Lipid Geometrical Isomerism: From Chemistry to Biology and Diagnostics

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1. INTRODUCTION

Lipid research in the life sciences has renewed its attractiveness in the last two decades or so, going from chemical, analytical, and biological subjects, to making important connections with nutrition, and with increasing societal relevance of health conditions related to lipid metabolism. Lipidomics, the discipline regarding chemical and metabolic lipid fates in living organisms, has brought about a successful innovation to lipid research, being one aspect of the scientific response to the need for understanding this part of biology, and offering a platform for gathering the studies of lipid changes in living organisms in a comprehensive and dynamic manner. Fatty acid-containing structures, such as triglycerides, phospholipids, and cholesteryl esters, are included in this reevaluation, adding many significant roles and functions to the canonical structural and biological activities formerly assigned to them. New insights have been obtained such that lipids can no longer be simply considered containers for cell activities or circulating molecules in plasma, but their reactivity and transformations make them critical players in biology, health, and diseases.1,2

The structures of fatty acids typically comprise a carboxylic acid function connected to a long hydrocarbon chain (up to 26 carbon atoms), which can be saturated or unsaturated with up to six double bonds. Examples of mono- and polyunsaturated fatty acid (MUFA and PUFA) structures are shown in Figure 1, with their common names, the abbreviations describing the position and geometry of the double bonds (e.g., 9cis or 9trans), as well as the notation of the carbon chain length and total number of unsaturations (e.g., C18:1). The geometry of the double bonds in naturally occurring fatty-acid-containing lipids of eukaryotes is exclusively cis as the key feature of the homeoviscous adaptation, because membrane homeostasis is obtained by a precise balance between saturated and cis-unsaturated structures. Because of its essentiality in living organisms, the cis geometry is strictly controlled by the
regioselective and stereoselective enzymatic activity of desaturases during MUFA and PUFA biosynthesis.\(^3\)

From the chemical point of view, unsaturated fatty acids are involved in oxidative and free radical processes, which also naturally occur during metabolic functions and signaling activities. The transformations due to peroxidation have been considered for many decades to be by far the most crucial events to natural lipids; they are involved in the chemical and biochemical fate of MUFAs and PUFAs, in signaling responses as well as in aging and health impairments at all tissue levels.\(^4\) In the past decade, the cis double bond geometry of MUFA and PUFA structures has received attention, pointing out that the less thermodynamically stable structure (i.e., the cis isomer) is the most represented in natural lipids, and its involvement in electrophilic free radical reactivity can produce a reversible addition with consequent isomerization and loss of the starting natural geometry.\(^5,6\)

Lipid geometry is an interdisciplinary research field, which involves chemical and mechanistic studies under biomimetic conditions, together with biophysical, biochemical, biological, and medical aspects. This Review will summarize recent research on these topics, starting from the analytical aspects treated in section 2, due to the fact that the performance of analytical procedures has been the first issue leading to the trans lipid discovery in all fields. Trans lipids can also be introduced into the body by the diet, and section 3 deals with “exogenous” sources such as industrial trans fatty acids (TFAs), well-known for their effects on health.\(^7\) Another exogenous source for humans is treated in section 4.1, that is, the microbial biohydrogenation that occurs in the stomach of ruminants and leads to some fatty acid isomers in dairy products and meat.\(^8\) The bacterial cis–trans isomerization treated in section 4.2 is instead considered an “endogenous” transformation occurring in Gram negative strains as a short-term adaptation response of these species to environmental toxicity or increased temperatures.\(^9\)

The free radical reactivity in different lipid molecules with formation of trans MUFAs and PUFAs is considered in section 5 as a chemical reaction in homogeneous solutions. This part describes the chemical, mechanistic, and synthetic aspects of the lipid cis–trans isomerization, highlighting the modes of different sulfur-containing substrates to generate thiol radicals (RS•), as well as discussing nitrogen dioxide (NO2•) as a possible isomerizing agent. In section 6.1, the lipid geometrical isomerization is carried out in aqueous systems, exploring the behavior of fatty acids and phospholipids in organized supramolecular structures, such as micelles and liposomes, respectively. These organized assemblies are mimics of well-known biological structures, such as circulating lipids and cell membranes, and free radical reactivity can be studied under more biologically relevant conditions using these systems. Section 6.2 describes how the model studies carried out in biomimetic radical chemistry became crucial for the development of an integrated vision of protein–lipid damages. By these studies, the trans lipid modifications could be correlated to a wider scenario of biological stress involving other biomolecules, such as sulfur-containing peptides and proteins, as well as small diffusible thiol radicals generated by the degradation of methionine, H2S, and metal–sulfur clusters. Section 7 focuses attention on the membrane models, which provided the most representative examples of the profound influence of lipid geometry on biological functions. Section 8 describes data obtained so far on trans lipids used in diagnostics, in relation to conditions where trans lipids cannot occur by the diet, and some health conditions, indicating the importance of expanding knowledge in this field. This Review aims at giving readers an overview of lipid geometry in an interdisciplinary fashion and connected to free radical production under natural or stress conditions, underlining the need for preventive and repair strategies for protecting the natural cis geometry so important for membrane functions and signaling.

2. ANALYTICAL RECOGNITION OF TRANS LIPIDS

The resolution of trans isomers, as positional and geometrical isomers, is a significant research challenge because it can offer several complications even with sophisticated mass spectrometry facilities. This section deals with trans lipid analysis, particularly in view of setting analytical protocols for complex biological samples and diagnostics. Although the field of lipid analysis has involved decades of research with the development of an array of methodologies to address the recognition of different lipids, the state-of-the-art of cis and trans fatty acid resolution still involves intense research into finding efficient analytical methods suitable for biological samples and biomarker discovery. An array of methodologies such as NMR, HPLC, LC/MS, GC, and GC/MS need to be effectively complemented to reach the optimal discrimination among different types of trans isomers. In most cases, these methods require preliminary derivatization steps to have fatty acid esters in hand. On the other hand, with IR and Raman spectroscopies, samples can be directly examined, but in these methods the resolution for each trans structure cannot be obtained, only the total trans content. The diversity of natural lipids offers an enormous analytical challenge, with the overall goal being the resolution of different types of fatty acids and the quantification of single isomers present in biological samples. In this respect, lipidomics aims at setting up an integrated analytical platform for the rapid and reliable detection and quantification of fatty acid isomers.
for reaching satisfactory and successful results, especially for applications in molecular diagnostics and biomarker discovery.

In this section, the latest advances in each methodology will be described, whereas more detailed aspects can be found in published reviews and books on these subjects.10–12

2.1. GC, GC/MS, and MS

Gas chromatography (GC) is the easiest and fastest technique for FA analysis.12,13 It is still the only technique that can separate positional and geometrical isomers of different FAs.14 using protocols that clearly avoid the overlaps between cis and trans isomers of different MUFA and PUFA. In fact, especially in complex mixtures (e.g., cell membranes or food lipid analysis containing cis, trans, and conjugated FA) can possible superimposition of peaks occur.

GC analysis requires the fatty acid-containing lipids to be converted to more volatile compounds, such as methyl ester (FAME) or other ester derivatives.15–17 FAMEs are easy to produce, and reviews have already reported and discussed different reaction conditions.12,13 Efficient methods exist for converting phospholipids, triglycerides, and cholesterol esters to FAMEs under base-catalyzed conditions. The base and other reaction parameters have to be appropriately chosen to have quantitative results. Although frequently used, acidic transesterification can afford byproducts, such as oxidized derivatives and positional isomers, especially if strong acid and high temperatures are at work.15 The transesterification protocols have been collected and discussed elsewhere.16 As regards mass spectrometric analysis, the most commonly used fatty acid derivatives are FAMEs, as well as the nitrogen-containing esters (i.e., 3-pyridylcarbinol (‘picolinyl’) esters, 4,4-dimethyloxazoline derivatives, and pyrrolidines).18

The trans-octadecenoic acids present in both ruminant fats and partially hydrogenated vegetable oils are quantitatively and nutritionally the most important group of trans fatty acids in the human diet.19 In this Review, we do not focus on their GC separation, which is well described in other publications, covering the screening of different operative conditions and all possible overlapping problems.11,20 Below, the most relevant achievements of the cis–trans isomer resolution give particular emphasis to the PUFA isomers.

For separation of all of the FAME components in a mixture, the choice of the chromatographic column and the carrier gas play a crucial role. For GC analysis of fatty acid isomers, fused silica capillary columns of 60–100 m length must be used, coated with highly polar cyanopolysiloxane stationary phases containing various polar substituents. They work by separation of fatty acid derivatives, which elute according to increasing carbon atom chain (e.g., retention time: C14 < C16 < C18 < C20 < C22) and number of double bonds (e.g., 0 < 1 < 2, etc.), trans isomers always being eluted before the cis with the exception of the C20–C22 PUFA. In contrast, GC columns coated with nonpolar or moderately polar phases give a completely different trend of separation.21,22 For the PUFA isomer recognition, it is strongly recommended to use a reference library, to identify the position of each isomer before performing analysis of complex biological samples. This approach includes the development of synthetic methods for isomers that are not commercially available. By this strategy, the EPA Methyl Ester (EPA-ME) library and their mono-trans isomers were created, and the elution proceeded as follows: 17trans < 14trans < 5trans = all-cis < 8trans < 11trans.23 In Figure 2 is shown an optimal separation between different omega-3 PUFA, such as all-cis C20:3, C20:4, and C20:5 fatty acids, the EPA mono-trans isomer (17trans), and the analysis of a commercial sample of deodorized tuna oil (trace I).23,24

As far as the carrier gas is concerned, helium and hydrogen are both used for gas chromatography. Although they show similar performances in the separation of different fatty acids as well as the separation of cis and trans isomers, hydrogen presents some advantages that make it the gas of choice. In fact, GC run time is shortened and the peaks are sharper with respect to helium.20 Because it gives a better resolution of peaks in the C18:1 and the C18:3-C20:1 regions, hydrogen is used for the routine analysis of vegetable and animal fats and oils.11,14 The good separation of EPA-ME from its 17trans EPA isomer and C20:3 ω-3 using hydrogen gas as carrier (Figure 2) allowed the determination of the presence of trans isomers incorporated after a diet with deodorized tuna oil in rat liver mitochondrial membranes.23 As previously mentioned, preparation of the trans MUFA and PUFA fatty acid standards can be performed by free radical synthetic methods to obtain exclusively geometrical isomers. Mono-trans arachidonate isomers provided the first example of the validity of this approach. Samples of human urine and platelets treated with a chemical reaction with nitrogen dioxide (NO2•24 showed the formation of mono-trans arachidonate, but in this work the analysis was poorly resolved.25–27 Further investigation by amelioration of GC analytical conditions, and by use of the synthetic approach with free radical methodology, provided the possibility of assessing a reliable protocol for recognition of arachidonate isomers.28 Efforts toward identification of these isomers in human blood plasma were carried out by comparison of different GC conditions.22

The assignment of the geometrical mono-trans arachidonate isomers is specifically connected to the geometry and position of the four double bonds present in the structure, which are important for allowing their implementation as biochemical markers of an in vivo isomerization process by radical stress conditions. In fact, the mono-trans arachidonate isomers in the

Figure 2. GC region relative to the elution of the omega-3 FAME present in deodorized tuna oil (trace I) using hydrogen as the carrier gas. The peak assignment was obtained due to the commercial availability of pure references of all-cis omega-3 fatty acids C20:5 (trace II, peak d), C20:4 (trace III, peak c), and C20:3 (trace V, peak a) and the synthesis of 17trans isomer of EPA (trace IV, peak b).23
5 and 8 positions (see Figure 1) have been identified as the most favored products of a free radical isomerization process occurring in phospholipid structures, with an interesting discrimination of the trans isomers in the 11 and 14 positions that may come from the industrially isomerized fats of the diet. It is worth recalling that desaturase enzymes operate in eukaryotic cells by a chemo- and regio-specific mechanism, forming exclusively the cis geometry in specific positions of the fatty acid chain during lipid biosynthesis. Therefore, the formation of the Strans and Strans isomers can be reasonably assigned only to the conversion of naturally occurring cis geometry by the action of endogenous radical stress. This highlights the importance of the unambiguous determination of arachidonate isomers in the biological samples.

GC/MS can be considered as the “gold standard” technique for FA analysis. In fact, because mass spectrometry, which is a powerful tool for lipidomic analysis, is not able to distinguish cis and trans geometrical isomers per se, the coupling with GC allows for a preliminary peak separation followed by recognition based on molecular mass.

For the sake of the preciseness, it must be said that in a few cases MS of fatty acid methyl esters can provide some structural information for the position and the geometry of double bonds, and different techniques have been exploited. A limiting issue with geometrical isomers is the fact that the ion pattern shows similar characteristics, whereas recognition is mainly based on comparison of the relative abundance of specific ions. This analysis can be used exclusively with monoenoic and isolated dienes, where the number of geometrical isomers is 2 and 4, respectively. For PUFA with more than 2 double bonds, the analysis presents some drawbacks due to the high number of possible geometrical isomers. Analyses of bacterial lipids demonstrated the usefulness of a derivatization procedure to study positional and geometrical isomers of MUFA. This consists of two steps: (i) reaction with dimethyl disulfide (DMDS), which adds two CH₃S moieties to the double bond, and (ii) MS analysis and examination of the fragmentation patterns. This allows the splitting of the DMDS adduct and the localization of the two fragments, thus furnishing unquestionable information on the original position of the double bond.

As far as the use of mass spectrometric features is concerned, an interesting paper reported the ability of EI using high electron energy (70 eV) ionization to identify the geometry of the central double bond in trienes and tetraenes of FAMEs. In this work, they also observed a slight variation between the cis and trans geometries of the terminal double bonds of trienes, identifying the ω-3, ω-6, and ω-9 positions from the intensity of peaks at m/z 108, 150, and 192, respectively.

More recently, a complete discrimination of the α-linolenic geometrical isomers using a low electron energy (30 eV) spectrometer was also reported. The geometry of the central double bond was ascertained by the recognition of the base peak in the mass spectrum: cis geometry gives rise to a base peak at m/z 79, while trans has a peak at m/z 95, which is more pronounced than in the cis isomer spectrum. Obviously, these small peak intensity differences can become useless in complex lipid mixtures such as those of biological samples.

A new tool is gaining attention for revealing the class distribution of complex lipids in tissues through imaging techniques, matrix-assisted laser desorption/ionization imaging mass spectrometry (MALDI IMS). Different lipid classes can be distinguished by their different masses, and a future goal will be to improve this technique for distinguishing also geometrical and positional isomers.

### 2.2. Argentation Techniques

Since their discovery in 1962, argentation techniques have been one of the most important tools for lipid analysis, allowing the separation of different lipid classes based on the unsaturation degree of molecules. The technique is based on the chemical ability of silver ions to form weak charge-transfer complexes with electron-rich olefinic double bonds. These complexes are stronger with cis than trans double bonds, allowing their separation by thin-layer (TLC) and high-performance liquid (HPLC) chromatography.

Silver-thin layer chromatography (Ag-TLC) is a well-known technique that fractionates FAMEs based upon the number and the configuration of the double bonds. Thus, SFA, MUFA, and PUFA, as well as cis and trans isomers, can be separated on a silica gel plate impregnated with a silver ion solution. Although Ag-TLC is considered a time-consuming technique with a long and tricky procedure, it has the great advantage of being inexpensive and highly reproducible in any laboratory. The Ag-TLC plates are easily obtained by dipping the silica TLC glass-plates face-down in a 5% solution of silver nitrate in acetonitrile. The cis isomer forms a strong complex with silver ions so it is retained to a greater extent by silica gel (lower Rₜ), while the weaker complex between trans isomer and silver ions produces less retention of the substrate from silica gel (higher Rₜ). This technique is useful for the separation of different fractions of PUFA isomers, where cis isomers can be separated from mono-trans, di-trans isomers, and so on, depending on the number of double bonds. A successful example of separation is given by of the mono-trans isomer fraction of AA and EPA, each containing 4 and 5 regioisomers, respectively. It is worth noting that it is not possible to further separate each regioisomer contained in the mono-trans fraction.

On the basis of the different molecular shapes of cis and trans isomers and the possibility of Ag complexation, a silver-exchanged zeolite Y material was created combining these two effects for differential elution of mixtures of geometrical isomers.

### 2.3. HPLC and LC/MS

HPLC methodologies using silver-ion stationary phases have been developed to separate cis and trans geometrical isomers of FA. Currently, drawbacks still include the low reproducibility and the contamination by traces of silver salts into the fraction, which are not easy to separate. Separation of FAMEs is described by small cartridge columns packed with a bonded benzene sulfonate medium that can be loaded with silver ions. Separation is obtained according not only to the geometrical configuration of the unsaturated bond but also to the degree of unsaturation. For food analysis purposes, such columns are reported for separating cis from trans monoenoic FAMEs.

Reversed-phase high performance liquid chromatography (RP-HPLC) was also proposed for the separation of cis–trans long-chain monounsaturated fatty acids. However, it presents several problems, such as the multiple use of HPLC columns and/or a HPLC gradient with polar organic solvents and water. In general, the retention time for fatty acids increases with an increasing number of carbon atoms of the fatty acid, and decreases with an increasing number of double bonds in its structure. The separation obtained in RP-HPLC has the advantage of using the lipid extracts directly...
without a derivatization procedure, as needed for GC analysis. Therefore, this is an active field in analytical lipid research. A tandem methodology with RP-HPLC separation coupled with derivatization and GC analysis should provide an effective tool for the examination of biological samples.

Reversed-phase liquid chromatography coupled with mass spectrometry (RS-LC/MS) is another robust analytical tool implemented for lipidomics. In 2012, the first contribution concerning the separation of cis/trans phospholipid isomers was reported. Previously, other studies had reported the use of LC/MS—GC and Ag-HPLC for analyzing hydrolyzed fatty acids, but none of them was applicable to intact lipid extracts. RP-LC possesses the great advantage of being able to separate lipids based on the polarity of the headgroup and the hydrophobicity of the carbon side chain. Particularly the latter property is responsible for the separation of saturated and unsaturated lipids. In fact, the chemical and physical properties of the whole molecule change due to the presence of a bend and the corresponding decrease in flexibility that comes about from a cis unsaturation in the hydrocarbon chain. In a RP-LC column, the unsaturated fatty acid chains are less well retained, so they can be separated from the saturated ones. A similar effect is observed with the trans isomers, which behave similarly to a straight saturated molecule in comparison to their bent cis equivalents.

### 2.4. NMR Spectroscopy

Nuclear magnetic resonance (NMR) spectroscopy is widely applied in a number of related fields: the identification of vegetable and fish oil components, iodine value, and, more recently, metabolomics analysis. NMR techniques have diagnostic potential for recognizing lipid classes. Both 1H and 13C NMR have been exploited for quantitative purposes as the primary analytical technique, one that is able to distinguish between cis and trans unsaturation and determine saturated, monounsaturated, and polyunsaturated components in fats and oils. Limitations of the technique are related to the sensitivity (proton NMR having some advantages with respect to carbon NMR) combined with the high cost of adequate NMR instrumentation (at least 14.1 T, ≥600 MHz) needed for resolving the signals. A critical region is that near the allylic proton resonances of cis and trans double bonds. A solution was proposed by applying an argentation technique to NMR spectroscopy (300 MHz). This study exploited the ability of silver ions to form a stronger complex with cis MUFA isomers with respect to the trans isomers, and this difference allowed better separation of the NMR allylic proton region. However, the more unsaturation is in the fatty acid, the less resolved becomes the multiplet in the allylic hydrogen region (usually at 1.9–2.2 ppm depending on the structure). On the other hand, carbon NMR can offer more detailed structure information gathered from the optimal distinction of the ethylenic carbon atom resonances; thus diagnostic signals can be found and used for recognition purposes. In comparison with the reported use of C4 and C16 resonances of arachidonic acid methyl ester (AA-ME), more interesting results were obtained by evaluating the ethylenic 13C region. In particular, the C15 resonance, which is the most deshielded carbon among the olefinic peaks in the arachidonate moiety, is influenced by the cis or trans double bond geometries, thus giving a distinct peak for each of the four mono-trans isomers of AA-ME, without overlaps with the all-cis isomer, allowing for the correct assignment of each signal (Table 1).

### Table 1. 13C NMR Assignment of the Most Deshielded Ethylenic Carbon Atom of Arachidonic Acid and EPA Methyl Esters (AA-ME and EPA-ME, Respectively)

<table>
<thead>
<tr>
<th></th>
<th>AA-ME21</th>
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<tbody>
<tr>
<td></td>
<td>14trans</td>
<td>11trans</td>
<td>all-cis</td>
<td>5trans</td>
<td>8trans</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C15, ppm</td>
<td>131.16</td>
<td>130.73</td>
<td>130.55</td>
<td>130.51</td>
<td>130.46</td>
<td></td>
<td></td>
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<tr>
<td>EPA-ME21</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>17trans</td>
<td>14trans</td>
<td>5trans + all-cis</td>
<td>8trans</td>
<td>11trans</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C17, ppm</td>
<td>132.57</td>
<td>132.24</td>
<td>132.03</td>
<td>132.01</td>
<td>131.96</td>
<td></td>
<td></td>
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</table>

The resonance of carbon-15 (C15) was used for AA-ME and its four mono-trans isomers, whereas the C17 resonance was utilized for EPA-ME and its five mono-trans isomers.

Similar results were evidenced in the case of EPA-ME isomers, using the C17 resonance of the 13C NMR spectrum (the most deshielded ethylenic carbon atom) for resolving the mono-trans geometrical mixture. As previously indicated, by combined GC and NMR analyses, the order of elution of these isomers was determined. Table 1 reports the resonance values (in ppm) of the ethylenic carbon atoms of AA and EPA methyl esters. These assignments and characteristics for omega-6 and omega-3 long-chain PUFAs are promising for the future application of NMR in metabolomics.

The analytical characterization of mono-trans isomers recently included the cholesteryl esters of linoleic and arachidonic acid, and in this case NMR, IR, Raman, and GC determinations were combined, providing exhaustive information likely to be useful for the development of high-resolution techniques utilized in diagnostics.

### 2.5. Infrared (IR) Spectroscopy

The historical importance of IR spectroscopy in the elucidation of the molecular structure of lipids and the present interest in the analysis of edible fats and oils both derive from the high information content of the spectra and the ability to assign specific absorption bands to particular functional groups. As early as the 1950s, IR was used to detect the presence and the amount of isolated trans double bonds in fats and oils and to monitor oil oxidation. Recently, there has been a renewed interest in this technique to measure the total trans content in food products and dietary supplements. Many IR spectroscopic procedures and official methods with increasingly improved levels of accuracy and reproducibility have been published in recent years (Table 2). IR is a reliable method for the measurement of trans fats and oils, based on the unique spectroscopic properties of fatty acid molecules. The intensity measurement of the absorption band between 976 and 956 cm\(^{-1}\) due to the deformation of the C–H bond adjacent to the isolated trans double bond is used for quantitative IR methods for the determination of isolated trans content. In fact, the band at ~966 cm\(^{-1}\) is a unique characteristic of isolated trans fatty acids, but the overlap with the broad absorption of triglycerides or other bands from conjugated (cis or trans) double bonds in the sample can interfere, especially at low concentrations of total trans. The accuracy of the IR method is also affected by the difficulties regarding the baseline boundaries. In fact, the 966 cm\(^{-1}\) band is broad, with a down-sloping baseline (an example is shown in Figure 3), and measurements of its height become increasingly less accurate as the trans levels decrease. However, many methods have been identified for overcoming these and other limitations (Table 2).

In particular, Fourier transform infrared-attenuated
total reflectance (FTIR-ATR) is consistently growing in popularity among analytical laboratories due to its simplicity and ease of use, as well as the elimination of the uncertainty associated with the location of the baseline.82–86

A newly proposed negative second derivative (2D) procedure succeeded in improving the accuracy and sensitivity of the FTIR-ATR methodology.81,87 The second derivative of an absorbance spectrum enhances the resolution of IR bands and allows a user to notice small shifts in IR band position and the presence of interference. In this way, potential interference attributed to conjugated fatty acids, and saturated fats can be easily recognized even at very low trans levels (below 1%).81,88,89 This 2D ATR-FTIR procedure is useful for rapid determination of the total trans fat content of commercial fats and oils for compliance verification with food labeling regulations.89

IR spectra can also be processed by multivariate statistical analysis. A common quantitative chemometrics tool used for data analysis is partial least-squares (PLS) regression (Table 2).90,91 PLS has been applied to the monitoring and controlling of industrial processes,92 because the determination of total SFA, trans FA, MUFA, and PUFA and trans FA (<5 min) is rapid and allows a user to notice small shifts in IR band position and the presence of interference. In this way, potential interference attributed to conjugated fatty acids, and saturated fats can be easily recognized even at very low trans levels (below 1%).81,88,89 This 2D ATR-FTIR procedure is useful for rapid determination of the total trans fat content of commercial fats and oils for compliance verification with food labeling regulations.89

FT-IR spectroscopy can also be used to detect trans conjugated double bonds. Cis-, trans- (or trans-, cis-) dienes

<table>
<thead>
<tr>
<th>band (cm⁻¹)</th>
<th>band assignment</th>
<th>limitation(s) and advantage(s)</th>
<th>ref</th>
</tr>
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<tbody>
<tr>
<td>~966</td>
<td>C–H out of plane of trans isolated double bond</td>
<td>reported as AOCS method Cd 14-95 replacing the Cd 14-61 method</td>
<td>14</td>
</tr>
<tr>
<td>990–945</td>
<td>C–H out of plane of trans double bond</td>
<td>reported as AOCS Official Method Cd 14d-99 to be carried out with the sample directly charged on the ATR crystal</td>
<td>82</td>
</tr>
<tr>
<td>~966</td>
<td>C–H out of plane of trans isolated double bond</td>
<td>reported as AOAC Official Method 2000.10; this is a universal method for detecting trans fats in all types of vegetable oils</td>
<td>83</td>
</tr>
<tr>
<td>~950 and ~980</td>
<td>C–H out of plane of trans-, cis-conjugated double bond</td>
<td>no derivatization and no solvent dilution; determination of geometrical composition; interference from other C–H bands</td>
<td>79</td>
</tr>
<tr>
<td>1670</td>
<td>C–C stretch of isolated trans double bond</td>
<td>rapid analysis</td>
<td>100</td>
</tr>
<tr>
<td>~1655</td>
<td>C–C stretch of isolated cis double bond</td>
<td>determination of cis–trans isomer ratio, iodine value, and oil adulteration</td>
<td>102</td>
</tr>
<tr>
<td>1640</td>
<td>C–C stretching of conjugated dienes</td>
<td>determination of conjugated double bond content</td>
<td>104</td>
</tr>
<tr>
<td>232–235 (nm)</td>
<td>conjugated dienes</td>
<td>reported as AOCS Official Method Cd 7-58 for detection of polyunsaturated fatty acids in animal and vegetable fats</td>
<td>115</td>
</tr>
<tr>
<td>270 (nm)</td>
<td>conjugated trienes</td>
<td>requires sample preparation</td>
<td>116</td>
</tr>
<tr>
<td>302 (nm)</td>
<td>conjugated tetraenes</td>
<td>interference from pigment absorption</td>
<td>118</td>
</tr>
</tbody>
</table>

The advantage of this technique is its ability to provide IR spectra for each separated FAME.90

FT-IR spectroscopy can also be used to detect trans conjugated double bonds. Cis-, trans- (or trans-, cis-) dienes...
have a characteristic doublet at 948 and 982 cm\(^{-1}\), whereas the corresponding trans\(_{1}\) trans\(_{1}\)-diene has a strong absorption band at 988 cm\(^{-1}\).\(^6\)

FT-IR spectroscopy has been also used for the analysis of fatty acids in biological samples. IR analyses were performed on dried films of intact liver cells,\(^7\) for quantifying the levels of total trans FAME in human adipose tissue\(^6\) and in microalgae extracts.\(^8\) FT-IR as well as Raman spectroscopy have been recently used for the analysis of cholesteryl esters of unsaturated fatty acids from plasma samples.\(^6\) The marker bands of isolated trans double bonds have been identified in both vibrational spectra, thanks to the comparison with the IR and Raman spectral library of cis and mono-trans cholesteryl ester isomers and a curve-fitting analysis (Figure 4). Exploiting this procedure, the analysis of the IR spectra from human plasma cholesteryl esters revealed a weak peak at \(\sim 970\) cm\(^{-1}\) (Figure 4A), observed in the same position in the mono-trans isomers of cholesteryl linoleate and arachidonate. Although this band is indicative of the presence of trans isomers, its detection is limited by several overlaps with other IR components. The band superimposition issue, together with the fact that the \(\sim 970\) cm\(^{-1}\) band is not always apparent in the raw spectra (because its visibility depends on the amount of trans fatty acid present), represent a drawback for the use of this IR band in the analysis of cholesteryl esters of biological samples.\(^6\)

2.6. Raman

The potential of Raman spectroscopy for the analysis of fats and oils has been known for decades, and the new generation of FT-Raman spectrometers has drastically increased its applications in food science.\(^9\) Raman spectroscopy is suitable for determining simultaneously the cis and trans content of unsaturated fatty acids, because the C=C stretching vibration for isolated cis isomers is near 1655 cm\(^{-1}\) and that of the trans isomer is near 1670 cm\(^{-1}\). A precision of ca. 1% can be obtained in the analysis of pure methyl esters and mono-, di-, and triunsaturated triglycerides, not conjugated in their native state.\(^10\) Raman spectroscopy has been applied to characterization and authentication of edible oils,\(^10\) to quantify total unsaturation, the cis/trans isomers, and conjugated double bond content,\(^10\) because the C=C bond conjugated with another double bond gives rise to bands shifted toward lower frequencies (Table 2). The formation of conjugated double bond systems and the isomerization of cis to trans double bonds due to thermal oxidation of unsaturated oils can be observed by analyzing the C=C stretching region.\(^10\) Formation of aldehydes, \(\alpha,\beta\)-unsaturated aldehydes, and hydroperoxides can be identified.\(^10\) In all oils, the formation of the band around 1670 cm\(^{-1}\), due to the isolated trans isomer olefins, can be seen during the oxidation processes.

In biology, Raman analysis can be used to examine fatty acids in membranes and evaluate the unsaturation degree,\(^10\) taking into account that the protein Amide I band in the 1650–1670 cm\(^{-1}\) region can obscure the sharp and unequivocal C=C band of unsaturated fatty acids and phospholipids. Therefore, a preliminary lipid/protein separation is recommended.

Raman spectroscopy of trans unsaturated cholesteryl esters seems promising, because also low trans isomer concentration gives rise to an intense marker band at \(\sim 1670\) cm\(^{-1}\) (Figure 4B), and this can be distinguished from the vibrational mode of the C=C steroid ring of the cholesterol moiety, which gives a band in the same spectral region of the trans C=C isomers. This analytical tool was used for the determination of the presence of trans isomers in the cholesteryl ester fraction of human plasma.\(^6\) Because cholesteryl esters have been associated with atherosclerosis and vascular inflammation,
screening of plasma fractions by Raman spectroscopy could afford an interesting and efficient method to develop for health applications.

In biological samples such as cells, micro-Raman spectroscopy provides possibilities for higher spatial resolution than FT-IR microscopy.\(^{111}\) Potentially, all constituents, including proteins, lipids, and nucleic acids, can be assigned,\(^{112}\) and minute differences at the subcellular level can be evidenced.\(^{113}\)

### 2.7. Ultraviolet (UV) Spectroscopy

The ultraviolet (UV) spectrum is principally used to detect or confirm the presence of fatty acids containing conjugated double bonds.\(^{114,117}\) In fact, isolated cis-double bonds exhibit an absorbance at 206 nm with a relatively low extinction coefficient, which renders this feature not very useful as a diagnostic tool in natural oils. UV spectroscopy is also used to detect the formation of conjugated systems during chemical or enzymatic isomerization of fatty acid double bonds.\(^{114,118}\) Conjugated double bonds in fatty acids give rise to a series of broad bands at successively higher wavelengths (Table 2). Different geometrical isomers have slightly different spectra, where the greater is the number of trans-double bonds, the higher is the extinction coefficient and the shorter are the wavelengths of the band maxima.\(^{119}\) The differences in the absorption maxima for the geometric isomers are so subtle that they would not likely be discernible in biologically or chemically produced fatty acid isomer mixtures.

### 3. DIET-DRIVEN LIPIDS CONTAINING TRANS FATTY ACIDS

The food industry switched from the production of animal-based fats to plant-based oils during the last century. This change was due to the belief that palm oil-based products are heart-healthier than the previous (animal-based) ones. In the early 1990s, it was discovered that plant-oil solidification normally utilized in margarine production generates harmful trans fatty acids (TFA). Moreover, neither of the two sources of fats contain good quantities of long-chain \(\omega-3\) PUFAs, which are mainly obtained from fish oils. The debate over the beneficial effects of animal-based fats and plant-oil fats is still a hot topic in the scientific community; aside from this, many ecological issues have lately been raised, due to the destruction of tropical forest for palm-oil production.\(^{120}\)

It is now accepted worldwide that TFAs have negative health effects.\(^{7}\) TFAs, both geometrical and positional isomers with unshifted and shifted double bond positions, were first identified as side products of industrial oil processing that can enter the food chain. Their presence in the human lipidome has been evidenced in connection with the wide use of partially hydrogenated and deodorized oils that occur in industrialized countries. TFAs can enter the food market by three main sources: (i) partial hydrogenation of fats, (ii) high-temperature processing of edible oils (deodorization processes), and (iii) use of ruminant meats and dairy products that have a trans content due to the biohydrogenation process (see section 4.1) taking place during animal metabolism. In the first two cases, industrial methodologies are involved: partially hydrogenated vegetable oils are obtained by several methods using hydrogen and metal catalysts, where radicals are not involved and the chemical transformations only afford positional isomers;\(^{110}\) the deodorization process is also a commonly used industrial procedure for eliminating the unpleasant flavors of fish oil by exposure of the oils to high temperatures (180—220 °C) under low pressure (1—10 mbar).\(^{122}\) In case of thermal isomerization, geometrical isomers are almost exclusively formed in which the position of the double bonds remains the same as those of the original fatty acids.\(^{7}\) The number of geometrical trans isomers that can be formed during this process is equal to \(2^n\), where \(n\) represents the number of double bonds in the molecule. Under normal operating conditions, mono-trans isomers are prevalently formed.

In both industrial processes, treatments can also render the natural oil sources more malleable and agreeable for food production, but at the expense of the natural \(\text{cis}\) fatty acid structure, which is converted to unnatural isomers. Because of the extensive use of these fats in foods, the trans intakes increased in industrialized countries was found to be correlated with the curves of coronary heart disease mortality tracked over the time. In the early 1900s, angina and myocardial infarction were unusual clinical events, but over the century they rapidly increased, becoming the major cause of death by midcentury.\(^{123}\)

Among other factors, TFA consumption was discovered to play a crucial role in this increase, raising serious concerns mainly in North America and northern Europe, where there is an estimated intake of 2—5 g/day. On the other hand, Mediterranean countries (Italy, Portugal, Greece, and Spain), which mainly use olive oil, reported lower TFA intake (1.4—2.1 g/day) and lower cardiovascular risk.\(^{124,125}\) As a result of a long debate, in 2003 the Food and Drug Administration (FDA) issued a regulation requiring manufacturers to list trans fat on the Nutrition Facts of foods and some dietary supplements.\(^{126}\)

In Europe, the label information of food products has been revised recently (EU Regulation 1169/2011) and will apply from December 2014; however, the trans content of foods has not been included, raising a debate over consumers’ safety.\(^{127}\)

Research in this field can be addressed to new processes and separation procedures to reduce the final trans content of the industrial chain, and to high performance analytical procedures to detect and characterize trans fatty acid isomers in the final products. Taking into account the health concerns over the problems caused by the low \(\omega-3\) intake in the diet of the general population,\(^{128}\) the enrichment of foods such as milk and fats with these essential fatty acids is becoming more frequent, deodorized oils being the source of EPA and DHA without unpleasant odor. However, particular attention has to be given to the quality of marine oils, as the refining processes that involve thermal treatment can affect the geometrical integrity of long-chain \(\omega-3\)-PUFAs.\(^{96}\) Recently, deodorized fish oils have been highlighted for their trans EPA content, and the follow-up in rats fed a low (5%) and high (30%) fat content diet including fish oil showed that trans EPA isomers, prevalently \(\text{17trans}\) isomer, can be incorporated at the level of liver mitochondria.\(^{23}\)

In previous studies, it was observed that the trans isomers of EPA and DHA resulting from the metabolism of isomerized \(\alpha\)-linolenic acid (obtained through diet) are basically the isomers with trans geometry in the n-3 position (\(\text{17trans EPA and 19trans DHA}\)).\(^{129}\) Intake of isomerized \(\alpha\)-linolenic acid led to incorporation of \(\text{trans DHA isomers in the retina and cerebral phospholipids in rats.}\(^{130}\) The analytical issues related to the distinction of omega-3 and omega-6 trans isomers have been treated in section 2 of this Review.

Because \(\omega-3\) PUFAs are well-known for their beneficial effects, such as protection against cardiovascular diseases,\(^{131}\) anti-inflammatory properties,\(^{132}\) positive effects on physiological and cognitive functions,\(^{133}\) and their key role in the brain and in neurodevelopment,\(^{134}\) the interference of trans \(\omega-3\)}
4. BACTERIAL SOURCES OF TRANS FATTY ACIDS

Most of the monounsaturated fatty acid (MUFA) residues present in plants and animals display cis geometry of the double bond. The significance of the ubiquitous cis structural feature of the unsaturated lipid double bond is based on its contribution to the organization of phospholipids in one of the most important units of living organisms: the cell membrane. Indeed, life needs the compartmentalization given by the phospholipid bilayer that surrounds the cell, the cis fatty acid necessary to provide the hydrophobic part with favorable properties to organize proteins and other components, rendering membranes active components with several functionalities rather than being merely a wall. The bend corresponding to the cis double bond gives quite typical physical characteristics, such as melting points or phase transition temperatures, as well as biophysical and biochemical properties, which will be discussed in section 7. It is intriguing that the trans geometry, despite its thermodynamic stability, is almost excluded from most of the unsaturated fatty acid structures involved in cellular compartments of living organisms.

A simple example of how membrane properties are tuned deliberately, by altering the proportions of saturated and monounsaturated fatty acids, is given by bacteria and their adaptation to environmental conditions. In these forms of life, the first detection of trans MUFA has been reported to occur by two main processes: (i) microbial hydrogenation (biohydrogenation) of unsaturated fatty acids; (ii) activity of a cis–trans isomerase enzyme.

4.1. Microbial Biohydrogenation in Ruminants

The trans geometry is displayed by conjugated linoleic acids (CLAs), which are natural compounds. These are mixtures of positional and geometrical isomers with two conjugated double bonds (C9, C10, C11) in the fatty acid chain. Of all possible isomers, the 9cis,11trans-C18:2 isomer (rumenic acid) represents 90% of the total CLAs in food products from ruminants. The trans geometry of CLAs can be considered endogenous for ruminants, whereas for humans this geometry has a dietary (exogenous) origin. The transformation of trans-vaccenic acid (11trans-C18:1) by Δ9-desaturase in animal tissues and mammary glands also affords rumenic acid. The percentage of the conversion of trans-vaccenic acid to CLA has been estimated to range from 5% to 12% in rodents and from 19% to 30% in humans. trans-Vaccenic and rumenic acids are observed as intermediates of unfinished ruminal biohydrogenation of linoleic acid and α-linolenic acid (Figure 5).

The biohydrogenation process involves two classes of microbial enzymes: isomerases and reductases. The initial step of biohydrogenation of linolenic and linoleic acids involves an isomerization of the 12cis double bond to 11trans, giving conjugated dienoic or trienoic fatty acids. The mechanism of this conversion is poorly understood. Wallance et al. recently suggested a radical-based mechanism initiated by hydrogen abstraction from the C11 atom. The conversion of rumenic acid to trans-vaccenic acid is catalyzed by a reductase acting on both linoleic and α-linoleic acids. The final step is the reduction to stearic acid (18:0). This is generally the rate-limiting step, giving an accumulation of trans-vaccenic acid in the rumen.

Extensive studies have drawn a consistent conclusion on the potential health effects of naturally derived trans-vaccenic acid and CLAs such as reduced risk of atherosclerosis, hypotriglyceridemic benefits, suppressing tumor development during initiation, promotion, and progression phases of carcinogenesis, anticarcinogenic and antiatherogenic activities, and antidiabetic properties. Although evidence has been reported that CLAs are also effective antioxidants, the mechanism of action is not yet clear.

4.2. Enzymatic cis–trans Isomerization in Bacteria

In the early 1990s, it was discovered that the cis and trans geometries of unsaturated lipids were significantly involved in adaptation responses. The response of psychrophilic bacterium Vibrio sp. strain AB-E and Pseudomonas putida P8 to an increase in temperature or the presence of toxic phenol concentrations, respectively, gave an immediate increase of monounsaturated trans isomers in their membrane phospholipids. These trans isomers were not dependent on growth, so that they did not derive from a de novo synthesis. So, when lipid biosynthesis is absent and the total saturated and unsaturated fatty acid content cannot vary, bacteria can have a short-term response known as a “geometrical stress response”. In several obligate aerobic and anaerobic bacteria, biosynthesis provides oleic or palmitoleic acid, respectively, but trans isomers were not found to derive from biosynthetic routes. Geometrical isomers (in particular 9trans-C16:1) were formed in anaerobic bacteria Pseudomonas sp. strain E-5, Pseudomonas putida, and Vibrio sp. strain AB-E, as could be determined by GC analyses. The analytical procedure to investigate trans MUFA isomers has been described in section 2.1. The biological path is carried out by a specific enzyme, which works without changing the position of the double bond but only its geometry, and does not require ATP or any other cofactor such as NADP(H), glutathione, or oxygen.

![Figure 5. Biohydrogenation of linoleic and linoleic acids in rumens and transfer of ruminant fatty acids to mammary glands.](image-url)
The higher thermodynamic stability of trans isomers is the driving force of the transformation.

The cis–trans isomerase (Cti) gene from Pseudomonas strains was cloned, purified, and characterized.\textsuperscript{155} Cti isomersases are proteins of ca. 80 kDa molecular weight whose sequences have been deduced from sequencing the corresponding gene.\textsuperscript{156} Comparison among several Cti protein sequences identified them as heme-containing proteins of the cytochrome c-type.\textsuperscript{155,157–159}

The adaptive response, for example, in the case of high temperature or in the presence of toxic compounds, implies that membrane fluidity has to decrease, and this effect is rapidly obtained either by changing the saturated/unsaturated ratio, activating fatty acid biosynthesis, or by a mechanism independent from the biosynthesis that exclusively acts on the cis compound, which is the cis–trans isomerase. In this way, the organism can rapidly cope with the emerging environmental stress using the different properties of membranes tuned by the lipid structures: trans lipid packing can occur in a more ordered state as compared to the cis isomers, so that a substantial effect on membrane fluidity and thus on the rigidity of the membrane is obtained, similar to an increase of saturated lipids. The change from cis to trans unsaturated double bonds does not have the same decreasing effect on membrane fluidity as does the change from cis to saturated fatty acids.\textsuperscript{160} It is intriguing that Pseudomonas and Vibrio species, which are widely occurring species in all niches of a great number of ecosystems such as soil, human skin, and water, have the isomerase pathway, also in comparison with other Gram-negative bacteria or species lacking such adaptation.\textsuperscript{9}

Studies of the molecular mechanism of enzymatic isomerization on Pseudomonas sp. strain E-3 evidenced the similarity between Cti and LOX at least due to the common inhibition given by antioxidants. However, chemists, which instead do not affect the isomerization, also inhibit LOX. A second mechanistic hypothesis was then formulated, consisting of the hydration–dehydration mechanism, similar to the formation of 3-trans-enoyl-CoA from the corresponding cis isomer.\textsuperscript{161}

Another mechanism was proposed on the basis of the analysis of the carbon isotope fractionation experiment of the cis–trans isomerization\textsuperscript{162} and the site-directed mutagenesis experiments on Cti of Pseudomonas putida P8, which caused the loss of isomerization activity.\textsuperscript{163} This implies the binding of the cis fatty acid to the active center of heme-containing proteins of the cytochrome c-type. Scheme 1 shows the proposed mechanism of Cti based on the enzyme–substrate complex as an intermediate, which allows the rotation of the carbon–carbon double bond to occur.

5. GEOMETRICAL ISOMERIZATION CATALYZED BY RADICALS IN HOMOGENEOUS SOLUTION

5.1. Early Work

The early work on geometrical isomerization using free radical chemistry concerned the reactivity of iodine atoms\textsuperscript{164} and thyl radicals (RS\textsuperscript{•}).\textsuperscript{165,166} These are able to add reversibly to double bonds, and, consequently, their efficiency in transforming the less stable cis into the thermodynamically more stable trans isomer was envisaged. The cis–trans isomerization of olefins by the addition–elimination sequence of the PhS\textsuperscript{•} radical is now an established methodology in fine chemical synthesis.\textsuperscript{167} Other radicals such as sulfonyl (RSO\textsubscript{2}\textsuperscript{•}),\textsuperscript{168} selenyl (PhSe\textsuperscript{•}),\textsuperscript{169} stannyl (R,Sn\textsuperscript{•}),\textsuperscript{170,171} germyl (Ph,Ge\textsuperscript{•}),\textsuperscript{172} and silyl ((Me\textsubscript{3}Si),Si\textsuperscript{•})\textsuperscript{173} radicals, as well as nitrogen dioxide (NO\textsubscript{2}\textsuperscript{•}),\textsuperscript{173} are also known to add to a double bond reversibly and, therefore, to isomerize alkenes. The efficiency of isomerization is strictly associated with the nature of the attacking radical; for example, it was shown for cis- and trans-3-hexen-1-ol that equilibration (cis/ trans = 18/82) is reached at 80 °C with PhS\textsuperscript{•}, Bu\textsubscript{3}Sn\textsuperscript{•}, and (Me\textsubscript{3}Si),Si\textsuperscript{•} radicals in 1, 4, and 10 h, respectively.\textsuperscript{174} This early work in chemistry was only later connected with the in vivo reactivity of biological relevant RS\textsuperscript{•} and NO\textsubscript{2}\textsuperscript{•} radicals toward lipid molecules.

5.2. Monounsaturated Fatty Acids (MUFA)

5.2.1. Isomerization by Thiyl Radicals. The radical-based thiol additions to cis and trans olefins are chain reactions and are accompanied by isomerization (Scheme 2).\textsuperscript{165} In the case of unsaturated fatty acids, the reaction with thiols has been carefully studied due to the importance of the cis double bond for the biological activity. Starting from the simple monounsaturated substrate, the reaction was initiated by various methodologies: the thermal decomposition of azo compounds,\textsuperscript{174} γ-rayolysis of tert-butanol solutions,\textsuperscript{174,175} or photolysis of di-tert-butyl ketone.\textsuperscript{176} Experimental evidence was also presented for the cis–trans isomerization occurring at 90–120 °C without adding initiator, the initiation step being a complex reaction between thiol and alkene through molecule-assisted homolysis of the S–H bond.\textsuperscript{177}

The time-dependence of the addition of 2-mercaptoethanol to methyl oleate and the isomerization catalyzed by the corresponding HOCH\textsubscript{2}CH\textsubscript{2}S\textsuperscript{•} radical were investigated in detail.\textsuperscript{175,176} On the basis of Scheme 2, kinetics were analyzed on the basis of the initiation rates, the known values of thyl radical self-termination rate constant, and the rate of hydrogen abstraction from the thiol, obtaining rate constants at room temperature for all four reactions involved in the isomerization of methyl oleate to methyl elaidate promoted by the HOCH\textsubscript{2}CH\textsubscript{2}S\textsuperscript{•} radical (see Scheme 3). The rate constants for thyl radical addition (k\textsubscript{i} in blue) to cis or trans isomers were found to be rather similar (k\textsubscript{i,trans}/k\textsubscript{i,cis} = 1.8), whereas for fragmentation of adduct radicals (k\textsubscript{f} in red) they were
of the HOCH_2CH_2S radical-catalyzed isomerization of methyl oleate

The addition of HOCH_2CH_2S radical to the cis and trans forms should be small. This is in accordance with the experimental rate constants in Scheme 3, which showed comparable rate constants for the addition reactions, whereas the $\beta$-fragmentation to the trans isomer was found to be 1 order of magnitude faster than that leading to the cis isomer. \(^{176}\)

5.2.2. Isomerization by Sulfonyl Radicals. The addition of thyl radicals to molecular oxygen is fast and reversible. The equilibrium constant of the oxygen addition (eq 1/−1) is about 3000, meaning that in air-saturated aqueous systems ([O_2] = 2.4 × 10^{-4} M) both RS* and the thylperoxyl radical RSOO* equilibrate at about equal concentrations, while under typical physiological conditions in cells ([O_2] ≤ 10^{-3} M) the equilibrium remains overwhelmingly on the thyl radical side. The reversible oxygen addition to thyl radicals (eq 1/−1) becomes significant also because of the known rearrangement of the thylperoxyl radical RSOO* to sulfonyl radical RSO_2* (eq 2). \(^{180}\) The effectiveness of the isomerization processes of the

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Scheme 3. HOCH_2CH_2S* Radical-Catalyzed Isomerization of Methyl Oleate

C(OMe) + HOCH_2CH_2S* ⇌ C(OMe) + HOCH_2CH_2S

k_{cis} = 1.6 \times 10^8 \text{M}^{-1} \text{s}^{-1}

k_{trans} = 2.9 \times 10^8 \text{M}^{-1} \text{s}^{-1}

k_{cis} = 1.7 \times 10^7 \text{s}^{-1}

k_{trans} = 1.6 \times 10^6 \text{s}^{-1}
```

The large preference of fragmentation to the trans isomer was attributed to different barriers for the formation of the two transition states from the equilibrium radical structure (see below). Using thiol in catalytic amounts (up to 0.5 equiv with respect to unsaturated substrate), it was shown that the HOCH_2CH_2S* radical induces cis–trans isomerization of methyl oleate in tert-butanol solutions saturated with 10% oxygen (corresponding to 0.23 mM), behaving similarly to the same reaction under anaerobic conditions.

The effectiveness of the isomerization processes in the presence of various additives has been addressed in homogeneous solution. In tert-butanol solution, the inhibition of the HOCH_2CH_2S* radical-catalyzed isomerization by known antioxidants was initially studied. The ability of thyl radical scavenging was found to increase along the series α-tocopherol < ascorbic acid 6-O-palmitate < all-trans retinol acetate. Vitamins E and C had a retarding effect, whereas conjugated polyunsaturated compounds, such as all-trans retinol, strongly inhibited the isomerization process by forming covalent bonds. The “anti-isomerizing” effect of all-trans retinol and, presumably, of other retinoids and carotenoids is based on their abilities of trapping several thyl radicals for each molecule.

Similar results were obtained for a variety of MUFAs, indicating that in solution these reactions do not depend on the double bond position in the alkyl chain (Figure 6). \(^{175, 178}\) The equilibrium constant of $K = 5.15$ for MUFAs is larger than that of 2-butenes ($K = 3.4$), presumably because of the large alkyl chain residues. A temperature dependence of $K$ for oleate in tert-butanol provided values of $\Delta H = -5.4 \text{kJ/mol}$ and $\Delta S = -5.5 \text{J/mol} \cdot \text{K}$ for the cis to trans conversion.\(^{175}\)

The enthalpy profile shown in Figure 7 refers to the reaction of HOCH_2CH_2S* radical with but-2-enes and was obtained by DFT-BB1K calculations. \(^{176}\) It is worth underlining that the difference in the enthalpy barrier for $\beta$-elimination of thyl radical depends on the formation of cis and trans isomers, the latter being 1.0 kcal mol$^{-1}$ lower. This difference is similar to the computed energy difference between the cis and trans isomers (1.2 kcal mol$^{-1}$). This indicates that the $\beta$-fragmentation involves late transition states, where the dominant steric effects are similar to those in the parent isomers. Consequently, the addition steps involve early transition states. There is only a small variation in the steric interactions, going from each single isomer to the transition states, so that the difference between the enthalpy barriers for

Figure 6. Isomerization of different monounsaturated fatty acid methyl esters (0.15 M) by 2-mercaptoethanol-derived thyl radicals in tert-butyl alcohol starting from the cis isomers at 75 mM thiol concentration. \((\blacktriangle)\) oleate; \((\blacksquare)\) palmitoleate; \((\blacklozenge)\) cis vaccenate; \((\bullet)\) oleic acid. Reprinted with permission from ref 175. Copyright 2002 American Chemical Society.

Figure 7. Enthalpy profile in kcal mol$^{-1}$ for the cis–trans isomerization of 2-butene by the HOCH_2CH_2S* radical.
MeSO₂⁺ radical was addressed in some detail.²¹² Again, the position of unsaturation and the hydrocarbon chain length have no influence on the cis–trans isomerization process (Figure 3, left side). The right side of Figure 8 shows no loss of unsaturated esters because the loss of cis isomer is exactly balanced by the formation of the trans geometrical isomer. It is worth mentioning that in the presence of oxygen the isomerization is completely suppressed, because sulfonyl radicals are effectively trapped by oxygen (eq 3).²¹²

\[
\text{RS}^+ + \text{O}_2 \rightleftharpoons \text{RSO}_2^* \\
\text{RSOO}^* \rightarrow \text{RSO}_2^* \\
\text{RSO}_2^* + \text{O}_2 \rightarrow \text{RSO}_2\text{OO}^*
\]

5.2.3. Isomerization by Nitrogen Dioxide. Reliable kinetic data for the reaction of the NO₂⁺ radical with olefins are limited to the gas phase, because both addition and hydrogen abstraction reactions are very slow processes.²¹³ Scheme 4 shows the analogous cis–trans isomerization of 2-pentene, the rate constants being obtained at 25 °C. The fragmentation of the adduct radical occurs with a rate constant of \( k_l \approx 8 \times 10^4\ \text{s}^{-1} \) for both isomers; therefore, molecular oxygen likely intercepts the carbon-centered radical intermediate prior to fragmentation. Evidence that H-atom abstraction from the allylic position (∼20 kJ/mol endothermic) is of the same order of magnitude as the addition step is also available. A detailed study of the reaction of NO₂⁺ with cyclohexene showed that the two reaction paths are also competitive in solution.²¹⁴

5.3. Polysaturated Fatty Acids (PUFA)

5.3.1. Reaction with Thiyl Radicals. While the understanding of MUFA isomerization is quite complete, the same does not hold true for the analogous reaction with PUFA. Using identical reaction conditions with regard to MUFA and catalytic amounts of thiol, the time profiles of linoleic acid methyl ester (9cis,12cis-C18:2; LAME) disappearance and formation of mono-trans and di-trans isomers in these experiments indicated that the cis–trans isomerization occurs stepwise (Scheme 5).²¹⁴ However, careful quantitative work indicated that side reactions are also at work and byproducts containing “conjugated diene” moieties were isolated. Their effect as inhibitors in the cis–trans isomerization via the trapping of thiyl radicals by addition was also envisaged.²¹⁴

Cis–trans isomerization reactions of methyl γ-linolenate (6cis,9cis,12cis-C18:3),²¹⁴ arachidonate (5cis,8cis,11cis,14cis-C20:4),²¹⁵ and EPA (5cis,8cis,11cis,14cis,17cis-C20:5),²³ catalyzed by HOCH₂CH₂S⁺ radicals, were studied in some detail, not only for the importance of the cis geometry for these biologically active compounds and the need for understanding the effects of the configurational change, but also in view of the ease of free radical synthetic routes for providing better availability of PUFA isomers. Indeed, the mono-trans isomers deriving as the first products from the step-by-step process (Scheme 5) have a very high potential for applications in biological studies and diagnostics (see section 8). Trans PUFA isomers were obtained either by stereospecific multistep synthesis,¹⁸⁵,¹⁸⁶ which is not an affordable methodology, or by heating under acidic conditions (p-toluenesulfonic acid),¹⁸⁷ which does not represent a specific synthesis of geometrical isomers. Instead, the free radical isomerization reaction could represent a valuable tool for the synthesis of geometrical trans lipid molecular libraries, taking into account the PUFA complexity and the greater number of possible geometric isomers, calculated as \( 2^n \), where \( n \) is the number of double bonds. EPA offers a seminal example, where a dual synthetic strategy with the key steps shown in Scheme 6 was followed. The step of epoxide preparation starting from EPA-ME allowed the separation of the different epoxide regioisomers by silica gel chromatography. The subsequent elimination step from each epoxide fraction afforded the corresponding mono-trans regioisomer with reasonable purity. On the other hand, EPA-ME free radical isomerization stopped at an early stage afforded the mixture of five mono-trans EPA-ME isomers in one step.
**Scheme 6. Dual Synthetic Strategies for the Preparation of Mono-trans PUFA Isomers**

After comparison of the isomers from both routes and examination of the analytical data, a complete and satisfactory recognition of the five mono-trans EPA methyl esters was obtained, so that the free radical route can now be successfully used as a rapid methodology for preparing mono-trans PUFA lipids, even in laboratories not specialized in chemical synthesis.23

As far as PUFA reactivity is concerned, it must be noted that PUFAs also have methylene-interrupted double bonds and bisallylic positions with a low C–H bond dissociation enthalpy, so that the reaction with alkanethiyl radicals (RS*) is strongly exothermic (eq 4). This reactivity attracted immediate interest for its connection with the role of thiols as biological repair agents. In fact, cysteine and glutathione are quite efficient hydrogen donors toward carbon-centered radicals generated by various pathways of damage in vivo, affording cysteinyl (CyS*) and glutathionyl (GS*) radicals. These two radicals were reported to react with PUFA bisallylic positions (eq 4) by two independent groups.188,189 Rate constants of \( k_1 \approx 10^7 \text{M}^{-1} \text{s}^{-1} \) were assigned using pulse radiolysis techniques for the competition between eq 4 and the reaction of GS* with ABTS,189 and for the direct formation of pentadienyl radicals by the action of CyS radicals.188 Other interesting facts are that (i) the rate constants increase in the series linoleic < linolenic < arachidonic (i.e., by increasing the number of bisallylic moieties)188,189 and (ii) an upper limit for the reverse rate constant of \( k_2 \approx 10^4 \text{M}^{-1} \text{s}^{-1} \) is given.188 It was suggested that \( \sim 50\% \) of thyl radicals abstract the bisallylic hydrogen, whereas the remaining RS* add to the double bond to form a radical adduct, and the presence of molecular oxygen efficiently scavenges all of the formed carbon-centered radicals.190

It was also reported that the trans isomers of linoleate moieties represent the fate of the pentadienyl radical, which reacts with thiols in different conformations.191 The same group later revised their conclusions in accordance with Scheme 5.192 Because of possible competitive pathways between hydrogen abstraction from the bisallylic positions and the reversible thyl radical addition to the double bonds, it is clear that the specific conditions and reactant concentrations are important for the outcome. Further chemical work will be useful to place the PUFA reactivity as a radical stress signal or damage and contribute to the full understanding of the possible fates of these biologically important molecules.

### 5.3.2. Reaction with Nitrogen Dioxide

The reaction of NO\(_2^*\) with methyl linolate in the presence and absence of oxygen attracted considerable attention because the NO\(_2^*\) radical has been proposed to initiate lipid peroxidation.153 The rate constant for the abstraction of bisallylic H-atoms by NO\(_2^*\) (almost thermoneutral) is expected to be much faster than the abstraction from the allylic positions in MUFA. Scheme 7 shows the hydrogen abstraction versus addition mechanism for the reaction of NO\(_2^*\) with PUFA that fits the experimental findings.183,194 The reaction products strongly depend on the reaction conditions. At low NO\(_2^*\) concentrations, for example, hydrogen abstraction predominates under anaerobic conditions with the formation of allylic products derived from radical 1 and small yields of trans isomers, whereas, in the presence of oxygen, trans isomers were not detected due to the trapping of radical 2 by oxygen.194 Therefore, in the presence of 0.1 mM oxygen the cis–trans isomerization became unimportant, because both paths generate carbon-centered radicals that react with O\(_2\) to give peroxy radicals that propagate peroxidation.

### 5.4. Cholesteryl Esters (CE)

Cholesteryl linolate and arachidonate esters are interesting targets for their biochemical connection with membrane phospholipid turnover, which takes place with the enzymatic activity of LCAT (lecithin cholesterol acyl transferase). They enter into lipoprotein formation via the enzymatic activity of the CETP (cholesteryl ester transfer protein), and both enzymatic activities are involved in atherosclerosis and vascular inflammation.195 The only data related to trans fatty acids were reported for the isomers taken from the diet, showing that the lipid geometry can affect the transfer of cholesteryl esters from HDL to LDL by CEPT.196 It is worth noting that most of the cholesteryl esters have unsaturated moieties, linoleic acids being the most represented fatty acids (ca. 50% of the total fatty acid content) in this lipid class. Trans double bonds in cholesteryl esters can derive from membrane phospholipids; therefore, it can be important to have information on the composition of plasma cholesteryl esters. For this reason, the synthetic build-up of a library of cholesteryl esters was proposed, with the preparation of mono-trans isomers of cholesteryl linolate and arachidonate using the convenient and fast approach of thyl radical-catalyzed cis–trans isomerization, starting from the natural cis substrates.84 The photoisomerization reaction is shown in Scheme 8. A degassed solution of cholesteryl ester 3 or 4 in 2-propanol was photoirradiated in the presence of catalytic 2-mercaptoethanol, and was stopped at an early stage favoring the mono-trans isomer formation. The Ag-TLC procedure allowed the isomer fractions to be isolated with the recovery of the unreacted cis isomers. The latter can undergo subsequent isomerization rounds, leading to overall yields of 80% in case of mono-trans cholesteryl linolate isomers and of 30% in case of mono-trans cholesteryl arachidonate isomers. The characterization of these isomers...
can be performed by an array of analytical methodologies described in section 2. By this approach, it was possible to establish the presence of cholesteryl ester isomers in human plasma (ca. 12 nM) for the first time, opening further developments in diagnostics.68

5.5. Triglycerides

The reactivity of unsaturated fatty acid moieties of triglycerides contained in natural oils attracts interest from several scientific fields, due to their multidisciplinary applications including material science, energy sources, and nutrition. This latter aspect is treated in section 3. The conversion of triglycerides containing unsaturated fatty acid moieties to their corresponding geometrical trans isomers can represent a convenient way to modulate the properties of natural oils, conferring new characteristics with possible convenient applications. Moreover, trans-containing oils can attract attention because they combine two factors: the positive environmental impact for the recycling of waste vegetable oils after their use in frying pans, and the more favorable properties as materials or biofuels due to the changes in viscosity accompanying the cis to trans conversion occurring at high temperatures.197

By applying high temperatures in frying pans, the cis to trans conversion can occur, thus creating an important concern over the quantities of trans fatty acids obtained from the diet (see section 3). It is not the scope of this Review to examine the literature related to the dietary impact of frying oils and the studies connected to the thermal isomerization, which is an active field of research. Model studies of triglyceride transformation by heating were performed,199 and it was found that the suppression of the isomerization is due to the natural antioxidant content of the vegetable oil.199

On the other hand, for technological applications, a tunable preparation of trans-containing oils is required. Trans-containing oils can be appropriately prepared by the action of photolytically or thermally generated HOCH2CH2S radicals in alcoholic solutions.200 This RS* radical-catalyzed approach was originally designed for building up a simple and economic lipid library for application in lipidomics. It was also shown that, apart from the fact that free radical reaction conditions can be appropriately tuned to control the trans content, the isomerization mixture can be treated by winterization to further increase the trans FA content. This is a practical and versatile way to allow variation of the trans content. Moreover, hydrolysis and methanalysis of trans triglyceride mixtures were carried out enzymatically to obtain the corresponding trans-free FA and trans FA methyl esters. This simple synthetic route could facilitate and expand the applicability of trans triglycerides in various fields.

The free radical reaction of double bonds with thiols has also found successful application in the so-called thiol–ene chemistry (TEC), in particular for the modification of the fatty acid moieties of polysaturated vegetable oils. In this case, the goal is not to obtain the isomerization, but to effect the thiol addition to influence the lubricating properties of surfaces. Several examples can be found in the recent literature. The coupling between the unsaturated bonds of linseed oil (~70% linoleic acid content) and thiol-functionalized surfaces was obtained by a photoinduced process as a strategy to attach a vegetable oil to a metal surface pretreated with mercapto silane.201 Butanethiol was added to corn oil (~60% linoleic acid content) by UV-initiated reaction to produce sulfide-modified vegetable oils. Using a thiol:double bond ratio of 6:1, up to 97% of double-bond conversion and no evidence of formation of any conjugated double bond residues were attained.202

Another full investigation was carried out on the addition of 2-mercaptoethanol to oleic acid moieties under UV light and room temperature, at different thiol:double bond and solvent:double bond ratios, as well as changing the irradiation intensity (0.5 to 15.0 W/cm²).203 In this way, optimal conditions were individuated for grafting the 2-mercaptoethanol unit to the vinyl group of rapeseed oil to yield bio-based polyols to be used for further transformation into new elastomeric products.

5.6. l-α-Phosphatidylcholines (PC)

In l-α-phosphatidylcholines (PC), the most abundant phospholipids in nature, a three-carbon glycerol bridge links the two fatty acid chains with a phosphoryl choline moiety. Scheme 9 shows the three distinct regions of PCs in different colors. In the sn-1 and sn-2 positions of the glycerol moiety the two fatty acid residues are attached, whereas in the sn-3 position the polar headgroup is connected. Lecithin from natural sources is a mixture of l-α-phosphatidylcholines with different fatty acid chains (see insets in Scheme 9). Because of the structural and functional roles of PCs in living organisms and the fact that all natural PCs display only the cis geometry of the double bonds, the change of this geometrical feature is an important event, which has to be fully considered in terms of its molecular and biological consequences.

The cis–trans isomerization of unsaturated fatty acid residues of POPC (1-palmitoyl-2-oleoylphosphatidylcholine),204 DOPC (1,2-dioleoylphosphatidylcholine),174 SAPC (1-stearoyl-2-arachidonoylphosphatidylcholine),205 soybean lecithin184 and egg lecithin185 catalyzed by HOCH2CH2S radicals was first studied in alcohols where these compounds are soluble. After the reaction, phospholipids were isolated and transesterified, obtaining their fatty acid residues as FAMEs to be examined by
and circulation, whereas vesicles mimic the supramolecular arrangement of lipids in cell membranes of most biological tissues. Obviously, the models can be added along with proteins, antioxidants, and other elements to better simulate physiological or pathological conditions. On the other hand, using these models under a variety of conditions, it can be expedient to gain information on the scenarios working in vivo. In all cases, fatty acids in the models are considered with their natural structures; therefore, the cis double bond has been mostly evaluated for aggregation and biophysical properties, also related to biotechnological applications and the evolutionary concept of the protocell. From a chemical point of view, for many years known fatty acid reactivity in natural lipids was represented exclusively by lipid peroxidation of PUFA residues, based on the natural (enzymatic) process to yield prostaglandins and other signaling mediators, and on the free-radical-mediated process starting from the reactivity of bisallylic hydrogen atoms, which ultimately leads to fatty acid chain degradation. It is worth mentioning that the field of lipid peroxidation as signal or damage in the biological environment is a very active subject of research and is addressed by several reviews and books in an interdisciplinary context, making up some of the oxidative stress pathways in biology and medicine.

However, as described in the previous sections, the discovery of fatty acid isomerization drew attention to the role of the natural cis lipid geometry and its reactivity under free radical conditions to give trans isomers. Therefore, the actual scenario of radical stress cannot overcome the question related to the maintenance of the thermodynamically fragile cis geometry. In this context, biomimetic chemistry provides models related as strictly as possible to the cellular environment, using natural substrates and biological concentrations, and taking into account the highly competitive scenario that is constantly present in the in vivo environment. Micelles can be obtained from the free fatty acid dispersed in aqueous medium together with a surfactant and an initiator, and up to now have been studied for reaction rate constants and outcomes of oxidative processes. On the other hand, vesicles can be obtained in the form of multilamellar vesicles (MLVs) and small or large unilamellar vesicles (SUVs, LUVs) and can be used for studying peroxidation and isomerization processes. Unilamellar vesicles are more appropriate models for the cellular environment than multilamellar vesicles, due to the presence of a single bilayer such as in natural membranes. Reports on the peroxidation process emphasized that the kinetic rate law for the oxidation of lipids in compartmentalized systems can be significantly different from that observed when the oxidation takes place in homogeneous solutions.

6. GEOMETRICAL ISOMERISM IN BIOMIMETIC RADICAL CHEMISTRY

6.1. Biomimetic Models (Micelles and Vesicles)

Fatty acids are ideal compounds for biomimetic chemistry, either as “imitations of a natural chemical process” or “mimics of a biological material in its structure or function.” In the interdisciplinary field created by life sciences, biomimetic chemistry has the very important role of providing mechanistic and molecular information useful to help the full understanding of biological processes and metabolic consequences. In particular, in the last two decades, lipid reactivity has emerged as an important piece of information to be connected with multiple signaling and functional pathways in living organisms. As far as fatty acid-based structures are concerned, in an aqueous environment they exist as organized systems, such as micelles and vesicles. Micelle aggregations mostly model the lipid organization involved in the digestive or pulmonary tracts

GC analysis and to have their trans content determined. The results can be summarized as follows: (i) the efficiency of isomerization depends mainly on the method of generation of thiyl radicals, (ii) for a particular fatty acid, the isomerization trend in solution is similar to the analogous isomerization of fatty acid methyl esters, that is, it produces only geometrical isomers, (iii) for PUFA residues, it is envisaged from the isomerization profile of mono-trans and di-trans isomers that the step-by-step mechanism shown in Scheme 5 operates, and (iv) each cis double bond isomerizes with the same efficiency independently of the position on the fatty acid chain, that is, the two mono-trans isomers of linoleate residues are formed in the same amounts as occur with the four mono-trans isomers of arachidonate residues. Evidence that the effectiveness of the cis–trans isomerization in the presence of 0.1–0.2 mM of oxygen is the same has also been obtained in the experiments with SAPC and soybean lecithin.

Scheme 9. Structure and Composition of Fatty Acid Residues of Various L-α-Phosphatidylcholines

<table>
<thead>
<tr>
<th>L-α-Phosphatidylcholines (PC)</th>
</tr>
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<tbody>
<tr>
<td><strong>Soybean Lecithin</strong></td>
</tr>
<tr>
<td>Palmitoyl</td>
</tr>
<tr>
<td>Stearoyl</td>
</tr>
<tr>
<td>Oleoyl</td>
</tr>
<tr>
<td>Vaccenoyl</td>
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<tr>
<td>Linoleoyl</td>
</tr>
<tr>
<td>γ-Linolenoyl</td>
</tr>
<tr>
<td><strong>Egg Lecithin</strong></td>
</tr>
<tr>
<td>Palmitoyl</td>
</tr>
<tr>
<td>Stearoyl</td>
</tr>
<tr>
<td>Oleoyl</td>
</tr>
<tr>
<td>Vaccenoyl</td>
</tr>
<tr>
<td>Linoleoyl</td>
</tr>
<tr>
<td>Arachidonoyl</td>
</tr>
</tbody>
</table>

POPC: R₁ = Palmitoyl, R₂ = Oleoyl
DOPC: R₁ = R₂ = Oleoyl
SAPC: R₁ = Stearoyl, R₂ = Arachidonoyl
6.1.1. Isomerization of Linoleic Acid in Micelles. There is only one report that evaluates peroxidation and isomerization in a biomimetic model under competitive conditions, showing that one process does not exclude the occurrence of the other. Using ionizing radiation as initiating conditions, aqueous suspensions of micelles using linoleic acid (LH) reacted under a variety of conditions, where thyl radicals are the main reactive species. Under anaerobic conditions, only the cis–trans isomerization was observed, and the addition/elimination of thyl radicals was calculated with a catalytic turnover number of 370, and a substantial amount of hydroperoxides (LOOH) were also produced; for example, 500 mM of LH gave 20 mM of LOOH and 10% conversion of LH into mono-trans isomers after γ-irradiation with 400 Gy (Figure 9). These results confirmed the endogenous mono-major isomerization in PUFAs under oxygen-free conditions. Nonetheless, it is clear that other pathway exists, which may result in the formation of di- and tri-trans isomers.

6.1.2. Isomerization of Unsaturated Residues of PC in Multilamellar Vesicles. Initial studies of cis–trans isomerization of unsaturated moieties of DOPC vesicles catalyzed by thyl radicals were performed in multilamellar vesicles (MLVs), using HOCH₂CH₂SH and varying the nature of initiation (the hydrophilic azo compound AAPH at 37 °C or γ-irradiation). Using ionizing radiation as initiating conditions, or UV-photolysis). The role of different thiols in the endogenous trans fatty acid formation will be discussed in section 6.2. Radiolysis of neutral water leads mainly to three reactive species: solvated electrons (e⁻aq), hydrogen atoms (H•), and hydroxyl radicals (HO•) as shown in Figure 10 (the values in parentheses represent the radiation chemical yields (G) in units of μmol J⁻¹). In a N₂O-saturated solution (~0.12 M of N₂O), e⁻ are transformed into HO• radicals (k = 9.1 × 10⁹ M⁻¹ s⁻¹); therefore, the main reactive species are HO• radicals and H• atoms (in a 9:1 ratio). In the presence of thiols, like the amphiphilic HOCH₂CH₂SH, hydrogen abstraction by HO• and H• directly produces thyl radicals (k = 6.8 × 10⁹ M⁻¹ s⁻¹ for HO• and kH = 1.7 × 10⁸ M⁻¹ s⁻¹ for H•).

Initial studies of cis–trans isomerization of unsaturated moieties of DOPC showed that the isomerization rate followed the lipophilicity order of thiols. For example, DOPC with HOCH₂CH₂SH in LUVET gave the highest e⁻ trans major isomerization in PUFAs under oxygen-free conditions. Nonetheless, it is clear that other pathway exists, which may result in the formation of di- and tri-trans isomers.

6.1.3. Isomerization of Unsaturated Residues of PC in Large Unilamellar Vesicles. Liposomes can be represented as shown in Figure 10, that is, a double layer formed by the spontaneous organization of phospholipid components in water, which delimits an aqueous cavity. Fatty acid tails can be saturated or unsaturated, and the disposition of the double bonds in the vesicle depends on the supramolecular arrangement of the bilayer. Unilamellar vesicles are the closest models of biological membranes, and they can be formed by different techniques, such as the extrusion methodology.

The biomimetic model, made of a cis fatty acid-containing glycerophospholipid in the form of an LUV of ca. 90 nm diameter, was studied by varying the lipophilicity of the thyl radical and its method of generation (AAPH at 37 °C, γ-irradiation, or UV-photolysis). The role of different thiols in the endogenous trans fatty acid formation will be discussed in section 6.2. Radiolysis of neutral water leads mainly to three reactive species: solvated electrons (e⁻aq), hydrogen atoms (H•), and hydroxyl radicals (HO•) as shown in Figure 10 (the values in parentheses represent the radiation chemical yields (G) in units of μmol J⁻¹). In a N₂O-saturated solution (~0.12 M of N₂O), e⁻ are transformed into HO• radicals (k = 9.1 × 10⁹ M⁻¹ s⁻¹); therefore, the main reactive species are HO• radicals and H• atoms (in a 9:1 ratio). In the presence of thiols, like the amphiphilic HOCH₂CH₂SH, hydrogen abstraction by HO• and H• directly produces thyl radicals (k = 6.8 × 10⁹ M⁻¹ s⁻¹ for HO• and kH = 1.7 × 10⁸ M⁻¹ s⁻¹ for H•).

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is, it was unable to reach the hydrophobic fatty acid chains. The reactivity of hydrophilic thiols as well as other sulfur-containing compounds will be reinterpreted later in the context of tandem protein–lipid damage (section 6.2.2). The presence of 0.2 mM of oxygen did not influence the effectiveness of this geometric isomerization. Evidence that no positional preference for isomerization of fatty acid residues at the sn-1 and sn-2 positions of PCs was also obtained for DOPCs in LUVET catalyzed by HOCH$_2$CH$_2$S$^•$ radicals.\textsuperscript{174,222} Like the MLV experiments, the catalytic turnover number of LUV isomerization demonstrated in vitro, and the trans–cis isomerization is driven by both the supramolecular arrangement of the hydrocarbon tails and the highly defined lateral diffusion. The preferential order of double bonds starts with positions 5 or 8 of arachidonate residues, followed by position 9 of oleate and linoleate, etc. In particular, the isomerization rate of the 9<sup>cis</sup> position of linoleate is very similar to that of 9<sup>cis</sup> in the oleate residues.\textsuperscript{205} For a particular PUFA residue, high regioselectivity is also observed for the formation of mono-trans isomers: in the linoleate residues, the 9<sup>trans</sup> isomer prevailed over the 9<sup>cis</sup> Isomer, whereas in the arachidonate residues the isomerization preferentially occurred in the 5 and 8 positions. These findings are important for monitoring the endogenous formation of trans lipids (vide infra).

### 6.2. Possible Biological Sources of Isomerizing Radical Species

Experimental results supported the correlation of the origin of an endogenous formation of trans lipids with radical stress conditions and formation of isomerizing reactive species. However, the most feasible culprits for the cis–trans isomerization in vivo are still a matter of discussion. Thiyl radicals RS$^•$ are favored on the basis of the efficiency of the thiyl radical-catalyzed cis–trans isomerization demonstrated in vitro, and the presence of several sulfur-containing compounds at millimolar concentration in the cell. Indeed, the role of thiyl radicals as radical stress inducers emerged from research that counteracts the long-standing and most well-known beneficial effects of thiols.\textsuperscript{223,224} The damage starts from the consideration that, in the so-called “repair reaction”, thiols can efficiently stop the radical cascade, by donating a hydrogen atom and trapping the radical intermediate of the chain. However, by donating a hydrogen atom, the thiol is transformed into the corresponding thiyl radical (RS$^•$), which can in turn attack a substrate. The location of the Cys residue, the most common thiol moiety in biological substrates, has relevance, because membrane proteins containing sulfur moieties are known. However, free cysteine and glutathione are not located in the bilayer.

A crucial question arose as to the most likely candidates for the biological production of diffusible thiyl radicals. Experiments were designed to answer this question and contribute to the understanding of how radical stress conditions can work in the biological environment. In the course of this investigation in our group, we developed the concept of an “integrated vision of protein–lipid damage”, which is relevant for lipidomics and proteomics and is explained in Figure 11.\textsuperscript{235–238} Diffusible thiyl radicals generated by radical stress are thought to be mainly responsible for the isomerization of membrane unsaturated lipids, with two principal processes for their generation: (i) from small sulfur-containing molecules, and (ii) from proteins or peptides containing sulfur moieties (Figure 11).\textsuperscript{226,229,231} In the latter case, a radical reaction with a primary target amino acid will lead to modified proteins or peptides and secondary diffusible radicals that cause isomerization of membrane lipids. These species may also be engaged in radical- and/or electron-transfer processes with additional protein amino acids, so that the ultimate location of radical damage may potentially be far from the site of initial radical attack.\textsuperscript{232}

NO$_2^•$ radicals have already been discussed above with respect to their isomerization potential (sections 5.2.3 and 5.3.2). In biological systems, NO$_2^•$ radicals could be assumed to initiate cis–trans isomerization, but their realistic potential appears to be very poor.\textsuperscript{226} Considering the complexity of the cellular environment and the fact that NO$_2^•$ is in the presence of biologically important substrates such as thiols and urate, a scenario for this reactivity emerges; indeed, with the former antioxidant it generates thiyl radicals, whereas the latter is an important “sink” for NO$_2^•$ in the vascular compartment, where the thiol concentration is very low, except within erythrocytes.\textsuperscript{233} Therefore, it can be confidently predicted that production of NO$_2^•$ in the cell (e.g., the cytosolic compartment) is followed within a few tens of microseconds by reaction with (mainly) glutathione and the formation of thiyl radicals. It could be useful to develop effective biomimetic models for this reactivity, and in this context a recently proposed delivery system of NO$_2^•$ for biological media could be of some help.\textsuperscript{234}

![Figure 11. Integrated vision of protein–lipid damage.](image)

#### 6.2.1. Small Diffusible Sulfur-Centered Radicals

Considering biologically relevant sulfur-containing species, the simplest thiol, hydrogen sulfide (H$_2$S), attracted interest as a naturally occurring gas with roles in nervous and cardiovascular systems, and in pathological conditions such as inflammation. At least three enzymes are responsible for hydrogen sulfide formation in vivo, which provide up to millimolar concentrations.\textsuperscript{235–238} Chemically, the reactions of the simplest S-centered radical (HS$^•$) are not as well-known as those of other
thiyl radicals, although a few papers have characterized some key reactions in the aqueous phase, and in liposomes. Indeed, different neutral radicals (e.g., HS· and HSS·) and radical anions (e.g., S−, HSS−, and HSSH−) can be produced, but not all of them are isomerizing agents. The use of the biomimetic model of vesicle suspension demonstrated the potential of thiol radicals (HS·/S−) derived from H2S to reach the hydrophobic fatty acid chains and attack double bonds, thus producing phospholipids containing trans-fatty acid residues in high yield (Scheme 10).

Scheme 10. Sulphydryl Radical-Catalyzed Isomerization of Cis Phospholipids in DOPC-LUVET

\[ \text{HS}^+ \leftrightarrow \text{S}^{2+} + \text{H}^+ \quad [\text{pK}_a (\text{HS}^+) = 4] \]

\[ \text{S}^{2+} + \text{R}_1 \text{R}_2 \rightarrow \text{H} \cdot \text{H} \cdot \text{R}_1 \text{R}_2 \rightarrow \text{S}^{2+} + \text{R}_1 \]

In the initial work on the thiyl radical-mediated isomerization of oleic moieties in DOPC large unilamellar vesicles, the geometric isomerism with hydrophilic cysteine under γ-radiolysis at natural pH was observed. In comparison with homocysteine, in which the SH moieties are separated by an extra CH₂ group, it was suggested that H-abstraction from the activated α-C position (affording radical 5 and subsequent β-fragmentation) generates HS· radicals, which are able to migrate into the lipophilic compartment and isomerize the double bond (Scheme 11).

Scheme 11. Proposed Mechanism for the Formation of Sulphydryl Radicals from the γ-Radiolysis of Cysteine

Experimental evidence has been recently presented that the thiyl radical generated in peptidic Cys residues undergoes various hydrogen transfer reactions with the C–H bonds, including the 1,3-hydrogen transfer reported in Scheme 12. An important experimental result, in connection with sulphydryl radical formation, is the ultimate conversion of the Cys residue into dehydroalanine residue, likely via β-elimination following a 1,3-hydrogen transfer of the initial thiyl radical. Although the rate constant for the reaction 7 → 8 is missing, those for the analogous reactions of radicals 9 and 10 were estimated by pulse radiolysis techniques to be 3 × 10⁴ and 5 × 10³ s⁻¹, respectively.

Scheme 12. Proposed Mechanism for the Transformation of Cysteinyl Radicals to Sulphydryl Radicals

The interaction of hydrated electrons (e⁻aq) and H⁺ atoms (primary species of γ-radiolysis) with disulfide-containing compounds in aqueous solution is summarized in Scheme 13. The disulfide radical anion derived from the direct electron attachment is generally in equilibrium with the sulfuryl radical, which is also obtained by the direct H⁺ atom attack on the sulfide moiety. Both reactive species dissociate reversibly into two entities, RS· and RSH (or RS⁻). Dibutyl disulfide was used in the isomerization of methyl oleate, and dimethyl disulfide in POPC. In the table inset of Scheme 13, the resulting elaidate residues after irradiation of 2 mM POPC in LUVET and 75 μM CH₃SSCH₃ are reported at different irradiation doses.

Scheme 13. Mechanism of Reaction of e⁻aq and H⁺ Atoms with Dialkyl Disulfide in Aqueous Solution

<table>
<thead>
<tr>
<th>Dose, Gy</th>
<th>Trans, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>84</td>
<td>82.2</td>
</tr>
<tr>
<td>126</td>
<td>90.9</td>
</tr>
</tbody>
</table>

In the case of the thioether function of methionine, hydrogen atom attack caused the formation of a sulfuryl type radical 12 (Scheme 14), which then undergoes a rapid desulfurization; thus, radical 13 is produced with formation of CH₃SH, a diffusible species that under radical stress conditions exists as the corresponding CH₃S· radical. Radical 13 is the precursor of α-aminobutyric acid (Abu).

Scheme 14. Mechanism of Reaction of H⁺ Atoms with Methionine in Aqueous Solution and the Formation of Diffusible CH₃S· Radical

6.2.2. Tandem Protein–Lipid Damage. Tandem lesions can be generated by a consecutive process occurring on two different molecules and can involve two different cell compartments, such as in the case of lipids with DNA or with proteins. The biomimetic models described above for cysteine, glutathione, and methionine were further developed to address lipid–protein damage and extrapolate to the more complex situations present in vivo. In this context, ionizing radiation also allowed specific damage to be examined as...
consequences of the reactivity of H-atoms and hydrated electrons, thus establishing the molecular basis of reductive radical stress. This concept complements the well-known oxidative stress and gives a better understanding of the global consequences of radical stress in living systems.

In the tandem protein—lipid damage process, as depicted in Figure 11, diffusable thyl radicals can derive from initial damage to sulfur-containing proteins/peptides causing the release of diffusible species, which are able to effect a second damage by isomerization of membrane lipids. The model mimicked two biological compartments, the membrane and the extracellular protein domain. In particular, peptide/proteins at micromolar concentration levels were added to a vesicle suspension (with lipids in millimolar concentration), the vesicles being formed by 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) or 1,2-dioleoylphosphatidylcholine (DOPC), where one or both fatty acid chains present the double bond in the cis configuration. The resulting proteo-liposome suspension was exposed to a radical stress condition, followed by isolation and trans-esterification of the phospholipids to the corresponding FAME, which allow the trans isomer content to be obtained by GC analysis. Figure 12 shows the integrated vision of radical stress as a combination between the lipid modification and the protein modification libraries.

Several sulfur-containing proteins have been chosen as models for these biomimetic studies: (i) Met-enkephalin, which contain a single Met residue, (ii) medium-sized proteins like RNase A and lysozyme (Lyso) that contain both Met residues and disulfide bridges with no thiol group present, (iii) metallothioneins (MTs) rich in Cys content that contain labile sulfide anions as nonproteic ligands, and (iv) large proteins like human serum albumin (HSA) that contain Met residues, disulfide bridges, and free thiol groups. Thyl radicals were found to be readily generated from various functionalities: from a free SH group by hydrogen abstraction, by cleavage of disulfide linkages, by attack at SMG groups in methionine residues, or at the inorganic sulfur of metal—sulfur clusters. In the latter two cases, a desulfurization process takes place with formation of S-centered radicals (CH_{3}S or S•), which in turn can migrate from the aqueous to the lipid bilayer, causing cis—trans isomerization of unsaturated fatty acid residues through their reversible addition to double bonds (Figure 12). The modification of S-containing proteins under radical stress was treated in recent reviews. Here, it is only mentioned that reductive stress occurs under anaerobic or hypoxic conditions, where H• and electrons cannot be rapidly trapped by oxygen. Under these conditions, for disulfide bonds one cysteine function was found to be mutated into alanine (Ala), whereas methionine (Met) mutated into α-amino butyric acid (AbA). The first case of Ala is a mutation very rarely reported in vivo for cysteine, whereas the second modification affords an amino acid that is not genetically coded, thus representing a chemical post-translational modification into another natural structure, the biological consequences of which are still not known.

The comparison of two models shown in Figure 13 involved use of pancreatic ribonuclease A (RNase A) and lysozyme (Lyso), the importance of the sequence and secondary structure for the reactivity and potential to generate small radicals involved in tandem damage was evidenced. Lyso is a globular protein containing 129 amino acids, including two Met residues and four pairs of Cys residues, forming four intramolecular disulfide bonds, whereas RNase A is a small globular protein with a single polypeptide chain, containing a total of 124 amino acid residues including four Met residues, four disulfide bonds, and no free Cys. Under similar conditions using the proteo-liposome systems, the percentages of trans isomers formed in DOPC vesicles with the two proteins as a function of irradiation dose are shown in Figure 13. Isomerization trends were different, with a 10-fold greater efficiency of RNase A in inducing lipid isomerization as compared to Lyso at the same irradiation doses.

On the other hand, the proteo-liposomes revealed different reactivities of metallothioneins (MTs). These proteins are a unique class of low-molecular weight metalloproteins, which have different arrangements of metal—thiolate clusters formed by cysteine or histidine with endogenous sulfur content, and regulate the exogenous concentration of sulfide anions (S2−). Moreover, metal ions present in the metal—MT complexes influence the extent of isomerization. For example,
substantial differences were observed in the behavior of QsMT depending on the presence of copper, zinc, or cadmium ions (Figure 14). Cd\(^{2+}\)-MTs generally have a higher capacity to induce cis–trans isomerization than Zn\(^{2+}\) or Cu\(^{2+}\)-MTs.\(^{260}\) The higher isomerization yields obtained in the presence of Cd\(^{2+}\) MT complexes can be due to the larger number of acid-labile S\(^{2-}\) ligands in the Cd aggregate (3.0 S\(^{2-}/\)MT) than in the Cu\(^{2+}\)-MT and Zn\(^{2+}\)-MT preparations (the S\(^{2-}/\)MT ratios are, respectively, 0.26 and 1.0).\(^{257}\) This suggested a possibly major role of the S\(^{2-}\) present in the metal clusters as a precursor of diffusible isomerizing radicals (HS\(^{-}/\)S\(^{2-}\)). In addition, the different content of acid-labile S\(^{2-}\) ligands in the metal complex induces also a slightly different folding of the Zn\(^{2+}\)-MT and Cd\(^{2+}\)-MT complexes, as evidenced clearly for QsMT and TpyMT, which can also affect the isomerization yield.\(^{262}\) Inorganic sulfides were also found to be effective in the cis–trans isomerization of lipid vesicles.\(^{257}\)

An interesting case for further applications in diagnostics can be found with human serum albumin (HSA), which is the most abundant carrier protein and accounts for over 50% of the total plasma protein content. HSA contains 6 Met and 35 Cys residues that form 17 disulfide bridges, leaving one free SH group. The coupled lipid–protein damage was identified by using POPC suspensions containing HSA.\(^{258}\) In parallel, proteomic analysis of the irradiated protein was carried out and revealed desulfurization sites of specific Met and Cystine residues in the sequence. MALDI-TOF and semiquantitative LC-ESI-IT-MS/MS peptide mapping experiments on different enzymatic digests demonstrated that desulfurization selectively affects Cys34, Met123, Met298, Cys514, Met548, and Cys567 to form Ala and Aba, as well as the isomerizing species (Figure 15).\(^{258}\) This model can find an interesting application in diagnostics, for the examination of biological samples of human plasma through an integrated analytical panel combining protein and lipid characterization, that is, detection of trans lipids and desulfurized protein residues. In this context, the model studies can provide rapid access to libraries of modified substrates to be used as references in the analytical protocols. It is also worth noting that the experiments with proteoliposomes suggest an interesting application for a very sensitive model system to assay radiation exposure, via the proteic sulfur reactivity and the amplification effect given by the catalytic cycle of the thyl radical-based cis–trans isomerization.

Figure 16 depicts the reaction of reducing radical species with protein cystine moieties, emphasizing the possible occurrence of both disulfide radical anion (in the case of eaq\(^{-}\)) or sulfuranyl radical protein intermediates (in the case of H\(^{+}\) atoms), which are present in a protonation equilibrium. The modification of Cys to Ala was highly indicative of the formation of the perthyl radical as a reaction intermediate,\(^{254,258}\) thus confirming previous EPR observations.\(^{263}\) We envisaged that the key feature is the equilibrium between the sulfuranyl radical and the couple RS\(^{+}/\)RSH, which is favored by the juxtaposition of reacting centers within the protein tertiary structure (cf., Scheme 13). This topotactic effect overcomes the unfavorable thermodynamic and kinetic factors, thus leading to a perthyl radical that was unexpected from a random distribution of reacting centers.\(^{263}\)

From biomimetic chemistry in aqueous suspensions, a conclusive and overall picture has emerged pointing out the role of thyl radicals as efficient catalysts for cis–trans isomerization of lipids in bilayers, with a potentially important route to radical damage of biological components as well as to sensitive tools for detecting early radical stress in cells.

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**Figure 14.** Dose dependence of the formation of elaidate (trans isomer) residues from \(\gamma\)-irradiation of N,O-saturated, 10 mM H\(_2\)PO\(_4\)^− solutions containing POPC vesicles (1.7 mM) and Cd\(^{2+}\), Zn\(^{2+}\), or Cu\(^{2+}\)-QsMT complexes (30 mM). The reactive species are HO\(^{+}\) radicals and H\(^{+}\) atoms.

**Figure 15.** Sulfur-containing amino acid residues (Met and Cys) are modified by attack of H\(^{+}\) and/or eaq\(^{-}\), yielding diffusible S-centered radicals such as CH\(_{3}\)S\(^{-}\) or HS\(^{-}/\)S\(^{2-}\), which are capable of migrating to the lipid bilayer and inducing cis–trans isomerization of unsaturated fatty acids.

**Figure 16.** Proposed mechanism for the formation of alanine residues and S\(^{2-}\) radicals in irradiated proteins.
7. MEMBRANE MODELS AND REGULATION OF PROTEIN FUNCTIONING

Biological membranes present a large variety of fatty acids with different carbon chain lengths and numbers of double bonds, their assembly being crucial for the tuning of biophysical properties connected to biological responses. The use of model systems for gathering quantitative information on the lipid organization and corresponding effects have greatly contributed to the realization that biological functions are controlled by basic physical principles. Model studies on trans fatty acids were carried out, in particular the trans isomer of oleic acid, because it is the most common monounsaturated fatty acid in natural sources. The structures of oleic acid (9cis-C18:1) and its trans geometrical isomer, elaidic acid (9trans-C18:1), are shown in Figure 1.

From the large difference in the melting points of the corresponding free fatty acids (13.4 °C for the cis isomer and 44 °C for the trans isomer, whereas the corresponding saturated fatty acid, stearic acid C18:0, melts at 72 °C), one would expect tremendous differences for this geometric change, and it is easy to understand why the trans configuration has a completely different effect on the fluidity of the phospholipid bilayer, as compared to the cis and saturated structures at physiological temperature. The replacement of one cis acyl chain by a trans fatty acid in phosphatidylethanolamines increases the transition temperature by 18–31 °C, depending on the structure of the other acyl chain of the lipid molecule. Change of cis unsaturated fatty acids to those obtained by trans configuration gives a significant reduction of the membrane fluidity, which is however smaller than the replacement of cis by saturated fatty acids. The effect of saturated, cis, and trans monounsaturated lipids was evaluated on the basis of the dimensions of the corresponding unilamellar vesicles. In Table 3 are compared liposome dimensions obtained from 1,2-

Table 3. Dimension of Liposomes Using Cis, Trans, and Saturated Phospholipids

<table>
<thead>
<tr>
<th>phospholipid</th>
<th>diameter (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>POPC</td>
<td>97 ± 10</td>
</tr>
<tr>
<td>PEPC 83%</td>
<td>72 ± 8</td>
</tr>
<tr>
<td>DPPC</td>
<td>45 ± 8</td>
</tr>
</tbody>
</table>

dipalmitoylphosphatidylcholine (DPPC, with two saturated fatty acid residues of palmitic acid, 16:0) to those obtained from 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) and with 1-palmitoyl-2-elaidoylphosphatidylcholine (PEPC).

It is clearly seen that the three vesicles have different sizes, trans fatty acids forming the compartment with an intermediate dimension between saturated and cis lipids. Only the cis configuration results in the largest vesicles, whereas the critical aggregation concentrations (CAC) of the three lipids are almost the same (in the range of (4–5) × 10^{-10} M). The different lipid structures influence the size of the resulting vesicles more than the critical number of molecules needed for the aggregation process. From this experiment, the role of cis geometry emerged for the first time as crucial for ensuring variations in the biological compartment sizes, as required for eukaryotic cells. It would be interesting to estimate the lipid contribution to the cell size, which is a matter of a lively scientific debate. As matter of fact, the presence of PUFA molecules containing two or more double bonds is the lipid signature of life evolution from prokaryotes, where unsaturation is provided only by cis and trans MUFAs (see section 4.2), to eukaryotes.

The presence of trans lipids in membranes also has a profound influence on temperature sensitivity as tested by insertion of a probe sensitive to the lipid environment, such as cis-parinaric acid, using accurate stopped-flow fluorescence measurements. Vesicles containing cis and trans phospholipids (POPC and PEPC) in different proportions were compared. In particular, using different temperatures in the range 10–35 °C and by monitoring the fluorescence decay of the probe, trans-containing vesicles were found to be the most sensitive to thermal conditions with a "switch" point for fluorescence increase at temperatures <25 °C. By further reducing the percentage of trans isomers, the response to the temperature factor becomes less and less, disappearing below the 5% trans content. This limit value may also be affected by the type of fatty acid residues present in the membranes. The transition temperature for POPC is −3 °C, whereas for 83% PEPC-containing vesicles this is 22.7 °C; therefore, only the trans isomer is in an ordered crystalline phase at ambient temperature, which reduces the interaction of the probe with the lipid bilayer and with itself, slowing the florescence decay. Another investigation on model membranes containing different monounsaturated trans fatty acid residues (9trans-C14:1, 9trans-C16:1, and 9trans-C18:1) determined that the affinity for cholesterol was 40–80% higher than their cis analogues, probably due to a better interaction between the straight trans acyl chain and the cholesterol molecule.

The trans geometry was also evaluated with respect to its influence on membrane protein behavior. The influence of the trans geometry on rhodopsin, a prototypical member of the G-protein coupled receptor family, was evaluated. In trans membrane models, the level of rhodopsin activation was diminished, in particular at lower temperatures (5 °C) at which trans isomers are in the gel state, whereas cis isomers are in the fluid state. Monounsaturated trans isomers of different chain length (C14, C16, C18) were also tested for their effect on the activity of γ-secretase, an enzymatic complex that hydrolyzes the β-amyloid precursor protein (APP), generating the amyloid β-peptide (Aβ) of Alzheimer’s disease. The complex needs to move within the lipid layer to allow substrate docking, lateral gating, cleavage, and release of products. By using different proportions of the fatty acid isomers in a mixture with POPC, it was determined that the γ-secretase activity is always affected. Going back to the late 1980s, it can be seen that studies in cellular and murine models drew attention to the effects of trans fatty acids on membrane functioning, including channels and membrane proteins. Newer insights have been more recently provided in connection with cellular signaling cascades. In human aortic endothelial cells, trans fatty acids (elaidic and linoleic acid, 9trans,12trans-C18:2) and stearic acid (C18:0) increased phosphorylation of the ICAM-1 transcriptional regulator, nuclear factor-κB (NF-κB), inducing a pro-inflammatory response due to the elevation of the ICAM expression. Analogously, differential activation of endothelial superoxide production, NF-κB activation, and reduction in NO production by different C18 isomers were observed in endothelial cells, suggesting that the location and number of trans double bonds are important determinants to target the NF-κB response. The effects of cis and trans phospholipids were also appreciated by comparing the effects of DEPC and DOPC on the sarcoplasmic reticulum (SR) Ca^{2+}-ATPase (SERCA) parameters, in particular V_{max} and K_{Ca}.

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finding different behavior that could be mainly attributed to the difference in fluidity of the corresponding bilayer organizations.276 The activity of NADPH oxidase was also evaluated, which is an integral protein complex producing non-mitochondrial reactive oxygen species in the form of the superoxide anion in phagocytes, as an indispensable event for the destruction of engulfed pathogens. The functioning of NADPH oxidase is regulated spatially and temporally by the translocation of subunits from cytoplasm to the membrane partner, the second messenger involved being arachidonic acid. As previously described (see sections 5.3 and 5.6), this omega-6 PUFA is an important target for the isomerization reaction, because it contains four double bonds and is highly susceptible to radical attack, producing mono-trans arachidonic acids (mono-trans AA). These isomers can target both of the membrane fractions, containing the cytb558 and the cytosolic p67phox, causing a down-regulation of enzymatic activity and superoxide production.277 It was suggested that the loss of the natural cis geometric feature induces substantial structural modifications of p67phox that prevent its translocation to the complex.

Another interesting application of the different biological effects of trans fatty acid isomers was proposed with the all-trans PUFA structures. In fact, PUFAs have many important biological activities connected to the presence of all-cis double bonds, which were already affected by the geometrical change of one double bond. All-trans PUFA represent a novel “anti-sense” strategy, because they can still target lipid pathways but do not display different effects or enzymatic blockage, such as described for DNA agents. The synthesis of all-trans arachidonic acid was carried out, and the first experiments demonstrated its inhibiting effect on PAF-induced rabbit platelet aggregation, thus showing a precise difference from the natural cis isomer.278 These compounds were also recently tested in comparison with arachidonic acid on an enzymatic pathway, the xanthine dehydrogenase/xanthine oxidase interconversion, which responds to oxidative stress.279 The all-trans isomer demonstrated its activity to potentiate the enzymatic pathway in rat liver cytosol in vitro, and promotes the interconversion, with an opposite effect as compared to the natural isomer.

Overall, research results draw attention to how activity modulation and responses can be achieved through mechanisms involving membrane fluidity and specific interactions of fatty acids with membrane constituents, highlighting the role of the trans geometry as a distinct and distinguishable input among the possible conformational changes of natural lipids. Biological effects of free radicals and molecular mechanisms involved in the lipid geometric isomerism make up part of the cellular and tissue impairment behavior under stress conditions.280,281 So far, lipid isomerization has been underestimated as compared to the effects of lipid peroxidation, as free radical processes involved in cell signaling and tissue functions. It is also expected that the topic of lipid geometry can be extended to other interesting applications, as initially shown in the case on active membrane drugs282 and nanostructure organization.283

8. LIPID GEOMETRICAL ISOMERISM IN DIAGNOSTICS

The application of “fatty acid-omics” to the evaluation of metabolic and nutritional pathways is growing, also in view of the direct application to health and prevention. It is clearly shown that both dietary and metabolic factors influence the resulting lipid balance in the organisms; therefore, lipidomic profiles can be useful to evaluate an individual condition in a comprehensive way.284,285 In the past decade or so, research into lipidomics in health and pathologies began to focus on free radical pathways; however, the availability of reliable analytical methodologies is considered a key factor in facilitating continuing developments in this very lively research area.286 One of these fields is oxidative lipidomics, which studies oxidized fatty acid moieties in cellular lipids to identify new biomarkers in signaling and degenerative processes.287,288 After the basic research on trans fatty acids in membrane models (see section 6.1.3), geometrical stress also started to involve lipidomic studies, attracting more interest in the analytical separation of the different cis and trans isomers (see section 2). Indeed, lipidomics of radical stress is an emerging research field, which stems from the evidence that membranes are well-known to be the cell compartment most involved in stress adaptation and signaling cascades. In this context, lipidomic investigations can be directed either to the membrane lipid remodeling occurring after the stress, or to the endogenously formed trans fatty acid residues as markers of the effects of free radical species, diffusing in the membrane bilayer and having the potential to isomerize the double bonds, as thoroughly discussed in section 6.2. Concerning the role of trans fatty acid as markers of free radical stress, the first studies focused on the levels of these isomers (in particular, oleic, linoleic, and arachidonic acid isomers) in cultured cells289 and in rat tissues.290,291 detected under rigorous trans-free diet conditions. It is worth noting that in the former case trans isomers were present at a low levels in the membrane phospholipids, and increased by supplementing with 10 mM thiols as well as by using radical initiation conditions.289 In rats, a clear difference in the trans percentages of different tissues was noted, erythrocyte membranes having the highest content, likely due to the presence of a significant percentage of arachidonic acid residues in their membrane phospholipids.290 Other in vivo models were examined, finding the significance of trans content also in rats performing physical activity. In this case, several animal groups were compared, differing by the age, the length, and intensity of the exercise and the post-training period, which also influences the life span.292 Interestingly, the decrease in the amount of trans fatty acids and in the inflammatory pathways (i.e., α6/ω3 ratio) in high-intensity trained rats was evidenced, underscoring the protective effect of high intensity aerobic training. In humans, the levels of trans fatty acid isomers are largely reported in consideration of their presence in foods containing partially hydrogenated or manipulated fats and oils. This research is ongoing, raising many concerns in different fields, from pregnancy to cancer to metabolic and neurological disorders.293–299 In most cases, the monitoring carried out in dietary studies was limited to commercially available trans fatty acid references, that is, to structures with one or two double bonds with C16 and C18 carbon atom chains, whereas the most significant endogenous markers, such as mono-trans isomers of arachidonic acid (see sections 5.2.1 and 6.1.3), are available only through synthesis. The use of appropriate references and the effectiveness of analytical protocols are necessary for the thorough evaluation of the trans content and the correct placement of the geometrical isomerism in diagnostics.295,299 Indeed, the two research groups that published the synthesis of the four mono-trans arachidonic acid isomers also reported on their detection studying health conditions that imply free radicals and inflammation, such as...
smoking, dermatological diseases, and cancer in humans.26,300–302 The four mono-trans isomers were found in equivalent amounts in the plasma of smokers,300 whereas different amounts were detected in membrane phospholipids, whether in cells, animals, or humans.21,289–291,302 An interesting observation came also from the analyses of nonagerians and centenarians located in the Cilento area of Italy; in fact, the erythrocyte membrane phospholipids in this cohort of 41 subjects showed a significant increase of elaidic acid (9-trans-C18:1), as compared to the population of the same geographical area and to the general population.303 The specific role of each trans isomer in cell signaling and metabolism still has to be revealed; however, these preliminary data can draw more attention to the effect of each isomer connected to a geometrical change, which must have a precise, yet undiscovered, significance for eukaryotic life, that relies upon the ubiquitous cis geometry.

9. CONCLUSIONS AND PERSPECTIVES

This Review summarized results on trans lipids in diverse research areas, demonstrating the importance of lipid geometrical isomerism and its great applicability to very different fields, from molecular processes to technological advancements. This subject originated from the merger of lipid and free radical research and fostered the development of biomimetic radical chemistry, to provide models for conducting mechanistic and product studies, in conditions strictly related to the complexity of the biological systems. The state of the art in trans lipid research indicates that models have so far provided very useful information transferable to applications in biological, biotechnological, and medical areas. This approach is also important for chemists involved in interdisciplinary research teams aiming at solving puzzling scenarios related to free radicals, the role of membrane stress, and lipid transformations.

Geometrical isomerism has wide significance for chemical, physical, and biological features, as pointed out in the various subjects of this Review. Expansion of trans lipid research can be easily foreseen in analytics, chemical mechanisms, liposome and oil technology, lipidomics, and diagnostics. Innovative fields can also include the design of molecular switches, taking advantage of the different sensitivity of trans- and cis-containing membranes to physical and chemical stimuli, as well as the determination of biological and pharmacological strategies based on the tunable molecular interactions produced by the exchange of the natural cis with the unnatural trans geometry.

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Notes

The authors declare no competing financial interest.

Biographies

Chryssostomos Chatgilialoglu was born in Greece. He received a doctorate degree in chemistry from the University of Bologna in 1976 and completed his postdoctoral studies at York University (UK) and the National Research Council of Canada, Ottawa. He has been research director at the Consiglio Nazionale delle Ricerche (Bologna) since 1991. He received the Fluka Prize “Reagent of the Year 1990”. His research interests lie in free radical reactions and in the past decade have been increasingly addressed to applications in life sciences. He is the author and editor of several books, including the Encyclopedia of Radicals in Chemistry, Biology and Materials published by Wiley in 2012. He is the author or coauthor of over 250 papers. He chaired the COST Action CM0603 on Free Radicals in Chemical Biology (2007–2011), and he is the Chairman of COST Action CM1201 on Biomimetic Radical Chemistry (2012–2016). He is President and cofounder of the spin-off company Lipinutragen.

Carla Ferreri started her scientific career in synthetic organic chemistry, being appointed as research fellow at the University of Napoli “Federico II” in 1984. In 2001 she moved to the Consiglio Nazionale delle Ricerche in Bologna, where she is currently Senior Researcher. Her present research interests are in the field of biomimetic chemistry, investigating free radical transformations of biomolecules related to the molecular pathways of radical stress, and in the transfer of basic mechanisms to biomarker discovery and health applications. She is cofounder of the spin-off company Lipinutragen and leads the R&D in nutrilipidomics and nutraceuticals.
Michele Melchiorre was born in 1980 in Camerino (Italy). He earned his M.Sc. in Chemistry and Pharmaceutical Sciences from the University of Bologna in 2006. He then moved to the University of Florence where he received his Ph.D. in Medicinal Chemistry in 2010, under the direction of Prof. M. N. Romanelli. In 2009, he spent 10 months at Harvard University (Cambridge, MA) as a visiting Ph.D. student in Chemical Biology, under the direction of Prof. S. L. Schreiber and the supervision of Dr. D. W. Young. After his doctoral studies, he joined the Medicinal Chemistry Team at the D3 Department of the Italian Institute of Technology (Genoa) for a few months. Since September 2010, he has worked as a postdoctoral fellow in the BioFreeRadicals group at ISOF-CNR Bologna. His current research interests are focused on trans lipids and free radical stress. He is currently synthesizing molecular libraries for lipid transformation, focusing on lipidomic analysis and biomarker development.

Anna Sansone was born in 1979. She studied Pharmacy at the University “La Sapienza” in Rome, where she graduated in 2004 and in 2008 received her Ph.D. in Pharmaceutical Science, working in the design, synthesis, and biological evaluation of peptides, pseudopeptides, and peptidomimetics. After some collaboration grants conferred by the CNR-Crystallographic Institute in Monterotondo-Rome, the Cenci Bolognetti Foundation in Rome, and the Chemical Science Department of Padua University, in 2011 she became a permanent researcher at the ISOF-CNR in Bologna where she joined the group of Dr. Chatgilialoglu. Her research interests concern free radicals from chemical aspects to biological involvements in life sciences, with particular attention to the characterization of protein structures by Raman spectroscopy and evaluation of free radical damage on proteins. She is a coauthor of over 80 papers in international journals.

Armida Torreggiani received her degree in Chemistry in 1992 and her Ph.D. in Biochemistry in 1997 at the University of Bologna (Italy). After several fellowships from the National Research Council, University of Bologna, and Brown University in Providence, RI, she became a permanent researcher in 2001. She is a member of the scientific committee of the European Conference on the Spectroscopy of Biological Molecules and a member of the Management Committee of the Cost Action CM1001 focused on the chemistry of non-enzymatic protein modification-modulation of protein structure and function. In 2011-2012 she was the chair of the Workshop on Oxidative Stress: Damage, Consequences and Repair” of the Italian Society for Research on Radiation and of the II National Congress on Raman Spectroscopy and Nonlinear Effects. Her research interests are centered on chemical biology, with particular reference to characterization of protein structures by Raman spectroscopy and evaluation of free radical damage on proteins. She is a coauthor of over 80 papers in international journals.

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ABBREVIATIONS

AA arachidonic acid
AAPH $\alpha,\alpha'$-azodisobutyramidine dihydrochloride
Ag/HPLC silver high-performance liquid chromatography
Ag/TLC silver thin-layer chromatography
Ala alanine
AOCS American Oil Chemists’ Society
APP $\beta$-amyloid precursor protein
BHT butylated hydroxytoluene
CAC critical aggregation concentration
CE cholesteryl esters
CETP cholesteryl ester transfer protein
CholLin cholesteryl linoleate
CLA conjugated linoleic acid
Cti cis-trans isomerase
Cys cysteine
DEPC 1,2-dieyldoylphosphatidylcholine
DHA docosahexaenoic acid

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DMDS  dimethyldisulfide
DOPC  1,2-dioleoylphosphatidylcholine
DPPC  1,2-dipalmitoylphosphatidylcholine
EI    electron ionization
EPA  eicosapentaenoic acid
EPR  electron paramagnetic resonance
ESI  electrospray ionization
FA   fatty acid
FAE  fatty acid ethyl ester
FDA  Food and Drug Administration
FID  flame ionization detector
FT   Fourier transform
FTIR Fourier transform infrared
FT-IR Fourier transform infrared attenuated total reflectance
FT-NIR Fourier transform near-infrared
GC   gas chromatography
HDL  high-density lipoprotein
HPLC high-performance liquid chromatography
HSA  human serum albumin
IMS  imaging mass spectrometry
IR   infrared
LAME linoleic acid methyl ester
LC   liquid chromatography
LCAT lecithin cholesterol acyltransferase
LDL  low-density lipoprotein
LF   linolenic acid
LOOH lipid hydroperoxides
LOX  lipoxygenase
LUV  large unilamellar vesicles
LUVET large unilamellar vesicles by extrusion technique
Lyso lysozyme
MALDI matrix-assisted laser desorption/ionization
ME   methyl ester
MI   matrix isolation
MLV  multilamellar vesicles
MS   mass spectrometry
MTs  metallothioneins
MUFA monounsaturated fatty acid
NADPH nicotinamide adenine dinucleotide phosphate
NF-xB nuclear factor-xB
NMR  nuclear magnetic resonance
PAF  platelet-activating factor
PC   L-α-phosphatidylcholines
PEPC 1-palmitoyl-2-oleoylphosphatidylcholine
PEPC 1-palmitoyl-2-oleoylphosphatidylcholine
PEPC 1-palmitoyl-2-oleoylphosphatidylcholine
PEPC 1-palmitoyl-2-oleoylphosphatidylcholine
PEPC 1-palmitoyl-2-oleoylphosphatidylcholine
PEPC 1-palmitoyl-2-oleoylphosphatidylcholine
PEPC 1-palmitoyl-2-oleoylphosphatidylcholine
PEPC 1-palmitoyl-2-oleoylphosphatidylcholine
PEPC 1-palmitoyl-2-oleoylphosphatidylcholine
PF  partial least-squares
POPC 1-palmitoyl-2-oleoylphosphatidylcholine
POMGA polyunsaturated fatty acid
QsMT Quercus suber metallothionein
RNase A ribonuclease A
RP   reversed-phase
SAPC 1-stearyl-2-arachidonoylphosphatidylcholine
SB-ATR single-bounce attenuated total reflectance
SERCA sarcoplasmic reticulum Ca2+-ATPase
SFA  saturated fatty acid
SR   sarcoplasmic reticulum
SUV  small unilamellar vesicles
TEC  thiol–ene chemistry
TFA  trans fatty acid
TLC  thin-layer chromatography
TOF  time-of-flight
TpyMT Tetrahymena pyriformis metallothionein
UV    ultraviolet

REFERENCES