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Food Chemistry 72 (2001) 301–307

Food Chemistry

www.elsevier.com/locate/foodchem

Determination of free fatty acids produced in filled-milk emulsions as a result of the lipolytic activity of lactic acid bacteria

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Received 3 November 1999; received in revised form 26 July 2000; accepted 26 July 2000

Abstract

Extraction procedures were optimised for quantitatively separating free fatty acids (FFAs) from other fat fractions and from lactic and benzoic acids in extracts from fermented filled-milk emulsions. Examination of FFAs from fermented milk-based emulsions indicated a high level of C16:0 which was attributed to selective hydrolysis of the C16:0 monoacylglycerol component in the emulsifier.

Keywords: Free fatty acids; Filled-milk emulsions; Lactic acid bacteria

1. Introduction

Many methods have been developed to determine the concentration of free fatty acids (FFAs) in foods as an indication of lipolysis and hence the quality of the food. The best of these methods are able to detect changes at a level before lipolytic off-flavours occur (Stepanik & Sorhaug, 1991). Ikins, Kwak, Zink and Jeon (1988) compared different methods and demonstrated the precision of and preference for gas–liquid chromatographic methods over methods determining the total acidity. Chromatographic methods enable the identification and quantification of individual FFAs. This is important because specific short- and medium-chain FFAs contribute to the flavour of products, with excessive levels being associated with characteristic, undesirable off-flavours (Woo, Kollodge & Lindsay 1984).

FFAs have to be separated from the extraction solvent in which the original fat is also dissolved. Kaluzny, Duncan, Merritt and Epps (1985) used bonded-phase columns to perform the extraction and isolation of FFAs. This technique was subsequently used by de Jong and Badings (1990) who first employed a mixture of chloroform and 2-propanol (2:1 v/v) to elute the neutral lipids and then a 2% formic acid in diethyl ether solution to elute the FFAs from a solid phase column. However, the second eluent contained not only FFAs but a mixture of other organic acids that could interfere with the subsequent analysis by gas–liquid chromatography. Nevertheless, this method has been found to give satisfactory results for milk and cheese (Deeth & Fitz-Gerald, 1995).

In the fermentation of milks there is the possibility of lipolysis brought about by lipases from the starter bacteria. Other metabolites will include a range of organic acids, such as lactic, acetic and propionic acids, plus traces of aromatic acids, such as benzoic and phenylacetic acids. These organic acids should be excluded from the determination of FFAs. In the same way, the precipitated protein in fermented products must not be allowed to inhibit the extraction of FFAs from the fat globules embedded in these precipitates (IDF, 1991). Where filled milks are fermented, the system may be more complex, as not only are different fats present but the formation of the emulsion may involve the use of surface-active agents.

This investigation sought to develop a method suitable for investigating the production of FFAs in fermented filled milks.

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0308-8146/01/$ - see front matter
PII: S0308-8146(00)00230-2

2. Materials and methods

Skim milk powder was obtained from Dairy Crest Ingredients (Surbiton, UK). Recodan™ RS, an integrated blend of food-grade emulsifier and stabilisers for the production of recombined, filled and imitation milk, was kindly donated by Danisco Ingredients UK Ltd (Northampton, UK). Anhydrous milk fat was obtained from Dairy Ingredients UK Ltd (Slough, UK). Fully refined and deodorised palm oil, maize oil and hydrogenated palm kernel oil were supplied by Cargill plc. (Bootle, UK). Deionised water (17 MΩ), obtained from a Purite HP Analyst and Still HP system (Purite Ltd, Thame, UK), was used for all the experiments. All monoacylglycerols, diacylglycerols and fatty acids used as standards for the trials were obtained from Sigma-Aldrich Co. Ltd (Poole, UK), against a specification of minimum 99% purity. Solvents used for gas chromatography (GC) were capillary GC grade, while the remaining reagents were of analytical grade, also obtained from Sigma-Aldrich Co. Ltd (Poole, UK). Sep-Pak Vac cartridges containing aminopropyl (500 mg/3 c.c.) were obtained from Waters Ltd (Watford, UK).

2.1. Preparation of emulsions

Reconstituted filled-milk emulsions were prepared according to the formula in Table 1. The emulsions were made by emulsification of ingredients at 70–75°C using a Rannie single-stage homogeniser at 100 bar homogenisation pressure, followed by sterilisation at 121°C for 15 min. Fat-droplet size was determined with a Malvern particle sizer, series 2600c (Malvern Instruments, Malvern, UK), to ensure that all emulsions were within the same range of fat-droplet size (mode 1.23–1.58 μm). When smaller batches of emulsions containing pure monoacylglycerols or diacylglycerols were prepared, emulsification was achieved with a Silverson high-shear mixer type L2R (Silverson Machines, Chesham, UK).

2.2. Fermentation of emulsions

Lyophilised DVI starter cultures, supplied by RP Texel (Stockport, UK), were added to 10 ml of reconstituted skim milk at 25°C to give 4×10^9 to 6×10^9 cfu/ml. Each culture was grown at its optimum temperature: *Lactococcus lactis* subsp. *lactis* var. *diacetylactis* at 30°C, *Streptococcus thermophilus* at 37°C, *Lactobacillus delbrueckii* subsp. *bulgaricus* at 40°C and *Lactobacillus rhamnosus* at 45°C. After incubation for 1 h at optimum growth temperature, 2 ml of each suspension were added to 248 ml of each emulsion to give 5×10^7 cfu/ml. The milk emulsions were allowed to ferment at the respective optimum growth temperature for 8 h, then cooled and held at 4°C.

Table 1
Composition of emulsions for study of lipid–bacterial interactions

	Ingredients (%, w/w)			
	Skim milk powder	Emulsifier	Fat	Water
Reconstituted skim milk (RSM)	8.5	0	0	91.5
RSM + emulsifier	8.5	0.2	0	91.3
RSM + emulsifier + fat	8.5	0.2	4	87.5

2.3. Determination of FFAs in filled milks

Lipid extraction, isolation of FFAs and capillary gas chromatographic analysis were based on the method proposed by de Jong and Badings (1990) but with refinement of the solvent extraction for isolation of FFAs to minimise interference from other organic acids and to optimise the extraction for recovery of short-chain FFAs.

Two series of experiments were carried out to optimise the elution of neutral lipids by the chloroform/2-propanol (2:1 v/v) solvent system and the subsequent elution of the FFA by 2% formic acid in diethyl ether.

Samples (10.00 g) of fermented filled-milk were weighed and mixed with 10 ml ethanol, 1 ml of 2.5 M sulphuric acid plus 1 ml of an internal FFA standard solution (0.5 mg/ml each of C5:0, C7:0, C13:0 and C17:0). The spiked sample was extracted with 15 ml diethyl ether/heptane (1:1 v/v) in a screw-capped centrifuge tube and then centrifuged for 2 min at 1100 ***g***. The upper solvent layer was transferred to a conical flask containing 1 g anhydrous sodium sulphate to remove residual water. The extraction was repeated twice with 10-ml aliquots of the solvent mixture and all the extracts were combined.

The aminopropyl column was conditioned with 10 ml heptane. The bulked lipid extract (approximately 35 ml) was then applied to the column. The neutral lipid components were extracted with varying quantities, 4, 8 or 12 ml, of chloroform/2-propanol (2:1 v/v). The second elution from the column used 2% formic acid in diethyl ether, the eluate being collected in 1-ml aliquots. Nonanoic acid was added as an injection standard at 500 μg/ml to each aliquot, which was then analysed by GC as described below. Data from examination of the aliquots were used in the subsequent investigation to optimise the recovery of FFA whilst minimising the extraction of other organic acids by use of the optimal volumes of each solvent system.

At the capillary GC stage, modifications to the original method were as follows: the initial sample was measured by weight instead of volume and 2.0 μl (instead of 0.5 μl) of extraction solution were injected into the Hewlett Packard 5890 Series II Gas Chromatograph equipped with a direct injector and flame ionisation

detector (FID). The capillary column was a Megabor® DB-FFAP column, 15 m×0.53 mm internal diameter, coated with FAP (d.f. = 1.0 μm) (Jones Chromatography Ltd, Hengoed, UK). The helium carrier-gas flow rate was 9 ml/min. After injection, the oven temperature was held at 60°C for 45 s and then raised to 245°C at 9°C per min. Confirmation of signal identities in the gas chromatograms was made by mass spectrometry of selected samples. An HP 5972 mass-selective detector attached to an HP 5890 series II gas chromatograph was used. In this case, automatic on-column injection was performed instead of the manual injection normally used for routine samples. The helium carrier-gas flow rate was 2 ml/min but the temperature programme remained as above. The mass spectra obtained were identified by comparison with those of standards.

Corrections were made for extraction yields and for relative response factors (RRFs), calculated with respect to the standards.

2.4. Analysis of the commercial emulsifier

Total lipid content and moisture content of Recodan™ RS were determined by the method proposed by Christie (1992a), followed by determination of the acylglycerol esters and FFA fractions using bonded-phase columns (Kaluzny et al., 1985) and by thin-layer chromatography (Christie, 1992b). The fatty acid composition of each acylglycerol fraction was determined by capillary GC of its fatty acid methyl esters (Christie, 1992b) using a Sigma 3B Gas Chromatograph with a column type CP Sil 88: 25 m×0.25 mm i.d. from Chrompack UK Ltd and connected to a FID. Determinations were at constant temperature and flow rate. Detector, injector and oven temperatures were 230, 230 and 180°C, respectively, with a helium flow rate of 2 ml/min.

3. Results

3.1. FFA extraction solvent system

The recovery of FFAs by elution with 2% formic acid in diethyl ether is shown in Table 2. Recovery of virtually all of the FFAs was achieved with 3–4 ml. Only in the case of hexanoic acid was there a significant residue in the fifth millilitre of eluent. The first millilitre did not contain significant amounts of any FFAs and, if collected, would only increase the dilution of the FFAs.

The degree of contamination of the second eluate by lactic and benzoic acids was dependent on the volume of the first eluent system. With both lactic and benzoic acids, the elution of the acid by the second solvent occurred earlier when a larger quantity of the first, chloroform/2-propanol, solvent system was used, as shown in Figs. 1 and 2. Lower volumes of chloroform/2-propanol are not recommended because lower initial extraction of neutral lipids leads to contamination of the subsequently eluted FFAs.

Collection of only the second, third and fourth millilitre was sufficient to achieve a high recovery of FFAs while minimising the carry over of benzoic acid and halving the level of lactic acid. Therefore, the experimental approach adopted was to discharge the first millilitre and to collect only the second, third and fourth millilitres.

Acetic acid was eluted early in the GC run and did not interfere with resolution of the individual fatty acids. Traces of lactic acid were resolved as a peak between that for nonanoic and decanoic acids while traces of benzoic acid gave a peak between decanoic and dodecanoic acids. These peaks were too small to interfere with resolution of the fatty acids. RRFs and Extraction Efficiencies for the capillary GC technique, based on the internal standards, are given in Table 3. RRF values showed that the detector displayed a slight change in response for the different FFA standards, the highest RRF being found with C9:0 and the lowest with C5:0. This was similar to the findings of de Jong and Badings (1990), their system achieving a better reproducibility (S.D.±0.01–0.04) in the model system but similar variability (S.D.±0.01–0.06) in recoveries from milk and cheese. After correcting for variation in RRFs, the extraction efficiencies indicated virtually complete recovery of FFA from the fermented filled milk.

3.2. Changes in FFAs during fermentations

Concentrations of FFAs generated during 6-h fermentations with *Streptococcus thermophilus* are summarised in Table 4(a). These data indicate a preferential release of palmitic acid from all fat sources, including the emulsifier. Release of stearic acid was also relatively high, while levels of linoleic acid were elevated in the case of maize oil. On deducting the FFA values obtained by fermentation of skim plus emulsifier, the values for release of palmitic acid remained high, while the values for free stearic acid were of the same order as for myristic and oleic acids, as shown in Table 4(b). Similar patterns were observed when repeating the fermentations with *Lactococcus lactis* subsp. *lactis* var. *diacetylactis, Lactobacillus delbrueckii* subsp. *bulgaricus* and *Lactobacillus rhamnosus*.

3.3. Composition of the commercial emulsifier

Table 5 shows the results obtained from analysis of the emulsifier. Monoacylglycerols were found to form the highest proportion of the lipid fraction with monopalmitin and monosteann as the main components. A small proportion of monomyristin was also detected.

Table 2
Percentage recoveries of individual free fatty acids in the eluate from final extraction by 2% formic acid in diethyl ether (means of three determinations)

Carbon number	Percentage recovery in relation to the volume of eluate (ml)					
	1	2	3	4	5	6
C4:0	0.0	47	71	86	100	100
C5:0	0.0	68	99	100	100	100
C6:0	0.0	28	46	61	77	100
C7:0	0.6	67	99	100	100	100
C8:0	0.0	61	100	100	100	100
C10:0	0.0	46	89	100	100	100
C12:0	1.1	60	98	100	100	100
C13:0	0.6	62	98	99	100	100
C14:0	1.1	61	92	96	98	100
C16:0	0.5	68	99	100	100	100
C17:0	0.6	70	99	100	100	100
C18:0	0.6	68	96	98	99	100
C18:1	0.0	70	98	99	99	100
Unweighted mean	0.4	60	91	95	98	100

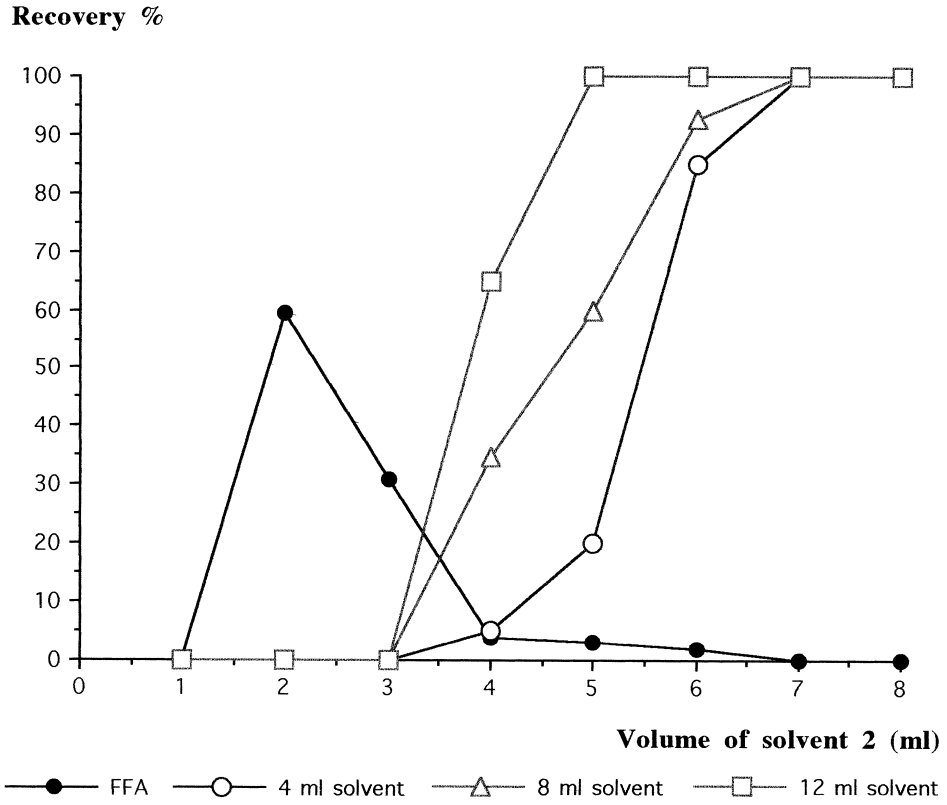

Fig. 1. Effect of the volume of the first solvent (chloroform/2-propanol) on the elution of benzoic acid expressed as cumulative per cent. Elution of free fatty acid (FFA) is shown as an incremental per cent.

3.4. Fermentation of emulsions with emulsifier components

Fermentation of emulsions containing pure diacylglycerides by *S. thermophilus* did not result in production of FFAs. However, when monoacylglycerides were used at equivalent levels to those present when the commercial emulsifier was included, the production of FFAs was observed as indicated in Table 6. These values were indistinguishable from those obtained by incubation of milk plus emulsifier, given in Table 4(a).

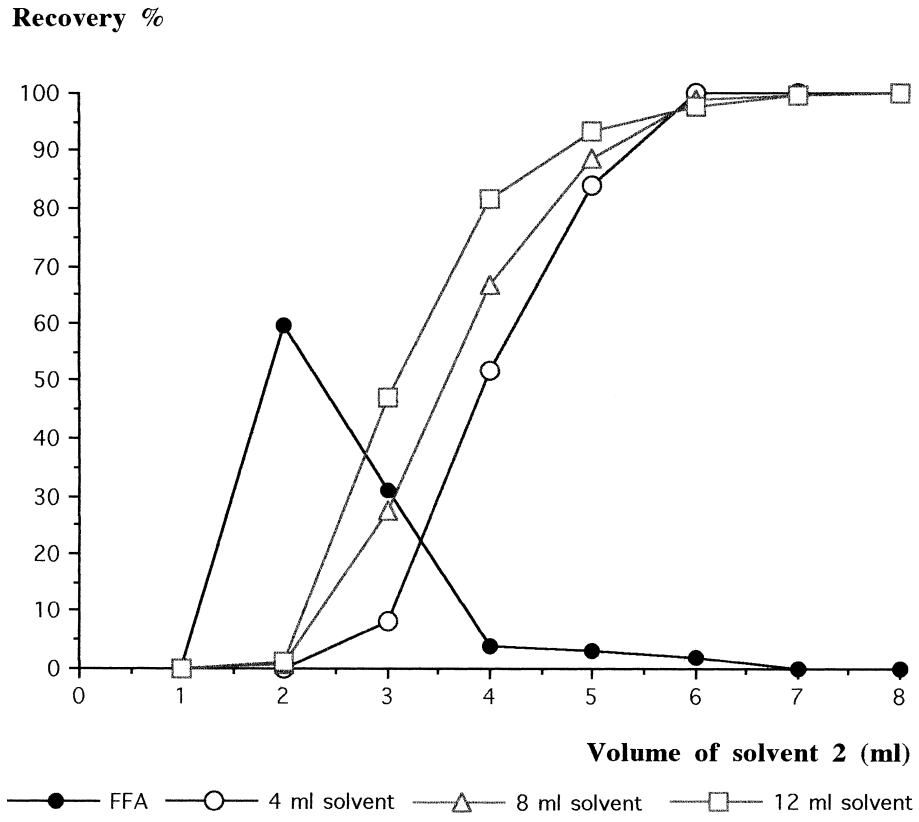

Fig. 2. Effect of the volume of the first solvent (chloroform/2-propanol) on the elution of lactic acid expressed as cumulative per cent. Elution of free fatty acid (FFA) is shown as an incremental per cent.

Table 3
Relative response factors (RRFs) and per cent extraction efficiencies of internal standards used in the capillary gas chromatography analysis (means and standard deviations for 25 replicates)

Carbon number	RRF compared to C17:0	RRF compared to C9:0	Extraction efficiency (%)
C5:0	0.72±0.06	0.60±0.05	100
C7:0	1.01±0.08	0.83±0.08	100
C9:0	1.22±0.07	1	
C13:0	1.18±0.08	0.97±0.08	99
C17:0	1	0.82±0.07	100

4. Discussion and conclusions

In fresh milk, lactic acid would only be present in trace quantities while, in cheese, there may be 1% lactic acid but 30% fat. However, in fermented milks, lactic acid is the major metabolite, being present at up to 1% with a typical fat content of 3.5–4.0%. Elution of lactic acid occurs between hexanoic and octanoic acids and could interfere with their quantification. This was not a problem for de Jong and Badings (1990), probably because they were working with systems containing a much lower ratio of lactic acid to fat.

Recovery of lactic acid was reduced by limiting the volumes of solvent used in the separation and recovery of FFAs. The optimisation of the volumes of solvents, in this method 4 ml of chloroform/2-propanol and then 2% formic acid in diethyl ether (collecting the second, third and fourth ml), maximised recovery of FFAs, while minimising interference by lactic and benzoic acids. Compared to using 6 ml of the second solvent, the preferred method yields a solution with twice the FFAs and half the lactic acid concentration, an overall four-fold improvement in the ratio of FFA to lactic acid. This would be accompanied by a 38-fold improvement in the FFA to benzoate ratio, though this is not as important, since benzoate is seldom a major component.

The methodology developed to determine the FFA content of fermented emulsions proved to be both sensitive and reproducible. This is important because very

Table 4
Release of free fatty acid (FFA) (ppm) from media in a 6-h fermentation by *Streptococcus thermophilus*[a]

Carbon number	RSM + E (ppm)	RSM + E + AMF (ppm)	RSM + E + HPKO (ppm)	RSM + E + MO (ppm)	RSM + E + PO (ppm)
(a)					
C14:0	4.1±1.0	5.4±1.3	5.5±1.3	5.0±1.4	4.5±1.2
C16:0	136±10	149±17	144±15	164±13	160±9.8
C18:0	17±3.4	16±6.5	18.2±3.4	23±2.2	19±5.9
C18:1	0	2.1±1.3	2.7±0.4	3.7±1.5	4.7±1.8
C18:2	0.3±0.2	0.5±0.5	0.1± < 0.1	7.0±1.1	1.1±1.7
		AMF	HPKO	MO	PO
(b)					
C14:0		1.3	1.4	0.9	0.4
C16:0		13	12	28	24
C18:0		−1	1.2	6	2
C18:1		2.1	2.7	3.7	4.7
C 18:2		0.2	−0.2	6.7	0.8

[a] (a) Means and standard deviations based on three determinations. (b) Net release of FFA attributed to the fat source. RSM, reconstituted skimmed milk; E, emulsifier; AMF, anhydrous milk fat; HPKO, hydrogenated palm kernel oil; MO, maize oil; PO, palm oil.

Table 5
Analysis of fatty components of the emulsifier (means and standard deviations from six determinations)

Major components		Lipid fractions		Fatty acids (% of fatty component)		
				C14:0	C16:0	C18:0
Fat	90.5±2.62	Monoacylglycerols	80.8±1.7	3.2±0.4	43.7±0.4	33.9±0.3
		Diacylglycerols	16.8±2.3	1.8±0.1	8.1±0.1	6.9±0.03
		Triacylglycerols	2.3±0.5	0.53±0.04	0.94±0.03	0.82±0.03
		Free fatty acids	< 0.1		< 0.04	< 0.06
Stabiliser	7.9±2.5					
Moisture	1.6±0.06					

Table 6
Production of free fatty acids by *Streptococcus thermophilus* in emulsions containing either commercial emulsifier or equivalent amounts of monoacylglycerols (means and standard deviations for four replicates)

Monoacylglycerol source	% Addition	Free fatty acid liberated (ppm)		
		C14:0	C16:0	C18:0
Monomyristin	0.06	4.3±1.1		
Monopalmitin	0.08		131±18	
Monostearin	0.06			18±3.7
Emulsifier	0.2	3.5±1.1	130±5	17±2.0

low levels of short-chain FFAs can be implicated in flavour and flavour defects in milk-based foods, the effect depending upon the concentration. More general methods, based on simple titration of the total liberated fatty acids or their derivatives (Ikins et al., 1988), are incapable of detecting such subtle changes, the titres being predominantly based on the primary metabolite, lactic acid.

Investigation of the changes occurring during the fermentation of milk-based emulsions indicated that there was very little lipolytic attack on the fat itself. In most cases, the generation of the generated FFA was characteristic of the emulsifier and not of the type of fat, only the unsaturated FFA being associated with the triacylglycerols in the emulsions. Predominantly, the lipolysis was associated with the monoacylglycerol fraction of the emulsifier with hydrolysis of the ester linkage to C16:0 being preferred to C14:0 and particularly the C18:o in the monoacylglycerols, the C16:C18 FFA ratio being 1.3:1 in the emulsifier and 8–9:1 in the emulsions.

Preferential hydrolysis of the C16 monoacylglycerol may be due to a combination of preference for that monoacylglycerol plus the tendency for monoacylglycerols to be located at the lipid–water interface which will make them more prone to attack. The presence of traces of unsaturated FFAs would indicate a potential for hydrolysis of triacylglycerols, particularly on extended storage, when lysis of starter organisms could lead to release of intracellular lipases. While this should not be a major problem for short shelf-life products, problems may occur when products such as imitation cheeses are stored for maturation.

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