Quantitative and Qualitative Analysis of Free Fatty Acids in Meat and Meat Products using Ion Exchange Resin

GANDEMER G.[1], MORVAN-MAHI B.[2], MEYNIER A.[1], LEPERCQ M.[2]

1. Laboratoire d'Etudes des Interactions des Molecules Alimentaires, INRA, BP 527, 44 072 Nantes cédex 03, FRANCE
2. Centre Technique de la Salaison, de la Charcuterie et des Conserves de Viande, 7 avenue du Gal de Gaulle, Maisons Alfort, FRANCE

SUMMARY : A method for quantitative and qualitative analysis of free fatty acids (FFA) in total lipid extracts from meat products using an anionic exchange resin (Amberlyst) is described. The optimized working conditions were : 50 to 100 mg of lipids were dissolved in 15 ml of acetone/methanol (2/1 v/v) and were stirred with 100 mg of resin during 30 minutes. Non fixed lipids were removed by washing the resin with 5 x 5 ml of solvent on a sintered glass filter. FFA were esterified directly on resin using methanol/BF3 before quantification by GLC. Using this procedure, FFA recovery was almost complete whatever the fatty acid chain length or unsaturation were. FFA contamination by other lipid fractions was weak (less than 2% for triglycerides, phospholipids or monoglycerides).

INTRODUCTION

FFA are involved in many chemical reactions such as lipid oxidation, myoglobin oxidation or flavor of dry and cooked meat products (GIRARD and BUCHARLES, 1985). Therefore more knowledge of FFA composition is required to a better understanding of the relationships between this lipid fraction and meat products quality. At the present time, little is known on the FFA composition of meat products and most of the results were obtained with time consuming procedure (MARION and WOODROOF, 1965) or methods suspected to cause glyceride hydrolysis (McCARTHY and DUTHIE, 1962 ; HORNSTEIN *et al.*, 1960)

Almost all of the methods were developped for FFA analysis in plasma or milk products. Most of these methods required a preliminary purification of FFA to eliminate interfering substances. It seems that these methods should be adapted to FFA purification in the total lipids extracted from meat products according to the methods widely used for quantitative lipid extraction (FOLCH *et al.*, 1957). Among the numerous methods published, we have chosen a method involving a FFA purification on strong anion exchange resin (amberlyst A26). This choice is based on the following facts :

1) Purification step is based on a specific property of FFA to adsorb on strong anion exchange resin reducing the risk of contamination by other lipid classes ;

2) Reagents and working conditions are mild enough to not induce lipid alterations ;

3) This type of method was successfully used for FFA analysis in milk (NEEDS *et al.*, 1983).

The aim of this study was to optimize the main steps of the method in order to determinate FFA composition of meat products.

MATERIALS AND METHODS

Chemicals. Solvents were analytical grade (Carlo Erba). Anion exchange resin was Amberlyst A26 (Sigma A-5522). Resin was treated as described by NEEDS *et al.* (1983). The resin (20 mg) was stirred with 200 ml of 1 M sodium hydroxide during 5 minutes and was washed free of alkali with 3 x 100 ml of distilled water and once with 150 ml of methanol.

The steps of the procedure were optimized using standard lipid mixtures. Each lipid class contained a specific fatty acid : triglycerides were pure triolein, FFA was linoleic acid, monoglycerides were 1-monolauroyl-glycerol, phosphatidyl-choline was di-palmitoyl-phosphatidyl-choline, phosphatidyl-ethanolamine was di-stearoyl-phosphatidyl-ethanolamine. So it was easy to estimate the FFA contamination by other lipid classes containing bound fatty acids. The standard lipids were dissolved in heptane at the following concentrations : triolein - 50 mg/ml, linoleic acid - 500 µg/ml, heptadecanoic acid - 50 µg/ml (internal standard), monoglycerides - 1 mg/ml, phosphatidyl-choline - 1 mg/ml and phosphatidyl-ethanolamine - 1 mg/ml. Purity and concentration of each lipid solution were assessed by GLC of their methyl esters. The various lipid mixtures used in this work were described in the result section.

To determine the recoveries of the fatty acid according to their chain length and unsaturat2ion, a mixture of long chain fatty acids were prepared by saponification of 100 to 500 mg of cod oil or muscle lipids with potassium hydroxide in alcoolic solution (10%) during one night at room temperature. After the unsaponifiable lipids were eliminated, chlorhydric acid was added and FFA were extracted with hexane. Fatty acid composition of these mixtures was determined by GLC of their methyl esters.

Purification of FFA. Lipids were dissolved in 15, 30 or 45 ml of solvent (ether/methanol or acetone/methanol, 2/1) in a 100 ml round bottom flask. After addition of resin (100 or 200 mg) and heptadecanoic acid (internal standard), the mixture was shaked using a magnetic

stirrer during 15, 30, 60 or 120 minutes. Non resin bound lipids were removed by washing the resin with solvent. Three procedures of washing were compared (see result section). Resin was transferred in clean and dry tube for the FFA methylation.

Gas Chromatography of FFA. FFA methylation was performed directly on the resin using BF3/methanol (14%) according to the method of MORRISON et SMITH (1964). Gas liquid chromatography of methyl esters was carried out using a DI 700 chromatograph (Delsi Instruments) equiped with split-splitless injector and flame-ionization detector paired with a CR3A integrator (Shimadzu). The capillary column (30 m long, 0.32 mm i.d.), coated with polyethylene stationary phase (econocap, Alltech) was maintained in the oven at 180 °C. Injector and detector temperatures were 250 °C. Head pressure of hydrogen carrier gas was 0.5 bar. Split injection mode was used (split flow rate = 40 ml per min.). The amount of each fatty acid was calculated by comparison with the peak area of the internal standard. FFA recovery was expressed as mg or % of the amount of FFA present in lipid standard mixture. FFA contamination was estimated by the calculation of the amount of the fatty acid of each lipid class bound on resin. Results were expressed as mg or % of the amount of FFA bound on resin.

Statistical analysis. To optimize working conditions, six factors with 2 or 3 levels were studied in two factor experiments (resin/solvent amounts, FFA amount/ length of shacking period, type of solvent/procedure of non resin bound lipis elimination). Four replication of each experiment were performed. Results were compared using a two way variance analysis. Other results were compared using a one way variance analysis.

RESULTS AND DISCUSSION

1 - Optimization of working conditions.

Resin and solvent quantities (Table 1). Volumes of solvent (ether/methanol) tested were 15, 30 and 45 ml and resin amounts were 100 or 200 mg. The lipid mixture used contained 200 mg of triolein, 2 mg of linoleic acid and 0.2 mg of heptadecanoic acid. Non resin bound lipids were removed by washing the resin on filter paper using 5 x 5 ml of solvent. The results showed that both the solvent volume and the resin amount had no significant effect on the recovery of linoleic acid and on the FFA contamination by triolein. Thus, the linoleic acid recoveries were on average 85% and the contamination by oleic acid accounted for about 4.5% of the linoleic acid amount.

Effects of FFA amount and length of the shaking period (Table 2). Lipid mixtures were composed of 0.2 , 0.5 , 1 or 2 mg of linoleic acid with 20, 50, 100 or 200 mg of triolein respectively. An appropriate amount of heptadecanoic acid was added (10% of the linoleic acid amount of the sample). Lipids were dissolved in 15 ml of solvent (ether/methanol) and stirred during 15, 30, 60 or 120 minutes. The recoveries of linoleic acid were similar whatever the length of the shaking period. They were on average 86%. Conversely, this parameter is affected by the amount of FFA. So, the recovery of linoleic acid was lower (83%) when FFA amount was 200 µg as compared of the linoleic acid recoveries obtained for larger amounts of FFA (87.5% for FFA amounts of 0.5 to 2 mg). The average of the FFA contamination by oleic acid was 12%. It should be noted that a considerable variability was observed in FFA contamination by triolein (1 to 40%). These results suggested that the washing procedure of the resin on filter paper was not suitable to eliminate the non resin bound lipids.

Effects of type of solvent and procedure of non resin bound lipid elimination (Table 3). The lipid mixtures contained of 200 mg of triolein, 2 mg of linoleic acid and 0.2 mg of internal standard. Lipids were dissolved in 15 ml of ether/methanol or acetone/methanol (2/1, v/v). Non resin bound lipids were eliminated according to one of the following procedures :

- the first one was that used by NEEDS *et al.* (1983) who removed the solvent and washed the resin in the 100 ml bottle 5 times with 15 ml of solvent ;
- in the second one, the content of the flask was tranferred on a filter paper and the resin was washed with 5 x 5 ml of solvent ;
- and the last procedure was similar to the second one but sintered glass filter was used instead of filter paper.

The results showed that the linoleic acid recoveries and its contaminations by triolein were similar whatever the type of solvent (98 to 99% and 2 to 3% respectively). On the contrary, procedure to eliminate non resin bound lipids affected largely the results. Thus, when resin was washed on filter paper, linoleic acid recoveries were lower as compared to the values obtained with the two other procedures (88% versus 99 to 102%). Conversely, contamination of linoleic acid by triolein was higher (6.3% versus 0.7-0.8%). These results could be explained by the absorption of a variable amount of lipid solution in the filter paper.

Although both the solvent mixtures gave similar results, the use of acetone/methanol is more suitable than ether/methanol because acetone is easier to manipulate. Within the two procedures to eliminate non resin bound lipids which gave accurate results, the one involving the washing of the resin on sintered glass filter is less time consuming than the one proposed by Needs *et al.* (1983)

The present results indicate that the optimized working conditions are the following ones : a lipid fraction containing at least 500 µg of FFA should be dissolved in 15 ml of acetone/methanol. After adding 100 mg of resin, the mixture is shaked during 30 minutes using a magnetic stirrer. Non resin bound lipids are removed by washing the resin on sintered glass filter with 5 x 5 ml of solvent. FFA are methylated directely on the resin using methanol/BF_3 before individual fatty acid quantification by GLC. This procedure has been used in all the following studies.

2 - FFA analysis in total lipid extracts of meat products.

Method evaluation over the range of FFA in meat products (Table 4). This study was performed with the following standard lipid mixtures : 50 mg of triolein and various FFA amounts (100, 200, 300, 400 or 500 µg of linoleic acid) or an increase amount of triolein (20, 50, 100 or 200 mg) containing a constant proportion of linoleic acid (1%). The results showed that linoleic acid recoveries were similar whatever the amounts of linoleic acid or triolein in the standard mixtures. The amount of oleic acid bound on resin varied from 4 to 19 µg. The linoleic acid contamination by triolein increased from 1 to 19% when the amount of linoleic acid adsorbed on the resin decreased from 2 mg to 100 µg. These results indicate that FFA contamination by triglycerides is negligible (less than 2%) when the samples contain at least 500 µg of FFA.

Interference from monoglycerides and phospholipids. This study was performed using four samples composed of 100 mg of triolein, 1 mg of linoleic acid, 100 µg of heptadecanoic acid and 1 mg of monolaurate and four samples composed of 50 mg of triolein, 500 µg linoleic acid, 50 µg of heptadecanoic acid, 2 mg of di-palmitoyl-phosphatidyl-choline (PC) and 2 mg of di-stéaroyl-phosphatidyl-éthanolamine (PE). Linoleic acid recoveries were complete whatever the lipid mixtures used (98 to 102%). On average, 8 µg of lauric acid were bound on resin. FFA contamination by monoglycerides accounted for less than 0.8% of linoleic acid. Five µg of palmitic acid and 2,3 µg of stearic acid were bound on resin which accounted for less than 0.5% of the amount of linoleic acid. So, it was concluded that monoglycerides and phospholipids did not affect significantly quantitative and qualitative analysis of FFA. These results corroborate those obtained by NEEDS *et al.* (1983). Moreover, these authors suggested that the elimination of phospholipids from total lipid extracts using silicic acid as proposed by SALIH *et al.* (1977) may cause a FFA loss.

Recoveries of individual fatty acids (Table 5)**.** This study was performed using 1 mg of FFA mixtures prepared by saponification of cod oil or muscle phospholipids. After adding 0.1 mg of heptadecanoic acid, the mixture was submitted to the procedure of FFA isolation described above. Recoveries of all the FFA were complete whatever the chain length and insaturation. These results are in a good agreement with those obtained previously for 4 to 18 carbon fatty acids (NEEDS *et al.*, 1983).

Individual free fatty acid quantification in some meat samples (Table 5). The method was applied to total lipid extracts from pig Trapezius and to lard samples. FFA of muscle lipids contained a large proportion of polyunsaturated fatty acids which indicate that lipolysis affect largely phospholipids. FFA of lard samples exhibited a fatty acid composition close to that of triglycerides.

REFERENCES

FOLCH J., LEES M., SLOANE-STANDLEY G.H., 1957. A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. 226, 497-509.

GIRARD J.P., BUCHARLES C., 1985. Evolution post-mortem des gras animaux. Bull. Techn. CRZV, Theix-INRA, 62, 81-92.

HORNSTEIN I., ALFORD J.A., ELLIOTT L.E., CROWE P.F., 1960. Determination of free fatty acids in fat. 32, 540-542.

McCARTHY R.D., DUTHIE A.H., 1962. A rapid quantitative method for the separation of free fatty acids from other lipids. J. Lipid Res., 3, 117-119.

MORRISON W.R., SMITH L.M., 1964. Preparation of fatty acid methyl esters and dimethyl acetals from lipids with boron-fluoride methanol. J. Lipid Res., 5, 600-608.

MARION J.E., WOODROOF J.G., 1965. Lipids fractions of chicken boiler tissues and their fatty acid composition. J. food Sci., 30, 38-43.

NEEDS E.C., FORD G.D., OWEN A. J., TUCKLEY B., ANDERSON M., 1983. A method for the quantitative determination of the individual free fatty acids in milk by ion exchange resin adsorption and gas-liquid chromatography. J. Dairy Res., 50, 321-329.

SALIH A.M.A., ANDERSON M., TUCKLEY B., 1977. The determination of short and long chain free fatty acids in milk. J. dairy Res., 44, 601-605.

Table 1 : Effects of resin amount and solvent volume on the FFA recovery and their contamination by triglycerides

	Recovery 1	Cont. 2
Resin amount (mg)		
100	86,0	3,2
200	84,8	2,9
Solvent volume (ml)		
15	86,1	2,8
30	86,2	2,8
45	83,9	3,7

Table 2 : Effects of FFA amount and lenght of shaking period on the FFA recovery and their contamination by triglycerides

	Recovery 1	Cont. 2
FFA amount (mg)		
0,02	83,1 a	11,7 a
0,05	88,2 b	13,1 a
0,1	87,4 b	17,6 a
0,2	87,1 b	8,9 a
Shaking time (min)		
15	86,1	13,7
30	86,2	14,4
60	88,3	11,4
120	86,3	11,8

Table 3 : Effects of solvent type and of procedure of elimination of non-resin bound lipids on the FFA recovery and their contamination by triglycerides

	Recovery 1	Cont. 2
Solvent type		
Ether/ Methanol	96,2	3,3
Acetone/ Methanol	96,4	2,0
Washing method		
Needs et al.(1983)	98,7 a	0,7 b
Filter paper	88,4 b	6,3 a
Filter sintered disc	101,8 a	0,8 b

For a given parameter, within the column, means superscripted by differents letters are significantly different at a level of 5%.

1) FFA recovery is expressed as % of FFA amount in lipids standard mixtures.
2) FFA contamination by triolein is expressed as % of FFA adsorbed on resin.

Table 4 : Effects of triglycerides (TG) and FFA amounts on the FFA recovery and their contamination by triglycerides

TG (mg)	FFA (mg)	Recovery 1	Cont. 2
50	0,1	97,4	19,3 a
50	0,2	99,0	9,1 b
50	0,3	98,3	5,6 bc
50	0,4	97,3	3 c
50	0,5	99,7	2 ,0 c
20	0,2	104,5	1,5 ab
50	0,5	103,2	1,0 b
100	1,0	101,4	1,7 a
200	2,0	99,2	0,8 b

Within the column, means superscripted by differents letters are significantly different at a level of 5%.

Table 5 : Effects of chain length and insaturation of FFA on their recovery

Fatty acids	3	Recovery 1	
14:0	4	101,5	[6,0] 4
16:0	6	100,7	[3,2]
18:0	6	99,8	[3,5]
16:1	6	103,9	[2,6]
18:1	6	98,2	[1,2]
20:1	6	97,8	[2,9]
22:1	2	103,0	[4,7]
18:2 n-6	6	98,2	[1,9]
18:3 n-3	6	100,2	[0,3]
18:4 n-3	2	97,8	[2,9]
20:4 n-6	6	101,3	[1,8]
20:5 n-3	2	98,7	[4,0]
22:5 n-3	2	99,1	[4,5]
22:6 n-3	2	98,0	[3,6]

Table 6 : FFA composition of muscle and lard

Fatty acids	Muscle (n=2)	Lard (n=4)
14:0	1,2	1,9
16:0	13,9	25,4
18:0	8,0	6,6
Saturated	**23,7**	**33,9**
16:1	3,4	6,1
18:1	29,7	32,4
Monounsat.	**33,1**	**38,5**
18:2 n-6	28,7	25,7
18:3 n-3	1,3	1,9
20:4 n-6	11,7	tr
Polyunsat.	**44,0**	**27,6**

1) Recovery is expressed as % of the amount of this fatty acid in FFA mixture.
2) FFA contamination by triolein is expressed as % of FFA adsorbed on resin.
3) Number of each fatty acid determinations
4) Standard deviation