high initial residue amounts of 2,4-D may reflect either a more rapid uptake and/or metabolism of bromoxynil by wheat seedlings or a greater susceptibility of bromoxynil to photodecomposition.

Application of 2,4-D and bromoxynil to wheat seedlings as a tank mixture did not result in significant differences in residues when compared to the residues observed when the chemicals were applied singly.

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Determination of the Triglyceride Composition of Olive Oil by a Multistep Procedure

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Olive oil was analyzed by separating the total triglycerides into fractions, different in the number of double bonds per mole, by means of Ag^+ TLC. The fatty acid composition—overall and at the β position—was then determined both of total triglycerides and of single fractions; moreover, each fraction was oxidized and the products were separated by means of TLC into classes, each containing molecules having the same number of azelaic acid residues (A_3; A_2S; AS_2). The acids contained in each class were then quantitated by means of GLC. From the obtained data, the triglyceride composition of olive oil was determined (21 molecular species = 92.5% of total triglycerides), without the many assumptions usually conceived by other methods.

Many methods have been described to determine the glyceride composition of fats.

In some of these, the analytical procedure is carried out by working directly on the fat (Dutton and Cannon, 1956; Quimby et al., 1953; Riemenschneider, 1954; Scholfield and Dutton, 1958; Scholfield and Hicks, 1957) or on the oxidized products of unsaturated fatty acid components of the fat (Hilditch and Lea, 1927; Kartha, 1961; Youngs, 1961); other methods are based either on the ozonization of double bonds, followed by the catalytic reduction of ozonides and by their separation and TLC quantification (Privett and Blank, 1961, 1963), or on the action of pancreatic lipase in the 1-3 positions of the triglycerides (Vander Wal, 1960).

Otherwise, the component glycerides of some seed oils were separated by Ag^+ TLC on two chromatoplates and then quantitatively determined by GLC of the methyl esters of their fatty acid components, with methyl eptadecanoate as an internal standard (Gunstone and Padley, 1965); the results agree with those obtained by other methods, but the isomeric molecular species are not determinable.

Some GLC methods directly operating on triglycerides (Freyer et al., 1960; Huebner, 1961; Kuksis and McCarthy, 1962; McCarthy et al., 1962, Pelick et al., 1961) have been entirely successful, though they do not allow for the separation of unsaturated from saturate glycerides with the same carbon number.

This has been partly overcome with an oxidation process that converts the unsaturated glycerides into compounds having smaller carbon numbers; GLC analysis of these esterified compounds gives the distribution of the individual saturated fatty acids in the glycerol mojety, whereas the unsaturated fatty acids (palmitoleic, oleic, linoleic, and linolenic) are estimated together as azelaoglycerides (Youngs and Subbaram, 1964).

An improved method (Subbaram and Youngs, 1964) based on the fractionation of glycerides into groups dif-

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Table I. Olive Oil Triglyceride Fractions Separated by Ag^+ TLC

fraction	mol % in the total triglycerides
1 double bond/mol	3.9
2 double bonds/mol	25.1
3 double bonds/mol	50.7
4 double bonds/mol	12.8
5+ double bonds/mol	7.5

fering in the total unsaturation followed by GLC analysis of oxidized, esterified glyceride groups gives the distribution of both saturated and unsaturated fatty acids but does not allow for the separation of isomeric glyceride species.

However, as reported by others (Subbaram et al., 1964), the results obtained by the above methods might disagree, especially when the glyceride composition of oils containing unusual acids is determined.

In our publication (Damiani et al., 1977), a thin-layer chromatographic separation of oxidized derivatives of the olive oil triglycerides into fractions (different in the number of azelaic acid residues per mole) was described; the amounts of mono- and dicarboxylic (azelaic) acids in each fraction were then determined by means of standard gas chromatography, and from these data the molar percentages of the A_3, A_2P, A_2St, AP_2 and APSt classes of oxidized triglycerides were obtained (A is azelaic acid residue, P is palmitic acid residue, and St is stearic acid residue).

With this procedure it is impossible to obtain further data and to determine, i.e., the molar percentages of the single species—OOO, OLO, OOL, LLO, LOL, LLL, etc.—from which the A_3-oxidized triglyceride originates.

For this purpose, in the present work an analogous procedure was carried out on the triglyceride fractions separated according to the number of double bonds per molecule, using Ag^+ thin-layer chromatography.

These data were then compared with those calculated by using the assumptions of Vander Wal (1960) and Coleman (1961), after the pancreatic lipase hydrolysis of the triglycerides by the procedure described by Coleman (1961) was carried out.

EXPERIMENTAL PROCEDURES

Isolation of Total Triglycerides. The sample of olive oil (1 g) was dissolved in petroleum ether, bp 30–50 °C (20 mL); 1 mL of this solution was applied as a band across the base of a silica gel G plate (20 × 20 cm, layers 0.5 mm thick), developed in petroleum ether (bp 30–50 °C)–diethyl ether–99% formic acid (70:30:1.5). Bands were visualized by spraying the plate with 2′,7′-dichlorofluorescein in ethanol (0.1% w/v).

The band containing the total triglycerides (R_f ~0.85) was scraped off and exhaustively extracted with $CHCl_3$ (~20 mL); $CHCl_3$ was then evaporated and the obtained triglycerides were stored in a desiccator, in an atmosphere of nitrogen and away from light.

Several separations were carried out to get enough materials for the following operations.

Ag^+ Thin-Layer Chromatography of Total Triglycerides and GLC Analysis of Fatty Acid Methyl Esters. An amount of 50–60 mg of total triglycerides (dissolved in 1 mL of $CHCl_3$) was separated into fractions by TLC on silica gel G impregnated with silver nitrate (Litchfield et al., 1964).

Each of these fractions, containing glycerides having the same number per mole of double bonds, was exhaustively extracted from the adsorbent and quantitated by the chromotropic acid method (Vanhandel, 1961).

The obtained values, reported in Table I, are an average of 10 determinations from as many chromatographic separations.

An amount of 5–20 mg both of total triglycerides and of each fraction was then refluxed with 10 mL of the mixture anhydrous CH_3OH–C_6H_6–concentrated H_2SO_4 (75:25:1).

After 30 min, 10 mL of water was added, the methyl esters were extracted with ethyl ether (2–4 mL), and the obtained solutions were directly analyzed.

GLC was carried out with a Varian 3700 gas chromatograph, equipped with a flame ionization detector and a Varian CDS 111 C. The column (6 ft × $^1/_8$ in.) packed with 20% LAC-886 on Chromosorb W 60/80 was operated isothermally at 180 °C with a carrier gas flow (N_2) of 30 mL/min.

Quantitative response factors were obtained from mixtures of known composition. The fatty acid composition of each fraction and of total triglycerides is reported in Table II.

Pancreatic Lipase Hydrolysis. Pancreatic lipase hydrolysis both of total triglycerides and of each fraction was carried out as suggested by Coleman (1961). The partial glycerides were separated on a thin layer (0.25 mm) of silica gel G, developed in petroleum ether (bp 30–50 °C)–diethyl ether–99% formic acid (70:30:1.5).

The β-monoglyceride band (R_f ~0.04), detected with 2′,7′-dichlorofluorescein, was scraped off the glass TLC plate and refluxed as described above; the obtained methyl esters were also analyzed as indicated. The fatty acid composition of each fraction and of total triglycerides at the 2 position is reported in Table II.

Oxidation of Triglyceride Fractions. An amount of ~50 mg of each triglyceride fraction was oxidized as indicated by von Rudloff (1956).

The oxidized glycerides were extracted with four 25-mL portions of $CHCl_3$ from each reaction mixture. The collected extracts were then concentrated (1–2 mL) in a current of air.

Thin-Layer Chromatography of Oxidized Triglyceride Fractions. The chloroformic solution of each oxidized glyceride fraction was applied as a band across the base of a precoated TLC plate with silica gel 60, Merck (20 × 20 cm, layers 0.25 mm thick); the separation was carried out by developing the plate for 1 h (~17.5 cm) in petroleum ether (bp 60–80 °C)–diethyl ether–99% formic acid (70:30:2). One, two, or three bands were visualized

Table II. Olive Oil Fatty Acid Composition of the Total Triglycerides and of the Fractions Having One, Two, Three, and Four Double Bonds per Mole, Separated by Ag^+ TLC (mol %)

	overall ($C_{1,2,3}$)					C_β (C_2)				
		double bonds/mol					double bonds/mol			
fatty acid	total triglyc.	1	2	3	4	total triglyc.	1	2	3	4
P	12.1	54.3	27.2	2.5	0.9	1.2	6.2	2.3	0.4	0.3
P′	0.5	0.2	0.3	0.6	0.6	0.4	0.6	0.6	0.4	0.4
St	2.2	8.0	4.9	0.4	0.1	0.1	0.9	0.3	0.1	
O	77.4	37.5	67.1	93.4	65.1	87.5	92.3	94.5	94.5	56.2
L	7.8		0.5	3.1	33.3	10.8		2.3	4.6	43.1

Table III. Olive Oil Acid Composition of the Oxidized Triglyceride Fractions

fraction	classes (produced by oxidn)	palmitic acid			azelaic acid			stearic acid			% C[e]	% F[f]
		a[a]	b[b]	c[c]	a[a]	b[b]	c[c]	a[a]	b[b]	c[c]		
1 double bond/mol	AS_2	2.2	56.4	56.4	1.4	35.9	35.9	0.3	7.7	7.7	3.9	3.9
2 double bonds/mol	A_2S	6.7	27.3	26.6	16.6	67.8	66.2	1.2	4.9	4.8	24.5	25.1
	AS_2	0.3	50.0	1.2	0.2	33.3	0.8	0.1	16.7	0.4	0.6	
			total:	27.8		total:	67.0		total:	5.2		
3 double bonds/mol	A_3	–	–	–	46.5	100	91.7	–	–	–	46.5	50.7
	A_2S	1.3	30.9	2.6	2.7	64.3	5.3	0.2	4.8	0.4	4.2	
	AS_2	–	–	–	–	–	–	–	–	–	–	
			total:	2.6		total:	97.0		total:	0.4		
4 double bonds/mol	A_3	–	–	–	12.5	100	97.7	–	–	–	12.5	12.8
	A_2S	0.1	33.3	0.8	0.2	66.7	1.6	–	–	–	0.3	
			total:	0.8		total:	99.3					
5+ double bonds/mol	A_3, A_2S		n.d.[d]			n.d.[d]			n.d.[d]		7.5	7.5

[a] Micromoles of P, A, and St (determined via GLC in the class) related to the % F values. [b] Mole percent in the class. [c] Mole percent in the fraction. [d] n.d. = not determined. [e] % C = mole percent of each class (sum of micromoles of P, A, and St determined in each class) in the total triglycerides. [f] % F = mole percent of each fraction in the total triglycerides.

Table IV. Olive Oil Glyceride Fractions, Relative Molecular Species, and Classes Produced by Oxidation

double bonds/mol of the original fraction	possible initial molecular species	classes[a]	mol % of total triglycerides	
1	POP, StOP, PPO, *StPO, StOSt, StStO, StPP', StP'P, StP'St, StStP', PPP', PP'P*	AS_2 (ASS + SAS)	3.9	3.9
2	OOP, OPO, OOSt, P'OP, *OStO, P'PO, P'OSt, P'StO, OP'P, OP'St, P'P'P, P'PP', P'P'St, P'StP'*	A_2S (AAS + ASA)	24.5	25.1
	PLP, PLSt, *LPP, LPSt, LStP, LStSt, StLSt*	AS_2 (ASS + SAS)	0.6	
3	OOO, OP'O, P'OO, *P'OP', P'P'O, P'P'P'*	A_3	46.5	50.7
	POL, PLO, OPL, StOL, StLO, *OStL, PPL, PLP', StP'L, StLP', P'StL, P'PL*	A_2S (AAS + ASA)	4.2	
	PPLn, PLnP, StLnSt, StStLn, PStLn, StPLn, PLnSt	AS_2 (ASS + SAS)	trace	
4	OOL, OLO, P'LO, *P'OL, OP'L, P'P'L, P'LP'*	A_3	12.5	12.8
	PLL, *StLL, LStL, LPL*	A_2S (AAS + ASA)	0.3	
5+	*LOL, OLL, LP'L, P'LL*, etc. *LnLP, LnPL, LLnP, LnLSt, LnStL, LLnSt*, etc.	A_3, A_2S (AAS + ASA)		7.5
			total:	100.0

[a] Produced by oxidation of initial molecular species.

after spraying the plate with 2',7'-dichlorofluorescein in ethanol (0.1% w/v); that is

band	recoverable in the triglyceride fraction originally having the following number of unsaturated acid residues/mol	$R_f \times 100$ values	class (type of oxidized triglycerides in the band)
1°	3	1-2	A_3
2°	2	10-12	A_2S (AAS + ASA)
3°	1	24-26	AS_2 (ASS + SAS)

where A is the azelaic acid residue and S is the saturated acid residue, palmitic or stearic.

Quantitation of Acids (A, P, and St) Contained in the A_3, A_2S, and AS_2 Classes of Each Oxidized Glyceride Fraction. Each of the bands obtained from TLC of each oxidized fraction was scraped off the glass TLC plate; the recovered materials, mixed with known and suitable weights of oleic acid (internal standard), were refluxed as indicated above.

GLC analysis of obtained methyl esters was also carried out as described above. The results are shown in Table III.

RESULTS AND DISCUSSION

The triglyceride composition of the olive oil sample was determined according to the experimental data in Tables I–III, as well as the indications in Table IV (for the determination of glyceride composition, it was not necessary to know absolute quantities of acids contained in the classes of each oxidized fraction—values reported in the a columns of Table III, determined by using oleic acid as an internal standard—but it was necessary to know the molar percentages of acids in those classes; since the above-mentioned absolute quantities were determined for confirming the data of Table I, they consequently are reported in Table III).

Table V. Outline for the Determination of Triglyceride Composition of Olive Oil[a]

fraction	classes (produced by oxidn)	C	F	data and relationships considered	species determined	mol %
1 double bond/mol	AS_2	0.039	0.039	(a) StOP + POP + PPO = 3.9		
				(b) StOP = 3 × St_C × C (or 3 × St_F × F) = 3 × 7.7 × 0.039 (or 3 × 8 × 0.039)	I, StOP	0.9
				(c) PPO = P_β × F = 6.2 × 0.039	II, PPO	0.2
				(d) POP = (a) − [(b) + (c)] = 3.9 − (0.9 + 0.2)	III, POP	2.8
2 double bonds/mol	A_2S	0.245		(e) OOP + OPO + OOSt + P′OP = 24.5		
				(f) P′OP = 3 × P'_F × F = 3 × 0.3 × 0.251	IV, P′OP	0.2
				(g) StOO = 3 × St_C × C = 3 × 4.9 × 0.245	V, StOO	3.6
			0.251	(h) OPO = P_β × F = 2.3 × 0.251	VI, OPO	0.6
				(i) POO = (e) − [(f) + (g) + (h)] = 24.5 − (0.2 + 3.6 + 0.6)	VII, POO	20.1
	AS_2	0.006		(l) PLP + PLSt = 0.6		
				(m) PLSt = 3 × St_C × C = 3 × 16.7 × 0.006	VIII, PLSt	0.3
				(n) PLP = (l) − (m) = 0.6 − 0.3	IX, PLP	0.3
3 double bonds/mol	A_3	0.465		(o) OOO + P′OO + OP′O = 46.5		
				(p) P′OO + OP′O = 3 × P'_F × F = 3 × 0.6 × 0.507 = 0.9		
				(q) OP′O = P'_β × F = 0.4 × 0.507	X, OP′O	0.2
			0.507	(r) P′OO = (p) − (q) = 0.9 − 0.2	XI, P′OO	0.7
				(s) OOO = (o) − [(q) + (r)] = 46.5 − (0.2 + 0.7)	XII, OOO	45.6
	A_2S	0.042		(t) POL + PLO + OPL + StLO = 4.2		
				(u) StOL + StLO = 3 × St_C × C = 3 × 4.8 × 0.042 = 0.6		
				(v) PLO + StLO = L_β × F = 4.6 × 0.042 = 2.3		
				(w) OPL = P_β × F = 0.4 × 0.507	XIII, OPL	0.2
				(z) POL + StOL = (t) − [(w) + (v)] = 4.2 − (0.2 + 2.3) = 1.7		
				(x) POL/StOL = P_C/St_C = 30.9/4.8 = 6.4		
				(x′) POL = 6.4 × StOL		
				(y) StOL = (z) − (x′) = 1.7 − 6.4 × StOL	XIV, StOL	0.2
				(a′) StLO = (u) − (y) = 0.6 − 0.2	XV, StLO	0.4
				(b′) PLO = (v) − (a′) = 2.3 − 0.4	XVI, PLO	1.9
				(c′) POL = (t) − [(b′) + (w) + (y) + (a′)] = 4.2 − (1.9 + 0.2 + 0.2 + 0.4)	XVII, POL	1.5
4 double bonds/mol	A_3	0.125		(d′) OOL + OLO + P′LO = 12.5		
				(e′) OOL = O_β × F = 56.2 × 0.128	XVIII, OOL	7.2
			0.128	(f′) P′LO = 3 × P'_F × F = 3 × 0.6 × 0.128	XIX, P′LO	0.2
				(g′) OLO = (d′) − [(e′) + (f′)] = 12.5 − (7.2 + 0.2)	XX, OLO	5.1
	A_2S	0.003		(h′) PLL = 3 × P_C × C = 3 × 33.3 × 0.003	XXI, PLL	0.3

[a] Symbol meaning: P_F, St_F, etc. = mole percent of acids P, St, etc. in the considered fraction; P_β, St_β, etc. = mole percent of acids P, St, etc. in the 2-monoglyceride of the considered fraction; P_C and St_C = mole percent of acids P and St in the considered (oxidized) class; C = molar fraction of the considered triglyceride class (with respect to the total triglycerides); F = molar fraction of the considered triglyceride fraction (with respect to the total triglycerides).

Among the possible initial species—from which the oxidized triglycerides were obtained—the ones in italics (Table IV) were not taken into consideration because they depended either on the rather low molar percentage in the various fractions of some of the constituent acids of the aforementioned species or on the low molar percentage of the fraction under consideration in relation to the total triglycerides or for both reasons.

The molar percentages of the remaining species were obtained as indicated in Table V. This table verifies that it was possible to obtain molar percent values of 21 triglyceride species (92.5% of the total) through the experimental data related to the classes of the various oxidized fractions as well as those related to the nonoxidized fractions.

The determination of these two data categories is of great analytic significance because it allows for a completely experimental evaluation without any preconceived assumptions, which is extremely interesting.

In Table VI, our values have been compared to those calculated by using the assumptions of Vander Wal (1960) and Coleman (1961), by means of the fatty acid composition of the positions 2 and 1–3 of total triglycerides; the fatty acid composition at the 1–3 positions was obtained by using the relationship

$$\alpha - \alpha'(1\text{–}3) = [3T - \beta(2)]/2$$

where $\alpha - \alpha'(1\text{–}3)$ = mole percent of each acid in the 1–3 positions, $\beta(2)$ = mole percent of each acid in the 2 position, and T = mole percent of each acid in the total triglycerides. [Using this relationship, the positions 1 and 3 are considered to have the same fatty acid compositions, while Brockerhoff and Yurkowski (1966) showed that the distribution is never completely symmetrical. However, it is easy to demonstrate that the molar percentages of the different molecular species, calculated by using the two series of data of the 1–3 acidic compositions, are very similar].

This comparison emphasizes how close the results were between the two procedures.

The olive oil used here was shown to contain nine molecular species with percentages higher than 1 (88.7 mol % of total triglycerides); the other twelve species make up 3.8% mol of the total.

These data agree fairly well those obtained by other authors (Kaufmann and Wessels, 1966; Fedeli and Jacini,

Table VI. Olive Oil Triglyceride Composition of Examined Sample (mol %)

molecular species	present procedure	Vander Wal-Coleman procedure
I, StOP	0.9	1.0
II, PPO	0.2	0.3
III, POP	2.8	2.7
IV, P′OP	0.2	0.2
V, StOO	3.6	4.0
VI, OPO	0.6	0.6
VII, POO	20.1	22.3
VIII, PLSt	0.3	0.1
IX, PLP	0.3	0.3
X, OP′O	0.2	0.2
XI, P′OO	0.7	0.8
XII, OOO	45.6	45.7
XIII, OPL	0.2	0.4
XIV, StOL	0.2	0.4
XV, StLO	0.4	0.5
XVI, PLO	1.9	2.7
XVII, POL	1.5	1.9
XVIII, OOL	7.2	8.0
XIX, P′LO	0.2	0.1
XX, OLO	5.1	5.6
XXI, PLL	0.3	0.2
total:	92.5	97.7

1967) using different procedures and, of course, different samples.

As reported in Table IV, the species OLL and LOL, which belong to the fraction containing glycerides having five double bonds/mol (class A_3), were not determined in this work. According to the procedure of Vander Wal and Coleman, they should have been present in our sample at the rate of 1.0 and 0.3 mol %, respectively.

This omission seemed irrelevant to us, as we were able to prove experimentally that the fraction containing molecules with five double bonds was present in our sample in very low molar percentage.

In order to obtain sufficient quantities of this fraction for the operations described in the experimental part, dozens of chromatographic separations would have been necessary and, although this would been quite possible with slight experimental modifications where necessary, it was completely unwarranted here.

In any case our aim was to reach, by completely experimental means and without any preconceived assumptions, the results that actually were obtained by using the above-described combination of multiple operations (Ag^+ TLC, followed by single fraction lipolysis, oxidation of these fractions, TLC of oxidized products, and then GLC of the methyl esters of the acids present in each of the classes A_3, A_2S, and AS_2 separated by TLC).

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Analysis of Total Phenols Using the Prussian Blue Method

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The method for determining vegetal phenolics based on the formation of the Prussian Blue complex has been reassessed. The color development has not interfered with the common naturally occurring substances except for ascorbic acid. The method is ~20 times as sensitive as the acidified vanillin method and 3 times that of the titanium method. The Prussian Blue method, plus a simple extraction procedure, has been used in order to provide a rapid estimation of the total phenol content in strawberries. Since a remarkable decrease in the levels of phenols was noted at progressive stages of ripeness, they can be considered as a chemical index of ripening.

Our investigation was aimed at finding one or more "warning" substances of the ripening process of fruits and vegetables which may be easily employed by agricultural producers. The search for ripeness indexes comes from the recurrent need to decide when fruits should be harvested. For this purpose total phenols were examined. Phenols which are widely distributed in fruits and vege-

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