Detection of Volatile Spoilage Metabolites in Fermented Cucumbers Using Nontargeted, Comprehensive 2-Dimensional Gas Chromatography-Time-of-Flight Mass Spectrometry (GC×GC-TOFMS)

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Abstract: A nontargeted, comprehensive 2-dimensional gas chromatography-time-of-flight mass spectrometry (GC×GC-TOFMS) method was developed for the analysis of fermented cucumber volatiles before and after anaerobic spoilage. Volatile compounds extracted by solid-phase microextraction were separated on a polyethylene glycol 1st-dimension column and 14% cyanopropylphenyl 2nd-dimension column. Among 314 components detected in fermented cucumber brine, 199 had peak areas with coefficients of variation below 30%. Peak identifications established by mass spectral library matching were 92% accurate based on 63 authentic standards. Analysis of variance of analytes' log peak areas revealed 33 metabolites changed in concentration after spoilage ($P < 0.05$), including increases in acetic, propanoic, and butyric acids, n-propyl acetate, several alcohols, and a decrease in furfural. GC×GC-TOFMS with a nontargeted, semi-automated approach to data analysis made possible the separation, identification, and determination of differences in polar volatile components, facilitating the discovery of several metabolites related to fermented cucumber spoilage.

Keywords: comprehensive 2D GC-MS, fermented cucumber volatiles, GC×GC-TOFMS, metabolites, nontargeted data analysis, 2-dimensional gas chromatography

Practical Application: An optimized method for the chemical analysis of volatile food components is described and applied to the profiling of volatile compounds in fermented cucumbers, resulting in the identification of 137 components, many of which are being reported for the first time in fermented cucumbers. This nontargeted GC×GC-TOFMS method and inclusive data analysis platform facilitated the discovery of several metabolites that were formed or utilized during anaerobic spoilage of fermented cucumbers. Further study of these metabolites will enhance our ability to understand and potentially control the metabolism of spoilage bacteria that can degrade lactic acid under the restrictive environmental conditions present in fermented cucumbers.

Introduction

Fermentation and storage in bulk tanks is used to preserve cucumbers for extended periods of time. The fermented cucumbers are then converted into a variety of processed pickle products, most notably hamburger dill chips. Fresh cucumbers of various sizes are typically brined in sodium chloride (NaCl) solutions so that the equilibrated concentration of NaCl is between 5% and 8% (wt/wt). This concentration of salt inhibits softening enzymes (Bell and Etchells 1961) and favors the growth of the naturally occurring lactic acid bacteria (Etchells and Jones 1943). Cucumber fruits contain approximately 2% to 3% fermentable sugars (Lu and others 2002), which are metabolized by lactic acid bacteria to predominantly lactic acid, thereby reducing the pH and the readily available energy sources for microbial growth. The combination of salt, acid pH, and lack of sugars results in a naturally preserved product that can typically be held for many months prior to final processing into pickle products.

One disadvantage of this fermentation process is the high concentration of NaCl in the waste stream. Efforts to reduce the NaCl used in fermentation and storage of cucumbers have resulted in the increased incidence of fermented cucumber spoilage. This spoilage has been characterized by a normal lactic acid fermentation followed by a gradual rise in pH and decrease in lactic acid concentration (Fleming and others 1989, 2002; Kim and Breidt 2007). The production of volatile compounds and increased pH compromise

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the quality of the product, often necessitating early processing of the tank or discarding the product if spoilage proceeds. If the pH rises above 4.6, clostridial spoilage may occur (Fleming and others 1989), so the possibility of germination and growth of *Clostridium botulinum* spores cannot be ruled out. The currently unpredictable nature of this spoilage contributes to increased production costs for the pickling industry, mainly in the form of increased monitoring of fermentation tanks. In cases where the pH has risen beyond control, product losses and increased waste disposal costs are also incurred.

Given the potential diversity of chemical components in a food fermentation system, a discovery-based approach may provide new insight into the changes in volatile compounds that occur due to microbiological spoilage after the normal fermentation process has been completed. Advances in gas chromatography-mass spectrometry (GC-MS) systems and data collection capability provide the potential to carry out separations of volatile chemical components using 2 different separation mechanisms by connecting columns with different bonded phases in series. The benefits and challenges associated with this technology have been the subject of recent reviews (Marriott and Shellie 2002; Adahchour and others 2008; Mondello and others 2008; Cortes and others 2009). This comprehensive 2-dimensional (2D) GC (GC×GC) methodology has been applied to the analysis of volatiles in a number of complex food matrices, including roasted coffee beans, butter, essential oils, grapes, roast beef, sugarcane spirits, honey, pepper, roasted barley, hazelnuts, olive oil, potato chips, basil, and Chinese liquor. Compared to chromatography with a single column, 2D chromatography resulted in resolution of more components and in improved mass spectral matches when a time-of-flight (TOF) MS detector was used for the analysis of butter volatiles and grape monoterpenoids (Adahchour and others 2005; Rocha and others 2007). Analysis of lavender essential oil using an orthogonal 2D separation consisting of a nonpolar 1st-dimension column followed by a polar 2nd-dimension column resulted in a 25-fold increase in sensitivity and a 3-fold increase in the number of resolved components as compared to traditional GC analysis (Shellie and others 2001). Orthogonal 2D separation of Cheddar cheese volatiles using a comprehensive, 2D GC-TOFMS (GC×GC-TOFMS) showed that separation in the 2nd dimension was necessary to resolve octane from hexanal and ethyl lactate from 3-octanol (Gogus and others 2006). Several other studies have reported separation of volatile compounds from complex food matrices with a nonpolar 1st-dimension column followed by a polar 2nd-dimension column (Cardeal and others 2006, 2008; Čajka and others 2007; Eyres and others 2007; Rocha and others 2007; Rochat and others 2007; Klimánková and others 2008; Cardeal and Marriott 2009; de Souza and others 2009; Lojzova and others 2009; Torres Vaz-Freire and others 2009). However, the reverse column combination as well as nonorthogonal polar-semipolar column combinations have also been demonstrated as viable alternatives for separation of volatile compounds in foods (Adahchour and others 2004, 2005; Mondello and others 2004; Ryan and others 2004; Bianchi and others 2007; Zhu and others 2007; Cordero and others 2008). Although the orthogonal, nonpolar-polar column combination was suitable for separating coffee bean volatiles, the reversed column combination showed a comparable structured order of the components and yielded a volatile compound profile that utilized more of the available separation space (Ryan and others 2004). Adahchour and others (2004) found that improved peak shapes and retention behavior for acids and alcohols were obtained on a polar-semipolar column combination. In addition, a useful pattern of separation for homologous series of compounds with different functional groups was obtained (Adahchour and others 2004; Cordero and others 2008). Therefore, this "reverse-type" GC×GC separation may have advantages for some analyses of food volatiles.

While GC×GC-TOFMS offers greatly increased capability for separating and detecting volatile components present in complex samples, the datasets generated are large and cumbersome. In metabolite profiling studies, target compounds are unknown and the goal is to identify a set of metabolites associated with a particular treatment or phenotype (also known as biological markers) among the hundreds to thousands of metabolites detected. The size and complexity of these types of datasets requires automation of the data analysis process. This study describes a nontargeted, comprehensive GC×GC-TOFMS method for separating and identifying volatile compounds in fermented cucumbers, and detecting changes in volatile metabolites occurring as a result of fermented cucumber spoilage.

Materials and Methods

Cucumber fermentation

Size 2B cucumbers (32 to 38 mm in diameter) were washed, packed into 3 3.84-L glass jars, and covered with brine (55:45 cucumber:brine ratio) containing calcium chloride ($CaCl_2$) and NaCl so that the equilibrated concentrations were 0.25% and 6% (w/w), respectively. Brined cucumbers were inoculated with 10^6 CFU/g *Lactobacillus plantarum* MOP3 starter culture (Culture Collection ID LA0219, USDA-ARS Food Science Research Unit, Raleigh, N.C., U.S.A.). Jars were closed with lids that were heated in boiling water to soften the plastisol liner, and a rubber septum was inserted into the lid of each jar to allow sampling of the brine with a syringe. The jars were stored at ambient temperature (21 to 25 °C) for 11 mo. Fermentation progressed normally in all 3 jars as indicated by decreases in pH and changes in organic acids and sugars as measured by high-performance liquid chromatography (HPLC) with ultraviolet light (UV) and refractive index (RI) detection (McFeeters and Barish 2003). Seven replicate samples of fermented cucumber brine from a single fermentation jar were analyzed in random order among 12 other fermented cucumber brine samples over the course of a 3-d run of the instrument to assess the analytical reproducibility for the nontargeted analysis of volatile components. Volatile compounds were also analyzed in triplicate for brine samples from the other 2 replicate fermentation jars.

Media preparation

Fermented cucumbers as described above were cut into pieces and blended into a slurry to prepare sterile, fermented cucumber slurry (FCS) as a medium for inoculation with spoilage microorganisms. The FCS was pressed through cheesecloth and centrifuged in 250-mL bottles at 12000 rpm for 15 min to remove particulate matter. The pH of the clarified slurry was raised from 3.1 to 3.8 by addition of 6 N NaOH to increase the rate at which spoilage occurred (Fleming and others 2002; Kim and Breidt 2007). The pH-adjusted, clarified FCS was sterile-filtered with a Nalgene FAST PES 0.2-μm pore size, 90 mm dia membrane, bottle-top filter apparatus (Daigger, Vernon Hills, Ill., U.S.A.). Twelve mL of sterile-filtered FCS was then aseptically transferred into sterile 15-mL conical tubes. The loosely capped tubes were placed into an anaerobic chamber (Coy Laboratory Products, Inc., Grass Lake, Mich., U.S.A.) for 3 d prior to inoculation to remove dissolved oxygen from the media.

Spoilage inoculum source

Brine from a laboratory cucumber fermentation that had undergone an undesirable secondary fermentation was used as spoilage inoculum. Two 5-gallon plastic pails with tightly fitting lids were packed with 9.5 kg size 2B cucumbers (38 to 44 mm in diameter) and covered with an equal volume of brine. One cover brine contained 4% NaCl, 36 mM $CaCl_2$, and 50 mM acetic acid from 20% vinegar to equilibrate at 2% NaCl, 18 mM $CaCl_2$, and 25 mM acetic acid during the fermentation. The 2nd cover brine contained NaCl, $CaCl_2$, KCl, $MgCl_2 \cdot 6H_2O$, and acetic acid to equilibrate at 1.2% NaCl, 0.8% KCl, 30 mM $CaCl_2$, 20 mM $MgCl_2$, and 25 mM acetic acid (McFeeters and Fleming 1997). The pails were inoculated with *L. plantarum* starter culture and fermented normally as indicated by a decrease in pH to 3.2 and typical utilization of sugars and production of lactic acid determined by HPLC 1 mo after initiating the fermentations. However, when the fermentations were sampled after 11-mo storage at ambient temperature, it was noted that the lactic acid had decreased substantially and the pH had risen to 4.1 (Table 1), which is typical of the anaerobic cucumber spoilage described by Fleming and others (1989, 2002) and Kim and Breidt (2007). Brine from the spoiled fermented cucumbers (2% NaCl) was used as the inoculum to reproduce spoilage in filter-sterilized FCS (6% NaCl, pH 3.8). In addition, solid-phase microextraction (SPME) GC×GC-TOFMS was carried out on frozen aliquots of these 1- and 11-mo brine samples that were thawed, diluted, and randomized for run order prior to analysis. Changes in volatile metabolites that occurred during spoilage were determined by comparison of the volatile profile of brine samples taken from the pails after the primary fermentation (1-mo storage) and after the lactic acid had decreased (11-mo storage).

Reproduction of spoilage

Conical centrifuge tubes containing 12-mL sterile-filtered FCS were inoculated in triplicate with 1 mL of spoilage brine and incubated anaerobically at ambient temperature along with triplicate noninoculated FCS controls. Samples were taken immediately after inoculation and after 3 wk, 2 mo, and 6 mo of incubation and stored at −80 °C until analysis. A significant decrease in lactic acid concentration, as measured by HPLC, was used to indicate the appropriate samples to use for analysis of changes in the volatile components that occurred upon spoilage (Table 1). SPME-GC×GC-TOFMS was carried out on initial and 6-mo samples that were thawed, diluted, and randomized for run order. Components that changed during anaerobic incubation of noninoculated FCS were presumed to have been formed as a result of chemical changes that occurred during the extended incubation period and were excluded from the group of compounds that changed as a result of microbial spoilage.

SPME of volatile components

Fermented cucumber brines or spoilage samples (200 μL) were diluted 1:5 with deionized water (796 μL) and acidified with 3 N H_2SO_4 (4 μL) in 10 mL screw-cap headspace vials (Microliter Analytical Supplies, Inc., Suwanee, Ga., U.S.A.). NaCl (0.40 g) was added to "salt out" volatile components from the samples. Spoilage samples were also analyzed at a 1:250 dilution to account for volatile components present in amounts that resulted in column overloading at the 1:5 dilution. Samples were randomized for analysis order (PROC PLAN, version 9.1.3 SAS® software, SAS Inst., Cary, N.C., U.S.A.) and placed into a refrigerated sample tray (2 °C). Automated sampling was performed using a CombiPal autosampler (Model CTC Analytics (Switzerland), LEAP Technologies, Carrboro, N.C., U.S.A.). Headspace vials containing the diluted samples were agitated at 500 rpm (5 s on and 2 s off) for 15 min at 40 °C prior to extraction. Volatile compounds were collected by insertion of a 1-cm, 50/30 μm DVB/Carboxen™/PDMS StableFlex™ SPME fiber (Supelco, Bellefonte, Pa., U.S.A.) into the headspace above the sample for 30 min at 40 °C with 100 rpm agitation (5 s on and 2 s off). Extracted volatile compounds were desorbed from the SPME fiber into the GC inlet at 250 °C for 15 min. A blank sample (1.0-mL deionized water containing 6 mM sulfuric acid and 0.4 g NaCl) was run between each fermented cucumber sample to reduce carry-over of components on the SPME fiber.

Comprehensive, GC×GC-TOFMS

A LECO® Pegasus III® GC×GC-TOFMS instrument (Model# 614-100-700, Leco Corp., St. Joseph, Mich., U.S.A.) included an Agilent GC (Model# 6890N, Agilent Technologies, Santa Clara, Calif., U.S.A.) fitted with a secondary oven and cryogenic modulator. The 2D separation was achieved using a SolGel-Wax™, 30 m × 0.25 mm i.d. × 0.25 μm film thickness (SGE, Austin, Tex., U.S.A.), polyethylene glycol 1st-dimension column in the primary oven and an RTX 17-01, 1.0 m × 0.1 mm i.d. × 0.1 μm film thickness (Restek, Bellefonte, Pa., U.S.A.), 14% cyanopropylphenyl-86% dimethyl polysiloxane 2nd-dimension column in the secondary oven. Columns were conditioned according to manufacturer recommendations prior to use. A 0.75 mm i.d. Siltek deactivated SPME liner (Restek, Bellefonte) was used in the inlet. It was set at 250 °C and operated in pulsed splitless mode with a pulse pressure of 37 psi for 1 min. The split vent was opened 2 min following injection, and the GC was operated in constant flow mode with 1.3 mL/min helium carrier gas. The primary oven temperature was maintained at 40 °C for 2 min and then increased at 5 °C/min to 140 °C. The temperature ramp was then increased to 10 °C/min to 250 °C and the temperature was held at 250 °C for 3 min. The secondary oven followed the same temperature program except the temperature was maintained at 10 °C higher than the main oven until

Table 1–Changes in organic acids and pH as an indicator of spoilage.

	Time (mo)	pH	Lactic acid (mM)	Acetic acid (mM)	Propanoic acid (mM)
Fermented cucumber spoilage					
After primary fermentation	1	3.17 ± 0.01	116.8 ± 5.6	27.6 ± 0.9	None detected
After spoilage	11	4.08 ± 0.01	10.4 ± 1.0	80.0 ± 1.9	39.5 ± 1.8
Reproduction of spoilage in fermented cucumber slurry					
Noninoculated control	0	3.79 ± 0.00	125.1 ± 12.1	5.7 ± 1.1	None detected
Noninoculated control	6	3.80 ± 0.00	128.6 ± 0.7	5.9 ± 0.1	None detected
Inoculated with spoilage brine	0	3.82 ± 0.00	106.2 ± 2.5	13.2 ± 1.8	2.9 ± 0.8
Inoculated with spoilage brine	6	4.46 ± 0.01	51.8 ± 0.9	62.5 ± 3.8	16.1 ± 1.3

the temperature reached a maximum of 250 °C in the secondary oven. The transfer line temperature was maintained at 250 °C. The modulator offset was +30 °C with a 1.5 s 2nd-dimension separation time and 0.3-s hot pulse. Compressed air (35 psi) was used for the hot pulses, and liquid nitrogen-cooled nitrogen gas (18 psi) was used for the cold pulses.

The mass spectrometer was operated with −70 eV and an ion source temperature of 200 °C. The detector voltage was set at 1500 V and masses 25 to 500 were collected at 200 spectra per second. No solvent delay was employed.

Data processing and analysis

Data analysis involved a series of steps that made use of the instrument software, ChromaTOF® version 3.25 (Leco Corp.) for data processing, Excel® 2003 (Microsoft Corp., Redmond, Wash., U.S.A.) for data compilation, and SAS® version 9.1.3 (SAS Inst.) for statistical analysis. At the time of data acquisition, user fields were created in the ChromaTOF® acquisition menu to include information that uniquely identified each sample injected as to treatment type, replicate number, time of sampling, and so on. This information was then accessible in the peak tables for every peak associated with that sample. Inclusion of this information at the acquisition step was a key element contributing to efficiency in subsequent review and statistical analysis of the peak table data.

ChromaTOF® software data processing methods were used to detect and quantify peaks based on unique masses as determined by the deconvolution algorithm. Data processing parameters are shown in Table 2. A library search of the NIST/EPA/NIH Mass Spectra Library (National Inst. of Standards and Technology [NIST], Gaithersburg, Md., U.S.A., 2005) was utilized for tentative identification of deconvoluted chromatographic peaks. Chemical names were assigned to peaks that had a minimum mass spectral similarity ≥800 (1000 is an exact match). The unique mass (U) for each peak, as assigned by the ChromaTOF® deconvolution algorithm, was used for peak area calculations. All samples were processed in comparison to a single run of a composite brine sample. The composite sample for each experiment was prepared by mixing equal volumes of samples from each treatment of the experiment. Therefore, the composite sample peak table should theoretically contain most components that are present in the experimental samples. In ChromaTOF®, a reference table was created using the composite sample peak table as a standard. Criteria for the reference table were set as detailed in Table 2, and peak tables for each sample were standardized against this reference using the compare function in the ChromaTOF® data processing method. The resulting standardized peak tables containing each peak associated with a quantification name and peak area, based on the respective unique mass, were copied into an Excel® spreadsheet for further analysis. Creation of a reference in ChromaTOF® was necessary to standardize the name assignment for a given peak (including unknowns that were named unknown 1, unknown 2, and so on) and to allow standardized quantification of the peak area with the same specific unique mass for each component in all chromatograms of an experiment. Even in replicate chromatograms of brine from a single sample, the ChromaTOF® algorithm may select different unique masses for quantification of the same analyte, resulting in the inability to make comparisons of peak areas among chromatograms for a given component. This inconsistency is beyond the control of the instrument operator and has been noted by other researchers (O'Hagan and others 2007). In addition to stipulating a single mass per analyte for peak area quantification, employing the reference chromatogram for standardizing peak tables had the advantage of assigning the same unknown number to the matching components in all chromatograms. Therefore, it was possible to do peak area comparisons of unidentified metabolites that without standardization would have been variably numbered depending on the number of unknowns detected in each chromatogram.

Table 2–Data processing parameters used to create standardized peak tables in ChromaTOF®.

Data step	Parameter	Value
Peak detection	Baseline offset	0.8
	Number of points averaged for smoothing	3
	Peak width (second)	0.1
	Signal to noise (S/N)	250
	Number of apexing masses	2
GC×GC parameters	Match required to combine	500
	Override the allowed retention time shift for combine (early and late) (second)	0.1
	First-dimension peak width (second)	15
Library identification	Search mode	Normal, forward
	Number of library hits to return	10
	Molecular weight range	40 to 1000
	Mass threshold	10
	Minimum similarity match before name is assigned	800
	Library	NIST mainlib
Quantification	Mass to use for area/height calculation	U (unique mass)
Reference (compare criteria)	Name, 1st-dimension retention time (s), 2nd-dimension retention time (s), and masses (unique mass in this case)	Fields populated from peak table of the composite sample
	R. T. deviation (s)	4.5
	Quantitate	Area
	Match threshold	500
	S/N threshold	5.0

Peaks not found in a sample chromatogram that were included in the reference table resulted in blank cells for the peak area value of that analyte. These missing values represented the absence of a component within the detection limits of the analytical method, referred to as left-censored data, and needed to be replaced prior to statistical analysis to avoid the loss of fundamental information. Substitution of left-censored data with a random number between zero and the detection limit has been shown to be an adequate statistical alternative in environmental data analysis where observations below the instrumental detection limit constituted less than 70% of the data (Antweiler and Taylor 2008). To obtain an estimate of the experiment-wide detection limit, the minimum reported peak area from all chromatograms within an experiment was located. For example, from the fermented cucumber spoilage experiment, this area was 196. Therefore, blank peak area cells for undetected analytes in the dataset were replaced with a random number between 1 and 195 (<196) to provide substitution data that reflected possible responses below the method's detection limit for undetected components.

Peak areas of volatile components ranged from 196 to $>10^8$ in magnitude and peak area variability within replicate analyses increased as peak area increased. Since the standard deviation of peak areas was generally found to be proportional to the mean peak

areas, log transformation was used to homogenize the variances prior to analysis of variance (ANOVA) (Steel and Torrie 1980). An ANOVA of log peak areas by quantification name was conducted to detect differences in volatile compounds among treatments (version 9.1.3 SAS® software, SAS Inst.). Significance was established at $P < 0.05$ after adjustment of P-values to control the false discovery rate using the method of Benjamini and Hochberg (1995).

Reference compounds

With the exception of the following, all chemicals were obtained from Sigma-Aldrich in their purest available form (Sigma-Aldrich, St. Louis, Mo., U.S.A.). Pentane, methyl propionate, 3-methyl-2-butanone, benzene, methyl isobutyl ketone, 3-penten-2-ol, 1-pentanol, and 3-hydroxy-2-butanone were acquired from Fluka (Sigma-Aldrich, St. Louis). 3-octanol was sourced from Alfa Aesar (Ward Hill, Mass., U.S.A.), 3-pentanol was obtained from Riedel-de-Haen (Seelze, Germany), and 4-methyl-2-heptanol was purchased from ChemSampCo (Trenton, N.J., U.S.A.).

Results and Discussion

Volatile components in fermented cucumbers

Approximately 477 peaks with S/N ≥250 were detected in the brine of cucumbers fermented with 6% NaCl (Figure 1). Of these, 314 peaks were attributed to the fermented cucumber brine based on manual inspection of the chromatograms and peak table data for brine samples compared to water blank chromatograms. The 163 artifact peaks included siloxanes, other system contaminants,

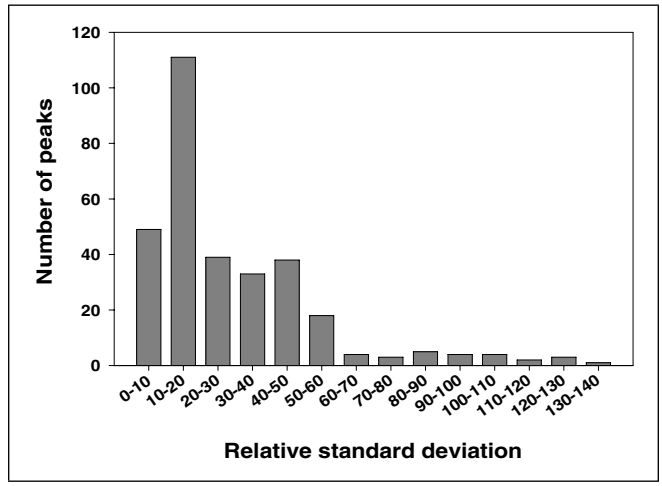

Figure 2–Peak area reproducibility ($n = 7$) for volatile components detected in fermented cucumber brine with SPME GC×GC-TOFMS.

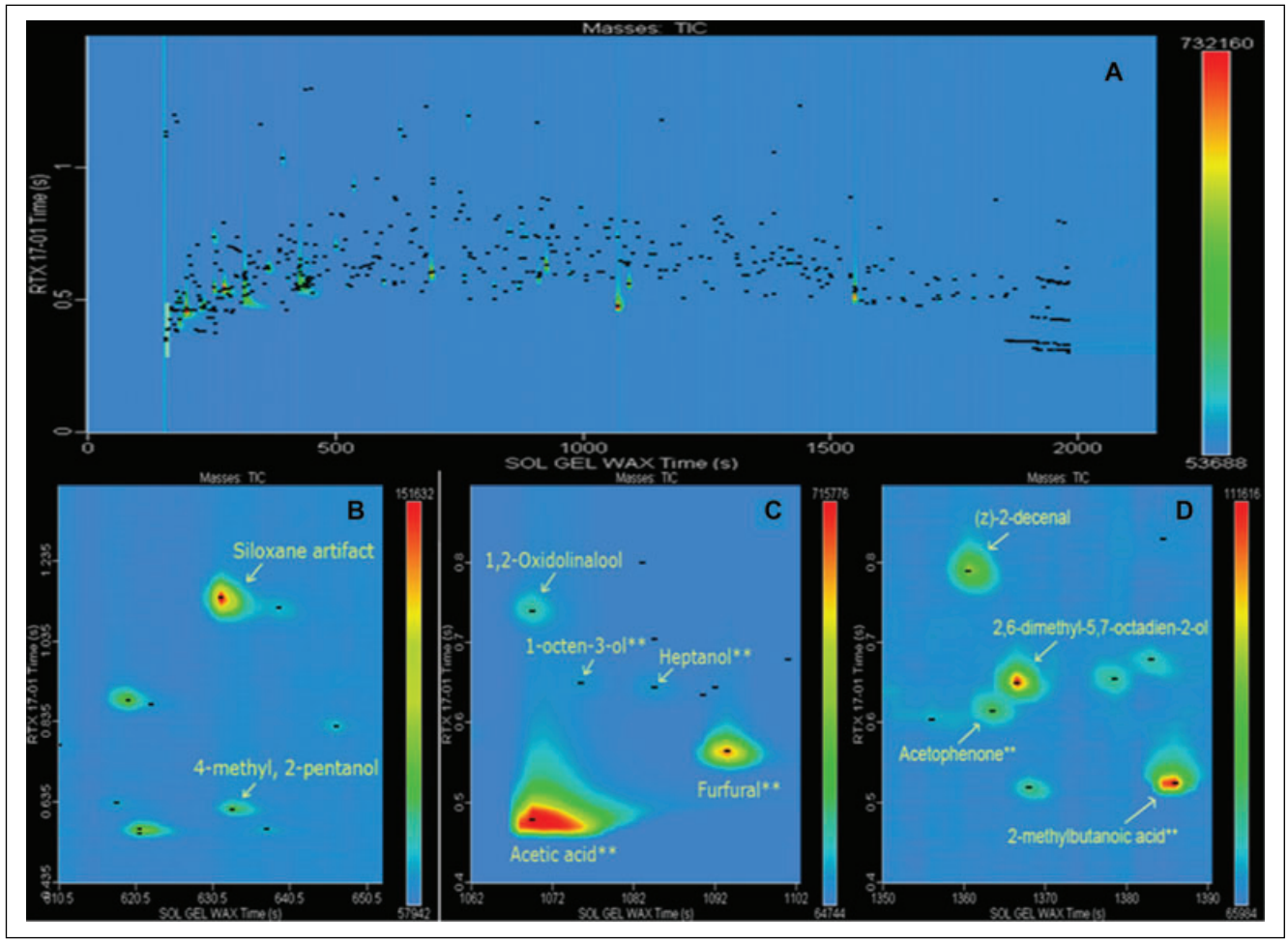

Figure 1–GC×GC-TOFMS total ion current (TIC) contour plot of volatile components in fermented cucumber brine (A). Three detail regions of the 2D separation of volatile components in fermented cucumber brine with a polar-semipolar column combination are shown, illustrating increased separation capacity (B), resolution of siloxane artifacts from metabolites of interest (C), and resolution of low intensity metabolite peaks in the 2nd dimension from an overloaded acetic acid peak (D). Peaks detected with S/N ≥ 250 are indicated by black peak markers.

Table 3–Volatile compounds in fermented cucumber brines detected using SPME GC×GC-TOFMS.

Compound[1]	CAS[2] registry #	Method of identification[3]	Similarity	RI^{4}_{calc}	RI^{5}_{lit}	Unique mass[6]	Average area	RSD[7]
Hydrocarbons								
Pentane	109-66-0	MS, RI, ST	934	500	500	41	45602	17.6
Hexane	110-54-3	MS, RI, ST	925	600	600	41	655816	27.2
1,4-pentadiene	591-93-5	MS, ST	943	646	nf	67	37400	19.7
Ethylcyclobutane	4806-61-5	MS	896	692	nf	56	472102	17.0
Cyclohexane	110-82-7	MS, RI, ST	904	742	737	56	88364	15.1
cis-5,5-dimethyl-2-hexene	39761-61-0	MS	901	757	nf	41	29255	16.7
Benzene	71-43-2	MS, RI, ST	970	938	936	78	743703	12.6
Toluene	108-88-3	MS, RI, ST	911	1042	1040	91	909212	9.1
m-xylene	108-38-3	MS, RI	909	1144	1132	91	17265	16.5
Cardene	694-87-1	MS, RI	926	1272	1269	104	64928	18.1
Alcohols								
2-methyl-2-pentanol	590-36-3	MS, RI	885	1110	1101	59	200446	11.0
3-pentanol	584-02-1	MS, RI, ST	938	1116	1112	59	81102	17.0
2-pentanol*	6032-29-7	MS, RI, ST	922	1129	1142	45	223939	14.4
2,4-dimethyl-2-pentanol	625-06-9	MS, ST	875	1147	nf	59	40578	18.0
Butanol*	71-36-3	MS, RI, ST	876	1153	1152	56	794956	13.7
2-methyl-3-pentanol	565-67-3	MS, RI	907	1167	1121	59	21068	21.2
1-penten-3-ol	616-25-1	MS, RI, ST	891	1169	1176	57	318459	4.6
3-penten-2-ol	1569-50-2	MS, RI, ST	841	1181	1182	71	11164	27.6
2,4-dimethyl-4-penten-2-ol	19781-53-4	MS	866	1195	nf	59	38954	15.7
Eucalyptol*	470-82-6	MS, RI	852	1223	1216	81	29202	10.5
2-hexanol	52019-78-0	MS, RI, ST	908	1238	1238	45	49865	17.0
3-methyl-3-buten-1-ol	763-32-6	MS, RI	883	1264	1263	68	37794	13.7
Pentanol*	71-41-0	MS, RI, ST	920	1265	1256	42	477300	12.8
2-methyl-2-heptanol	625-25-2	MS	812	1265	nf	59	47995	14.4
trans-2-penten-1-ol	1576-96-1	MS, RI, ST	929	1325	1335	57	22334	4.9
2-methyl-2-buten-1-ol	4675-87-0	MS, RI	862	1333	1315	71	55966	29.2
2-heptanol*	543-49-7	MS, RI, ST	942	1334	1334	45	299671	12.8
2-methyl-2-propen-1-ol	513-42-8	MS	808	1337	nf	72	11391	12.4
Hexanol*	111-27-3	MS, RI	891	1362	1354	43	2564937	9.5
4-methyl-2-heptanol	56298-90-9	MS, ST	928	1369	nf	45	120208	21.1
trans-3-hexen-1-ol	544-12-7	MS, RI	926	1371	1371	67	20351	12.4
2,3-dimethyl-1-pentanol	10143-23-4	MS	834	1388	nf	85	3623	10.8
cis-3-hexen-1-ol	928-96-1	MS, RI, ST	951	1389	1388	67	335213	11.4
2-methyl-2-octanol	628-44-4	MS, ST	893	1397	nf	59	71673	10.8
3-octanol	589-98-0	MS, RI, ST	917	1399	1395	55	19815	16.1
trans-2-hexen-1-ol	928-95-0	MS, RI	862	1408	1410	57	31238	24.4
2-octanol	5978-70-1	MS, RI	904	1421	1430	45	29309	13.6
1-octen-3-ol	3391-86-4	MS, RI, ST	932	1451	1456	57	248344	13.4
Heptanol	53535-33-4	MS, RI, ST	900	1457	1460	56	106479	10.2
2-ethyl-1-hexanol*	104-76-7	MS, RI	932	1495	1492	57	291814	12.3
cis-3-hepten-1-ol	1708-81-2	MS, RI	877	1509	1491	81	18911	15.5
2-nonanol	628-99-9	MS, RI, ST	840	1528	1528	45	34665	17.4
Octanol*	111-87-5	MS, RI	900	1568	1561	56	63772	8.0
4-terpineol	562-74-3	MS, RI	827	1614	1617	93	4391	9.1
Myrcenol	543-39-5	MS, RI	860	1622	1604	59	28202	17.4
cis-2-octen-1-ol	26001-58-1	MS, RI	896	1626	1616	57	15126	12.0
cis-ocimenol	5986-38-9	MS, RI	847	1662	1662	93	55339	12.7
trans-ocimenol	5986-38-9	MS, RI	837	1685	1688	93	72282	11.9
α-terpineol*	98-55-5	MS, RI, ST	914	1703	1718	59	345764	13.3
cis-6-nonen-1-ol	35854-86-5	MS, RI	941	1720	1711	67	19277	11.8
Benzyl alcohol	100-51-6	MS, RI, ST	900	1900	1874	79	48419	9.6
Phenylethyl alcohol*	60-12-8	MS, RI, ST	942	1939	1939	91	54906	10.5
Aldehydes								
Acetaldehyde	75-07-0	MS, RI, ST	928	727	727	44	7442303	12.2
Pivaldehyde	630-19-3	MS, RI	872	807	809	41	146801	11.0
2-methylbutanal	96-17-3	MS, RI, ST	878	914	914	57	90940	17.4
3-methylbutanal	590-86-3	MS, RI, ST	864	918	917	41	431295	5.8
Hexanal*	66-25-1	MS, RI, ST	923	1084	1080	57	664650	7.9
trans-2-methyl-2-butenal	497-03-0	MS, RI	912	1098	1094	84	29451	26.2
2-pentenal	1576-87-0	MS, RI, ST	884	1137	1135	55	175677	6.8
2,4,4-trimethyl-2-pentenal	53907-61-2	MS	800	1254	nf	55	17749	23.2
cis-2-heptenal*	57266-86-1	MS, RI	932	1340	1331	41	540286	14.7
Nonanal*	124-19-6	MS, RI, ST	902	1402	1396	41	283024	16.4
trans-2-octenal	2548-87-0	MS, RI, ST	873	1432	1432	55	317319	19.4
Furfural	98-01-1	MS, RI, ST	892	1464	1474	96	2442243	4.5
2,4-heptadienal	5910-85-0	MS, RI	873	1469	1468	81	30513	10.8

Continued

Table 3–Continued

Compound[1]	CAS[2] registry #	Method of identification[3]	Similarity	RI^{4}_{calc}	RI^{5}_{lit}	Unique mass[6]	Average area	RSD[7]
(E,E)-2,4-heptadienal	3/5/4313	MS, RI	873	1501	1497	81	156003	12.0
Benzaldehyde*	100-52-7	MS, RI	872	1530	1528	77	77720	3.1
2-decenal	2497-25-8	MS, RI	926	1658	1652	41	77800	23.2
3,5-dimethyl-benzaldehyde	5779-95-3	MS	912	1837	nf	133	100878	13.6
Ketones								
Acetone*	67-64-1	MS, RI, ST	922	814	814	58	1363820	8.2
3-methyl-2-butanone	563-80-4	MS, RI, ST	847	929	929	39	25302	27.9
3,3-dimethyl-2-butanone	75-97-8	MS, RI, ST	870	949	978	57	32611	10.4
2-methyl-3-pentanone	565-69-5	MS, RI	863	997	1003	57	37274	7.6
2,4-dimethyl-3-pentanone	565-80-0	MS, RI, ST	880	1000	995	71	10099	11.9
Methyl isobutyl ketone	108-10-1	MS, RI, ST	926	1008	1008	43	524065	14.1
3-methyl-2-pentanone	565-61-7	MS, RI, ST	902	1019	1016	43	70287	6.4
1-penten-3-one	1629-58-9	MS, RI, ST	837	1024	1024	55	698188	9.8
4,4-dimethyl-2-pentanone	590-50-1	MS	886	1025	nf	43	144329	10.2
3-hexanone	589-38-8	MS, RI, ST	914	1055	1052	57	65095	12.7
2-methyl-1-penten-3-one	25044-01-3	MS, RI	907	1069	1069	69	40626	10.4
trans-3-penten-2-one	3102-33-8	MS, RI, ST	844	1134	1123	69	29502	17.1
4-methyl-3-penten-2-one	141-79-7	MS, RI	886	1140	1131	98	8040	14.0
4-methyl-2-heptanone	6137-06-0	MS, RI	902	1224	1206t	58	124174	13.9
3-hydroxy-2-butanone	513-86-0	MS, RI, ST	863	1301	1289	45	983577	11.4
1-octen-3-one	4312-99-6	MS, RI	905	1319	1299	55	210558	16.0
6-methyl-5-hepten-2-one	110-93-0	MS, RI	838	1351	1340	43	112007	22.2
2-hydroxy-2,4-dimethyl-3-pentanone	3212-67-7	MS	865	1376	nf	59	7580	9.9
Acetophenone	98-86-2	MS, RI, ST	935	1660	1660	77	42629	9.8
p-methylacetophenone	122-00-9	MS, RI	890	1789	1794	119	19819	10.1
Acids								
Acetic acid*	64-19-7	MS, RI, ST	927	1446	1450	60	9802352	17.5
Propanoic acid	79-09-4	MS, RI, ST	938	1543	1534	45	230741	7.3
Pivalic acid	75-98-9	MS, RI	863	1586	1579	57	106234	11.5
Butanoic acid*	107-92-6	MS, RI, ST	846	1636	1620	60	55323	9.8
2-methyl-butanoic acid	116-53-0	MS, RI, ST	861	1677	1682	74	136728	9.5
Pentanoic acid	109-52-4	MS, RI, ST	913	1734	1734	60	81854	8.9
Hexanoic acid*	142-62-1	MS, RI, ST	864	1865	1841	60	721279	6.4
Octanoic Acid	124-07-2	MS, RI, ST	891	2071	2053	60	375641	9.2
Nonanoic acid	112-05-0	MS, RI, ST	892	2157	2157	60	501018	17.3
Decanoic acid	334-48-5	MS, RI	869	2219	2263	60	52296	14.4
Esters								
Methyl acetate	79-20-9	MS, RI, ST	882	825	828	74	1012974	13.7
Methyl propionate	554-12-1	MS, RI, ST	815	905	911	57	57501	14.0
Ethyl propionate	105-37-3	MS, RI	880	956	957	57	81551	11.7
Ethyl nitrate	625-58-1	MS	934	969	nf	76	4708	14.7
Isoamyl acetate	123-92-2	MS, RI, ST	851	1127	1127	43	33883	28.2
Methyl lactate	2155-30-8	MS, RI, ST	948	1331	nf	45	1719881	9.6
Ethyl lactate	97-64-3	MS, RI, ST	949	1354	1353	45	7808306	7.4
Isoamyl lactate	19329-89-6	MS, RI	852	1580	1583	45	27084	22.9
trans-3-hexenyl butanoate	53398-84-8	MS, RI	826	1621	1602	71	42111	20.8
2-methyl-, 3-hydroxy-2,4,4-trimethylpentyl propanoate	74367-34-3	MS	894	1902	nf	71	281380	6.0
2-methyl-, 2,2-dimethyl-1-(2-hydroxy-1-methylethyl) propyl propanoate	74367-33-2	MS	854	1921	nf	71	185822	5.4
Ethers								
Oxetane	503-30-0	MS, ST	897	790	nf	58	305941	5.2
tert-amyl methyl ether	994-05-8	MS	871	790	nf	73	32913	14.2
1,2-oxidolinalool	76985-29-0	MS	896	1446	nf	59	379361	8.0
Diphenyl ether	101-84-8	MS, RI	863	2038	2017	51	37843	8.2
Furans								
2-methylfuran	534-22-5	MS, RI, ST	895	864	876	82	54359	9.0
2-ethylfuran	3208-16-0	MS, RI, ST	900	950	945	81	60954	8.8
trans-linalool oxide*	34995-77-2	MS, RI	886	1477	1484	59	112235	7.9
2-acetylfuran	1192-62-7	MS, RI	911	1509	1511	95	19327	12.7
Butyrolactone	96-48-0	MS, RI	962	1637	1635	42	276487	11.3
5-pentyl-γ-lactone	104-61-0	MS, RI	882	2056	2055	85	100505	6.8
Pyrans								
Linalool 3,7-oxide	7392-19-0	MS, RI	868	1111	1109	71	255202	10.4
trans-rose oxide	876-18-6	MS, RI	822	1365	1341	139	7646	13.9
Nerol oxide	1786-08-9	MS, RI	831	1476	1466	83	10567	13.3

Continued

Table 3–Continued

Compound[1]	CAS[2] registry #	Method of identification[3]	Similarity	RI^4_{calc}	RI^5_{lit}	Unique mass[6]	Average area	RSD[7]
Phenols								
Butylated hydroxytoluene	128-37-0	MS, RI	858	1946	1902	205	49914	19.8
p-propylguaiacol	2785-87-7	MS, RI	919	2117	2103	137	11239	11.8
3,5-di-tert-butyl-4-hydroxybenzaldehyde	1620-98-0	MS	834	>2219	nf	219	5239	15.9
Nitrogenous compounds								
Methyl isocyanide	593-75-9	MS	980	1002	nf	41	302897	9.9
3-methyl-butanenitrile	625-28-5	MS, RI	797	1132	1120	41	23517	8.5
3,3-dimethyl-butanamide	926-04-5	MS	813	1205	nf	59	134717	14.0
5-methyl-isoxazole	5765-44-6	MS	878	1215	nf	43	25887	14.4
Acetaldoxime	107-29-9	MS	941	1301	nf	59	14668	21.9
Hexanenitrile	628-73-9	MS, RI, ST	872	1315	1303	54	58876	21.4
4-O-acetyl-2,5-di-O-methyl-3,6-dideoxy-d-gluconitrile	N/A	MS	848	1335	nf	129	4639	13.5
2-methoxy-3-isopropyl-pyrazine*	25773-40-4	MS, RI	863	1432	1443	137	51619	8.8
Sulfur compounds								
Dimethyl disulfide*	624-92-0	MS, RI, ST	981	1072	1075	94	128995	27.9
3-methylthiophene	616-44-4	MS, RI, ST	920	1120	1120	97	14082	17.8
Dimethyl sulfoxide	67-68-5	MS, RI, ST	935	1576	1582	63	249017	16.3

[1]Compounds reported previously in fermented cucumber brine are designated with an *.
[2]Chemical Abstracts Service registry number.
[3]MS = identification based on mass spectral match to the NIST 05 library with >800 similarity, RI = comparison with published retention indices on polyethylene glycol column phase, ST = mass spectral and retention index match to authentic standard.
[4]Retention indices based on 1st-dimension retention of components on a SOL-GEL-WAX (polyethylene glycol) column using SPME GC×GC-TOFMS.
[5]Retention indices reported in the literature (nf = not found); References available at the NIST Chemistry WebBook database, http://webbook.nist.gov.
[6]Mass selected by ChromaTOF software during automated data processing to represent an interference free mass for each analyte; The unique mass for each component was used for calculation of peak area.
[7]Relative standard deviation ($n = 7$).

and column bleed at the higher end of the temperature program. Fortunately, with the polar-semipolar column combination, these artifacts were well resolved from sample volatile components (Figure 1), making it possible to detect low-level volatile metabolites in the midst of system contaminants. The presence of contaminant compounds is not unusual and often creates a mass spectral background that can interfere with identification and quantification of sample analytes in one-dimensional (1D) GC chromatograms.

Of the 314 sample peaks detected in fermented cucumber brine, 214 (68%) were tentatively identified by ChromaTOF® data processing based on the best spectral match to the NIST05 library with similarity ≥800. To evaluate the quality of these tentative identifications, authentic standards of 63 compounds were individually chromatographed. The 63 test compounds were chosen from throughout the chromatographic run subject to commercial availability. Based upon retention time and mass spectral matches with components detected in the fermented cucumber brine samples, the best library match was a correct identification in 58 of the 63 cases (92%). The incorrect identification of acetic acid was most likely due to column overload, which has been demonstrated to create problems with the ChromaTOF® deconvolution algorithm (Lisec and others 2006). Although it was incorrectly identified, the overloaded acetic acid peak would have interfered with detection of at least 3 other components in the 1st dimension. These components were clearly resolved in the 2nd dimension, enabling their detection and identification (Figure 1).

Among the 314 volatile components in fermented cucumber brine, 199 had <30% relative standard deviation (RSD) in their peak areas for 7 replicate analyses randomized among 12 other fermented cucumber brine samples over the course of a 3-d run of the instrument (Figure 2). This volatile compound profile was representative of fermented cucumbers in all 3 of the 6% NaCl fermentations analyzed. The 199 reproducibly detected volatile components in fermented cucumber brines included 40 unknowns and 159 tentatively identified compounds. Further manual inspection of the data and comparison with retention indices reported in the literature resulted in the identification of 137 volatile compounds in fermented cucumber brine (Table 3). Comparison of these metabolites with previously reported volatile compounds in fermented cucumber brines (Zhou and McFeeters 1998; Marsili and Miller 2000) indicated that this method may be more sensitive in the detection of plant terpenoids, esters, alcohols, highly volatile aldehydes, and light hydrocarbons as greater numbers of these compounds were found in the present study. Several volatile compounds previously identified in fermented cucumber brines using 1D GC-MS on nonpolar columns were also found in this study, including butanol, pentanol, hexanol, octanol, 2-pentanol, 2-heptanol, 2-ethyl-1-hexanol, eucalyptol, α-terpineol, phenylethyl alcohol, hexanal, nonanal, 2-heptenal, benzaldehyde, acetone, acetic acid, butanoic acid, hexanoic acid, linalool oxide, 2-methoxy-3-(1-methylethyl) pyrazine, and dimethyl disulfide (Zhou and McFeeters 1998; Marsili and Miller 2000).

Nontargeted detection of volatile metabolites associated with spoilage fermentations

Fermented cucumber slurries before and after spoilage with a mixed culture inoculum obtained from spoiled fermented cucumbers were subjected to the GC×GC-TOFMS analysis described. ANOVA of analyte log peak areas revealed 33 metabolites that changed significantly ($P < 0.05$) in concentration after spoilage (Table 4). The nontargeted data analysis approach narrowed the field of approximately 500 peaks per sample to 33 metabolites of interest without extensive manual inspection of the 2D peak table data and chromatograms. The only manual inspection required was review of representative chromatograms to be certain that an appropriate sample dilution was chosen for analysis. Since several compounds of interest were overloaded at the low dilution and many compounds were undetected at higher dilutions, it was necessary to run the FCS samples at 2 dilutions and compile the results to avoid floor and ceiling effects. Noninoculated, sterile-filtered

FCS controls were used to exclude volatile compounds that may have changed due to chemical reactions during the extended incubation time. Changes in compounds tentatively identified as 1-(2,4-dimethyl-furan-3-yl)-ethanone, 2-methyl-2-pentanol, and amylene hydrate were similar in magnitude and direction in both control and spoilage samples. Therefore, they were excluded from the list of potential spoilage metabolites.

Volatile metabolites that changed during spoilage (Table 4) included increases in acetic acid, propanoic acid, butanoic acid, n-propyl acetate, several alcohols, and a decrease in furfural. The observed increases in acetic, propanoic, and butanoic acids were in accordance with previously published studies that showed increases in these components in fermented cucumbers that had undergone secondary spoilage fermentation, as measured by HPLC and tentatively identified based on retention time match (Fleming and others 1989, 2002; Kim and Breidt 2007). In the present study, the increase in butanoic acid detected by GC×GC-TOFMS was below the detection limit of the HPLC. However, quantitative analysis of acetic and propanoic acids by HPLC confirmed the fold increases detected with this nontargeted volatile analysis method in the anaerobic reproduction of the spoilage in FCS (Table 1). In addition to confirming these 2 primary spoilage metabolites, several other target spoilage metabolites were discovered. Comparison of these metabolite changes to the original brine samples from spoiled fermented cucumbers showed that 11 of the 33 metabolites that changed in cucumber slurries upon controlled reproduction of spoilage coincided with metabolites that were formed or utilized in the brines of spontaneously spoiled fermented cucumbers. In addition to acetic, propanoic, and butanoic acids, there were increases in n-propyl acetate, isoamyl acetate, and 1-butanol. Decreases were observed in components identified as ethyl lactate, acetonitrile, methyl lactate, tetrahydrofuran, and 1-penten-3-ol. The other 22 metabolites observed to change when the spoilage was transferred to sterilize FCS in an anaerobic chamber showed that the transferred bacteria were able to utilize or produce a number of metabolites differently than in the original spoilage. The significance of these metabolites in the overall spoilage process remains to be determined.

Table 4–Changes in volatile metabolites associated with anaerobic spoilage of fermented cucumber slurry (pH 3.8, 6% NaCl).

Metabolite[1]	CAS[2] registry #	RI[3]	Fold change[4]	Direction of change
4-methyl-benzenemethanol	589-18-4	1960	579.4	Increase
Unknown A	N/A	1371	342.2	Increase
4-methyl-2-heptanol	56298-90-9	1375	188.2	Increase
4-methyl-2-pentanol	108-11-2	1173	183.5	Increase
2-hexanol	626-93-7	1231	33.4	Increase
4-methyl-3-hepten-2-one[MS]	22319-25-1	1015	29.4	Increase
Unknown B	N/A	1902	11.7	Increase
2-pentanol	71-41-0	1126	11.4	Increase
3-methylene-2-pentanone[MS]	4359-77-7	1137	6.3	Increase
Isoamyl acetate*	123-92-2	1127	6.0	Increase
n-propyl acetate*	109-60-4	961	6.0	Increase
Propanoic acid*	79-09-4	1543	4.8	Increase
2-heptanol	543-49-7	1334	4.7	Increase
Tert-butyl ethyl ether	637-92-3	710	4.6	Increase
Acetic acid*	64-19-7	1449	4.5	Increase
Unknown C	N/A	920	3.7	Increase
Unknown D	N/A	962	3.3	Increase
Butanoic acid	107-92-6	1636	3.0	Increase
3-pentanol	584-02-1	1110	2.8	Increase
1-butanol*	71-36-3	1153	2.5	Increase
2-methyl-2-propanol	75-65-0	897	2.5	Increase
Methyl acetate	79-20-9	825	2.3	Increase
unknown E		882	1.6	Increase
Ethyl lactate*	97-64-3	1353	1.7	Decrease
Methyl propionate	554-12-1	905	1.9	Decrease
Acetonitrile*	75-05-8	988	2.3	Decrease
Methyl lactate*	2155-30-8	1328	3.2	Decrease
Tetrahydrofuran*	109-99-9	854	3.5	Decrease
1-penten-3-ol*	616-25-1	1166	4.5	Decrease
Unknown F	N/A	1535	6.2	Decrease
Furfural	98-01-1	1464	30.1	Decrease
Unknown G	N/A	967	85.0	Decrease
2,3-butanedione	431-03-8	965	100.1	Decrease

[1]Identification based on mass spectral and retention index match to authentic standards except where noted. [MS]indicates a tentative identification based on mass spectral match to the NIST library. Metabolites marked with an * symbol indicate those compounds that were also found to increase or decrease in the brines from the original spoilage of fermented cucumbers.
[2]Chemical Abstracts Service registry number.
[3]Retention indices based on 1st-dimension retention of components on a SOL-GEL-WAX (polyethylene glycol) column using SPME GC×GC-TOFMS.
[4]Fold change based on ratio of unique mass peak area of a given metabolite in spoiled fermented cucumber slurry as compared to initial fermented cucumber slurry.

Conclusions

Comprehensive GC×GC-TOFMS provides the analytical capability to resolve and identify many more volatile components from food samples than traditional GC-MS methods. However, complex datasets are generated for each sample such that standard approaches to data analysis are impractical when the target analytes are unknown. Optimization of the 2D separation combined with a semi-automated approach to data reduction using the instrument software and basic statistical analysis made it feasible to detect and identify many volatile components in fermented cucumbers. Fermented cucumber brines were found to contain 137 reproducibly detected and identified volatile compounds from a variety of chemical classes including hydrocarbons, aldehydes, ketones, alcohols, acids, esters, furans, and terpenoids. The nontargeted GCGC-TOFMS method and data analysis made possible the separation, identification, and determination of differences in polar volatile components, facilitating the discovery of several metabolites that were formed or utilized during anaerobic spoilage of fermented cucumbers. Further study of these metabolites will enhance our ability to understand and potentially control the metabolism of spoilage bacteria that can degrade lactic acid under the restrictive environmental conditions present in fermented cucumbers.

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