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Changes in the Concentrations of Free Fatty Acid, Monoacylglycerol, and Diacylglycerol in the Subcutaneous Fat of Iberian Ham during the Dry-Curing Process

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Changes in diacylglycerols, monoacylglycerols, and free fatty acid composition of subcutaneous fat of six Iberian hams during the dry-cured process were investigated. In addition, an analytical method for simultaneous quantification of diacylglycerols, monoacylglycerols, and free fatty acid by solid-phase extraction–gas chromatography was developed. The different molecular species of free fatty acids, monoacylglycerols, and diacylglycerols and 1,2- and 1,3-isomers of diacylglycerols have been described for the first time in this type of sample. A logarithmic increase of the 1,3-diacylglycerol profile throughout the processing time has been found, reaching a balance value of 62% around 500 days. The formation of diacylglycerol isomers takes place, although the 1,3-/1,2-diacylglycerol ratio increases during the process to 1.65 due to isomerization of the 1,2-form toward the 1,3-form. The profiles of monoacyl- and diacylglycerols and free fatty acids follow the same trend. The experimental values of free fatty acid are greater than theoretical prediction, probably due to phospholipid and monoacylglycerol hydrolysis.

INTRODUCTION

Dry-cured Iberian ham is one of the few traditional meat products. Since 1998, this product has been included in the European Union list of the Designation of Origin (*1*), which has survived due to its highly demanded characteristics, with long taste and rich nutty flavor (*2, 3*). These appreciated organoleptic properties are due to a long-term maturing process which requires between 12 and 24 months. Among the biochemical reactions occurring during the ripening process, those which affect the lipid fraction are of great importance since the degradation products contribute to the overall organoleptic properties of the final product (*4*).

The glyceridic fraction of Iberian dry-cured ham represents a large amount of the total lipid content, and it is characterized by a predominance of monounsaturated fatty acids, followed by saturated fatty acids (*5*). Several studies have evaluated the changes in the intramuscular and subcutaneous lipid fraction during the ripening of Iberian dry-cured ham. Martín et al. (*6*) observed that oleic (C18:1) and palmitic (C16:0) acids were the main fatty acids of the acylglycerols, but they did not differ between mono-, di-, and triacylglycerols. Larrea et al. (*7*) studied the changes in total triacylglycerols, monoacylglycerol fraction, and the total amount of diacylglycerols. For this fraction, an increase was reported during the ripening process. Besides this study, a few have been conducted on the diacylglycerol fraction of the subcutaneous fat of raw and dry-cured hams. Delgado et al. (*8*) reported a decrease in the diacylglycerol fraction during the ripening period. Coutron-Gambotti and Gandemer (*9*) investigated the changes in triacylglycerols, fatty acid, diacylglycerols, and monoacylglycerols of subcutaneous adipose tissue during ripening of Corsican dry-cured ham. However, none of these studies evaluated the different isomeric forms of diacylglycerols.

Diacylglycerols are generated by acidic and enzymatic hydrolysis of triacylglycerols during transformation and storage of oils and fats. During the storage of oil, the changes in this fraction are due to an isomerization process. The 1,2-diacylglycerols spontaneously change to 1,3-diacylglycerols since it is a more thermodynamically stable molecular specie (*10*). In olive oil, the 1,3-/1,2-diacylglycerol ratio has proved to be useful for assessing the oil aging, conditions of storage, and handling. Besides this, the kinetics of diacylglycerol formation allows the estimation of the storage time of virgin

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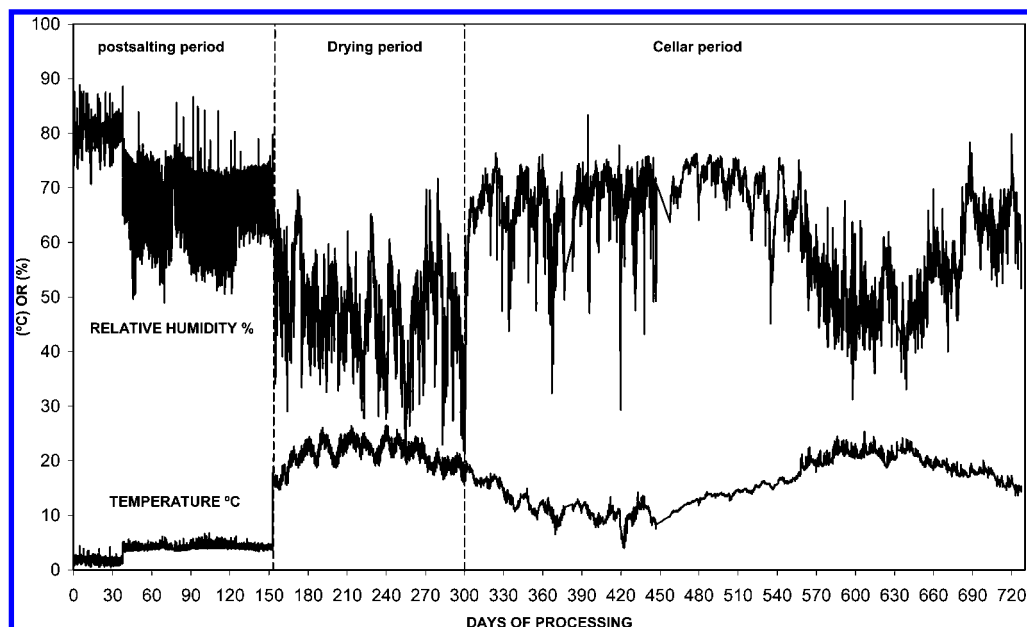† Instituto de la Grasa.
‡ University of Seville.

Figure 1. Evolution of environmental temperature and relative humidity during ripening of Iberian dry-cured ham.
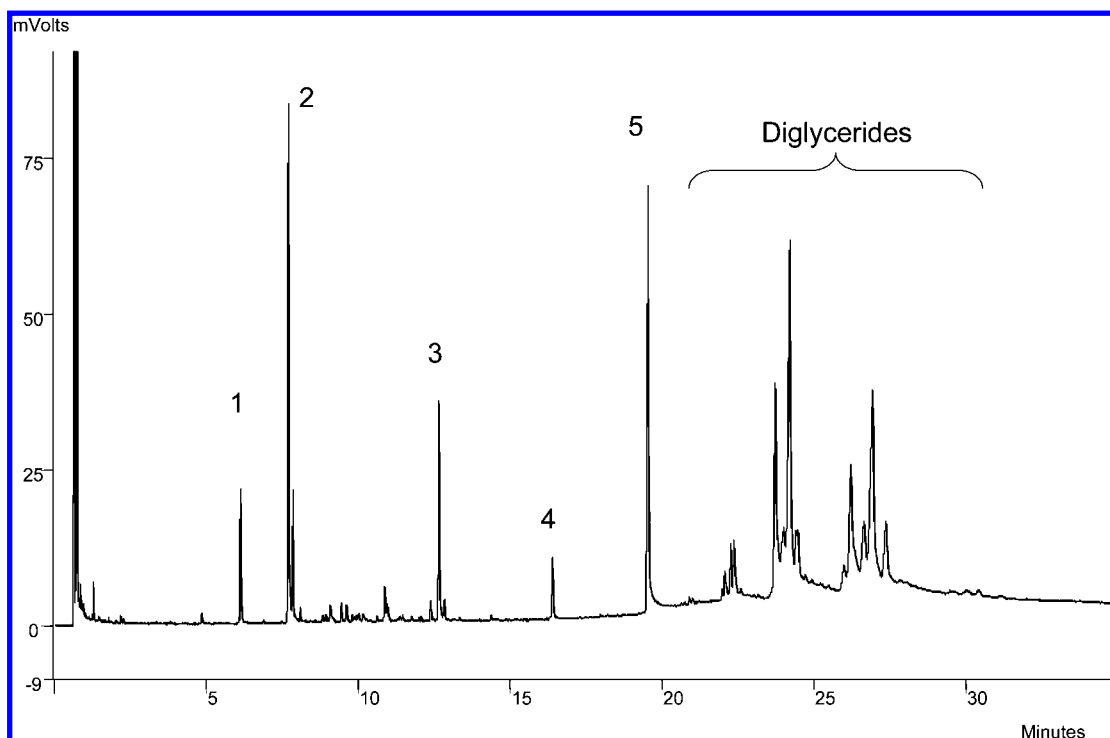

Figure 2. Gas chromatogram of the polar fraction from 0 to 35 min corresponding to subcutaneous fat of Iberian dry-cured ham. Labeled peaks are identified as follows: 1, palmitic acid; 2, oleic acid, 3, 1-monoolein; 4, cholesterol; 5, 1,3-dimyristin (IS).

olive oil (*11*). Both the total diacylglycerol content and the ratio between isomeric forms have also been used as indicators of palm oil quality (*12*). The diacylglycerol composition has been studied in the lipidic fraction of different foods during its transformation or storage. A decrease in diacylglycerol content has been observed in some food processing, such as fermentation of pork sausage (*13*) or during ice storage of sardine (*Sardinella gibbosa*) (*14*). In other cases, a significant increase is observed, as in the salting-fermentation process of certain fishes (*15*). Milk-derived products also show a significantly higher content of diacylglycerols than milk (*16*).

Therefore, the quantitative study of the composition of diacylglycerols appears to be of great interest to evaluate the quality of the fats and, consequently, of the treatment to which they have been subjected. To our knowledge, a detailed study of the changes occurring in this fraction during the ripening process of dry-cured ham has not been undertaken.

The aim of this work was to investigate the changes exclusively in free fatty acids and monoacyl- and diacyl-glycerols of the subcutaneous fat and, hence, for a better understanding of the Iberian ham dry-cured process. As a previous step to achieve this objective, it was necessary to improve the experimental fractionation procedure of the fat, with a reliable analytical method that allows both recovery and quantification of the free fatty acids, monoacylglycerols, and diacylglycerols simultaneously.

Fatty Acid and Monoacyl/diacylglycerol in Fat of Iberian Ham

J. Agric. Food Chem., Vol. 55, No. 26, 2007 **10955**
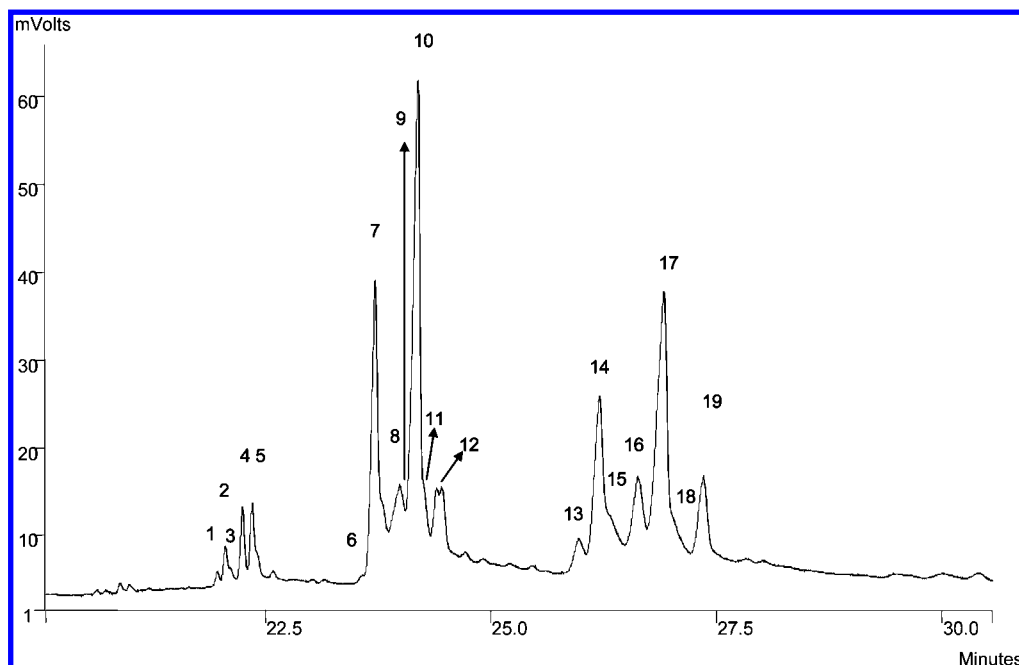
Figure 3. Gas chromatogram (expanded plot) from 20 to 30 min as taken from Figure 2. Labeled peaks are identified as follows: 1, 1,2-PP; 2, 1,2-PPo; 3, 1,2-MO; 4, 1,3-PP; 5, 1,3-PPo + 1,3-MO; 6, 1,2-PS; 7, 1,2-OP; 8, 1,2-OPo; 9, 1,2-LP; 10, 1,3-OP; 11, 1,3-OPo; 12, 1,3-LP; 13, 1,2-OS; 14, 1,2-OO; 15, 1,3-OS; 16, 1,2-OL; 17, 1,3-OO; 18, 1,2-LL; 19, 1,3-OL.

Table 1. Response Factors (RFs) Obtained for Representative Monoacylglycerols and Fatty Acids Relative to 1,3-Dimyristin as Internal Standard

	RF mean[a]	SD	RSD (%)
1-monopalmitin	0.299	0.0060	2.00
oleic acid	0.418	0.0181	3.36

[a] Response factor (RF) calculated as the mean of four replicates.

MATERIALS AND METHODS

Processing and Sampling of Hams. Six hams (between 8.8 and 9.1 kg) were obtained from three castrated Iberian pure f14-month-old pig males, fattened extensively with acorns and pastured for 90 days prior to slaughter, and were processed in an industry for 24 months. The stages and the number of days from the beginning of the processing were as follows: After the slaughter, hams were removed from the carcasses after 24 h refrigerated storage at 1 °C. Then they were placed in piles completely covered only with marine salt (they were not in contact with each other) at low temperature (1.0 °C) and high relative humidity (about 80%) for 9 days. After being washed to remove salt from the surface, the hams were hung at 2.0 °C and a relative humidity of 80% for 38 days and then at 4.0 °C and a relative humidity of 70% to 153 days (postsalting). Then, they were taken to a dryer at temperatures varying from 4 to 27 °C and a relative humidity ranging from 70% to 43% for 147 days. Next, the hams were left to mature during 430 days in a cellar at temperatures ranging from 10 to 27 °C and 58–80% relative humidity. The environmental conditions (temperature and relative humidity) were recorded continuously throughout the whole period of maturing (**Figure 1**).

A sample of each ham (about 1 g) was taken across of subcutaneous adipose tissue covering the *Biceps femoris* muscle, without touching it, about once a month since the animal was slaughtered (raw sample) until the dry-cured process was finished (cured ham). Samples were stored at −25 °C before analysis. The samples of subcutaneous fat coming from both hams of each animal were mixed and melted in an oven microwave (*17*) during 3 min at 360 W of power. Possible isomerizations in the microwave extraction process of the samples are not reported for subcutaneous fat of the Iberian pig. However, some authors have made similar studies on human adipose tissue fat, using the method of Folch as the extraction method under cold conditions (*18*) and obtained mean values for 1,3-diacylglycerols around 20%,

Table 2. Repeatability Results of Diacylglycerols, Monoacylglycerol, and Free Fatty Acid of Raw and Dry-Cured Samples Analyzed by SPE-GC

			raw			dry-cured		
peak[a]	RT[b]	diacylglycerols[c]	mean (n = 6)	SD	RSD (%)	mean (n = 6)	SD	RSD (%)
1	21.950	1,2-PP[d]	2.69	0.43	16.11	0.61	0.20	32.18
2	22.050	1,2-PPo[d]	3.62	0.78	21.54	0.88	0.14	15.34
3	22.101	1,2-MO[d]	1.74	0.25	14.55	0.32	0.12	37.85
4	22.240	1,3-PP[d]	0.24	0.20	81.43	1.79	0.35	19.53
5	22.347	1,3-PPO + 1,3-MO[d]	0.04	0.10	244.95	1.87	0.44	23.35
6	23.559	1,2-PS[d]	4.28	0.47	11.04	0.63	0.41	64.99
7	23.711	1,2-OP[d]	28.63	1.96	6.85	10.86	0.84	7.76
8	23.985	1,2-OPo[d]	4.34	0.44	10.05	4.38	0.86	19.72
9	24.050	1,2-PL[d]	4.88	0.44	9.02	0.00	0.00	
10	24.187	1,3-OP[d]	2.18	0.40	18.37	22.94	1.53	6.69
11	24.248	1,3-OPo[d]	0.30	0.34	113.09	2.41	1.30	53.97
12	24.394	1,3-PL[d]	1.24	0.23	18.24	2.52	0.56	22.20
13	25.971	1,2-OS[d]	5.40	1.20	22.16	1.65	0.23	14.02
14	26.199	1,2-OO[d]	19.57	1.12	5.73	9.03	2.44	27.02
15	26.298	1,3-OS[d]	4.59	0.53	11.51	1.88	1.25	66.59
16	26.628	1,2-OL[d]	8.29	0.51	6.12	6.51	0.65	9.97
17	26.912	1,3-OO[d]	5.02	0.41	8.26	22.43	3.48	15.53
18	27.006	1,2-LL[d]	0.87	0.39	45.16	3.26	2.13	65.40
19	27.351	1,3-OL[d]	2.06	0.40	19.59	6.05	0.67	11.01
		total diacylglycerols[e]	4.85	0.09	1.82	30.80	0.72	2.34
		free fatty acid[e]	18.86	0.24	1.28	16.57	0.34	2.05
		monoacylglycerol[e]	0.38	0.03	7.71	5.49	0.40	7.21

[a] See Figure 3. [b] RT = retention time. [c] M = myristic, P = palmitic, Po = palmitoleic, S = stearic, O = oleic, and L = linoleic. [d] Expressed as %. [e] Expressed as mg/100 g of fat.

which is greater than that found by us in raw samples (15.67%). Therefore, it is assumed that the microwave extraction did not produce any appreciable isomerization. The sample of fat was then immediately filtered through Albet filter paper ref 1300 before analysis.

Materials and Reagents. All reagents were of analytical reagent grade, unless otherwise specified. The standards oleic and palmitic acid, 1-palmitoyl-*rac*-glycerol, 1,3-dimyristin, 1,2-dimyristoyl-*rac*-glycerol, 1,3-dipalmitin, 1,2-dipalmitoyl-*rac*-glycerol, 1,3-distearin, 1,2-distearoyl-*rac*-glycerol, 1,2-dioleoyl-*rac*-glycerol, 1,3-diolein, and trilinolein were purchased from Sigma-Aldrich (St. Louis, MO). To obtain 1,2-dilinolein a enzymatic hydrolysis of porcine pancreas was done using lipase

10956 *J. Agric. Food Chem.,* Vol. 55, No. 26, 2007

Narváez-Rivas et al.

Table 3. Effect of the Dry-Curing Process on the Free Fatty Acid, Monoacylglycerol, and Diacylglycerol Profile[a]

diacylglycerols[b]	% raw	% dry-cured	mg/100 g of fat raw	mg/100 g of fat dry-cured
1,2-PP	3.11 ± 0.61[c]	0.37 ± 0.11	0.07 ± 0.02	0.11 ± 0.01
1,2-PPo	3.50 ± 0.02[c]	0.79 ± 0.21	0.08 ± 0.04	0.24 ± 0.06
1,2-MO	1.42 ± 0.13	0.31 ± 0.07	0.03 ± 0.01	0.09 ± 0.03
1,3-PP	0.00 ± 0.04[c]	2.15 ± 0.20	0.00 ± 0.00[c]	0.64 ± 0.02
1,3-PPO + 1,3-MO	0.00 ± 0.00[b]	2.14 ± 0.29	0.00 ± 0.00[c]	0.64 ± 0.03
1,2-PS	5.24 ± 2.00	0.26 ± 0.15	0.06 ± 0.00	0.08 ± 0.03
1,2-OP	25.70 ± 3.70[c]	11.15 ± 0.38	0.62 ± 0.21[b]	3.36 ± 0.45
1,2-OPo	4.74 ± 0.51	4.92 ± 0.43	0.11 ± 0.05[a]	1.49 ± 0.31
1,2-PL	4.18 ± 1.03[b]	0.00 ± 0.00	0.08 ± 0.02[a]	0.00 ± 0.00
1,3-OP	2.00 ± 1.52[b]	25.17 ± 1.76	0.06 ± 0.04[c]	7.52 ± 0.40
1,3-OPo	1.21 ± 1.88[b]	1.74 ± 0.47	0.01 ± 0.02	0.53 ± 0.20
1,3-PL	0.00 ± 0.00[a]	2.20 ± 0.89	0.00 ± 0.00	0.68 ± 0.31
1,2-OS	5.38 ± 0.13[b]	1.69 ± 0.14	0.12 ± 0.06[b]	0.51 ± 0.03
1,2-OO	24.26 ± 1.42[b]	8.80 ± 0.85	0.54 ± 0.25[a]	2.66 ± 0.55
1,3-OS	6.85 ± 3.07	1.74 ± 0.94	0.15 ± 0.09	0.54 ± 0.34
1,2-OL	6.73 ± 0.35	7.40 ± 0.97	0.16 ± 0.07[c]	2.21 ± 0.20
1,3-OO	4.99 ± 2.10[b]	21.39 ± 1.76	0.17 ± 0.11[c]	6.39 ± 0.27
1,2-LL	0.06 ± 0.39	2.04 ± 1.02	0.01 ± 0.01	0.64 ± 0.37
1,3-OL	0.62 ± 1.19[b]	5.73 ± 0.40	0.05 ± 0.03[b]	1.73 ± 0.31
total 1, 2-DGs	84.33 ± 2.92[c]	37.74 ± 1.74	1.89 ± 0.73[b]	11.37 ± 1.81
total 1, 3-DGs	15.67 ± 2.92[c]	62.26 ± 1.74	0.44 ± 0.26[c]	18.67 ± 1.73
total diacylglycerols			2.33 ± 0.99[c]	30.04 ± 3.49
monoacylglycerols			0.80 ± 0.47[c]	5.06 ± 2.00
palmitic acid			4.15 ± 0.68[b]	2.66 ± 0.33
oleic acid			14.54 ± 3.00	14.09 ± 2.49
total free fatty acid			18.69 ± 3.54	16.75 ± 2.76

[a] Data are the means ± standard deviation (*n* = 3). Different letters indicate significant differences between raw and dry-cured data (a for *p* < 0.01, b for *p* < 0.001, and c for *p* < 0.0001). [b] M = myristic, P = palmitic, Po = palmitoleic, S = stearic, O = oleic, and L = linoleic.
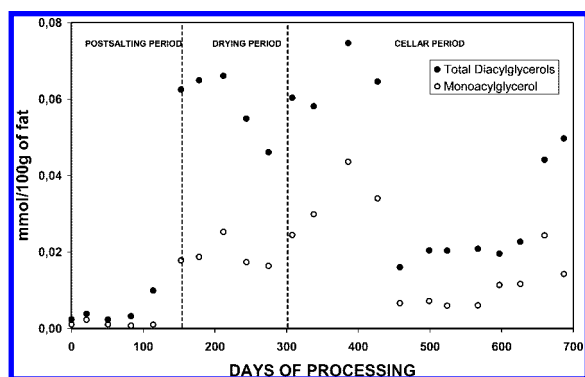
Figure 4. Evolution of total diacylglycerols (1,2 and 1,3) and monoacylglycerol amount (mmol/100 g of fat) during the dry-curing process.

according to a procedure previously described in the literature (*19*). After a short reaction time, 1,2-dilinolein was isolated by thin-layer chromatography in silica gel impregnated with boric acid to prevent the isomerization (*20*). 1,3-Dilinolein was purchased from Nuchek-Prep (Elysian, MN). The solid-phase extraction cartridge (3 mL), packed with a diol-bonded phase, was purchased from Supelco (Bellefonte, PA). Silylating reagent was prepared by adding 3 mL of hexamethyl-disilazane and 1 mL of trimethylchlorosilane to 9 mL of anhydrous pyridine.

Free Fatty Acid and Mono- and Diacylglycerol Analysis. Free fatty acids and mono- and diacylglycerols form a part of the fat polar fraction, and consequently, their analysis can be carried out following a methodology for suitable separation and quantification (*21*).

Free fatty acid and mono- and diacylglycerol fractions were isolated by solid-phase extraction (*22, 23*). The solid-phase extraction column was placed in a vacuum elution apparatus and washed under vacuum with 4 mL of hexane. The vacuum was released immediately after the wash to prevent the column from becoming dry. 1,3-Dimyristin standard

Table 4. Levels of Experimental and Calculated Free Fatty Acids (mmol/100 g of Fat) and Experimental/Calculated Ratio of Iberian Pig Hams during the Dry-Curing Process in Days[a]

days	palmitic (*n* = 3)	oleic (*n* = 3)	total_exptl (*n* = 3)	total_calcd (*n* = 3)	ratio
0	0.01 ± 0.00	0.03 ± 0.01	0.04 ± 0.01	0.00 ± 0.00	7.93 ± 2.04
21	0.01 ± 0.00	0.04 ± 0.01	0.05 ± 0.02	0.01 ± 0.01	6.28 ± 1.68
51	0.01 ± 0.00	0.02 ± 0.00	0.03 ± 0.01	0.00 ± 0.00	7.95 ± 2.34
83	0.02 ± 0.01	0.04 ± 0.01	0.06 ± 0.02	0.00 ± 0.00	11.62 ± 2.84
114	0.02 ± 0.00	0.05 ± 0.01	0.07 ± 0.01	0.01 ± 0.00	6.23 ± 0.56
153	0.04 ± 0.03	0.12 ± 0.09	0.17 ± 0.11	0.10 ± 0.07	1.74 ± 0.30
178	0.04 ± 0.03	0.11 ± 0.05	0.15 ± 0.08	0.10 ± 0.07	1.57 ± 0.52
212	0.03 ± 0.02	0.10 ± 0.06	0.13 ± 0.08	0.12 ± 0.08	1.17 ± 0.05
244	0.02 ± 0.00	0.09 ± 0.01	0.11 ± 0.01	0.09 ± 0.04	1.42 ± 0.53
275	0.02 ± 0.01	0.07 ± 0.02	0.09 ± 0.02	0.08 ± 0.03	1.26 ± 0.62
308	0.04 ± 0.02	0.09 ± 0.06	0.12 ± 0.08	0.11 ± 0.02	1.08 ± 0.49
338	0.04 ± 0.01	0.15 ± 0.02	0.19 ± 0.03	0.12 ± 0.02	1.58 ± 0.29
386	0.02 ± 0.02	0.12 ± 0.07	0.14 ± 0.09	0.16 ± 0.08	0.83 ± 0.15
427	0.06 ± 0.02	0.18 ± 0.03	0.24 ± 0.04	0.13 ± 0.02	1.78 ± 0.31
458	0.02 ± 0.00	0.07 ± 0.01	0.10 ± 0.00	0.03 ± 0.01	3.64 ± 1.25
499	0.03 ± 0.01	0.08 ± 0.01	0.10 ± 0.02	0.03 ± 0.01	2.92 ± 0.37
524	0.03 ± 0.01	0.08 ± 0.02	0.11 ± 0.03	0.03 ± 0.01	3.34 ± 0.27
567	0.03 ± 0.01	0.09 ± 0.01	0.12 ± 0.02	0.03 ± 0.01	3.69 ± 0.74
597	0.05 ± 0.00	0.12 ± 0.01	0.16 ± 0.01	0.04 ± 0.01	3.87 ± 0.48
626	0.04 ± 0.02	0.10 ± 0.01	0.14 ± 0.03	0.05 ± 0.02	3.42 ± 1.59
660	0.02 ± 0.01	0.10 ± 0.03	0.12 ± 0.04	0.09 ± 0.05	1.58 ± 1.04
687	0.01 ± 0.00	0.04 ± 0.01	0.04 ± 0.01	0.08 ± 0.01	0.55 ± 0.03

[a] Data are the means ± standard deviation (*n* = 3).

solution (200 μL) (1.02 mg mL^{-1}) and 500 μL of fat solution in *n*-hexane (0.2 mg mL^{-1}) were applied to the column, and the solvent was pulled through, leaving the standard and the sample on the column. A volume of 6 mL of hexane−methylene chloride−ethyl ether (89:10:1 v/v) was applied to the column, and a first fraction was collected. Subsequently, 4 mL of chloroform−methanol (2:1 v/v) was applied to the column and a second fraction collected. This fraction was evaporated to dryness in a rotary evaporator under reduced pressure. The residue was treated with 200 μL of the silylating reagent and left at room temperature for 15 min.

One microliter of the silylated fraction was injected into the gas chromatographic system. The chromatographic analysis was performed using a Varian 3800 (Varian Co., Palo Alto, CA) equipped with a split/splitless injector and a flame ionization detector; a fused silica capillary DB-17HT column (30 m × 0.32 mm i.d., 0.15 μm film thickness) and Varian 8100 automatic injector were used. Hydrogen was used as carrier gas at a column constant flow of 4.0 mL min^{-1}.

Injector conditions were as follows: split/splitless mode, split ratio 1:40, and temperature 300 °C. The liner used for split mode injection in the 1079 type injector was a Gooseneck-type liner (i.d. = 3.4 mm) which was deactivated and packed with a glass frit.

The oven conditions were as follows: The initial oven temperature was kept at 100 °C and was then raised to 180 °C at a rate of 15.0 °C min^{-1}. Next it was raised to 296 °C at a rate of 7.0 °C min^{-1} and held isothermally for 13.10 min. While the detector temperature was 360 °C, air and hydrogen with flow rates of 300 and 30 mL min^{-1}, respectively, were used for the detector, which had an auxiliary flow of 30 mL min^{-1} nitrogen.

A representative chromatogram report of subcutaneous fat and the corresponding peak identification is shown in **Figure 2**. **Figure 3** shows more detail of the diacylglycerol profile shown in **Figure 2**.

For quantification of the diacylglycerols, 1,3-dimyristin was used as internal standard. The internal standard was selected because no components eluted were found in this region. For quantification of the free fatty acids and monoacylglycerols, 1,3-dimyristin was used, too; then the relative response factors were determined using solutions of 1.140 and 1.012 mg mL^{-1} pure standard of oleic acid and 1-mono-palmitin, respectively, following the derivatization procedure described above. Relative response factors determined for free fatty acids and monoacylglycerols are listed in **Table 1**. An accurate determination of
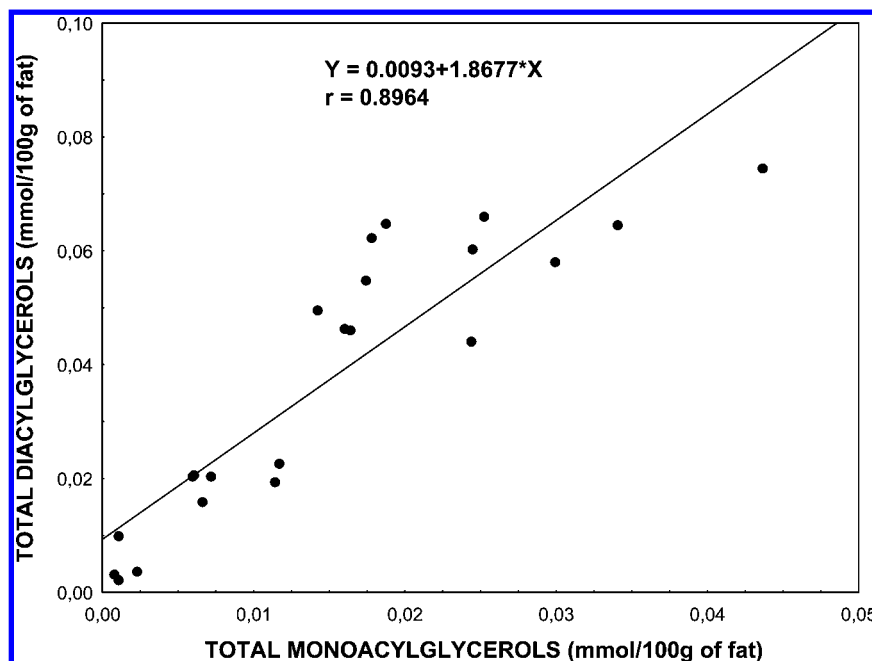
Figure 5. Plot of the relationship of total diacylglycerol (1,2 and 1,3) amount vs monoacylglycerols (mmol/100 g of fat) during the dry-curing process.

the response factors was necessary for obtaining a good quantification of the different components.

In order to verify the absence of contamination by high molecular weight compounds or decomposition products, we have injected silylating reagent between injections of samples, observing that these compounds do not elute.

The assignment of the chromatographic peaks was done by means of standards of 1,2- and 1,3-isomers of dimyristin, diplamitin, diolein, distearin, and dilinolein, which allowed to deduce the carbon number of the components associated with each peak group, as well as the difference between the retention times of the 1,2- and 1,3-isomers (**Table 2**).

RESULTS AND DISCUSSION

Recovery and Repeatability of the Method. The recovery has been previously described, because the SPE procedure for isolating the polar glyceridic fraction was used by other authors (*22*). This is complete (104%), and therefore, the separation obtained with the SPE diol column is adequate.

The repeatability of the method was studied using a raw and a dry-cured sample and a new SPE column for each replicate. These results are shown in **Table 2**; they indicate a good repeatability for the assay.

Free Fatty Acid, Monoacylglycerol, and Diacylglycerol Profile in Raw and Cured Ham. Table 3 shows the mean values of the diacylglycerols analyzed in the subcutaneous fat as a relative percentage of total diacylglycerols and as mg/100 g of fat they correspond to in the initial and final stages of the dry-curing process. Several interesting observations can be deduced from these data. First, the diacylglycerol content in the raw sample is significantly ($p < 0.0001$) lower than in the dry-cured ham. This result is in agreement with previosly published data (*6, 8*). They could be explained by the intense hydrolysis of the triacylglycerol fraction during the dry-curing process (*9*). At the same time, diacylglycerols are also affected by hydrolysis to monoglyceride, as supported by the increase in the monoacylglycerol content observed in the dry-cured ham vs the raw ham. The only specie of monoglyceride found was the monoolein. Second, the major diacylglycerols (considered as a relative percentage of the total) in the raw sample are 1,2-

OP and 1,2-OO while in the cured sample are the isomeric forms 1,3-OP and 1,3-OO. Considering the total isomeric forms, the 1,2-diacylglycerols predominate in the raw sample while the 1,3-diacylglycerols are the most abundant in the dry-cured one. No data are available in relation to the diacylglycerol profile of raw or dry-cured hams. However, the same changes have been detected in olive oil during the storage process. It has been suggested that 1,2-diacylglycerols isomerize toward 1,3-dia-cylglycerols, which are more thermodynamically stable molecular species (*10, 11*).

Evolution of Free Fatty Acid, Monoacylglycerol, and Diacylglycerol Fractions during the Dry-Cured Process. As mentioned above, the lipids of the subcutaneous adipose tissue of the Iberian ham were subjected to an intense chemical and enzymatic hydrolytic process. Some authors suggest the possibility that the hydrolysis processes of subcutaneous fat lipids are due to the activity of endogenous lipase, which has not yet been studied (*23*). Concerning the present results, **Figure 4** shows the evolution of the total diacylglycerols and monoa-cylglycerols (expressed as mmol/100 g of fat). It can be observed that during the postsalting stage (up to 150 days) the levels of the polar acylglycerols remained at a low level, indicating that they both have a slow formation rate (**Figure 4**). Although previous research (*9, 24*) indicates that the highest lypolytic enzyme activity occurs during this stage, it has also been emphasized that there is a temperature dependence for the enzyme activity. The low temperatures during the postsalting period could be a limitation factor for the enzyme activity. During the drying period there is an intense increase in the levels of both acylglycerols, which could be related to an increase in the enzyme activity due to a rise in temperature up to 15–25 °C, which also favors the chemical hydrolysis. This increase remains during drying and the first 150 days of the cellar stage. These results are in agreement with previous research (*9*). The highest values of diacylglycerols, monoacylglycerols, and free fatty acids were obtained during this period of time (**Figure 4** and **Table 4**). From this point (450 days), it can be observed that both fractions suffer a sharp decrease, but with an increasing tendency toward the end of the process. This fact is in agreement

10958 *J. Agric. Food Chem.,* Vol. 55, No. 26, 2007

Narváez-Rivas et al.
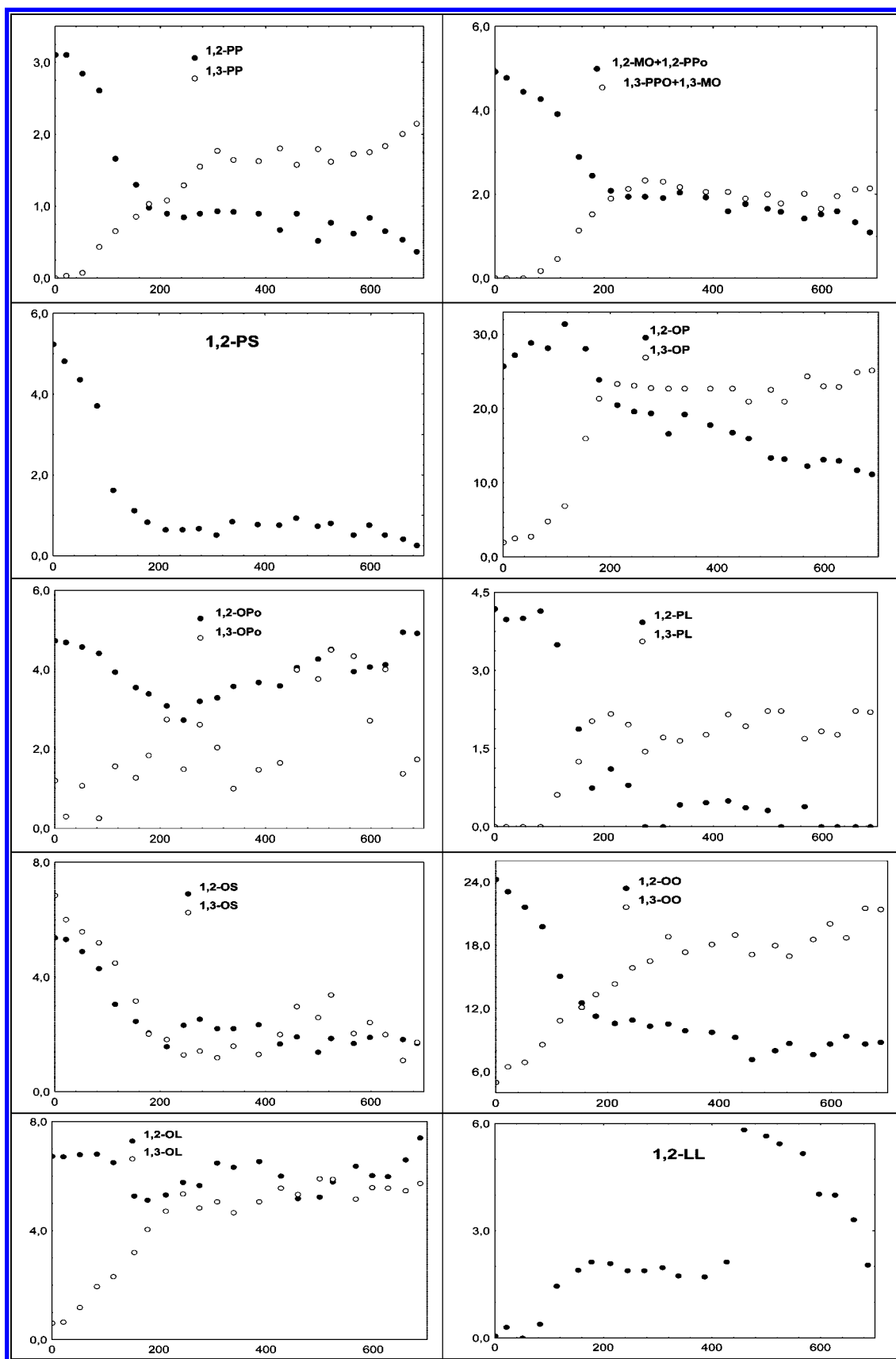
Figure 6. Evolution of total diacylglycerols (1,2 and 1,3) as a relative percentage of total diacylglycerols vs time of dry-curing (in days).

with the disappearance of lipoprotein lipase activity described in previous papers (*9, 24*) and a remaining chemical hydrolysis. **Figure 5** shows the concentration of the diacylglycerols versus monoacylglycerols during the whole process. From this figure, the relative formation rate between these species can be deduced. A linear trend is observed which indicates that the formation rate of diacylglycerols is almost double (1.87) that of monoacylglycerols.
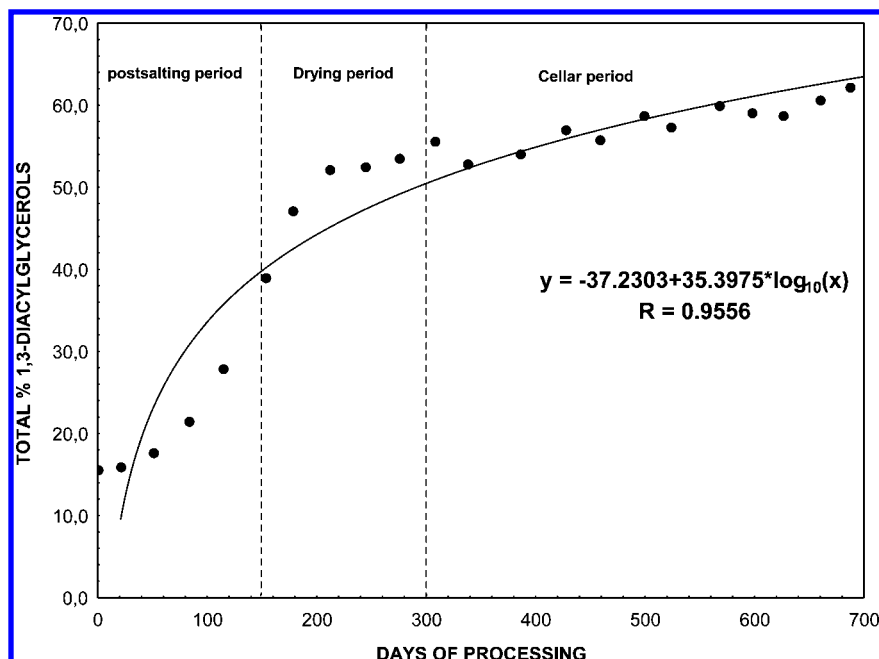
Figure 7. Effect of dry-curing time on the total 1,3-diacylglycerol contents, as a relative percentage of total diacylglycerols, as found in Iberian pig hams.
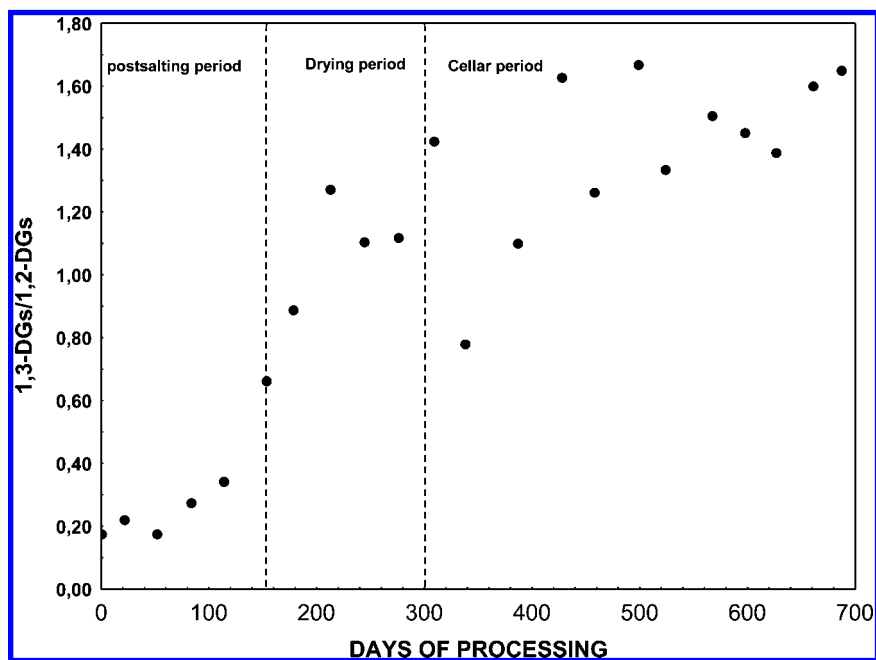

Figure 8. Evolution of 1,3-diacylglycerol/1,2-diacylglycerol ratio as a function of dry-curing time (in days).

Table 4 shows the values in mmol/100 g of fat of palmitic and oleic acids for each of the samplings carried out during the whole dry-curing process. It can be observed that the free fatty acids show a similar tendency as that described for diacylglycerols and monoacylglycerols. The higher levels are observed during the dryer stage, according to the intense hydrolytic activity, as previously suggested.

However, the final acidity value is not significantly different from the value obtained for the raw sample. The decrease in free fatty acid content in the dry-cured ham compared to the raw sample can be explained in part by the preferred loss of free fatty acids with respect to mono-, di-, and triacylglycerols by the sweated process observed during curing that really happens. Also, **Table 4** depicts the total

amount of free fatty acids formed during the ripening process and the total calculated amount according to the equation FFA (mmol/100 g) = 2(monoacylglycerol) (mmol/100 g) + diacylglycerol (mmol/100 g); this equation is only applied to the enzymatic hydrolytic process. It can be observed that the stoichiometric ratio of free fatty acids (experimental/ calculated) is greater than 1 in almost all of the measuring points. This can be explained considering that, besides the enzymatic hydrolysis of tri- and diacylglycerols, there is a chemical hydrolysis of monoacylglycerols and other chemical compounds, such as phospholipids, cholesterol esters, and tocopherol esters. However, the phospholipid level in subcutaneous fat is very low, about 0.46 g/100 g of fat (*5, 25*), and their hydrolysis is more relevant in the intramuscular

10960 *J. Agric. Food Chem.,* Vol. 55, No. 26, 2007

Narváez-Rivas et al.

fat (*7*). The high experimental values found here could also be due to the hydrolysis of monoacylglycerols, which could be important in the last stage (450 days) of curing, where both diacylglycerols and monoglyceride decrease.

Isomerization of Diacylglycerols. As mentioned before, the diacylglycerols formed by the hydrolysis of triacylglycerols are affected by an isomerization process. **Figure 6** shows the evolution (in relative percentage) of the diacylglycerols during the whole process (from 0 to 687 days). The two isomeric forms, the 1,2- and 1,3-diacylglycerols, are represented in the same figure. In general, it can be observed that the percentages of 1,2-diacylglycerols decrease sharply during the postsalting process (about 150 days), and then a moderate decrease or stabilization occurs during the dryer and cellar period. On the contrary, the relative percentages of the corresponding 1,3-diacylglycerol increase sharply during the postsalting period, and afterward, a moderate increase or a stabilization is observed. This tendency is in accordance with data reported in the literature for olive oil during storage (*10, 11*).

Figure 7 shows the evolution of the relative percentage of total 1,3-diacylglycerols during the time of ripening. A logarithmic increase can be observed up to a value of approximately 62% of the total fraction (at about 500 days of process or half of that in the cellar), remaining almost constant or with slow changes from this point. These changes are explained because initially there are only 1,2-diacylglycerol isomers but the isomerization process is rather quick, reaching an equilibrium in a short time.

Figure 8 represents the molar relationships between 1,3-diacylglycerols and 1,2-diacylglycerols during the process. It can be observed that there is an increase from 0.20 (initial value) to 1.65 at the end of the process. This increase in the relative formation rate cannot be considered linear along the whole process as found in olive fruit (*10*). The higher increase is observed during postsalting and dryer stages (about 300 days), and then a moderate increase occurs during the cellar period. At about 450 days, this ratio reaches a possible equilibrium. This fact can be explained, in agreement with the previous reports found in the literature, which reports besides the 1,3-diacylglycerol formation from triacylglycerols hydrolysis there is a isomerization from 1,2-diacylglycerols to 1,3-diacylglycerols. This process is an acid-catalyzed reaction by the free fatty acids liberated from triacylglycerol hydrolysis (*10, 11*).

In conclusion, from the results above mentioned, it is deduced that the analytical method applied is a good alternative to quantify this fraction. Also, the dry-curing of the Iberian ham process is characterized by a least three simultaneous reactions: (a) triacylglycerol hydrolysis to form diacylglycerols; (b) diacylglycerol isomerization to give an important increase of 1,3-diacylglycerols, and (c) diacylglycerol hydrolysis. However, other competitive reactions such as oxidation of acylglycerols and fatty acids and hydrolysis of monoacylglycerols cannot be excluded.

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Fatty Acid and Monoacyl/diacylglycerol in Fat of Iberian Ham

J. Agric. Food Chem., Vol. 55, No. 26, 2007 **10961**

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