A Rapid Method for the Quantitative Determination of Short-Chain Free Volatile Fatty Acids from Cheese

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The determination of free volatile fatty acids (FVFA) is of interest in the analysis of cheeses. As these compounds are components of taste and flavor, they give indications on metabolic reactions taking place during cheese ripening and can provide an evaluation of cheese defects and their causes. One of the most widely used methods for the determination of FVFA in cheese involves preliminary recovery from the matrix by steam distillation, followed by gas chromatography separation. Relatively high distillate volumes must be collected to achieve a quantitative yield of all the compounds of interest, so that, as a result, the solution is too diluted to achieve good instrumental sensitivity. In this paper, an alternative method for the determination of C2−C6 free carboxylic acids in cheeses involving the use of a Nukol capillary column and crotonic acid as internal standard is described. This method is quick and cheap, as the sample preparation is a simple extraction with water. The underivatized FVFA are then directly separated by gas chromatography. Using this method, all FVFA in cheeses can be quantified with good repeatability and excellent recovery.

Keywords: *Cheese; free volatile fatty acids; gas chromatography*

INTRODUCTION

In cheeses, varying quantities of short-chain free fatty acids (C2−C6) can be found, originating from the fermentation of lactose, lipolysis, or amino acid degradation processes. Free volatile fatty acids (FVFAs) have a qualitative and quantitative profile that varies according to the degree and type of cheese maturation and can therefore give indications of some metabolic reactions taking place during maturation. The analytical determination of these components is also a useful parameter for the evaluation of the quality of specific types of cheese characterized by accentuated lipolysis or for spotting anomalous fermentations in other types of cheese (Chavarri et al., 1997). For example, as reported by Battistotti et al. (1993), the sensorial evaluation of provolone cheese is related to the ratio C4:C3. In ripened provolone cheese with good flavor, this shoud range between 2.5 and 4.

Most of the methods for determining FVFAs in cheese described in the literature comprise steam distillation starting from an aqueous acidified suspension of the sample (Ceccon et al., 1981; Parliment et al., 1982; Stancher et al., 1982; Battistotti et al., 1984; Ceccon, 1990). The main drawback of these methods is that, to obtain a quantitative yield of all the desired components, it is necessary to collect large amounts of distillate. The alkalinized distillate has to be concentrated back, and the fatty acids, freed from the corresponding salts by adding a strong acid, can be extracted in an adequate solvent for gas-chromatographic analysis. Moreover, according to some authors (Ceccon et al., 1981; Stancher et al., 1982; Ceccon et al., 1983), distillation speed and the recovery of volatile fatty acids can be affected, apart from the characteristics of the distillation apparatus, by some composition variables of the product (cheese salt and fat contents) and by the pH of the reaction environment. Finally, not all fatty acids comply with a linear law between the logarithm of the quantity collected and the volume of the corresponding distillate, and for some cheeses a deviation from this linearity has been observed, particularly for butyric and caproic acids. The distillation method, although performed under strictly monitored conditions to minimize possible sources of error can lead to wrong quantitative evaluations. It also takes long times and involves operational problems due to the high volumes with which it is necessary to work. The alternative proposed by some authors (Ceccon et al., 1983), which aims at minimizing such errors by the application of adequate correcting factors on subsequent volumes of collected distillate, entails further complication of the method. As an alternative to steam distillation, De Jong and Badings (1990) described a method requiring a preliminary extraction of fat from the cheese followed by an SPE separation of free fatty acids from neutral lipids. This preparative method was faster than the distillation method, since it requires about 50 min.

The aim of this work was to propose a new rapid procedure involving a simple water extraction and GC determination of FVFAs in cheese.

MATERIALS AND METHODS

Isolation of Free Volatile Fatty Acids. To 10 g of grated cheese were added 35 mL of water and 1 mL of internal standard solution (crotonic acid). This mixture was homogenized by a Politron (Kinematica, 6014 CH Lucerne, Switzerland) for 2 min and then centrifuged at 5000 rpm for 20 min to obtain an aqueous extract deprived of the triglycerides which floated and proteins which pelleted. This extract was acidified to pH 3−4 to free FVFAs present as salts. The FVFAs

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Table 1. Influence of the Volumetric Ratio Aqueous Extract:Ether on the Recovery of the FVFAs Added from an Aqueous Solution

	recovery (% at various aqueous extract:ether volumetric ratios			
FVFA	5 mL:1 mL	5 mL:2 mL	1 mL:1 mL	1 mL:2 mL
acetic acid	9	22	48	87
propionic acid	18	37	69	92
isobutyric acid	45	70	90	101
butyric acid	42	67	89	101
isovaleric acid	72	89	97	103
valeric acid	73	89	97	102
isocaproic acid	92	99	100	103
caproic acid	93	99	100	102

were then extracted from 1 mL of the acidified aqueous solution using 2 mL of diethyl ether, the GC determination of which was then taken. We also examined the possibility of directly injecting the acidified aqueous extract in the column. The procedure requires 30 min of work before GC injection.

Chromatographic Conditions. The quantitative analyses of FVFAs were achieved by high-resolution gas chromatography using a Varian model 3700 instrument (Varian, Sunnyvale, CA 94598) equipped with a split injector, a flame-ionization detector (FID) and a Hewlett-Packard 3396 A integrator (Hewlett-Packard, Palo Alto, CA 94304). A Nukol fused-silica wide-bore column (15 m × 0.53 mm i.d.) with a film thickness of 0.5 μm (Supelco, Bellefonte, PA 16823-0048) was employed. The carrier gas was helium with a flow rate of 25 mL/min, and the split ratio was 1:1. The oven temperature was maintained at 80 °C for 5 min and then increased to 180 °C at a rate of 8 °C/min. Injector and detector temperatures were 180 and 200 °C, respectively.

RESULTS AND DISCUSSION

Sample Preparation. The method assessed in this study consisted of a simple homogenization of cheese in an aqueous solution with an adequate internal standard (crotonic acid). Initially, the extraction was performed in a basic environment (adding sodium hydroxide until the phenolphthalein turns in color) with the aim of obtaining FVFAs in salt form. This prevented losses during the homogenization step. However, recovery tests performed on a standard mix of free fatty acids diluted in water showed that, in the absence of sodium hydroxide, even the most volatile free fatty acids are not lost in significant quantities during homogenization by Politron. Therefore, the precaution of salifying the free fatty acids to prevent volatilization of the lower molecular weight components proved unnecessary. The aqueous extract obtained from centrifuging had a pH of 5−6, and it was therefore necessary to acidify it to pH 3−4, so as to free FVFAs. A 1 mL sample of the acidified aqueous extract was then partitioned with 2 mL of diethyl ether. An attempt to concentrate back the aqueous extract (in a vacuum oven at 75 °C), to increase sensitivity, created problems such as caramelizing of the residue (owing to the presence of sugars and residues with amino groups in the extract) and consequent difficulties in redissolving the dried residue. Similar problems of dissolving the dried residue were also noticed when the extract was evaporated by rotavapor after an alcohol was added to form an azeotrope.

Effect of the Volumetric Ratio Aqueous Extract: Ether on Partitioning. In the liquid−liquid partition step, the volumetric ratio between the aqueous extract and diethyl ether proved to be crucial for obtaining quantitative recoveries of FVFAs, particularly those with shorter chains. Table 1 shows the recovery percentages of the single FVFAs, obtained by subjecting a standard mixed aqueous solution to partitioning with different ether volumes. It can be seen that only the aqueous extract:ether ratio of 1:2 proved adequate for obtaining quantitative yields also for the most water-soluble fatty acids such as acetic and propionic acids. FVFA recoveries, especially for acetic acid and propionic acid, can be improved by saturating the aqueous extract with NaCl. In this way, when an increased sensitivity is needed, we can use a larger aqueous extract:diethyl ether ratio.

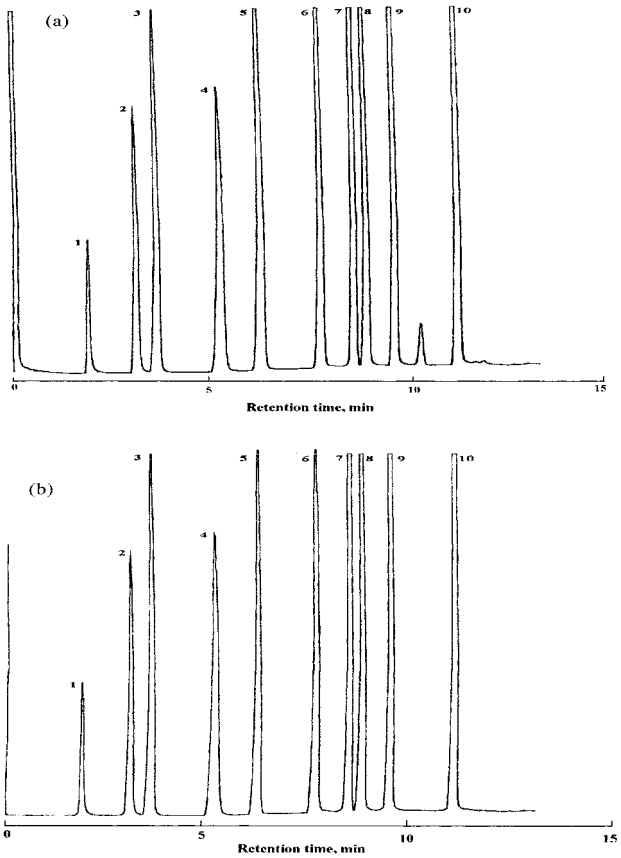

Figure 1. GC chromatograms of free volatile fatty acids: (a) injection of the ether extract after water:ether repartition; (b) direct injection of the acid aqueous extract; (1) acetic acid; (2) propionic acid; (3) isobutyric acid; (4) butyric acid; (5) isovaleric acid; (6) valeric acid; (7) crotonic acid (internal standard); (8) isocaproic acid; (9) caproic acid; (10) heptanoic acid.

As an alternative to partitioning with ether, the aqueous extract could be injected directly into the column. The fused-silica column used in this experiment had a good tolerance of the introduction of small quantities of water, and the presence of salts, amines, and amino acids in the extract did not seem to be a problem for injection: the gas-chromatographic trace appeared to be free of interfering peaks (Figure 1). The drawback of direct injection of the aqueous extract is that it was necessary to frequently clean the insert from salts and heavy components which did not vaporize. Moreover, it remains to be verified whether the column undergoes a more rapid deterioration and loss of efficiency under such conditions of use.

Recovery. The method described above enabled us to analyze short-chain (C2 − C6) free fatty acids, as those with longer chains were too apolar to remain in the aqueous extract and were incorporated into the separated fat during centrifuging.

Table 2. Recovery of the Individual FVFAs Added to Montasio Cheese[a]

FVFA	amount determined in sample (mg/100 g)	amount of standard added (mg/100 g)	amount found in sample after addition (mg/100 g)				recovery (%)	
			1	2	3	mean	mean	CV
acetic acid	3.62	12.02	16.95	15.98	14.70	15.87	102	5.8
propionic acid	0.81	14.82	19.24	19.03	18.51	18.93	122	1.6
isobutyric acid	0.50	17.62	21.35	21.57	21.96	21.63	119	1.2
butyric acid	5.58	17.62	25.09	24.63	26.14	29.29	112	2.5
isovaleric acid	0.83	20.42	22.66	22.57	22.92	22.72	107	0.7
valeric acid	0.47	20.42	21.56	20.62	21.05	21.08	101	1.8
isocaproic acid	0.00	23.24	23.17	22.81	23.12	23.03	99	0.7
caproic acid	2.49	23.24	23.36	22.58	21.92	22.62	87	2.6

[a] The internal standard was crotonic acid. The results are the means of three replicate analyses of the same sample. CV = coefficient of variation.

Table 3. Analytical Repeatability in FVFAs Determination[a]

FVFA	1	2	3	4	5	6	average (mg/100 g)	SD	CV (%)
acetic acid	419.9	412.2	394.22	388.7	447.3	391.6	409.0	22.5	5.5
propionic acid	670.5	660.8	685.15	660.9	703.6	657.5	673.1	18.1	2.7
isobutyric acid	traces	traces	traces	traces	traces	traces	traces		
butyric acid	125.2	123.6	128.43	126.5	128.8	124.7	126.2	2.1	1.7
isovaleric acid	2.9	2.9	3.0	3.2	3.1	2.9	3.0	0.1	4.2
valeric acid	1.3	1.2	1.3	1.3	1.4	1.3	1.3	0.1	5.4
isocaproic acid	traces	traces	traces	traces	traces	traces	traces		
caproic acid	4.5	4.8	4.2	4.5	4.5	4.5	4.5	0.2	4.0

[a] Results are the means of six replicate analyses of the same sample. SD = standard deviation. CV = coefficient of variation.

The free fatty acids showed, as the length of their hydrocarbon chain increases, a growing affinity to fat, and it was therefore important to choose an internal standard with a behavior as similar as possible to that of the fatty acids studied. At first, we chose heptanoic acid (C7), which proved inadequate for the purpose. In fact, heptanoic acid was too apolar when compared to FVFAs, and some passed into the fat, leading to estimates of the recoveries much higher than 100%. The situation improved significantly when a more polar fatty acid such as crotonic acid (C4:1) was used as internal standard. Table 2 shows the recoveries obtained for the single volatile fatty acids with crotonic acid as internal standard. This recovery test was performed in triplicate, by adding a known quantity of FVFAs to a sample of Montasio cheese. Recovery was calculated by comparing the peak area of FVFAs present in the Montasio sample with that from the fortified sample. The recoveries showed a decreasing trend related to an increasing apolarity of the fatty acids, and consequently, the fatty acids more polar than crotonic acid have recoveries higher than 100%. However, this phenomenon was more contained than that recorded for heptanoic acid. Anyway, it is sufficient to correct the gross data for the relevant recoveries.

Repeatability. The repeatability of the method was tested on a single sample of Emmenthal cheese by replicating the entire analysis six times. Average contents (mg/100 g) and coefficients of variation (CVs, %) are reported in Table 3. The results showed a good repeatability.

In conclusion, the method here proposed proved valid in determining FVFAs (C2−C6) and was quick and cheap as sample preparation was a simple extraction in water.

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