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Mass spectrometry of compounds with high molecular mass – Part II

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ABSTRACT

The second part of this article will discuss mass spectrometer's analyzers and application of high resolution spectrometry to identification and separation of complex mixtures [1]. Today's mass analyzers with resolution to fifth decimal point are quite common and offer unusual ability to analyze complex mixtures without prior separation. Separation techniques are required when low resolution equipment is used. Modern MS analyzers can assess components in complex mixture without prior isolation from the matrix, only limitation is ability to ionize those compounds. Additionally, detecting system lowered detection limit of MS to the level of femtograms and for some systems even lower.

Spektrometria mas związków chemicznych z dużymi masami cząsteczkowymi – część II

Słowa kluczowe: rozdzielczość, analizatory, TOF, Orbitrap, białka, mikroorganizmy, cykloproteiny, rozkład fitosteroli

STRESZCZENIE

W drugiej części niniejszego artykułu charakteryzowane są spektrometryczne analizatory mas i opisywane jest zastosowanie wysokorozdzielczej spektrometrii mas do rozdzielania i identyfikacji składników złożonych mieszanin [1]. Współcześnie analizatory z rozdzielczością określaną piątym miejscem po przecinku są powszechnie stosowane i dają niezwykłą możliwość analizy złożonych mieszanin bez wcześniejszego ich rozdzielania innymi metodami. Jedynym ograniczeniem jest możliwa trudność jonizacji tych składników. Dodatkowo, układy detekcji mają tak małą granicę detekcji, ze możliwe jest wykrywanie femtogramowych a nawet mniejszych iości związków chemicznych.

1. MASS SPECTROMETRY – ANALYZERS

Only ionized molecules can be analyzed by mass spectrometer analyzer for their molecular mass, whereas neutral molecules are not detected. Mass spectrometer analyzers evolve into high resolution systems, it means that these spectrometers can measure molecular mass up to fourth or sixth decimal point. Why this accuracy is required? Higher mass accuracy is leading to easier identification of a compound, because based on molecular mass elemental structure of compound is established. When molecular mass is established with high accuracy it leads to lower number of possible specific compounds (Tab. 1). This way it is possible to eliminate compounds having similar masses however different at third and fourth decimal point.

Table 1 Relationship between mass spectrometer resolution and the number of possible elemental molecular structures

Tabela 1 Zależność pomiędzy rozdzielczością analizatora a ilością potencjalnych związków o specyficznej strukturze

Resolution (Da)	Number of possible elemental structures
1	5264
0.001	38
0.00001	3

Explanation of this relation is discussed in Figure 1, compounds different in molecular mass on third and fourth decimal points are not separated by low resolution mass spectrometer and they are coming as wide peak. Whereas, on the high resolution MS these compounds are separated and their molecular masses established individually.

The typical analyzers currently offered in the MS are: (1) Quadrupole; (2) Ion Trap; (3) Time of Flight (TOF); (4) Orbitrap; (5) Magnetic Sector; (6) Ion Cyclotron Resonance (ICR). Among these analyzers only TOF, Orbitrap, magnetic sector and ICR can operate at high resolution mode, others listed above are low resolution MS. Together with resolution it is important accuracy of mass assessment and stability of system calibration and typical mass assessment accuracies in Table 2 are presented.

TOF instrument requires constant calibration of the system, which is usually achieved by injection of the standard at constant intervals or with sample. This limitation is related to the measurement of time with assumption that distance of the ions path is constant. The ions' path length is affected by environmental conditions mainly by temperature due to the dilation effect of materials used for construction of a flight tube. Even a few millimeters difference in the length of ion's path

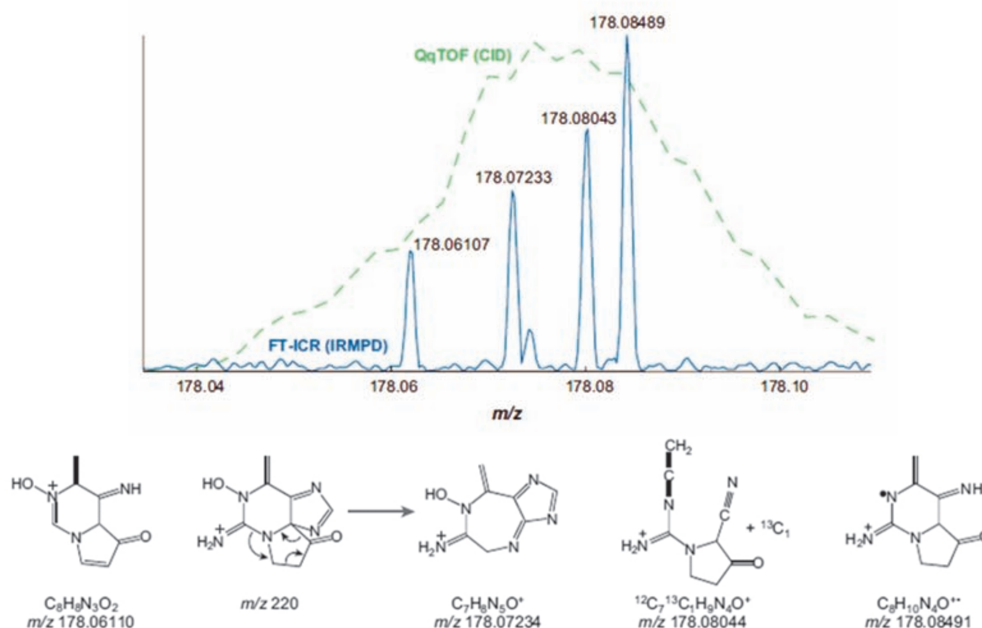


Figure 1 Effect of resolution on identification of compounds. Broken line represents mass spectrum peak of lower resolution MS [2]

Rysunek 1 Wpływ rozdzielczości na możliwość identyfikacji związków chemicznych o zbliżonej strukturze. Linia przerywana przedstawia spektrum uzyskane na spektrometrze o małej rozdzielczości

Table 2 Accuracy of molecular mass assessment by different mass analyzers

Tabela 2 Dokładność oznaczania mas molekularnych przez różnego typu analizatory

Type	Mass Accuracy (ppm)
FTICR	0.001 - 1
Orbitrap	0.5 - 1
Magnetic Sector	1 - 2
TOF-MS	3 - 5
Q-TOF	3 - 5
Triple Quadrupole	3 - 5
Linear Ion Trap	50-200

Mass spectrometer: FTICR – Fourier Transform Ion Cyclotron Resonance; TOF – Time of Flight MS; Q-TOF – Combined Quadrupole and Time of Flight.

cause important difference in flight time, mainly because differences in flight time are measured in micro and nano seconds. Most instruments have limited length of flight path due to limitation of the instrument size to fit into laboratory, and if flight tube is longer it require strict temperature control in lab environments and more often calibration. ICR, Orbitrap and magnetic sector instruments offer extremely good stability of calibration and insignificantly are affected by laboratory environment. Orbitrap used in my laboratory is keeping it calibration within specified range for up to 45 days.

Currently magnetic sector mass spectrometer are replaced by ICR and Orbitrap instruments, which are smaller and easier to operate, and these instruments are disappearing from the market fast, and are not included in discussion in this paper.

1.1 Time of Flight (TOF)

This analyzer was one of the first used in mass spectrometry, developed in 60th together with magnetic sector. In the 80th new generations of TOF were designed with much better resolution, mainly by increasing distance for ions to fly and with it was increased accuracy of flight time measurement to allowing assessment of molecular mass up to the fourth decimal point.

Mechanics of TOF is very simple: ions are sent into flightpath by "Kicker", receiving the same amount of energy. According to the kinetic energy formula, speed is reversely proportional to the mass of ion. Though ions with smaller mass will fly faster and will have shorter flying time, whereas ions with bigger mass will be detected later (Fig. 2). Of course it is based on the following premise: that distance between "Kicker" and detector have to be the same for all molecules during the whole analysis time.

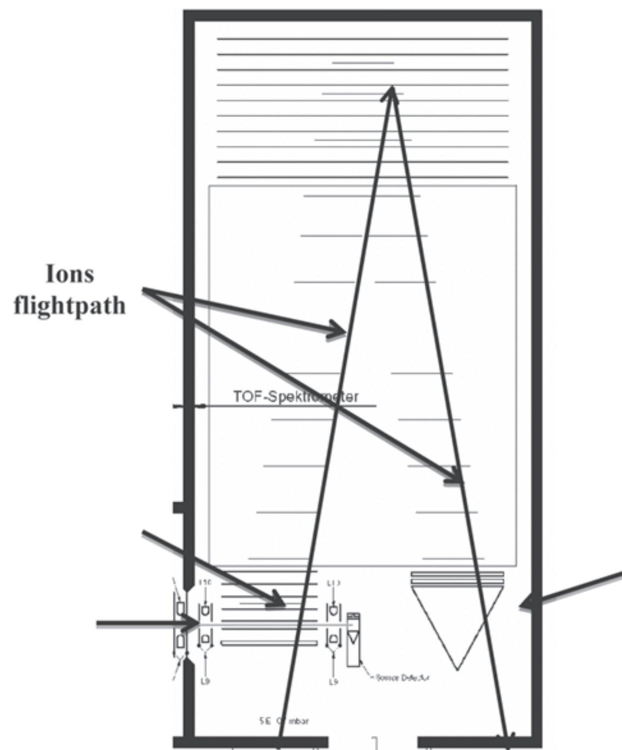


Figure 2 Typical configuration of time of flight mass spectrometer (TOF)

Rysunek 2 Typowa konfiguracja czasu przelotu spektrometru (TOF)

TOF instruments are very sensitive to the temperature changes, and with it changes of dimensions of the flying tube occurs changing length of flightpath. The longer flightpath, the more stable temperature is required, even a few degree change, can make results inaccurate. To prevent changes in accuracy of mass assessment, TOF instruments require constant calibration, even when analysis is running. Currently offered instruments have built-in continuous calibration system, to better control mass accuracy and to achieve higher reproducibility.

1.2 Orbitrap (Exactive)

Orbitrap MS system operates similarly to cyclotron, where ionized molecules receive strictly controlled amount of energy by applied electrical field and making them orbiting around electrodes. Since ions are injected into trap tangentially, its start to orbit between external and internal electrodes. At the same time high frequen-

cy electrical field is applied by excitation electrodes, causing ions to orbit inside Orbitrap (Fig. 3). In the system ions receive specific amount of energy and ionized molecules speed is related to mass, smaller molecules have higher speed than larger one. Detection electrodes record current generated by orbiting ions, which is transformed into mass spectrum. The Orbitrap system is patented by Thermo Scientific and is available in a few iterations, offering resolution above 100,000 (1-2 ppm) and is characterized by extremely stable calibration.

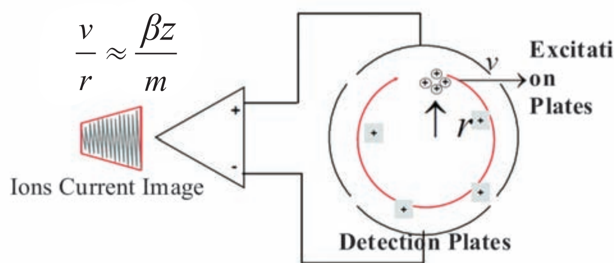


Figure 3 Operation and detection in ICR and Orbitrap systems

Rysunek 3 System działania spektrometru Orbitrap i cyklotronu jonów

Typical configuration of the bench-top Orbitrap system, named Exactive, in Figure 4 is presented. Sample is introduced into mass spectrometer directly by pumping it with low flow by syringe pump, or it ionization system is connected directly to HPLC. In both cases molecules present in a sample are ionized as described in the previous section and ions are extracted into MS by vacuum and charge set on extracting and focusing lenses (Fig. 4). Curved lens play a function of eliminating neutral molecules which also gets into system due to high vacuum in MS. Ions are transported into C-Trap from which are tangentially introduced into Orbitrap, where are separated and detected according to their mass to charge ratio. Additionally this system is equipped in high energy dissociation cell, where MS/MS is done by applying neutral gas and controlled amount of energy to dissociate molecules and equilibrate distribution of energy among molecules. It means that excited molecules, or having high energy, loosing energy and forming stable ions. These dissociated ions are detected by Orbitrap, this way it is, for example very easy to establish sequence of amino acids in protein molecule.

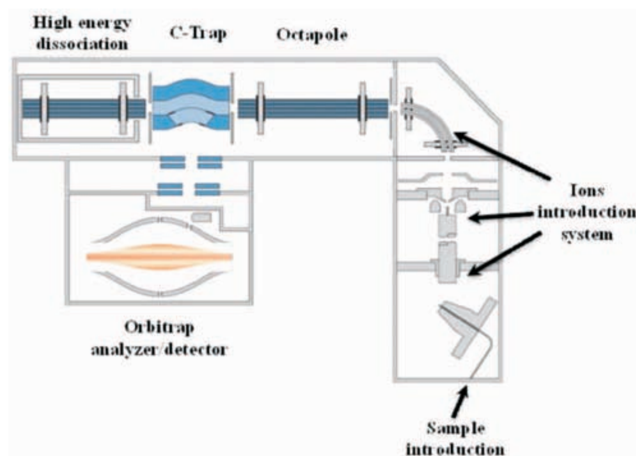


Figure 4 Schematic of Orbitrap Mass Spectrometer (Exactive)

Rysunek 4 Schemat spekrometru masowego Orbitrap

1.3 Ion Cyclotron Resonance (ICR)

ICRMS operates similarly to Orbitrap system, however the ions are kept “floating” in the ICR cell by very strong magnetic field. In this system magnetic field is formed by cryogenic superconductive magnet and field strength is in the range of a few to more than a dozen of Tesla’s (Fig. 5). The magnet is usually very heavy and system requires constant supply of liquid nitrogen. Generally, higher strength of magnetic field offers better resolution, and resolution of these systems is usually above a million (1,000,000).

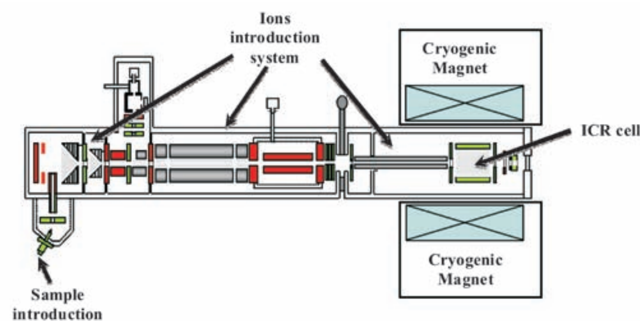


Figure 5 Schematic of Fourier Transform Ion Cyclotron Resonance Mass Spectrometer (ICRMS)

Rysunek 5 Schemat spekrometru masowego cyklotronu jonów z fourierowską transformacją danych

Sample is introduced directly into ICR as “shotgun” or by separation system such as HPLC similarly as discussed for Orbitrap. Ionized molecules through set of focusing lenses are transferred into ICR cell, where they are “floating” in magnetic field (Fig. 5). Then ions are spun out as described for Orbitrap and current formed by ions measured (Fig. 3). As next step, current is transformed into mass spectrum and molecular mass of ions is detected. The most important advantage of ICR is very high resolution and sample do not

require prior separation and multitude of ions can be separated by mass.

2. FOOD COMPONENTS AND CONTAMINANTS DATA

In this section some examples of high resolution mass spectrometry applications will be discussed, to show advantages in comparison to low resolution MS, including MS/MS. The latter one is to some extend good tool for indirect identification, where parent molecule is dismantled to specific fragments, however chemistry of degradation is complicated and often require background work to establish it for particular group of chemical components. This identification method became particularly difficult when complex mixture of compounds, as is present in food system, in most cases require efficient separation of the compounds prior identification with MS/MS. Applying high resolution MS allowing us to separate and relatively easy identify large number of components in complex mixture without prior separation. Additionally preliminary preparation of the sample or isolation of compounds of interest is not required when high resolution MS is utilized.

2.1 Proteins

Proteins are the most common components playing a different functions in every living organism and analysis of it molecular patterns can be used as identification of product or assessment of it identity. Also proteins can be utilized for identification of bacteria strains, particularly pathogenic type of microbes. In Figure 6 specific pattern of protein compounds is presented, which can be used for identification of proteins, including source, because pattern of protein parts is source dependent. Numbers with plus sign indicate how many charges each fragment has, multiple charges in protein molecules is normal because nitrogen atoms are very easy to be ionized.
Selected examples of proteins represent typical metabolically involved proteins. The cytochrome C complex is a small heme protein found loosely associated with the inner membrane of the mitochondrion. Whereas transferrin is an iron transporting protein, present in all organisms having blood, including human where absorption of iron for example from supplement is limited or is happening at the very low level due to the limited number of carrying it proteins.

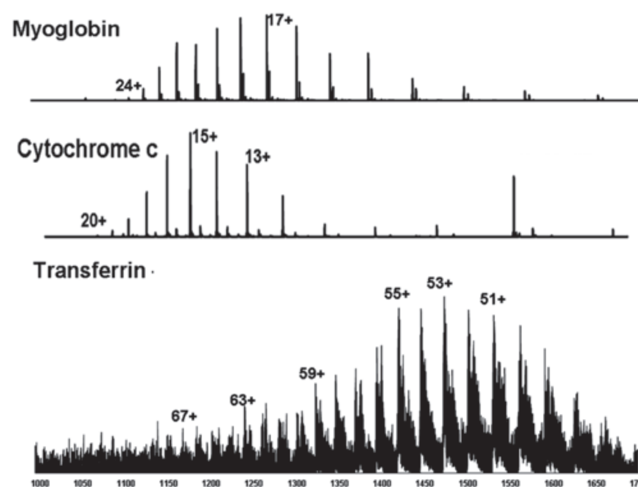


Figure 6 Patterns of myoglobin, cytochrome C and transferrin proteins [3]
Rysunek 6 Analiza mioglobiny, cytochromu C i białka transferiny

Pattern of protein components is usually the same for specific source of it, however distribution of fragments is affected by source. Also from fragments it is possible to establish size of whole protein molecule and specific protein databases are available where typical fragments of specific proteins are described.

2.2 Identification of microorganisms

Microorganisms are identified by analyzing protein or ribonucleic acids patterns. Similarly to described above protein identification the same technique is used for nucleic acids composition or pattern.
Utilizing those patterns it is possible not only to differentiate microorganisms by type but also by strain within the same type of bacteria. This ability is presented in Figure 7, where different strains of E. coli are identified when whole cells are introduced into MS, however this can be only done when high resolution MS is applied.

2.3 Analysis of the very complex mixtures

Presented below mass spectrum of light crude oil is the very good example of the ability of high resolution MS. Crude oil is probably the most complex mixture of organic compounds available in the nature, usually thousands of components are present in it. In this particular case 12500 compounds were identified with simple direct injection of the oil, which is called “shotgun” approach, it means that it is not necessary to do any preparation of a sample, neither separation of compounds or group of components prior running on MS.

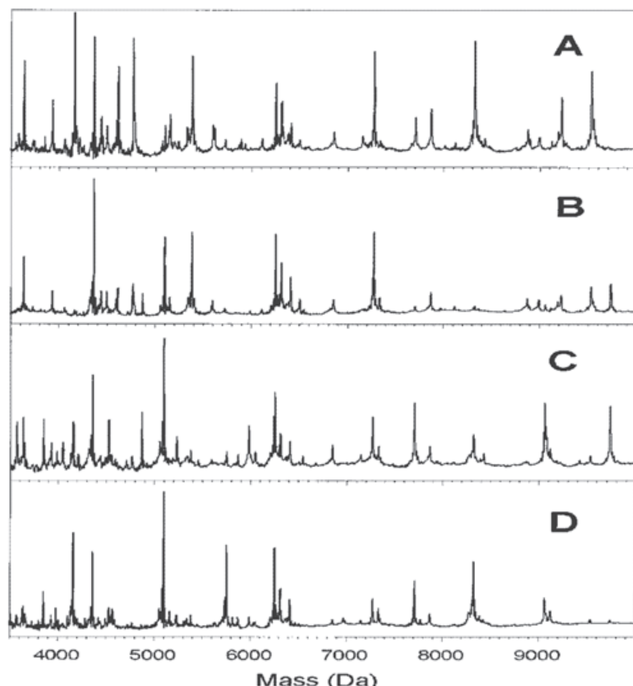


Figure 7 MALDI-TOF mass spectra and identification of four strains of E. coli [4]

Rysunek 7 Spektra masowe i identyfikacja bakterii E. coli za pomocą systemu MALDI-TOF>

As specified on the graph, in this case resolution was only 400000, but mass in Figure 8 accuracy at 0.2 ppm. This analysis was done on ICR spectrometer, which in the new version can very easily achieve resolution of 1.5 to 2 million, providing even better separation of components and the same sample run on this MS was able to separate about 45000 individual compounds.

2.4 Flaxseed oil cyclopeptides

Flaxseed oil is unique in many characteristics and it contains the highest amount of linolenic acid among all oilseeds, also unique proteins which are oil soluble and has cyclic structure, where amino acids are connected together forming circle (Fig. 9). Most of cyclopeptides contains methionine residue in their structure, this amino acid has sulfur atom in the structure, which is oxidized to form methionine sulfoxide (Fig. 9). Oxidized form of methionine is causing bitterness of flaxseed oil, oxidation of this amino acid is happening before oxidation of linolenic acid. That is why, cold

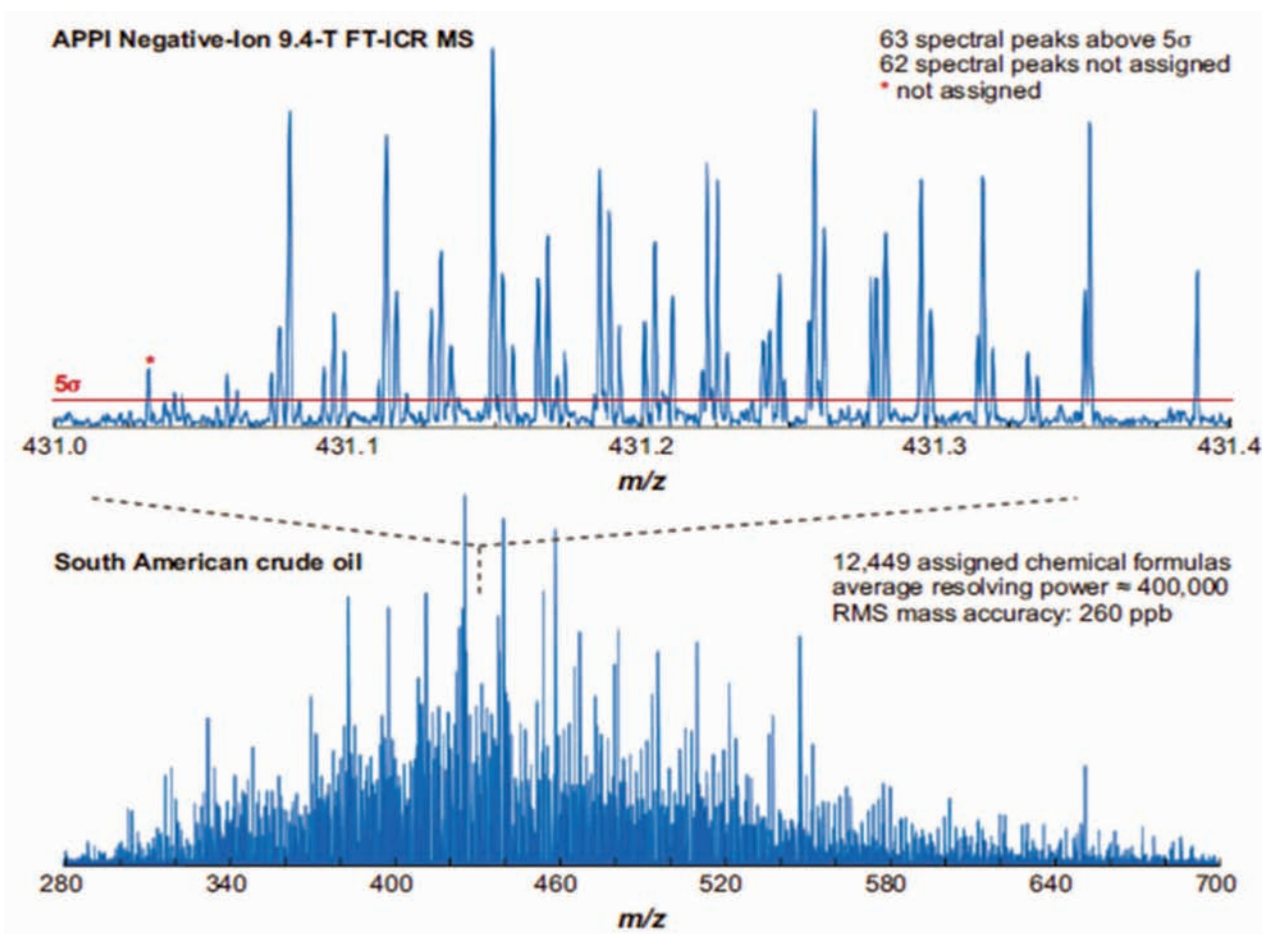


Figure 8 Fourier transform–ion cyclotron resonance mass spectrum of a South American crude oil. Upper spectrogram shows compounds with molecular mass within a range of 0.4 m/z [2]

Rysunek 8 Spektra masowe ropy z Południowej Ameryki uzyskane za pomocą spekrometrii rezonansu cyclotronowego jonów

pressed flaxseed oil is bitter because often is produced from immature and physically damage seeds, and oxidation methionine is happening within seed and even during pressing when is done improperly. Flaxseed oil contains usually fourteen different cyclopeptides, not all have methionine, and it is difficult to separate them for quantification due to very similar properties.

Applying new HPLC packing, the core system Kinetex from Phenomenex, we were able to separate all cyclopeptides, and MS was used to verify compounds identity. In Figure 9 the upper chromatogram shows separation of all cyclopeptides, whereas on the bottom part, sequence of amino acids for cyclopeptides A [5]. To achieve sequencing of individual amino acids, peptide was treated with high energy nitrogen in high energy dissociation cell of Exactive system. Individual amino acids were removed from peptides and identified by Orbitrap when all were injected into it (Fig. 10).

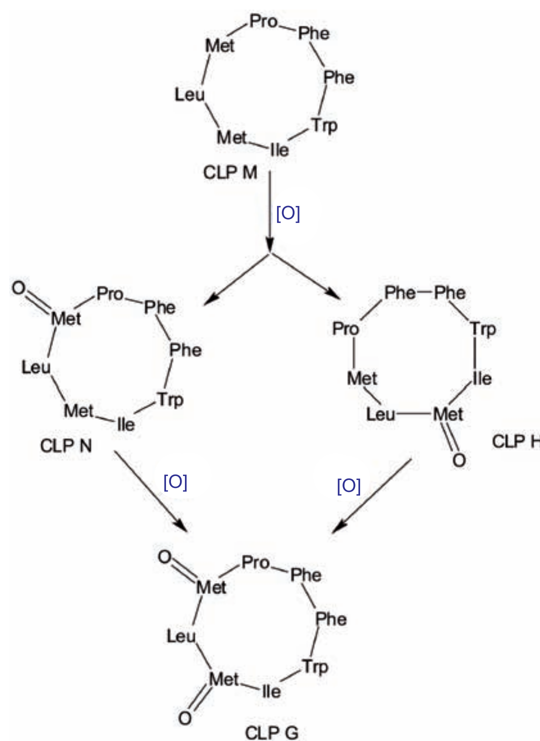
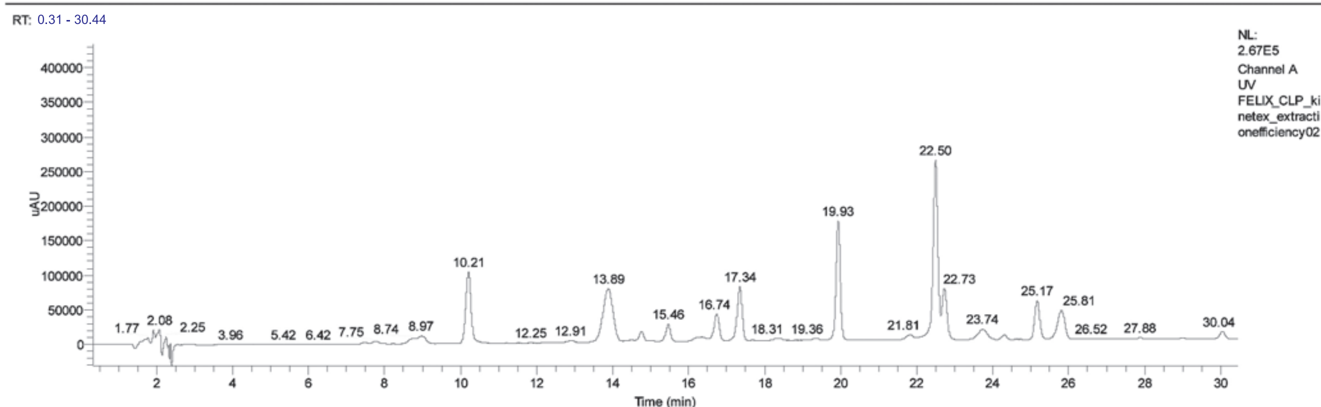


Figure 9 Sequential oxidation of CLP M containing two methionine residues. CLP – cyclopeptides with letters represents different types

Rysunek 9 Oksydacja cykloproteiny M posiadającej dwie cząsteczki metioniny. Cyklopeptydy z różnymi literami reprezentują różne ich typy



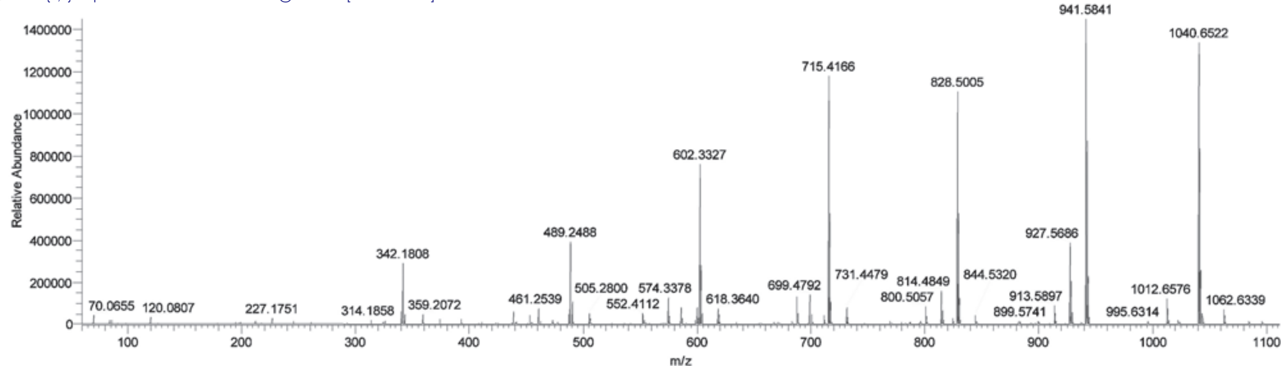


Figure 10 Analysis and identification of flaxseed oil cyclopeptides

Rysunek 10 Rozdział i identyfikacja cyklopeptydów siemienia lnianego

2.5 Phytosterols degradation during frying

Phytosterols are the important components of all vegetable oils, these compounds have ability to limit absorption of cholesterol in human digestive tract, and positively affecting human's blood lipids. However, those compounds have chemical structure similar to cholesterol and are prone to oxidative degradation, particularly this process is accelerated during oil processing, storage and frying. Work done in my laboratory showed that sterols under frying and processing conditions are forming oligomers, where many sterol monomers are chemically bonded together, by simple condensation reaction between hydroxyl groups. Utilizing high resolution MS we were able to identify oligomers formed up to the pentamer during the degradation of sitosterol (Fig. 11). On the bottom part of the graph molecular masses of different oligomers are presented, which are affected by the type of sterols combined together.

Concluding the above discussion it is clear that high resolution mass spectrometry it is a very powerful analytical tool to be applied in food and metabolic sciences to decipher components present in our foods and how their affect human metabolism. This methodology also has potential to be used for detection of compounds which positively and negatively affect human health, including verifying food source and adulterations of foods.

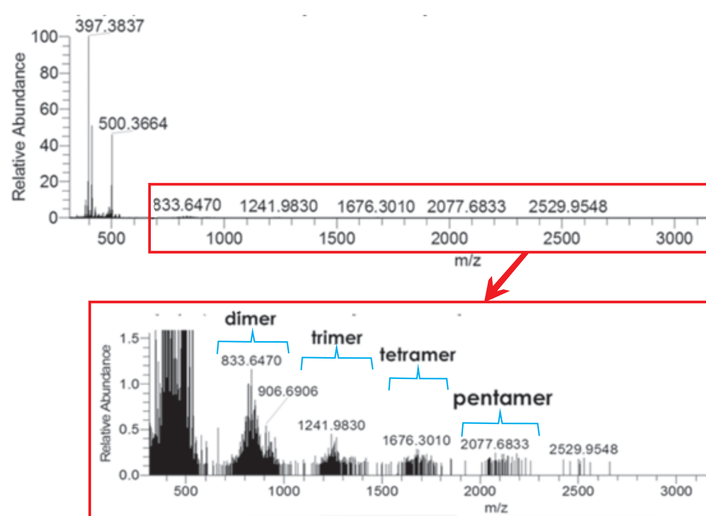


Figure 11 Identification of oligomers formed during thermal degradation of sitosterol

Rysunek 11 Identyfikacja oligomerów powstających w czasie termicznej degradacji sitosterolu

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