Semiquantitative Determination of Short-Chain Fatty Acids in Cane and Beet Sugars

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Abstract

Some sugars, specifically white beet sugar and raw cane sugars, possess off-flavors and off-odors. Although not necessarily the source, the presence of short-chain fatty acids serves as an indicator of an off-odor problem in sugar. Solid-phase microextraction (SPME) is used to collect the volatile compounds from the headspace of sugar. The temperature, moisture, and type of SPME fiber are varied to optimize recovery. Sugars analyzed in the absence of water using an incubation temperature of 70°C with a divinylbenzene–carboxen–polydimethylsiloxane fiber yield the most reproducible results. Data from depletion analyses report a recovery level of 38% for the first injection. The semiquantitative analysis of butyric acid is accomplished using injected standards to develop a calibration curve.

Introduction

Some sugars, specifically white beet sugar and raw cane sugars, possess off-flavors and off-odors (1). Microorganisms, pollutants in the environment, or lipid oxidation are responsible for these off-odors and off-flavors (2). Sugar beets may acquire off-odor compounds from the presence of fungi and bacteria, which can cause a "musty" or "earthy" aroma (3). Processing the sugar at a high temperature with very concentrated solutions usually minimizes microbiological reactions and removes off-odor compounds (1). However, enzymes, acids, bases, or salts may catalyze acidic hydrolysis during processing and storage. Subsequent reactions may produce other compounds that result in off-odors and off-flavors (4).

Off-flavor sugar directly affects the value of the commodity and poses a problem to the sugar industry and consumers. Quality control involves using instrumental testing procedures to identify the off-flavors and determine and eliminate their sources (3). Iron from the processing equipment may produce acidic or metallic off-flavors (1). Burnt and bitter flavors may result from overheating the sugar (5). Volatiles from the packing material often contribute to off-flavors, and excess fertilization or drought during the planting season may also cause the sugar to taste salty or bitter (1).

Microbial activity is one source of fatty acid production in sugar (3). Olfactory sensory testing and quantitative results suggest that a combination of short-chain fatty acids is the most significant source of off-flavors and off-odors in sugar (4). Three short-chain fatty acids in particular contribute to the overall flavor profile of off-flavor sugars. These are acetic, propanoic, and butyric acid (3). Hexanoic and especially 2-methyl-butanoic acid (isovaleric acid) are also suspected of contributing to off-flavor. Sugars with large amounts of these fatty acids have characteristic sour and rancid aromas and flavors (6).

Sugar analysis for flavor quality control involves isolating and identifying trace amounts of compounds that contribute to off-flavors in products. Many studies have used solid-phase microextraction (SPME)–gas chromatography (GC)–mass spectrometry (MS) for analysis in a variety of fields, including the food and agriculture industries (7). Despite the extensive use of this method, research on sugar has not been conducted using this technique. Therefore, SPME–GC–MS would appear to be an ideal technique for the analysis of aromas in sugar. This versatile technique could allow for the analysis of aromas in sugar in the form of crystals, powders, or syrups.

SPME was developed to identify the analytes that are present in the headspace or liquid phase of samples such as food, soil, or water (8,9). This technique was originally intended for the analysis of environmental samples but was quickly adopted by other industries, specifically the food and agriculture industries. SPME is often coupled with other techniques such as GC–MS. This technology is simple, accurate, and efficient (10,11) and is suitable for quantitative analysis. Quantitative analysis using SPME is possible, yet problematic (12,13).

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The objective of this study was to optimize the parameters for the analysis of short-chain fatty acids in white cane and beet sugar for relative quantitative analysis using SPME–GC–MS. Slight differences in analytical parameters (such as the type of fiber used, moisture content, and incubation temperature) affect the accuracy of results.

Experimental

Analyses were performed using sugars provided by Sugar Processing Research Institute, Inc. through various proprietary industrial sources. A total of 78 sugars (including raw cane sugar and white cane and beet sugar) were ranked based on butyric acid content and divided into three categories. Sugars remained at room temperature until sample preparation, which involved placing 0.75 g of sugar into 2-mL vials. Milli-Q water (0–80 µL) was added beneath the sugar with a syringe. Vials were sealed with a crimp cap fitted with a Teflon septum. Samples were run in triplicate and stored at room temperature (for up to 2 h) until analyzed.

Samples were placed in a CTC SPME autosampler (Leap Technologies, Carrboro, NC) and individually heated in a sample agitator at 50°C, 60°C, or 70°C for 15 min. One of three types of fibers, divinylbenzene (DVB)–carboxen (CAR)–polydimethylsiloxane (PDMS) (50- and 30-µm film), polydimethylsiloxane (PDMS) at 7.30- and 100-µm film, or polyacrylate (PA) at 85-µm film, was used during the 15-min extraction period (Supelco, Bellefonte, PA). Fibers varied with respect to stationary phase and film thickness. Sample volatiles were desorbed into the injection port of an Agilent (Palo Alto, CA) 6890 GC equipped with a 5973 MS system. Helium was used as the carrier gas under a constant flow of 36 cm/s through a 30-m, 0.25-µm DB-5 capillary column (J&W Scientific, Folsom, CA). The initial GC temperature (50°C) was held for 1 min. The temperature was ramped first at 5°C/min to 100°C and then at 15°C/min to 270°C and held for 5.67 min. Each 30-min GC run was followed by a 5-min cooling period.

Table I. Average Responses and RSDs of Butyric Acid in Three White Sugars at Three Temperatures

	Acceptable sugar		Borderline sugar		Unacceptable sugar	
	Average response	%RSD	Average response	%RSD	Average response	%RSD
50°C	1781	173	7286	35	21048	19
60°C	1506	25	9056	27	24336	48
70°C	2522	27	12609	11	29451	10

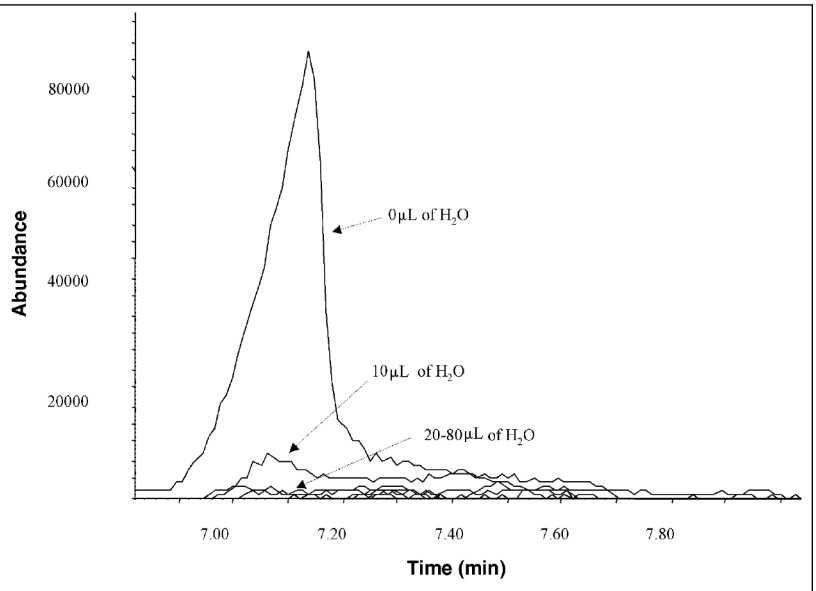

Figure 1. Butyric acid peak of unacceptable white sugar analyzed at 70°C with a DVB–CAR–PDMS fiber with nine different water levels.

The MS was operated in scan mode. With the exception of propanoic acid, the base peak of the short-chain fatty acids was found at *m/z* 60. The integrated peak areas of *m/z* 60 were used for the quantitation of volatile fatty acids, and qualifier ions were examined with regard to retention time and ion ratios. The molecular ion and base peak for propanoic acid was found at *m/z* 74 and used for quantitation.

Following optimization of the fiber, temperature, and water, sugar samples were analyzed using the optimal conditions determined by the experiment. Compounds in the headspaces of the samples were tentatively identified using the Wiley Mass Spectral Library (7th Edition) (Palisade Corp., Newfield, NY). In order to determine the recovery of volatile short-chain fatty acids in the headspace, a single sugar sample was repeatedly analyzed until no analyte was observed. Samples were analyzed in triplicate to give standard deviations. In order to generate a calibration curve, an authentic standard of butyric acid was obtained and a stock solution of 1 ppt was made in methanol. Serial dilutions of 0.1, 0.5, 1, 5, 10, 50, 100, 500, 1000, and 5000 ng/µL were prepared, and a 1-µL injection of each dilution was made in triplicate. A calibration curve was developed based on the integrated peak area of *m/z* 60 as a function of the mass of butyric acid injected. No response was observed for the 0.1-ng/µL injections, and the 0.5-ng/µL injections were detectable but not quantitatable. A correlation of 0.9750 was found for the range of 1 to 5000 ng/µL.

Results and Discussion

SPME is an equilibration technique and dependent upon a number of sampling parameters including fiber stationary phase, temperature, mechanical mixing, and exposure time. The time required to achieve equilibration varies directly with the thickness of the coating on the fiber. The thickness of the fiber coating limits the amount of material that is adsorbed. For fatty acid analysis, extended adsorption periods generally result in greater loading, especially for the longer-chain fatty acids (14,15). For competitive cases in which the fiber quickly becomes saturated, extended

adsorption periods can actually result in a decrease in the adsorption of some compounds (16). Extended SPME adsorption periods are not practical when using an autosampler and running large numbers of samples. In these cases, the SPME adsorption period needs to be less than the GC run time, and the optimal recovery may not be achieved. Consequently, adsorption time was not optimized in this study. Instead, it was set at 15 min. This trade-off not only affects the amount recovered, but may increase the error associated with precision if equilibration is not achieved.

Polar molecules such as the short-chain fatty acids are generally detected more readily using a polar coating such as PA (16), whereas the analyses of nonpolar molecules is accomplished using a nonpolar coating such as PDMS. For fiber selection, identical samples of a borderline-unacceptable white sugar were analyzed in triplicate under the same conditions using three fibers that differed in the content of their stationary phase and film thickness. The chromatograms generated with the PDMS and PA fibers did not show any significant peaks for the short-chain fatty acids in the sugar samples at the three incubation temperatures. The DVB–CAR–PDMS fiber extracted the short-chain fatty acids more readily than the PA and PDMS fibers. Although little difference was observed between the PDMS and PA fiber, previous reports have shown that the PA fiber is superior to the PDMS fiber for the recovery of fatty acids under slightly different conditions (16). All subsequent analyses were conducted using the DVB–CAR–PDMS fiber.

A method previously used for the analysis of volatile compounds in sugar involved the use of direct thermal desorption (3). Results indicated that the addition of small amounts of water increased the detection of volatile compounds released from the sample (3). However, in SPME the addition of water to the sample can suppress some volatile compounds while enhancing others (17). Varying amounts of water (0, 10, 20, 30, 40, 50, 60, 70, and 80 µL) were introduced to a borderline-unacceptable white sugar sample to determine butyric acid responses at varying moisture contents. More butyric acid was extracted by the DVB–CAR–PDMS fiber at all three incubation temperatures in the absence of water. The presence of water inhibited the recovery of the short-chain fatty acids. Figure 1 depicts the butyric acid peak using reconstructed ion chromatograms (RICs) (*m/z* 60) at 70°C with various amounts of water added to the sample. Appreciable amounts of butyric acid were observed only in the traces containing 0 and 10 µL of water added.

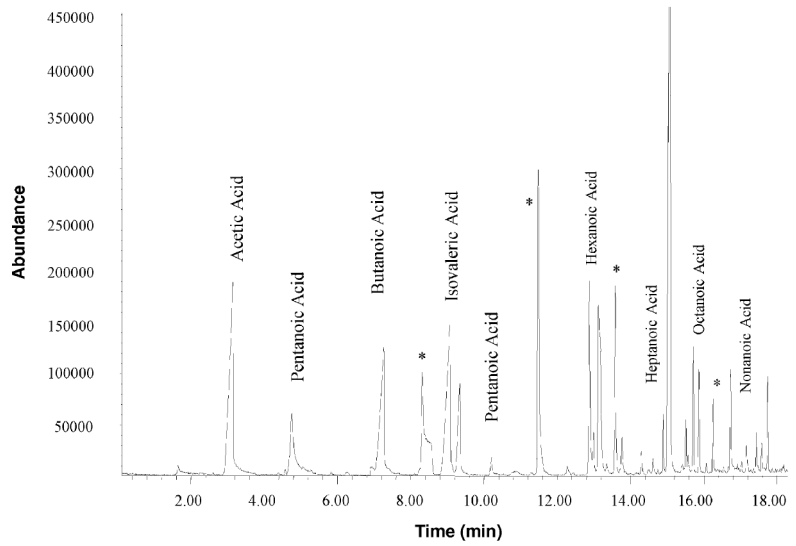

Figure 2. TIC of compounds in an unacceptable white sugar. Peaks marked with an asterisk represent contaminants from the fiber/septa.

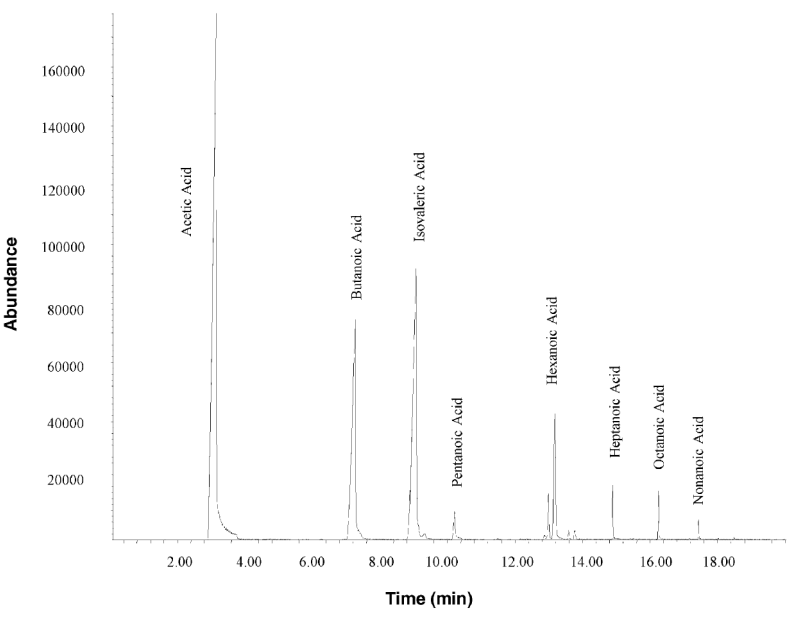

Figure 3. An RIC of identified short-chain fatty acids in an unacceptable white sugar (*m/z* 60).

Acceptable, borderline-acceptable, and unacceptable sugars were analyzed in triplicate with a DVB–CAR–PDMS fiber in the absence of water at three incubation temperatures (50°C, 60°C, and 70°C). The error represented by the standard deviation of the acceptable sugar was lowest for the samples analyzed at 60°C (Table I). However, the integrated peak areas (*m/z* 60) for the acceptable sugar were all less than 6000 counts, which was barely above the baseline (thus the sample contained a negligible amount of butyric acid, < 1 ng). The standard deviation of the samples analyzed at 70°C was less than the standard deviation of the samples analyzed at 50°C and 60°C for both the borderline-unacceptable and unacceptable sugars. Greater precision was observed at an adsorption temperature of 70°C.

Following optimization of the fiber, temperature, and moisture, an unacceptable white sugar was analyzed in the absence of water with the DVB–CAR–PDMS fiber at an incubation temperature of 70°C. A total ion chromatogram (TIC) is presented in Figure 2. Prominent peaks were identified based on the library searches of their mass spectra. The first half of the chromatogram

Table II. Results from the Depletion Analysis of Butyric Acid

Injection No.	Average abundance	%RSD	Percent recovery
1	120412	0.91	38.13
2	94541	6.40	29.94
3	57364	14.45	18.16
4	27063	3.44	8.57
5	11646	9.68	3.69
6	3850	98.84	1.22
7	496	132.07	0.16
8	277	95.58	0.09
9	85	37.67	0.03
10	80	46.55	0.03

is dominated by the short-chain fatty acid peaks. Several siloxane contaminants routinely seen in SPME analysis are marked with an asterisk. Straight-chain fatty acids (C_2 through C_9) were observed as well as three branched short-chain fatty acids (3-methyl butanoic acid, 2-methyl-butanoic acid, and 2-ethyl-hexanoic acid). The presence of some short-chain alcohols and aldehydes has been attributed to lipid oxidation (9) and microbial activity. Short-chain fatty acids are not the sole source of off-flavor in sugar. However, their presence serves as a marker for an unacceptable product. An RIC of *m/z* 60 is presented in Figure 3. With the exception of propanoic acid, the C_2–C_9 acids were readily observed.

In order to determine the amount of butyric acid recovered from a single sampling event, a depletion study was conducted by continually sampling the same vial. After 8 SPME adsorption

Table III. Amount of Butyric Acid Detected in 78 Sugar Samples*

Sugar No.	Butyric acid (ng)	Butyric acid (ppb)	Sugar No.	Butyric acid (ng)	Butyric acid (ppb)
0	13	18	39	117	156
1	5488	7317	40	134	178
2	10828	14438	41	30	40
3	167	222	42	31	42
4	177	236	43	52	70
5	6846	9129	44	110	147
6	6402	8536	45	103	137
7	33	44	46	55	74
8	32	43	47	172	229
9	22	29	48	35	46
10	24	32	49	123	164
11	18	24	50	94	126
12	18	23	51	63	84
13	23	30	52	5	6
14	127	169	53	30	40
15	19	26	54	186	248
16	49	66	55	74	98
17	277	369	56	194	259
18	67	90	57	367	490
19	14	18	58	200	267
20	92	123	59	91	122
21	5	7	60	273	365
22	13	18	61	14	19
23	107	143	62	17	23
24	96	128	63	5	6
25	98	131	64	5	6
26	12	16	65	6	8
27	23	30	66	5	6
28	154	201	67	5	6
29	87	116	68	5	6
30	242	323	69	139	186
31	104	139	70	10	13
32	100	133	71	12	16
33	168	224	72	16	21
34	80	107	73	57	76
35	31	41	74	23	30
36	44	58	75	70	93
37	122	163	76	25	33
38	149	199	77	20	27

* Concentration based on a recovery value of 38%.

Table IV. Response Factors Relative to Butyric Acid Based on *m/z* 60 and Amounts of Fatty Acids in an Unacceptable White Sugar

Acid	Response factor	Nanograms level	Parts-per-billion level
Acetic acid	0.11	2726	3634
Propanoic acid	0.37	49	65
Butyric acid	1.00	151	201
Isovaleric acid	0.82	178	237
Pentanoic acid	1.07	27	36
Hexanoic acid	0.78	40	54
Heptanoic acid	0.41	24	32
Octanoic acid	0.41	7	9
Nonanoic acid	0.21	5	7

periods, only trace levels of butyric acid could be observed with 98.5% of the headspace butyric acid recovered after 5 samplings (Table II). As expected, the relative standard deviation (RSD) increased with decreasing concentrations of butyric acid, ranging from less than 1% to over 100% error. The first sampling resulted in a recovery of 38% and was the value used for estimating the concentration of butyric acid in the headspace of the sugar samples.

For quantitation using SPME, a calibration curve was generated using known amounts of butyric acid injected into the GC. Assuming that both the liquid injection and SPME injection techniques are similar in efficiency, a plot of the integrated peak area versus the amount of butyric acid injected should serve as a calibration curve. No discernible detector response was observed for the 0.1-ppm (0.1 ng) standards. The 0.5-ppm (0.5 ng) standards were detectable, but the signal-to-noise ratio was less than 2. This set the detection limit between 0.5 and 1 ng. The volatile and semivolatile compounds in the headspace of the remaining 77 beet and cane sugar samples were analyzed by SPME–GC–MS. Table III lists the amount (in nanograms) of butyric acid and the calculated concentration found in each of the sugars. The concentration was an estimate and served as the minimum amount present. Values above 5000 ng have been extrapolated using the calibration curve and are tentative at best. Acceptable sugars tend to contain less than 75 ppb, borderline sugars contain from 75 to 150 ppb, and unacceptable sugars contain greater than 150 ppb butyric acid.

Relative mass spectral response factors of the C_2–C_9 straight-chain fatty acids and isovaleric acid are presented in Table IV. The responses were normalized on the integrated peak area of butyric acid (*m/z* 60). With the exception of propanoic acid, all other chromatographic peaks were based on *m/z* 60. Propanoic acid lacks a proton on the gamma carbon necessary for the McLafferty rearrangement and *m/z* 60 is not observed. The base peak and molecular ion at *m/z* 74 is generally used instead. Acetic acid also lacks a gamma carbon but readily forms a molecular ion as the base peak at *m/z* 60. These response factors do not take into account the differences in the recovery of the short-chain fatty acids in the SPME equilibration process. However, this does allow for a semiquantitative comparison between similar samples. Acetic acid is the most abundant short-chain fatty acid. A general trend of decreasing concentration is observed with increasing chain length. This may well be an artifact because of the SPME adsorption process with the more-volatile compounds being recovered more efficiently.

Conclusion

This study identifies the optimal fiber, moisture content, and incubation temperature for the analysis of white beet and cane sugar via SPME–GC–MS. The short-chain fatty acid series from acetic to nonanoic acid was observed along with the branched-chain fatty acids 3-methyl butanoic acid, 2-methyl-butanoic acid, and 2-ethyl-hexanoic acid. A value of 38% was estimated for the recovery of butanoic acid in the headspace of sugar based upon depletion analysis. Semiquantitative information for comparing relative amounts of short-chain fatty acids in off-flavor sugar was provided.

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