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By C.M. Zorzut and P. Capella

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RESEARCHES ON THE ISOMERS OF MONOUNSATURATED FATTY ACIDS OF VEGETABLE OILS BY CHROMATOGRAPHY - MASS SPECTROMETRY (*)

by C.M. Zorzut and P. Capella
Institute of Agrarian Chemistry and Agrarian Industry of the University of Bologna.

RESEARCHES ON THE ISOMERS OF MONOUNSATURATED FATTY ACIDS OF VEGETABLE OILS BY CHROMATOGRAPHY - MASS SPECTROMETRY

The isomers of monounsaturated fatty acids of some vegetable oils were studied by the combination of gas chromatography-mass spectrometry after conversion of the monounsaturated fatty acids into the corresponding trimethylsilyloxy (TMSO) derivatives.

The results allowed us to show that - with the only exception of parsley seed oil - the most widely represented isomers are the 9-cis-hexadecenoic, the 9-cis-octadecenoic, the 11-cis-eicosenoic and the 13-cis-docosenoic. While in general the data previously known in the literature have been confirmed the presence of isomers not yet reported has been demonstrated, such as the 6-cis-hexadecenoic, the 8-cis and 9-cis-eicosenoic in parsley seed oil, and the 13-cis-octadecenoic in rapeseed and mustard seed oil.

The results are then discussed in the light of the present knowledge on unsaturated fatty acid biosynthesis.

(*) Paper read at the IX Italian Congress on Studies of Fatty Substances, Bologna, 10-12 June, 1968.

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TRANSLATION NON REVUE
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Introduction:

The study of the structure of monounsaturated fatty acids of oils and of natural fats has led to the conclusion that, except for rare exceptions, these have the double bond in positions 9, 10 (acids of 16 and 18 carbon atoms) or in the position 11, 12 and 13, 14 (acids of 20 and 22 carbon atoms respectively). The knowledge with regard to the presence of their isomers of position is both inadequate and fragmentary.

An examination of the most recent literature has led us to the conclusion that numerous insoluble structural components are widely diffused in the natural oils (1-3).

The knowledge of the composition, in structural isomers of monounsaturated fatty acids, presents the following interesting aspects:

1) **Biologically** with regard to the natural mechanisms of their synthesis and their relaboration and therefore of the vegetable and animal physiology in the catabolic and anabolic processes of the fatty substances; in this context, studies on filogenesis and taxonomy are also important, especially in regard to the vegetable organisms.

2) **Industrially** both in relation to the modifications undergone by the fatty acids in the course of the processes of preparation of the edible oil products (4) and also the exploitation of sources of the composts with a particular structure.

In the study of the monounsaturated fraction of the fatty acids, previously isolated, the methods most commonly used
are based on the oxidated breaking-up of the molecule in the positions of the double bonds and gas chromatographic analysis on chromatography of the oxidized fragments (2, 5-12). The aforesaid methods are complicated, above all in their quantitative aspect, with regard to the possibility of formation of products with further oxidative domolition of the primary fragments and from the fact that acids with different numbers of carbon atoms but having the same position of unsaturation, give place to a product in common; the method of the oxidative domolition, moreover, often imposes difficult processes of enriching and purification, when the monounsaturated fatty acids present in small percentages in the mixture must be isolated. Other methods (13) provide for the use of the tubular open column (capillary) for gas chromatographic analysis of the esters of the monounsaturated fatty acids: the determination is in this case possible only for the acids having the double bonds close to the aliphatic extremity of the molecule.

New prospects for a brilliant solution of the problem have opened up with the application of mass spectrometry in the study of the fatty acids.

This method of experimentation has produced definite results in the definition of the structure of saturated esters (14-18), while it has been halted when faced with the esters of the monounsaturated fatty acids in cases such as: the molecule undergoes in fact, under an electronic impact, phenomena of breaking up of the double bond which leads to a constant statistical distribution of the position of unsaturation along the
molecule. Numerous research workers have as a consequence turned their attention to the various derivatives of the esters of monounsaturated fatty acids obtaining, in some cases, a good spectral characterization of the composts with a different position of the double bond and with different steric configuration (19-22). The examination of the spectrums of the mass of pure composts did not however give any hope for the possibility of determining either the presence or the structure of composts represented in small percentages in a mixture of fatty acids.

Recent research (23) undertaken by us on the suitable derivatives of the esters of some pure monounsaturated fatty acids has enabled us to determine how far these derivatives could be similar in order to determine the position of the double bond and has suggested to us the possibility of defining the structure of their isomers present in complex mixtures.

The procedure adopted requires the preparation of di-trimethyl silyloxy (TMSO) derivatives of the methyl esters of monounsaturated fatty acids, according to the following reaction method:

\[
\begin{align*}
\text{OsO}_4 & \rightarrow C = C \rightarrow C - C - \\
2 \text{Na}_2\text{SO}_4 & \rightarrow \text{HMDS} \rightarrow C - C - \\
& \text{TMCS} \rightarrow \text{TMSO} \rightarrow \text{OTMS}
\end{align*}
\]

dove HMDS = esametildisilasano; TMCS = trimetilchlorosilano.

where HMDS = hexamethyldisilasane
TMCS = trimethylchlorosylane
These composts were subsequently analysed by mass spectrometry. The main fragmentation took place because of the breaking up of the C-C bond in the original position of the double bond, as illustrated in the following formula:

\[
\begin{align*}
\text{CH}_3 - \text{[CH}_2]_n - \text{CH} - \text{CH} - (\text{CH}_2)_m \text{ COOR} \\
\text{TMS} & \quad \text{OTMS}
\end{align*}
\]

The mass spectrums show, in fact, two peaks of high intensity corresponding to the fragments a and b of each isomer. In figures 1, 2, and 3 there are reproduced, for example, the mass spectrums of TMSO derivatives of the methyl esters of oleic acid, palmitoleic and petroselineic acids.

In these mass spectrums, which facilitate considerably the determination of the position that the double bond occupied prior to oxidization of the fatty acids, the intensity of the main fragments (a & b) is always sufficient for the purposes of their identification, even in cases where the product analysed contains numerous isomers of position and when these are present in small percentages. It is interesting further to note, in order to make a quantitative examination of the spectrums of the mixture of the monounsaturated fatty acids, that the relative intensity of the ions a and b varies only slightly with the position of the double bond, and therefore in a
manner which we consider to be regulated by a precise law.

The mass spectrums of the derivatives of monounsaturated fatty acids'stereoisomers do not show such significant differences as to define their geometrical structure.

This however does not constitute a handicap in the method, since the isomers cis-trans can be easily separated for purposes of chromatography on a thin strata (24), before the preparation of the derivatives.

The favourable aspects of fragmentation under electronic impact of the composts examined in the aforementioned research has induced us to study the isomers of position and the geometrical ones, of the monounsaturated acids present in the following oils: rapeseed, parsley, soya, olive, flax, mustard, sunflower, maize, peanuts and hemp.

Experimental Part.

The samples of the oils analysed were extracted with hexane from seed or from the oily fruit. The fatty acids, obtained for purposes of saponification, were then esterified with methanol- BF₃ at 12%.

By means of chromatography on a thin strata of silica gel G/AgNO₃ (24) the corresponding fractions were then separated from the methyl esters of the cis-monounsaturated and trans-monounsaturated acids.
These fractions were then transformed into the corresponding di-trimethylsilyloxy derivatives according to the method described previously (23) and analysed directly by means of the combination of gas chromatography-mass spectrometry.

Figure 1 - Mass spectrum of TMSO derived from methyl oleate.

Figure 2 - Mass spectrum of TMSO derived from methyl-petroselinate.
Figure 3 - Mass spectrum of TMSO derived from methyl palmytholeate.

For this purpose a gas chromatograph-mass spectrometer LKB model 9,000 was utilized (*). The gas chromatographic apparatus for the introduction of the samples was furnished with a spiral column in glass measuring 2 m X 3 mm, filled with SE 30 at 2.5% on gas Chrom P, 100-120 mesh previously washed with acids and silanated. Temperature of the column: 220 degrees C; temperature of the evaporator: 270 degrees C; carrier gas: He, 30 ml/min. The molecular separator was maintained at 240 degrees C and the ionizing source at 310 degrees C. Power of ionization 70 eV; current of ionization 20 μA. The spectra were registered in 5 sec (m/e 1 - 400) on an apex of gas chromatographic peaks.

(*) Apparatus in use at the Centre of mass spectrometry of the University of Milan. We wish to offer our thanks to the directors of the Centre for their kind permission to use the above and Dr. Tito Salvatori for his courteous collaboration.
Results and review

Figure 4 is a reproduction of a gascromatogram of TMSO derived from the monounsaturated acids of rapeseed oil. The separation of the derivatives with differing numbers of carbon atoms is total and it is therefore possible to record the spectrums of the mass of single peaks without interference.

Figure 4 - Gascromatogram of TMSO derivatives of the methyl esters of monounsaturated fatty acids of rapeseed oil.

Three main peaks are present due to the homologous acids with 18, 20 and 22 carbon atoms.

The central zones of their mass spectrums are reproduced in Figure 5.

These confirm that the peak C\textsubscript{18} contains, side by side with the predominant cis-9-octadecenoic acid, small quantities of the isomers cis-11 - and cis-6-octadecenoic. This last
isomer was not referred to in the work of Kuemmel (2) whose researches were undertaken using the method of oxidative demolition.

The isomer cis-11-eicosenoic, and small quantities of the isomer cis-13 are present, instead, in the peak C_{20} while in the peak C_{22} there is only the isomer cis-13-docosenoic. In this last case we were unable to confirm the presence of the isomer cis-15-docosenoic, which was found instead by Kuemmel.
Figure 5 - Mass spectrums of TMSO derivatives of the methyl esters of monounsaturated fatty acids of rapeseed oil.

Figure 6 - Mass spectrums of TMSO derivatives of methyl esters of monounsaturated fatty acids of parsley oil.
We must mention however, even if our reservations are valid with regard to the possibility of errors resulting from the method of analysis by means of oxidative demolition in the case of small quantities of isomers, that our research was done on one sample only and has, therefore, no statistical validity.

The composition of the monounsaturated fatty acids of parsley oil is of considerable interest. The mass spectrums of TMSO derivatives of the acids C\textsubscript{16}, C\textsubscript{18}, C\textsubscript{20} are reproduced in Figure 6.

It may be pointed out that the hexadecenoic acids are composed of quantities which have more or less equal isomers cis-6-hexadecenoic and cis-9-hexadecenoic. The presence of the cis-6-hexadecenoic acid, though this is not surprising, was as far as we know never traced before in oils and vegetable fats, but only in the fat of hair (25) and in the fat of human excretion (26). The spectrum of the acids with 18 carbon atoms, confirms, instead, what has been stated previously (27, 28): as in all the oils of umbelliferous seeds, the petroselinic (cis-6-octadecenoic) forms the major part, together with a lesser quantity of oleic acid (cis-9-octadecenoic). The composition of the peak C\textsubscript{20}=-, instead, appears more complex where four isomers are present: the cis-6, the cis-8, the cis-9, and the cis-11 eicosenoic, as can be pointed out from the couples of peaks at m/e 285-217, 257-245, 243-259 and 215-287.
In Figure 7 is reproduced the isomeric composition of the acids C\textsubscript{16} - and C\textsubscript{18} - of the oil of virgin olives: the isomers which are most represented, confirming the previous researches (2), are respectively the cis-9-hexadecenoic and the cis-9-octadecenoic together with a small quantity of the isomers cis-11-hexadecenoic and cis-11-octadecenoic.

Fig. 7 - Mass spectrums of TMSO derived from the methyl esters of the monounsaturated fatty acids of olive oil.

In Table 1, finally, there is a representative recapitulation of the isomers of position present in the homologous monounsaturated fatty acids of all the oils examined.
A perusal of the aforesaid data enables us to show that for acids with 16 and 18 carbon atoms there predominate largely the isomers with the double bond in the position 9, 10. The octadecenoic acids contain moreover smaller quantities of the isomer Δ11 which does not seem to be a normal constituent of the hexadecenoic acids: this is present in olive oil, but has not been found, for example, in the oil of parsley seeds; in this last, there is present instead, as we have examined, the isomer Δ6.

The acids with 20 carbon atoms are composed in a large part of the isomer Δ11 with small quantities of the isomer Δ13. The only isomer Δ13 was found in the acids with 22 carbon atoms.

The position of the double bonds in the monoenic acids examined so far seems to be in a large measure in accord with the most recent views on the biosynthesis of unsaturated acids. The formation of every isomer could in fact, be reasonably explained by the suggested mechanism of enzymatic de-hydrogenation of the corresponding saturated acids and the elongation of the chain by addition of two carbon atoms.

The data reproduced in Table 1 seems to agree with what has been suggested by Kishimoto and Radin (29), who have postulated the existence of two distinct de-hydrogenated enzymes, capable of moving respectively on the positions 6 and 9 from the carboxyl group of palmitic and stearic acids. These enzymes
would take into account the formation of the acids cis-6 and cis-9-hexadecenoic, and cis-6 and cis-9-octadecenoic.

It may however be noted that the knowledge on the de-hydrogenated enzymes is still somewhat limited and only from yeast there has been isolated an enzyme which is capable of de-hydrogenating the palmitic and stearic acids in position 9 with the formation of the palmitoleic and oleic acids (30). The mechanism of elongation of the chain - demonstrated with the help of the composts marked in the case of the monoenic acids (31) and dienic acids (32) of the sphingo-lipids and, in vitro, with olein CoA (33) - would explain the formation of the monoenic acids with 20 and 22 carbon atoms and the isomers cis-11-octadecenoic, according to the following pattern:

\[
\begin{array}{c}
\text{C}_{16}^\Delta^6 \\
\text{C}_{20}^\Delta^8
\end{array} \rightarrow \begin{array}{c}
\text{C}_{16}^\Delta^9 \\
\text{C}_{20}^\Delta^8
\end{array} \rightarrow \begin{array}{c}
\text{C}_{16}^\Delta^8 \\
\text{C}_{18}^\Delta^8 \\
\text{C}_{18}^\Delta^7 \\
\text{C}_{18}^\Delta^6
\end{array} \rightarrow \begin{array}{c}
\text{C}_{18}^\Delta^9 \\
\text{C}_{18}^\Delta^8 \\
\text{C}_{20}^\Delta^9
\end{array} \rightarrow \begin{array}{c}
\text{C}_{20}^\Delta^6
\end{array}
\]

The isomers enclosed in the squares are those formed by de-hydrogenation of the corresponding saturated acids. It may be pointed out that this arrangement would take into account the formation of all the isomers, with the exception of the acids \( C_{16} \Delta^{11} \), \( C_{20} \Delta^{6} \) and \( C_{20} \Delta^{9} \) for which direct de-hydrogenation of the corresponding saturated acid would seem to be the
answer. The acids cis-6 and cis-9-eicocenoic have been found however, only in the oil of parsley seed.

We would like to point out, finally, that we could find no trace in this oil of the acid cis-8-octadecenoic, which should have resulted from the addition of a unit of C$_2$ to the acid cis-6-hexacenoic, while the acid cis-8-eicocenoic is present and is most likely derived from the acid cis-6-octadecenoic with a similar mechanism.

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<th>Vegetable oil</th>
<th>Relative heights of fragments a and b for groups of isomers of acids</th>
<th>Δa</th>
<th>Δb</th>
<th>Δc</th>
<th>Δd</th>
<th>Δe</th>
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<tr>
<td>Colza (Brassica campestris)</td>
<td>C$_{16}$</td>
<td>3,5</td>
<td>10,2</td>
<td>100</td>
<td>98</td>
<td>4,2</td>
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<tr>
<td>Rapeseed</td>
<td>C$_{16}$</td>
<td>88</td>
<td>100</td>
<td>90</td>
<td>61</td>
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<tr>
<td></td>
<td>C$_{16}$</td>
<td>77</td>
<td>100</td>
<td>14</td>
<td>18</td>
<td></td>
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<tr>
<td></td>
<td>C$_{16}$</td>
<td>34</td>
<td>100</td>
<td>93</td>
<td>85</td>
<td></td>
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<tr>
<td>Parsley</td>
<td>C$_{16}$</td>
<td></td>
<td></td>
<td>100</td>
<td>93</td>
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<tr>
<td>Soja (Soja hispida)</td>
<td>C$_{16}$</td>
<td>100</td>
<td>79</td>
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<td></td>
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<tr>
<td>Soya</td>
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<td>99</td>
<td>100</td>
<td></td>
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<td>Linio (Linum usitatissimum)</td>
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<td>100</td>
<td>96</td>
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<td>Maiz (Zea mays)</td>
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<td>100</td>
<td>95</td>
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<td>Arachide (Arachis hypogea)</td>
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<td>2,8</td>
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<tr>
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Conclusions:

The method of determining the position of the double bond in the monoenic acids by means of gas chromatography-mass spectrometry has been found to be simple and quick to operate and enables one to work with very small quantities, of one-two mg.

And small quantities of isomers can be readily shown up when these are present in quantities exceeding or equal to 1% of the most abundant isomer.

The results obtained have confirmed basically the data already noted in the literature on the subject, but in some cases it has been possible to show up the presence of isomers which were not noted previously, such as the acids cis-6-hexadecenoic, cis-8 and cis-9-eicosenoic in the oil of parsley seed, and cis-13-octadecenoic in rapeseed and mustard oils.

The slight variations that are found in the relative intensity of the fragments a and b in the different isomers of position (23) make it necessary to determine the mass spectrum of the pure single isomers, other than the standardization of experimental conditions, when exact quantitative results are to be obtained.

From an examination of the results obtained by us, it is possible, nevertheless, to show with a percentage error of around 15%, the quantitative ratio of the isomers present in the different acids, based on the ratio of the height of the main fragments corresponding to the different isomers on the mass spectrum.
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DISCUSSION RECORDED

PRESIDENT: PROF. CANTARELLI (Institute of Agrarian Industries of the University of Perugia).

PRESIDENT

We congratulate most heartily Dr. Zorzut for the excellent demonstration of an extremely refined technique, which I consider most useful since it has a very wide application. The next time we shall have a direct quantitative tabulation. This has been a most oriented and extremely interesting paper; and then there are applications of a biochemical nature which are yet another horizon that is opening up in relation to this research. My warm thanks again.

A discussion then took place in which among those taking part were Prof. Privett (Hormel Institute of the University of Minnesota) and a resumé of which was provided by Prof. Capella:

PROF. CAPELLA (Institute of Agrarian Industries of the University of Bologna).

As everybody knows, Prof. Privett has put forward a most refined method for the determination of the position of the double bonds by means of ozonolysis of the unsaturated acids and determination by means of gas chromatography of the fragments that are obtained by this method. Now according to Prof. Privett, his method has above all the advantage of being a very rapid one and also a better deal than the method used by us; naturally by deal I refer to the fact that we must have the mass spectrometer.
I am certainly in agreement with the second of these objections but cannot say the same for the first; our method is more or less equally rapid and above all the point where we believe our method is different from the method of ozonolysis is this: we are not forced to proceed with the prior separation of the homologues present in a complex mixture of fatty acids in order to study the different isomers of position of each homologue.

For example if we have a series from C14 to C20 of mono-unsaturated acids, we can effect a gas chromatography of the derivatives, take the mass spectrum of each peak and thus see in each peak how many isomers and what sort of isomers are present.

Naturally, in the method outlined by Prof. Privett also which does not provide for the separation by means of chromatography on a thin strata, but provides instead a separation by means of liquid-liquid chromatography of the different isomers which according to him, brings the things to a parity, i.e. we are forced to effect a chromatography on a thin strata in order to separate the monounsaturated acids from the polyunsaturated ones and Prof. Privett makes a liquid-liquid chromatograph which separates the homologues. Thus, he states we bring the "things to parity". I believe that on this position of parity we can all be satisfied.