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ADVANCED APPLICATIONS OF MATRIX ASSISTED LASER DESORPTION IONIZATION – TIME OF FLIGHT (MALDI-TOF) MASS SPECTROMETRY IN FOOD LIPIDOMICS

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el estudio es en sí mismo un acto de esperanza

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PREFAZIONE

La presente Tesi di Dottorato di Ricerca riporta una parte delle acquisizioni e dei progressi a livello analitico che sono stati ottenuti nel corso di diversi anni, a partire dai primi test effettuati verso la fine del 2003, presso l'Istituto di Scienze dell'Alimentazione del Consiglio Nazionale delle Ricerche di Avellino e il Dipartimento di Scienza degli Alimenti, Facoltà di Agraria, Università di Napoli "Federico II". La gran parte dei dati presentati è comunque il risultato del lavoro effettuato nel corso del triennio di Dottorato di Ricerca.

Quando - oramai quasi dieci anni fa - il Prof. Francesco Addeo mi chiese di provare a distinguere con la tecnica di spettrometria di massa (MS) MALDI-TOF la composizione di alcuni olii vegetali, accolsi la sua proposta con perplessità. La frazione lipidica di interesse alimentare è studiata e oramai ben conosciuta da decenni, e mi chiedevo quali nuove possibili acquisizioni lui stesse immaginando.

La tecnica MALDI, che io utilizzavo da qualche anno per l'identificazione e caratterizzazione di peptidi e proteine, ha il grande vantaggio della rapidità di analisi. Eppure l'ottenimento di uno spettro realmente informativo e la sua completa interpretazione richiese qualche mese di impegno e di prove. All'epoca, i pionieristici lavori di letteratura relativi all'analisi di grassi alimentari con la tecnica MALDI non erano molti di più delle dita delle mani e le procedure analitiche tra l'altro piuttosto nebulose e controverse. I nostri risultati sull'applicazione della tecnica MALDI MS per discriminare i grassi di deposito animale e per ottenere un rapido *profiling* della complessa miscela lipidica del latte, pubblicati sulla rivista *European Journal of Lipid Science and Technology* (Ed. Wiley) nel 2007, mostrano con ogni probabilità i primi spettri MALDI-TOF MS di queste miscele lipidiche comparsi in letteratura scientifica.

L'assenza del supporto - o meglio del "conforto" - di altre ricerche effettuate sull'argomento, mi rendeva vagamente incerto sull'affidabilità dei dati pubblicati, nonostante avessi ripetuto le analisi decine di volte e i risultati fossero più che plausibili, considerato che la composizione di queste miscele lipidiche era caratterizzata in dettaglio già da decenni. Così, quando poco dopo la pubblicazione mi fu recapitata una e-mail con la richiesta di un reprint dell'articolo, rimasi di ghiaccio di fronte allo schermo del computer. La richiesta era da parte del Prof. Paul Finch della Royal Holloway University of Surrey (UK), il quale dopo una serie di complimenti relativi al lavoro di ricerca concludeva testualmente "*I hope to try your technique on lipid extracts of pottery*", ossia "spero di potere testare la sua tecnica sugli estratti lipidici della ceramica". Non ero sicuro di aver compreso e verificai su più di un dizionario di lingua inglese il significato del termine "pottery"… Gli estratti lipidici della ceramica!!...Quella richiesta mi sembrò immediatamente una sarcastica bocciatura dei risultati pubblicati nonché una pungente and insolente provocazione. D'istinto pensai di rispondergli male, o almeno chiedere spiegazioni. Poi una rapida ricerca in internet mi fece raggiungere la pagina web di Paul Finch. Era un Professore Emerito e chimico-analitico che stava cercando di mettere a punto un metodo analitico rapido e sensibile per caratterizzare i grassi alimentari residui in vasi antichi di terracotta rinvenuti nel corso di scavi archeologici.

Ovviamente, l'inedita prospettiva applicativa che assumeva la strana richiesta del Prof. Flinch mi inorgoglì e rappresentava un esempio fulgido di come il mare della conoscenza sia fatto di gocce che seguono il loro imponderabile percorso e vanno a bagnare lidi assolutamente impensati.

La successiva messa a punto delle metodologie di preparazione del campione e di analisi strumentale, combinate alla mole di letteratura scientifica che veniva via via prodotta sull'impiego della tecnica MALDI-TOF MS in vari campi della *system-biology*, mi hanno permesso di apprezzare appieno il suo elevato livello informativo e gli enormi vantaggi nelle applicazioni ai lipidi, in termini di messe di informazioni composizionali e strutturali ottenibili, in relazione all'esiguità dei tempi di preparazione del campione. Negli anni è diventato gradualmente evidente che questa tecnica è potente e versatile dato che la sua l'applicazione razionale può contribuire a decifrare una serie pressoché illimitata di problematiche analitiche.

L’intuito e l’esperienza del Prof. Addeo avevano in qualche modo precorso i tempi, considerando che nell’ultimo decennio il numero di studi “lipidomici” in cui si fa un utilizzo esclusivo o comunque sostanziale della tecnica MALDI è cresciuto a ritmi esponenziali. L’applicazione di una tecnica innovativa a campi anche oramai consolidati, come quello dello studio della frazione lipidica degli alimenti, apre nuove prospettive e spalanca orizzonti imprevedibili.

Grazie anche al progresso degli apparati strumentali e al supporto del “data mining” bioinformatico, attualmente la tecnica MALDI MS è, quale metodologia a sé stante o quale strumento complementare, una delle *core technologies* nell’ambito di quelle che si sono oramai affermate quali “piattaforme di analisi lipidomica”.

Le tecniche evolute di MALDI “imaging” che, monitorando la distribuzione spaziale di specifici lipidi, forniscono immagini tridimensionali ad alta risoluzione di un tessuto o di un intero organo, sono la prova eclatante della realizzazione di quanto sarebbe sembrato fantascienza solo qualche anno fa.

Questa Tesi rappresenta una goccia nel mare attuale dell’ “analisi lipidomica”.

Gianluca Picariello

Marzo 2013

LIST OF ABBREVIATIONS

9-AA 9-aminoacridine
AA arachidonic acid - C20: 4, ω-6
APCI atmospheric pressure chemical ionization
API atmospheric pressure ionization
CHAC α-cyano-4-hydroxy cinnamic acid
CI chemical ionization
CID collision induced decay
CN carbon number
DAG diacylglycerols
DE Delayed Extraction
DHA docosahexaenoic acid C22: 6, ω-3
DHB 2,5-dihydroxybenzoic acid
EI electron ionization
EPA eicosapentaenoic acid - C20: 5, ω-3
ESI electrospray ionization
EVO oil - extra-virgin olive oil
F20TPP *meso*-tetrakis pentafluorophenyl porphyrin
FA fatty acids
FAB fast atomic bombardment
FID flame ionization detector
FT-ICR Fourier transform-ion cyclotron resonance
GC gas chromatography
GP glycerophospholipid
HAP hydroxyapatite
HPLC high performance liquid chromatography
HP-SEC high performance-size exclusion chromatography
IT ion trap
LC-MS liquid chromatography-mass spectrometry
LIT or **LTQ** lineari on trap
MAG monoacylglycerols
MALDI matrix-assisted laser desorption ionization
MS mass stectrometry
MS/MS tandem massspectrometry
MUFA monounsaturated fatty acids
NC nitrocellulose
NMR nuclear magnetic resonance
PC phosphatidylcholine
PE phosphatidylethanolamine
PI phosphatidylinositol
PL phospholipid
PS phosphatidylserine
PSD Post-Source Decay
PTV programmed temperature vaporizer
PUFA polyunsaturated fatty acids
Q quadrupole
SM sphingomyelin
STM synthetic TAG mixture
TAG triacylglycerol
TLC thin layer chromatography
TOF time-of-flight
TPC total polar compounds
SM sphingomyelin

ADVANCED APPLICATIONS OF MATRIX ASSISTED LASER DESORPTION IONIZATION – TIME OF FLIGHT (MALDI-TOF) MASS SPECTROMETRY IN FOOD LIPIDOMICS

ABSTRACT

The application of matrix assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry (MS) to the study of lipids has been long underestimated due to a series of intrinsic limitations associated to the technique, including the interference of matrix ion signals in the low *m/z* region, the natural tendency of lipids to fragment even under the "soft" ionization conditions of MALDI, the scarce reproducibility of the analysis, the inability in discriminating positional regioisomers or to characterize structural details such as position and geometry of double bonds in unsaturated lipids. In the last decade, because of several technical improvements, both the single-lipid targeted and the lipidome-wide extended investigations, carried out with the exclusive use or the substantial support of MALDI MS, have exponentially increased. MALDI-TOF has become one of the core tools of the recently born "lipidomics" and has proved to boast a series of unarguable advantages in the analysis of complex lipid mixtures, such as i) minimal sample handling; ii) no derivatization needed; iii) short analysis time required (1-2 min); iv) very high specificity, resolution and reliability; v) high sensitivity and dynamic range; vi) relative straightforward assignment of signals in the mass spectra; vii) possibility of performing analysis in mixture, without any previous chromatographic step.

We have explored the possibility of extending the application of MALDI-TOF MS to food-related analytical concerns, with progressively increasing challenges. Several quick strategies of sample pre-treatment have been developed to significantly enhance the capabilities and the analytical applicability of MALDI-TOF in food lipidomics, especially aimed to the analysis of triacylglycerols (TAG) in edible oils and fats. For instance, bromine addition to double bonds isolates the contribution of saturated TAGs from animal fats in the mass spectra. Similarly, the catalytic hydrogenation of TAGs allows to profile the pattern of the TAG families in complex mixtures (i.e. milk fat, fish oils) and to detect very low-abundance TAG classes. The silica gel pre-fractionation prevents ion suppression, allowing to profile polar separately from non-polar fractions. This analysis has allowed to identify several categories of neo-formed components in thermo-oxidized edible vegetable oils (sunflower and virgin olive oil) which underwent deep-frying conditions, as well as to discover TAG-derived species non detected before. Silica gel pre-partition has been preliminarily utilized also to investigate lipolysis in fermented meat products such as salami.

Several successful tests were also performed to isolate and analyze by MALDI-TOF MS non-TAG lipids. To this purpose, phospholipids have been selectively enriched from complex food lipid mixtures, using hydroxyapatite as an innovative support for solid-phase extraction.

A very promising strategy of sample deposition, that consists of the co-crystallization of lipids and matrix over a thin nitrocellulose (NC) substrate film, minimizes or completely abolishes TAG fragmentation. In addition, NC MALDI-TOF MS suppresses matrix ionization and improves the analytical repeatability. These aspects enable MALDI MS to the reliable quantitative evaluation of TAG and TAG-related species which derive from oxidative and hydrolytic scission, provided that suitable internal standards are selected.

Although the strategies outlined in this Thesis are virtually of general applicability in lipidomics, they have been specifically tailored for addressing analytical issue related to the study of food lipids. The rationale and targeted application of the MALDI MS-based strategies exhibits wide applicative capabilities to support the food science and technology under several aspects which include food quality, traceability, authenticity, nutritional properties, legal purpose assessment and monitoring of the modification induced by technological treatments on foodstuff.

1 GENERAL INTRODUCTION

1.1 Lipid classification

The “lipid” family embrace a huge class of compounds, many of which having chemical structures mutually not related. The class of lipids is so heterogeneous that even an exhaustive definition does not actually exist. Some estimates place the number of distinct naturally occurring chemical entities within the lipid class between 10000 and 100000. Lipids have been loosely defined as biomolecules that are generally hydrophobic in nature and in many cases soluble in organic solvents (Smith, 2000). Such a definition is not universally accepted and does not include all the components that are normally classified as lipids. Indeed, while lipids are usually extracted from the biological source by organic extraction, without special precautions some lipid families (such as the very polar phosphoinositides) can escape into the aqueous phase during phase partitioning (Wenk, 2005).

A more accurate definition is possible when lipids are considered from a structural and biosynthetic perspective, and many different classification schemes have been used over the years. In this sense, lipids can be defined as hydrophobic or amphipathic small molecules that may originate entirely or in part by carbanion-based condensations of thioesters (fatty acids, polyketides, etc.) and/or by carbocation-based condensations of isoprene units (prenols, sterols, etc.) (Fahy et al., 2005). According to the most widely used classification lipids are catalogued into eight classes: fatty acyls, glycerolipids, glycerophospholipids, sphingolipids, sterol lipids, prenol lipids, saccharolipids and polyketides (Fahy et al., 2005).

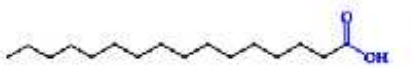
Fatty acyls constitute structurally the simplest subclass, including fatty acids (FAs), fatty alcohols, fatty aldehydes, fatty esters, fatty amides, fatty nitriles, fatty ethers, eicosanoids and hydrocarbons. Many components of this class, especially the eicosanoids derived from n-6 and n–3 polyunsaturated FAs, have potent biological activities. FAs are also the building blocks of lipids with a higher level of structural complexity, such as glycerolipids, i.e. monoacylglycerols, diacylglycerols, and triacylglycerols (TAGs) that, according to an obsolete nomenclature, were commonly referred to as triglycerides. In acylglycerols, FA chains attached to a glycerol moiety through an ester bond, but ether bonded FAs can be also found in glycolipids in minor amounts. Glycerophospholipids (GPs) are key components of cellular membranes although they are also involved in distinct functionalities such as metabolism and signaling. In addition to phosphatidylglycerol, in which a glycerol position is esterified by a simple phosphoryl group, in the most abundant GPs of biological membranes, phosphate group can be bound to an amino-alcohol, as in phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylserine (PS). The

glycerol-phosphate group binds a poli-alcohol in phosphatidylinositols (PIs). Sphingolipids are a complex class of lipids that share a sphingoid base backbone as the common structural trait. Sphingomyelins (SMs) and ceramides are classified within the sphingolipid family and are constituents of cellular membranes as well. Overall, SMs and GPs are commonly referred to as phospholipids (PLs) due to a phosphate group in the headgroup. The polar phosphate head confers PLs the typical amphipatic chemico-physical features. Sterols contain the cholestan core made of four condensed cycles. Cholesterol is the primary sterol lipid in animal fat. It is the precursor of bile acids and sexual hormones and play a critical structural role in the lipid membrane.

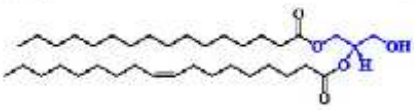
A gross classification and the exemplificative scheme of the basic structures of each lipid class is reported in **Table 1**.

Table 1. Lipid categories, abbreviations, sub-categories and exemplificative structures

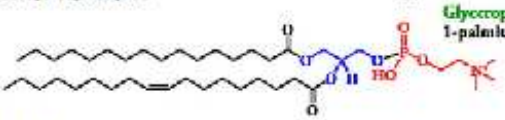
Category	Abbreviation	Sub-categories
Fatty acyls	FA	Fatty acids and conjugates [FA01] Octadecanoids [FA02] Eicosanoids [FA03] Docosanoids [FA04] Fatty alcohols [FA05] Fatty aldehydes [FA06] Fatty esters [FA07]
Glycerolipids	GL	Monoacylglycerols [GL01] Diacylglycerols [GL02] Triacylglycerols [GL03]
Glycerophospholipids	GP	Phosphatidic acids [GP10] Phosphatidylcholines [GP01] Phosphatidylserines [GP03] Phosphatidylglycerols [GP04] Phosphatidylethanolamines [GP02] Phosphatidylinositols [GP06] Phosphatidylinositides [GP07-09] Cardiolipins [GP12]
Sphingolipids	SP	Sphingoid bases [SP01] Ceramides [SP02] Phosphosphingolipids [SP03] Phosphonosphingolipids [SP04] Neutral glycosphingolipids [SP05] Acidic glycosphingolipids [SP06]
Sterol lipids	ST	Sterols [ST01] Steroids [ST02] Secosteroids [ST03] Bile acids and derivatives [ST04]
Prenol lipids	PR	Isoprenoids [PR01] Quinones and hydroquinones [PR02] Polyprenols [PR03]
Saccharolipids	SL	Acylaminosugars [SL01] Acylaminosugar glycans [SL02] Acyltrehaloses [SL03] Acyltrehalose glycans [SL04]
Polyketides	PK	Macrolide polyketides [PK01] Aromatic polyketides [PK02] Non-ribosomal peptide/ hybrids [PK03]



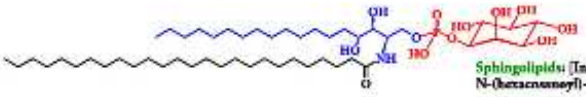
Fatty Acyls [FA(16:0)]
Palmitic acid



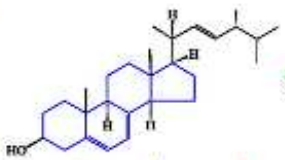
Glycerolipids [DAG(16:0/18:1(9Z))]
1-palmitoyl-2-oleoyl-sn-glycerol



Glycerophospholipids [PC(16:0/18:1(9Z))]
1-palmitoyl-2-oleoyl-sn-glycerophosphocholine

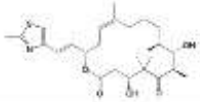


Sphingolipids [1m-1-P-Cer(t18:0/26:0)]
N-(hexacosanoyl)-4R-hydroxysphinganine-1-phospho
-(1'-myo-inositol)



Sterol Lipids [Ergosterol]
Ergosta-5,7,22E-trien-3B-ol

Prenol Lipids [Farnesol]
2E,6E-farnesol



Biosynthesis of lipids does not occur under a genic control. Nevertheless, lipids are produced, transported and recognized by the concerted actions of numerous enzymes, binding proteins, and receptors that are influenced by endogenous and exogenous (e. g. diet and temperature) factors. Although it is unclear how and why nature generates and perpetuates this incredible diversity, there is an increasing awareness of the critical importance of lipids in all aspects of life.

The need of analytical tools that can readily tackle such a complex range of molecular diversity is one of the key reasons why the systematic study of lipids has lagged behind the related system-biology "omic" disciplines, such as genomics and proteomics. However, the impressive technical advances introduced in the last decade have promoted the standardization degree of lipid analysis at levels that are comparable with those of genomics and proteomics, consequently moving extraordinarily forward the current knowledge about lipids.

1.2 Lipidomics

In analogy to the definition of other biomolecular subsets (e. g. genome, proteome, metabolome), the entire repertoire of chemically distinct lipid species in a biochemical system has been referred to as a "lipidome", which first appeared into the literature in 2001 (Kishimoto et al., 2001). Therefore, lipidomics has to be intended as the comprehensive characterization of the lipid entities within a biochemical system. With the term lipidomics, however, it is usually indicated also the array of the analytical platforms that have been developed for the systematic, high-throughput analysis of lipids.

In general, following the track of the recently-borne "system-biology" approach, lipidomics goes well beyond the simple complete characterization of lipid molecules in a biological system, but it also includes the understanding of the functions that lipids carry out within a biological system, taking into account their primary role in cell signalling, membrane architecture, transcriptional and translational modulation, cell-cell and cell-protein interactions and the dynamic response to environmental changes. The complete definition of these aspects is obviously a very challenging task, but the basic knowledge of the lipidomic profile entails the systematic characterization of the lipid components of a given system. Like proteomics, lipidomics is a subject which is both technology driven and technology driving as it has prompted the development of a series of dedicated analytical platforms and tools for data mining. Lipidome is part of the metabolome, intended as the complete panel of the primary and secondary metabolites of a given biological apparatus. In consideration of the relevance of lipids for biological systems and of the specific methodologies that are progressively developed, lipidomics is emerging as self-a standing "omic" science from the wider perspective of metabolomics (Griffiths and Wang, 2009). One of the most

promising and appealing purposes of the lipidomic investigations are aimed to identify potential biomarkers for establishing preventive or therapeutic approaches for several diseases, including, among the others, diabetes, obesity, atherosclerosis and Alzheimer's disease (Hu et al., 2009). Current research on lipids tends to shift the subject of investigations from determining the molecular structures of single lipids in biochemical samples to characterizing global changes of lipid metabolites in a systems-integrated context, with an approach that might be defined "functional lipidomics" (Feng and Prestwich, 2006). The pattern recognition methodologies, such as nuclear magnetic resonance (NMR) and the up-to-date mass spectrometric techniques, enable the so-called "molecular profiling" that represents inclusive snapshots of the entire lipid complements.

1.3 Food lipidomics

Foods are in general heterogeneous matrices of very different chemical compounds and reflect the complexity of the animal or vegetal organism they arise from. Just like for any other biological system, the recently born "omic" sciences, intended as the systematic definition of subsets of specific biomolecules, are finding applications also to foods. Thus, for instance, food proteomics include the complete cartography of the proteins, while metabolomic studies the panel of small molecules (low molecular weight arbitrarily assigned below 1-2 kDa) of a food product. The specific topic of food lipidomics only slightly differs from the general aspects of lipidomics, taking into account that alimentary lipids, once regarded simply as energetic reservoirs, do have biological and nutritional roles, many of which are now well established. Furthermore, in the majority of foods the lipids and their interaction features drastically affect the overall physical characteristics, such as flavor, texture, taste and appearance. Under a different perspective, food lipids "hold the memory" and reflect the history of the technological treatments foodstuff has undergone.

Generally, food lipids are largely dominated by TAGs. Food-derived TAGs are major sources of energy and provide essential lipid nutrients. This is the case of all the vegetable oils, such as those from olive, corn (maize), palm, soybean and sunflower as well as animal fats, such as tallow, lard and butter, and commercial products such as ghee or margarines. Seed oils naturally serve as a source of energy and structural fatty acids for the developing embryo. The most abundant animal TAGs are depot fats (from adipose tissue) or milk fats, and in any case their main function may be as a store of energy. Partial glycerides such as diacylglycerols (DAGs) and monoacylglycerols (MAGs), FAs and other minor components, like PLs and sterols, contribute to the lipid fraction of foods at a much lower extent. The lipid fraction of foods may greatly vary as a results of a series of dynamics. Variability factors of the edible oil composition include among the others genetics, meteorological conditions, geographical origin and technological parameters, such as extraction

methods and storage. Similarly, the composition of animal fats is affected by a series of endogenous and exogenous effects, these latter including feed and season.

The TAG fraction, in turn, is far to be an homogeneous set of components. In fact food TAGs and consist of complex mixtures differing by several basic attributes: (i) carbon number (CN), depending on the FAs esterified to glycerol; (ii) the degree of unsaturation, considering that FAs can be saturated, mono- or poly-unsaturated; (iii) the position and *cis/trans* configuration of FA double bonds; (iv) the regioisomerism of FA on the glycerol backbone (*sn*-2, *sn*-1/*sn*-3); and (v) the enantiomeric asymmetry at the *sn*-2 glycerol position when primary glycerol hydroxyl groups are esterified with different FAs. In fact, asymmetric TAGs can display optical activity, though this is usually too low to be measured. The conventional D/L or R/S systems could designate such enantiomers without ambiguity with simple molecules, but problems arise in application to the complex mixtures of naturally occurring TAGs. Such problems are prevented by describing the stereochemistry of TAGs and other glycerolipids with the "stereospecific numbering" (*sn*) system as recommended by a IUPAC-IUB commission (IUPAC-IUB Commission on Biochemical Nomenclature, 1967).

Because of the lipogenesis mechanisms, in which aceyl-CoA is exploited as building block to progressively construct the aliphatic chain, the most abundant FAs contain an even number of CN. However, especially animal fats or fish oils can contain odd-numbered FAs, which derive from rumen bacteria or water microorganisms, respectively. Some particular fats or some oils, such as castor oil, can also contain hydroxylated FAs (e. g. ricinoleic acid: 12-hydroxy-9-*cis*-octadecenoic acid).

Due to a similar complexity, the comprehensive and complete characterization of a food TAG mixture is often technically challenging. On the other hand a precise determination of the lipid composition of a food product is of primary importance for the knowledge of the foodstuff composition, for assessing the nutritional properties and for several reasons, some among which are listed below:

- *Legal.* Government regulations often demand that the amounts of saturated, unsaturated and polyunsaturated lipids, as well as the amount of cholesterol, be specified on food labels.
- *Nutritional.* The amount of specific FAs (e. g. saturated or *trans*-FAs) has to be maintained below opportunely defined threshold values. On the other hand higher relative amounts of polyunsaturated FAs are often desiderable.
- *Food Quality.* Many desirable physical characteristics of foods, such as appearance, flavor, mouthfeel and texture, depend on the type of lipids present.

• *Lipid oxidation.* Foods which contain high concentrations of unsaturated lipids are particularly susceptible to lipid oxidation, which can lead to the formation of undesirable off-flavors and aromas, as well as potentially toxic compounds (*e.g.*, cholesterol oxides and hydroxynonenal)
• *Adulteration.* Adulteration of fats and oils can be disclosed by targeting the lipid fraction, referring it to the profile expected for a genuine sample.
• *Food Processing and traceability.* Processing conditions of many foods affect the lipid composition, that determines important quality parameters of the food products.

"Lipidomics" has been practiced for years with differing methodologies with increasing degrees of analytic refinement (Han et al., 2012). Actually, in consideration of the complexity of the food lipid systems and of their technological and nutritional relevance, in the years many analytical protocols have been used for specifically addressing the issues that now can be comprehensively embraced by the "food lipidomics" topic. The analytical approaches are now progressively evolving toward the platforms of the modern lipidomics.

1.4 Analytical methods in lipidomics

Unlike other classes of biomolecules that can be considered as permutations on a common and finite set of monomers (e.g., proteins and oligonucleotides), complex lipids, such as for example glycerophospholipids and sphingolipids, are constituted of a large number of building blocks that can give rise to an astonishing array of combinations. Permutations that arise only from common eukaryotic lipid motifs may give rise to more than 180,000 phospholipid structures that could be present in a given cell or tissue extract. This number does not include other sources of heterogeneity introduced by isomeric lipids that differ in double-bond position, backbone substitution regioisomerism, *sn*-stereochemistry or *cis* and *trans* geometry of double bonds. The challenging ultimate aim of lipidomics can be achieved only if robust and high-throughput analytical and bioinformatic tools are made available.

Traditional methods for analyzing lipids rely on preliminar steps of prefractionation into lipid classes or polar and nonpolar lipids. Historically, gas chromatography (GC) also coupled to mass spectrometry (MS) detection has been by far the dominant technique in lipid research (Ryhage and Stenhagan, 1960; Sjövall, 2004). GC has successfully contributed to most of the current knowledge about lipids and allows to overcome the specificity problems as the great part of compounds in a sample can be resolved and detected. On the other hand, GC-based analysis suffer from several shortcomings, primarily due to the relative low volatility of some lipid classes, that render sometime insufficient to tackle the extreme complexity of the current lipidomic questions. In fact, lipid samples require handling pre-fractionation and derivatization steps that are time

consuming and rise the issue of sample "integrity". Lipid oxidation occurring over the time course of the analysis is also of concern and can greatly diminish sample integrity (Lerno et al., 2010). Chromatography-based lipid separations, either GC or high performance liquid chromatography (HPLC), especially with classical systems, also deliver limited resolution and can rarely resolve all the components of complex lipid mixtures. The lost of information about intact native lipid molecules, for instance in food samples containing simultaneously TAGs and partial acylglycerols, is one of the main pitfalls of the GC based analysis.

Other analytical techniques have been applied to lipid analysis with varying degrees of success, primarily Fourier transform infrared spectroscopy, NMR and MS (Hu et al., 2009; Carrasco-Pancorbo et al. 2009). While each of these methods has its own strengths and weaknesses, the most recently developed MS methods based on atmospheric pressure ionisation (API), electrospray ionization (ESI) (Han and Gross 2003; Han and Gross 2005; Schwudke et al., 2007) and matrix-assisted laser desorption ionization (MALDI) (Fuchs and Schiller, 2009) have emerged as the most powerful platforms for lipidomics.

1.5 The role of Mass Spectrometry in lipidomics

Recent advances in MS and progresses in chromatographic methodologies have largely driven the development of lipidomics. In turn, the analytical "issues" related to the omic sciences, also including lipidomics, have prompted MS technology and MS-related bioinformatics to push forward. Therefore, omics and omic platforms have evolved at the same rate. The greatest impulse to the borne of the omics has been undeniably prompted by the "soft" MS techniques, i. e. ESI (Fenn et al., 1989) and MALDI-TOF MS (Karas and Hillenkamp, 1988) , that have been introduced almost simultaneously in the late 1980s. Unlike conventional ionization sources, such as electron ionization or chemical ionization, the soft ionization techniques ESI and MALDI yield minimal or very limited in-source fragmentation under ordinary operating conditions. By this way, ESI and MALDI generate spectra that are of straightforward assignment, as virtually each signal correspond to a single component or to a set of isobaric compounds, and preserve the information about the intact molecules. This latter aspect has important nutritional implications when edible oils/fats are investigated, as many nutritional properties seem to dependo on how the FA components are grouped together as DAGs or TAGs rather than on the overall FA composition (Murase et al., 2001; Mu and Porsgaard, 2005). Both the techniques do not necessarily require preventive chromatographic separation and provide very quickly a profiling of complex lipid mixtures.

Nevertheless, analytical protocols in lipidomics are still far to be standardized. The initial enthusiasm of being able to harvest a great amount of compositional data with biochemical interest

is being gradually replaced by the demand for enhanced analytical precision and more accurate consistency (Shevchenko and Simons, 2010). It is not uncommon that lipidomics data conflict among from different laboratories. It also sounds unpractical or unimaginable to impose "golden standard" MS devices and related software for automation and data processing. In fact, due to the large diversity in molecular structures there is no single mass spectrometric approach which could cover an exhaustive molecular characterization of the whole lipidome of an organism, but usually techniques are selected on the basis of specific analytical requests and often different experimental platforms have to be combined.

The Lipid Metabolites and Pathways Strategy (LIPID MAPS) initiative constitutes the first broad scale attempt of overcoming those hurdles that still hinder lipidomics to become a true systems biology approach on par with the other "omic" disciplines. A new comprehensive classification system for lipids, along with a recommended nomenclature and structural drawing representation, and the adoption of advanced MS techniques as the instrumental platform to investigate lipidomics, are among the aims of LIPID MAPS (Schmelzer et al., 2007).

1.6 Basics of Mass Spectrometry

MS-based platforms constitute the core technologies in current lipidomics. The use of MS for analyzing lipids is not new as the classical electron ionization (EI) and chemical ionization (CI) techniques have largely been utilized, since the to elucidate the structure of relatively volatile lipids. However, far from all the lipids are amenable for EI and CI analysis because of the absolute requirements that the molecule must be insensitive to heat and have a sufficient vapour pressure to enter as a gas into the ion source of the mass spectrometer. Because of their characteristics, EI and CI are frequently used in combination with GC separation methods (GC-MS).

A massive impact on our understanding of the complex nature of the lipid components of biochemical systems has been produced by the recent evolution of MS technologies. Modern MS enables a rapid, accurate, reproducible, highly-sensitive and highly-specific analysis of either a chromatographically separated or even a crude complex lipid extract. Since some decades, MS is actually emerging as a new self-standing science on the border between chemistry and physics, whose field of investigation is the behaviour of ions in the gas phase. Indeed, to analyze a molecule by MS it has to be both volatilized and ionized. These two conditions are achieved by the source of a mass spectrometer. The gas phase ions produced in the source are sorted according to their *m/z* (mass-to-charge ratio) in the mass analyzer. Finally, ions are detected and processed by elaborating systems that yield the "mass spectrum", i. e. a two-dimensional plot of ion-abundance *vs. m/z* values. Therefore, a mass spectrum provides an highly accurate measurement of the *m/z* ratio of the

ions, from which it is generally immediate to infer the molecular mass of the individual compounds in a sample. The measurement of the *m/z* of ion fragments, which are naturally produced in the EI source or generated by collision induced decay (CID) in the tandem MS (MS/MS) experiments, yields structural information. Importantly, under opportunely controlled conditions MS can deliver quantitative data.

Typically, a mass spectrometer can be schematized as a device containing three main blocks which are an ionization source, a section of mass analyzers, and an ion detector (**Figure 1**). These components are operated by electronics and a computer.

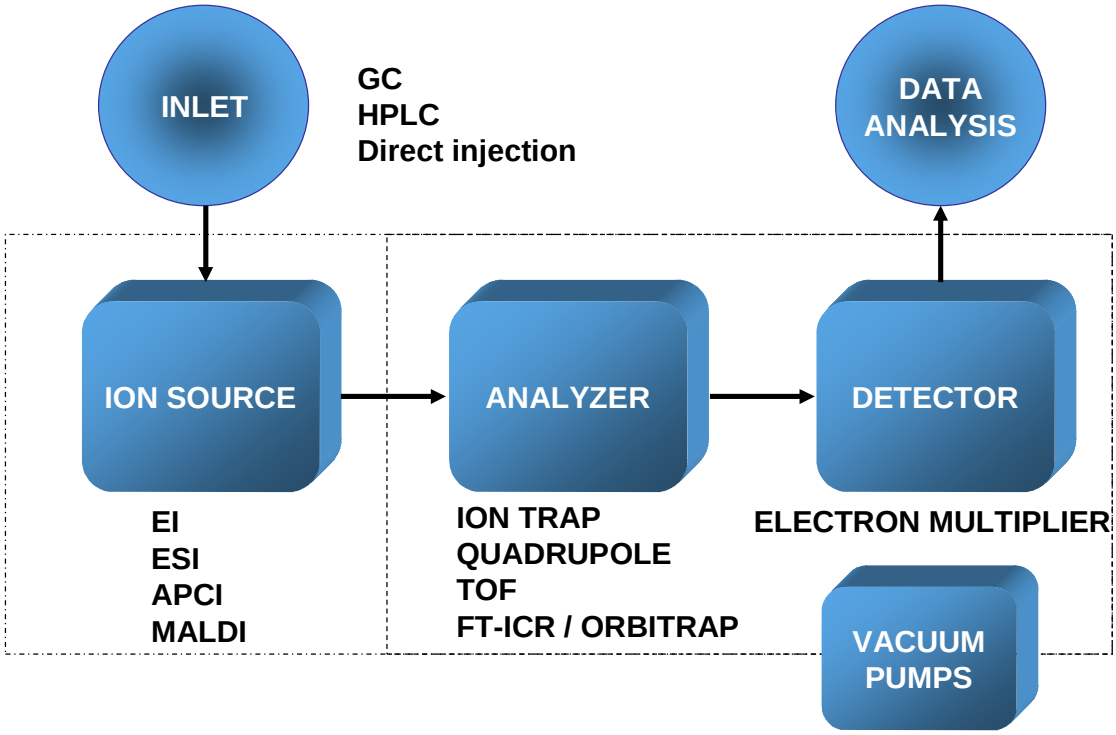


Figure 1. Block diagram of a typical mass spectrometer. MS/MS configuration contains more than one analyzer

1.6.1 Mass spectrometry ion sources

Currently, the ESI interface is the principal source enabling technology for tackling complex lipidomes. Increasing evidence is demonstrating that MALDI source is very useful in lipidomic research alone or complementarily to the ESI methods. ESI generates gas phase-ions at atmospheric pressure from a liquid solution that is forced through a capillary, made in conductive material, at a low flow rate (0.2-200 μl/min). The application of a strong electric field (0.8-5 kV) results in a fine spray of charged droplets with the same polarity as the applied voltage. As these charged particles move, the solvent continues to evaporate, thereby increasing the internal electric field density. When the mutual repulsive force of the charges exceeds the liquid surface tension, then fission occurs. Essentially, two models have been proposed to explain the ESI production of gas phase ions, i. e. the charged residue and the ion evaporation models (Cole, 2010). The real process is a merge of these two extreme models as a series of fission events and a continuous expulsions from distorted liquid droplets (Taylor cone), ultimately yielding "naked" charged isolated (gas phase) ions.

ESI-ionized molecules preserve almost unmodified the charge status they had in solution. Thus, molecules are ionized primarily *via* protonation (in positive ion mode) or deprotonation (in negative ion mode), or *via* the formation of adduct cations (*e.g.* ammonium, alkali metals) or adduct anions (*e.g.* Cl^-). Poly-electrolytes, such as proteins or peptides, ionize characteristically as multi-charged ions in an ESI MS spectrum. Aside from rare exceptions (de Souza et al., 2009; Liang et al., 2007), ESI-ionized lipids are singly-charged species. Atmospheric pressure chemical ionization (APCI), that is an additional source used in lipidomics, vaporizes solvent and sample molecules by spraying the sample solution into a heater using a inert gas, such as N_2, while molecules are ionized through corona discharge. APCI has for many aspects a source design similar to ESI. Unlike MALDI which is a pulsed, ESI is a continuous ion source that is well suitable to be interfaced on-line with HPLC separation (HPLC-ESI MS).

The MALDI source is somehow an evolution of the fast atom bombardment (FAB) that firstly allowed to directly analyze non volatile lipids such as PLs (Jensen et al., 1987; Murphy and Harrison, 1994). Although, fast atomic bombardment (FAB) afforded a major contribution to lipid MS, it suffered from several drawbacks such as (*a*) low overall sensitivity; (*b*) the presence of interfering matrix ions; (*c*) significant source fragmentation that biased quantification. For this reason FAB has fallen into disuse.

In the case of MALDI, analytes, homogeneously dispersed in proper solvents, are mixed with a solution of an opportune ultraviolet absorbing species - the so-called "matrix" – and are deposited on an apposite steel target and then let dry. One microliter or less of the analyte solution is generally

employed. The target is placed in the instrument source that operated under vacuum conditions. A pulsed laser beam (usually a N_2 laser emitting at λ=337 nm) is tuned to the appropriate energy and is directed in order to hit the co-crystals. Laser pulses transfer an elevated energy density to the analyte-matrix system, which in turn results in desorption subsequent to a sublimation at a temperature that has been estimated to range between 409 and 455 K (Stevenson et al., 2000). The high matrix to analyte ratio ensures that most of the energy is absorbed by the matrix and minimizes direct irradiation of the analyte. In fact, one of the primary effect of the matrix is to create a plume that adsorbs most of the laser energy, thereby preventing analyte dissociation. Therefore, the MALDI process of desorption (to the gas phase) and ionization starts from a "solid solution" of the analyte in the matrix. Most of the used matrix are weak organic acids (*e.g.* α-cyano-hydroxy-cinnamic acid; 2,5-dihydroxybenzoic acid; sinapinic acid) that are able to charge the analytes through a proton transfer due to the laser-induced molecular vibrations. Indeed, the MALDI effect depends upon the close proximity of the sample and matrix in the expanding gas cloud. In other cases, molecules can be ionized by different mechanisms as it is the case of the formation of metal ion adducts (exchange of metal ions) or the negative analysis mode (proton abstraction). The analyte ions produced are called ''adducts" or ''quasimolecular ions" (sometimes the term ''pseudomolecular" ions may be also found, although IUPAC discourages its use). Analytes with oxygen functionality are readily cationized with alkali metal salts like LiCl, NaCl, KCl, while analytes with unsaturated hydrocarbon functionality are readily cationized with transition metals like silver and copper. Due to protonation or formation of metal adducts recording positive-ion mode spectra is much more common. It even seems that MALDI detection of negative ions is much less sensitive than positive ones (Fuchs et al., 2010). However, addressing cationization of lipids is not a trivial task and it is a critical part of the sample preparation method.

The MALDI source almost exclusively produces singly-charged ions (Karas et al., 2000). The reason why even very large proteins, poly-electrolites or protonable polymers are predominantly (or exclusively) singly-charged it is not perfectly clear. In any case, the precise mechanism(s) of the MALDI ionization is far to be completely elucidated (Chang et al., 2007).

1.6.2 Mass analyzers

ESI and MALDI sources are combined in various ways with a number of mass analyzers. Currently, four types of mass analyzers are routinely used: quadrupole (Q), ion-trap (with its variants quadrupole ion-trap, QIT; linear ion trap, LIT), time-of-flight (TOF) and Fourier transform-ion cyclotron resonance (FT-ICR) mass analyzers. The structural information or the definitive identification about a selected ion can be obtained by tandem MS/MS. The ion of interest is

subjected to collision-induced dissociation by interaction with a collision gas. In tandem mass instruments, the first mass analyzer is used for the selection of the ion of interest, which is then fragmented in the collision cell; a second mass analyzer is used to separate the fragment ions on the basis of their *m/z* values, thus creating a product MS/MS spectrum where the ion count is plotted against the *m/z* of "daughter" ions. An MS/MS spectrum affords structural information and can be utilized for either structural confirmation or *de novo* assignment. Complex hybrid multiple-stage mass analyzers such as triple Q (QQQ), hybrid Q-TOF and tandem TOF (TOF-TOF) instruments have been combined for the purpose of MS/MS experiments. A typical QQQ mass spectrometry in both full-scan or tandem MS (MS^2) configurations is chematized in **Figure 2**.

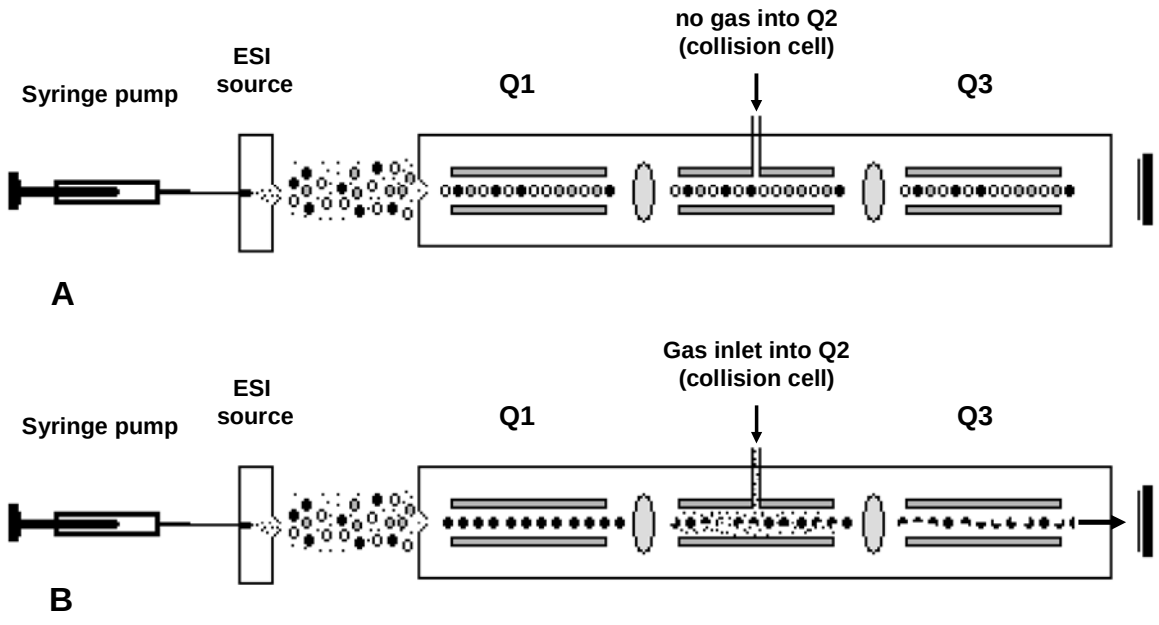


Figure 2. Typical triple quadrupole (QQQ) instrument in Q1 full scan (A) and tandem mass spectrometry (B) configurations. In full scan Q1 or Q3 are the scanning analyzers. In MS/MS configuration, Q1 and Q3 quadrupoles are operating as mass analyzers, and Q2 is operating as a collision cell. The precursor ion of interest is selected by Q1 and filtered to enter the collision cell (Q2), where it is fragmented by collision-induced dissociation (CID). The fragments are *m*/*z* scanned by Q3

One of the primary characteristics of an analyzer is its resolving power that is quantified through the resolution (R), whose most accepted definition is: $R = m/\Delta M$, where m is the lower value of two adjacent mass spectral peaks and ΔM is the mass difference between two adjacent mass spectral peak of about equal height, with an overlay (valley) of 10%. Q-TOF instruments deliver high resolution (up to 40,000) and mass accuracy of better than 5 ppm, which is sufficient

for obtaining many of lipidomic compositional details. In addition, TOF analyzers have a very high scan rate to acquire full product ion spectra at a very fast rate. Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR) MS offers a virtually unlimited mass resolution (even >500,000) and sub-ppm mass accuracy. Owing to the extremely high accuracy, many lipid structures can be assigned even in the absence of MS/MS spectra. Its combination with a linear ion trap (LTQ-FT) has become a upper edge standard instrumentation in proteomics, but its use is limited by the cost-effectiveness and by the often problematic instrument maintenance. Thus, very a few groups use it in lipidomic research. Triple Q instrument, as well as recent commercial configurations of Q hybrid instruments, are particularly adequate for quantitative analysis and allows the targeted monitoring through the selected reaction monitoring (SRM) or multiple reaction monitoring (MRM) MS mode. The performances of mass analyzers vary in sensitivity, resolution, mass accuracy and ability to produce high quality MS and MS/MS spectra. A special mention is deserved by a recently introduced type of hybrid instrument, referable to the Fourier-transform technology (commercially the LTQ-Orbitrap system), because of its high resolution (up to 150,000) and accuracy in molecular mass determination, although amore practical instrument management if compared to LTQ-FT instruments.

All the mass analyzer and ion detector sections (in MALDI also the source) operate at low pressures (10^{-2} -10^{-7} Torr) to prevent perturbed transmission and detection of the gas phase ions. While ESI is better interfaced with Q or IT analyzers, MALDI is generally interfaced to a TOF, that suites the pulsed nature of the MALDI source. Furthermore, TOF has a nearly unlimited mass range and this matches the MALDI production of singly-charged ions, especially when larger biomolecules are to be analyzed. The recent improvement of the performances of the analyzers, such as advanced TOF speed acquisition have prompted also performance improvement in the "ancient" MS methods such EI, enabling the introduction of ancillary techniques such as the GC x GC two dimensional separation, that permit permits, at least in some cases, the *de novo* identification of lipids from EI mass spectra, even within a very complex matrix (Mondello et al., 2008).

1.7 ESI MS approaches in lipidomics

The approaches that have been developed to deal with complex mixtures of lipids can basically divided in two categories: the first one involves little, if any, pre-separation of the lipids other than by simple solvent extraction, followed by direct infusion of the complex mixture of many different lipid classes in the mass spectrometer. This approach has been referred to as "shotgun" method and certainly has many advantages as well as some limitations.

The shotgun lipidomics arises from the intrinsic soft-ionization nature of ESI. It is particularly effective for polar lipids because these species readily undergo protonation $[M+H]^+$ or deprotonation $[M-H]^-$, depending on their molecular structure. Negative ion mode shotgun lipidomic analysis is also very informative for the analysis of glycerophospholipids. Under typical ESI conditions each lipid in a mixture corresponds to an ion in the spectrum. The resulting spectrum represents, therefore, a "profile" of the lipids in the extract. The major concern with the shotgun lipidomics is ion suppression, especially if very complex crude lipid extract containing a broad range of lipids classes is directly infused into the mass spectrometer. However, ion suppression can be prevented with several strategies, including pre-fractionation by differential extraction of lipid classes.

The second lipidomic approach makes use of chromatographic separation of crude lipid extracts to isolate specific lipid classes prior to analysis with or without further separation of the individual molecular species. This approach is substantially the HPLC-ESI MS analysis of lipids. HPLC-ESI MS is the technique of choice when low abundant compounds, such as signalling lipids, are to be detected. By this way, suppression events are minimized by decreasing the number of competing analytes entering the MS ion source at the same time and more accurate characterization and quantification is possible. In addition, the LC part of an LC-MS or LC-MS/MS method can, within certain limits, enables the separation of the isobaric and isomeric lipid species (Hu et al., 2009). The time-consuming concern has been limited by the introduction of ultrahigh pressure LC (with run times of ~10 min) with improvements also for the sensitivity and resolution. Recent developments of nanospray technologies have further increased the sensitivity of ESI, and chip-based nanospray-array devices have further significantly increased sample throughput (Hu et al., 2009).

The LC-ESI MS methodologies are well suited for targeted detection, monitoring and quantification of selected lipid markers in complex mixtures. One often does not know what precise lipid components can be present or formed under particular conditions. Thus, first surveys are generally exploratory ''untargeted'' readouts and are followed by progressively refined targeted analyses. As these approaches generate a huge amount of data, bioinformatics gives an invaluable contribution to "systems biology" initiatives that may help further advance into our understanding of biological systems.

1.8 MALDI-TOF MS for lipidomics

Since the very first "lipidomics" paper in 2003 (Han and Gross, 2003), the number of papers dedicated to this topic is steadily increased.

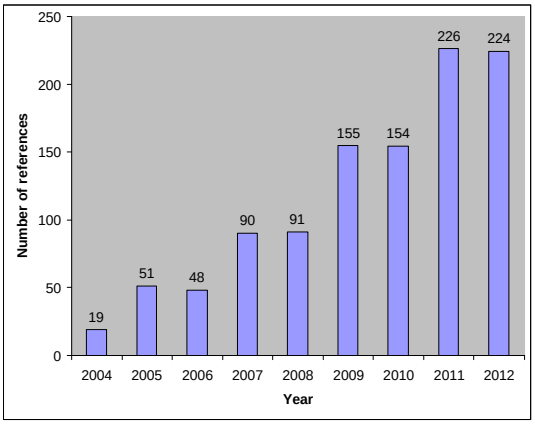


Figure 3. Yearly histogram of lipidomics-related scientific articles obtained by Scopus with "lipidomics" as a keyword (March 2013).

A simple inspection of a yearly histograph of the literature published in last eight years (**Figure 3**) with Scopus database with "lipidomics" as a keyword displayed a rapidly increasing rate of publications, which now numbers more than 200 annually. Although the number of "lipidomics" papers is much lower than papers dealing with genomics (~ 25000) and proteomics (~13000), this demonstrates the significantly increased interest in lipid research during the last decade. In particular, the applications of MALDI MS to lipidomics, are gaining extended interest.

In a review of 2004, Schiller et al. verbatim stated: "It is surprising how little attention MALDI-TOF MS has so far attracted in the field of lipids" (Schiller et al., 2004). In effect, for many years, the potentiality of MALDI MS in lipidomics has been largely underestimated due to several intrinsic drawbacks that include: (a) interference of cluster of matrix signals in the low *m/z* region; (b) ion suppression: most intense signals suppress the lower ones, complicating the detection of minor components. In this sense, a major concern is the detection of phospholipids classes in mixture (Pektovic et al., 2001); c) natural tendency of lipid to fragment, even under the "soft" ionization conditions of MALDI, thereby biasing quantitative determinations; (d) no capability in discriminating positional regioisomers or to characterize structural details such as position and geometry of double bonds in unsaturated lipids; (e) cost of the MS instruments. **Figure 4** reports the number of yearly published articles containing the words MALDI and lipid or phospolipids, demonstrating the continuously increasing interest in MALDI-MS applications in lipid analysis.

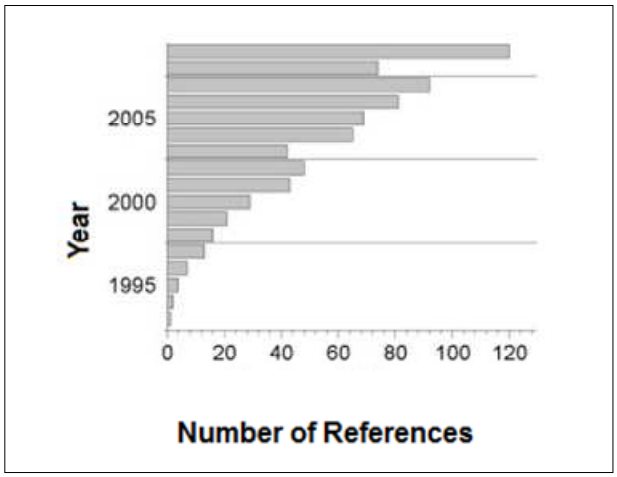


Figure 4. Number of scientific papers containing the term "MALDI" and the term "lipid" or "phospholipid". Figure adapted from: *Fuchs B, Süss R, Schiller J. An update of MALDI-TOF mass spectrometry in lipid research. Prog Lipid Res. 2010;49(4):450-75. Review*

Indeed, it has turned clear that MALDI MS can offer a series of relevant advantages if compared to other techniques, mainly consisting of: (a) minimal sample handling; (b) no need for hydrolysis or derivatization prior to analysis, so that the information about the intact molecules is preserved; (c) profile of an entire lipid fraction in time scales ranging from seconds to minutes; (d) relatively high tolerance to buffer and polar contaminants in the sample; (e) relative straightforward assignment of lipid components, due to production of unique or a few ion signals for each compounds; (f) high sensitivity, in the range of pico-femtomoles; (g) high dynamic range, as under controlled conditions of analysis low-abundance components can be detected simultaneously with the very-high abundance ones. Owing to its speediness, MALDI-TOF MS is useful for the routine analysis of a large number of samples. In addition, provided that the instrument is available, MALDI analyses are to be considered relatively inexpensive if compared to biochemical assays.

As already stated above, MALDI MS- based procedures for the analysis of lipids are far from the degree of standardization they have reached in the analysis of peptides and proteins. Nevertheless, a fervent research in the last decade has made available several convenient protocols for the MALDI analysis of both non-polar and polar lipids. The research in this field is also finding renewed motivations because of the successful application of MALDI-TOF MS to tissue imaging, through the monitoring of lipids-derived ions (Murphy et al. 2009; Gode and Volmer, 2013). The specific issue of the MALDI imaging is beyond the scope of this thesis.

Two major improvements have to be considered the milestones that have advanced the use of MALDI MS in the analysis of lipid mixtures:

1) the use of proper matrices. Dozens of matrices have been proposed for the MALDI analysis of lipids. However, only a handful are practically and routinely used. Primary requirements of a "good" matrix are its absorption properties at the laser-wavelength and efficient mixing properties with the analyte to give homogeneous co-crystallization. The analysis of lipids that are characterized by rather low molecular weights (in comparison to proteins, polymers, polynucleotides) requires additional properties of the matrix, such as the low yield of matrix ions. Matrices derived from cinnamic acid such as a-cyano-4-hydroxycinnamic acid and sinapinic acid, tend to undergo gas phase clustering, producing many interfering signals within the operating *m/z* values. These strong signals also provoke suppression and detector saturation. DHB (2,5-dihydroxybenzoic acid) is the most widely used and seems to be the most appropriate matrix for the analysis of lipids. DHB is a "cold" matrix, that reduce the prompt in-source fragmentation of lipids, which is particularly intense in the case of "hot" matrixes- With DHB both positive and negative ion mass spectra can be recorded from the same sample. Differently, *p*-nitroaniline (Estrada and Yappert, 2004) and recently, 9-aminoacridine (9-AA) have been proposed as alternative matrices of choice to record negative ion MALDI mass spectra (Fuchs et al., 2009). Other matrices, such as, 4-chloro-α-cyano-cinnamic acid (Jaskolla et al., 2008), 2,4,6,trihydroxyacetophenone (Stübiger and Belgacem, 2007) and α-cyano-2,4-difluorocinnamic acid (Teuber et al., 2010) have been proposed as convenient MALDI matrix in lipidomics. Laser/desorption ionization of TAGs has been demonstrated possible even in the absence of matrix (Calvano et al., 2005). The properties of the most used matrices, including advantages and pitfalls in the analysis of lipids, are summarized in **Table 2**.

2) the progress of electronics with the introduction of reflector TOF analyzers and the Delayed Extraction (DE) technology. Following their formation, ions are accelerated by an electric field (typically of the order of 20 kV). After passing a charged grid, the ions drift in a field-free region, the tube of flight, where mass separation occurs: low *m/z* reach the detector faster than high *m/z* ions. This is the typical simple ''linear" geometry of a TOF analyzer. Resolution and peak widths may be improved by using a reflectron, which by means of a "electric" mirror that deflects and reverses the ion beam, enlarges the path length. The linear and reflector geometries of the MALDI-TOF mass spectrometers are schematized in **Figure 5**. The DE consists in a delayed (within the range of nanosecods) and a pulsed rather than a continuous extraction of ions from the source to the analyzer. By this way, through mutual collisions ions average the root mean speed and compensate the relatively broad kinetic energy distribution due to the ablation process.

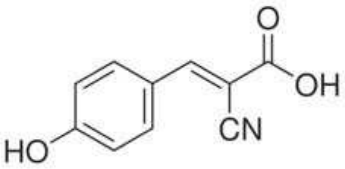
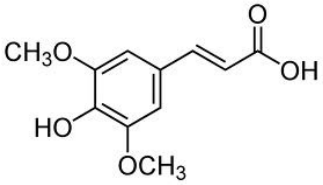
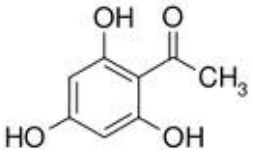
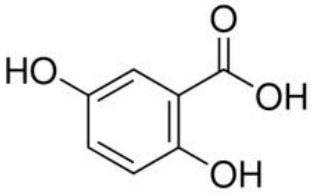
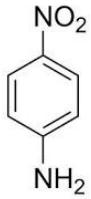
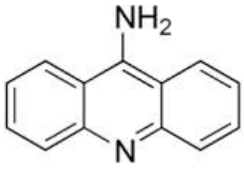
Matrix	Molecular formula – $[MH]^+$	Molecular structure	Properties and drawbacks
α-cyano-4-hydroxy cinnamic acid (CHCA)	$C_{10}H_7NO_3$ 189.04		Common matrix for peptides. In lipid analysis it produces many interfering signals. It tends to form clusters. Superior properties if the hydroxyl group is replaced by a chlorine (Jaskolla et al., 2008)
sinapinic acid (SA)	$C_{11}H_{12}O_5$ 224.07		Adequate for large proteins. Drawbacks as for CHCA. Scarcely used for lipids
2,4,6-trihydroxy Acetophenone (THAP)	$C_8H_8O_4$ 168.04		Neutral matrix. Suitable for recording negative-ion spectra of neutral or negatively charged lipids. Indicated as capable of selective detection of a certain lipid class in mixture (Stübiger et al., 2007)
2,5-dihydroxy benzoic acid (DHB)	$C_7H_6O_4$ 154.03		So far most largely used matrix in lipid research. Suitable for positive- and negative-ion detection. Lower background in the case of positive-ion spectra
para-nitroaniline (PNA)	$C_6H_6N_2O_2$ 138.04		Due to its basicity, most suitable for negative-ion detection. Can selectively detects certain PLs (Estrada and Yappert, 2004). Not completely stable under high-vacuum conditions
9-aminoacridine (9AA)	$C_{13}H_{10}N_2$ 194.08		Very promising matrix for lipid research. Particularly indicated for negative ion detection. Higher sensitivity than DHB, but spectra are affected by the solvent systems.

Table 2. Overview of the most common matrices used for the MALDI-MS analysis of lipids

The combined effect of reflector and DE enables a much better ion separation and drastically enhances both accuracy and resolution.

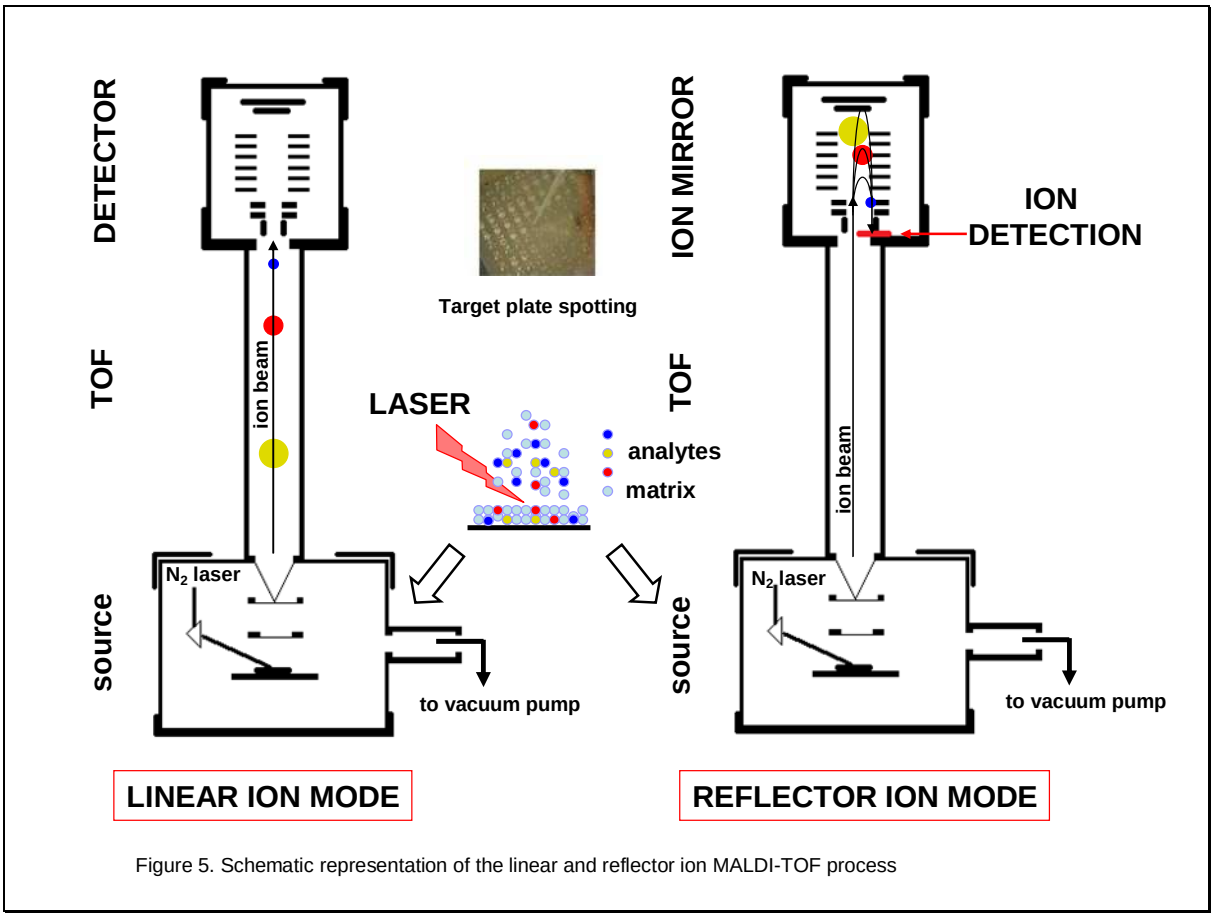


Figure 5. Schematic representation of the linear and reflector ion MALDI-TOF process

1.9 General aims of the Doctoral Thesis

As a prosecution of a research activity undertaken some years ago in collaboration with the supervisor Prof. Francesco Addeo, the current Doctoral Thesis has aimed to develop a series of analytical strategies in order to contribute to the standardization of the operative practices and to expand the informative level of MALDI-TOF MS analyses in food lipidomics. For the most part, these methodologies have been applied to the analysis and characterization of many food TAG mixtures of both plant and animal origin, addressing a variety of analytical enquiries. A significant section concerns the MALDI-based profiling of thermo-oxidized glycerolipids in edible oils. Preliminary experiments have been carried out to investigate the profiles of lipolysis-generated species as well as to isolate GPs in order to detect them separately from non-polar lipids in complex food lipid matrices.

The developed strategies essentially consist of sample preparation/pre-treatment methods and operative procedures, that have been designed with the attempt of providing responses to specific analytical issues, including:

(a) mass spectral isolation of the contribution of saturated TAGs, with respect to the unsaturated counterparts;

(b) simplified profiling of very complex lipid mixtures and enhanced detection of minor TAG families, such as very low or very high carbon number (CN) TAGs;

(c) pre-fractionation and analysis of very complex mixtures generated by thermo-oxidation of edible oils or by lipolysis during ripening of fermented food products;

(d) minimization of the in-source prompt fragmentation of TAGs in order to simplify the detection of low MW components and to reduce the bias in quantitative determinations;

(e) reduction of the matrix background, especially in the low *m/z* range, to include in the TAG profiling the informative spectral range with $m/z < 500$;

(f) application of already described MALDI MS-based methodologies (Ayorinde et al.; 2000; Hlongwane et al., 2001) to saponified oils and fats, in order to replace time-consuming GC analysis of fatty acids;

(g) attempts to develop fast procedures, based on the MALDI MS profiling, to assess the authenticity of dietary oils/fats, such as butterfat, and to detect possible adulterating lipids;

(h) fast selective enrichment/isolation of the GPs from the TAG fraction that almost always largely pre-dominant in foods.

Importantly, the procedures that have been set up are easy, inexpensive and of general applicability, even relevant for non food-related lipid mixtures. These methodologies have been tested and validated for a large number of oils or fats. However, for the sake of briefness only

selected applications will be shown and discussed to exemplify the main outcomes and the extended capabilities of MALDI-TOF MS-based analysis.

It has to be underlined that the potentialities of MALDI MS are not expected to exceed those of other lipidomic techniques, such as HPLC-ESI MS which at the moment has to be considered the "golden standard" in the field of lipid analysis. Nevertheless, MALDI, either alone or complementary to ESI MS-based methods, can afford a major contribution to the characterization of complex lipid mixtures and in general to the field of food science and technology, especially if opportune standardized procedures will be set up and rationally tailored to bridge specific analytical gaps.

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2 MATERIALS AND METHODS

A large number of edible oils and fats have been used to test and to tune the numerous MALDI MS-based methodologies, that in turn were designed to meet the specific requirements of the lipid mixtures.

Anhydrous bovine milk fat BCR-519 certified material was from the Institute for Reference Materials and Measurements (Geel, Belgium). This reference milk fat certifies a single TAG percentage. Lard and tallow body fat samples were obtained from local farms and were each a mixture (in weight) of ten bulk samples. Two months-ripened Napoli type salami was obtained from a local farm. Refined sunflower oil, hazelnut, walnut, castor oil and grape seed oil were obtained from the local market. Extra-virgin olive (EVO) oil samples, obtained by only cold pressing of olives, were furnished by a local oil mill (Avellino, Italy). Fish oils (including tuna, salmon, squid) and two fish oil based mixtures, used as dietary supplements named Eskimo-3 (mainly anchovy oil) and Pikasol (anchovy oil partially trans-esterified with ethanol), were purchased from Pharma Marine (Norway).

Human milk samples were obtained from 10 health donors, at lactation age in the 7-90 gg. Analytical standard TAGs including triolein, tripalmitin, trilaurin, and tricaprine, were purchased from Sigma-Aldrich (Milan, Italy). Standard 1-monoolein and 1,3-distearin were furnished by Larodan AB (Malmö, Sweden).

All solvents and reagents were of the highest grade commercially available and were for the most purchased from Carlo Erba (Milan, Italy). Acetic acid glacial and methanol were from J.T. Baker (Deventer, The Netherlands). All aqueous solutions were prepared using Milli-Q (Millipore, Bedford, USA) water. Rhodamine 6G and fatty acid (palmitate) were purchased from Sigma. Thin-layer chromatography (TLC) plates (No. 11845; 20636 20 cm) were from Merck (Darmstadt, Germany).

2.1 Fat/oil bromination

Bromination was carried out by drop-wise addition of 5% Br_2 (v/v) in $CHCl_3$ to TAG mixtures (10 mg/1 mL of $CHCl_3$ or $CHCl_2$) until solution turned to red. The glass tubes containing the TAG solutions were kept in an ice cold bath during the bromine addition. Afterwards, bromine excess was reduced with 10% aqueous sodium thiosulphate e vigorous vortexing. The aqueous phase was discarded and fat/oil solutions used for MS analysis.

2.2 Fat/oil hydrogenation

For TAG hydrogenation 10 mg of fat/oil were dissolved in 1 mL of $CHCl_2$ in the presence of a catalytic amount of platinum powder, under 1 bar H_2 pressure. Reaction proceeded 2h at room temperature. After hydrogenation lipid solutions were paper filtered, dried under a nitrogen stream and stored at -20 °C until use.

2.3 Thermo-oxidation of sunflower and extra-virgin olive oil

To simulate a deep-frying process, refined sunflower oil and extra-virgin olive (EVO) oil were continually heated at 180°C for 6 h, respectively, using a thermostatic household-frying bath (Tefal, Milan, Italy). Oil samples were collected in duplicate at different times during the heating; 0 (initial time), 60, 120, 240, and 360 min, and stored in glass bottles at -20°C until analyzed.

2.4 Separation of polar and non-polar fraction of thermo-oxidized oils

Polar and non-polar fractions were separated by chromatography on silica gel according to the official IUPAC procedure for the determination of the total polar compounds (IUPAC: Determination of polar compounds in frying fats, 1992). Briefly, 10 mg of oil dissolved in 2 mL hexane-diethyl ether 90:10 (v/v) was loaded onto a hand packed silica gel column (onto a Pasteur pipette plugged with a wisp of cotton wool). The non-polar fraction primarily containing the unoxidized TAGs was eluted with hexane-diethyl ether 90:10 (v/v), and the solvent was evaporated. The polar compounds were then eluted by diethyl ether and finally dried. The efficacy of separation was checked by thin layer chromatography (TLC), using pre-coated silica gel plates eluted with hexane-diethyl ether-acetic acid 80:20:1 (v/v/v) visualized by exposure to iodine vapors. A neat separation between the two fractions was achieved by silica gel chromatography.

2.5 Separation of polar and non-polar lipid fractions from Napoli type salami

Lipids pieces of Napoli type salami were separated from meat using a scalpel and suspended (100 mg/mL) in $CHCl_3$/methanol 2/1. Suspension was kept 1 h an ultrasonic bath, vigorously vortexed and finally centrifuged (Minifuge centrifuge, Heraeus, Osterode, Germany, 8000 rpm, 10 min 15°C). The supernatant was dried under a nitrogen stream and lipids were re-dissolved in $CHCl_3$ prior to chromatography separation on silica gel. Chromatographic step was carried out as described above for the separation of polar and non-polar fractions of thermo-oxidized oils.

2.6 Preparation of TAG samples for mass spectrometric analysis

To address the preferential cationization of TAGs as sodium adducts, lipid in $CHCl_3$ or CH_2Cl_2 solution (generally at the approximate concentration of 5 mg/mL) were extensively debated on a Vortex mixer with 0.5 M sodium acetate solution (1 mL). In tests of Li^+-cationization, triolein solution was debated with a 0.5 M LiCl aqueous solution. The aqueous phase was then discarded and the organic layer used for the MS analysis.

2.7 Hydroxyapatite chromatography enrichment of phospholipids

Preliminary tests based on the hydroxyapatite (HAP) chromatography were carried out to enrich phospholipids from both bovine and human milk. Total lipid extraction from both freshly collected bovine and human milk was performed by adapting the method by Bligh and Dyer (1959). Lipid fraction was obtained by milk centrifugation, followed by 5 min of cream hardening at -20°C and removal with a spatula. Approximately 0.1 mL of the floating cream, containing intact milk fat globule membranes (MFGM), were re-suspended with water in a glass tube to 0.2 mL. To this, 0.750 mL of $CHCl_3$/methanol 1/2 (v/v) was added and the resulting suspension was vigorously debated. He solution was finally adjusted to the final $CHCl_3$/methanol/water 1/1/0.9 (v/v/v) ratio by addition of 0.250 mL of pure chloroform and 0.250 mL of water. The final biphasic system was allowed to separate into two layers. The lower (chloroform) phase was washed three times with milliQ water and dried under a nitrogen stream. The lipid extract was re-dissolved in 0.5 mL of $CHCl_3$/methanol 2/1 (v/v) and used for HAP chromatographic separation. HAP gel (Bio-gel HTP, Biorad, Milan, Italy) was suspended in $CHCl_3$ (1 gr. in 5 mL) and packed by gravity onto a Pasteur pipette plugged with a small wisp of cotton wool. TAGs were washed away by stepwise flushing 3 mL of $CHCl_3$ and 3 mL of $CHCl_3$/methanol 2/1 (v/v). Phospholipids were eluted with a ternary mixture $CHCl_3$/methanol/1 M sodium phosphate 6/3/1. The eluates were directly used for the MALDI MS analysis.

2.8 MALDI-TOF MS spectrometry analysis

MALDI-TOF MS experiments were carried out on a PerSeptive BioSystems (Framingham, MA, USA) Voyager DE-Pro instrument, equipped with an N_2 laser (337 nm, 3 ns pulse width, 20 Hz repetition rate). In every cases, mass spectra have been acquired in the reflector positive ion mode using Delayed Extraction technology with a delay time of 150 ns applied between laser pulses and

activation of the ion-extracting high voltage. The instrument operated with an accelerating voltage of 20 kV. Even if several matrices have been tested (including CHCA, SA and THAP), in agreement with most of the literature, DHB provided clearly superior performances. The matrix solution was prepared by dissolving 10 mg of crystalline 2,5-dihydroxybenzoic acid (DHB) (Sigma, St. Luis, MO, USA) in 1 mL methanol containing 0.1% trifluoroacetic acid (TFA, Sigma, Milan, Italy). Alternatively, especially in the cases when adducts other than Na^+ ones (*e. g.* K^+), were detected, matrix was 10 mg/mL DHB in 50% aqueous acetonitrile (v/v) containing 5 mM sodium acetate. The fat/oil solutions (1 μL) was mixed (1/1, v/v) with 1 μL of the matrix directly onto the apposite stainless-steel plate (Perseptive BioSystems) and air-dried. Spectra of the phospholipid enriched fraction were acquired in the same conditions, using DHB in 50% acetonitrile/5 mM sodium acetate as the matrix. Typically, 250 laser pulses or more were acquired for each mass spectrum. In order to minimize source fragmentation, the laser power was generally kept at a value not higher than 10% above threshold. External mass calibration was performed with a separate acquisition using a mixture of standard TAG (obtained from Sigma) and low mass standard peptides. To check for the repeatability, samples have been analyzed at least in triplicate. Mass spectra were analyzed using the Data Explorer 4.0 software (PerSeptive BioSystems).

Post-Source Decay (PSD) MALDI ion spectra were acquired after isolation of the appropriate TAG precursor ions using timed ion selection. Fragment ions were refocused onto the final detector by stepping the voltage applied to the reflector. All precursor ion segments were acquired at low laser power to avoid saturating the detector; the laser power was increased for all of the remaining segments of the PSD acquisitions. Typically, 200 laser shots were acquired for each fragment-ion segment. The individual segments were finally stitched together using software developed by PerSeptive BioSystems.

2.9 Nitrocellulose MALDI TOF MS experiments

The nitrocellulose (NC) solution (1-20 mg/mL) was obtained by dissolving a piece of Transblot-NC pure membrane (Biorad, Milan, Italy) with either acetone or the acetonitrile/methanol 7/3 (v/v) mixture. Five-eight mg/mL was established as the optimal NC concentration range for MALDI analysis. NC solution (1 μL) was dropped to individual wells of the stainless steel MALDI target. The solvent almost instantaneously evaporated leaving a thin and homogeneous film covering the area of the well over which matrix and analytes were spotted.

Matrix was DHB 10 mg/mL in 35% acetonitrile (v/v)/5 mM sodium acetate. Matrix solutions containing percentages of acetonitrile higher than 50% in part re-dissolved the NC layer yielding detrimental effects on the spectrum.

Since the ability to obtain good mass spectra strongly depended on several factors among which the crystal formation, different protocols were tested for various types of samples.

We tested three methods to spot the TAG solutions, essentially based on the following procedures:

1. the "dried droplet" method: a premixed solution of matrix (1 µL) and sample (1 µL) was deposited over a thin preformed NC layer;
2. the "sandwich" method: sample (1 µL) was spotted over the pre-coated and solvent free NC layer on which matrix (1 µL) was applied and allowed to evaporate.
3. the "multilayer" method: matrix (1 µL) was applied over the pre-coated NC and let to dry; finally, sample solution (1 µL) was applied and allowed to evaporate.

Alternatively, the "dried droplet" spotting method was tested with DHB dissolved in ethanol containing 5 mM sodium acetate. The most convenient spotting procedure, in terms of reduced fragmentation, sensitivity, repeatability and mass accuracy resulted the "multilayer" deposition method. This finding is in agreement specific spotting methods that have been tested for the MALDI-TOF MS characterization of wax esters (Vrkoslav at al., 2009). For comparison, the same matrix and spotting procedures described above without using NC pre-coating were employed to acquire MALDI-TOF spectra.

2.10 NC MALDI-TOF MS based quantitative determinations

The point-to-point and shot-to-shot repeatability was evaluated by acquiring 10 replicated point-to-point and 10 spot-to-spot NC MALDI-TOF MS and MALDI-TOF MS spectra of EVO oil. The variability was evaluated from the relative standard deviation of the intensities of the most representative signals of EVO oil (*m/z* 853.7, 855.7, 877.7, 879.7, 881.8, 903.8, and 905.8) normalized with respect to the *m/z* 907.8 base peak of the triolein.

Tripalmitin was used as internal standard (IS) to spike EVO oil in several quantitative ratio in the exemplifying quantitative analysis of triolein in EVO oil. This choice is justified by the complete absence or presence in undetectable amounts of tripalmitin in EVO oil. The tripalmitin-to-EVO oil ratio varied in the range of 1:50 to 1:1 (w/w), with the IS ranging from 0.1 to 10 μg/mL. The tripalmitin-to-triolein signal intensity ratio (*m/z* 829.7-907.8) - an average of 10 measurements – versus the tripalmitin-to-EVO oil (w/w) ratio demonstrated a linear relationship (R^2 = 0.995) over the explored concentration range. The regression analysis allowed the measurement of the amount

of each TAG occurring in the oil sample, taking into account the IS signal according to the following equation: (W_{TAG}/W_{oil}) (I_{TAG}/I_{IS}) (W_{IS}/W_{oil}) (MW_{TAG}/MW_{IS}) R_z where W is the weight, I_{TAG} and I_{IS} are the relative signal intensities of TAG and IS read from the spectrum, respectively, and MW is the molecular weight. R_z is a correction factor that takes into account the ionization efficiency of the target TAG relative to the IS. In the case of triolein, R_z was 0.82. This correction factor needs to be determined empirically, also taking into consideration that it may non linearly vary when unequal TAG amounts occur simultaneously in the samples.

2.11 MALDI-TOF MS analysis of fatty acids deriving from saponified milk fat and olive oil

Saponification of human milk fat and EVO oil was carried out as described by Ayorinde et al. (2000) and Hlongwane et al. (2001) with minor modifications. Briefly, 1 g of NaOH pellets in 20 mL of methanol were refluxed in a 50 mL round-bottomed flask equipped with a water condenser, until complete dissolution. Lipid mixtures (200 mg) were included to the hot methanolic solution. The resulting brownish solution was stirred with a magnetic bar for 45 min. The mixture was then transferred to a clean bottom flask containing ~5 g of cruched ice. The gelatinous soap obtained was paper-filtered, air dried and ground into a fine powder which was stored at -20 °C until used. Prior to analysis saponified fats/oils were dissolved 20 mg in 1 mL of methanol/water (4/1, v/v).
For the MALDI-TOF MS analysis of fatty acids 10 mg/mL in chloroform of *meso*-tetrakis(pentafluorophenyl)porphyrin (F20TPP) with MW 974 (Sigma), was used as the matrix. Samples (1 µL) and matrix (1 µL) were mixed directly onto the MALDI target and dried under vacuum.

2.12 Gas chromatography analyses

TAGs from human milk fat and fish oil samples (1% w/v in n-hexane) were analyzed by GC-chromatography, in order to verify the consistency of the MALDI MS-based quantitative determinations. GC analyses of TAGs were carried out with a Agilent 6850 II-series (Palo Alto, CA, USA) instrument, equipped with a flame ionization detector (FID) and a PTV (programmed temperature vaporizer). TAGs were separated with a cyanopropyl methyl silicon (Quadrex 0-07-23) capillary column (50 m, 0.25 µm i.d.; 0.25 µm film thickness) from (Quadrex corporation, CT, USA) . High-purity helium was as a gas carrier at 1.2 mL/min. The injection was performed with a split ratio of 1/60 and a heating program of 50 °C for 0.1 min, followed by a 400 °C/min

increase to 370 °C with a 5 min hold. The column oven temperature program was 250 °C for 2 min, followed by an increase of 6 °C/min to 360 °C for 10 min.

Fatty acid composition of TAGs was determined by analyzing fatty acids methyl esters (FAME) after TAG trans-esterification with KOH in anhydrous methanol. To this purpose 100 mg of extracted oil/fat in a 10 mL glass-stoppered test-tube was dissolved in 2 mL n-hexane. A solution of 2 N potassium hydroxide (KOH) in methanol (300 µL) was added. The tube was vortexed for 30 s and allowed to react for 4 min at room temperature. Aliquots (1 µL) of the upper organic phase were analyzed by high-resolution gas chromatography.

GC of FAME were carried out using a Perkin Elmer Auto-system XL model gas chromatograph equipped with a PTV, a FID detector and a fused silica capillary column (Supelco Bellofonte, USA) (100 m, 0.25 µm i.d.; 0.20 µm film thickness; mod. SP 2380). The oven was set at an initial temperature of 100 °C for 5 min. The temperature was then increased at a rate of 3 °C/min to 165 °C and held for 10 min. This procedure was followed by a second increase in temperature at a rate of 3 °C/min to a final temperature of 260 °C, held for 28 min. The split ratio selected was 1:30. Helium with a linear velocity of 20 cm/s was the carrier gas. The FID conditions were a 10:1 ratio of air:hydrogen and temperature 260 °C. Chromatographic peaks were assigned and quantified by comparison with external analysis of the standard Supelco 37 Component FAME Mix (Supelco Bellofonte, PA, USA). All the GC analysis were carried out in the laboratory of Prof. Raffaele Romano, University of Naples "Federico II", Portici (Napoli).

2.13 ^{13}C Nuclear Magnetic Resonance spectroscopy-based detection of synthetic TAGs adulterating butterfat.

MALDI-TOF MS was tentatively used to detect foreign synthetic TAGs in authentic butterfat. A synthetic TAG mixture (STM) mimicking the saturated short- and medium-Cn TAGs of butter fat was obtained by adapting the strategy of Neises and Steglich (1979), according to which FA are esterified to glycerol by activation with odiimide in the presence of 4-dimethylaminopyridine. STM was prepared by Dr. Olga Fierro of the Institute of Food Sciences, Italian National Research Council (CNR) in Avellino, Italy. As explained below, MALDI-TOF MS profiling, as a "pattern recognition technique" was insufficient to reliable detect the addition of synthetic TAGs at percentage lower than 30% (w/w). Therefore, in collaboration with Prof. Raffaele Sacchi of the University of Naples "Federico II", Portici (Naples) a ^{13}C-NMR strategy was set up to assess the authenticity of butterfat and to disclose possible adulteration with synthetic TAGs.

High-resolution natural abundance ^{13}C-NMR spectra, acquired at the NMR Service of Istituto di Chimica Biomolecolare ICB of CNR (Pozzuoli, Italy) by Dr. Dominique Melck, were recorded on a

Bruker DRX-600 spectrometer, equipped with an inverse TCI CryoProbe™ (Bruker BioSpin, Rheinstetten, Germany) fitted with a gradient along the Z-axis at a probe temperature of 27°C, operating at 150.90 MHz ^{13}C frequency. TAG samples (25 mg) were dissolved in 0.7 mL of $CDCl_3$ and transferred to 5 mm NMR tubes. Each free induction decay was acquired over a 36 kHz spectral width using a 90° pulse of 14.7 μs and inverse-gated decoupling to avoid the nuclear Overhauser effect of the signals. Chemical shifts were compared to $CDCl_3$ as the internal standard, which was assumed to resonate at δ_C = 77.00 ppm. Carbonyl resonances were assigned on the basis of chemical shifts recorded on standard triacylglycerols (Fluka, Switzerland) and of literature data (Andreotti et al, 2000; Wellenberg, 1990). For the determination of the limit of detection (LOD) and the limit of quantification (LOQ), at least n=5 replicate samples of reference butterfat spiked with variable amount of STM were analyzed. Signals were manually integrated and the linearity of the calibration curve was confirmed by linear regression analysis.

2.14 References

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3 RESULTS AND DISCUSSION

3.1 Potentiality of MALDI-TOF MS as a pattern recognition technique for profiling edible triacylglycerols

3.1.1 Premises and aims

After performing a number of preliminary tests to establish a set of operative procedures, substantially in order to find the best analytical conditions in terms of sensitivity, resolution and reproducibility, the MALDI-TOF MS techniques have been exploited to profile a large varieties of edible oils and fats of both animal and plant origin. The MALDI-TOF MS optimized methods were demonstrated capable of addressing several analytical concerns, and have been applied to a progressively increasing degree of research challenges with the aims of defining the boundaries of their capabilities and pitfalls in food lipidomics.

3.1.2 Optimization of the cationization conditions

In MALDI MS-based analyses, TAGs are generally detected as cation adducts of alkali metals (most commonly Na^+ or K^+). Under typical conditions, no signals for protonated TAGs are observed. It has been suggested that $[TAG+H]^+$ species do form, but undergo a fast gaseous hydrolysis, that provokes the appearance of several fragmentation signals in the MALDI-TOF MS spectrum. In contrast, $[TAG+M]^+$ adducts, where M^+ represents an alkali-metal ion, did also fragment, but over time scales longer than the drift time in the TOF tube (Al Saad et al., 2003), and therefore can be detected. Ammoniated triacylglycerols are rarely observed in MALDI-TOF mass spectra. MALDI ionization of TAGs is accompanied almost invariably by a certain degree of prompt in-source fragmentation, which is particularly intense in the case of “hot” matrixes. Prompt fragmentation of protonated TAGs gives rise to artefact “diacylglycerol (DAG)-like” ions, arising from the loss of the metal salt of a fatty acid. The commonly accepted mechanism of TAG fragmentation is reported in **Figure 6**.

Fragmentation has detrimental effects of the informative level of the spectra, especially because it increases the number of signals in the low *m/z* region and it biases quantitative inference. The extensive TAG fragmentation due to the laser fluence was attributed by the pioneering investigators at least in part to the incompatibility of the solvents for lipids and those normally used for dissolving matrices (e. g. aqueous 50% acetonitrile) (Asbury et al., 1999).

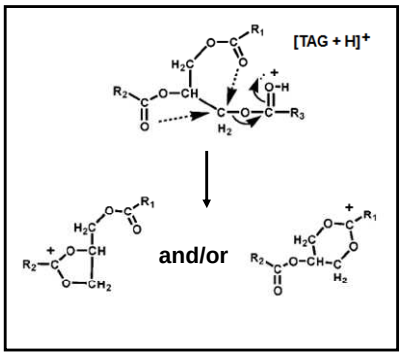

Figure 6. Mechanism of prompt fragmentation of protonated TAG. A similar pathway underlies the fragmentation patterns observed in collision induced decay or in Post-Source Decay (PSD) MS spectra. Figure adapted from *Al Saad et al. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry of lipids: ionization and prompt fragmentation patterns. Rapid Commun Mass Spectrom. 2003;17(1):87-96.*

These limitations have been overcome to some extent through the use of "softer" matrixes (for instance, DHB/*p*-nitroaniline) and/or by optimizing the procedures for sample preparation.

The nature of TAG adducts has critical importance on the quality of the MALDI MS detection. The cationization with alkali-metal ions promotes an enhanced detection of TAGs for the reasons explained above. The Li^+ adducts seem to be the most stable and have a lower tendency to fragmentation (Cvacka and Svatos, 2003). The strength of the dative bond between TAGs and alkali metals or NH_4^+ cations vary in the order of $Li^+ > Na^+ > K^+ > NH_4^+$ (Adams and Gross, 1986). The stronger bond with Li^+ reduces partly the phenomenon of the "mobile charge" that underlies the remote-charge fragmentation mechanism. Nevertheless, the use of lithium salts as cationizing agents or for the production of "doped" matrices (Cvacka and Svatos, 2003) has remained boundary because of several reasons: i) lithiation does not completely abolish fragmentation; ii) almost always Na^+ adducts are also detected, so complicating the spectra. In fact multiple cationization leads to ambiguity in identifying the individual lipid species in complex mixtures: iii) lithium naturally occurs with two isotopes, namely ^6Li and ^7Li (natural abundances 7.5% and 92.5%, respectively), thus introducing a novel source of signal dispersion and heterogeneity as well as possible interferences in the mass spectra.

In **Figure 7** the MALDI-TOF spectra of standard triolein (MW 884.8, monoisotopic) carried out after addressing Na^+ (panel A) and Li^+ (panel B) cationization are compared. Although fragmentation of Na+-triolein (*m/z* 907.8) is more pronounced (signal at *m/z* 603.5), spectrum of the Li+-triolein (*m/z* 891.7) is complicated by the presence of interfering significant signals of contaminating Na^+- and K^+-adducts (*m/z* 923.8) and by the simultaneous cationization with the ^6Li

natural isotope (*m/z* 890.7). Note that at the operating resolution the K^+-adducts (*m/z* 923.8) is undistinguishable on the basis of the nominal mass from possible Na^+-adduct of oxidized triolein. In fact, the difference between atomic weight of Na and K (16.1093) is very close to the atomic weight of oxygen (15.9994).

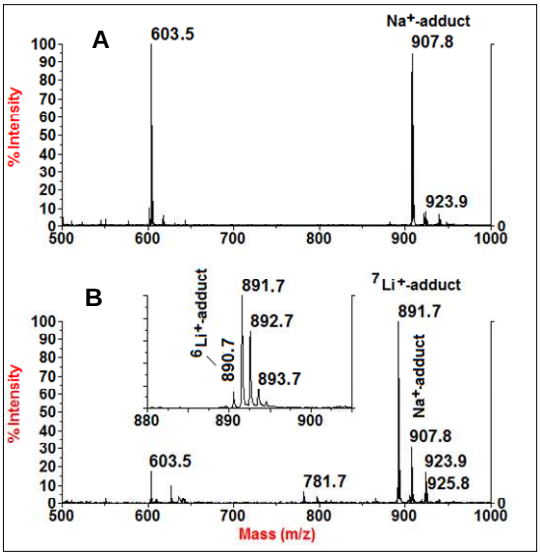

Figure 7. Comparison of MALDI-TOF MS spectra of standard triolein (MW 884.8, monoisotopic) cationized with Na (panel A) and Li (panel B) salts. Fragmentation of Na^+-triolein (*m/z* 907.8) is more pronounced (*m/z* 603.5). Nevertheless spectrum of the Li^+-triolein (*m/z* 891.7) is complicated by the presence of interfering significant signals of contaminating Na^+- and K^+-adducts (*m/z* 923) and by the simultaneous cationization with the ^6Li natural isotope (*m/z* 890.7).

The selective cationization with Na^+ provides the best compromise between clean spectra and low TAG fragmentation. Thus, in our analysis we have practically always addressed the cationization of TAGs with sodium salts. A complete cationization is critical to avoid confusion between possible oxidized TAGs and K^+-adducts of TAGs. For this reason, in addition to debating organic solution of TAGs with solution of sodium salts, in most of cases matrix also contained 5 mM sodium acetate. Differently form other reports, no major improvements were observed in the spectral quality when DHB, the matrix that we have most widely used in these investigations, was dissolved in a non-polar compatible solvent (*e.g.* ethanol, acetone) instead of a water/acetonitrile system.

3.1.3 MALDI-TOF MS as a pattern recognition techniques: lipid profiling

MALDI-TOF MS has been widely used in the past as a pattern recognition technique to profile oils and fats. The MALDI profile offers an immediate survey of the TAG families occurring in the

sample clusterized according to their CN. In **Figure 8** an exemplificative spectrum of EVO oil is reported. The TAG families are easily distinguished because of the signals that differs byof 26 or 28 mass units (28 corresponds to -CH_2-CH_2 ethylene insertion, while 26 reflects also a different unsaturation degree). The information about naturally occurring DAGs is also immediately deduced (low *m/z* region). This spectrum has been acquired with the NC MALDI-TOF MS method (see below); the DAG signals are real species in the mixture and not "DAG-like" artefacts due to fragmentation. Within each cluster, the TAGs with the same CN but with different degrees of unsaturation can be easily distinguished, because of the difference of 2 mass units/double bond. TAG and DAG ones occur as Na^+-adducts in the spectrum.

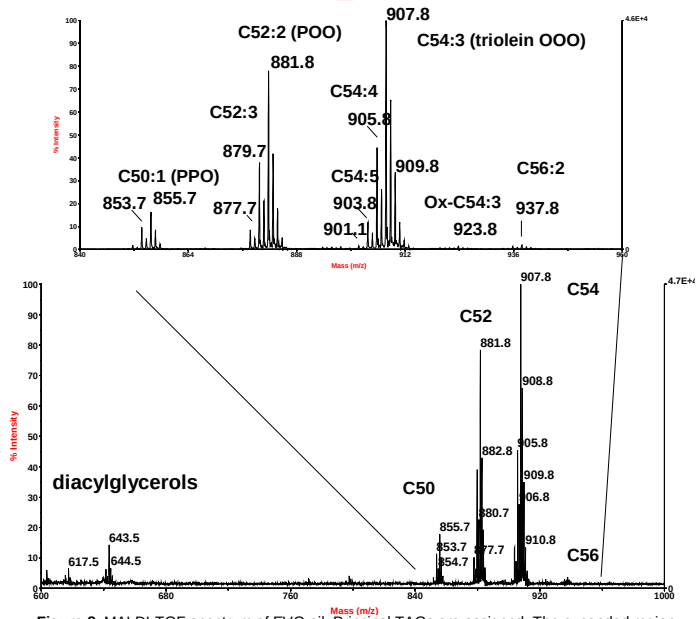

Figure 8. MALDI-TOF spectrum of EVO oil. Principal TAGs are assigned. The expanded region evidences within each family cluster several TAGs differing by the unsaturation degree. Spectrum has been acquired with the NC MALDI-TOF MS method (see below).

Figure 9 exemplifies the easy differentiation of two common edible oils, *e.g.* EVO and sunflower oil, on the basis of the higher amount of polyunsaturated fatty acids (PUFA) in sunflower oil. The most prominent signals of sunflower oil are C54:5 and C52:4 in the respective clusters, while in EVO oil the triolein (C54:3) and C52:2 are prevalent due to the high content of monounsaturated fatty acids (MUFA), primarily oleic acid. Obviously, the MW measurement alone does not give information about the regioisomerism of the TAGs (for example, POO and OPO, *m/z* 881.8, where P= palmitate and O= oleate, are undistinguishable), as well as it is not possible to obtain information about position and geometry of the double bonds in unsaturated FAs.

Spectra of **Figure 9** were subjected to a software assisted automatic "deisotoping", that is a procedure for selecting only the monoisotopic signals. The remaining signals of the isotopic distribution, arising because of the 1.1% natural occurrence of ^{13}C isotopes, are removed. Deisotoping renders a cleaner spectrum of very straightforward interpretation.

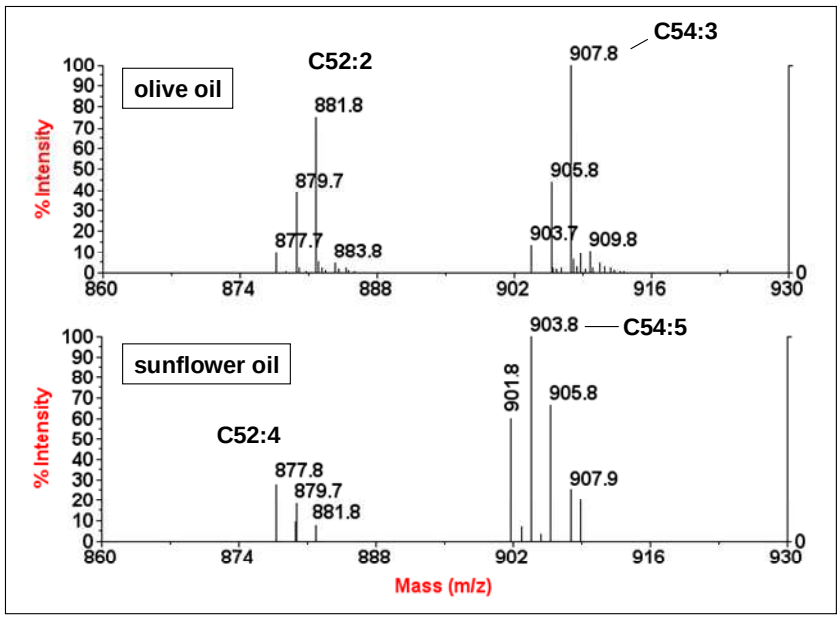

Figure 9. MALDI-TOF MS based comparison of high CN TAGs from olive and sunflower oils. It is clearly appreciated the higher content of PUFA in sunflower oil while MUFA prevail in olive oil. Spectra have been deisotoped.

In the current study, we have profiled many edible oils and fats of both common and uncommon utilize. Some selected examples are shown in the following. In **Figure 10** are compared the spectra of beef tallow (bovine deposit fat) and lard (swine deposit fat). MALDI-TOF MS analysis enables a clear and immediate distinction between these two fats. The tallow profile is more complex as it contains TAG families substantially in a wider range (C46-C54), while lard has exclusively C50-C54 TAGs. Averagely, lard has a "more unsaturated" character, as TAGs with a higher number of double bounds are prevailing. This observation is in agreement with the lower average content of saturated FAs in lard (~40%) if compared to tallow (~44-48%) and the higher amount of MUFA in lard (~55%) with respect to tallow (~50%) (data from: Christie at al., 1986). To this purpose, highly unsaturated C52:5, C54:5 and C54:6 TAGs were detected exclusively in lard. Signals corresponding to C55:0, C55:1, C55:2 and C54:0 TAGs typified tallow. Interestingly, tallow contains appreciable

odd-numbered CN TAGs, that are produced by ruminants according to the propanoic acid biosynthetic pathway. The odd-numbered CN TAGs are almost completely missing in monogastric animal species.

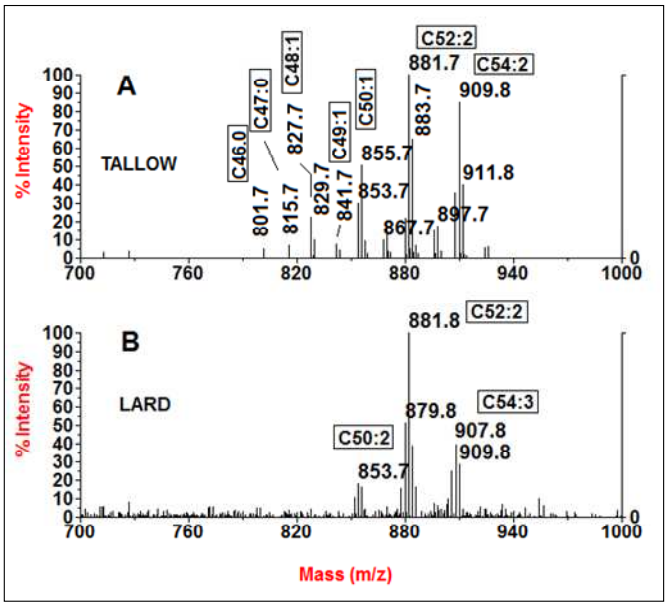

Figure 10. MALDI-TOF MS comparison of bovine (A) and swine (B) deposit fat. The profile of tallow is more complex due to the presence of TAGs in the C46-C54 range, while only C50-C54 TAGs occur in lard. Tallow also contains appreciable odd numbered-CN TAGs. Lard has averagely a "more unsaturated" character. Spectra have been automatically deisotoped.

As further examples of MALDI-TOF MS oil/fat profiling, in **Figure 11** are shown the spectra of coconut butter, that has plenty of short- and medium-chain saturate FAs belonging to low-medium CN saturated TAGs.

The MALDI-TOF MS spectrum of hazel nut oil (**Figure 12A**) is a quite interesting case of study as it shows a TAG profile that, as it is already known, is closely similar to that of EVO oil, with the base peak due to triolein, arising from the high MUFA content. For this reason hazelnut oil is frequently employed to deceptively adulterate the more expensive EVO oil. Spectra of EVO oil will be shown in other sections of this thesis. Several MALDI or GC-based determinations of the differential TAG content, combined with mono- or multivariate statistics, have been proposed even recently to detect hazelnut oil in EVO oil (Yang et al., 2013).

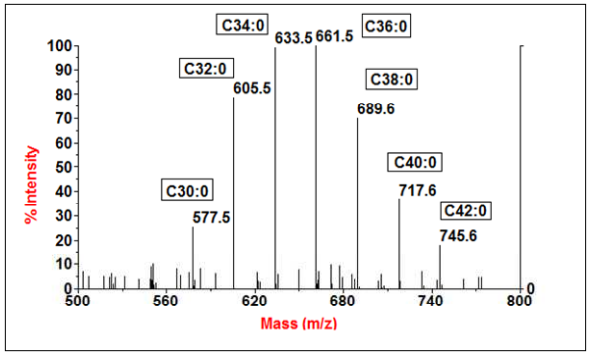

Figure 11. MALDI-TOF MS profiling of coconut butter, containing exclusively saturated low-/medium-CN TAGs

However, quantitative determinations by MALDI are quite complicated, so it should be more reliably used for monitoring specific "on/off" markers or different profiles of target compounds, rather than discrepancies in the amount of single compounds.

the most promising strategies seem to rely on MALDI MS differential analyses which target compounds other than TAGs, such as polar unsaponifiable species (Calvano et al. 2010) or phospholipids (Calvano et al., 2012). These methods enable the detection of up to 1% hazelnut in EVO oil. Walnut oil has a definitely different TAG profile with respect to hazelnut and EVO oil (**Figure 12B**).

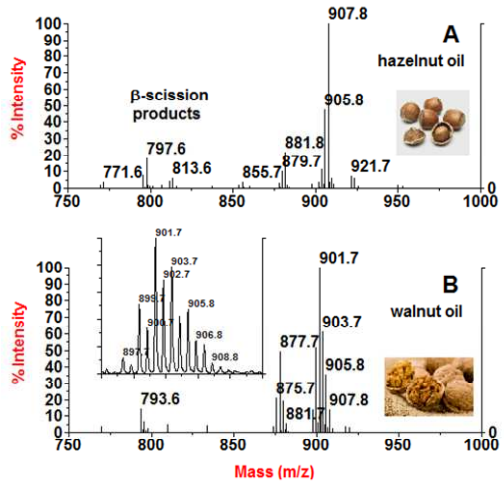

Figure 12. MALDI-TOF MS profiling hazelnut (A) and walnut (B) oils. Due to the high content of oleic acid, hazelnut has a TAG profile closely resembling that of EVO oil. Walnut has a different TAG content, with a "more unsaturated" character. Spectra are automatically deisotoped. Inset shows the non-deisotoped C54 TAG cluster of walnut.

The last example of MALDI-TOF TAG profiling is that of castor (*Ricinus communis*) oil (**Figure 13**), used in food industry as a food additive and a mould inhibitor. This oil offers an interesting example of TAGs containing hydroxylated FAs (~85 ricinoleic acid). Data are substantially in agreement with a previous MALDI determination of castor oil (Ayorinde et al., 2000), that showed triricinoleic (RRR) as the predominant species (*m/z* 955.8) and lower intensity signals of RRS (*m/z* 941.7, S= stearate), RRO (*m/z* 939.7), RRL (*m/z* 939.7, L=linoleate), RRP (*m/z* 913.8). Interestingly, we detected a low-intense signal at *m/z* 953.8, indicative of the occurrence of a di-insaturated derivative of ricinoleic acid ("ricinolinoleic acid"), that has not been described before.

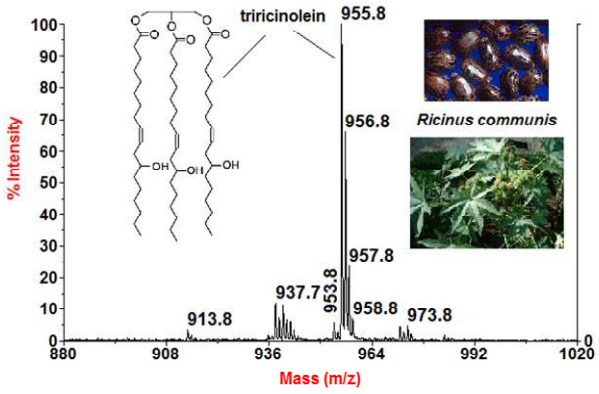

Figure 13. MALDI-TOF MS analysis of castor (*Ricinus communis*) oil. The most intense signal corresponds to triricinolein. Ricinoleic acid is an example of hydroxylated FA.

3.1.4 Capabilities and pitfalls of MALDI-TOF MS lipid profiling: identification of milk from several species

As emphasized before, the MALDI MS-based profiling is a powerful tool for discriminating typologies or oils and fats. However, the MALDI MS monitoring provides reliable a analytical response principally when specific ("on/off") monitorable markers have been already assessed. **Figure 14** shows the MALDI MS based comparison of the milk fat profiles from bovine (**Figure 14A**) and ewe milk (**Figure 14B**). The TAG patterns enable a clear distinction between the two fat mixtures. More in detail, the bovine milk fat exhibits the typical "bimodal" pattern that is also characteristic of the GC based separation of TAGs. On the contrary, ewe milk TAGs exhibit a distinct monomodal pattern. In addition, owing to a higher content of short chain FAs if compared to the bovine counterpart, ewe milk exhibits more intense signals in the low *m/z* range.

Although the two milk fat are easily distinguished as pure fats, the detection of their blends even arising from addition of as abundant as 50% of cheaper bovine milk to ewe milk, is problematic. In this sense, direct MALDI-TOF MS analysis is not reliable to disclose possible adulterations.

The extreme condition of this shortcoming is the failure in the differentiation between water buffalo and bovine milk fats. Also due to a certain fluctuation in the TAG content that adds to the variability of MALDI MS response, pure water buffalo and bovine milk fats are practically indistinguishable by MALDI profiling. We have carried out several attempts with mono- and multivariate statistical treatments of the signal intensity distributions to discriminate these two fats only on the basis of the MALDI MS, but every efforts have failed. Spectra of water buffalo and bovine milk fat are reported in **Figure 15**.

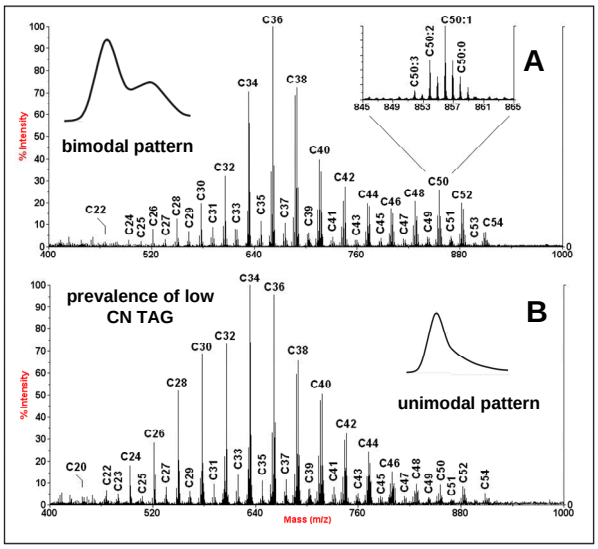

Figure 14. MALDI-TOF MS comparison of TAGs from bovine (A) and ewe (B) milk fat. The bovine TAGs exhibit the typical bimodal pattern observed also in the GC separations. In constrast ewe milk has a monomodal patter and exhibits higher intensity signals for low CN TAGs. Spectra acuired with the NC MALDI MS method

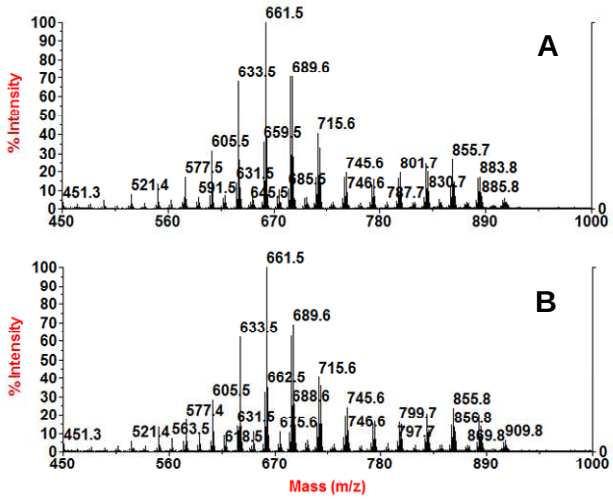

Figure 15. water buffalo (A) and bovine milk fat (B) are undistinguishable by the MALDI-TOF MS analysis of TAGs

On the other hand, also the capillary GC analysis (**Figure 16**) was inadequate to discriminate between bovine and buffalo milk fat due to their very close compositional similarity. GC analyses have been carried out of the laboratory of Prof. Raffale Romano, University "Federico II" of Naples. Intensity of MALDI signals and GC pear areas are quantitatively correlated, aside from the MALDI underestimation of saturated TAGs owing to the reduced efficiency to ionization of saturated TAGs if compared to the unsaturated counterparts.

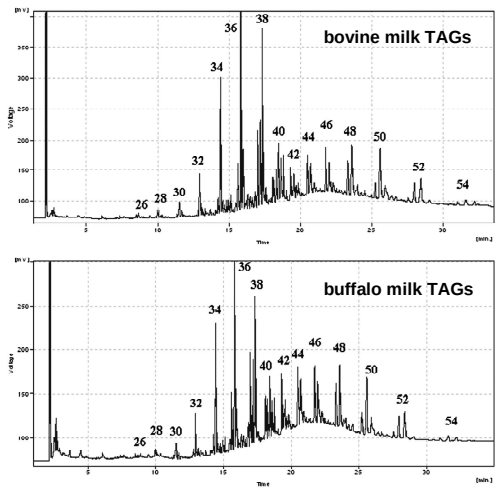

Figure 16. Capillary GC comparison of TAGs from bovine (A) and ewe (B) milk fat, demonstrating the close similarity of the two fats typologies. In order to average the fluctuations of TAGs in milk fat, bovine milk fat was the EU CRM-519 standard and buffalo counterpart was collected from a bulk milk of at least 50 individuals.

3.1.5 Capabilities and limits of MALDI-TOF MS profiling: detection of synthetic TAGs in butterfat

Because of its relatively high price, butter is frequently a target of adulteration, that are generally realized by adding less expensive deposit animal fats or vegetable margarine. According to the EU normative, edible spreadable fats have to be classified according to their dairy and non-dairy origin and labels must comply with the origin of the lipid material (Council Regulation (EC) No. 2991/94). The adulteration with plant oils or with deposit fats, that can be detected at very low levels with GC-based methods, such as the official Precht method (Commission Regulation (EC) No. 213/2001; Precht, 1992), some decades ago (Parodi, 1971), as well as recently, an analytical problem arose when high proportions of synthetic TAGs, even enzymatically or chemically esterified with high proportions of animal fats (mainly tallow), were used to create a butter surrogate. Such a fat can be produced in order to reflect the physico-chemical macroscopic properties of butterfat (van Ruth et al., 2009). To investigate the possibility of using MALDI-TOF MS for disclosing possible adulteration of butterfat with synthetic TAGs, we spiked authentic butterfat (standard anhydrous milk fat BCR CRM-519) with an opportune synthetic TAG mixture (STM).

The STM was synthesized by Dr. Olga Fierro of the Institute of Food Sciences - National Research Council of Italy (CNR), Avellino, Italy, by esterifying glycerol with short- and medium-chain FAs, in order to reproduce their average content in authentic milk fat. For explorative purpose, only saturated FAs were used to prepare the STM. The MALDI MS spectrum of STM is shown in **Figure 17**, along with the spectrum of EU BCR-519 spiked with STM (3:1, w/w). Because the STM only contains low- and medium-CN TAGs, the spectrum of the butter-STM blend exhibited more intense signals of C20-C36 TAGs compared to genuine butter. A careful inspection of the MALDI spectrum, especially if combined to the statistical evaluation of the signal intensities, allows for the qualitative assessment STM in butter.

However, the detection of percentages as low as 15-20% of STM in butterfat was quite problematic. Therefore, also in this case the MALDI-TOF MS, as a pattern recognition technique, suffers from intrinsic pitfalls in resolving the constituents of a multi-component lipid mixture. Furthermore, on a larger scale the composition of butter TAGs can be mimicked more closely by mixing proper amounts of interesterified saturated and unsaturated FAs. In cases like these and in absence of structural data that might be afforded by complex MS/MS experiments and sophisticated instrumentations, MALDI MS is insufficient to provide analytical response.

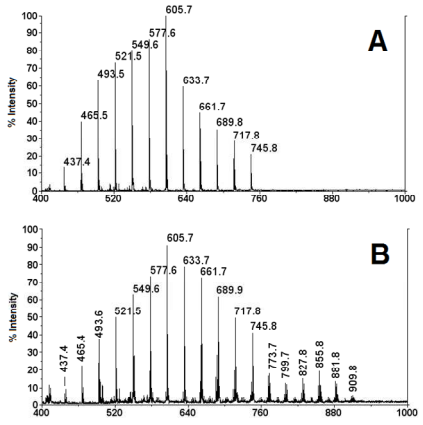

Figure 17. MALDI-TOF mass spectra of (A) synthetic mixture of TAG-Na^+ adducts, (B) EU BCR CRM-519 spiked with STM (3:1, w/w)

In collaboration with Prof. Raffaele Sacchi of the University "Federico II" of Naples and with Dr. Dominique Melck of the Institute of Biomolecular Chemistry – CNR, Pozzuoli (Naples), we developed a ^{13}C NMR spectroscopic methods to overcome the analytical shortcomings relevant to the issue of detecting synthetic TAGs in authentic butterfat.

3.1.6 ^{13}C-NMR spectroscopy based detection of synthetic triacylglycerols in butter

Milk fat TAGs are well-known to have a non-random distribution of FAs at the glycerol backbone, having C6-C8 FAs preferentially and C4 exclusively located at the *sn*-1,3 position (α and α' position) (Breckenridge and Kuksis, 1968; Kuksis et al., 1973). In synthetic TAGs the distribution of FAs on the glycerol backbone is randomized.

Thus, the determination of the regioisomeric distribution of butyrate (C4) on the glycerol backbone provides a diagnostic indicator to differentiate genuine butter from mixtures with synthetic TAG. Both ^{1}H and ^{13}C high resolution (HR) NMR have been long established as powerful tools for the study of the TAG regiosiomerism, offering significant advantages over other methods (Pfeffer et al., 1977; Gunstone 1993; Kalo et al., 1996). In particular, ^{13}C-NMR has been successfully exploited to establish the positional distribution of FAs in butter TAGs (Van Calsteren et al., 1996). Natural abundance $^{1}3C$ NMR, in particular, is more undemanding because of the wider range of chemical shifts and of the simpler spectra due to the lack of ^{13}C-^{13}C spin-spin couplings.

The expanded view of the carbonyl regions in the ^{13}C-NMR of BCR-519 standard butterfat (**Figure**

18) clearly evidences that the C_1 chemical shifts are separated into two clusters corresponding to the *sn*-1,3 and *sn*-2 positions. The C_1 resonances of saturated (S) and unsaturated (U) FAs are easily distinguishable. Chemical shifts were assigned on the basis of literature data (Andreotti et al., 2000) and by acquiring spectra of standard TAGs such as tributyrine. The exclusive diagnostic resonance at 173.04 ppm confirms the specific occurrence of C4 at the *sn*-1,3 position in authentic butter (**Figure 18A**). By contrast, no signal was detected at ~172.66 ppm that is diagnostic diagnostic of C4 esterified in the *sn*-2 position. The spectrum of standard tributyrin (**Figure 18B**) confirmed the assignment of the butyrate C_1 resonances. The STM exhibited chemical both chemical shifts at 173.29 and 172.87 ppm, indicative of C4 esterified at the *sn*-1,3 and *sn*-2 positions, respectively (**Figure 18C**), with area ratio *sn*-1,3/*sn*-2 ~2/1 close to the random occupancy of the α- and β-glycerol positions. The ^{13}C-NMR spectrum of the BCR-519 standard butterfat previously spiked with 2.5% STM exhibited the expected 173.25 ppm resonance corresponding to the C_1 of butyrate in the *sn*-1,3 position as well as a clearly detectable signal at 172.82 ppm due to *sn*-2 esterified C4 in synthetic TAGs (**Figure 18D**). In these conditions the signal of *sn*-2 esterified C4 was still integrable and exhibited a value 2.8%, in good agreement with the expected values. C_1 carbonyl signal of *sn*-2 esterified C4 was still detectable at spiking percentages of 1%, although not reliably integrable. Therefore limit of quantification (LOD) and limit of detection (LOD) of the method were quantified as 2.5% and 1% respectively.

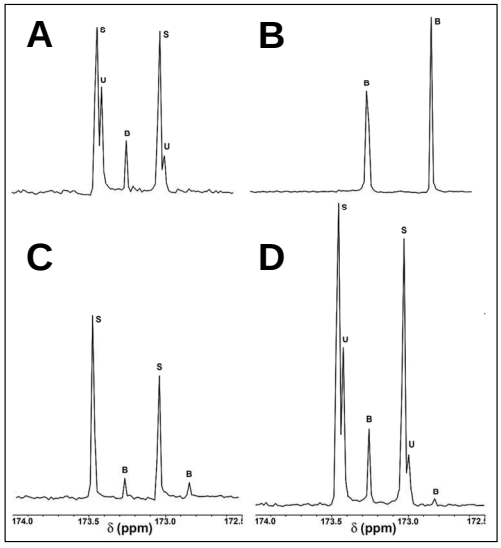

Figure 18. Proton decoupled natural abundance ^{13}C-NMR spectra expanded at the carbonyl region of (A) authentic butterfat BCR-519; (B) standard tributyrin; (C) STM; (D) BCR-CRM519 butterfat spiked with 2.5% (w/w) of STM. The resonances corresponding to saturated (S), unsaturated (U) and C4 (B) FAs esterified at the *sn*-1,3 and *sn*-2 positions are assigned in the figure.

3.1.7 Conclusions

MALDI-TOF MS is a powerful method to obtain rapid indications about of “native” TAGs from edible oils and fats. It furnishes accurate profiles that are in most of cases useful to infer the overall TAG composition. Exploiting the relatively high resolution of the reflector mode MALDI-TOF with clear detection of the molecular isotopic distribution, the TAG molecules (in reality, a set of isobaric regioisomers) within each TAG family, differing only by their degrees of unsaturation, can be easily assigned. On the other hand, by providing exclusively the MW (and relative signal intensities) of the single species, MALDI analyses are not capable of resolving the constituents of a multi-component lipid mixture, especially when one oil/fat component is highly prevalent over the other(s). Thus, MALDI measurements are insufficient in detecting lower amounts of foreign lipids in an oil/fat blend. Furthermore, MALDI MS does not provide information about structural details of TAGs, such as regioisomerism at the glycerol positions or geometry/position of double bonds in unsaturated FAs. In cases like these, when these structural details are diagnostic of nature of lipids or treatments they have undergone, MALDI needs to be replaced or supported by other specific techniques, such as for instance NMR.

3.2 MALDI-TOF MS for the determination of fatty acids in saponified oil and fat TAGs

3.2.1 Premises and aims of the MALDI MS-based determination of FAs

One of the major factors that have limited an extended application of the MALDI-TOF MS to the analysis of small molecules resides in the fact that the common matrices produce strong ion signals that interfere with the detection of the low MW analytes. Matrix ions suppress analyte signals and, due to the formation of non covalent cluster and several adducts, matrix “obscure” the low *m/z* spectral region. Ayorynde et al. (2000) proposed the use of the *meso*-tetrakis(pentafluorophenyl) porphyrin (F20TPP) as the matrix for the ionization of small molecules including FAs and amino acids. F20TPP has a MW = 974 and does not produce interfering ions in the low-mass region. According to the previous studies (Ayorinde et al., 2000; Hlongwane et al., 2001) the MALDI-TOF MS analysis of FAs were proved to superior to the GC-based determination of FAME, especially because of the lack of baseline resolution between FAME with the same CN but with different degree of unsaturation. In contrast, the resolution of MS at low *m/z* achieve unparalleled performances. We applied the already described procedure, with the precise purpose of targeting the very low-abundance long chain-PUFA in breast milk fats.

3.2.2 Determination of FAs in virgin olive oil and human milk fats

Preliminarly, we tested the methodology described by Ayorinde et al. (2000) on FAs arising from saponification with 2 N NaOH in methanol of olive oil (**Figure 19**), and clearly observed all the expected FAs with optimal resolution. FAs are detected as sodiated sodium carboxylates adducts $[RCOONa + Na]^+$.

It has been demonstrated by GC analysis of FAME that the MALDI signal intensity is quantitatively related to the FA relative abundance (Hlongwane et al., 2001). The correlation is particularly accurate if isotopic contributions and response factors of the MALDI analysis are taken into account.

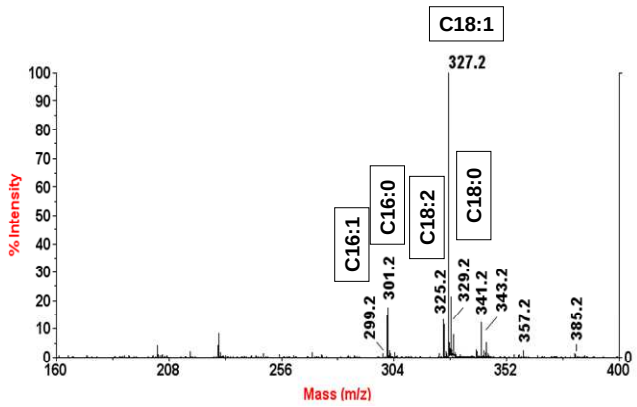

Figure 19. MALDI-TOF MS analysis of FAs of EVO oil. FAs are detected as sodiated sodium carboxylates $[RCOONa + Na]^+$ adducts. The main FAs are assigned in figure.

Therefore, we addressed this methodology to the study of the FA composition of human milk fat, and in particular to the detection of essential long chain-PUFA, such as arachidonic acid (AA, C20: 4, ω-6), eicosapentaenoic acid (EPA, C20: 5, ω-3) and docosahexaenoic acid (DHA, C22: 6, ω-3), that seem associated to a correct development of brain and retina of the newborn, even though their nutritional significance has been recently questioned (Keim et al., 2012) . Considering the variability in the FA content in human milk, that depends on a large number of factors, including stage of lactation, pre-term or full-term delivery, mother age and diet, we analyzed ten mature breast milk samples deriving from full-term donors. The MALDI-TOF MS analysis of a representative sample of human milk FAs, along with the assignment of FAs, is reported in **Figure**

20. The expected most abundant FAs of human milk (C10-C18) occurred as the most intense signals in the spectrum. A signal likely corresponding to trace amounts of C8 (*m/z* 189.2) occurred as well. AA, EPA and DHA were detected as minor species, also flanked by several possible interfering signals. Although their detection was clear in practically all of the ten samples analyzed, their precise quantification was hindered by the very low amount and by the production of signals only slightly higher (signal-to-noise ratio ~2-5) than the noise baseline. The approximate relative amount of the long-chain PUFA was estimated to range between 0.2 and 0.7%, in line with the values reported by other authors (Fleith and Clandinin, 2005).

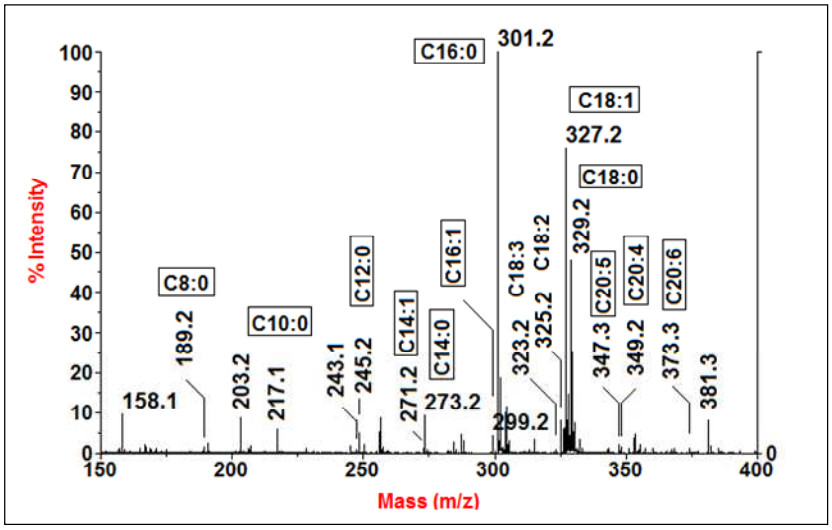

Figure 20. MALDI-TOF MS analysis of FAs in human milk. AA (C20: 4, ω-6, *m/z* 349.2), EPA (C20:5, ω-3, *m/z* 347.3) and DHA (C22: 6, ω-3, *m/z* 373.3) were detected, but not quantifiable.

3.2.3 Conclusion

MALDI-TOF MS can be exploited as a powerful and rapid tool to evaluate the "gross" FA composition of a lipid mixture and as a method alternative to GC-based ones. Although MALDI signal intensities is related to the quantitative amount of FAs and reliable estimations can be inferred (Hlongwane et al., 2001), the determination of very low-abundant components is quite problematic.

3.3 Strategies of sample pre-treatments for increasing the informative level of the MALDI MS analysis of specific TAG classes

3.3.1 Premises and aims

The global profiling of a complex lipid mixture can be complicated by the effects of ion suppression, that are well described for MS techniques in general. Namely, most abundant ions can suppress the ionization of less abundant molecules, compressing the dynamic range of the analysis. A further important concern that hinders the comprehensive detection of the components in a complex lipid mixture is related to the structure-dependent ionization efficiency, sometimes referred to as "response factor". Specific software-assisted procedure have been developed to compensate the effects of different response factors, that however need to be empirically established, and corrective parameters to compute the isotopic contribution, for quantitative purposes (Guyon et al., 2003). Similarly to ESI (Han and Gross 2001), in MALDI the ionization efficiency is inversely correlated to the equivalent CN of TAGs. Furthermore, unsaturated TAGs have a significant stronger tendency to be desorbed, if compared to the saturated counterparts (Ayorynde et al., 2009). Therefore, the ionization of completely saturated TAGs in animal fats, *per se* intrinsically not predominant, is suppressed for the most part. The signals of saturated TAGs is often difficult to isolate, especially when it is very poorly intense, as it can fall in the isotopic distribution of the mono-unsaturated species. Nevertheless, the identification of saturated TAGs in foods is a very relevant subject under a nutritional standpoint, as these compounds are known to be strictly associated to a number of cardio-vascular diseases and metabolic disorders.

A different complication arises when the components of very complex mixtures of poly-unsaturated oils, such as for instance fish oils, are to be assigned. The occurrence of a large number of TAGs covering a wide range of degree of unsaturations causes overlapping between adjacent signal clusters. In cases such as these, a straightforward identification of the individual TAGs is challenging, when not impossible. The different degree of unsaturations also produces signal dispersion, with the result that very low abundant TAG families may escape detection.

To overcome these concerns and to enlarge the informative level of the MALDI analyses, we developed two simple strategies of sample pre-treatments. Combined with the spectra of the "native" oil/fat mixtures, these methods provide a more detailed "snapshot" of the sample under examination.

3.3.2 Spectral isolation of saturated TAGs: bromination of animal fats

Completely saturated TAGs were barley or even not at all isolated in the MALDI-TOF MS spectra of bovine (tallow) and swine (lard) deposit animal fats (**Figure 10**), in spite of their already established presence. Tri-saturated TAGs of lard and tallow are estimated to be 2.50 and 14.5%, respectively (Luddy and Riemenschneider, 1946). Thus, they occur at a significative percentage, at least in tallow. To bridge this gap we performed tallow and lard bromination prior to MALD-TOF MS analysis. Bromination consists of a fast and quantitative addition of elemental bromine (Br_2) to double bonds. Reaction requires not more than 5 minute. Excess Br_2 is deactivated with aqueous sodium thiosulfate, while fats remain dissolved in chloroform or dichloromethane, kept in an ice cold bath. Debating organic phase with sodium thiosulfate also induce Na^+-cationization, so that samples can be directly analyzed. Bromine naturally occur in two isotopes, i.e. ^{79}Br and ^{81}Br, at nearly the same abundance. Because of bromination the MW of unsaturated TAGs shifts of ~160/double bonds. In contrast saturated TAGs are unaffected by Br_2 addition and their MW does not change. By this way, the detection of saturated species as Na^+ adducts becomes straightforward as signals do not undergo signal suppression or ^{13}C isotopic effects arising from mass proximity to unsaturated species. The MALDI spectra of brominated tallow and lard are compared in **Figure 21**.

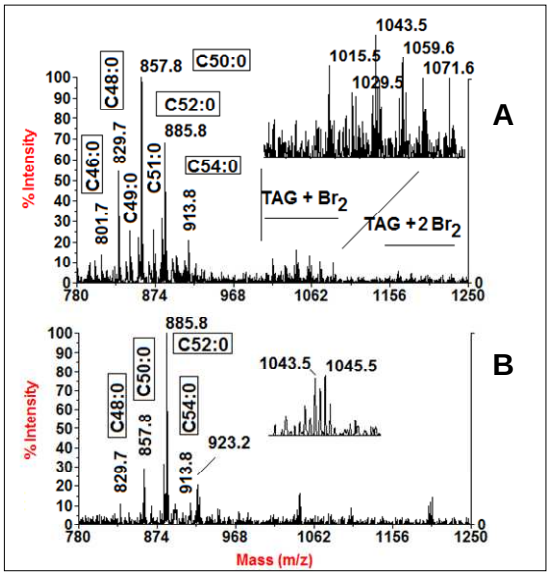

Figure 21. Tallow (A) and lard (B) were brominated prior to MALDI MS analysis. While unsaturated TAGs shifted of 160Da/double bonds, saturated TAGs were unaffected and easily detected. Insets show the typical isotopic patterns of brominated TAG.

Tallow was confirmed to possess a "more saturated" character (lower iodine number) and a TAG content slightly more disperse than lard (C46-C54 *vs.* C48-C54). While in tallow prevailed the C50:0, related to a higher content of palmitate (C16), in lard was prevalent the C52:0 due to a high

percentage of C18 FA. It was confirmed the occurrence of odd-numbered TAGs in tallow (C47-C51), in contrast to lard in which they were almost completely missing.

The assignment of brominated TAGs is not as linear as that of the native TAGs. Brominated TAGs have low ionization efficiency, tends to fragment and signals are dispersed because of the two naturally occurring Br isotopes (Picariello et al., 2007). The identification of poly-unsaturated TAGs are even more tricky, because of a linear overlapping of isotopic ions ensuing when the combination among several Br isotopes increases (inset of **Figure 21**).

Bromination of butterfat evidenced the expected distribution of saturated TAGs ranging from very low- to high-CN (**Figure 22**). Also in butter, because of its ruminant origin, odd-numbered TAGs did occur. It has to be underlined that, likely because of a suppression effect, brominated TAGs arising from unstaturated species were almost not detected at all in the spectrum.

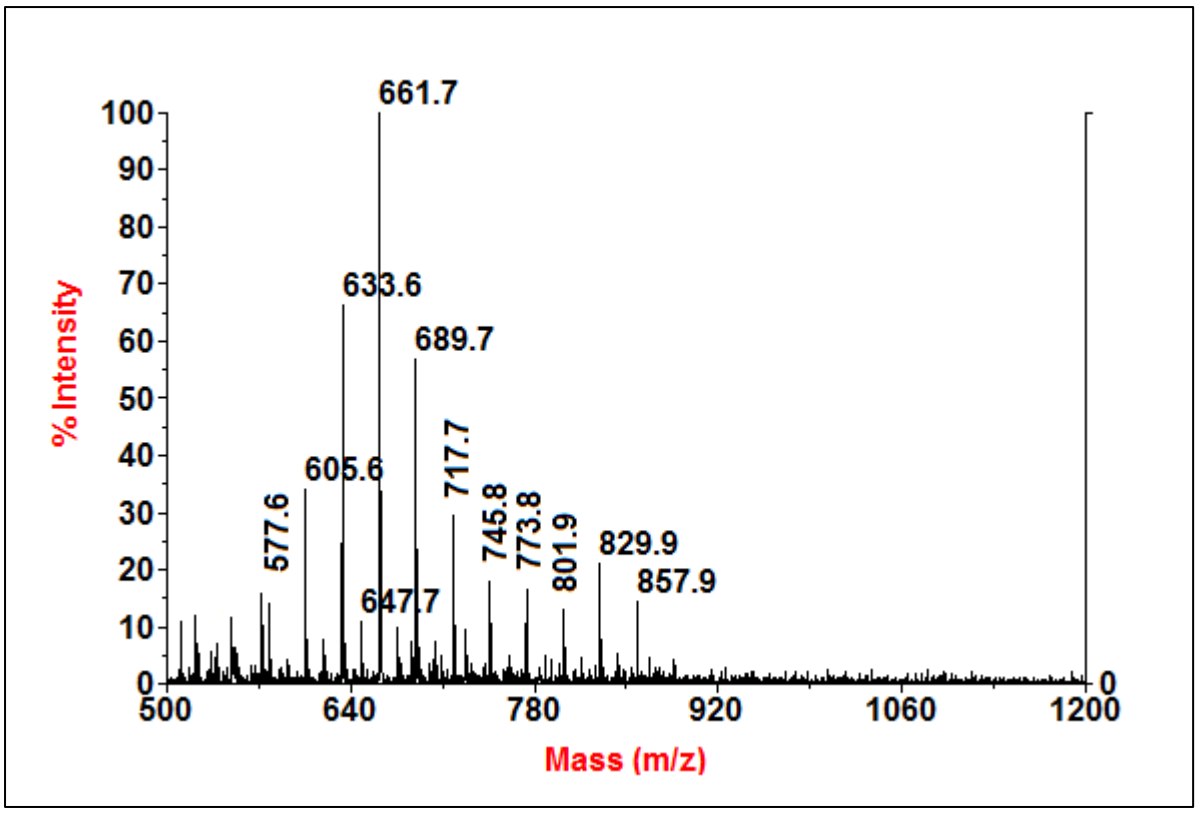

Figure 22. MALDI-TOF MS analysis of brominated butterfat.

3.3.3 Detection of the very-low abundant TAG families: the case study of fish oils

Depending on the degree of unsaturations, in many oils/fats TAGs families are distributed on a more or less large range. The signal dispersion in the MALDI-TOF MS spectra reflects the level of such a distribution that in many cases can be also very pronounced. For instance, in fish oils

co-exist a huge number of unsaturated TAGs, whose number of double bonds can make signal clusters to overlap, so to hinder the assignment of individual TAGs. Signal dispersion also reduces the possibility of assessing the presence of low abundant TAG families.

Hydrogenation of TAG is a widely performed reaction, utilized also at an industrial scale. It is an easy reaction to carried out at a laboratory scale and does not require particular skills.

By hydrogenating TAGs, the information about the unsaturation goes lost. On the other hand spectra are very simplified because only saturated TAGs, usually differing by 28 units corresponding to an ethylene group (or by 14 due to a methylene, in the case of odd-numbered CN TAG), are observed. In **Figure 23** the spectrum of native tuna oil is compared with its hydrogenated counterpart. The spectral simplification is evident, so that the entire distribution of TAG families can be easily elucidated.

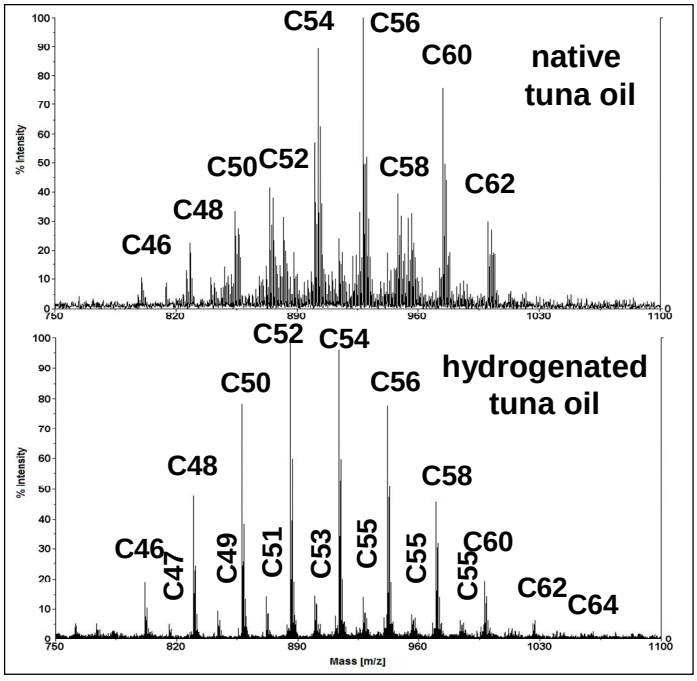

Figure 23. MALDI-TOF analysis of native and hydrogenated tuna oil

The effect of "coalescence" of the unsaturated species toward the respective saturated TAGs enhances the signal detection. In this way it is possible to ascertain the occurrence of less abundant TAG families, generally at very low- or very high-CN. After hydrogenation low abundance very high-CN TAG (C64) were detected in tuna oil, and the presence of odd-numbered TAGs, probably

arising from the plankton metabolic pathways, became evident. Overall we analyzed ten distinct fish oil samples, purchased from a Norwegian farm. We could observe TAG families as long as up to C66 in seal oil (not shown).

In the case study of fish oils, the relative intensity of MALDI signals was compared to the GC determinations carried out by capillary GC analysis of TAGs.

For the sake of briefness and for immediate inspection, quantitative determinations have been drawn in histograms, rather than tabulated. It has to be underlined that MALDI (carried out with the NC MALDI methods, see below) and GC-based evaluations were in close agreement. Interestingly, based on the peak area of GC or on the signal intensity of MALDI specta, no TAG species overcame the relative amount of 6 %. In **Figure 24** the relative abundance of TAGs obtained for tuna and seal oils by GC are plotted. Histograms closely resemble the MALDI-TOF MS patterns of respective oils.

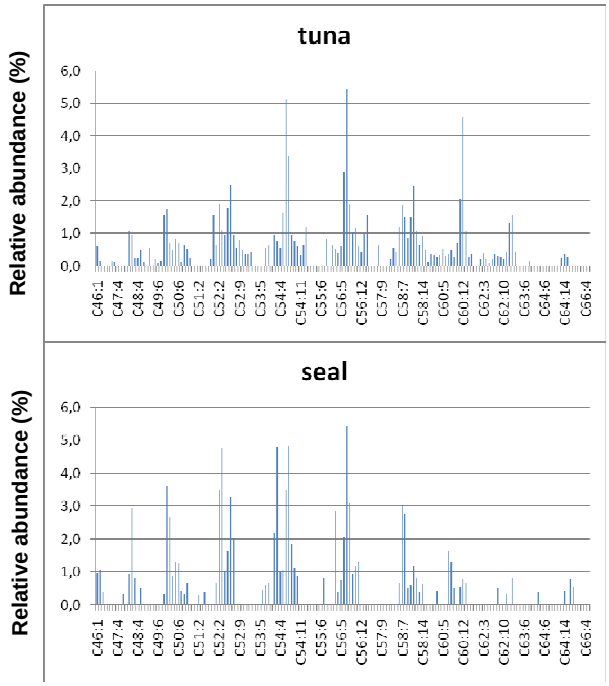

Figure 24. Relative abundances of TAGs in tuna and seal oil, determined by GC. Histograms closely resemble the NC MALDI-TOF MS patterns of respective oils

We also utilize hydrogenation for analyzing several oils/fats of different origin, including deposit animal fats and bovine and human milk fat. After hydrogenation a butterfat sample prior to MALDI MS analysis we were able to extend the range of the TAG families also in milk fat to the C20-C60 range. In fact low intensity signals were detected for C20-C22 and C56-C60 TAGs (Picariello et al., 2007). It is worth of note that the very low-CN C22 and C20 TAGs had not been

described before in bovine milk. C56 and C58 TAGs were also detected in MALDI-TOF MS spectra of hydrogenated lard and tallow (Picariello et al., 2007).

3.3.4 Post Source Decay (PSD)-MALDI-TOF MS of hydrogenated TAGs

As an additional benefit, the hydrogenation of oils/fats enables the possibility of easily carrying out PSD-MALDI-TOF MS experiments.

Although MALDI MS is a "soft" ionization source, a certain level of fragmentation can variably occur as a consequence of the high energy excess after the high-power transfer by laser. Fragment ions are mainly formatted through an unimolecular gas-phase mechanism of decomposition, after the ions are accelerated by the high-potential grid. Usually, fragmentation happens after the ions enter the tube of flight (post-source). The desorbed ions have a Gaussian distribution of the initial internal energy. Generally, relatively a few ions contain enough energy to fragment during their lifetime. After being accelerated, in the field-free region of the mirror (tube of flight) precursor and fragment ions have the same velocity (v), thus in a linear mode experiment they would heat the detector simultaneously, without any possibility to be differentiated. Due to a different mass, precursor and fragment ions have a different kinetic energy ($1/2\ mv^2$) and exhibit different degree of "penetrating" the reflector ion mirror. Thus due to the reflector deflection, they can be discriminated according to their mass. Roughly, PSD experiments furnish information similar to the MS/MS experiments. The ions entering the mirror can be chosen through a "timed ion selected".

PSD can be convenient for the analysis of TAGs, that, due to their intrinsic properties, have a significant ion production in the source. However, PSD has not been extensively used for routine experiments due to difficulty of selecting only a specific ion (both excluding or including its isotopic distribution) through the timed ion selector. The slit of the ion selector can be modulated, but it is not so effective to discriminate ions that differ by 1-2 mass units. Therefore, selecting a TAG ion in a cluster, makes also the closer ion TAGs, differing by the degree of unsaturation, to enter the mirror, thereby complicating much the final PSD spectrum.

In hydrogenated lipids, unsaturations have been compacted in a unique signal corresponding to the saturated species and in this way time ion selection is easy and effective. Thus, by PSD experiment it is possible to deduce the length of the different FAs that constitute a TAG.

The comparison of the PSD MALDI MS spectra of the ion *m/z* 885.8 (C52:0) from hydrogenated milk fat and lard (**Figure 25**), demonstrates that, while constituting FAs of lard are only C16 and C18, in milk fat there is an additional minor contribution of the C14 and C20 FAs. Fragmentation happens with the loss of FA carboxylate anions following two cooperating mechanisms according

to which Na^+ ions can be alternatively lost or retained (Al Saad et al., 2003). Fragment ions are assigned in **Table 3**. Obviously, the information about the double bonds is lost because of hydrogenation.

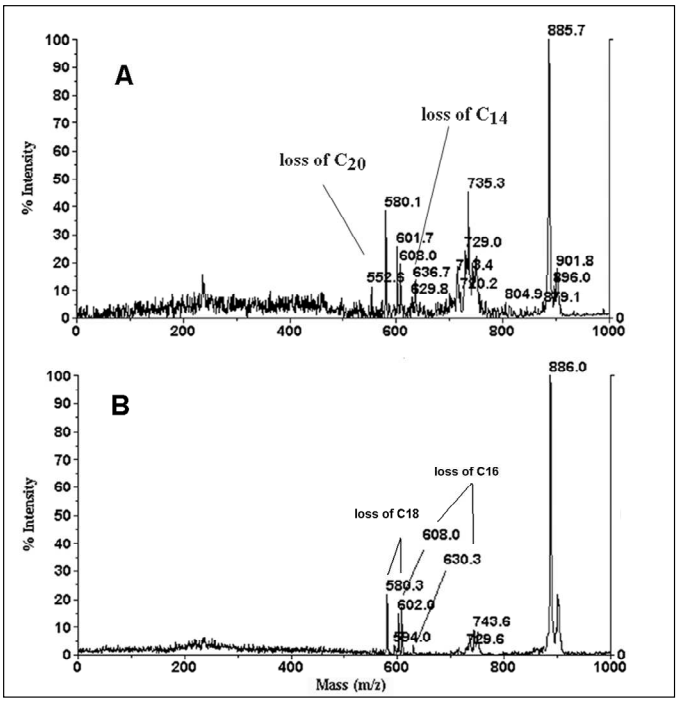

Figure 25. MALDI-PSD fragmentation spectra of the selected ion *m/z* = 885.8 (sodiated 52:0) from hydrogenated milk fat (A) and lard (B). Assignment of fragment ions is reported in Table 3. Figure adapted from Picariello et al. 2007.

Measured mass of fragment (*m/z*)	Theoretical mass of fragment (*m/z*)	Residue loss by the sodiated $C_{52:0}$
	MILK FAT	
552.6	551	arachidate + Na^+
580.1	579	stearate + Na^+
602.7	601	stearate
608.0	607	palmitate + Na^+
629.8	629	palmitate
636.7	636	myristate + Na^+
	LARD	
580.4	579	stearate + Na^+
602.1	601	stearate
608.0	607	palmitate + Na^+
630.5	629	palmitate

Table 3. Assignment of the peaks arising from the MALDI-PSD fragmentation of C52:0 TAG from hydrogenated milk fat and lard

3.3.5. Conclusions

Two simple and fast strategies of sample pre-treatment enlarge the informative power of the MALDI MS when complex lipid mixture are to be analyzed. Bromination enables the spectral isolation of tri-saturated TAGs. It is particularly effective when animal fats are to be comprehensively profiled, while it is obviously useless to analyze common edible plant oils, in which tri-saturated TAGs are completely missing. Hydrogenation of TAGs compacts the signals of the differently unsaturated TAGs to the corresponding tri-saturated species, simplifying the assignment of the same CN TAG families and enhancing the sensitivity toward very low-abundance TAG classes. As the information about the unsaturation of the lipids is lost, these two strategies require to be combined to the MALDI MS analysis of the native mixtures.
Through the possibility of performing PSD MALDI spectra, hydrogenation enables to access information about the length of the carboxylic FAs constituting TAGs.

3.4 Sample pre-fractioning prior to MALDI analysis of polar and non-polar lipids

3.4.1 Premises and aims

Both MALDI and ESI MS suffer from strong ion suppression effects when analyzing complex lipid mixtures, in which the components also cover a high range of concentrations (Petkovic et al. 2001; Byrdwell and Neff, 2004). Polar and non-polar lipids have a significant different ionization efficiency, with non-polar ones generally much more detectable than the polar species. TAGs, which are generally prevalent, suppress the ionization of minor amounts of oxidized TAGs or phospholipids in complex mixture. For this reason, in order to comprehensively profile oils/fats containing polar lipids, the lipid mixture needs to be pre-fractioned. We optimized the conditions of pre-fractioning and MALDI MS analysis of oxidized/hydrolyzed TAGs from several mixtures, such as thermal stressed vegetable oils and lipids of a fermented sausage. Preliminary attempts have been carried out to chromatographically partition TAGs and minor amounts of phospholipids in human milk.

3.4.2 Pre-partition and MALDI-TOF MS analysis of polar and non-polar lipids: the case study of thermo-oxidized oils

Thermal stress provokes drastic modifications in unsaturated vegetable oils, with consequent loss of nutrients and sensory attributes (Frankel, 1998). In overheated/fried oils the radical-mediated autoxidation of unsaturated/polyunsaturated TAGs leads to the formation of hydroperoxides, preferentially from conjugated FAs. Hydroperoxides, that are primary products of lipid oxidation,

are unstable at frying temperatures, and spontaneously undergo homolytical cleavage of O-O bonds, forming peroxy and alkoxy radicals. The radicals in turn decompose to a wide variety of secondary oxidation products. A series of decomposition TAG reactions (e.g., hydrolysis, polymerization, cyclization, and cracking) could occur to form a varieties of classes of compounds, including β-scission products, cyclic fatty acids, DAGs, MAGs and monomeric or oligomeric oxidized TAG (**Figure 26**).

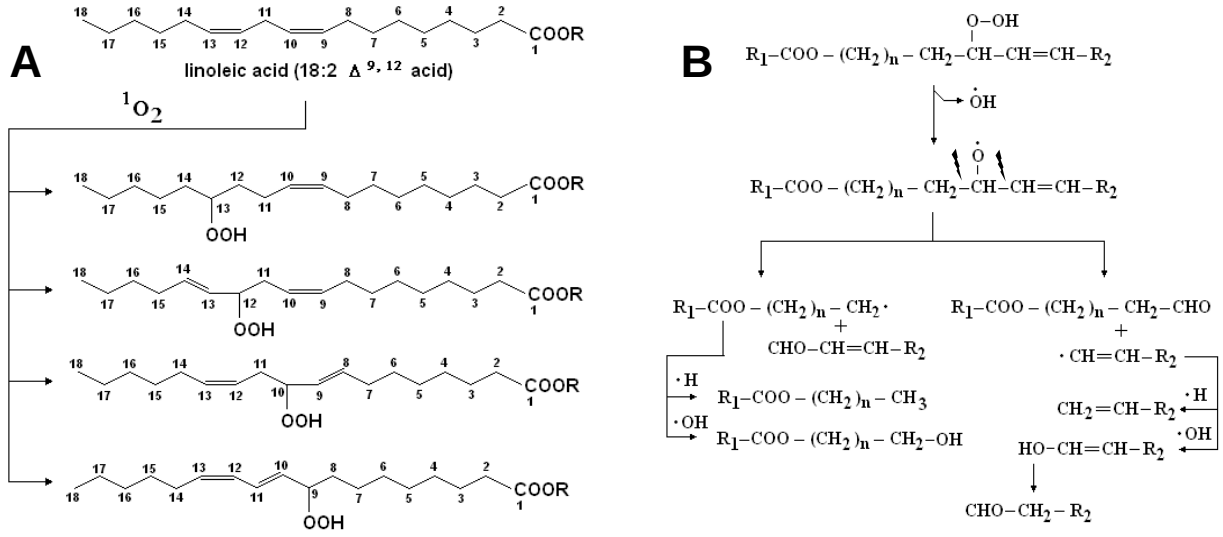

Figure 26. Scheme of the auto-oxidation and formation of hydroperoxides (A) and subsequent scission of hydroperoxides (B) that leads to a large series of products of the oxidative scission. Adapted from Picariello et al., 2009.

Both non-volatile and volatile compounds are formed during deep frying (Frankel, 1982). The degree of the thermally-induced damage strongly depend on the severity (temperature and time) of the thermal stress, in addition to other factors such as moisture, metal ions, frying foodstuff, oxygen accessibility. While most of volatile fried-flavors and off-flavors escape from the frying medium, non-volatile products, including non-polar non-oxygenated and polar oxygenated components, gradually accumulate in the oil, are absorbed by the fried foods and could be finally ingested.

The total polar compounds (TPC) amount is the most objective indicators for the evaluation of the thermal damage (Dobarganes et al., 2000). Generally, ~25% by weight is considered by IUPAC to be the safe upper limit of TPC (IUPAC. Determination of polar compounds in frying fats 2.507, 1992). TCP are gravimetrically measured after selective isolation on silica gel. Products of oil thermo-oxidation have been characterized with a large variety of techniques including high pressure-size exclusion chromatography (HP-SEC) (Dobarganes and Marquez-Ruiz, 1995), NMR

(Adhvaryua et al., 2000) and HPLC-ESI (or APCI) MS (Byrdwell and Neff, 2002; Byrdwell and Neff, 2004). Preliminary attempts carried out by MALDI-TOF MS (Schiller et al., 2002) were not sufficiently satisfactory due to the effects of suppression.

We exploited MALDI-TOF MS for comprehensively profile TAGs in deep-fried sunflower and EVO oils (4h, 180 °C and 6h, 180 °C, respectively), after pre-fractioning of polar and non-polar components by silica-gel chromatography. Polar and non-polar fractions were separated according to the official IUPAC procedure for the determination of TCP (IUPAC. Determination of polar compounds in frying fats 2.507, 1992).

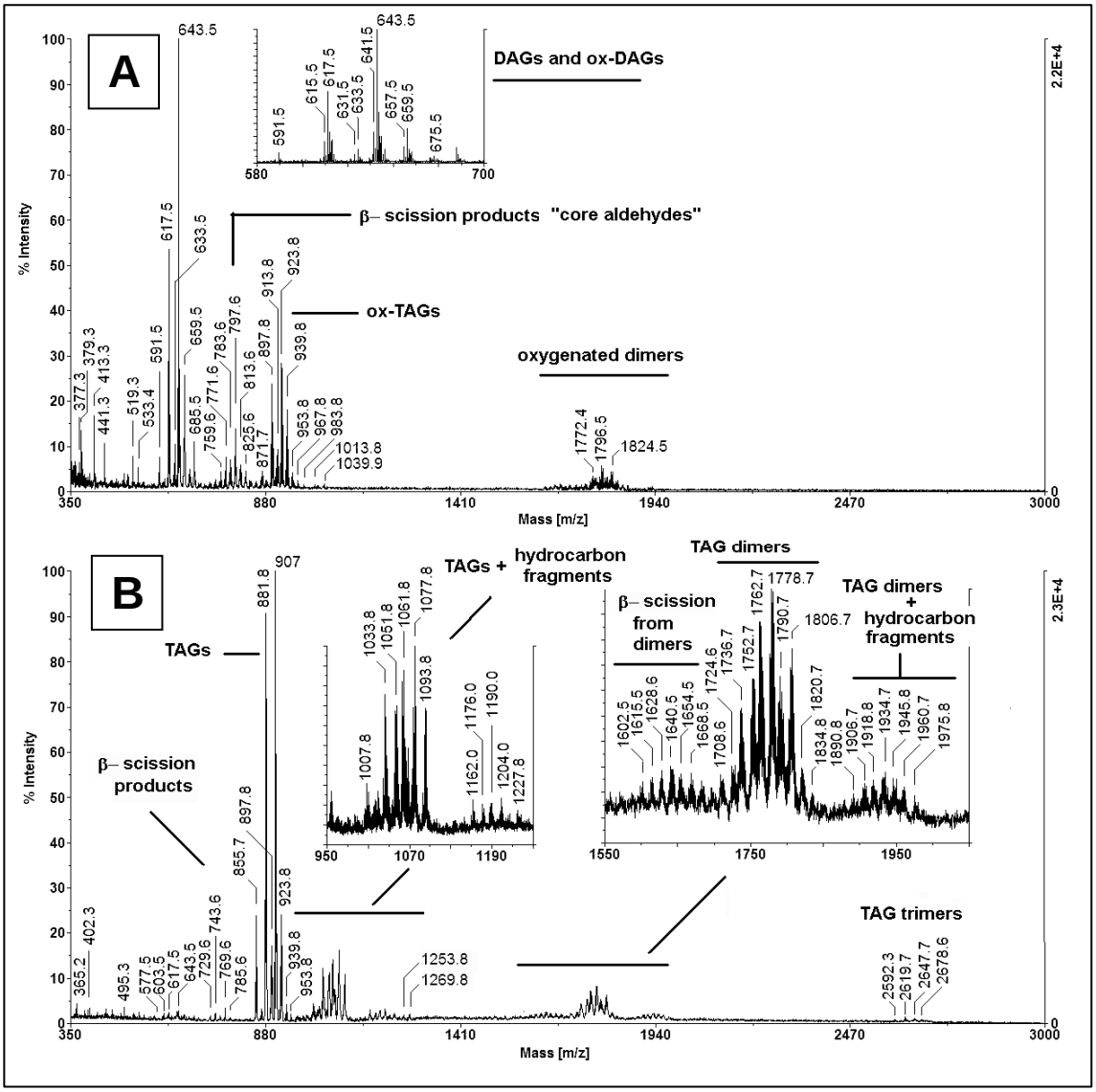

Figure 27. MALDI-TOF MS analysis of the polar (A) and non-polar (B) fractions of thermally stressed EVO oil. The insets are expanded views of spectral regions. The Figure has been adapted from the same mass spectra of the original figure in Picariello et al. 2010.

In **Figure 27** are shown the spectra of the polar and non-polar fractions of thermally-stressed (4h, 180°C) EVO oil. In the polar fraction it is evident the presence of mostly oxygenated products,

including mono or poly-oxygenated TAGs, DAGs (formed thorugh hydrolytic scission) and oxidized DAGs, β-scission products ("core aldeheydes) arising from TAGs but also in lower amount from DAGs, and oxygenated dimers. It has to be underlined that most of these compounds are not detected if the analysis is carried out without previous chromatographic steps, because of the TAG-induced suppression.

In the non-polar fraction there is a huge variety of products belonging to several classes such as, non-oxygenated β-scission products, native TAGs, inter-molecular covalent adducts of TAGs with low MW aliphatic hydrocarbons which derive from the scission of other TAG molecules, TAG dimers along with corresponding scission products and adducts with small hydrocarbons, TAG trimers. An exhaustive assignment of most of the individual species has been already published by us (Picariello et al. 2009). To the best of our knowledge, there was no previous evidence of minor β-scission products of dimeric TAGs (*m*/*z* 1602.5-1668.5), although they were predictable. Similarly, the expected adducts of TAG dimers with low MW fragments (*m*/*z* 1890.8-1975.8) were first time disclosed together with the expected non-oxidized dimeric TAGs (*m*/*z* 1708.6-1822.7). It needs to be emphasized that these spectra have been acquired in the reflector ion mode (with the NC method, see below), and all the species, even belonging to the class of trimers, have been singled out. Obviously, isobaric species can not be discriminated. For instance, it is not possible to distinguish between TAGs joined by an ether linkage with a bridge oxygen and dimers linked through a C-C bond in which one TAG carries an alcohol function. In the linear ion mode MALDI-TOF MS could be even more sensitive toward the low-abundance species, but with compromised resolution. The MALDI-TOF MS analysis of the polar and non-polar fractions of sunflower oil evidenced a more severe thermal damage, as sunflower oil undergo 6h of thermal stress (instead of 4h for EVO oil), as well as because of the higher content of PUFA and the absence of anti-oxidants such polyphenols or tocopherols that in contrast occur in EVO oil.

3.4.3 Pre-partition and MALDI-TOF MS analysis of polar and non-polar lipid fractions: lipolysis in Napoli type salami

The pre-partition followed by MALDI-TOF analysis can be exploited for analyzing the products of TAG modification during ripening of fermented foods. We have applied the analytical procedure to the study of lipolysis and lipid oxidation in a two-month ripened Napoli type salami. Total lipid fraction has been extracted from manually excised lipid spots, using the procedure of Bligh and Dyer (1959) and were subjected to successive partition on silica gel column chromatography. Chromatography has been carried out as previously reported for the separation of polar and non-polar lipids from heated oils. Just like in that case, the chromatographic fractionation was carried

out on a small scale and therefore implied a minimal consumption in terms of time, efforts and solvents. MALDI-TOF MS spectra of polar and non-polar are compared in **Figure 28**.

While the non-polar fraction is substantially composed only by unmodified high-CN TAGs, also found in lard, and very low amounts of DAGs (probably arising from an uncomplete silica gel selection), the polar one is more complex, being represented by oxidized-TAGs, major amounts of DAGs arising from hydrolytic lipolysis and by a complex set of products of β-scission products. Importantly, the β-scission products ("core aldehyde"-type), arising from the oxidative breakdown of mainly mono- and poly-unsaturated TAGs, are the non-volatile complementary moieties of the volatiles that play a key role in the development of flavors that are appealing for human consumers. These aroma compounds, along with biogenic amines that derive from the catabolic pathway of amino acids, concur to confer many organoleptic traits to fermented meat products (Singh et al., 2012). The identification of the individual non-volatile scission products would be interesting, but it is technically challenging and it would require a higher resolution separation step prior to MS analysis. By a quantitative standpoint the accumulation of lipolytic products is expected to be much less pronounced than in thermally stressed oils/fats. Due to a more complex lipid raw material, the study of lipolysis in dairy products by MALDI-TOF MS is expected to be even more challenging.

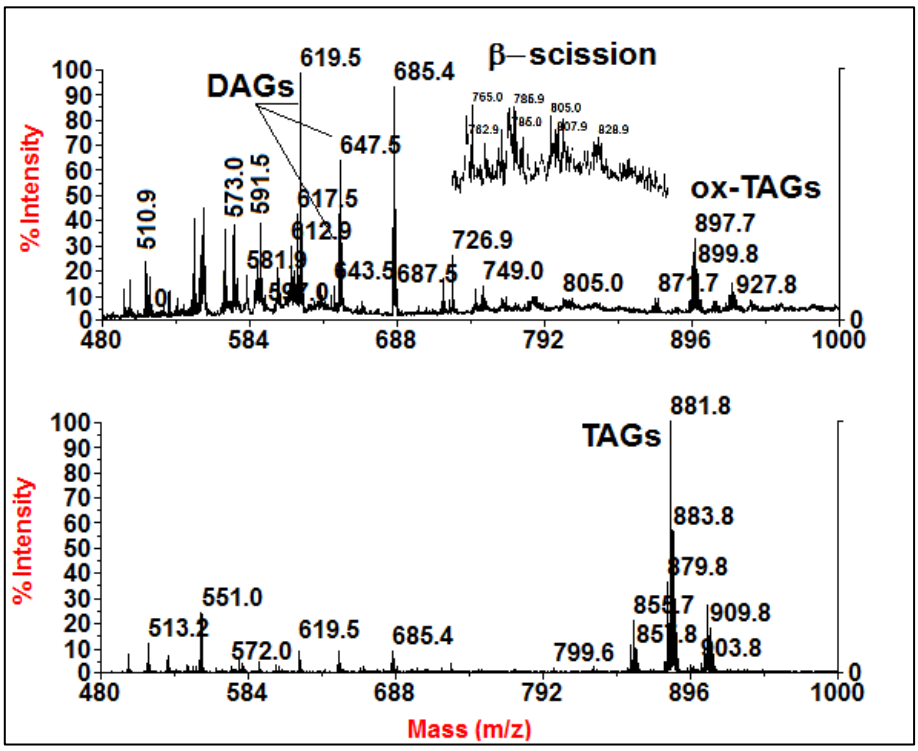

Figure 28. MALDI-TOF MS analysis of polar and non-polar fraction from a ripened Napoli tyoe salami.

The outcomes here presented are preliminary and need to be validated by a comparative GC analysis. Nevertheless, once optimal analytical conditions will be defined, the MALDI-TOF MS analysis of lipolytic products have a wide array of possible technological applications to foods, as they may allow to assess the ripening time, or to find lipid "signatures" associated to specific fermenting agents (*e.g.* autochthonous lactic acid bacteria, starters). Hence, they can support the process of typifying traditional productions or protected food preparations. The method can also contribute to the acquisition of basic knowledge about the lipolytic process that, under several perspectives, is still to be elucidated. The utilize of MALDI for the study of oxidative modifications in lipids has been recently reviewed (Fuchs et al. 2011). Despite the importance of the issue for the food and lipid science, to the best of our knowledge, this is the first attempt in studying by MALDI MS the lipolysis in ripened meat products.

3.4.4 MALDI-TOF MS analysis of non-TAG lipids: selective enrichment of phospholipids

Phospholipids (PLs) have been long considered the packing material of cells. Since some decades, it has progressively became clear that PLs have a central role as chemical mediators in signal transduction, prostaglandin synthesis, lipoprotein secretion and intestinal lipid absorption. Of particular relevance is the study of oxidized cell PLs, because it is the ultimate effect of oxidative stress. Although the specific features are still under study and need to be completely elucidate, PLs have also very relevant nutritional properties. Indeed, PLs are important constituent of aliments, also used at an industrial level as additives, that concur to confer functional properties to the foodstuff. In consideration of their relevance, many efforts have been carried out to develop strategies aimed to the comprehensive characterization of the PL repertoire. MALDI-TOF is emerging as one of the most helpful tools for studying PLs. In spite of a series of currently established protocols, the analysis of PLs remains rather tricky, due to their amphipatic physico-chemical properties. In fact, owing to the different chemistries, it is often difficult to characterize "one-step" more classes of lipids.

In foods the detection of PLs is often masked by the overabundant TAG components. Thus, many strategies have been proposed to isolate or selectively enrich PLs with respect to the neutral lipids. For instance, Calvano et al. (2009) proposed a solid-phase micro-extraction on TiO_2 beads that exploits the affinity of phosphate-containing molecules for the titanium dioxide. Similarly, Emerson et al. (2010) have proposed the use of solid-phase florisil extraction columns to separate PLs from TAGs. Also when the PL fraction has been effectively isolated, a relevant concern is raised by the much higher ionization efficiency of PCs with respect to other PLs (Petkovic et al., 2001). The

discrepancies in the detectability can be also very important: as an example, the detectability of PEs is 2% of PCs (Fuchs et al., 2010).

Recently, we have investigated the use of hydroxyapatite (HAP) for isolating phosphopeptides (Mamone et al., 2010). The method has been proved very effective and selective, due to the high affinity of phosphate-containing molecules for HAP, so that several research groups have applied the techniques also to analyze even cell system-wide extended "phosphopeptidomes" (Fonslow et al. 2012). HAP is a crystalline form of calcium phosphate with a chemical formula of $Ca_{10}(PO_4)_6(OH)_2$, which has been used since a long time for the separation of phosphoproteins and nucleic acids. It is known to have high affinity for phosphorylated species. Therefore, we have tried to extract selectively PLs by using solid-phase HAP gel and to elute in this way PLs from neutral TAGs that do not bind HAP. Our results are still preliminary and require several additional tests to establish the capabilities, the advantages and the drawbacks of this method with respect to those already published.

In any case, our preliminary tests seem to work. The bottle-neck step remains the release of PLs from HAP, due to a very strong complexations. In **Figure 29** is shown the spectrum of PLs from human milk, eluted from HAP after an extensive washing of the neutral TAGs with $CHCl_3$ and then $CHCl_3$/methanol 2/1 (v/v). It is evident the complete absence of TAGs and the presence of signals that have been tentatively assigned to PCs, PEs and SMs. The approximate relative contents of PL classes in human milk is: PCs ~ 30%; PEs ~ 26%; PSs ~ 6%; PIs ~ 4-5%; SMs ~30-32% (Jensen et al. 1978) We did not detect PSs and PIs probably because of their lower relative abundance and of the suppression effects by PCs. However, a TLC separation prior to MS analysis is expected to enlarge the coverage of the PL classes.

3.4.5 Conclusions

Lipids have physico-chemical traits very variegated. Therefore, the entire characterization of complex lipid mixtures, containing several lipid classes is often impracticable. The superiority of ESI MS in lipidomic studies originates for the most from the possibility of on-line interfacing ESI with high resolution separation chromatography. Several HPLC with automatic off-line MALDI spotter have been commercialized, but they have not paralleled the diffusion of the HPLC-ESI MS systems, because of their lower versatility and compliance. On the other hand, opportunely designed fast pre-fractionation steps for partitioning the lipid in classes, greatly enlarges the MALDI-TOF MS coverage of an entire "lipidome". The application of simple strategies of pre-fractioning has enabled the access to analytical details never penetrated before, as in the case of the profiling of thermo-oxidized oils.

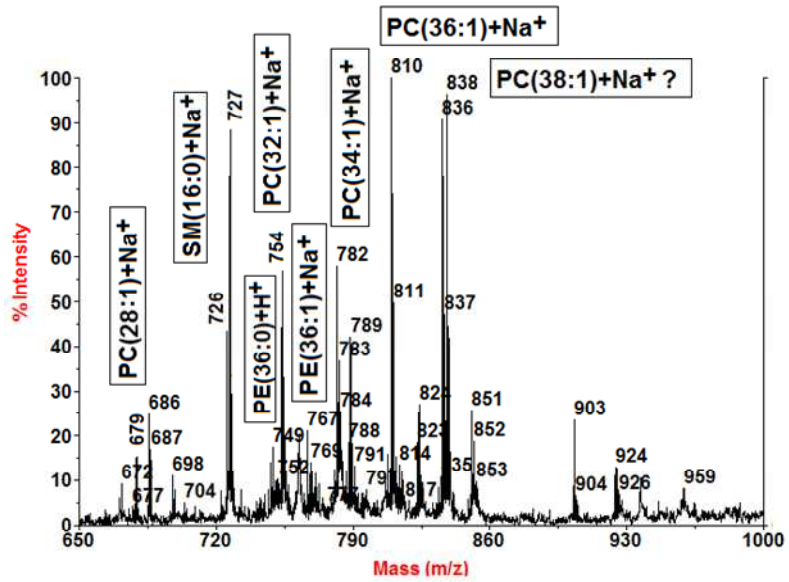

Figure 29. MALDI TOF MS analysis of phospholipids selectively extracted by HAP from human milk. Assigments are only tentative, as several isobaric compounds are expected to occur.

The use of HAP as a pre-fractioning step for isolating phospholipids has interesting perspectives if protocols will be standardized. Under opportune elution conditions, it can be envisaged that HAP is able to sub-fractionate PLs, for instance according to the phosphate heads on the basis of a possible their different binding affinity, in order to prevent the effect of MS ion suppressions among different PL typologies. This aspect will be the object of specific investigations in a next future.

3.5 Use of MALDI-TOF for quantitative applications: nitrocellulose (NC) MALDI-TOF MS

3.5.1 Premises and aims

Under typical conditions no signals of protonated species are observed in the MALDI-TOF MS spectra of TAGs ($[TAG+H]^+$). Harvey (1995) suggested that this the results of a fast gas-phase hydrolysis of protonate TAGs. Fragmentation appeared to be strongly dependent on the nature of the matrix and solvent systems and on the presence of water in the matrix solvent. Fragmentation has a drastic negative impact on the quality of the MALDI MS analysis of lipid mixtures, as it introduces ambiguity n the characterization of the individual components. The fragmentation degree

is variable, and for this reason, any attempts in inferring quantitative data of strongly biased. In addition, the presence of fragment "DAG-like" ions at the low m/z range, where also matrix ion occur, heavily hinder to get information about low MW species. Sample/matrix co-crystallization has also a critical impact on the reproducibility of the signal intensities, an issue that is related to the possibility of inferring MALDI quantitative data. Thus, we have aimed to find simple strategies to prevent fragmentation and enhance reproducibility in order to check the possibility of exploiting MALDI-TOF MS for the quantitative determination of TAGs.

3.5.2. Nitrocellulose (NC) minimizes fragmentation of TAGs in MALDI-TOF MS

Several methods have been proposed to reduce fragmentation as for instance the utilization of LiDHB matrix (Cvacka, Svatos, 2003). For a series of reasons already explained above, the use of LiDHB has been abandoned. Gidden et al. (2007) found a strict correlation between TAG fragmentation and acidity of the solvent/matrix system. Therefore they suggested the addition of NaOH, $NaHCO_3$, or NH_3, which act as "H+ scavengers", to the TAG solution for limitating fragmentation. However, Al-Saad et al. (2003) demonstrated that fragmentation does not arise only from protonated TAGs, but on a longer time scale also alkali-metal ion adducts do fragment, according to two concurring mechanisms in which the alkali metal can either be lost or retained. These mechanisms become important especially when analyzers other than TOF are used, due to the long time scale of the ion paths.

A couple of previous studies have demonstrated that with the use of a pre-formed NC substrate film prior to sample and matrix deposition, it is possible to enhance the ion yield in the MALDI MS of proteins/peptides and DNA, as well as improve the shot-by-shot and sample-to-sample reproducibility (Jonson et al., 1986; Tonkinson and Stillman, 2002; Preston et al., 1993). NC also enhanced the sensitivity of peptide analysis up to the low attomol range (Miliotis et al. 2002). The application of polymers intercalating MALDI matrices for analyzing even very small molecules, such as CHAC, was not new (Donegan et al., 2006). It was already known the effect of NC in suppressing the matrix ionization and preventing detector saturation by matrix ions, even though the underlying mechanism is not clear.

Thus, we applied the use NC substrate layers to the MALDI-TOF MS analysis of TAGs. Previously, we tested several procedures of a sample deposition and found the best performances with a strategy that consists in the sequential deposition of matrix and TAG over the stainless steel target pre-coated with a thin layer of NC.

NC MALDI-TOF spectra of TAG are almost completely free from interfering fragment and matrix ions at low *m/z*.

When applied to MALDI-TOF analysis of TAGs, outcomes were excellent (Picariello et al., 2010)
As an example, it is shown in **Figure 30** the MS spectrum of TAGs from human milk. Human milk contains medium- and high-CN TAGs because of the exclusive presence of medium- (C10-C14) and long chain (C16-18) FAs. The almost complete absence of "DAG-like" ions allows to easily appreciate the occurrence of minor amounts of physiologically formed DAGs (*m/z* 617 and 643). The performances of the NC MALDI-TOF MS were particularly striking when a more complex mixture such as bovine milk fat (CRM-519) was analyzed (**Figure 31**).
In this case a complete "profiling", including the characterization of variously unsaturated, low-, medium-, and high-CN and odd-CN TAG, were "one-step" obtained. Because of the absence of fragment and matrix ions, the small *m/z* range was completely clean, so that very low-CN TAGs up to C22 and C20, and probably even C18, were clearly identified.
The application of NC MALDI-TOF MS to the analysis of the polar fraction of EVO oil, evidenced the occurrence of low MW hydrolytic compounds, up to MAGs (Picariello et al., 2010).
The comparison between ordinary and NC conditions of MALDI analysis of standard MAGs, DAGs and TAGs demonstrated an almost complete absence of both fragment and matrix ions, when analytes were co-crystallized on NC substrates (Picariello et al., 2010).
The mechanisms underlying the almost complete absence of fragment ions when using NC have not been investigated. As a pure speculation, it is possible hypothesize that the metal plate could have a role in the prompt fragmentation of TAGs, catalyzing some how their laser-induced hydrolysis. The The NC has an effect of isolating TAGs from the contact with the metal target. The hypothesis of an enhanced desorption in the presence of NC seems to be not real, as, in the presence of NC, a significant increase in the laser power in required by TAGs to be desorbed (~20 % higher).

3.5.3 NC MALDI TOF MS enables reliable quantification of TAGs

Although well documented and exploited, the use of MALDI as a quantitative technique is still debated. MALDI MS is in fact considered to be an unreliable technique, due to a series of associated concerns, including linearity and precision of the data acquisition system, sample preparation and signal reproducibility. The main shortcoming in quantitative MALDI is sample preparation from which derives the sample-to-sample, point-to-point and shot-to-shot experimental reproducibility. The use of an internal standard (IS), opportunely selected, overcomes in part this concern. Aside from the negligible fragmentation, the NC substrate modified the crystallization of matrix/analyte solution and allowed for more even sample-matrix crystallization that can be appreciated also by naked eye inspection. The even sample deposition enhances the shot-by-shot and sample-by-sample reproducibility, enlarging the potential of MALDI-TOF MS to semi-

quantitative and quantitative determinations. We tested the reproducibility and the linearity of the response in terms of ion production using standard TAGs, in particular tripalmitin and triolein. The tripalmitin-to-triolein signal intensity ratio (*m*/*z* 829.7-907.8) - an average of 10 measurements – versus the tripalmitin-to-virgin olive oil (w/w) ratio demonstrated a linear relationship (R^2) 0.995) over a wide concentration range. As expected on the basis of literature data, equimolar amount of tripalmitin and triolein did not give a balanced MALDI ion production, with the unsaturated TAG having higher ionization efficiency. In this conditions we assessed the possibility of quantifying triolein in EVO oil, using tripalmitin as the internal standard (IS). The relative amount of the selected TAG in the mixture was calculated according to the following equation:

$$W_{TAG}/W_{oil} = (I_{TAG}/I_{IS})\ (W_{IS}/W_{oil})\ (MW_{TAG}/MW_{IS})\ R_z$$

where W is the weight, I_{TAG} and I_{IS} are the relative signal intensities of TAG and IS obtained from the spectrum, respectively, and MW is the molecular weight. *R*z is a correction factor that depends on the different ionization response of the target TAG relative to the IS. In the case of triolein, R_z, determined empirically, was 0.82 but it may non-linearly vary with the TAG-to-IS ratio. In test EVO oil samples, triolein was estimated to be 33-35 %, a range of values that are in good agreement for the expected ones.

3.5.4 Conclusions

A precoated NC film on the MALDI target minimizes fragmentation in the analysis of TAGs and suppresses interfering matrix ion signals. These interesting advantages enable NC MALDI-TOF MS to profile complex mixtures of acylglycerols, even in the presence of low MW hydrolytic compounds such as MAGs. The method extends the dynamic range of the MALDI analysis applied to acylglycerols. NC also induces a radical improvements of the repeatability of the MALDI measurements, especially through an homogeneous matrix-sample crystallization, thereby proposing MALDI MS as a good alternative to GC-based techniques for the evaluation of TAGs in fats and oils. Quantification is particularly reliable provided that a suitable IS is selected.

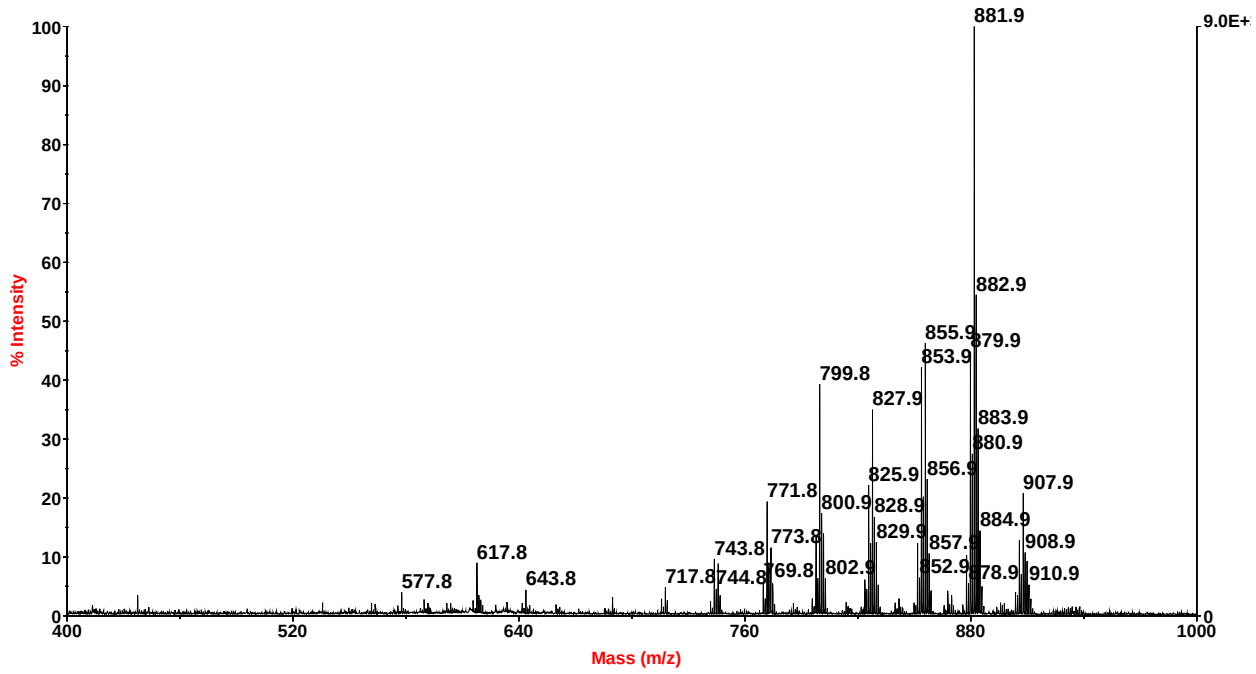

Figure 30. NC MALDI-TOF MS spectrum TAGs from human milk, showing no or very little fragmentation. Quantitation of TAGs is in good agreement with GC-based determinations

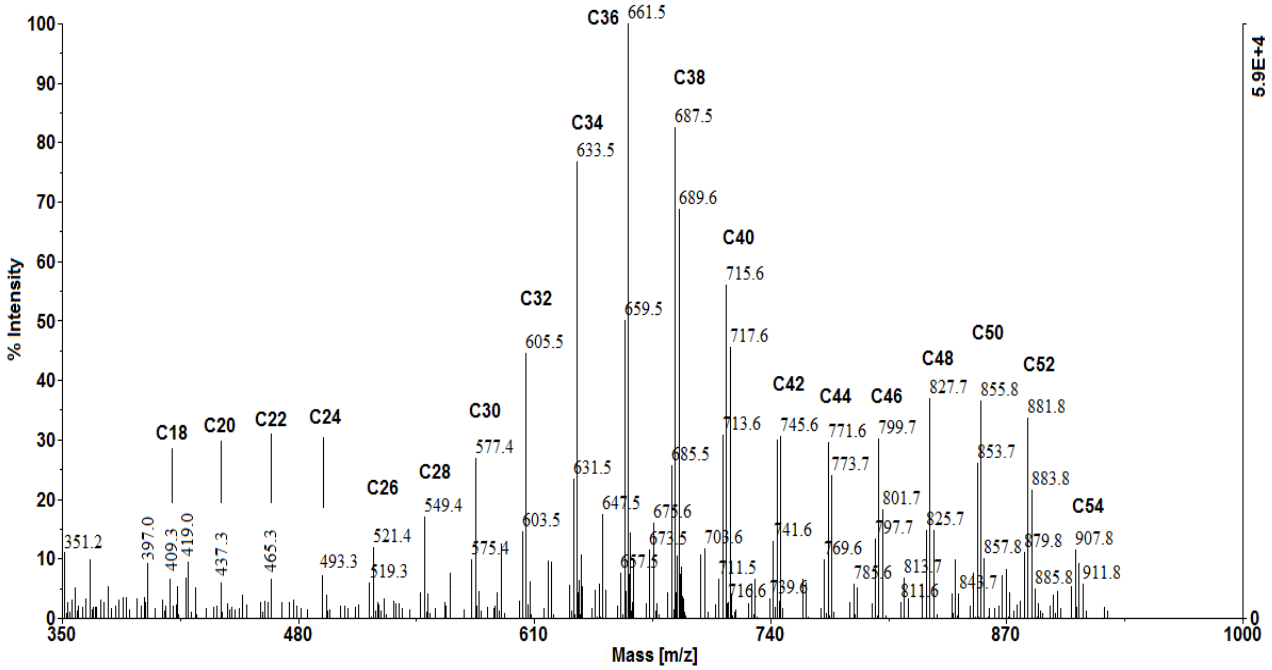

Figure 31. De-isotoped NC MALDITOF MS of butterfat. Because of the absence of matrix interfering and fragment ions very low-CN TAGs up to C22-C20 and probably C18 (needs to be confirmed) were identified. The typical bimodal pattern and the occurrence of odd-numbered CN TAGs was evident

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4 CONCLUDING REMARKS AND PERSPECTIVES

The astonishing advances in food science have prompted the development of totally new (bio)technological processes for food preparations. The growing knowledge about food functionality, bioactivity and toxicity is rising novel and urgent questions and is mirroring the increased awareness of consumers. The “omics”, alternatively intended as sciences or technological platforms that are aimed to the comprehensive characterization of biomolecular classes, are “holding the promises”, by demonstrating themselves capable to tackle the challenge of the complexity.

Mass spectrometry (MS) techniques, also including the recent advances of MALDI MS, are the core tools of the “omics”, “lipidomics” included. MS is not a unique technique, but an array of tools and strategies that can be variously combined, also hyphenated with other methodologies, enabling analytical access to many information levels. The large range of MS-based analytical procedures requires the deep familiarity with the potentiality and pitfalls of each approach in addition to a case-by-case application, carefully designed as a function of the analytical demand.

Due to degree of specialization that food analysis and food technology have attained, these two fields sounds only apparently separated. MALDI MS, for instances, has the capabilities to afford major contribution to the filed of food science and technology, provided that the competencies of researchers skilled in the technique are combined with those working in food technology and biotechnology. For advanced application it would also required relevant bioinformatic support, finalized to data acquisition and data processing. Synergistic and trans-disciplinary efforts are needed to provide solutions for the requirements of producers, food industry, regulatory agency as well as for consumers in order to improve food quality, functionality and storability, and to optimize the effects of technological processes on food components with the ultimate objective of promoting not only knowledge and the process optimization, but above all human health and wellness. In terms of technology, it means that significant improvements are to be achieved to define a panel of specific target compounds (or categories of compounds) and to make the detection and quantification more robust, automated and comprehensive. For many reasons, lipids areamong the ideal probes to assess the sensory/nutritional traits of a food or to trace back its “history”.

The instrumentation and maintenance costs and the required operative skills are among the primary factors that have currently prevented MS from a wider applications to protocols of food monitoring and control. These shortcomings could be at least in part overcome establishing unified analytical centers and realizing an appropriate policy of resource rationalization.

While several years will be still necessary to standardize the analytical procedures and to include MS in routine application protocols, it can be envisaged that MS, especially MALDI-based

MS due to the relative easy and quick utilization, will become in a next future a virtually irreplaceable tool to guarantee the safety of foods for consumers, to establish the nutritional role of food formulations or to sustain the development of new functional foods and ingredients for various application, not necessarily limited to the food industry.

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APPENDIX I

Scientific production (limited to the last 3 years: 2010-2013)

Original research articles published on peer-reviewed international Journals

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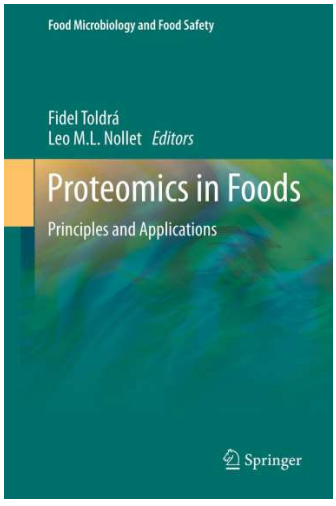

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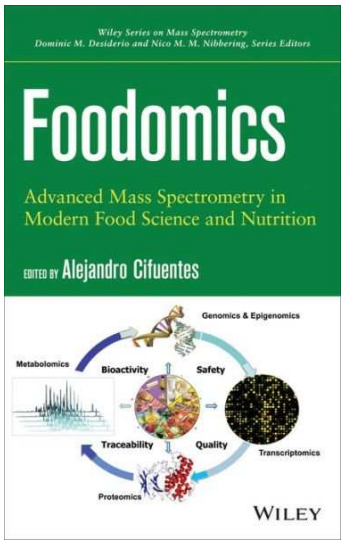

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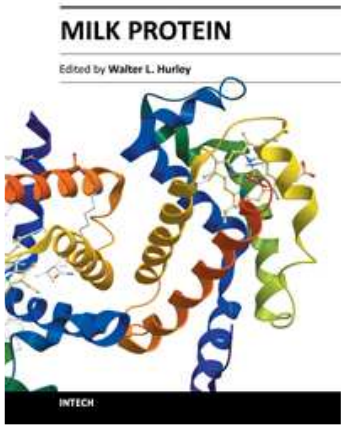

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Picariello G, Mamone G, Nitride C, Camarca A, Gianfrani C and Ferranti P. Shotgun proteome analysis of beer and immunogenic potential of beer polypeptides. proc. 7th ItPA Annual National Conference, Viterbo (2012). **Poster winning the 1st prize in the best poster award competition (out of 118 presented posters)**

Nitride, C., Mamone, G., **Picariello, G**., Mills, C., Berni Canani, R., Nocerino, R. and Ferranti, P. 2012 Identification of a new food allergen from hazelnut (Corylus avellana): proteomic and immunoassay based approach. Proc. EuPA 2012 Scientific Congress. New horizons and applications for Proteomics.

Nitride, C., Mamone, G., **Picariello, G**., Mills, C., Berni Canani, R., Nocerino, R., Ferranti, P. 2012 Proteomic and immunoassay characterization of a new food allergen from hazelnut (Corilus avellana). PO23 / Digestive and Liver Disease 44 Suppl. 4 S259–S292.

Picariello, G., Iacomino, G., Mamone, G., Fierro, O., Ferranti, P., Addeo, F. Peptidomics to trace the metabolic fate of milk proteins: Biological and immunological implications. 6th European Summer School. FEBS Advanced lecture Course – High Performance Proteomics. Bressanone 19-25 August 2012. **Poster shared with another the 1st prize in the best poster award competition (out of 72 presented posters)**

Picariello, G., Iacomino, G., Mamone, G., Fierro, O., Ferranti, P., Addeo, F. Peptidomics to trace the metabolic fate of milk proteins: Biological and immunological implications. Infogest Cost-Action Meeting, Leatherhead UK – October 2-4, 2012.

Selected abstracts of research articles with focus related to the Doctoral Thesis
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Eur. J. Lipid Sci. Technol. **109** (2007) 511–524 DOI 10.1002/ejlt.200600255 511

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One-step characterization of triacylglycerols from animal fat by MALDI-TOF MS

Comparative characterization of milk fat, lard, and beef tallow triacylglycerols (TAG) has been achieved by using matrix-assisted laser desorption/ionization–time-of-flight (MALDI-TOF) mass spectrometry (MS). The samples formed characteristic patterns, with major TAG signals differing in quantity and intensity according to the fat. In milk fat, the significant contribution of short-chain fatty acids (C_4–C_{10}) extends the TAG number to the C_{20}–C_{60} range. In lard and beef tallow, C_{12}–C_{18} fatty acids restrict the range to C_{48}–C_{54}, also typical of vegetable oils. Fats originating from ruminants contain odd TAG missing in lard. Signature TAG were identified for each animal fat. C_{20}–C_{46} specifically fingerprint milk fat; 52:5, 54:5 and 54:6 TAG mark lard; and 55:0, 55:1, 55:2 and 54:0 TAG typify beef tallow. Fats were also analyzed after hydrogenation or bromination; hydrogenation helped to fingerprint the low-abundant long-chain TAG and to distinguish short-chain native TAG from collision-induced fragments; bromination allowed clear separation of saturated and unsaturated TAG. Differences in the fatty acid composition amongst homologous isobaric TAG of different structures were identified by post-source decay analysis of hydrogenated precursors. MALDI-TOF has proven advantageous for simultaneously detecting TAG at various unsaturation degrees within different TAG classes (Cn). The data provide insight into animal fat differentiation on a molecular basis, increasing the analytical description to a new level, proving the so far underestimated capacity of MALDI-TOF MS in this field.

Keywords: Triacylglycerols, milk fat, lard, beef tallow, MALDI-TOF mass spectrometry.

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

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MALDI-TOF Mass Spectrometry Profiling of Polar and Nonpolar Fractions in Heated Vegetable Oils

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Triacylglycerol oxidation of thermally stressed (6 h at 180 °C, simulating deep-frying conditions) edible vegetable oil (sunflower and olive) was studied using matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF MS). Chromatographic separation of the nonpolar and polar components from the heated oil performed on silica gel prior to MS analysis significantly enhanced the detection of oxidized components. The spectra contained signals that were assigned to triacylglycerols (TAG), diacylglycerols (DAG), triacylglycerol oxidative dimers, oxidized TAG, and TAG fragments arising from the homolytic β-scission of linoleyl, peroxy, and alkoxy radicals. Enrichment of the polar compounds prevented mass spectrometric ion suppression, thus allowing the detection of minor species originating from thermal oxidation. In addition, this allowed the monitoring of polar compounds in vegetable oils undergoing mild thermal treatment. As such, chromatographic separation coupled with MALDI-TOF MS analysis provided a rapid, sensitive, and specific tool to assess the thermal oxidation of vegetable oils.

KEYWORDS: Sunflower oil; virgin olive oil; thermo-oxidation; polar and nonpolar fractions; MALDI-TOF MS

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Nitrocellulose Film Substrate Minimizes Fragmentation in Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry Analysis of Triacylglycerols

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The potential of matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) for the analysis of intact triacylglycerols (TAGs) is generally limited by the extensive in-source prompt fragmentation. The sequential deposition of matrix and TAGs over the stainless steel target precoated with a thin layer of nitrocellulose (NC) drastically reduced fragmentation in the MALDI-TOF MS profiling of oils and fats. The NC MALDI-TOF MS profiles of native and thermally stressed virgin olive oil and butter are reported as case studies, along with test analyses of a standard mixture of mono-, di-, and triacylglycerols. Mass spectra were almost completely devoid of both fragment and matrix ion signals, thus disclosing relevant information, especially in the low molecular mass range. The detection of several partial acylglycerols of low abundance and minor TAGs that are barely observed with other techniques also provided evidence for an increased dynamic range of NC MALDI-TOF MS that was due to the minimization of suppressive effects. The NC film substrate also improved the shot-to-shot and sample-to-sample reproducibility of the ion production through the exhibition of a more homogeneous matrix/analyte cocrystallization, thus enabling MALDI-based measurements to a consistent quantification of TAGs.

derivatization prior to analysis, (c) acquisition time scales ranging from seconds to minutes, (d) relatively high tolerance to buffer and salt contaminants in the sample, (e) production of unique ion signals for specific TAG−metal adducts, which simplifies the analysis and allows rapid profiling of complex TAG mixtures, and (f) high sensitivity in the range of femtomoles or lower. Recent advances in MALDI MS/MS have made it possible to obtain structural information regarding lipids in a way that is similar or complementary to ESI-MS or APCI-MS.[3−5]

In comparison to the analysis of peptides and proteins, standardized protocols for the MALDI MS analysis of TAGs are far from being established, and various matrix systems and sample preparation strategies have been developed. In practice, many matrixes are inadequate because of inhomogeneous crystallization (the so-called "sweet-spot phenomenon").[6] MALDI ionization of TAGs is generally accompanied by prompt in-source fragmentation, which is particularly intense in the case of "hot" matrixes.[1,2] In addition to matrix-derived ions, spurious signals derived from TAG breakdown can interfere with the detection of components with molecular masses lower than 700 Da.[7] Additional shortcomings such as suppressive effects degrade sensitivity and repeatability of the method and, combined with the current commercial unavailability of suitable internal standards (IS), hinder the use of MALDI MS for TAG evaluation.[8] The intrinsic limitations of the technique can be overcome to some extent through the use

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review

Novel Mass Spectrometry-Based Applications of the 'Omic' Sciences in Food Technology and Biotechnology

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Summary

The revolution of 'omic' sciences has introduced integrated high-throughput approaches to address the understanding of the biochemical systems and of their dynamic evolution. In the field of food research, 'omics' are depicting a comprehensive view which largely overcomes the merely descriptive approaches of the early proteomic and metabolomic era. Thus, the recently born 'foodomics' is to be intended as a global perspective of knowledge about foods, which covers the assessment of their composition, the effects of (bio)technological processes for their production, their modifications over time and the impact that food consumption has on human health. Food proteomics and metabolomics, along with their derived 'omic' branches such as peptidomics, lipidomics and glycomics, are still evolving technologies capable of tackling the nature and the transformations of foods. In the development of the advanced 'omic' platforms, because of their potential to profile complex mixtures of biomolecules, mass spectrometry techniques have assumed an unquestionable role. Because proteins are central molecules in all biological systems, proteomic platforms are pivotal among the 'foodomic' tools, as the proteomes and related peptidomes provide biomolecular subsets mostly informative about the history of a food product. Similarly, food interactomics and metabonomics aim to study the dynamics that occur in foodstuff. The ultimate aim of foodomics is the production of high-quality and safe food products for improving human health and well-being. In this review we critically present the recent research outcomes in the field of food sciences that have been achieved through the contribution of the 'omic' methodologies relying on mass spectrometry.

Key words: proteomics, peptidomics, metabolomics, interactomics, mass spectrometry, food proteins and peptides, food quality, food safety, food technology