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Review

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Lipid Geometrical Isomerism: From Chemistry to Biology and Diagnostics

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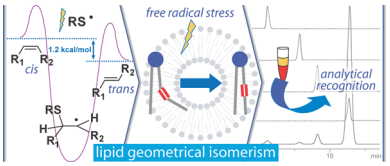

CONTENTS

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., 9*cis* or 9*trans*), 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

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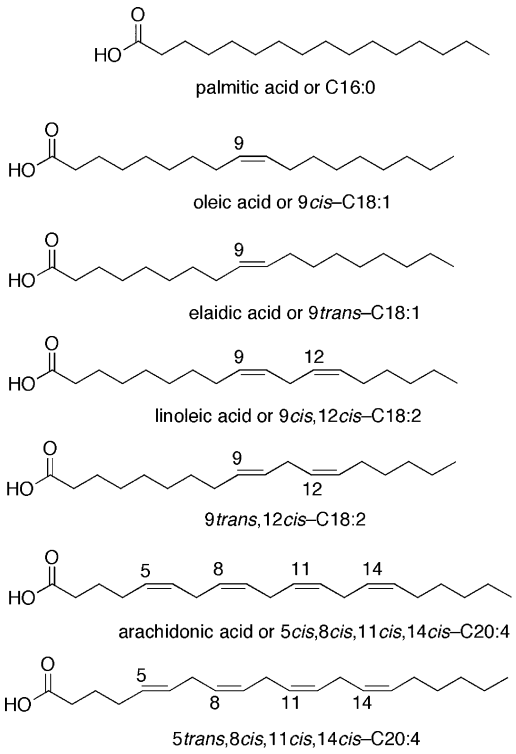

Figure 1. Examples of natural fatty acids and some *trans* isomers.

regiospecific 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 processes 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 thiyl radicals ($RS^{\bullet}$), as well as discussing nitrogen dioxide ($NO_2^{\bullet}$) 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 thiyl radicals generated by the degradation of methionine, H_2S, 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

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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 MUFAs and PUFAs. 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 cholesteryl 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 trans-esterification 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 vegetables 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 PUFAs. 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: 17*trans* < 14*trans* < 5*trans* = all-*cis* < 8*trans* < 11*trans*.[23] In Figure 2 is shown an optimal separation between different

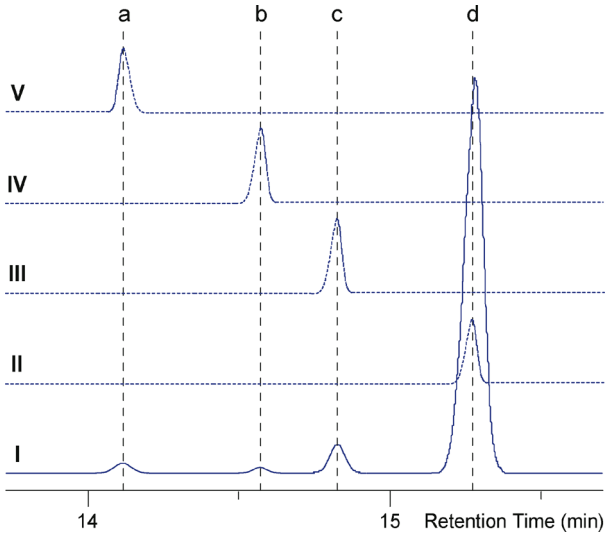

Figure 2. GC region relative to the elution of the omega-3 FAME present in deodorized tuna oil (trace I) using hydrogen as the carrier gas. The peak assignment was obtained due to the commercial availability of pure references of all-*cis* omega-3 fatty acids C20:5 (trace II, peak d), C20:4 (trace III, peak c), and C20:3 (trace V, peak a) and the synthesis of 17*trans* isomer of EPA (trace IV, peak b).[23]

omega-3 PUFAs, such as all-*cis* C20:3, C20:4, and C20:5 fatty acids, the EPA mono-*trans* isomer (17*trans*), and the analysis of a commercial sample of deodorized tuna oil (trace I).[23]

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 17*trans* 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 by a chemical reaction with nitrogen dioxide ($NO_2^{\bullet}$)[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.[21] 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

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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 5*trans* and 8*trans* 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,[28] and different techniques have been exploited.[29−33] 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 monoenes and isolated dienes, where the number of geometrical isomers is 2 and 4, respectively. For PUFAs 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 MUFAs.[34] This consists of two steps: (i) reaction with dimethyl disulfide (DMDS), which adds two CH_3S 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.[28] 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.[35] 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.[35] 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).[36,37] 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,[38,39] argentation techniques have been one of the more 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.[40,41] These complexes are stronger with *cis* than *trans* double bonds, allowing their separation by thin-layer (TLC) and high-performance liquid (HPLC) chromatography.[41,42]

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.[41] The *cis* isomer forms a strong complex with silver ions so it is retained to a greater extent by silica gel (lower R_f), while the weaker complex between *trans* isomer and silver ions produces less retention of the substrate from silica gel (higher R_f). 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.[21,23] 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.[43]

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.[44] 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.[45] For food analysis purposes, such columns are reported for separating *cis* from *trans* monoenoic FAMEs.[40,46,47]

Reversed-phase high performance liquid chromatography (RP-HPLC) was also proposed for the separation of *cis*−*trans* long-chain monounsaturated fatty acids.[48,49] 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.[44,48,49] The separation obtained in RP-HPLC has the advantage of using the lipid extracts directly

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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.[50−54] In 2012, the first contribution concerning the separation of *cis*/*trans* phospholipid isomers was reported.[55] Previously, other studies had reported the use of LC/MS−GC[48] and Ag-HPLC[56,57] 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.[55]

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,[58] iodine value,[59] and, more recently, metabolomics analysis.[60−62] NMR techniques have diagnostic potential for recognizing lipid classes. Both ^{1}H and ^{13}C 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.[63,64] 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).[65] This study exploited the ability of silver ions to form a stronger complex with *cis* MUFA isomers with respect to the *trans* isomers,[42] 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),[66,67] more interesting results were obtained by evaluating the ethylenic ^{13}C 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).[21]

Table 1. ^{13}C NMR Assignment of the Most Deshielded Ethylenic Carbon Atom of Arachidonic Acid and EPA Methyl Esters (AA-ME and EPA-ME, Respectively)[a]

	AA-ME[21]				
	14*trans*	11*trans*	all-*cis*	5*trans*	8*trans*
C15, ppm	131.16	130.73	130.55	130.51	130.46
	EPA-ME[23]				
	17*trans*	14*trans*	5*trans* + all-*cis*	8*trans*	11*trans*
C17, ppm	132.57	132.24	132.03	132.01	131.96

[a]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 ^{13}C NMR spectrum (the most deshielded ethylenic carbon atom) for resolving the mono-*trans* geometrical mixture.[23] 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,[68] 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.[61]

2.5. Infrared (IR) Spectroscopy

The historical importance of IR spectroscopy in the elucidation of the molecular structure of lipids[69] 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.[12,14,70] 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.[71−75] 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).[76,78−80] 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,[81] 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).[76,78−80] In particular, Fourier transform infrared-attenuated

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Table 2. Characteristic Bands Used with Limitations and Advantages of Each Spectroscopic Method

band (cm^{-1})	band assignment	limitation(s) and advantage(s)	ref
		Conventional IR	
~966	C−H out of plane of *trans* isolated double bond	reported as AOCS method Cd 14-95 replacing the Cd 14-61 method	14
		applied for fats and oils after calibration with standard references; CS_2 dilution and derivatization of the sample to the corresponding FAME; highly sloping background; low accuracy for *trans* level <5%	71
			72
			76
			77
		FTIR-ATR	
990−945 range	C−H out of plane of *trans* double bond	reported as AOCS Official Method Cd 14d-99 to be carried out with the sample directly charged on the ATR crystal	82
		no derivatization and no solvent dilution; minimizes the uncertainty associated with the baseline and thus improves precision and accuracy; rapid determination (5 min) in natural or processed oils and fats	83
			84
		(SB-ATR)FTIR	
~966	C−H out of plane of *trans* isolated double bond	reported as AOAC Official Method 2000.10; this is a universal method for detecting *trans* fats in all types of vegetable oils	83
		very simple to apply	
		-2D-ATR FTIR	
~966	C−H out of plane of *trans* isolated double bond	recognition of low *trans* levels (<1%)	87
		no interference from conjugated dienes and saturated fats	88
		rapid determination of *trans* fats in edible oils	89
		FTIR-PLS Regression	
~966	C−H out of plane of *trans* isolated double bond	rapid method for the determination of total SFA, MUFA, PUFA and *trans* FA (<5 min)	90
		used for monitoring industrial processes	91
			92
		FTIR	
~950 and ~980	C−H out of plane of *trans*-, *cis*-conjugated double bond	no derivatization and no solvent dilution; determination of geometrical composition; interference from other C−H bands	79
988	C−H out of plane of *trans*-, *trans*-dienes		81
		Raman	
1670	C═C stretch of isolated *trans* double bond	rapid analysis	100
		no sample preparation required; determination of *trans* fats in edible oils and *trans* cholesteryl esters in human plasma	101
			102
			68
~1655	C═C stretch of isolated *cis* double bond	determination of *cis*−*trans* isomer ratio, iodine value, and oil adulteration	102
			103
			104
			105
1640	C═C stretching of conjugated dienes	determination of conjugated double bond content	100
			103
			104
		UV	
232−235 (nm)	conjugated dienes	reported as AOCS Official Method Cd 7-58 for detection of polyunsaturated fatty acids in animal and vegetable fats	115
270 (nm)	conjugated trienes	requires sample preparation	116
		interference from pigment absorption	118
302 (nm)	conjugated tetraenes		119

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 is rapid (<5 min), fully automated, and unbiased by the operator. The interface of IR spectrophotometer with a GC allows the characterization of fatty acid double bond geometries directly after chromatography.[93−95] The advantage of this technique is its ability to provide IR spectra for each separated FAME.[96]

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

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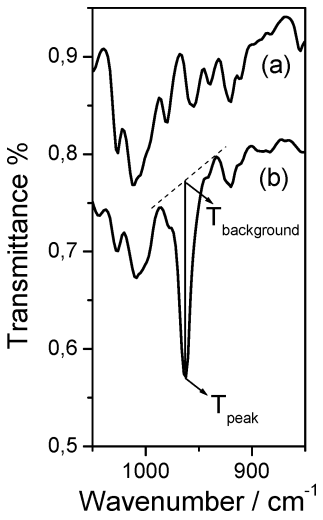

Figure 3. IR spectra of (a) methyl oleate (9*cis*-C18:1) and methyl elaidate (9*trans*-C18:1). The transmittance intensity of the 966 cm^{-1} band is obtained by subtracting the baseline value at the peak maximum ($T_{background}$) from the maximum transmittance at the peak (T_{peak}) (AOCS Method Cd 14-95).

have a characteristic doublet at 948 and 982 cm^{-1}, whereas the corresponding *trans*-, *trans*-diene has a strong absorption band at 988 cm^{-1}.[79]

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,[97] for quantifying the levels of total *trans* FAME in human adipose tissue[85] and in microalgae extracts.[98] FT-IR as well as Raman spectroscopy have been recently used for the analysis of cholesteryl esters of unsaturated fatty acids from plasma samples.[68] 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 ~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 ~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.[68]

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.[99] 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.[100] Raman spectroscopy has been applied to characterization and authentication of edible oils,[101] to quantify total unsaturation, the *cis*/*trans* isomers, and conjugated double bond

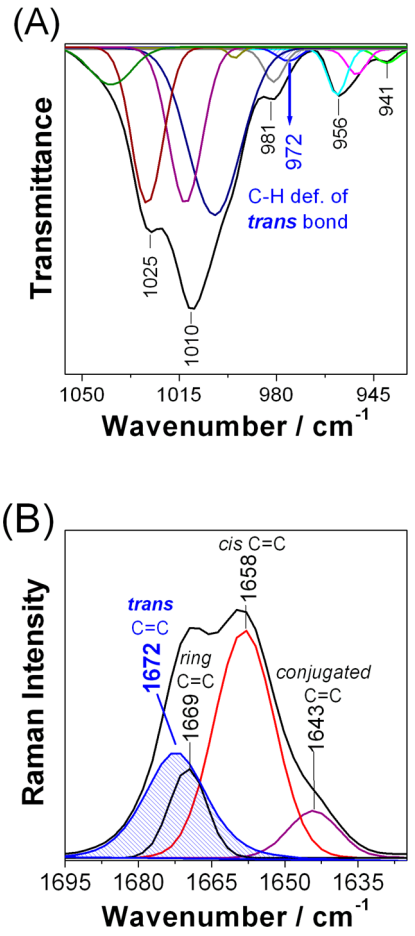

Figure 4. Curve-fitting analysis of IR (A) and Raman (B) spectra of human plasma cholesteryl esters. The analysis was performed on the corresponding spectral ranges where the vibrations of *trans* double bonds give rise to a marker band.[68]

content,[102−104] 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.[105−108] Formation of aldehydes, α,β-unsaturated aldehydes, and hydroperoxides can be identified.[103,108] 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,[109] 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 ~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.[68] Because cholesteryl esters have been associated with atherosclerosis and vascular inflammation,[110]

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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[116] 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 ω-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;[121] 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 *cis* fatty acid structure, which is converted to unnatural isomers. Because of the extensive use of these fats in foods, the *trans* intake increase 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 ω-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 17*trans* 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 α-linolenic acid (obtained through diet) are basically the isomers with *trans* geometry in the n-3 position (17*trans* EPA and 19*trans* DHA).[129] Intake of isomerized α-linolenic acid led to incorporation of *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 ω-3 PUFA are well-known for their beneficial health 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* ω-3

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strongly reduces the expected health benefits. Moreover, it is of great importance to establish the connection between the presence of TFAs and PUFAs; in fact, TFAs can reduce the *cis* PUFA availability in human metabolism by direct inhibition of desaturase (Δ5 and Δ6) and elongase enzymes.[135,136]

It is not the scope of this Review to focus on the incorporation and effects of *trans* fatty acids from the diet, as this has been described in several reviews and books.[7,129,137−139] However, it is worth underlining that from this research two interesting points emerge: (i) *trans* isomers can interact with most of the enzymes of the *cis* lipids, sometimes at the same rate; and (ii) the effects of the *trans* geometry cannot be generalized, but each isomer can have a specific activity that must be appropriately studied.

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.[140] 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.[141] Indeed, life needs the compartimentalization 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* MUFAs 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 9*cis*,11*trans*-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 (11*trans*-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.[140,141] *trans*-Vaccenic and rumenic acids are observed as intermediates of unfinished ruminal biohydrogenation of linoleic acid and α-linolenic acid (Figure 5).

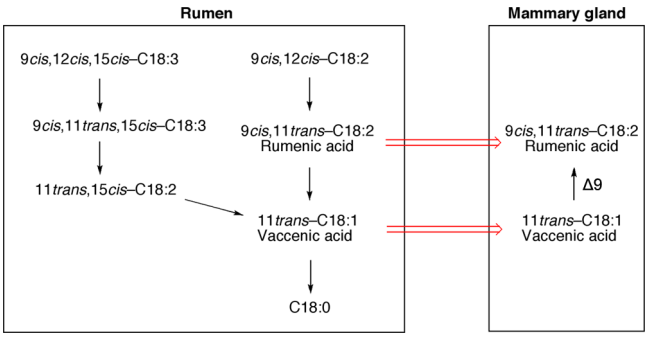

Figure 5. Biohydrogenation of linolenic and linoleic acids in rumens and transfer of ruminant fatty acids to mammary glands.

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 12*cis* double bond to 11*trans*, 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.[142,143]

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.[140,141]

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,[144] hypotriglyceridemic benefits,[145] suppressing tumor development during initiation, promotion, and progression phases of carcinogenesis,[146] anticarcinogenic and antiatherogenic activities,[147] and antidiabetic properties.[148] Although evidence has been reported that CLAs are also effective antioxidants, the mechanism of action is not yet clear.[149]

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.[150] The response of psychrophilic bacterium *Vibrio* sp. strain ABE-1[151] and *Pseudomonas putida* P8[152] 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.[153] Geometrical isomers (in particular 9*trans*-C16:1) were formed in anaerobic bacteria *Pseudomonas* sp. strain E-3, *Pseudomonas putida*, and *Vibrio* sp. strain ABE-1, 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.[151,152,154]

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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.[155] Cti isomerases are proteins of ca. 80 kDa molecular weight whose sequences have been deduced from sequencing the corresponding gene.[156] Comparison among several Cti protein sequences identified them as heme-containing proteins of the cytochrome *c*-type.[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* isomerization. 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 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.[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.[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, chelating agents, 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.[161] Another mechanism was proposed on the basis of the analysis of the carbon isotope fractionation experiment of the *cis*−*trans* isomerization[162] and the site-directed mutagenesis experiments on Cti of *Pseudomonas putida* P8, which caused the loss of isomerization activity.[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

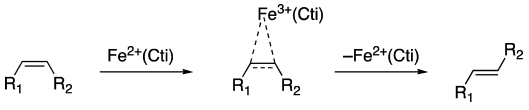
The early work on geometrical isomerization using free radical chemistry concerned the reactivity of iodine atoms[164] and thiyl radicals (RS•).[165,166] These are able to add reversibly to double bonds, and, consequently, their efficiency in transforming the less stable *cis* into the thermodynamically more stable *trans* isomer was envisaged. The *cis*−*trans* isomerization of olefins by the addition−elimination sequence of the PhS• radical is now an established methodology in fine chemical synthesis.[167] Other radicals such as sulfonyl ($RSO_2^•$),[168] selenyl (PhSe•),[169] stannyl ($R_3Sn^•$),[170,171] germyl ($Ph_3Ge^•$),[172] and silyl ($((Me_3Si)_3Si^•)$[171] radicals, as well as nitrogen dioxide ($NO_2^•$),[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•, $Bu_3Sn^•$, and $(Me_3Si)_3Si^•$ radicals in 1, 4, and 10 h, respectively.[171] This early work in chemistry was only later connected with the in vivo reactivity of biological relevant RS• and $NO_2^•$ radicals toward lipid molecules.

5.2. Monounsaturated Fatty Acids (MUFA)

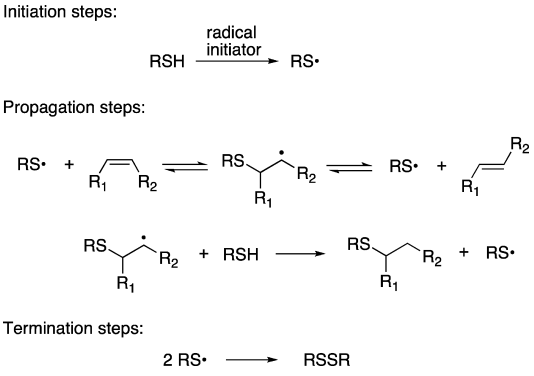
5.2.1. Isomerization by Thiyl Radicals. The radical-based thiol additions to *cis* and *trans* olefins are chain reactions and are accompanied by isomerization (Scheme 2).[165] In the case of unsaturated fatty acids, the reaction with thiols has been carefully studied due to the importance of the *cis* 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,[174] γ-radiolysis of *tert*-butanol solutions,[174,175] or photolysis of di-*tert*-butyl ketone.[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.[177]

The time-dependence of the addition of 2-mercaptoethanol to methyl oleate and the isomerization catalyzed by the corresponding $HOCH_2CH_2S^•$ radical were investigated in detail.[175,176] On the basis of Scheme 2, kinetics were analyzed on the basis of the initiation rates, the known values of thiyl 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 $HOCH_2CH_2S^•$ radical (see Scheme 3). The rate constants for thiyl radical addition (k_a in blue) to *cis* or *trans* isomers were found to be rather similar (k_a^{trans}/k_a^{cis} = 1.8), whereas for fragmentation of adduct radicals (k_f in red) they were

Scheme 1. Proposed Mechanism of *Cis*−*Trans* Isomerase (Cti)



Scheme 2. Addition of Thiols to Alkenes as Chain Reaction



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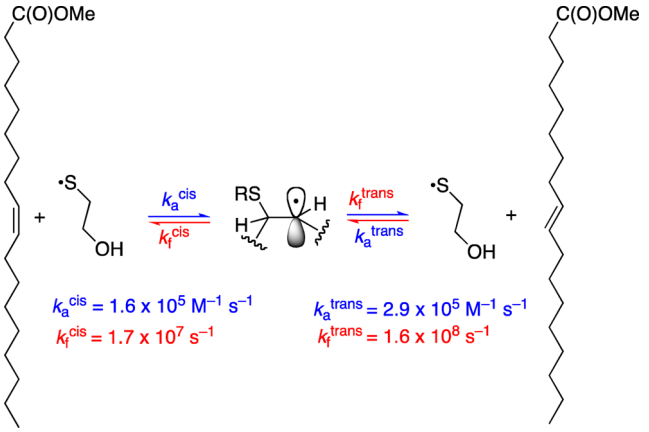
Scheme 3. $HOCH_2CH_2S^•$ Radical-Catalyzed Isomerization of Methyl Oleate



substantially different (k_f^{trans}/k_f^{cis} = 9.4).[176] 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 thiyl radical scavenging was found to increase along the series α-tocopherol < ascorbic acid 6-*O*-palmitate < all-*trans* retinol acetate.[178] 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 thiyl 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 ΔH = −5.4 kJ/mol and ΔS = −5.5 J/mol·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 β-elimination of thiyl 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 β-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

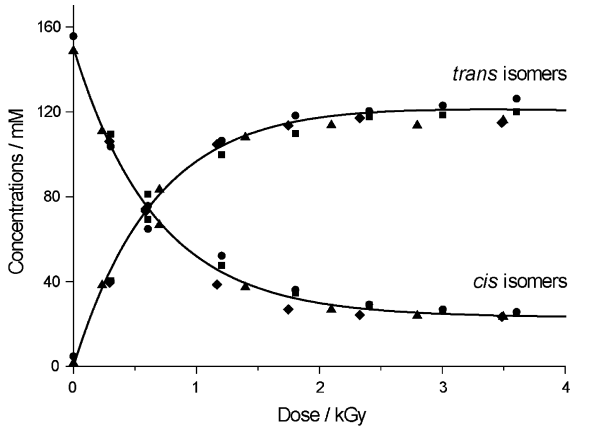


Figure 6. Isomerization of different monounsaturated fatty acid methyl esters (0.15 M) by 2-mercaptoethanol-derived thiyl radicals in *tert*-butyl alcohol starting from the *cis* isomers at 75 mM thiol concentration. (▲) oleate; (■) palmitoleate; (◆) *cis* vaccenate; (●) oleic acid. Reprinted with permission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

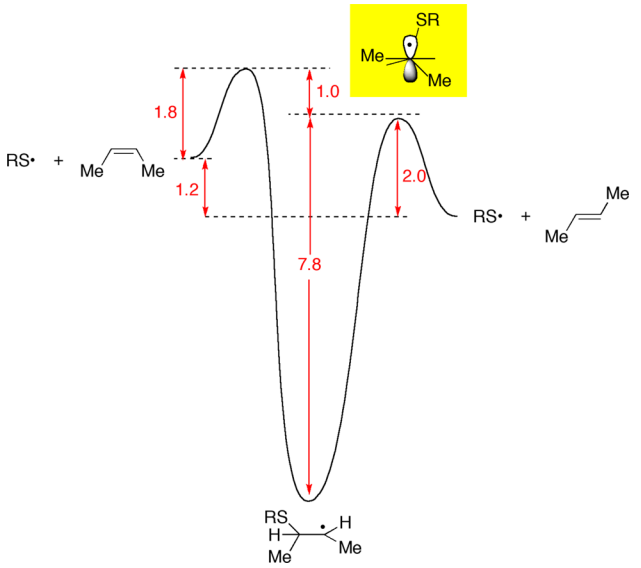


Figure 7. Enthalpy profile in kcal mol^{-1} for the *cis*–*trans* isomerization of 2-butene by the $HOCH_2CH_2S^•$ radical.

the addition of $HOCH_2CH_2S^•$ 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 β-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 thiyl radicals to molecular oxygen is fast and reversible. The equilibrium constant of the oxygen addition (eq 1/−1) is about 3000,[179] meaning that in air-saturated aqueous systems ($[O_2]$ = 2.4 × 10^{-4} M) both RS$^•$ and the thiylperoxyl radical RSOO$^•$ equilibrate at about equal concentrations, while under typical physiological conditions in cells ($[O_2] \leq 10^{-5}$ M) the equilibrium remains overwhelmingly on the thiyl radical side. The reversible oxygen addition to thiyl radicals (eq 1/−1) becomes significant also because of the known rearrangement of the thiylperoxyl radical RSOO$^•$ to sulfonyl radical $RSO_2^•$ (eq 2).[180] The effectiveness of the isomerization processes of the

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$MeSO_2^•$ radical was addressed in some detail.[181] 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

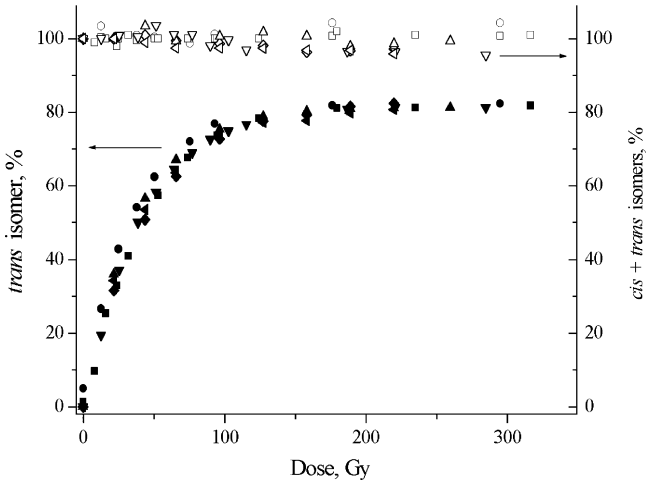


Figure 8. (Left side) Formation of *trans* isomers from γ-irradiation (dose rate = 10 Gy/min) of different monounsaturated fatty acid methyl esters (2.5 mM) with CH_3SO_2Cl (1.25 mM) in N_2-fluxed ethanol/water (2/1, v/v) at 22 °C: (●) 9*trans*-C14:1; (■) 9*trans*-C16:1; (◆) 6*trans*-C18:1; (▲) 9*trans*-C18:1; (▼) 11*trans*-C18:1; (◀) 11*trans*-C20:1. (Right side) Each point is the sum of *cis* and *trans* isomers. Reprinted with permission from ref 181. Copyright 2007 American Chemical Society.

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).[181]

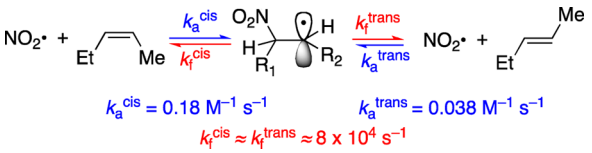
$$RS^• + O_2 \rightleftharpoons RSOO^• \quad (1/-1)$$

$$RSOO^• \rightarrow RSO_2^• \quad (2)$$

$$RSO_2^• + O_2 \rightarrow RSO_2OO^• \quad (3)$$

5.2.3. Isomerization by Nitrogen Dioxide. Reliable kinetic data for the reaction of the $NO_2^•$ radical with olefins are limited to the gas phase, because both addition and hydrogen abstraction reactions are very slow processes.[182] Scheme 4 shows the analogous *cis*–*trans* isomerization of 2-

Scheme 4. $NO_2^•$ Radical-Catalyzed Isomerization of 2-Pentene in the Gas Phase

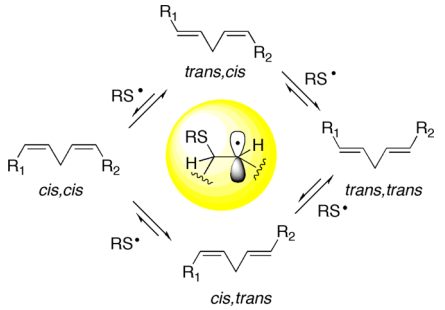


pentene, the rate constants being obtained at 25 °C. The fragmentation of the adduct radical occurs with a rate constant of $k_f \approx 8 \times 10^4$ s^{-1} for both isomers; therefore, molecular oxygen likely intercepts the carbon-centered radical intermediate prior to fragmentation. Evidence that 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 $NO_2^•$ with cyclohexene showed that the two reaction paths are also competitive in solution.[183]

5.3. Polyunsaturated 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 (9*cis*,12*cis*-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).[184] However, careful quantitative work

Scheme 5. *Cis*–*Trans* Isomerization of PUFA Catalyzed by Thiyl Radicals



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.[184]

Cis–*trans* isomerization reactions of methyl γ-linolenate (6*cis*,9*cis*,12*cis*-C18:3),[184] arachidonate (5*cis*,8*cis*,11*cis*,14*cis*-C20:4),[21] and EPA (5*cis*,8*cis*,11*cis*,14*cis*,17*cis*-C20:5),[23] catalyzed by $HOCH_2CH_2S^•$ 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*-toluenesulfinic 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.

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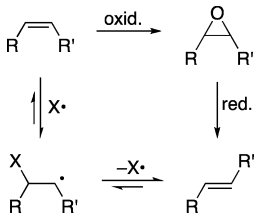
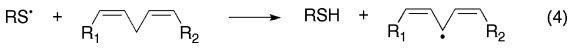
Scheme 6. Dual Synthetic Strategies for the Preparation of Mono-*trans* PUFA Isomers



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



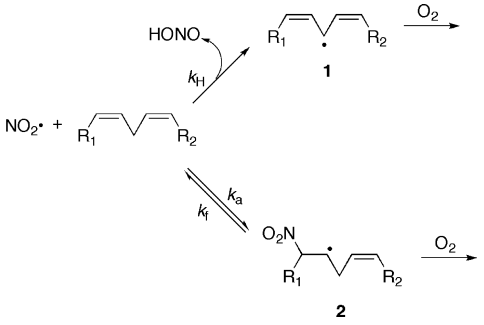
(4)

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 $k_1 \approx 10^7$ M^{-1} 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 $k_{-1} \leq 10^5$ M^{-1} s^{-1} is given.[188] It was suggested that ~50% of thiyl 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 thiyl 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 linoleate in the presence and absence of oxygen attracted considerable attention because the NO_2• radical has been proposed to initiate lipid peroxidation.[193] 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

Scheme 7. Hydrogen Abstraction versus Addition Mechanism for the Reaction of Nitrogen Dioxide with PUFA



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 peroxyl radicals that propagate peroxidation.

5.4. Cholesteryl Esters (CE)

Cholesteryl linoleate 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 cholesterol 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 linoleate and arachidonate using the convenient and fast approach of thiyl radical-catalyzed *cis*–*trans* isomerization, starting from the natural *cis* substrates.[68] 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 linoleate isomers and of 30% in case of mono-*trans* cholesteryl arachidonate isomers. The characterization of these isomers

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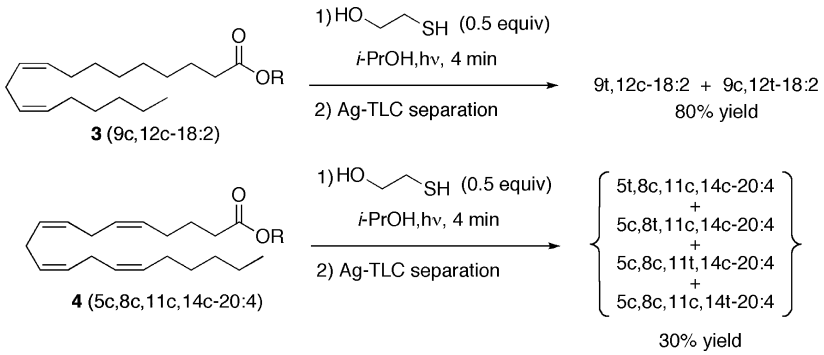
Scheme 8. Synthesis of the Mono-*trans* PUFA Cholesteryl Ester Library



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,[198] 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 $HOCH_2CH_2S^{\bullet}$ radicals in alcoholic solutions.[200] This $RS^{\bullet}$ radical-catalyzed approach was originally designed for building up a simple and economic *trans* 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 methanolysis 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 polyunsaturated 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/cm^2).[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 lecithin[184] and egg lecithin[205] catalyzed by $HOCH_2CH_2S^{\bullet}$ 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

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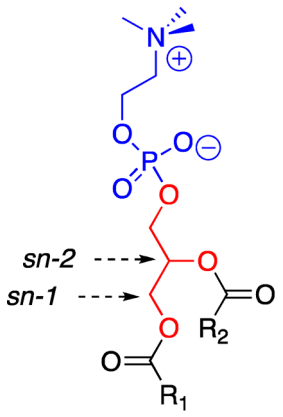
Scheme 9. Structure and Composition of Fatty Acid Residues of Various L-α-Phosphatidylcholines

L-α-Phosphatidylcholines (PC)



Soybean Lecithin

Palmitoyl	4.5%
Stearoyl	3.8%
Oleoyl	11.9%
Vaccenoyl	1.3%
Linoleoyl	63.5%
γ-Linolenoyl	6.3%

Egg Lecithin

Palmitoyl	32.0%
Stearoyl	14.1%
Oleoyl	27.0%
Vaccenoyl	1.2%
Linoleoyl	20.0%
Arachidonoyl	4.8%

POPC: R_1 = Palmitoyl, R_2 = Oleoyl
DOPC: $R_1 = R_2$ = Oleoyl
SAPC: R_1 = Stearoyl, R_2 = Arachidonoyl

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[205] and soybean lecithin.[184]

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".[206] 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.[207] Micelle aggregations mostly model the lipid organization involved in the digestive or pulmonary tracts 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.[208,209] 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.[4,210−212]

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.[213] On the other hand, vesicles can be obtained in the form of multilamellar vesicles (MLVs) and small or large unilamellar vesicles (SUVs, LUVs)[207] 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.[214−217]

In early studies of liposome peroxidation and the role of glutathione and cysteine, thiols were also considered protective,[218] and the *cis*−*trans* isomerization was not even considered in the experimental work. Thiols are efficient in the repair reaction, but the corresponding formation of thiyl radicals cannot be omitted in the resulting product studies, because the catalytic cycle of double bond isomerization can be operative, as previously described.

Biomimetic models strongly contributed to the assessment of thiyl radical reactivity toward membrane fatty acids, evidencing all important features connected with this heterogeneous system, such as substrate partitioning, initiation compartment, radical diffusibility, and supramolecular organization, all influencing the resulting lipid modifications.

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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.[219] Using ionizing radiation as initiating conditions, aqueous suspensions of micelles using linoleic acid (LH) reacted under a variety of conditions, where thiyl radicals are the main reactive species. Under anaerobic conditions, only the *cis*–*trans* isomerization was observed, and the addition/elimination of thiyl radicals was calculated with a catalytic turnover number of 420. In air-equilibrated solutions, the *cis*–*trans* isomerization process was still observed 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-

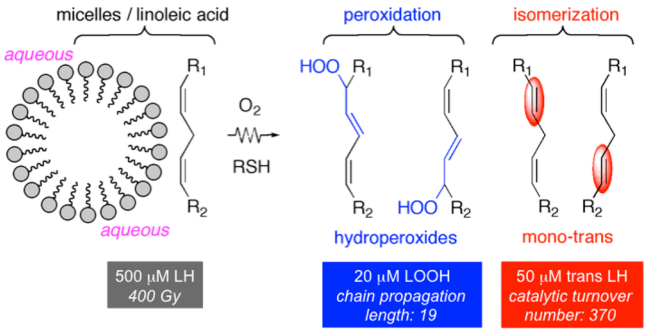

Figure 9. The parallel estimation of peroxidation and isomerization in micelles of linoleic acid.

trans fatty acid formation in PUFA residues as a relevant marker of thiyl radical reactivity also under competitive conditions. Moreover, they motivate further interest in the evaluation of the parallel involvement of oxidative and isomerization processes, either in complex models or in the scenario of signaling and damaging events during radical stress.

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 thiyl radicals were performed in multilamellar vesicles (MLVs), using $HOCH_2CH_2SH$ and varying the nature of initiation (the hydrophilic azo compound AAPH at 37 °C or γ-irradiation).[174] 2-Mercaptoethanol is an amphiphilic compound; therefore, neither the aqueous compartment of initiation nor the unrestricted diffusion of this molecule in lipid/water dispersions can influence the mode of thiyl radical formation or reactivity, respectively. As a consequence, observed differences in the efficiency of MLV *cis*–*trans* isomerization as compared to the behavior in large unilamellar vesicles (LUVs) (see below) can depend mainly on the peculiar diffusional effects associated with each of the two differently organized systems. It is worth mentioning that the experiments with MLVs and $HOCH_2CH_2SH$ were carried out using two different dose rates, that is, 1.4 and 30.6 Gy min^{-1}. It was calculated that the catalytic turnover numbers of isomerization by $HOCH_2CH_2S^{\bullet}$ radicals were ca. 250 and 20, respectively, at the initial phase.[174] Higher efficiency of the *cis*–*trans* isomerization at a low dose rate is due to the decrease of competitive reactions involving thiyl radicals. The effect of *trans* configuration on the physical properties of the lipid bilayer will be addressed in section 7. Here, it is only mentioned that MLVs made from different percentages of 1,2-dielaidoylphosphatidylcholine (DEPC) showed that the presence of *trans* fatty acid residues substantially decreases the membrane fluidity.[174] Analogously, permeability measurements of MLVs provided clear evidence that physical properties, such as permeability and fluidity, of *trans*-phospholipids are different from those of *cis*-phospholipids, due to the changes of the barrier properties of the bilayer with a tighter lipid packing.[178] The *cis*–*trans* isomerization of DOPCs in MLVs by amphiphilic thiyl radicals in the absence and presence of the various antioxidants was also addressed.[178] The ability of thiyl radical scavenging was found to increase along the series α-tocopherol ≪ ascorbic acid < all-*trans* retinol acetate.

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

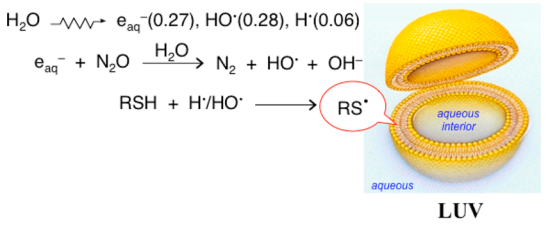

Figure 10. A large unilamellar vesicle (LUV) and radiolytic generation of thiyl radicals.

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[207] and the injection[220] methodologies.

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 thiyl 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 species: solvated electrons (e_{aq}), hydrogen atoms ($H^{\bullet}$), and hydroxyl radicals ($HO^{\bullet}$) as shown in Figure 10 (the values in parentheses represent the radiation chemical yields (G) in units of μmol J^{-1}).[221] In a N_2O-saturated solution (~0.02 M of N_2O), e_{aq}^- are transformed into $HO^{\bullet}$ radicals ($k = 9.1 \times 10^9$ M^{-1} s^{-1}); therefore, the main reactive species are $HO^{\bullet}$ radicals and $H^{\bullet}$ atoms (in a 9:1 ratio). In the presence of thiols, like the amphiphilic $HOCH_2CH_2SH$, hydrogen abstraction by $HO^{\bullet}$ and $H^{\bullet}$ directly produces thiyl radicals ($k = 6.8 \times 10^9$ M^{-1} s^{-1} for $HO^{\bullet}$ and $k_8 = 1.7 \times 10^9$ M^{-1} s^{-1} for $H^{\bullet}$).[221]

Initial studies of *cis*–*trans* isomerization of unsaturated moieties of DOPC showed that the isomerization rate followed the lipophilicity order of thiols.[174,222] Amphiphilic $HOCH_2CH_2SH$ in LUVET gave the highest efficiency of geometric isomerization, as compared to glutathione and cysteine, indicating that thiyl radicals generated in the aqueous compartment enter the hydrophobic region of the bilayer and isomerize the 9,10-double bond of oleic moieties. Instead, cysteinyl radical was not efficient due to its hydrophilicity; that

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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 $HOCH_2CH_2S^{\bullet}$ radicals.[174,222] Like the MLV experiments, the catalytic turnover number of LUV isomerization by $HOCH_2CH_2S^{\bullet}$ radicals increased by decreasing the dose rate.[174]

The isomerization of soybean lecithin in LUVET using $HOCH_2CH_2SH$ or GSH as $RS^{\bullet}$ radical precursors,[184] and egg lecithin or SAPC with $HOCH_2CH_2SH$,[205] was studied in some detail, to understand the PUFA residue behavior. It was found that the lipid supramolecular organization of vesicles has a profound effect on the isomerization outcome involving the differentiation of the various double bonds. Trends of the reactivity indicated the overall picture of geometric isomerization in model membranes by the action of diffusible free radicals. An amphiphilic thiyl radical, diffusing from the aqueous phase to the hydrophobic region of the membrane bilayer, starts to isomerize the double bonds nearest to the glycerol bridge, that is, nearest to the aqueous phase. The sequence of events 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*cis* position of linoleate is very similar to that of 9*cis* in the oleate residues.[205] For a particular PUFA residue, high regioselectivity is also observed for the formation of mono-*trans* isomers: in the linoleate residues, the 9*trans*,12*cis* isomer prevailed over the 9*cis*,12*trans* 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 $RS^{\bullet}$ 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.[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 ($RS^{\bullet}$), 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.[225−230] 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).[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.[232]

$NO_2^{\bullet}$ radicals have already been discussed above with respect to their isomerization potential (sections 5.2.3 and 5.3.2). In biological systems, $NO_2^{\bullet}$ radicals could be assumed to initiate *cis*−*trans* isomerization, but their realistic potential appears to be very poor.[226] Considering the complexity of the cellular environment and the fact that $NO_2^{\bullet}$ 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 $NO_2^{\bullet}$ in the vascular compartment, where the thiol concentration is very low, except within erythrocytes.[233] Therefore, it can be confidently predicted that production of $NO_2^{\bullet}$ 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 $NO_2^{\bullet}$ for biological media could be of some help.[234]

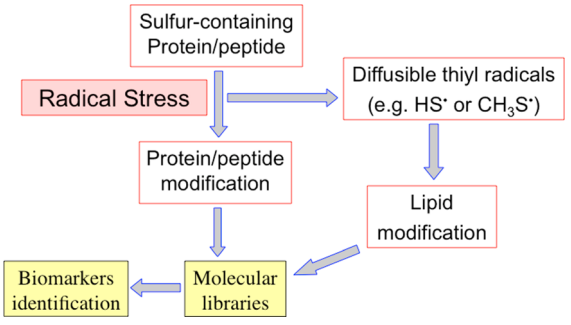

Figure 11. Integrated vision of protein−lipid damage.

6.2.1. Small Diffusible Sulfur-Centered Radicals.

Considering biologically relevant sulfur-containing species, the simplest thiol, hydrogen sulfide (H_2S), 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 millimilar concentrations.[235−238] Chemically, the reactions of the simplest S-centered radical ($HS^{\bullet}$) are not as well-known as those of other

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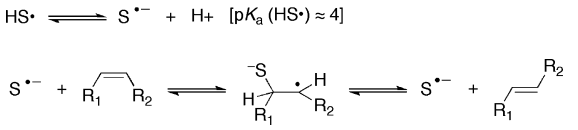
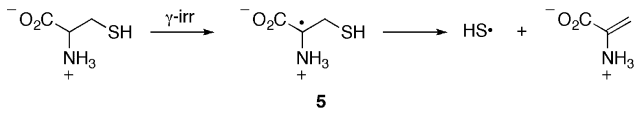
thiyl radicals, although a few papers have characterized some key reactions in the aqueous phase,[239] and in liposomes.[240] Indeed, different neutral radicals (e.g., $HS^{\bullet}$ and $HSS^{\bullet}$) and radical anions (e.g., $S^{\bullet-}$, $HSS^{\bullet 2-}$, and $HSSH^{\bullet-}$) can be produced, but not all of them are isomerizing agents.[240] The use of the biomimetic model of vesicle suspension demonstrated the potential of sulfhydryl radicals ($HS^{\bullet}/S^{\bullet-}$) derived from H_2S to reach the hydrophobic fatty acid chains and attack double bonds, thus producing phospholipids containing *trans*-fatty acid residues in high yield (Scheme 10).[240]

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



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.[174,222] In comparison with homocysteine, in which the SH moieties are separated by an extra CH_2 group, it was suggested that H-abstraction from the activated α-C position (affording radical **5** and subsequent β-fragmentation) generates $HS^{\bullet}$ radicals, which are able to migrate into the lipophilic compartment and isomerize the double bond (Scheme 11).

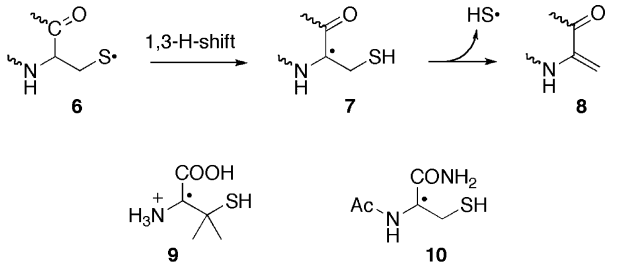
Scheme 11. Proposed Mechanism for the Formation of Sulfhydryl Radicals from the γ-Radiolysis of Cysteine



5

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.[241−244] An important experimental result, in connection

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

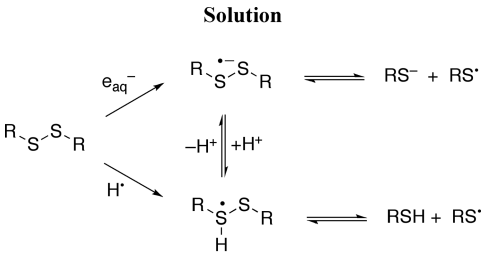


6 **7** **8** **9** **10**

with sulfhydryl radical formation, is the ultimate conversion of the Cys residue into dehydroalanine residue **8**, likely via β-elimination following a 1,3-hydrogen transfer of the initial thiyl radical **6**. 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 3×10^4 and 5×10^3 s^{-1}, respectively.[244]

The interaction of hydrated electrons (e_{aq}^-) and $H^{\bullet}$ atoms (primary species of γ-radiolysis) with disulfide-containing compounds in aqueous solution is summarized in Scheme 13.[245,246] The disulfide radical anion derived from the direct

Scheme 13. Mechanism of Reaction of e_{aq}^- and $H^{\bullet}$ Atoms with Dialkyl Disulfide in Aqueous Solution

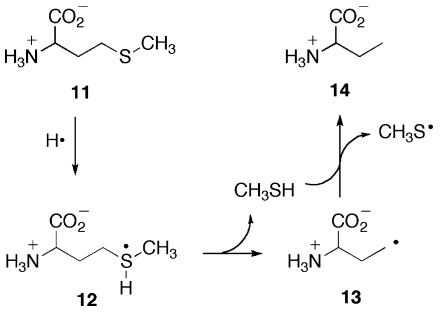


Dose, Gy	Trans, %
0	0
84	82.2
126	90.9

electron attachment is generally in equilibrium with the sulfuranyl radical, which is also obtained by the direct $H^{\bullet}$ atom attack on the sulfide moiety. Both reactive species dissociate reversibly into two entities, $RS^{\bullet}$ and RSH (or RS^-). Dibutyl disulfide was used in the isomerization of methyl oleate,[178] and dimethyl disulfide[247] in POPC. In the table inset of Scheme 13, the resulting elaidate residues after irradiation of 2 mM POPC in LUVET and 75 μM CH_3SSCH_3 are reported at different irradiation doses.

In the case of the thioether function of methionine **11**, hydrogen atom attack caused the formation of a sulfuranyl type radical **12** (Scheme 14), which then undergoes a rapid

Scheme 14. Mechanism of Reaction of $H^{\bullet}$ Atoms with Methionine in Aqueous Solution and the Formation of Diffusible $CH_3S^{\bullet}$ Radical



11 **14** **12** **13**

desulfurization; thus, radical **13** is produced with formation of CH_3SH, a diffusible species that under radical stress conditions exists as the corresponding $CH_3S^{\bullet}$ radical. Radical **13** is the precursor of α-aminobutyric acid (**14**, Aba).[248,249]

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[250] or with proteins.[251] 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

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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, diffusible thiyl 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 transesterification 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.

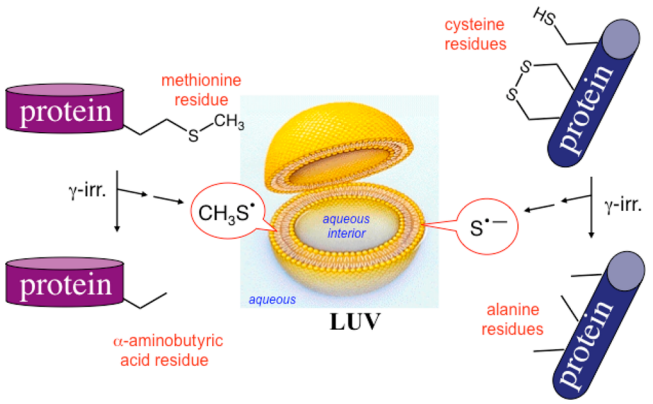

Figure 12. Tandem protein–lipid damage under reductive radical stress. Methionine and cystine residues are modified to α-aminobutyric acid and alanine/cysteine, respectively, by the attack of $H^•$ and/or e_{aq}^-. Concomitant formation of diffusible sulfur-centered radicals ($CH_3S^•$ or $S^{-•}$) able to migrate into the lipid bilayer induces *cis–trans* isomerization of unsaturated fatty acid residues.

Several sulfur-containing proteins have been chosen as models for these biomimetic studies: (i) Metenkephalin[252] and amyloid-β,[253] which contain a single Met residue, (ii) medium-sized proteins like RNase A[247,254,255] and lysozyme (Lyso)[256] 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,[257] and (iv) large proteins like human serum albumin (HSA) that contain Met residues, disulfide bridges, and free thiol groups.[258] Thiyl 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 SMe 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 ($CH_3S^•$ 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.[245,259,260] 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-aminobutyric 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)[247,254] and lysozyme

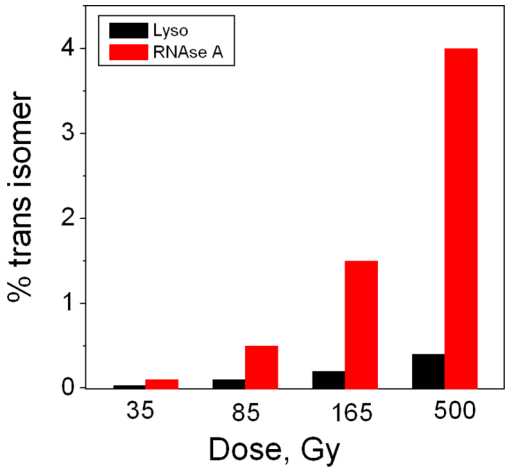

Figure 13. Percentages of *trans* lipid isomers found after γ-irradiation of N_2O-saturated suspensions of DOPC/Lyso and DOPC/RNase A at different dose exposure. The reactive species are $HO^•$ radicals and $H^•$ atoms.

(Lyso);[256] 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.[225]

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 (S^{2-}).[261,262] Moreover, metal ions present in the metal–MT complexes influence the extent of isomerization. For example,

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substantial differences were observed in the behavior of QsMT depending on the presence of copper, zinc, or cadmium ions (Figure 14). Cd^{II}-MTs generally have a higher capacity to

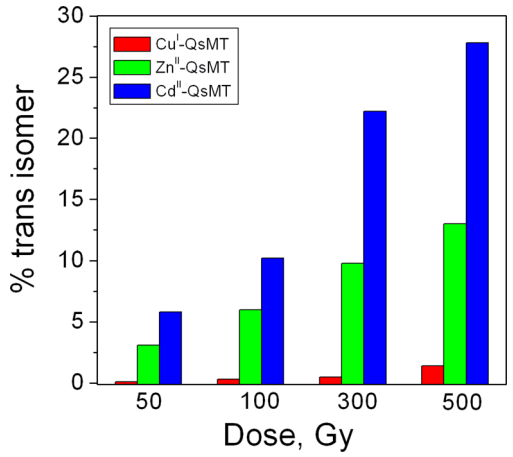

Figure 14. Dose dependence of the formation of elaidate (*trans* isomer) residues from γ-irradiation of N_2O-saturated, 10 mM $H_2PO_4^-$ solutions containing POPC vesicles (1.7 mM) and Cd^{II}-, Zn^{II}-, or Cu^{I}-QsMT complexes (30 mM). The reactive species are $HO^\bullet$ radicals and $H^\bullet$ atoms.

induce *cis*–*trans* isomerization than Zn^{II}- or Cu^{I}-MTs.[260] The higher isomerization yields obtained in the presence of Cd^{II}-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^{I}-MT and Zn^{II}-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 ($HS^\bullet/S^{\bullet-}$). In addition, the different content of acid-labile S^{2-} ligands in the metal complex induces also a slightly different folding of the Zn^{II}-MT and Cd^{II}-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 proteo-liposomes suggest an interesting application for a very sensitive

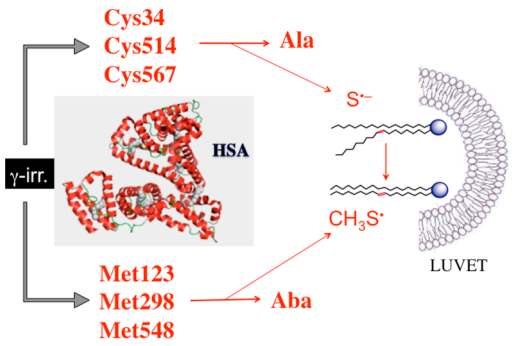

Figure 15. Sulfur-containing amino acid residues (Met and Cys) are modified by attack of $H^\bullet$ and/or e_{aq}^-, yielding diffusible S-centered radicals such as $CH_3S^\bullet$ or $HS^\bullet/S^{\bullet-}$, which are capable of migrating to the lipid bilayer and inducing *cis*–*trans* isomerization of unsaturated fatty acids.

model system to assay radiation exposure, via the proteic sulfur reactivity and the amplification effect given by the catalytic cycle of the thiyl radical-based *cis*–*trans* isomerization.

Figure 16 depicts the reaction of reducing radical species with protein cystine moieties, emphasizing the possible

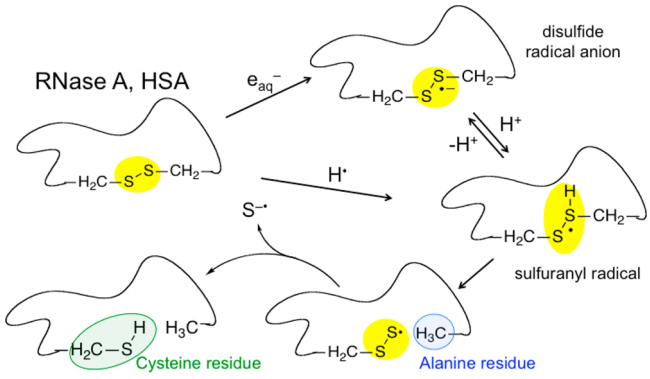

Figure 16. Proposed mechanism for the formation of alanine residues and $S^{-\bullet}$ radicals in irradiated proteins.

occurrence of both disulfide radical anion (in the case of e_{aq}^-) or sulfuranyl radical protein intermediates (in the case of $H^\bullet$ atoms), which are present in a protonation equilibrium. The modification of Cys to Ala was highly indicative of the formation of the perthiyl 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 $RS^\bullet$/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 perthiyl 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 thiyl 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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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 (9*cis*-C18:1) and its *trans* geometrical isomer, elaidic acid (9*trans*-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,[264] 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 with a *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-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).[204]

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

phospholipid	diameter (nm)
POPC	97 ± 10
PEPC 83%	72 ± 8
DPPC	45 ± 8

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^{-6} M). The different lipid structures influenced 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.[265] 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.[266] 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.[266] 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 (9*trans*-C14:1, 9*trans*-C16:1, and 9*trans*-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.[267]

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.[267] 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.[268] 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.[269−273] Newer insights have been more recently provided in connection with cellular signaling cascades. In human aorthic endothelial cells, *trans* fatty acids (elaidic and linoelaidic acid, 9*trans*,12*trans*-C18:2) and stearic acid (C18:0) increased phosphorylation of the ICAM-1 transcriptional regulator, nuclear factor-*kB* (NF-*k*B), inducing a pro-inflammatory response due to the elevation of the ICAM expression.[274] Analogously, differential activation of endothelial superoxide production, NF-*k*B 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-*k*B response.[275] 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 $cytb_{558}$ and the cytosolic $p67^{phox}$, 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 $p67^{phox}$ 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 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 drugs[282] 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 cells[289] 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., $\omega 6/\omega 3$ 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.[293−297] 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.[298,299] 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

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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

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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.

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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 modification of proteins and lipids due to free radical damage.

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	α,α'-azodiisobutyramidine dihydrochloride
Aβ	amyloid β-peptide
Aba	α-aminobutyric acid
Ag-HPLC	silver high-performance liquid chromatography
Ag-TLC	silver thin-layer chromatography
Ala	alanine
AOCS	American Oil Chemists' Society
APP	β-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-dielaidoylphosphatidylcholine
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
FAME	fatty acid methyl ester
FDA	Food and Drug Administration
FID	flame ionization detector
FT	Fourier transform
FTIR	Fourier transform infrared
FTIR-ATR	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 acyl transferase
LDL	low-density lipoprotein
LH	linoleic acid
LOOH	lipid hydroperoxides
LOX	lipoxygenase
LUV	large unilamellar vescicles
LUVET	large unilamellar vesicles by extrusion technique
Lyso	lysozyme
MALDI	matrix-assisted laser desorption/ionization
ME	methyl ester
Met	methionine
MI	matrix isolation
MLV	multilamellar vescicles
MS	mass spectrometry
MTs	metallothioneins
MUFA	monounsaturated fatty acid
NADPH	nicotinamide adenine dinucleotide phosphate
NF-κB	nuclear factor-κB
NMR	nuclear magnetic resonance
PAF	platelet-activating factor
PC	L-α-phosphatidylcholines
PEPC	1-palmitoyl-2-elaidoylphosphatidylcholine
PLS	partial least-squares
POPC	1-palmitoyl-2-oleoylphosphatidylcholine
PUFA	polyunsaturated fatty acid
QsMT	*Quercus suber* metallothionein
RNase A	ribonuclease A
RP	reversed-phase
SAPC	1-stearoyl-2-arachidonoylphosphatidylcholine
SB-ATR	single-bounce attenuated total reflectance
SERCA	sarcoplasmic reticulum Ca^{2+}-ATPase
SFA	saturated fatty acid
SR	sarcoplasmic reticulum
SUV	small unilamellar vescicles
TEC	thiol−ene chemistry
TFA	*trans* fatty acid
TLC	thin-layer chromatography
TOF	time-of-flight
TpyMT	*Tetrahymena pyriformis* metallothionein
UV	ultraviolet

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