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Detection and Prediction of Hydrolytic Rancidity in Milk by Multiple Regression Analysis of Short-Chain Free Fatty Acids Determined by Solid Phase Microextraction Gas Chromatography and Quantitative Flavor Intensity Assessment

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The objective was to establish a method for detecting and predicting hydrolytic rancidity in milk by correlating quantitative sensory data with individual short-chain free fatty acids (FFA) (C_4−C_{12}) in milk determined by solid phase microextraction and gas chromatography (SPME-GC). A FFA-based equation for determining rancid flavor intensities in milk was derived by stepwise regression analysis. A highly significant ($p < 0.001$) correlation coefficient (R^2) of 0.84 indicated that rancidity scores were dependent on FFA obtained by SPME-GC and that a good proportion of the variation in the rancidity scores was explained by the model. When rancidity scores were predicted for 19 commercial milks, one sample was found to be distinctly rancid by the statistical model and by the trained sensory panel. The rest of the samples were found to be nonrancid by either method. Thus, the predicting power of the model was shown because there was 100% correct flavor classification for the samples tested.

KEYWORDS: Solid phase-microextraction gas chromatography; short-chain free fatty acids; milk; rancid flavor

INTRODUCTION

Off-flavors in dairy products that are caused by the release of free fatty acids (FFA) from milk through the action of lipases are known as hydrolytic rancidity or lipolyzed flavors (*1*). Individual FFA concentrations were correlated to quantitative sensory data for the detection and prediction of hydrolytic rancidity off-flavors in butters (*1*). Similarly, the relationships among acid degree values (ADV), FFA concentrations, and sensory perceptions were established for the detection of rancid flavor in milk (*2*). Lipases, which may be inherent in milk or produced by psychrotrophic microorganisms, liberate FFA from glycerides. Increased concentration of FFA contributes to a rancid, bitter, unpleasant taste in milk that is objectionable to many consumers. FFA of shorter chain length, primarily C_4−C_{12}, are important in characterizing rancid flavor but longer chain FFA (C_{14}−$C_{18:1}$) are not associated with rancidity (*2*).

Milk flavor is so bland and mild that the presence of any off-flavor can easily overshadow its pleasant, slightly sweet flavor. Off-flavors directly affect consumer acceptance and enjoyment of milk. To avoid the occurrence of off-flavors in milk before it reaches the consumer, adequate testing procedures are needed. These methods must measure the flavor precursor, flavor component, or causative agent before the flavor has developed. After correlation with sensory analysis, these analytical methods can be used as predictive measures of off-flavor development (*3*). It is generally recognized that although analytical data can provide very useful information, sensory data are required for the importance of the analytical measurements in flavor research to be established. Indeed, what the researcher wants to know in the correlation of analytical and sensory data is the cause−effect relationship between a group of components and sensory qualities (*4*).

Rancid flavor in milk is frequently determined by the acid degree value (ADV). However, the accuracy of the ADV in predicting sensory detection of rancidity was questioned (*2*, *3*). ADV is frequently used to detect rancidity with no concurrent sensory evaluation of milk samples. If the ADV is high, the milk sample may be discarded, although it may not have a detectable rancid flavor (*2*). Apparently, the reason for this disparity in the relationship between ADV and prediction of rancid flavor intensity is the difference in solubility of the FFA in milk (*3*). The ADV does not recover the short-chain FFA (C_4−C_{12}) in the fat separation process and only partially recovers the medium-chain FFA (C_{10}−C_{16}) (*3*). Those fatty acids that are implicated in rancid flavor, C_4−C_{12}, are hydrophilic and, therefore, remain in the aqueous phase of the milk (*3*). Thus, the ADV is measuring only fat-soluble FFA, which remain in the fat during the separation procedure (*3*). A high ADV may reflect a change in concentration in long-chain fat-soluble FFA

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but does not necessarily indicate an increase in concentration of the volatile, flavorful short-chain FFA (*3*). Therefore, the ADV is not always a good measure of rancid flavor development because it does not detect the shor-chain FFA (C_4-C_{12}) responsible for the off-flavor (*2*, *3*, *5*).

Thus, several methods were reported for the quantitative determination of individual short-chain FFA (*6*−*11*). However, statistical approaches have been lacking in attempts to correlate FFA analytical data and rancid flavor in milk. The objective of this research was to establish an analytical system for detecting and predicting hydrolytic rancidity in milk by correlating quantitative sensory data with individual short-chain FFA (C_4-C_{12}) in milk by using stepwise multiple regression analysis. A rapid method for the quantitation of FFA using solid phase microextraction and gas chromatography (SPME-GC), which had been shown to provide the sensitivity, accuracy, and precision desired for discriminating between fresh and rancid milk, was used (*6*).

MATERIALS AND METHODS

Reagents. Free fatty acid standards (butanoic, hexanoic, octanoic, decanoic, dodecanoic, tetradecanoic, and hexadecanoic) were purchased from PolyScience, Co. (Niles, IL).

Collection of Samples. Raw milk was collected on two different occasions from a local dairy farm and stored at 4 °C until processed. Each of the raw milks was combined with pasteurized homogenized milk to prepare two different batches of laboratory-prepared rancid milk (LPRM). Batch to batch variability of LPRM was desired in order to have a wide range of rancid samples. High-quality fresh pasteurized homogenized milk was collected 2 days after processing or 12 days from the due date, purchased from the local market, and stored at 4 °C. Nineteen commercial pasteurized homogenized milk samples containing 3% milkfat, from four different dairies, were collected before the due date from the local market and kept at 4 °C.

Preparation of Rancid Milk Samples. Two different batches of LPRM were prepared as described by ref *12* with modifications. Raw and fresh pasteurized homogenized milk were mixed (1:1) and incubated at 5 °C for 24 h to allow rancidity to develop. LPRM was batch-pasteurized at 66 °C for 35 min and cooled rapidly. Subsamples of the LPRM were frozen in separate vials until use for analysis. Samples with different rancidity intensities were prepared by mixing aliquots of LPRM with high-quality fresh pasteurized homogenized milk (5:95, 10:90, 15:85, 20:80, 25:75, 30:70, 35:65, 40:60, 45:55, and 50:50). Mixtures were prepared as required for sensory and analytical testing. A total of 40 rancid samples were prepared by mixing the two different batches of lipolyzed LPRM and high-quality fresh pasteurized homogenized milk collected on four different occasions. Additionally, six more samples of high-quality fresh pasteurized homogenized milk were analyzed. Thus, a total of 46 samples were subjected to chemical and sensory analysis.

Sensory Evaluation. First, 12 panelists were selected from 25 interviewed individuals on the basis of their habits; they had to be nonsmokers and regular milk consumers. Five panelists of 12 initial judges were selected by using sequential sampling (*13*). The judges were four females and one male between 25 and 46 years of age. By applying sequential analysis, panelists' sensitivity was tested on the basis of their ability to discriminate between milk rancidity levels. Once the five panelists were selected, the absolute or detection sensory threshold for milk rancidity was determined. Milks that corresponded to the sensory detection threshold were labeled as distinctively rancid milks and were used as reference samples during training and sensory testing. According to the method of limits, the detection threshold is determined when 50% of the judges agree to assign to the same sample the sensation (*14*). When sensory judges were presented with milk samples of increasing rancidity levels prepared in the laboratory, it was determined that samples prepared with 15% LPRM in fresh pasteurized homogenized milk were the level required to bring the sensation of rancidity.

Training started by holding discussions between the panel leader and the panelists to understand the rancidity intensity scale and the use of the reference standards. The method used for training and testing was scoring on a 10-point rancidity intensity scale (from 0 = imperceptible to 10 = extremely pronounced) characteristic of descriptive sensory analysis (*13*). The samples used for training and testing were milk with different rancidity intensities prepared as described above. Training began by presenting panelists with the end-point references. Flavor notes associated with samples were described by the panel leader, and the technique to be used in the evaluation process was explained. Panelists were asked to recognize and record the flavor quality and intensity of rancid flavor in samples used in training sessions. At every testing session panelists were provided with three reference samples, a high-quality fresh milk, a distinctively rancid milk (15:85) that corresponded to the absolute or detection sensory threshold, and an extremely rancid milk (50:50). To stabilize data for unknown samples, assigned intensity assessments for reference samples were included as vertical marks on the rancid flavor intensity scale. Panelists were asked to first smell and taste the reference samples, although it was recommended that the rancid milks be tasted only if required, and then assess the samples accordingly. Panelists were instructed to rinse their mouths with water between samples and eat unsalted crackers on a free-choice basis for clearing the palate. Panelists were seated in individual sensory booths, and six samples coded with a three-digit number to remove bias were presented at every testing session. The 6 coded samples were milks with different rancidity intensities from the total of 46 samples prepared as described above.

The positions occupied by the reference samples on the scale corresponded to coded values of 0.0 (fresh milk), 3.0 (distinctly rancid milk), and 10.0 (extremely rancid milk). Thus, samples with a sensory score from 0 to 2.9 were classified as nonrancid milks, whereas samples with a score of ≥ 3.0 were classified as rancid milks. To assess the performance of the trained panelists, six coded samples prepared by mixing aliquots of LPRM with fresh pasteurized homogenized milk (0:100, 10:90, 15:85, 25:75, 35:65, and 50:50) were tested and analyzed by using the analysis of variance appropriate for a randomized complete block design. The absolute threshold for rancidity, which is the lowest stimulus capable of producing the sensation, was determined by the method of limits (*14*).

FFA Determination by SPME-GC Analysis. FFA in 40 samples of LPRM, 6 samples of high-quality fresh pasteurized homogenized milks, and 19 samples of commercial pasteurized homogenized milks were determined as described by ref *6*. Briefly, volatile FFA were collected by placing 40 mL of milk containing 28% NaCl and pentanoic acid (20 ppm) as an internal standard at pH 1.5 in a sealed vial, equilibrating for 30 min at 70 °C, and exposing a polyacrylate fiber (PA, 85 μm, Supelco Co., Bellefonte, PA) for 60 min to the vial headspace. Short-chain FFA were released to the headspace when the pH was adjusted to 1.5 with sulfuric acid (*6*). FFA adsorbed on the fiber were desorbed for 5 min into a Hewlett-Packard 6890 gas chromatograph (Wilmington, DE) and separated on a DB-FFAP (30 m × 0.32 mm i.d., 0.25 μm, J&W Scientific, Folsom CA) capillary column. FFA were tentatively identified by spiking samples with analytical standards. Calibration curves for individual FFA were constructed by spiking freshly pasteurized milk with aliquots of stock solutions (20000 ppm) of FFA analytical standards (C_4-C_{12}, PolyScience, Co.) to final concentrations of 10, 20, 40, and 100 ppm.

ADV determinations for 40 samples of LPRM, 6 samples of high-quality fresh pasteurized homogenized milks, and 19 samples of commercial pasteurized homogenized milks were determined in triplicate according to the *Standard Methods for the Examination of Dairy Products* (*15*).

Statistical Analysis. Quantitative individual FFA and corresponding rancid flavor intensity data for 46 samples prepared in the laboratory with different degrees of rancidity were correlated by stepwise multiple linear regression (MGLH) using Systat/Sygraph (Systat Inc., Evanston, IL). For the regression analysis, the dependent variable was the rancid flavor intensity score termed as the observed rancidity score (RS_{obs}) and was entered as the log transformed for each sample tested, and the independent variables were the concentrations of FFA in parts per million for each milk sample. Similarly, the ADV and $\log(RS_{obs})$, as

Table 1. Free Fatty Acid Concentrations in Fresh Pasteurized Homogenized and Distinctly Rancid Milks (Absolute Detection Sensory Threshold)[a]

	mean FFA (ppm)							
	fresh pasteurized homogenized[b]				distinctly rancid milk[b]			
FFA	sample 1	CV[c]	sample 2	CV	sample 3	CV	sample 4	CV
C_4	0.65	6.6	0.48	6.4	5.39	5.9	4.97	6.0
C_6	nq[d]		nq		2.47	2.5	3.67	3.0
C_8	nq		nq		0.70	4.3	1.22	5.2
C_{10}	2.28	7.3	2.52	7.6	3.93	7.5	4.46	7.0
C_{12}	1.92	1.5	1.97	1.4	3.16	1.0	3.36	1.34

[a] Distinctly rancid milk corresponds to the absolute detection sensory threshold as determined by the method of limits (*14*). [b] FFA determination by SPME-GC as reported in ref *6*. [c] CV, coefficient of variation (%), $n = 5$. [d] nq, not quantifiable, below the limit of quantification (LOQ) of 0.1 ppm.

Table 2. Summary of the Analysis of Variance for the Assessment of Sensory Panelists' Performance

source	DF	sum of squares	mean square	*F* value
samples	5	529.16	105.83	71.97[a]
panelists	4	21.75	5.43	3.69[b]
samples × panelists	20	82.36	4.11	2.80[c]
error	60	88.22	1.47	

[a] $p \leq 0.01$. [b] No significant difference ($p > 0.01$). [c] $p \leq 0.01$.

independent and dependent variables, respectively, were also correlated by applying regression analysis, and this equation was compared to the FFA-based equation. To assess the adequacy of the regression models for making predictions, the differences between the observed and predicted values of the dependent variable for 19 commercial milk samples were calculated.

RESULTS AND DISCUSSION

Sensory Evaluation. The FFA concentrations for milks that corresponded to the detection sensory threshold, as determined by SPME-GC, are shown in **Table 1**. These concentrations for the detection threshold were lower than the range (22.5−46.1 ppm) reported in the literature (*16*). The detection threshold reported in the literature was obtained by adding FFA standards to the milk (*16*), whereas in this work, FFA were naturally generated in the LPRM. It has been reported that the addition of FFA to milk does not result in the typical rancid flavor due to the inability to achieve the natural physical chemical state (*17*). Thus, the use of naturally generated rancid milks may be a better alternative for the determination of absolute sensory detection threshold.

ADVs for fresh milk (samples 1 and 2) were 0.6, which are within the range reported for normal milk (ADV < 0.7) (*15*). ADVs for distinctly rancid milk (absolute detection threshold), samples 3 and 4, were 2.1 and 2.2, respectively. According to the *Standard Methods for the Examination of Dairy Products*, samples with ADVs > 1.4 would be classified as extremely hydrolyzed milks (*15*), although others (*2*) reported that milks with ADVs > 1.5 did not taste rancid to trained panelists.

Sensory training consisted of 22 testing sessions before panel performance was evaluated. An analysis of variance for six treatments or coded samples prepared by mixing aliquots of LPRM with fresh pasteurized homogenized milk (0:100, 10:90, 15:85, 25:75, 35:65, and 50:50) and three replicates was applied to sensory data to assess the results (**Table 2**). The data were analyzed to identify significant variation among panelists and among samples. There were no significant differences ($p > 0.01$) among panelists, suggesting that the panel was subjected to enough training (**Table 2**). Similarly, significant differences among samples $p \leq 0.01$ indicated that the panel had enough training because in fact the samples tested had different rancidity intensities (**Table 2**). According to Watts et al., training is complete when panelists are comfortable with the evaluation procedure, can discriminate among different samples repeatedly, and can produce reproducible results (*18*). Because the interaction between samples and panelists was significant, sensory data for the individual judges were examined to find the source of interaction. Sensory data revealed that two of the judges were using slightly different parts of the scale; thus, to solve this problem, a more frequent use of reference samples was encouraged during testing.

The analytical scheme for the detection and prediction of milk rancidity consisted of the integration of the tools needed to permit the association of FFA chemical data to sensory responses, SPME-GC, and flavor scoring correlated by multivariate statistics. Because milk rancidity is ultimately detected by its sensory perception, calibration of an alternate method, such as FFA determination by SPME-GC, requires the input of a trained panel. However, once the relationships are established by a mathematical model, the objective evaluation of unknown samples can be accomplished without the need for human input.

The accuracy and precision of one of the analytical tools used in this study, SPME-GC for the determination of FFA, was reported inby ref *6*. Similarly, the performance of the trained sensory panel as an analytical tool was assessed by testing panelists' performance.

Statistical Correlation of Quantitative Rancidity Intensity Assessments and Individual Short-Chain FFA. The next step in the analytical scheme consisted of establishing the relationships between FFA quantitative data determined by SPME-GC and the observed rancidity score (RS_{obs}) generated by the trained sensory panel. The regression model generated from the data for the 46 samples prepared in the laboratory with different degrees of rancidity was expressed by the following equation:

$$\log (RS_{obs}) = -0.13892 + 0.00534\ C_4 + 0.01846\ C_6 + 0.0189\ C_8 + 0.1321\ C_{10} + 0.00091\ C_{12} + 0.00002\ C_4C_6 + 0.00027\ C_4C_8 + 0.00052\ C_4C_{10} - 0.00010\ C_6C_8 + 0.00003\ C_6C_{10} + 0.00027\ C_8C_{10} \quad (1)$$

A highly significant ($p < 0.001$) coefficient of multiple determination (R^2) of 0.84 for this model indicated that milk rancidity as determined by the sensory panel was dependent on the concentration of FFA. Although the R^2 indicated the adequacy of the regression equation, calculation of the residuals for additional samples was required to establish their predictive power (*19*). Thus, the FFA concentrations of 19 additional commercial milk samples were measured and used to calculate predicted rancidity scores (RS_{pre}) by using eq 1 (**Table 3**). According to the sensory panel, 18 samples were classified as nonrancid, because they presented an RS_{obs} within the range (0−2.9) characteristic for fresh, nonrancid milk, and only sample 19 was classified as distinctly rancid milk because it presented a sensory score of 3.0 (**Table 3**). When sensory scores were predicted (RS_{pre}) by the FFA-based model (eq 1), flavor classification of the 19 samples tested agreed 100% with the flavor classification obtained by the observed rancidity scores (RS_{obs}); 18 samples were classified as nonrancid with RS_{pre} ranging from 0 to 2.9, and sample 19 was classified as distinctly rancid with a RS_{pre} of 3.0 (**Table 3**). In this work, a highly significant correlation was found between free fatty concentra-

Table 3. Predictive Power of Equations Derived from Free Fatty Acid Data and Acid Degree Values (ADV) of Commercial Milks

		FFA-based equation[a]		ADV-based equation[a]		
sample[b]	RS_{obs}[b]	RS_{pre}[b]	residual[b]	RS_{pre}[b]	residual[b]	ADV
1	1.96	0.66	1.30	1.92	0.40	1.15
2	1.86	0.61	1.24	2.06	–0.20	1.28
3	1.62	0.47	1.15	2.05	–0.43	1.27
4	1.58	0.31	1.27	1.57	0.10	0.77
5	2.17	0.72	1.44	2.21	–0.40	1.41
6	1.56	1.00	0.56	2.24	–0.68	1.44
7	1.90	1.06	0.84	2.10	–0.20	1.32
8	1.70	0.98	0.72	2.05	–0.35	1.27
9	2.52	0.98	1.54	2.10	0.42	1.32
10	1.04	0.95	0.09	2.21	–1.17	1.41
11	2.32	2.92	–0.60	2.49	–0.17	1.64
12	2.10	1.93	0.17	2.55	–0.45	1.68
13	1.33	0.67	0.66	2.14	–0.81	1.35
14	1.01	2.25	–1.24	2.49	–1.48	1.64
15	1.96	1.19	0.76	2.04	–0.08	1.26
16	1.32	2.00	0.68	1.75	–0.43	0.98
17	1.43	1.02	0.41	2.16	–0.73	1.37
18	2.14	1.04	1.10	1.87	0.27	1.10
19	3.00	3.00	0.00	3.80	–0.80	2.43

[a] Equations 1 and 2. [b] Commercial milk samples; RS_{obs} = observed rancidity score; RS_{pre} = predicted rancidity score; residual = $RS_{obs} - RS_{pre}$.

tions and rancidity sensory scores. On the contrary, a nonsignificant low correlation among individual fatty acids or groups of fatty acids was reported (*2*). Poor correlation could have been due to incomplete recovery of the FFA, because these were extracted on an alumina column at neutral pH, whereas in the SPME-GC method used in this study, short-chain FFA were efficiently carried to the headspace when 28% NaCl was used and the pH was adjusted to 1.5 (*6*). It was reported that recovery of FFA depends on the pH of the system and that a low pH, between 1.3 and 3.0, is needed to recover short-chain (C_4−C_8) fatty acids (*2*). Thus, chemical methods that change the pH of the system may provide greater recovery of the short-chain fatty acids and have a stronger correlation to rancid flavor (*2*).

ADVs for the 46 milk samples used in the model ranged from 0.6 to 4.08, which corresponded to a wide range of observed rancidity scores (1.6−8.7). To establish the relationship between these 46 ADV data and observed rancidity scores (RS_{obs}), a regression model was generated and expressed by the following equation:

$$\log (RS_{obs}) = 0.041 + 0.532\ (ADV) \qquad (2)$$

A lower coefficient of multiple determination (R^2) of 0.79 than that for the FFA-based model, but highly significant ($p < 0.001$), was obtained for this model, indicating that milk rancidity as determined by the sensory panel correlated to the ADV values. When eq 2 was applied to predict sensory scores (RS_{pre}), the same flavor classification resulted as that obtained from the FFA-based equation (**Table 3**). However, according to the *Standard Methods for the Examination of Dairy Products* (*15*), milks with an ADV > 1.0 are rancid. Thus, if ADV values solely were used to classify the flavor of commercial milks, all but samples 4 and 16 would be classified as rancid milks because they presented ADV values > 1.0. For this reason, it has been suggested that sensory evaluation must be conducted to confirm the presence of rancid flavors in a milk sample that has a high ADV and that a milk should not be classified as rancid solely on the basis of its ADV (*5*).

Although a high correlation was found between ADV values and rancidity sensory scores, others (*2*, *3*, *5*) reported very poor correlations. It is possible that these differences may be due to the source of lipases releasing the FFA. The hydrolysis of fatty acids from glycerides in LPRM was primarily caused by milk lipase from the raw milk added to the homogenized milk (*2*). Milk lipase is a nonspecific lipase that releases various fatty acids in nearly the same proportion, whereas lipases from bacterial origin have varying specificities (*2*).

In conclusion, the analytical system established in this work has potential for application in the dairy industry for the detection and prediction of milk rancidity. Results confirmed previous findings that techniques based on the determination of short-chain free fatty acids highly correlate with rancid flavor. A high correlation between short-chain free fatty acids determined by solid phase microextraction gas chromatography and flavor scoring determined by a trained sensory panel indicated that a good proportion of the variation in rancid flavor was explained by the regression model. Although sensory evaluation was required for the calibration of the instrumental method, once the mathematical model was established, the objective evaluation of unknown samples was accomplished without the need for sensory panelists.

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