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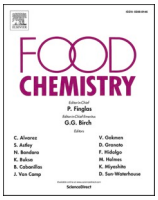

Validated method for the determination of free volatile carboxylic acids in cheese and bacterial cultures by GC-FID after esterification in aqueous extract and headspace injection

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ABSTRACT

A simple, rapid, sensitive and robust gas chromatographic method was developed for the simultaneous determination of free volatile carboxylic acids (FVCA) in cheese and bacterial cultures. The target analytes were extracted and converted directly from the aqueous phase to their ethyl esters using headspace. The lower detection limits for the volatile carboxylic acids in the cheese samples were less than 0.3 and less than 0.6 μmol kg^{-1} in the bacterial culture samples. The lower limits of quantitation in cheese were better than 0.001 mmol kg^{-1} for all analytes. The upper limits of quantitation varied from 39 to 136 mmol kg^{-1} in cheese and 78 to 272 mmol kg^{-1} in bacterial cultures depending on the analyte. The Horwitz ratio showed good precision for all analytes (less than 0.77). The proposed method is suitable for the determination of target metabolites directly from aqueous extracts and can also be validated for other matrices.

1. Introduction

Free volatile carboxylic acids (FVCA) such as those unbranched from C1 to C6, and isoforms of butyric, valeric and caproic acids, are formed during cheese ripening as desired and also as undesired metabolites. Although in the literature these acids are often also referred to as free volatile fatty acids, strictly speaking only applies to butyric acid and *n*-caproic acid which compose milk fat. All these compounds are formed by hydrolysis of milk fat and by lactate and protein, such as during heterofermentative lactic acid fermentation or amino acid deamination. In most cases, all mechanisms can be observed during cheese ripening to a greater or lesser extent depending on the type of cheese. In addition, the presence of undesirable microorganisms can lead to unintended fermentations in which FVCAs are formed in sometimes high concentrations (Fox, Guinee, Cogan, & McSweeney, 2017).

On the other hand, these compounds are contributors to the typical and desirable flavors of many cheeses. For example, the conversion of lactate into propionic acid by propionibacteria is a known metabolic pathway for the formation of the typical propionic acid flavor in Emmental cheese (Fröhlich-Wyder, Bisig, Guggisberg, Jakob, Turgay, & Wechsler, 2017).

The quantitative composition of FVCA is an important quality characteristic of a cheese. Variations in the composition indicate disturbances during ripening and are very helpful in the search for manufacturing defects. For example, *Obligat heterofermentative lactobacilli* form acetate and *Faktulativ heterofermentative lactobacilli* form formate and acetate (Fröhlich-Wyder, Guggisberg, Badertscher, Wechsler, Wittwer, & Irmler, 2013). In addition to propionate, propionic acid bacteria can also form acetate (Fröhlich-Wyder et al., 2017) while *Coliform* bacteria form lactate, formate and acetate (Eugster, Jakob, & Wechsler, 2012). An early butyric acid fermentation is observed with *Clostridium butyricum* or as late bloating with *Clostridium tyrobutyricum*. The Putrifikus (white rot) is caused by *Clostridium sporogenes* spores, which produces a whole spectrum of carboxylic acids such as acetate, propionate, butyrate, isovalerate, isobutyrate and isocaproate through their proteolytic activities (Gómez-Torres, Garde, Peirotén, & Ávila, 2015). Similarly, an atypical butyric fermentation with little gas (H_2 and CO_2) and butyric acid is induced by *Clostridium beijerinckii* (Klijn, Nieuwenhof, Hoolwerf, van der Waals, & Weerkamp, 1995). Certain yeasts can produce acetate at an early stage of ripening and cause undesirable eye formation.

The new method is based on an unpublished in-house development that has been continuously improved. In the previous method, 20 g of cheese was first distilled in an acidic medium with steam and the

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distillate titrated with NaOH to determine the total acidity. Subsequently, 1 mL of the over-titrated solution was esterified and the relative concentrations of each FVCA were determined by headspace injection on a GC-FID. Together with the total acidity, the individual absolute contents could then be calculated. Over the years, various optimizations have been made to improve recoveries and reduce workload. Initially, propanol was used as the esterification reagent. Later, due to blank value problems, a switch was made to ethanol. The optimal esterification and headspace conditions (time, temperature) were systematically determined. For the switch from steam distillation to basic extraction, the amount of ethanol used for esterification as well as different sodium hydroxide concentrations and extraction times were optimized. Special attention was paid to avoid possible saponification of the milk fat.

2. Materials and methods

2.1. Reagents

Formic acid (≥98 %); acetic acid (glacial 100 %); propionic acid (≥99.5 %); isobutanoic acid (2-methylpropanoic acid) (≥99.5 %); *n*-butanoic acid (≥99 %); isovaleric acid (3-methylbutanoic acid) (≥99 %); isocaproic acid (4-methylpentanoic acid) (≥99 %); *n*-caproic acid (≥98 %); 3,3-dimethylbutanoic acid (≥98 %); 2,2-dimethylpropanoic acid (≥99 %); zinc sulfate heptahydrate (≥99.5 %); hydrochloric acid fuming (37 %); sodium hydroxide solution (c = 2 mol/L) and ethanol absolute (≥99.5 %) were obtained from Merck (Darmstadt, Germany).

2.2. Samples

Hard and semi-hard Swiss cheese samples (Appenzell (n = 3), Emmental (n = 38), Gruyère (n = 19), Sbrinz (n = 4), Raclette (n = 8) and alp cheeses (n = 9) comes from our experimental cheese factory or were bought at local groceries in Bern, Switzerland.

Propionic acid bacteria are used for the manufacture of Emmental and Swiss cheeses. Therefore, the "propionic" cultures Prop 01 and Prop 96 are produced and sold by the Liebefeld Kulturen AG (Bern, Switzerland, http://www.liebefeld-kulturen.ch/). It is part of the quality assurance to determine the ratio of propionic acid to acetic acid content in these cultures after production. For the present study, 41 of these propionic cultures were analyzed.

2.2.1. Sample preparation

Cheese: 4.0 ± 0.1 g of grated cheese was weighed to the nearest 0.001 g in a 100 mL beaker and 20 mL deionized water was added. After addition of 1.0 mL internal standard solution (400 mg each of 2,2-dimethylpropanoic acid and 3,3-dimethylbutanoic acid added to 20 mL NaOH (c = 2 mol/L) and diluted to 100 mL with deionized water), the mixture was homogenized using a Polytron PT1300 D disperser (Kinematica AG, Malters, Switzerland) at 20′000 rpm for 30 s. Subsequently 1 mL zinc sulfate solution (zinc sulfate heptahydrate in deionized water at the final concentration of 0.3 g mL^{-1}) was added, mixed again briefly and then the suspension was filtered and clarified through a Whatman™ filter paper 589/3 into a 50 mL Erlenmeyer flask.

Cultures: 2.0 ± 0.1 g of culture solution are weighed to the nearest 0.001 g in a 100 mL beaker and 22 mL deionized water was added. After addition of 1.0 mL internal standard solution, the mixture was homogenized using a Polytron PT1300 D disperser (8′000 rpm, 30 s). Next, 1 mL zinc sulfate solution was added to the solution, before being briefly mixed again followed by filtering and clarification of the suspension through a Whatman™ filter paper into a 50 mL Erlenmeyer flask.

For the blank samples, deionized water was used instead of the sample material.

2.2.2. Esterification and instrumental conditions

To determine the free volatile carboxylic acids (FVCAs), the same esterification procedure was used for both sample types. A sample of 1 mL filtrate was transferred to a 20 mL headspace vial and 200 μL each of HCl 10 % and ethanol were added. The tightly capped vials were incubated at 95 °C for 3 h. Thereafter, 1 mL of the headspace was analyzed with an Agilent 8890 gas chromatograph (Agilent Technologies, Basel, Switzerland) equipped with a Agilent HP-5 cross-linked phenyl methyl silicone fused silica capillary column (50 m × 0.32 mm × 0.52 μm), a flame ionisation detector (FID) and a PAL3 autosampler (Agilent Technologies, Basel, Switzerland) in headspace mode. Injection was performed into a split/splitless injector in split mode (30:1) using helium as the carrier gas at a constant column flow rate of 1.4 mL min^{-1}. The GC temperature program started at 40 °C (4 min) then progressed at a rate of 8 °C min^{-1} to 144 °C followed by a rate of 30 °C min^{-1} to 279 °C (0.5 min). The temperatures of the injection port and detector were 110 °C and 320 °C. The Agilent OpenLab software version 3.2 supported the system and was also used for data processing. Results are reported in mmol kg^{-1} for the samples and in μg mL^{-1} for the control solutions.

2.2.3. Calibration procedure

A stock solution of all FVCAs was prepared by weighing 200 mg of each acid in 20 mL NaOH (c = 2 mol/L) and then diluting to 100.0 mL with deionized water. As an internal standard, 400 mg of 2,2-dimethylpropanoic acid and 3,3-dimethylbutanoic acid were weighed into 20 mL NaOH (c = 2 mol/L) and diluted to 100 mL with deionized water. For external calibration, a reference solution with a concentration of 200 μg mL^{-1} for each carboxylic acid was prepared by mixing 10.0 mL of stock solution FVCA with 10.0 mL of the internal standard solution and diluting to 100.0 mL with deionized water. A sample of 1 mL of the reference solution was esterified and measured like a sample at the beginning and end of each series. The measured retention times of each carboxylic acid was used for identification and the peak areas were required for a one-point calibration. For this purpose, the peak areas of the first five acids (C1, C2, C3, i-C4, n-C4) were normalized with the internal standard 2,2-dimethylpropanoic acid and the remaining acids (i-C5, i-C6 and n-C6) with the second internal standard 3,3-dimethylbutyric acid.

The mean values of the normalized peak areas of the external standard measurements at the beginning and end of a measurement series, including the zero point, result in a linear calibration function for each acid.

2.2.4. Spiking

For the determination of the recovery rates in cheese samples, 1 mL of the stock solution was added directly to the cheese samples.

2.2.5. Control solutions

For monitoring each series of measurements, 2 control solutions with a concentration for each carboxylic acid of 10 μg mL^{-1} and 40 μg mL^{-1} were prepared by mixing 500 μL. and 2000 μL, respectively, of the stock solution FVCA with 10.0 mL of the internal standard solution and diluting to 100.0 mL with deionized water. At the beginning and end of each series, 1 mL of the control solutions was esterified and measured.

3. Results

3.1. Cheese and bacterial cultures analysis

All FVCAs were detected in cheese and bacterial cultures with good resolution by the proposed method.

Fig. 1 shows typical chromatograms for target analytes in their ethyl ester form after analysis by the proposed method. Fig. 1A shows a standard calibration sample containing 200 μg mL^{-1} of each of the carboxylic acids. The chromatogram of a typical Emmental cheese shows the characteristically high concentrations of acetic acid and propionic acid (Fig. 1B). A similar chromatogram is also obtained when analyzing a propionic acid culture (Fig. 1C).

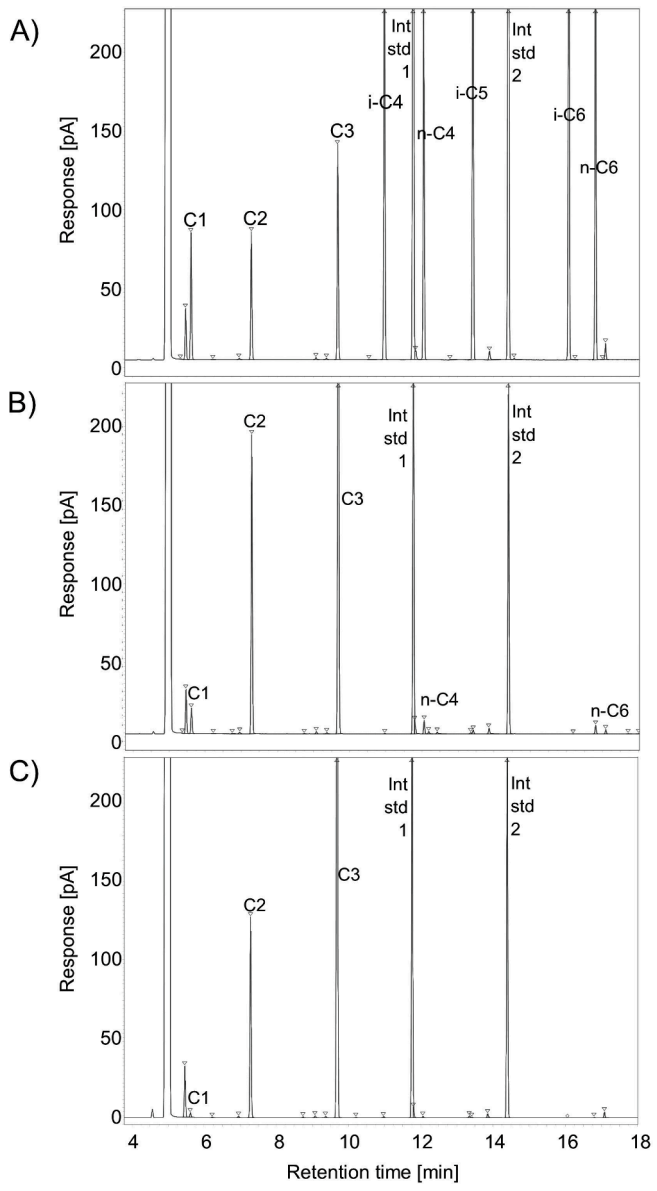

Fig. 1. Chromatogram of the free volatile carboxylic acids in their ethyl ester form of a standard calibration sample (A) containing 200 µg mL^{-1} each of formic acid (C1), acetic acid (C2), propionic acid (C3), isobutyric acid (i-C4), *n*-butyric acid (n-C4), isovaleric acid (i-C5), isocaproic acid (i-C6), *n*-caproic acid (n-C6), and 400 µg mL^{-1} each of 2,2-dimethylpropanoic acid (Int std 1) and 3,3-dimethylbutanoic acid (Int std 2), hard cheese sample (B) and bacteria sample (C).

3.2. Method validation

For the validation, the similar quality parameters were determined as for the method for the determination of the diols (Badertscher, Freiburghaus, Wechsler, & Irmler, 2017). The trueness, specificity, precision, limit of detection (LOD) and upper and lower limit of quantification (LOQ) were determined.

3.2.1. Trueness

Recovery measurements were performed with Sbrinz, Appenzeller and Gruyère cheeses. Each cheese was measured 10 times with and without spiking. As shown in Table 1, the recoveries obtained ranged from 90 % to 109 % across the cheeses measured.

The trueness was additionally checked by recovery measurements in two control solutions at 10 and 40 µg mL^{-1}. As shown in Table 2, recoveries for each analyte ranged from 92 % to 96 % for the 10 µg mL^{-1} control solution (n = 166) and from 99 % to 101 % for the 40 µg mL^{-1} control solution (n = 166).

3.2.2. Specificity

Potential interferences were assessed by analyzing calibration, blank and control solution samples. No interfering peaks were observed in the respective GC-FID chromatograms (Fig. 1A, B and C).

3.2.3. Precision

The data in Table 3 demonstrate a high precision of the presented method. This was demonstrated with a Horwitz ratio (HORRATr) of less than 0.77 for all analytes (Horwitz & Albert, 2006).

3.2.4. Limits of detection (LOD) and limits of quantification (LOQ)

The limits of detection (LOD) corresponded to the amount of analytes required to achieve a signal-to-noise (S/N) ratio of 3. The lower LOQs corresponded to a S/N ratio of 10. The peak area of a peak three times higher than the average noise was used to calculate the detection limits for each acid. The lower LOQs were calculated using the area of a peak

Table 2
Recovery rate of free volatile carboxylic acids in control solutions.

Control solution	Control 1 (10 µg mL^{-1}) (n = 166)		Control 2 (40 µg mL^{-1}) (n = 166)	
FVCA	Mean (µg mL^{-1})	Recovery ± SD (%)	Mean (µg mL^{-1})	Recovery ± SD (%)
C1	11.3	94 ± 3	47.8	100 ± 4
C2	9.7	92 ± 2	41.8	99 ± 4
C3	10.1	94 ± 2	42.5	100 ± 4
i-C4	10.3	96 ± 1	42.9	101 ± 4
n-C4	10.6	96 ± 2	44.3	101 ± 5
i-C5	11.4	96 ± 2	47.6	100 ± 4
i-C6	10.5	96 ± 2	43.7	100 ± 5
n-C6	10.5	96 ± 3	43.7	100 ± 5

Table 1
Recovery rate of free volatile carboxylic acids in different types of cheese.

Cheese		Sbrinz (n = 10)		Appenzeller (n = 10)		Gruyère (n = 10)		Mean (n = 3)
FVCA	Added level (mmol kg^{-1})	Sample (mmol kg^{-1})	Recovery (%)	Sample (mmol kg^{-1})	Recovery (%)	Sample (mmol kg^{-1})	Recovery (%)	Recovery ± SD (%)[a]
C1	13.29	0.70	109	1.91	106	1.82	110	108 ± 2
C2	8.44	9.90	96	9.39	83	16.35	92	90 ± 7
C3	7.12	0.70	109	0.80	107	0.45	110	109 ± 2
i-C4	5.23	0.00	108	0.86	105	0.16	110	108 ± 3
n-C4	5.45	1.05	108	0.19	106	1.57	108	107 ± 1
i-C5	4.53	<0.01	107	2.04	104	0.18	108	106 ± 2
i-C6	3.79	0.00	103	0.00	100	0.00	99	101 ± 2
n-C6	4.00	0.26	105	0.00	95	0.05	98	99 ± 5

Mean and standard deviation of 3 types of cheese.

Table 3
Precision data of the FVCAs at different levels in cheese and bacterial cultures.

Cheese	max. Level (mmol kg^{-1})	*n* Samples	RSD_r (%)	Repeatability *r* (mmol kg^{-1})	$HORRAT_r$
C1	9	78	1.5	0.4	0.35
C2	68	78	1.4	2.7	0.48
C3	100	78	0.9	2.5	0.31
i-C4	1	74	0.3	0.01	0.05
n-C4	10	78	0.4	0.1	0.11
i-C5	1	78	0.9	0.02	0.16
i-C6	0.4	42	0.8	0.01	0.14
n-C6	1	78	1.0	0.02	0.19
Bacterial cultures	**max. Level (mmol kg^{-1})**	***n* Samples**	**RSD_r (%)**	**Repeatability *r* (mmol kg^{-1})**	**$HORRAT_r$**
C1	4	41	3.7	0.4	0.77
C2	83	41	1.1	2.6	0.37
C3	164	41	0.7	3.4	0.28
i-C4	0.1	41	3.7	0.01	0.48
n-C4	0.17	41	3.1	0.01	0.44
i-C5	0.07	41	4.2	0.01	0.52
i-C6		0			
n-C6		0			

Precision is expressed as relative standard deviation (RSD_r) and repeatability values (r), calculated from duplicate measurements of real samples and obtained under repeatable conditions.

10 times higher than the average noise level. The upper LOQs for the given validation data were calculated using the peak areas from the measurement of a solution containing 600 μg mL^{-1} of each analyte (Fig. 2). Up to this concentration, linearity is still given for all analytes with a coefficient of determination $R^2 > 0.99$.

As shown in Table 4, the obtained limits confirm the suitability of the proposed method for the quantitative determination of these FVCAs over a wide measurement range of concentrations in cheese samples. This also applies to the extended limit values of the bacterial culture samples. Since the sample weight is only half as large for bacterial cultures, the limits must be extended by a factor of 2 of the cheese data.

4. Discussion

Many different approaches have been taken to determine free volatile carboxylic acids (FVCAs) in cheese and cultures samples. In the early 1980s, FVFAs in cheese were mostly determined by steam distillation followed by extraction and gas chromatography (Parliment, Kolor, & Rizzo, 1982). However, these methods have been continuously developed to improve the measurement sensitivity and additionally to

Table 4
LOD and LOQ of the FVCAs at different levels in cheese and bacterial cultures.

Cheese (n = 10)[a]	LOD		Lower LOQ		Upper LOQ	
	(μmol kg^{-1})	(μg kg^{-1})	(μmol kg^{-1})	(μg kg^{-1})	(mmol kg^{-1})	(mg kg^{-1})
C1	0.29	13.5	0.98	45.0	136	6270
C2	0.19	11.4	0.63	37.8	86	5160
C3	0.10	7.6	0.34	25.2	73	5370
i-C4	0.04	3.4	0.13	11.3	54	4730
n-C4	0.05	4.3	0.16	14.3	55	4890
i-C5	0.03	3.0	0.10	10.1	46	4710
i-C6	0.03	3.2	0.09	10.8	39	4510
n-C6	0.03	3.4	0.10	11.4	41	4770

[a] The limits for bacterial cultures must be extended by a factor of 2.

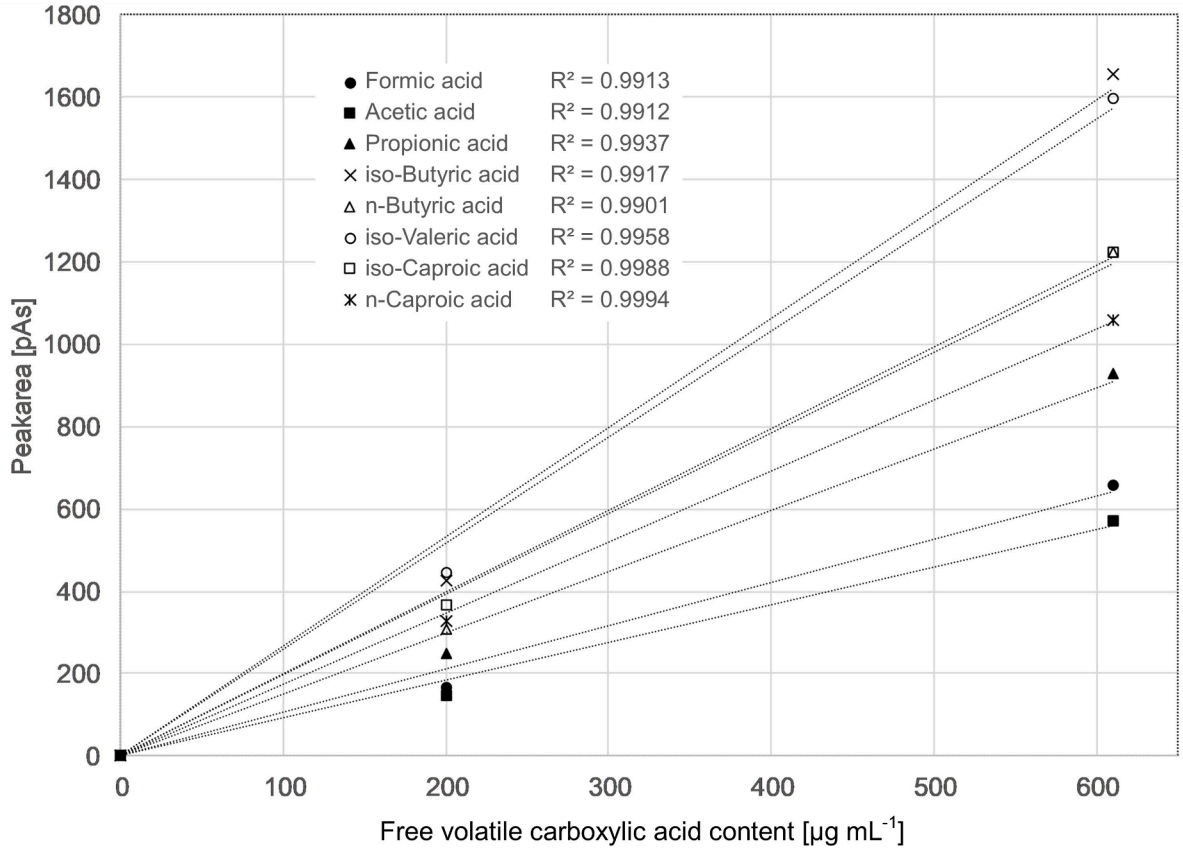

Fig. 2. Linearity test for the free volatile carboxylic acids in their ethyl ester form.

simplify the pretreatment of the samples. For example, volatile free carboxylic acids were extracted from the fat with apolar solvents and pre-purified with various adsorbents. This is often followed by additional purification steps such as simultaneous distillation or solid phase extraction. The isolated fatty acids can then be esterified before separation by gas chromatography on polar or nonpolar capillary columns and determined by FID or TCD (Attaie & Richter, 1995; De Jong & Badings, 1990; Kim & Lindsay, 1990). A rapid and inexpensive method involving a simple extraction of the FVCAs (C2 to C6) with water was presented by (Innocente, Moret, Corradini, & Conte, 2000). The acids are determined directly after extraction with diethyl ether on a gas chromatograph with FID. The recoveries for Montasio cheese samples ranged from 87 to 122 % and the coefficients of variation for a 6-fold determination in the same matrix were between 1.7 and 5.5 %. The LOD and LOQ were not described. The profile of C2-C12 volatile acids of a Spanish soft cheese during ripening by SPME-GC–MS was described without validation data by (Delgado, González-Crespo, Cava, García-Parra, & Ramírez, 2010). Among other volatile components, (Berard, Bianchi, Careri, Chatel, Mangia, & Musci, 2007) used the dynamic headspace extraction technique in conjunction with gas chromatography-mass spectrometry to determine the free carboxylic acids from C2 to C18:1 in 24 samples of "Fontina Valle d'Aosta", an Italian semi-hard cheese, with an RSD of less than 5 %. A method for the determination of free fatty acids (FFA) as ethyl esters was presented by (Perotti, Bernal, Meinardi, & Zalazar, 2005). In this method, the FFA (C6:0 to C18:2) from Reggianito Argentino cheese samples were extracted aqueous at different ripening times, converted into their sodium salts, dried and subsequently esterified with an ethanol-sulfuric acid mixture and extracted with hexane. The extract was analyzed by GC-FID. Validation data were not reported. A method for the quantification of volatile free fatty acids (C2-C7) in cheese using a multiple headspace solid-phase microextraction (MHS-SPME-GC-FID) was published by (Rincon, Pino, Ayala, & Afonso, 2014). The LOD and LOQ values for each acid ranged from 0.007 to 0.043 mg kg^{-1} and 0.018 to 0.109 mg kg^{-1}, respectively (Berard et al., 2007). (Kim, Kwon, Choi, & Ahn, 2019) report a quantitative determination of FVCAs (C2, C3, C4 and C5) in microbial samples. After extraction of FVCAs with ethyl ether-hexane (1:1, v/v) followed by aminopropyl solid phase extraction (SPE), the acids were analyzed by GC-FID. The limits of quantification (LOQs) of the analytical method ranged from 5.71 to 11.20 μg mL^{-1}. Total recoveries excluding acetic acid ranged from 96.51 to 108.83 % with relative standard deviations (RSDs) of less than 10 %.

In the present work, volatile carboxylic acids were acid-catalyzed esterified directly in the aqueous solution. By distributing the volatile apolar esters into the headspace, the ester yield could be improved. The esterification of formic acid also allowed sufficiently sensitive measurement with FID. The ethyl esters of the FVCAs separated well on an inert 5 % phenylmethylsilicone capillary column. The described procedure could be extended to a reference method by using isotopically labelled target components using a GC–MS. Initial experiments with C13- or D-labelled acetic and propionic acids have been successfully performed. The method should be applicable to other matrices as long as the FVCA can be extracted with a weakly basic aqueous solution.

5. Conclusion

The present work describes a new method for the simultaneous quantitative determination of free volatile carboxylic acids (FVCA) in cheese and bacterial cultures. The method is simple to apply and very robust and specific due to two selective separation procedures. After a weakly basic aqueous extraction, the esterification step is performed with ethanol new directly from the aqueous phase in a headspace vial. The ethyl esters thus formed are determined by gas chromatography and flame ionization detection. The method was optimized and validated for eight target analytes. The validation parameters of trueness, specificity, precision, LOD, and upper and lower LOQ demonstrate that the method is suitable for accurate quantification over a wide range of measurements in cheese and bacterial culture samples.

The method is successfully applied to real samples in practice. There is no reason not to use the method for other sample matrices. Additional validation work is required to confirm the latter.

CRediT authorship contribution statement

René Badertscher: Conceptualization, Methodology, Validation, Writing – original draft. **Carola Blaser:** Investigation, Writing – review & editing. **Priska Noth:** Investigation, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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