their phenolic content, related to their antioxidant capacity, measured by means of classical methods, allowing to define a characteristic PCs pattern of each matrix.

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Proteomic characterization and comparison of human platelet lysates for a standardized and high quality production

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Keywords: *human platelet lysate, cell culture, proteomics*

Since human platelet lysate (hPL) is rich in growth factors, it has been emerging as a possible substitute of fetal bovine serum (FBS) in cell culture, particularly in the expansion of mesenchymal stromal cells (MSCs) [1]. It presents several advantages, such as the elimination of the risk of bovine pathogens transmission and possible xeno-immunization [2]. However, there is a need to better investigate the composition and bioactivity of hPL and to develop a method which minimizes the variability in hPL production.
For this study, three different groups of blood donors (n=18 for each) have been used. hPL samples were prepared following two protocols based on different freeze/thaw cycles, one basic (hPLbasic) and a second with an increased mitogenic effect in *in vitro* model (hPLplus). Each of them, including the related platelet poor plasma (PPP), was albumin and IgG- depleted and enzymatically digested with FASP protocol [3]. Then they were "proteomically" analysed in triplicate using nLC-UHRTOF. Proteins were relatively quantified through label-free strategy based on PEAKS studio X+ platform.
At first, proteomic batch to batch variability of the same preparation was evaluated. Data were filtered with three increasing levels of stringency in data filtering. Independently from the set parameters, hPLplus showed a lower variability with respect to hPLbasic (15% vs 30% , 10% vs 31% and 14% vs 21% at poor, mild and high stringency, respectively), suggesting that the improvement of the propagation in cell culture for hPLplus is combined with an increase in the stability of the preparation. This result was, interestingly, confirmed by the study of cell expansion *in vitro* (data not reported). Moreover, the comparison between the two preparations (basic and plus) allowed to define a panel of proteins which could explain the beneficial effect in cell culture.
To conclude, this proteomic approach allowed to explore both the quality and the reproducibility of preparation methods, and simultaneously to gain an insight of the possible molecular actors actively involved in cell growth/differentiation in *in vitro* model for regenerative medicine.

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Cannabinoids analysis as powerful tool for the identification of commercial marijuana

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Keywords: *Cannabis sativa L.; HPLC-MS/MS analysis, Cannabinoids*

During the past decades, companies, research organizations and institutions focused their interest on industrial hemp, Cannabis sativa L. species with low contents of the psychoactive substance THC (Δ-9-tetrahydrocannabinol) [1]. The secondary metabolism of *Cannabis sativa* L. produces interesting molecules with different biological properties. Among them, cannabinoids represent the most investigated ones thanks to the ability to interact with some receptors of the endocannabinoid system, related to the control and modulation several physiological and pathophysiological processes [2, 3]. Cannabinoid class is mainly made up of neutral cannabinoids as cannabidiol (CBD), cannabigerol (CBG), cannabichromene (CBC), cannabinol (CBN), cannabidivarin (CBDV), and acidic cannabinoids as tetrahydrocannabinolic acid (THCA), cannabidiolic acid (CBDA) and cannabigerolic acid (CBGA). For the sample's treatment a fast and effective method was used, which consists in the first homogenization phase of the sample, by shredding with a chopper and sieving with a sieve, followed by extraction in ethanol. After suitable dilution was carried out the analysis of cannabinoids by means of HPLC-MS/MS. The dataset of 161 hemp samples sampled from four Italian retailers was analysed with ANOVA. To evaluate a correlation between the nine cannabinoids Pearson coefficients were calculated using the dataset of the 161 hemp samples. A partial positive correlation was found between decarboxylated cannabinoids (THC, CBD, and CBC) but no correlations between the 6 decarboxylated and 2 acidic cannabinoids THCA and CBGA. Tukey HSD multiple comparison test highlighted that the acidic cannabinoids had a lower capacity of discrimination than their neutral forms that showed a partial discrimination between the four hemp retailers, apart from CBDV. In conclusion, the present study contributes to support the thesis that the concentrations of THC and CBD are not sufficient to discriminate commercial marijuana that can be identified by the synergic contribution of the nine cannabinoids investigated.

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Development of a new analytical protocol by stable carbon isotope ratio to identify and characterize the authenticity of commercial cleaning products formulated with biobased surfactants.

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Keywords: *Carbon stable isotope ratio; Surfactants; Isotope Ratio Mass Spectrometry (IRMS)*

The fast growth of cosmetic and detergent market and the problems associated with their environmental, social and economic impacts have stimulated research and development of eco-friendly and biobased formulations [1,2]. Stable carbon isotope analysis could be used to determine the origin of commercial surfactants and cleaning products, especially in quality process by chemical companies. Radiocarbon methodology is the standard method to assess and check the authenticity of biobased surfactants, but the high cost and time consuming of this technique are relevant disadvantages to its commercial application. Very recently, a few pioneering studies have described the possibility to use IRMS (Isotope ratio mass spectrometry) as alternative method to radiocarbon methodology [3]. Isotope mass spectrometry is an automated and useful tool with many applications in different scientific areas. The $\delta^{13}C$ value was applied to commercial surfactants, such as ethoxylate alcohol, sodium lauryl sulfate, alkyl polyglucoside with different origin, that are the most common raw materials used in cleaning products. In this study, the isotopic analysis was performed on mixtures of commercial surfactants to simulate the commercial detergent formulations (handwashing, multisurface cleaner and degreaser) and then on bulk professional cleaning products to match relationship between isotope carbon composition and concentration of surfactants in real samples. This study demonstrated that $\delta^{13}C$ was correlated to the origin of surfactants. In particular we analysed five samples of biobased surfactants, with $\delta^{13}C$ value from -22,6 ‰ to -28,0‰, and six samples from carbon fossil raw material, with $\delta^{13}C$ value from -28,5 ‰ to -32,0‰, which were the most common raw material used in commercial cleaning products. Isotope carbon composition was also analysed on mixtures of biobased and fossil surfactants to simulate and perform the method for stable carbon isotope analysis of commercial cleaning products. Furthemore the results assessed the relationship between stable carbon isotope ratio values and surfactant concentration in mixtures: for example in 50% mixtures of biobased ($\delta 1^{3}C$ -22,6 ‰) and synthetic surfactant ($\delta^{13}C$ -32,5 ‰), we found a $\delta^{13}C$ value -28,00 ‰. The main advantage in using $\delta^{13}C$ analysis is related to cheapness and easy-to-operate method in comparison to radiocarbon methodology.

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Development of a Rapid LC-MS/MS Method for the Simultaneous Determination of Total Homocysteine, Methylmalonic Acid and 2-Methylcitric Acid in Dried Blood Spots: Application to Expanded Newborn Screening

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Keywords: *Organic Acidemias; LC-MS/MS; Newborn Screening*

Propionic acidemia, methylmalonic acidemias and combined remethylation disorders share abnormal propionylcarnitine (C3) and/or methionine levels by tandem mass spectrometry analysis in dried blood spot (DBS) samples from Newborn Screening (NBS) [1]. However, C3 and methionine are not good predictive markers due to their lack of disease specificity [2], leading to high false-positive rates. Here we developed and applied a rapid method for the simultaneous quantification of homocysteine (HCY), methylmalonic acid (MMA) and 2-methylcitric acid (MCA) in DBS samples by LC-MS/MS, providing a daily tool to make an accurate differential diagnosis. **Method.** The analytes were extracted from two 3.2-mm DBS disks with a solution containing dithiothreitol and internal standards (2H4-HCY, 2H3-MMA and 2H3-MCA). The extract was derivatized with 3N HCl in n-butanol and analysed by LC-MS/MS (ACQUITY UPLC I-Class/Xevo TQD IVD, Waters Corporation) operating in ESI+ mode under MRM acquisition. LC separation was carried out on a BEH C18 2.1 x 50mm column within 9 min, injection-to-injection. The analysis of calibrators within 5 consecutive days proved the method is linear with R2> 0.994 for HCY, R2> 0.995 for MMA, R2> 0.998 for MCA. The method reproducibility, accuracy and precision were assessed. **Application.** The method provided a fast, specific and reproducible second-tier test for the suspicion of propionic and methylmalonic acidemias, to be used in NBS clinical routine. The application led to cope with false positive results, discriminating between a high level of C3 related to inborn errors of metabolism, and those related to external factors, such as vitamin-B12 deficiency during pregnancy [3]. The daily use of this LC-MS/MS method performs more accurate analysis on the original sample provided for primary NBS, limiting the false positive rate and the psychological burden of the family, and supporting the differential diagnosis among organic acidemias [4].

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Characterization of 'superfoods' by means of metabolomic approach

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Keywords: *untargeted MS; superfood; metabolomics;*

In recent years, superfoods represent a billion-dollar global industry due to a push in marketing to promote these foods, advertising them as both healthy and exotic to appeal to consumers all over the world [1]. The popularity of these products is increasing, primarily due to increased incidence of health issues such as obesity, diabetes, heart disease, with a final goal of promoting general health. Concurrently the price of superfoods in grocery stores has increased, stimulating the interest of industry and research. The aim of this study is to provide evidence for potential molecular differences in the composition of superfoods by analysing the metabolomic patterns of fruit and vegetable samples. Samples were analysed using ultra-high performance liquid chromatography coupled (UHPLC-HRMS/MS). Bioactive compounds were annotated using network annotation propagation (NAP) available on Global Natural Product Social Molecular Networking (GNPS) [2] to improve in silico fragmentation predictions using FooDB and SUPNAT databases. Furthermore, the Dereplicator workflow, an algorithm designed to annotate the presence of known microbial peptides in MS/MS data, was used. Multivariate statistical analysis was performed to identify differences in the molecular diversity between superfood and common plant-based foods. For this purpose, superfood candidates were compared to the total dataset using metadata such as botanical taxonomy and ingredients. As shown through multivariate statistical analysis using PCoA to calculate diversity across all samples, there is no significant dissimilarity between the chemical composition of superfoods and non-superfoods categories , due to the full set of 565 foods is made up of a diverse array of samples with different sample matrices as well as properties and an array of many different botanical families. Molecular networking revealed the presence of several molecular families made up of superfood molecules not shared with the rest of the plant-based food samples. Since the exact causes of benefits from these plant-based foods are not well known, annotation propagation using the NAP tool was used to identify new putative compounds specifically.These results indicate that these foods contain significant amounts of potentially new bioactive microbial peptides. For the first time 21 types of food samples labelled as superfood were analyzed against a broad number of raw fruits and vegetables using UHPLC-HRMS and advanced data analysis tools. Metadata were used to inform sample analysis and identified the botanical family as a subcategorization revealing molecular differences between foods classified as superfoods and other fruits and vegetables.

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