FT-IR Spectroscopy Studies of Sphingolipids in Human Cells

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Sphingolipids have attracted significant attention due to their pivotal role in cellular functions and physiological diseases. A valuable tool for investigating the characteristics of sphingolipids can be represented via Fourier-transform infrared (FT-IR) spectroscopy, generally recognized as a very powerful technique that provides detailed biochemical information on the examined sample with the unique properties of sensitivity and accuracy.

sphingolipids sphingomyelin sphingosine ceramide human cells FT-IR spectroscopy

1. Introduction

Lipids are a heterogeneous organic group of biomolecules that are insoluble in water due to the hydrocarbon nature of a large part of their structure. Lipids have several essential biological functions: they represent structural components of membranes, they serve as storage and transport for energy-rich molecules, they act as a protective coating on the surface of many organisms, and they also participate in the recognition of specific characters. Some of them have significant biological activity, usually typified by vitamins and hormones. Although lipids are a distinct class of biomolecules, they are often bound with components of other biomolecules to form hybrid molecules, such as glycolipids containing carbohydrates, lipids, and lipoproteins, which contain lipids and proteins. In these biomolecules, their components' characteristic chemical and physical properties mix to perform specialized biological functions [1][2][3].

Recently, sphingolipids (SLs) received significant attention due to their multiple roles in cellular functions, such as cell migration and adhesion, formation of membrane domain, DNA damage response, senescence, aging autophagy, and apoptosis. SLs also have fundamental functions in cancer cell biology, diabetes, cardiovascular diseases, neurodevelopment, neurodegeneration, and in biological processes related to tissue and bones [4][5][6][7][8][9][10][11][12][13][14][15][16][17][18][19][20][21]. Given the relevance of this class of lipids, many different experimental techniques have been exploited for their characterization. Thin-layer chromatography (TLC), high-pressure liquid chromatography (HPLC), gas chromatography (GC), and capillary electrophoresis (CE) are the most primarily used techniques used to characterize the structural diversity of SLs [5][22][23]. These techniques have allowed for the development of a sphingolipidomics approach in which quantitative structural analysis of all SLs, or at least all members of a substantial subgroup, can be pursued in large samples [5][24][25][26].

Several studies about SLs used methodologies based on NMR and vibrational techniques, such as Raman and Fourier-transform infrared (FT-IR) spectroscopies [22]. This last technique represents a unique tool thanks to its sensitivity in acquiring information on biological samples [27][28][29][30][31][32][33] and the effects of their interactions with external agents [34][35][36][37]. In addition, FT-IR spectroscopy has been also adopted for differentiating between benign and malignant tumors in the colon [38][39], breast tissues [40][41], and prostate [42][43]. The analysis of biofluids (saliva, serum, urine, and blood) represent another interesting field of application of this technique [44][45], even though particular attention should be paid to the contribution of water content that can interfere with the signal coming from the examined samples [46][47].

As far as concerns lipids, great attention has been devoted to the acquisition of infrared spectra of lipids. as is evident from [22][48][49][50][51][52] and the references therein. The huge amount of work performed in this field has made available a certain number of databases dedicated to the FT-IR spectroscopy of lipids and similar compounds. In **Table 1**, the most cited ones are reported.

Table 1. Online databases with information on lipids and lipidomics.

Source	Url	Notes
Lipid Bank—Japanese Conference on the Biochemistry of Lipids (JCBL)	https://lipidbank.jp (accessed on 1 February 2023)	LipidBank is a free database of natural lipids including fatty acids, glycerolipids, SLs, steroids, and various vitamins.The database contains more than 6000 unique molecular structures, their lipid names, and spectral and literature information.
NIST Chemistry WebBook	https://webbook.nist.gov (accessed on 1 February 2023)	The NIST Chemistry WebBook provides access to: thermochemical data; IR spectra, mass spectra, UV/Vis spectra, and gas chromatography data. It is possible to search for data on specific compounds based on name, chemical formula, CAS registry number, molecular weight, chemical structure, or selected ion energetics and spectral properties.
Spectral Database for Organic Compounds (SDBS)	https://sdbs.db.aist.go.jp (accessed on 1 February 2023)	SDBS is an integrated spectral database system for organic compounds, which includes 6 different types of spectra: an electron impact mass spectrum (EI-MS), a Fourier-transform infrared spectrum (FT-IR), a 1H nuclear magnetic resonance (NMR) spectrum, a 13C NMR spectrum, a laser Raman spectrum, and an electron spin resonance (ESR) spectrum.

Given the potentialities of FT-IR spectroscopy in lipid characterization and quantification, many researchers have adopted FT-IR for investigating particular aspects of SLs and their role in different biological frameworks.

2. Basic Principles of FT-IR Spectroscopy

FT-IR spectroscopy analyzes the spectral infrared radiation absorbed by a sample. The energy of the absorbed infrared radiation equates the energy needed to transition between the vibrational states of functional groups. The radiation intensity absorbed by a sample produces the infrared absorption spectrum, in which there are peaks or bands related to a particular mode of the vibrations that is characteristic of functional groups in the sample [27][37]. The main component of a modern FT-IR spectroscopic apparatus is a Michelson interferometer (see **Figure 1** and [37] for further details), which substitutes the monochromator that was present in the old infrared spectrometers. FT-IR spectrometers enable the collection of absorption spectra quickly and precisely by obtaining qualitative and quantitative information on the structure of the compounds analyzed using modern software algorithms.

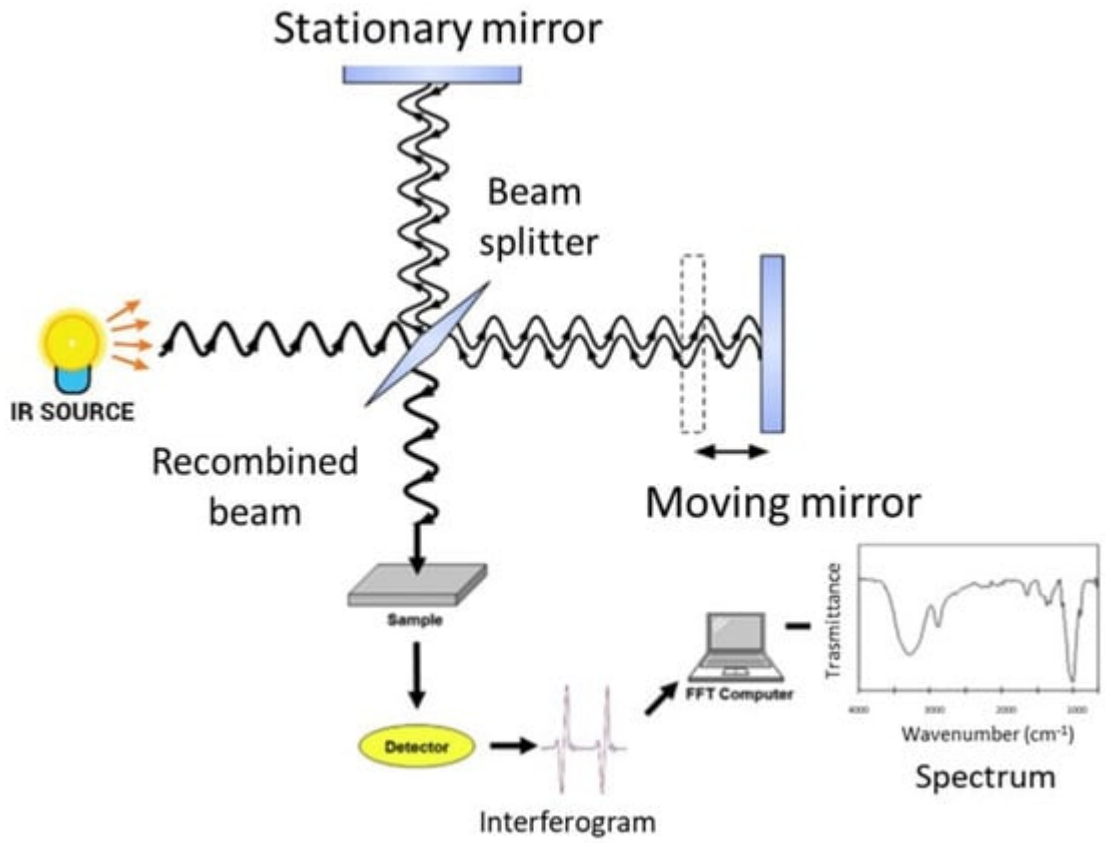


Figure 1. Schematic representation of an FT-IR spectrometer.

The sample under investigation is positioned in one of the two arms of the interferometer, the frequencies that are related to the excitation of vibrational states of the functional groups of the molecules of the sample are attenuated, and the obtained interferogram is Fourier transformed into an absorption spectrum in terms of the wavenumber, usually given in cm^{-1}.

FT-IR spectrometers offer many advantages compared to traditional infrared ones [28][29][30]. As previously said, the most relevant benefit is the ability to collect spectra in a short time. In fact, the detector can simultaneously analyze and record all frequencies and wavelengths. FT-IR spectroscopy also offers high precision and accuracy in the determination of wavenumbers thanks to the use of the monochromatic radiation of a laser source as an internal standard. Unwanted sample heating effects are excluded since the infrared source is sufficiently far from the

sample. Then FT-IR spectra can be obtained from samples at controlled temperatures using proper accessories [53]. The use of suitable cells also allows the acquisition of spectra from samples under different pressures [54]. Moreover, an FT-IR spectrometer can be equipped with a microscope to allow the analysis of samples or parts of samples at the microscopic level [55].

3. Experimental Aspects of FT-IR Spectroscopy

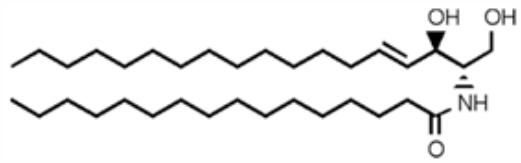
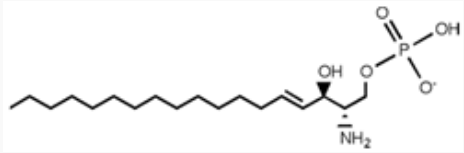
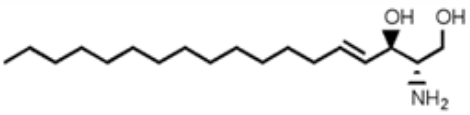
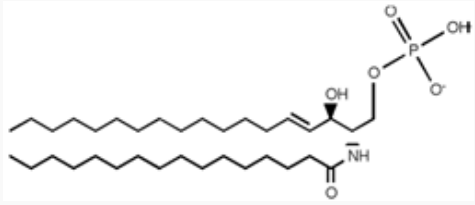
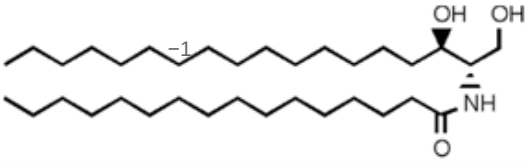
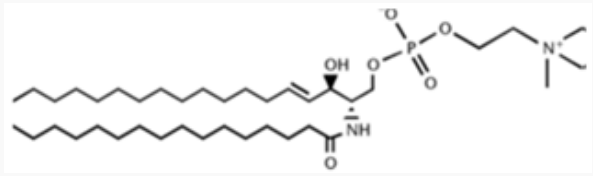
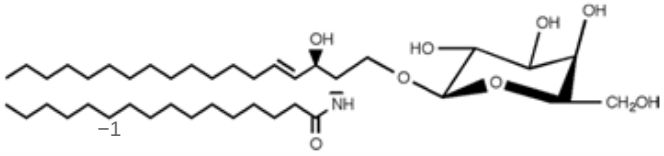
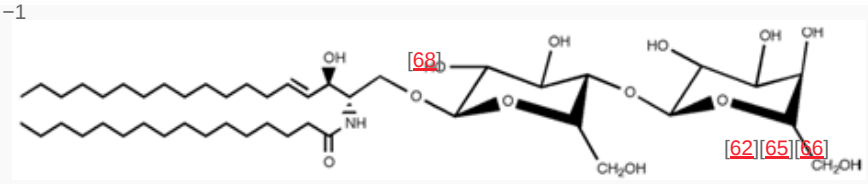
There are three main infrared spectroscopy sampling modes: transmission, reflectance, and attenuated total reflection (ATR), each with advantages and disadvantages [31][56]. For lipid analysis, the most largely used approaches are transmission and ATR modes. For transmission measurements, the samples are placed between two IR transparent windows. Generally, CaF_2, BaF_2, and KBr windows are used together with a proper sample holder in which the Teflon spacer can obtain the appropriate thickness, typically around 6–10 µm [57]. As an alternative, the samples can be included within a KBr matrix and pressed to form a pellet that can be subsequently analyzed after being dried [58].

The ATR approach is based on the presence of an evanescent wave at the reflecting interface between the sample and the crystal [59]. When a radiation beam penetrates inside a crystal, and the angle of incidence at the interface between the sample and crystal is greater than the critical angle, which is given by the refractive indices of the two surfaces, the beam penetrates a fraction of a wavelength beyond the reflecting surface, and when it comes into close contact with a material that selectively absorbs radiation, the beam loses energy at the wavelength where the material absorbs. The spectrometer measures and plots the attenuated radiation as a function of the wavelength, yielding the sample's absorption spectral characteristics. ATR employs crystal materials, such as diamond, ZnSe, and ZnS, in contact with the sample under investigation. A pressure plate is needed to ensure the connection between the samples and the crystal. Except for diamond objectives, excessive pressure can harm some crystals [60][61].

4. FT-IR Characterization of SLs

Before discussing the principal results reported in the literature about FT-IR studies on SLs, it is worthwhile to mention the fundamental paper of Fringeli and Gunthard [62] that provides the basis for all subsequent studies about lipids components in biological samples. Some other authors give relevant contributions in this field [59][63][64][65][66]. In **Table 2**, an almost exhaustive list of the peaks present in lipid samples is reported together with their assignments.

Table 2. Main sphingolipids present in human cells and their structures [21].

Sphingolipid Compound	Structure
Ceramide (Cer)	
Sphingosine 1-phosphate (S1P)	
Sphingosine (SP)	
Ceramide-1-phosphate (C1P)	
Dihydroceramide	
Sphingomyelin (SM)	 [67]
Galactosylceramide	
Lactosylceramide	 [68] [62][65][66]

Peaks Position (cm^{-1})	Assignments
892	C=C bending (fatty acid)
1050–1070	C-O-C stretching (nucleic acids and phospholipids)
1085–1090	PO^-_2 symmetric stretching (nucleic acids and phospholipid)

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Peaks Position (cm^{-1})	Assignments
1224–1240	PO^-_2 asymmetric stretching (nucleic acids and phospholipid)
1343	CH_2 wagging bending (phospholipid, fatty acid, and triglyceride)
1367	CH_3 symmetric bending (lipids)
1392–1400	CH_2 asymmetric bending, COO^- stretching (proteins and fatty acids)
1445–1470	CH_2 bending (mainly lipids and phospholipids, with little contribution from proteins)
1456–1467	CH_3 bending (lipids, cholesterol, and proteins)
1545–1549	N-H bending (lipids)
1660–1670	C=C stretching (lipids, fatty acids)
1730–1750	C=O stretching (fatty acid ester, triglycerides, and cholesterol esters)
2850–2865	CH_2 symmetric stretching (lipids, fatty acids)
2870–2874	CH_3 symmetric stretching (protein side chains, lipids, with some contribution from carbohydrates and nucleic acids)
2916–2925	CH_2 asymmetric stretching (mainly lipids, with little contribution from proteins, carbohydrates, and nucleic acids)
2956–2970	CH_3 asymmetric stretching (lipids, fatty acids, protein side chains, with some contribution from carbohydrates and nucleic acids)
3007–3015	C-H stretching (lipids, unsaturated fatty acids)

nctions; it would be very hard work to try to summarize all the number of contributions that can be found in the literature. For this reason, the following text presents a brief synopsis of some papers concerning SM, Cer, SP, and S1P that can contribute to evidence of the pivotal role of FT-IR spectroscopy in this field.

(a) Sphingomyelin (SM)

In **Table 4**, some significant papers on sphingomyelin are briefly summarized in chronological order. As far as concerns the class of investigated samples, commercial ones have been used in many cases. For some studies, lipids extracted from cells and tissues have been adopted. In these cases, Bligh and Dyer as well as Folch methods have been used [69][70]. Regarding the FT-IR geometry adopted for spectra collection, it is possible to notice that both transmission and ATR approaches have been used. For transmission measurements, in a large number of cases, CaF_2 windows have been used even though KBr pellets have shown also to be useful.

Table 4. Summary of some relevant results obtained on SM by using different FT-IR spectroscopy approaches.

References	Lipid Extraction Method/Sample Details	Spectra Collection Geometry	Aim	Main Findings
[71]	Commercial samples	Transmission geometry using CaF_2 windows	To investigate the molecular interactions between SM and PC in phospholipid vesicles.	The changes in the acyl chains and SM, conformation induced by PC are observed.
[72]	Commercial samples	Transmission geometry using CaF_2 windows	To study the effects of temperature and pressure on structural and conformational properties of PC/SM/cholesterol model raft mixtures.	The conformational properties of the lipid systems are monitored by examining the positions and intensities of infrared absorption bands.
[73][74]	Rat brain tissue samples	Transmission geometry using CaF_2 windows	To examine the spatial distribution of molecular changes associated with C6 glioma progression.	The concentrations of SM, nucleic acids, PS, and glucocerebroside are significantly affected during C6 glioma development.
[75]	Lipids extracted from brain tissues using Folch and Bligh and Dyer methods.	Transmission KBr pellets	To analyze the lipid extracts from the brain to identify their composition.	Lipid content can be evaluated via FT-IR spectroscopy, which may improve the differential diagnosis of brain cancers.
[76][77]	Commercial samples and lipids extracted from PC 3 cells using Bligh and Dyer method.	ATR	To analyze the changes in the lipidome of prostate cancer PC-3 cells after exposure to sub-lethal ouabain levels.	Lipid alterations induced by ouabain can be identified by variations in the ester/choline/phosphate ratios in FT-IR spectra.
[68]	Commercial samples and lipids extracted from PC-3 cells using Bligh and Dyer method.	Micro-ATR	To develop PLS models based on FT-IR spectra to determine the changes in the amounts of different lipids in extracts from PC-3 cells treated with four antitumor drugs.	After treatments with anticancer drugs, the spectral region of the polar headgroups of samples did not show any noticeable alterations. However, the developed PLS models can be used for high-throughput measurements.
[78]	Commercial samples	ATR	To investigate the changes occurring in detergent-resistant membranes (DRM) extracted from human breast cancer cells	FT-IR spectroscopy and multivariate analysis enable to monitor of the changes in the composition of DRMs. This approach can be useful for the

References	Lipid Extraction Method/Sample Details	Spectra Collection Geometry	Aim	Main Findings
			when treated with the omega-3 fatty acid docosahexaenoic acid.	label-free characterization of lipid components in cells.
[79]	Commercial samples	Transmission geometry using CaF_2 windows	To examine the interaction profile of carboplatin at varying concentrations with SM multilamellar vesicles.	Carboplatin affects the phase transition, enthalpy, the cooperativity parameter, the phase transition temperature, the lipid order, the lipid fluidity, and the hydrogen state of specific groups in hydrophilic parts of the examined samples.

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demonstrated that the gauche ratio to all-trans is not affected by SM and PC interaction. The authors also examined the characteristics of the C=O stretching band of PC located at 1735 cm^{-1}, evidencing that the phosphate group of SM takes part in hydrogen bonding between the molecules of SM and possibly PC. Particular attention was also given to the Amide I contribution at 1635 cm^{-1} and to bands related to the phosphate group and located at 1220 and 1080 cm^{-1} [71].

Dreissig et al. investigated the different SLs and phospholipids that are present in lipids extracted from the porcine brain. They presented the FT-IR spectra of some SLs, phospholipids, and neutral lipids. If the SM spectrum is considered in detail, it is possible to recognize the contribution of different vibration modes reported in **Table 3** and in **Table 2** of [75]. In the high wavenumber region related to hydrocarbon chains, peaks related to CH_2 asymmetric and symmetric stretching are positioned at 2924 and 2852 cm^{-1}, respectively. In the spectral region related to the lipid polar headgroup, contributions attributed to C=O stretching (1647 cm^{-1}), N-H bending (1545 cm^{-1}), CH_2 bending (1466 cm^{-1}), CH_3 symmetric bending (1378 cm^{-1}), PO^-_2 asymmetric stretching (1240 cm^{-1}), PO^-_2 symmetric stretching (1090 cm^{-1}), and C-O-C stretching (1055 cm^{-1}) can be noticed. In [75], the authors examined the difference in band position and intensities in the spectra of the different commercial samples. They also prepared lipid mixtures to train and validate a quantification model for determining the composition of brain lipid extracts. The results of the PLS regression of FT-IR spectra were successfully related to the lipid quantification obtained by using TLC. Even though the FT-IR spectroscopic approach presented in this paper cannot be generally used for the quantitative analysis of all lipids, it might contribute to the diagnosis of brain tumors by evaluating the changes occurring in the lipid composition of tumor cells [75].

The papers of Gasper et al. [76][77] represent a typical example of the use of FT-IR spectroscopy for studying the effects of drugs on SLs, a topic largely investigated by using this vibrational technique. In the present case, the authors aim to examine the number of changes in lipids in PC-3 cells exposed to sub-lethal levels of ouabain, a well-known cardiotonic steroid that demonstrates an anti-cancer activity in vitro and in vivo that indicates the possibility of adopting these compounds as chemotherapeutic agents in oncology. The authors examined the different spectra obtained between the average spectra for lipid extracts related to different experimental conditions. In [76], three curves are reported: the first is related to the changes occurring in the first 6 h after the

treatment, the second is related to the changes between 24 and 6 h of treatment and the last one concerns the changes between 36 and 24 h of treatment. The most relevant changes for lipids are present between 6 and 24 h. Principal component analysis (PCA) was also performed for evaluating the differences among the FT-IR spectra related to different experimental conditions. According to the findings of [76][77], FT-IR spectroscopy not only detects cellular changes caused by ouabain but also qualitatively assesses the evolution of these alterations during therapy. Ouabain's overall biological effect on PC-3 cell lipids increased SM and decreased PC [76][77].

A particularly interesting aspect of FT-IR spectroscopy is related to the possibility of its use for the quantitative determination of a single component in a mixture. This possibility has been evidenced by the paper of Derenne et al. [68], which developed PLS algorithms for quantifying lipids in complex mixtures of lipids. The lipid composition obtained via FT-IR spectroscopy was first validated by using HPLC and used for determining the lipid composition of lipids extracted from cells exposed to four different drugs. These treatments did not cause significant changes in the polar headgroups' spectral region. However, the authors suggested that their PLS models appear to be reliable and can be helpful for routine analysis [68].

(b) Ceramide (Cer)

Moore et al. [80] investigated the conformational order and phase behavior of hydrated Cers to provide the first complete investigation of the intermolecular and intramolecular chain and headgroup interactions in hydrated non-hydroxy fatty acid (NFA) and hydroxy fatty acid (HFA) Cers. In particular, the authors examined the temperature dependence of the methylene stretching, scissoring, and rocking mode frequencies. This study indicates the occurrence of two relevant phase transitions, one around 60 °C and the other around 80 °C. The behavior of the Amide I and II modes shows different contributions from NFA and HFA Cers. The results reported in this paper were also of relevance to the domain mosaic model of the stratum corneum lipid barrier that was proposed in the seventies of the last century.

Another paper addressing the analysis of the conformational order of Cer by using FT-IR spectroscopy is reported in [81]. In addition, these authors investigated the dependence on temperature of the conformational stability of Cers. They studied different Cers and mixtures and polar–nonpolar lipid interactions. Their study evidenced the impact of the polar headgroup on the conformation of the hydrocarbon chains when the temperature increases, with the role of the endogenous molecules also being more prominent.

More recently, de Arada et al. [82] used FT-IR spectroscopy to examine the interactions of polar headgroups from Cers and SM. These authors studied four regions of the FT-IR spectra of these compounds: C-H stretching and CH_2 scissoring vibrations, the Amide I region, and the phosphate vibration range. The study of the two latter areas was the novelty of the de Arata et al. paper. The temperature dependence of hydrated samples of pure SM and SM–Cer mixtures was examined and compared with the results of differential scanning calorimetry measurements. The data from the hydrocarbon chain region show a transition in agreement with previous observations. The results related to the Amide I region are more interesting since they evidence that SM and Cer carbonyl groups strongly interact, probably through H bonds. Furthermore, the results pertaining to the phosphate group suggest a relevant

role of H bonds in the interaction between SM and Cer. The results of [82] evidence that SM and Cer can have an interaction through their polar headgroups that is differently from what occurs to other lipids.

(c) Sphingosine (SP) and sphingosine 1-phosphate (S1P)

The contribution reported in [83] is related to the joint use of differential scanning thermometry and FT-IR spectroscopy for investigating the effect of SP and stearylamine on the interaction of SP with calcium. Additionally, in this case, spectroscopic observations confirm the phase transition observed by using differential scanning thermometry. The inspection of the ester C=O stretching mode appearing as a broad band at 1734 cm^{-1} suggests that the amino bases do not introduce hydrogen bonding with the C=O group and do not modify its hydration state. The same evidence is obtained by considering the band related to the phosphate group located at 1220 cm^{-1}. The degree of dehydration of the phosphate group can also be quantitatively determined by using a partial least-squares multivariate statistical analysis. A further study from the same research group [84] is devoted to the use of FT-IR for quantitative analysis of the dehydration process of the phosphatidylserine phosphate group in the presence of Ca^{2+} caused by various molecules, such as diacylglycerol, SP, and stearylarnine, by adopting a partial least-squares statistical method. FT-IR can also be used for estimating the apparent pKa of lipid carboxyl groups. The absorbance signals given by the protonated and the unprotonated forms of the specific group under investigation can be determined. In so doing, it is possible to evidence that diacylglycerol increases the dehydration of the phosphate group due to Ca^{2+} while the amino-bases SP and stearylamine avoid the dehydration of the phosphate group. The paper of Derenne et al. [68] that has been cited in the previous table also gives interesting results for SP and S1P.

(d) FT-IR Lipidomic studies involving sphingolipids

It is worth noting that, recently, great attention has been devoted to two aspects of FT-IR spectroscopy technology to broaden its field of application further. The former of these aspects are related to the development of experimental approaches allowing the characterization of a considerable number of samples by developing a high-throughput approach [85][86][87]. The second concerns the “omics” aspect. Usually, this term is used for indicating the analysis of genes (genomics), proteins (proteomics), and metabolites (metabolomics), but also the analysis of all lipids and the molecules with which they interact, and their function within biological systems assume a particular relevance since lipidomics represent one of the most critical aspects of modern analytical biochemistry. The most general techniques used in lipidomics are the techniques of TLC, HPLC, GC, and CE mentioned in the Introduction; however, also in this framework, FT-IR spectroscopy is increasingly appreciated [22][88][89]. Considering sphingolipids, Ramos-Garcia et al. [25] showed that FT-IR analysis could provide a qualitative and quantitative biochemical characterization of isolated exosomes, allowing for a fast and direct quantification of the total lipid content and evidencing sphingolipid contribution. Another contribution in this field has been given by Guleken et al. that individuated changes in sphingolipid metabolism in the blood serum of endometriosis-affected patients [24][26].

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