Capter 120

Applications of lipidomics by mass spectrometry in the search for molecular alterations in patients diagnosed with COVID-19

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ABSTRACT
Lipids are responsible for energy reserve, and vitamin absorption, besides being structural and functional components of biomembranes and signaling mediators. Due to the pandemic caused by the dissemination of the SARS-CoV-2 virus, recent researchers have sought to correlate changes in lipid metabolism in different biological matrices, including plasma, tracheobronchial secretions, and nasopharyngeal/oropharyngeal fluids. However, the collection/obtaining of these samples uses invasive protocols, which generates discomfort in patients, being desirable the search for alternative analytical methodologies that use samples collected in a non-invasive way, such as saliva. Because of this, this chapter aims to describe the application of lipidomics by ultra-high-resolution mass spectrometry to assess molecular changes in the spittle of patients diagnosed with COVID-19.

Keywords: Lipids, spittle, COVID-19, Microextraction, Mass Spectrometry.

1 INTRODUCTION

On December 31 of the year 2019, the World Health Organization (WHO), identified the spread of Severe Acute Respiratory Syndrome (SARS), in the city of Wuhan, China. This was a new coronavirus, SARS-CoV-2, and the associated disease was named coronavirus 2019 (COVID-19). SARS-CoV-2 is from the Coronaviridae family, and it is a ribonucleic acid (RNA). The two main proteins associated with the virus are the S protein known also as spike glycoprotein, which is responsible for the entry of the virus into the host cell by binding to the cellular receptor, and the N protein (viral nucleocapsid), which regulates the replication of the virus in the human body (UZUNIAN, 2020).

The symptoms of infected patients are very similar to those of influenza, such as dry nose, fever, fatigue, and loss of taste and/or smell. In several cases, a worsening of the disease may occur, and as a consequence the manifestation of more severe symptoms, such as acute respiratory distress syndrome (ARDS), hyperinflammation, and damage to organ functions, such as cardiac disorders, acute kidney damage, and liver dysfunction. Some specific groups of people are considered at risk for the worsening of

the disease, such as those with chronic diseases such as hypertension and diabetes, asthma, chronic obstructive pulmonary disease, smokers, pregnant women, immunosuppressed patients, and people over the age of 60 (TALEGHANI, TAGHIPOUR, 2021). The spread of the disease is extremely worrying since it has already affected about 481 million people worldwide, including more than 6.1 million fatalities (data as of March 2022), and is considered the most serious pandemic since the Spanish flu (BRAZIL, 2022, WHO, 2022).

It has been reported in the literature that some carriers of SARS-CoV-2 are asymptomatic, i.e., they have no signs of the disease or only mild symptoms. The high number of asymptomatic patients increases the risk of spreading the virus; therefore, early, rapid, and efficient testing of the population is very important as a form of prevention since people with positive results can be isolated and minimize the degree of contagion of the virus (TALEGHANI, TAGHIPOUR, 2021).

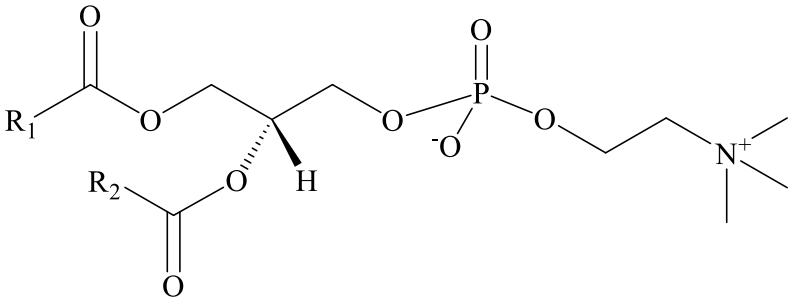
According to the center for disease control and prevention, molecular and antibody-based methods are two methods for detecting SARS-CoV-2. The polymerase chain reaction (RT-PCR) is a molecular test based on genetic material, whose main purpose is the identification of the virus genome in samples of nasal or throat secretions, which are collected with the aid of a device called a swab. However, these tests have some limitations, the RT-PCR for example requires adequate infrastructure on the part of clinical laboratories, technical expertise, and specific inputs to perform the tests, which results in high added value to the analysis. The serological tests for antibodies are simpler to be performed, but they depend directly on the moment of infection since the human body can take from one to three weeks to start producing the antibodies against COVID-19, which occasionally results in false-negative results (TALEGHANI, TAGHIPOUR, 2021).

Lipidomics Associated with SARS-CoV-2 Infection

Lipid metabolites have several functions in the human body and can provide information on cellular metabolism. Among the main functions of lipids are: a) energy reserve; b) vitamin absorption; c) tissue layer protection; d) structural and functional components of biomembranes. Lipids are hydrophobic or amphiphilic molecules that originated entirely or partially by condensation of thioester carbamides, and/or condensation of carbocations of isoprene units. Furthermore, they are divided into classes and subclasses according to the type of chemical bonding between the main group and the aliphatic chains (HAN, 2016).

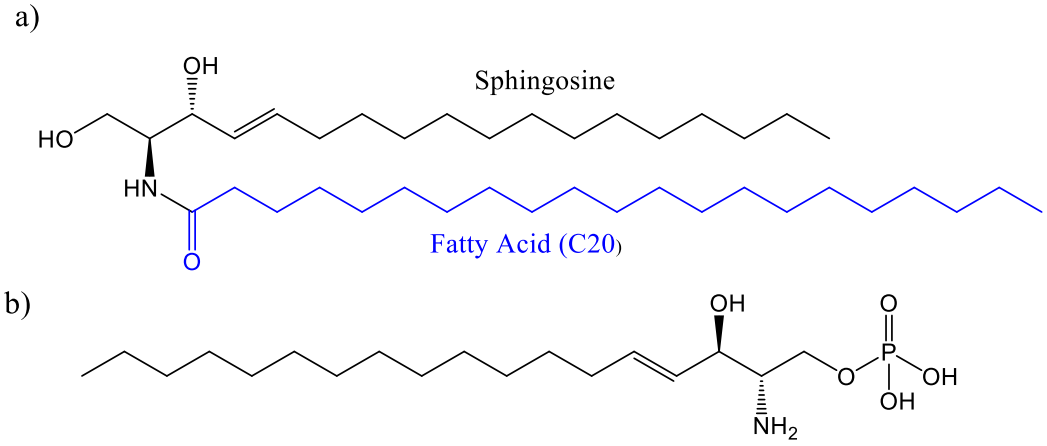
Glycerophospholipids or also known as phosphoglycerides are the largest class of membrane lipids and consist of glycerol, fatty acids, phosphate, and alcohol. It has been reported in the literature that activation of phospholipase A2 may be associated with lipotoxicity and inflammation. In addition, changes in homeostasis can influence membrane potential and ion transport (HAN, 2016). Figure 1 exemplifies a molecule belonging to the glycerophospholipid class, [1,2-diacyl-sn-glycero-3-phosphocholine].

Figure 1 - Structural representation of the molecule [1,2-diacyl-sn-glycero-3-phosphocholine], belonging to the glycerophospholipid class



The second largest class of membrane lipids are the sphingolipids, consisting of a long-chain amide, formed via serine and palmitate, which regulate the production of sphinganine and dihydroceramide. The amide in mammals helps regulate actin, a protein that constitutes the filaments of muscle cells. The simplest molecules of this group are ceramides, formed by fatty acids linked to the amino group (Figure 2), the concentration levels of ceramides are essential for understanding the metabolism of sphingolipids since this is an intermediate for the biosynthesis of complex sphingolipids. Another important molecule of this class of lipids is sphingosine-1-phosphate (S1P), which acts in cell maintenance, cell migration, and inflammatory response (OLIVEIRA FREDI; TINOCO, 2015; HAN, 2016).

Figure 2 - Chemical structure of the sphingolipid class: a) Ceramide [N-(eicosanoid)-sphing-4-enine]. b) Sphingosine-1-phosphate

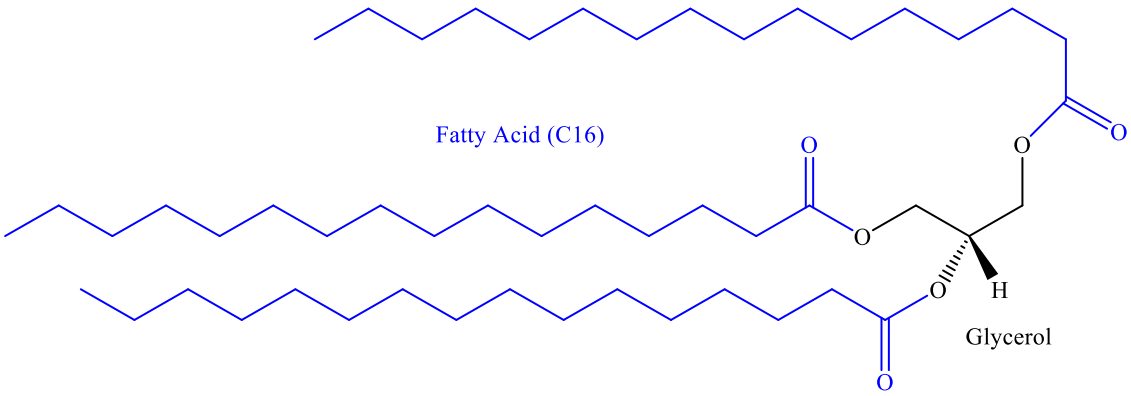


Ceramides are considered central molecules in the study of sphingolipid metabolism. In the studies by Seitz et al., (2015) it was reported that changes in ceramide levels are related to infections with Neisseria gonorrhoeae, Staphylococcus aureus, Listeria monocytogenes, Pseudomonas aeruginosa, Salmonella typhimurium, Escherichia coli, and Mycobacterium avium. Furthermore, it can be observed that the levels of ceramides and sphingosines are altered as the infection process progresses in cystic fibrosis lungs. Corroborating the role of ceramides in lung infection, Petrache et al., evidenced that ceramides may trigger signaling pathways associated with the development of emphysema-like disease. Therefore, the study of

ceramides and other sphingolipids in SARS-CoV-2 infection is important, since the worsening of the disease can generate ARDS (PETRACHE, et al., 2011; SEITZ, et al., 2015).

Glycerolipids are a class of lipids, composed of esters and/or ethers of fatty acids with glycerol (Figure 3), and are the most important form of storage and transport of fatty acids in the body. According to the number of hydroxyl groups attached to the glycerol, these can be classified as monoacylglycerols, diacylglycerols (DGs), and triacylglycerols (TGs). For understanding cellular metabolism, it is important to know that diacylglycerol can be produced in different cellular compartments, and its cycle is dynamically balanced, depending on cellular energy storage and use. Fatty acids are a class containing monocarboxylic acids from long hydrocarbon chains and can be saturated, or unsaturated (HAN, 2016).

Figure 3 - Structure of 1,2,3-trihexadecanoyl-sn-glycerol, an example of a triacylglyceride



Information on cellular structure and levels are important for understanding the molecular mechanisms underlying metabolic syndrome (HAN, 2016). Table 1 describes some studies involving metabolites, including lipids, directly identified in biological samples positive for SARS-CoV-2 infection; as well as, the analytical technique used for such identification.

Table 1 - Relationship between metabolites and SARS-CoV-2 infection

Matrices	Metabolites	Instrumentation	Reference
Fluids (nasopharynx/oropharynx)	DG; PC; LPC; PS; glycerol-3-phosphate ; AA;	PSI-MS	(DE SILVA, NAYEK, *et al.*, 2020)
Plasma	DG; TG; PC; OS; TCA; FAA; CE; AA; CP; Cer; LPC; PI; LPO; LPA.	LC-MS	(WU, SHU, *et al.*, 2020)
Plasma	TG; HDL; VLDL; NLR.	Immunoassay	(KIMURA, SANT'ANNA, *et al.*, 2021)

DG:			
Plasma	PC; Vit. E; CE; LCP; LPE; PCe; PI; SM; FAs; PUFAs; Cer; DG; TG; PEp; CoQ10; MUFAs; GM3; GlcSph; SULF; ACar; S1P; LacCer.	LC-MS	(DEI CAS, OTTOLENGHI, *et al.*, 2021)
Plasma	S1P; FAA; AA; Arabic; malted; trehalose; S-adenosylmethionine; desaminothyrosine; ribose; xylitol ; Phe; Tyr; Arg; ornithine; cytokines.	GC-MS UHPLC-MS	(DANLOS, GRAJEDA-IGLESIAS, *et al.*, 2021)
Saliva	Val; Leu; Pro; Phe.	LC-MS	(FRAMPAS, LONGMAN, *et al.*, 2021)

diacylglycerols; **PC:** phosphatidylcholine; **LPC:** lysophosphatidylcholine; **PS:** phosphatidylserine; **AA:** aspartic acid; **PSI-MS:** mass spectrometry with ionization by a spray formed on a paper support; **TG:** triglycerols; **TCA:** tricarboxylic acid; **FAA**: free fatty acids; **CE:** cholesterol ester; **CP:** Carbamoyl phosphate; **Cer:** ceramides; **PI:** phosphatidylinositol; **LPO:** lipid peroxide; **LPA:** lysophosphatidic acid; **LC-MS:** liquid chromatography coupled to mass spectrometry; **HDL:** high-density lipoprotein; **VLDL:** very low-density lipoprotein; **NLR:** neutrophils and lymphocytes; **SM**: sphingomyelins; **Vit. E:** antioxidant vitamin E; **LPE:** lysophosphatidylethanolamine; **PCe**: ester-linked phosphatidylcholine; **PI:** phosphatidylinositol; **FAs:** saturated fatty acids; **PUFAs:** polyunsaturated fatty acids; **PEp:** vinyl-linked phosphatidylcholines; **CoQ10:** Coenzyme Q10; **MUFAs:** monounsaturated fatty acids; **GM3:** glaglisides; **GlcSph**: glycosphingolipids; **SULF:** sulfatides; **ACar:** acylcarnitines; **S1P:** sphingosine-1-phosphate; **LacCer:** lactosylceramides; **GC-MS:** gas chromatography coupled to mass spectrometry; **UHPLC-MS:** ultra-high-pressure liquid chromatography coupled to mass spectrometry; **DC:** diacetylspermidine; **Val:** valine; **Leu:** leucine; **Pro:** proline; **Phe:** phenylalanine. **Tyr:** tyrosine; **Arg:** arginine.

During the SARS-CoV-2 infection process, metabolic abnormalities involving mainly lipids were observed. This information may contribute to a better understanding of the physiology and pathology of the virus (Table 1). Another major contribution of these studies is attributed to the use of these molecules as biomarkers, enabling their use in the development of new rapid tests for diagnosis. The research of Silva et al., proposed a diagnostic test based on mass spectrometry with ionization through a spray formed on paper support (PSI-MS), for the detection of lipids and other low molecular weight molecules in samples of nasopharyngeal and oropharyngeal fluids from SARS-CoV-2 infected patients (SILVA et al., 2020).

Wu et al., (2020) reported a study in plasma samples where a total of 431 metabolites and 698 lipids were identified and quantified, which were responsible for classifying the SARS-CoV-2 infected samples. Among the lipids identified, fatty acids, diacylglycerols, and triacylglycerols were found in high concentrations in patients who died from COVID-19.

Lipids showed a characteristic and highly influential behavior in individuals infected with SARS-CoV-2, as shown in the study by Kimura et al., (2021). By analyzing plasma samples, the authors showed changes in lipid profiles, generating dyslipidemia, mainly by decreasing high-density lipoprotein cholesterol (HDL) and increasing the concentration of TGs according to disease severity. This same dyslipidemia was observed in the group of SARS-CoV-2 infected patients with Diabetes Mellitus, type II (DM2), or obesity, confirming that this alteration is not due to the existence of comorbidities. As reported

by the authors, the COVID-19 virus has a predilection for people with DM2 and this is a risk factor for the worsening of the disease. Parallel to this, according to Kimura, obesity favors the development of DM2 (KIMURA, et al., 2021).

Another recent study reported that patients with COVID-19 who have altered renal function exhibited high concentration levels of phosphatidylcholine, phosphatidylethanolamines, acylcarnitines, cholesterol, and sphingolipids. Concerning advancing inflammation, hypoxia , coagulation status and age, decreased sphingosine-1-phosphate, sphingomyelins, lysophosphatidylethanolamine, diacylglycerols, glycosphingolipids and phosphatidylcholine were found compared to SARS-CoV-2 infected patients without altered renal function (DEI CAS, et al., 2021). Comparing patients with COVID-19, Danlos et al., (2021) inferred that about 77 metabolites (amino acids, lipids, polyamines and sugars) were differentiated in the plasma of critically ill patients compared to patients with mild symptoms.

As can be seen in the studies presented here, the samples of plasma and nasopharyngeal and oropharyngeal fluids present very important molecular information, but the collection of these samples is extremely invasive, causing some discomfort to patients, which reduces the adherence of the population to the tests for pandemic control. Thus, an alternative would be to use saliva, a biofluid rich in information and easy to obtain. Recently, Frampas et al. (2021) performed a study of metabolites in saliva using liquid chromatography coupled with mass spectrometry (LC-MS). In this study, the authors employed partial least squares discriminant analysis (PLS-DA) with a sensitivity of 0.74 (95% confidence interval 0.60 -0.86) and specificity of 0.75 (0.55 - 0.89), enabling the separation of some metabolites essential for distinguishing between the groups of patients with high and low severity of COVID-19.

Microextraction of Lipids in Biological Matrices

Sample preparation is a fundamental step for the determination of lipids in biological matrices since these samples have a high content of endogenous compounds that may interfere with the analytical signal of systems such as chromatography and mass spectrometry. Thus, it is important to choose the right procedure for the extraction and/or separation of the target analyte, taking into account some characteristics, such as chemical stability, solubility and polarity. The most common sample preparation methods for lipid extraction in biological samples are single organic solvent extraction (SOSE), liquid-liquid extraction (LLE), liquid-liquid microextraction (LLME), solid phase extraction (SPE) and solid phase microextraction (SPME) (TEO, CHONG, et al., 2015).

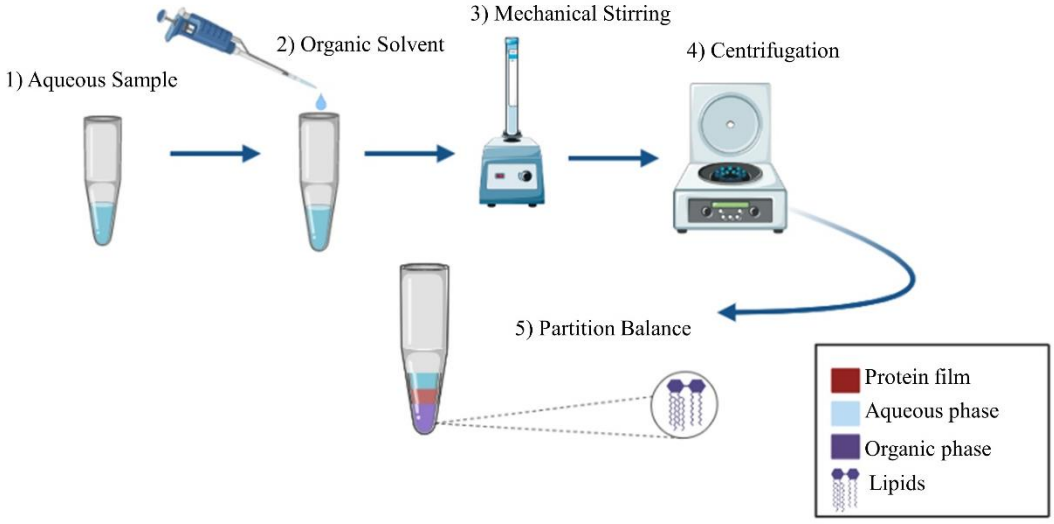
LLME consists of the addition of an immiscible extracting solvent to the sample, followed by mechanical stirring and centrifugation, which are required to increase the separation between the phases and the extraction efficiency of the method. This technique favors the extraction of several classes of lipids which include phospholipids, glycolipids, fatty acids, DGs and TGs.

The methodology developed by Folch et al., (1957) (chloroform/methanol/water 8:4:3 v/v/v), and subsequently modified by Bligh and Dyer (1959) (chloroform/methanol/water 1:2:0.8 v/v/v) has been by

far the most widely employed for the extraction of lipids in biological matrices. Different researchers have already performed adaptations of the method according to each objective, such as modifications in the solvent proportions, the addition of acid (acetic or hydrochloric), and even the replacement of chloroform by dichloromethane to minimize the toxic effect (MEDVEDOVICI; BACALUM E; DAVID, 2018).

Although the methodologies are adapted, the rationale involved in liquid-liquid microextractions (Figure 4) still prevails, being still an extraction based on the equilibrium between immiscible phases. The principle of the technique is based on the partition of a solute and/or analyte between two immiscible solvents, usually water (aqueous phase) and organic solvent (organic phase). In this case, the partition equilibrium is strongly determined by the physicochemical parameters of the two liquids and can be advantageously used to pre-concentrate the analytes of interest and eliminate endogenous compounds. The success of the technique is determined by the appropriate choice of the extracting solvent and the use of additives, such as pH adjustment, which strongly determines the solubility in water (MEDVEDOVICI; BACALUM E; DAVID, 2018).

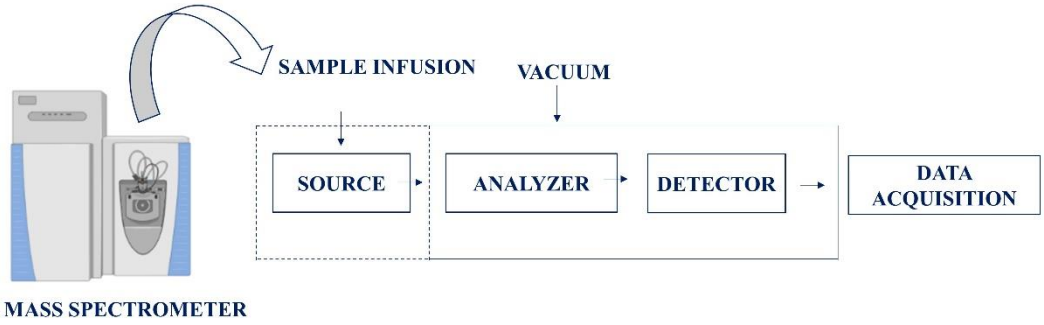
Figure 4 - Liquid-Liquid MicroExtraction of Lipids in Biological Matrices



3 MASS SPECTROMETRY: FUNDAMENTALS AND APPLICATIONS IN LIPIDOMICS

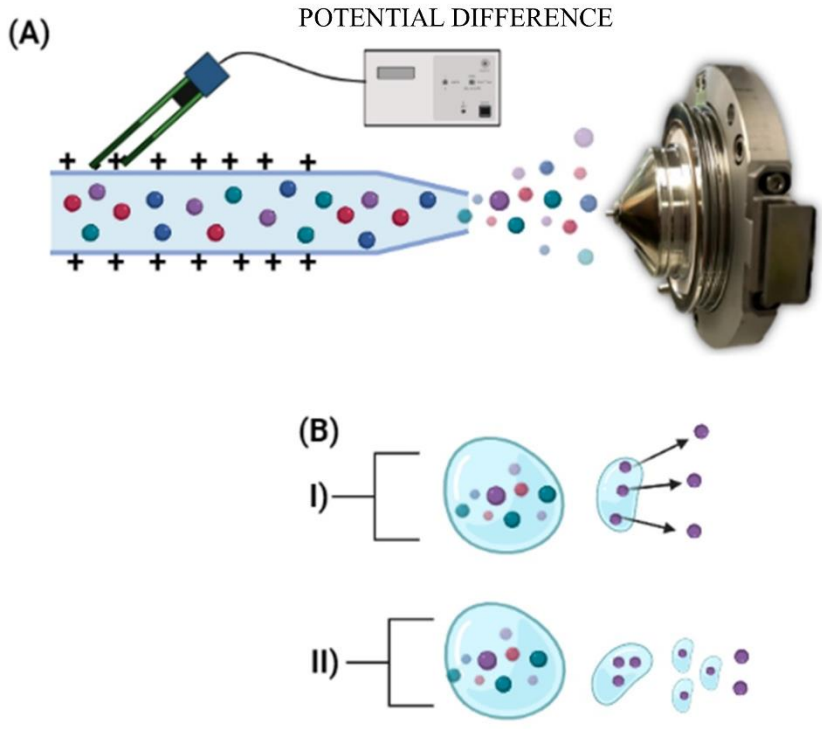
Several analytical techniques can be employed for the analysis of lipids in biological matrices after the extraction process, as presented in Table 1. However, mass spectrometry (MS) has stood out in this type of analysis because it provides valuable information about the structural composition and exact mass when it comes to high-resolution MS, of the lipids under study. The technique is based on the measurement of the mass-to-charge ratio (m/z) of charged species, and as a consequence identifies unknown compounds, quantifies known compounds, and elucidates the structure of molecules (LANÇAS, 2019). Figure 5 shows a scheme of the main components of the mass spectrometer.

Figure 5 - Main components of a mass spectrometer (LANÇAS, 2019)



The operation of a mass spectrometer is based on the introduction of the sample, which can be done by a chromatographic system, electrophoretic or direct infusion through a syringe pump. Then, the sample goes to the ionization source, which has the objective of ionizing the sample constituents, except electrospray ionization (ESI) and its analogous sources, since in this type of system the sample is previously ionized in solution and the ionization source acts as a nebulizer of the sample solution. In ESI, the sample solution is infused through a metallic capillary to which a potential difference is applied between the entrance of the mass spectrometer and the capillary. This potential difference promotes an electric field between the capillary and the entrance of the mass spectrometer, which provides the formation of an electrolytic spray of the sample solution. In this system, there is the use of auxiliary gases, sheath and drying to aid the nebulization and evaporation of the solvent (HOFFMANN, STROOBANT, 2007, KEBARLE, VERKCERK, 2009). Figure 6 shows a schematic representation of an ESI source.

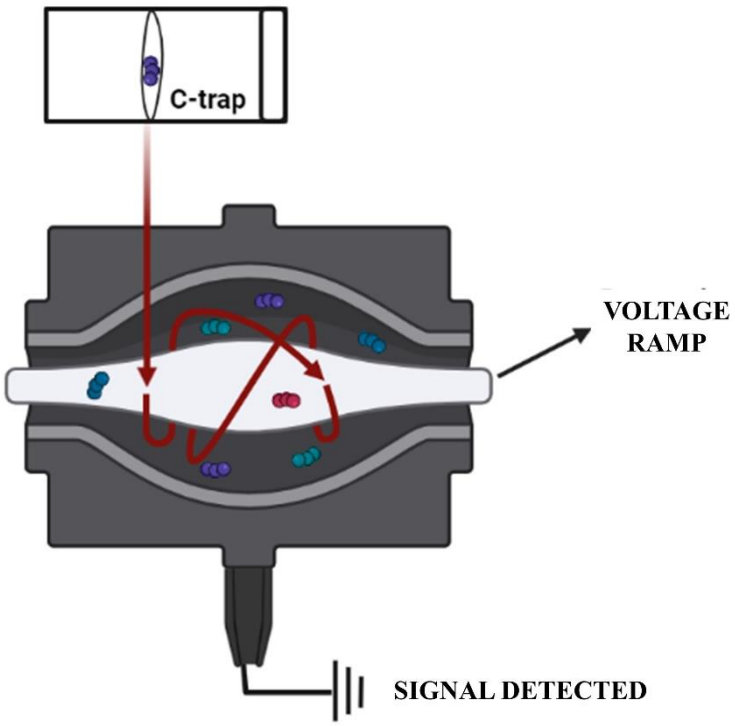
Figure 6 - (A) Representation of ions formed in the Electrospray process and (B) respective ion formation approaches: I) Ion evaporation model; II) Residual charge model



Currently, two mechanisms explain the formation of gas phase ions in ESI. The first was proposed by Dole (1968), known as the Residual Charge Model, which theorizes that as the solvent present in the droplets evaporates, the ionic density within the droplet increases, causing the coulombic repulsion to cause the droplet to crack, reducing its size. This process repeats until the point where only one ion remains per droplet, which finally has its solvent evaporated leaving only the ion in gas phase. The other model known as the Ion Evaporation Model, proposed by Iribarne and Thomson (1976), suggests that as the solvent in the droplets evaporates the ionic density and hence the coulombic repulsion increases, ejecting an ion into the gas phase. There is no consensus on which effect occurs mostly, but the most accepted is that both models occur simultaneously (KEBARLE, VERKCERK, 2009, LANÇAS, 2019).

After the process of ionization of the sample, it is forwarded to the analyzer which has as its main objective to discriminate the ions according to the m/z ratio. The analyzer must be able to distinguish minute mass differences and must allow the passage of a sufficient number of ions to produce ionic currents that are readily measured. One of the models of ultra-high resolution (mass accuracy < 5 ppm) analyzers is the commercially available Orbitrap (Figure 7) in 2005 based on Makarov's research (ZUBAREV, MAKAROV, 2013).

Figure 7 - Orbitrap ion analyser



This analyzer is preceded by a device called C-trap that accumulates and sends ion packets into the Orbitrap cell. The ions have their kinetic energies decreased inside the C-trap through collisions with the nitrogen gas, and when all ions reach equivalent energies, they are ejected tangentially into the analyzer being trapped around the central electrode that is under a high voltage. The electrostatic field, together with the high kinetic energy of the ions, causes the ions to initiate axial oscillations, thus preventing them from

colliding with the central electrode. Thus, ions with the same m/z ratio will oscillate at the same frequency and their image currents will be detected by sensors present on the external walls of the analyzer. These signals are converted into frequencies by Fourier transform and then converted into a mass spectrum (ZUBAREV, MAKAROV, 2013).

4 CONCLUSION

As illustrated in this chapter, it is possible to infer that lipids are important signals in inflammatory processes, and with the outbreak of the COVID-19 pandemic, several researchers have turned their research to better understand the metabolic pathways of lipids by SARS-CoV-2 infection. In contrast, most experiments performed were on invasive samples (plasma, nasopharyngeal and oropharyngeal fluids) which generate discomfort to patients during the sampling process. Bligh and Dyer extraction has been one of the most valuable sample preparation techniques, allowing the concentration of the highest number of lipid classes. The investigation of these lipid signals in saliva samples can be an important tool in the diagnosis and understanding of metabolic processes involved in COVID-19. Among the analytical techniques, mass spectrometry is extremely important, especially for the combination of ESI with the Orbitrap analyzer which brings results with high-resolution power for the structural elucidation of complex lipids.

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