Determination of Furan Fatty Acids in Extra Virgin Olive Oil

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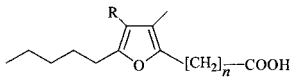
The presence of 4 different furan fatty acids (F-acids) was detected in 18 samples of transmethylated monovarietal extra virgin olive oil: methyl 10,13-epoxy-11,12-dimethyloctadeca-10,12-dienoate [diMeF(9,5)], methyl 12,15-epoxy-13,14-dimethyleicosa-12,14-dienoate [diMeF(11,5)] and both olefinic derivatives of diMeF(11,5) with one unsaturation on the side chains conjugated with the furan ring. Transmethylated oils were analyzed by normal phase high-performance liquid chromatography coupled on-line with capillary gas chromatography. After the gas chromatographic separation step, a more selective detection of F-acids was achieved by using a photoionization detector mounted in series with a flame ionization detector. The concentration of F-acids ranged between 50 ppb (detection limit of the method) and 2.1 ppm in the oil. The olefinic derivatives of diMeF(11,5) acids detected were not artifacts created during the sample preparation or during the chromatographic analysis.

Keywords: *Furan fatty acids; on-line LC-GC; photoionization detector; extra virgin olive oil; mass spectrometry; squalene*

INTRODUCTION

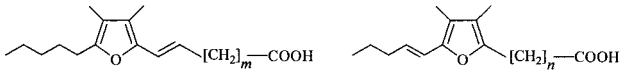
Furan fatty acids (F-acids) (Figure 1) are present in plants, fishes, amphibians, reptiles, and mammals, including man (Hannemann et al., 1989). Their physiological role has been studied because they are also isolated in appreciable amounts in commonly used foods such as fish oil, seed oils, and butter (Guth and Grosch, 1992). In some fish F-acids can represent up to 25% of the acids in the liver lipids (Glass et al., 1975). Because they accumulate during the spawning season, a biological role of F-acids is assumed in fish. Moreover, their structure is similar to that of some prostaglandins (a five-membered ring with an alkyl chain and an alkyl carboxylic chain). Nevertheless, F-acids are not biosynthesized in rats (Wahl and Liebich, 1994), and their role in mammalian metabolism is still unknown. F-acids were found to prevent oxidation of linoleic acid (Okada et al., 1990) and to act as antioxidants in plants (Batna and Spiteller, 1994). A relationship seems to exist between photosynthesis and F-acid concentration in plants, because parts of the plants that are exposed to daylight show a higher content of F-acids (Hannemann et al., 1989). Furthermore, F-acids are found in some vegetables such as soybeans (Guth and Grosch, 1991) and wheat, whereas they are known to be absent (or they were only identified but not determined quantitatively) in olives, sesame, walnut, grape seed, or sunflower (Wahl et al., 1994). For these reasons some attention was given to the determination of F-acids as quality markers for soybean oil. Their role as precursors of a light-induced off-flavor was first reported (Guth and Grosch, 1991) but recently questioned (Kao and Wu, 1998). However, there are no references about the possible use of F-acids in the quality control of other valuable oils, such as extra virgin olive oil, because the characterization of F-acids in olive oil has not been carried out. Preseparation of F-acids from predominantly straight-chain fatty acids is a critical step and renders analytical methods long and laborious. Procedures described in the literature are not suitable for routine analysis. They can involve a two-step urea fractionation (Guth and Grosch, 1991), hydrogenation, and thin-layer chromatography (Puchta et al., 1988) or bidimensional GC-MSD (Wahl and Liebich, 1994) for the identification. In this work a new analytical method for the determination of F-acids in edible oils (e.g., olive oils)

Non olefinic furan fatty acids



Non olefinic furan fatty acid	Abbreviation	*n*	R
8,11-epoxy-9,10-dimethylhexadeca-8,10-dienoic acid	diMeF(7,5)	6	CH_3
9,12-epoxy-10,11-dimethylheptadeca-9,11-dienoic acid	diMeF(8,5)	7	CH_3
10,13-epoxy-11-methyloctadeca-10,12-dienoic acid	MeF(9,5)	8	H
10,13-epoxy-11,12-dimethyloctadeca-10,12-dienoic acid	diMeF(9,5)	8	CH_3
11,14-epoxy-12,13-dimethylnonadeca-11,13-dienoic acid	diMeF(10,5)	9	CH_3
12,15-epoxy-13-methyleicosa-12,14-dienoic acid	MeF(11,5)	10	H
12,15-epoxy-13,14-dimethyleicosa-12,14-dienoic acid	diMeF(11,5)	10	CH_3

Olefinic furan fatty acids



Olefinic furan fatty acid	Abbreviation	*m*	*n*
10,13-epoxy-11,12-dimethyloctadeca-10,12,14-trienoic acid	diMeF(9, 5:1)	-	8
10,13-epoxy-11,12-dimethyloctadeca-8,10,12-trienoic acid	diMeF(9:1, 5)	6	-
12,15-epoxy-13,14-dimethyleicosa-12,14,16-trienoic acid	diMeF(11, 5:1)	-	10
12,15-epoxy-13,14-dimethyleicosa-10,12,14-trienoic acid	diMeF(11:1, 5)	8	-

Figure 1. Non olefinic and olefinic furan fatty acids

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was developed by using high-performance liquid chromatography (HPLC) coupled on-line with capillary gas chromatography (GC) (on-line LC-GC). To improve the sensitivity and selectivity of the method, a photoionization detector (PID) was mounted in series with a flame ionization detector (FID) after the GC separation step.

MATERIALS AND METHODS

Reagents and Standards. All of the solvents were of analytical grade. *n*-Hexane was passed through basic aluminum oxide and then distilled. The standard diMeF(9,5) acid methyl ester (purity ~94%) was synthesized (Pompizzi, 1999) and provided by Dr. R. Pompizzi (Zürich, Switzerland) and contained impurities of diMeF(8,5), diMeF(7,5), diMeF(10,5), and diMeF(11,5), which were useful for the identification of F-acids.

Oil Samples. Extra virgin monovarietal oil samples (18) were obtained from the following Italian cultivars: Ascolana Tenera, Carolea, Cima di Melfi, Coratina, Frantoio, Gentile di Larino, I-77, Itrana, Leccino, Maiatica, Maiolice, Nocellara del Belice, Nocellara Etnea, Nociara, Pendolino, Peranzana, Rosciola di Rotello, and Termite di Bitetto. Olives were cold-pressed in October 1997. The acidity of the obtained oil ranged between 0.26 and 0.65 (expressed as milligrams of oleic acid in 100 mg of oil), and the peroxide value ranged between 1.1 and 6.2 (milliequivalents of active O_2 in 1 kg of oil). The oil obtained from Maiatica olives showed a peroxide value of 7.5, whereas Rosciola di Rotello olives gave an oil with a peroxide value of 9.8. After cold pressing, samples were frozen and kept in the dark until the analysis was performed. Soybean oil was obtained in the laboratory by mechanical cold compression of 50 g of soybeans.

Sample Preparation. Extra virgin olive oil (500 mg) was transmethylated according to the method of the Official Food Control Authority of the Canton of Zürich (Suter et al., 1997a,b). Only two modifications were adopted to suit this method to the LC-GC apparatus: the organic solvent was *n*-hexane instead of pentane, and the transmethylated sample was directly injected in the LC-GC system without dilution.

LC-GC-PID-FID Configuration. Transmethylated samples were directly analyzed by on-line LC-GC. The LC-GC system was a Dualchrome 3000 by Fisons (Rodano, Italy). The 250 × 2 mm i.d. LC column was packed with Lichrospher silica gel 60, 5 μm (Stagroma, Switzerland). A mixture of MTBE (1.2%) in hexane served as mobile phase at a flow of 500 μL/min. The LC injection loop had a volume of 100 μL. The LC column was automatically backflushed with 2 mL of MTBE after each analysis. The LC detector was a MicroUVis (Carlo Erba, Milan, Italy) spectrophotometer set at a wavelength of 226 nm [maximum absorbance of the standard diMeF(9,5)]. The on-column interface was a transfer line consisting of a 30 cm × 0.17 mm o.d. silica capillary tubing. The transfer volume of the LC window was 1500 μL.

The retention gap for the GC was a 10 m × 0.53 mm i.d. precolumn deactivated with an ultrathin layer (1 nm) of OV-1701 OH stationary phase (van der Hoff et al., 1994). No retaining precolumn was used. The 30 m × 0.32 mm i.d. GC separation column was coated in the laboratory with a 0.2 μm film of PS-255, a methylsilicone (apolar) stationary phase (Fluka, Buchs, Switzerland). Hydrogen was used as the carrier gas at a flow of 2 mL/min with a closed vapor exit. The LC-GC transfer occurred at 80 °C; after 8 min, the temperature was increased to 120 °C at a rate of 20 °C/min; it was then programmed to increase to 260 °C at a rate of 2 °C/min. The detection was performed by a PID 80 (CE, Rodano, Italy) equipped with a 8.4 eV lamp. The PID was mounted in series with an FID.

Two digital thermometers monitored the temperature at the outer walls of the retention gap; the first thermocouple was mounted 7 m from the injector and the second one 8 m. The flooded zone did not reach the second thermocouple during LC-GC transfer. The time for closing the solvent vapor exit (SVE) was estimated from the cooling effect (Hyötyläinen et al., 1997) registered by the first thermocouple.

PID Selectivity toward F-Acid Methyl Esters. Soybean oil (50 mg) spiked with diMeF(9,5) (2.5 mg) was transmethylated and analyzed by GC-PID-FID using the same instruments and precolumn as described above (Figure 3). The GC separation column was substituted with a 60 m × 0.25 mm i.d. Restek (model 2330) column coated with a 0.2 μm film consisting of 90% biscyanopropylsilanol. Carrier gas (H_2) flow was 2 mL/min. Amounts of 0.3 μL were injected on-column at 60 °C. After 5 min, the oven temperature increased to 100 °C at 20 °C/min and then to 240 °C at 3 °C/min.

Identification of F-Acids by Large Volume Injection (LVI)-GC-MS. The LC-GC identification of F-acids was confirmed by mass spectrometry (MS) analysis. An Ultra Trace 8060 (Fisons, Rodano, Italy) gas chromatograph equipped with an electronically controlled carrier gas supply and a pneumatically actuated SVE valve was used. The mass detector was a Fisons 800 with electronic impact (70 eV) mode. The source temperature was set at 210 °C. The LC fraction was manually collected and reconcentrated under a gentle N_2 flow to 200 μL. The fraction was then injected onto the GC-MS with an autosampler. The same GC retention gap and separation column as described earlier were temporarily mounted into the GC oven.

Quantitative Determination of F-Acids. Three different amounts of diMeF(9,5) were added to aliquots of the same oil sample before transmethylation, resulting in the following concentrations of added standard to the oil: 0, 6.5, 65, and 650 ppm. A calibration curve was obtained from 11 different injected solutions.

RESULTS AND DISCUSSION

HPLC Separation. LC preseparation of the furan acid methyl esters was the critical step. A sufficient separation of F-acids from straight-chain fatty acids should be obtained; otherwise, partial GC coelution would be unavoidable. At the same time, the volume of LC eluent should be small enough to enable an optimum LC-GC transfer. With reversed phase LC there was no separation of F-acid methyl esters from the straight-chain fatty acids. The diMeF(9,5) standard was not well separated from linoleic acid methyl ester (C18:2Me). F-acids are eluted, in normal phase LC (NPLC), with decreasing carbon chain length [e.g., first diMeF(11,5) and then diMeF(9,5)]. Straight-chain fatty acids are eluted in NPLC with increasing unsaturation degree; thus, stearic [which is not revealed by UV detection (Baker and Melhuish, 1984)] precedes oleic, linoleic, and linolenic acid methyl esters, respectively (Figure 2). This resulted in coelution of linolenic acid methyl ester (C18:3Me) and diMeF(11,5). However, diMeF(9,5) is rather well separated from C18:3Me by LC and is eluted after it. Therefore, a broad LC window beginning with the peak for C18:3Me and ending with the short-chain F-acids was transferred in GC (Figure 2A).

LC-GC Transfer. Owing to the large retention volume and the partial preseparation of the F-acids, the LC window (Figure 2A) was very broad (1500 μL). Using the loop-type interface, some of the most volatile components were expected to be lost. Transfer of the 1500 μL fractions by the on-column technique presupposes an extremely large proportion of concurrently evaporating solvent and was not carried out so far. Therefore, a detailed study of the solvent trapping process was necessary and is reported in previous works (Boselli et al., 1998; Grolimund et al., 1998). Use of thermocouples to determine the front end of the flooded zone in the uncoated precolumn helped to adjust the LC-GC transfer by reducing concurrent evaporation to ~87%.

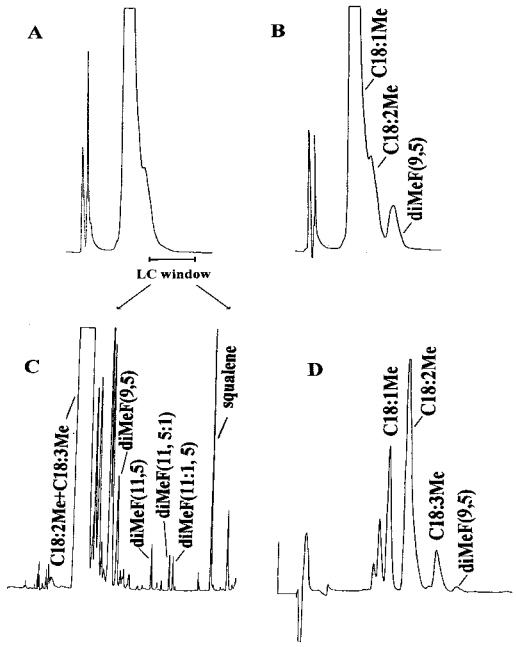

Figure 2. (A) LC trace of 2 mg of extra virgin olive oil; (B) LC trace of sample A spiked with 650 ppm of diMeF(9,5) methyl ester; (C) LC-GC-PID trace of the LC fraction reported in (A); (D) LC trace of 200 μg of cold-pressed soybean oil. C18:1Me, C18:2Me, and C18:3Me are the methyl esters of oleic, linoleic, and linolenic acid, respectively.

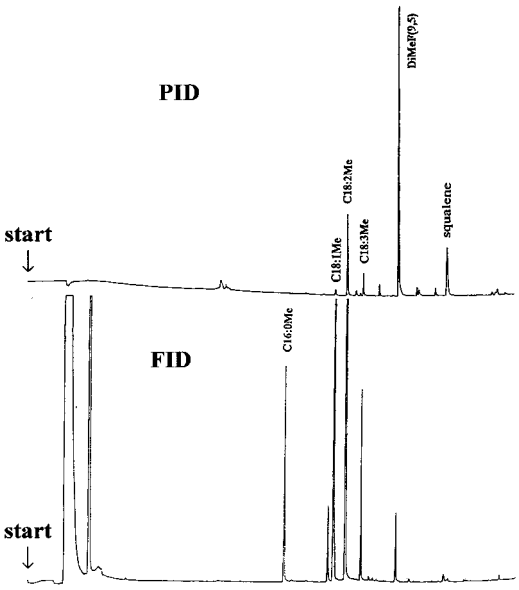

Figure 3. GC-PID-FID trace of a transmethylated soybean oil sample spiked with diMeF(9,5) methyl ester.

Table 1. Relative Retention Time ($\bar{T}_x$) of Some F-Acid Methyl Esters, Linolenic Methyl Ester (C18:3Me), and Squalene in GC with PS-255 as a Stationary Phase

compound	$\bar{T}_x$	compound	$\bar{T}_x$
diMeF(7,5)	1.43	MeF(11,5)	0.78
C18:3Me	1.40	diMeF(9:1, 5)	0.76
diMeF(8,5)	1.23	diMeF(11,5)	0.63
MeF(9,5)	1.17	diMeF(11, 5:1)	0.43
diMeF(9,5)	1	diMeF(11:1, 5)	0.38
diMeF(10,5)	0.83	squalene	0
diMeF(9, 5:1)	0.81		

Finally, the LC-GC injection of a standard solution of *n*-alkanes under the same conditions as described earlier provided for a recovery of >97% of C_{16} hydrocarbon without using a retaining precolumn. This result was in agreement with previous work (Boselli et al., 1998). Thus, short-chain F-acids such as diMeF(7,5) should be efficiently recovered if present in samples.

Table 2. F-Acids (Olefinic and Non olefinic) Detected in 18 Samples of Monovarietal Extra Virgin Olive Oils[a]

sample	diMeF-(9,5)	diMeF-(11,5)	diMeF-(11, 5:1)	diMeF-(11:1, 5)
Ascolana Tenera	0.39	0.40	0.54	0.47
Carolea	0.42	2.10	0.54	0.43
Cima di Melfi	0.49	0.08	0.65	0.60
Coratina	0.36	0.33	0.65	0.73
Frantoio	0.73	0.73	0.87	0.88
Gentile di Larino	0.41	0.38	0.45	0.44
I-77	0.35	0.05	0.35	0.35
Itrana	0.73	0.06	0.23	0.23
Leccino	0.36	0.38	0.52	0.45
Maiatica	0.48	0.24	0.67	0.67
Maiolice	0.23	0.05	0.42	0.48
Nocellara del Belice	0.23	0.22	0.48	0.50
Nocellara Etnea	0.42	0.40	0.41	0.39
Nociara	0.88	0.08	0.58	0.66
Pendolino	0.16	0.08	0.70	0.90
Peranzana	1.54	0.05	0.40	0.37
Rosciola di Rotello	0.80	0.24	0.61	0.53
Termite Bitetto	2.00	0.59	0.30	0.29

[a] Data are expressed in ppm of fatty acids in oil. Each sample was analyzed in duplicate and the average value is reported. The relative standard deviation (rsd) was within the limits reported under Repeatability of the Method.

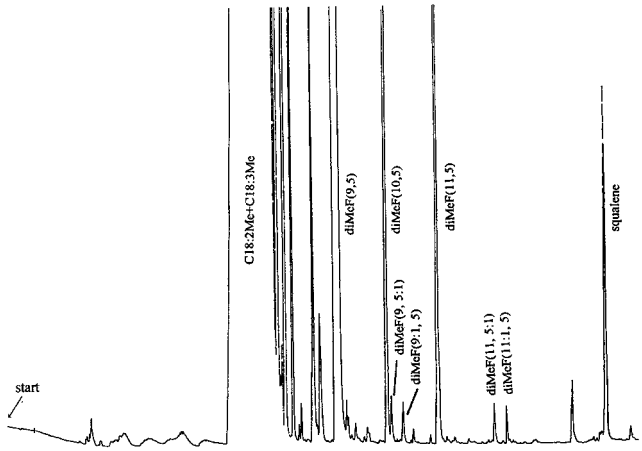

Figure 4. PID trace of an extra virgin olive oil sample spiked with 650 ppm of diMeF(9,5) methyl ester and analyzed by LC-GC-PID-FID (see previous figures for abbreviations).

PID. Photoionization with a low ionization energy (8.4 eV) improved qualitative and quantitative detection. The PID selectivity toward different fatty acid methyl esters could be easily evaluated by using a capillary column coated with a polar GC stationary phase. The GC traces obtained from a sample of soybean oil analyzed with a 90% biscyanopropylsilanol GC stationary phase are shown in Figure 3; PID and FID were recorded in series. Saturated fatty acids are not ionized at 8.4 eV. Oleic, linoleic, and linolenic acid methyl esters are strongly attenuated in comparison with the FID trace, whereas diMeF(9,5) and squalene are amplified. The selectivity of PID can be expressed in terms of the ratio between the PID peak area and the FID peak area for a single substance. The ratio depends on the makeup and purge gas flows of the PID and on the age of the detector's lamp; in the worst case the PID selectivity ratio ranged between 4.0 and 4.5 for F-acids. Obviously these results are not dependent on the GC stationary phase.

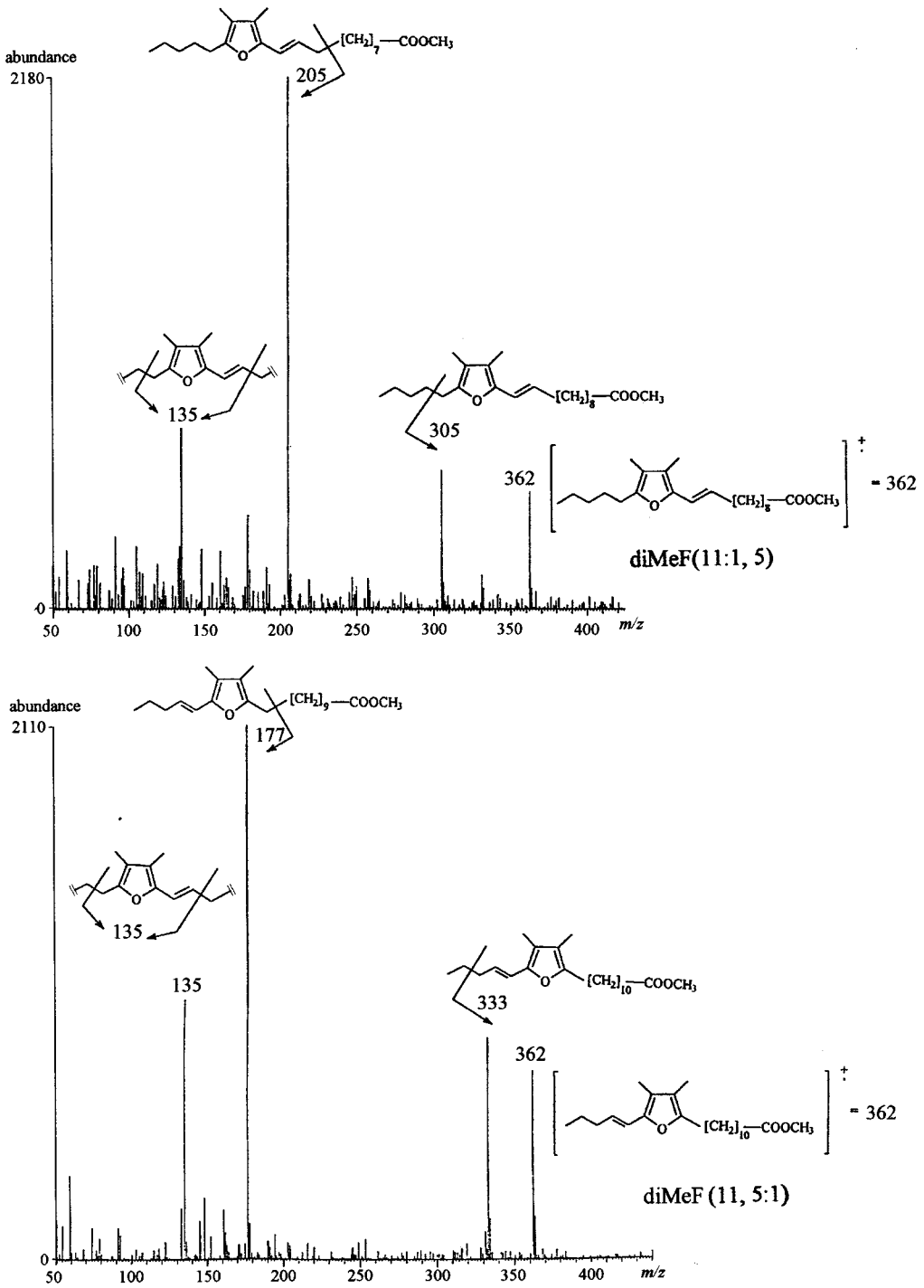

Figure 5. Mass fragmentation of diMeF(11, 5:1) and diMeF(11:1, 5) methyl esters.

Repeatability of the Method. The method showed an acceptable repeatability. One oil sample was analyzed in four replicates; the resulting relative standard deviation (rsd) was 8.3% for diMeF(9,5) and 11.5% for diMeF(11,5). All other samples were analyzed in duplicate, and the average values are reported in Table 2.

Response Linearity of the Method. The following regression line

$$y = mx + q \qquad (m = 8152;\ q = 23216)$$

was obtained with the method of standard addition (Snyder and Kirkland, 1979) by injecting the solutions spiked as described under Quantitative Determination of F-Acids. In the equation, y is the peak area of diMeF-(9,5) and x is the concentration of diMeF(9,5) added to the sample (expressed as parts per million of F-acid in the oil sample). The coefficient of determination (r^2) for the regression line was 0.9926 (for 11 injections). m is the regression coefficient, and q is the peak area of diMeF(9,5), which is naturally present in the oil sample.

F-Acids and Olefinic F-Acids in Olive Oil. The detection limit is the key point for the determination of F-acids. Precedent methods (Guth and Grosch, 1992; Hannemann et al., 1989) seem to quantify F-acids above 1 ppm, whereas the LC preseparation coupled with the selective PID allows for a low detection limit, ~50 ppb of F-acids in the oil. This is mainly the reason F-acids have not been detected so far in olive oils.

Identification of F-acids was confirmed by LVI-GC-MS analysis. For practical reasons, the following proportion was developed to normalize chromatographic retention times:

$$\bar{T}_x = \frac{T_x - T_{sq}}{T_{diMeF(9,5)} - T_{sq}}$$

where $\bar{T}_x$ = relative retention time of the x F-acid, T_x = reported retention time of the x furan acid, T_{sq} = reported retention time of squalene, and $T_{diMeF(9,5)}$ = reported retention time of diMeF(9,5).

$\bar{T}_x$ is dependent only on the GC stationary phase, provided that a constant oven temperature rate is set. Peaks for both squalene and diMeF(9,5) were used for the standardization of retention times. Squalene is present as an impurity and is eluted at high temperatures in GC with the PS-255 stationary phase. Thus, it does not interfere with the GC analysis. On the contrary, squalene is eluted at a temperature that can hinder the determination of long-chain F-acids when using a GC column coated with the polar 90% biscyanopropyl stationary phase. Table 1 shows $\bar{T}_x$ values for the detected F-acids.

Concentrations of diMeF(9,5) and diMeF(11,5) found in extra virgin oil samples are reported in Table 2. These are the most abundant naturally occurring F-acids. Average concentrations were 0.62 ppm for diMeF(9,5) and 0.36 ppm for diMeF(11,5). Nevertheless, experimental data show a high dispersion among the 18 samples.

In the same LC heart cut, the presence of two peaks corresponding to the olefinic derivatives of diMeF(11,5) was registered: they show a double bond conjugated with the furan ring in one or the other side chain (Figure 1). Both olefinic derivatives of diMeF(9,5) were detected only in the olive oil sample to which had been added 650 ppm of diMeF(9,5) standard (Figures 2B and 4). All of the data were confirmed by GC-MS and were comparable with the mass fragmentations reported in the literature (Guth and Grosch, 1992; Ishii et al., 1988). Mass fragmentations of olefinic derivatives of diMeF-(11,5) are reported in Figure 5.

Also, diMeF(11:1, 5) and diMeF(11, 5:1) were detected in all 18 samples, but in smaller and rather constant amounts; the average concentration was 0.52 ppm for both isomers with relative standard deviations (rsd%) of 29 and 35%, respectively. Thus far, not much attention has been given to the determination of olefinic F-acids. DiMeF(9, 5:1) and diMeF(9:1, 5) were found to be artifacts (Ishii et al., 1988) produced during GC analysis with split−splitless injection (injection temperature = 250 °C). They were not found in the 18 olive oil samples but were detected in a sample to which had been added 650 ppm of diMeF(9,5) standard, which was used for the determination of the calibration curve (concentrations were low enough not to affect calculation). This seems to confirm what is reported in the literature. The GC trace is shown in Figure 4.

In contrast, there are no data about the possibility that diMeF(11:1, 5) and diMeF(11, 5:1) can be formed by the autoxidation of diMeF(11,5). Experiments carried out during this work lead to following results:

(a) DiMeF(11:1, 5) and diMeF(11, 5:1) were not present [<0.2% of the pure diMeF(9,5)] in the standard solution injected with the on-column GC-PID-FID configuration (injection temperature = 80 °C).

(b) A direct consequence of (a) is that these compounds are formed neither during GC nor after photoionization or flame ionization.

(c) Sample preparation did not affect olefinic diMeF-(11:1, 5) and diMeF(11, 5:1). In fact, the addition of diMeF(11,5) standard to an oil sample before transmethylation did not increase the concentration of olefinic F-acids after LC-GC-PID-FID analysis.

For these reasons it can be confirmed that diMeF(11:1, 5) and diMeF(11, 5:1) are naturally occurring in oil and are not formed during sample preparation.

Conclusions. F-acids are found in olive oil at low levels (some parts per million). Thus, only the improvement of the analytical method (preseparation and detection steps) allows for the determination of F-acids in food samples. However, the presence of olefinic F-acids as naturally occurring components in plant oils should let us reject some separation methods that involve hydrogenation of the sample as a method for a better separation between F-acids and straight-chain fatty acids (see Introduction). These methods cause the elimination of unsaturated furan fatty acids [e.g., diMeF(11:1, 5) and diMeF(11, 5:1)], which are indeed natural components of some food lipids.

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