Determination of Major Aroma Impact Compounds in Fermented Cucumbers by Solid-Phase Microextraction–Gas Chromatography–Mass Spectrometry–Olfactometry Detection

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Abstract

Purge-and-trap, solid-phase extraction, and solid-phase microextraction–gas chromatography–mass spectrometry (SPME–GC–MS) sample preparation techniques for the analysis of odor impact chemicals in fermented cucumber brine are compared. SPME–GC–MS is coupled with detection frequency olfactometry experiments to determine key impact odor compounds in the brine. The most potent odorants that define the typical characteristic brine aroma are *trans*-4-hexenoic acid and *cis*-4-hexenoic acid. Confirmation of key impact odorants in brine is confirmed by recombination experiments.

Introduction

Most commercially processed dill pickle products are produced from cucumbers that are naturally fermented and stored in large open tanks with 5–12% salt. The high salt level inhibits the growth of undesirable microorganisms and allows the salt-tolerant lactic acid bacteria to ferment the sugars to lactic acid. Sugars equilibrate between the cucumber and the brine and are used as a food source by the microorganisms. The sugars that diffuse from the cucumbers are fermented sequentially by *Leuconostoc mesenteroides*, *Pediococcus cerevisiae*, *Lactobacillis brevis*, and *Lactobacillis plantarum*. Depending on the condition of fermentation, approximately 0.6–1.2% lactic acid is formed in 7–14 days. As the pH is lowered to 3.2, the metabolism of *L. plantarum* is inhibited, and approximately 0.25% sugar remains after lactic acid fermentation has stopped. It is important to ferment as much of the sugar as possible to retard the growth of yeasts. The formation of sufficient lactic acid is an important factor in the quality and preservation of the fermented pickle.

Numerous secondary fermentation reactions also occur in fermenting cucumbers that generate byproducts such as fusel oil components, carboxylic acids, esters, and carbonyl compounds that impart unique flavor and aroma attributes to the pickle and brine. The characteristic aroma of the fermented pickle and brine before the addition of spices and flavoring is difficult to describe but is generally characterized as silage-like with a sour, slightly sweet, green note. Production of the primary and secondary fermentation byproducts and the lack of cucumber flavor development are responsible for the formation of the typical aroma and flavor of fermented pickles. It is believed that the production of the cucumber flavor impact chemicals—most importantly (*E,Z*)-2,6-nonadienal and 2-nonenal), which are formed from the action of lipoxygenase on linolenic and linoleic acids in the cucumber—is inhibited in the pickle because the low pH of the brine inactivates the lipoxygenase. Inactivation of lipoxygenase destroys the pathway for the biogenesis of cucumber flavor compounds (1).

Few studies have been conducted to determine the chemicals responsible for the typical aroma of fermented pickles. Zhou and McFeeters (2) studied volatile compounds present in cucumbers fermented in 2% salt using purge-and-trap (P&T) gas chromatography–mass spectrometry (GC–MS) methods (2). They observed over 100 volatile compounds in GC chromatograms and identified 37 of them by MS. Monitoring these volatiles over the fermentation period (0–10 days), Zhou and McFeeters noted a loss of (*E,Z*)-2,6-nonedienal and 2-nonenal and an increase in linalool to levels several times its odor threshold. Linalool increased to 44 ppb, approximately 10 times greater than its odor threshold level in water (3). Zhou and McFeeters concluded that the contribution of linalool and other compounds to the flavor of fermented cucumbers remains to be determined.

The present study compares volatiles and semivolatiles in a typical fermented cucumber brine sample analyzed by various GC–MS sample preparation techniques including purge-and-trap (P&T), solid-phase extraction (SPE), and solid-phase microextraction (SPME). In order to determine potential aroma and flavor impact compounds and to assess their contribution to the characteristic brine flavor, brine samples were

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further analyzed with an olfactometry detector (OD, sniff port) using the detection frequency method (4,5). Once suspect odorants were identified and their concentrations in brine estimated, synthetic brines were prepared by combining pure chemicals of suspected odorants at a range of concentration levels typically present in brine. The synthetic brines were then evaluated by a sensory panel to determine the closeness of match to authentic tankyard brine. A recombination study of this type was previously used to confirm which odor-active compounds in beet sugar were responsible for its characteristic stale, musty aroma (6).

Using SPME–GC–MS–OD in conjunction with the detection frequency method and recombination studies, it was possible to objectively determine the most important compounds that impact the aroma of fermented pickles and brine. Several of these odor impact chemicals have not been previously reported in the literature.

Experimental

Sampling

In previous unpublished studies, approximately 24 samples (500 mL) of tankyard brine were taken from four different pickle production facilities located in four different states (Michigan, Wisconsin, Arkansas, and North Carolina) over a period of three years. These samples were analyzed by P&T and SPE. At the time, SPME fibers were not commercially available. All brine samples studied had the characteristic aroma of fermented pickles, with only slight odor nuances detected between samples. In this earlier work, *trans*-4-hexenoic acid was detected as a major chromatographic peak in all brine samples in the range of 5 to 114 ppm by SPE, and it was undetectable in all but two of the samples when analyzed by P&T. In the two samples in which *trans*-4-hexenoic acid was detected by P&T, the levels were in excess of 100 ppm and the chromatographic peaks were relatively small.

Data reported in the present work are based on one brine sample taken from a fermentation tank in a Wisconsin pickle processing plant. The odor of the brine was similar to the 24 samples previously analyzed, and chromatographic profiles determined by P&T and SPE were similar to profiles observed in earlier studies, with a few minor differences.

SPE–GC

Twenty milliliters of fermentation brine were added to a 360-mg Waters (Milford, MA) C_{18} Sep-Pak. The SPE cartridge was preconditioned with 2 mL methanol and then rinsed with 5 mL of distilled water prior to the addition of brine. SPE was performed using a 10-mL syringe rather than a vacuum manifold technique. When quantitation was desired, the 20-mL brine sample was spiked with 10 µL of internal standard solution (0.1 mg/mL of 2-ethylhexyl acetate) prior to the addition to the SPE cartridge. Extracted brine components were eluted from the SPE cartridge with 0.5 mL of methanol. P&T-grade methanol (Fisher Scientific, Itasca, IL) was used for prewetting the SPE cartridge and eluting analytes from the cartridge. The volume of injected SPE eluate was 0.5 µL. Splitless injections were made using a 30-m × 0.25-mm-i.d. FFAP column (J&W Scientific, Folsom, CA) with a film thickness of 0.25 µm. FFAP was selected because a significantly higher level of organic acids was extracted by SPE than by P&T. The column temperature program for the FFAP column was as follows: an initial temperature of 50°C was maintained for 3 min, increased at a rate of 6°C/min to 240°C, and then held for 4 min.

SPME–GC

One milliliter of brine, 0.7 g of sodium chloride, and a microstirring bar (Fisher) were placed in a 6-mL glass GC vial (38 × 22 mm) and capped with 20-mm PTFE–silicone septa (Wheaton Scientific Products). The injector was operated in split mode (3:1 split ratio) at a temperature of 275°C. In order to achieve sharp chromatographic peaks with acceptable resolution, it was necessary to use the injector in the split mode of operation. The GC injection port was fitted with a special insert for SPME analysis (Varian, Walnut Creek, CA). The SPME fiber was a 75-µm Carboxen–PDMS (Supelco, Bellefonte, PA). With the fiber exposed, the sample vial was placed in a 50°C water bath (fiber exposure started immediately with the sample at 19°C), and the sample was extracted for 20 min while stirring at 350 rpm. The fiber was placed in the headspace above the sample. To facilitate the thermal desorption of extracted volatiles from the fiber, the SPME fiber remained in the Varian 1078 injector for 3 min. A 30-m × 0.25-mm-i.d. DB-5 fused-silica capillary column (J&W Scientific, Folsom, CA) with a film thickness of 1 µm was used, and the flow rate of the helium carrier gas was 1.0 mL/min. The following column temperature programming sequence was used: an initial temperature of 50°C was maintained for 1 min, increased at a rate of 6.0°C/min to 180°C, held at 180°C for 4 min, increased at a rate of 6.0°C/min to 230°C, and then held for 4 min.

The DB-5 column was selected for SPME because it provided a better resolution of two important odorants (*trans*-4-hexenoic acid and phenyl ethyl alcohol) than the FFAP column. However, acid peaks tended to be broader and less symmetrical with the DB-5 column than with the FFAP column.

P&T–GC

P&T instrumentation employing a Tenax trap and all method parameters (e.g., purge gas volume and rate, purge time and temperature, valve oven temperature, bake temperature and time, type of purge vessel, etc.) have been previously reported (7). The only difference with the previously published P&T method is that the sample size was 3 mL fermentation brine, and no internal standard was added. The same DB-5 column and column temperature program used for SPME were also used for P&T, except that P&T injections were made in the splitless mode and the carrier gas flow rate was 2.28 mL/min for the SPME injections.

MS conditions

The electron ionization mode of the Varian Saturn 3 ion trap detector (ITD) was used. The mass range was set at *m/z* 40–300, the manifold temperature was 190°C, and the transfer

line temperature was 200°C. These MS conditions were used for all P&T, SPE, and SPME analyses.

Olfactometry detection frequency methodology

Olfactometry experiments were conducted on brine samples analyzed by P&T, SPE, and SPME. However, detection frequency olfactometry experiments were all based on samples extracted by SPME. The olfactometry detector (Microanalytics, Round Rock, TX) was heated to 140°C. Connections of the DB-5 analytical column, the sniff port transfer line, and the ITD transfer line to the variable outlet splitter are illustrated in Figure 1. The outlet splitter was set in the 100% open mode. The sniff port transfer line (approximately 1.6 m in length) consisted of 0.021-inch-i.d. × 0.029-inch-o.d. Silcosteel tubing (Restek, Bellefonte, PA). The transfer line to the ITD was a 2.0-m DB-5 capillary column. The helium carrier flow was 2.28 mL/min to the ITD and 4 mL/min to the sniff port. Therefore, the split ratio (sniff port to ITD) was approximately 1.75:1. No make-up gas was connected to the variable outlet splitter. Normally, helium make-up gas at a flow rate of 1–2 mL/min is used to help sweep analytes from the dead volume space in the connection to the splitter. In our case, however, this port on the splitter valve was capped; no significant reduction in peak resolution or broadening of peak shapes was observed.

The intensity and duration of aromas emitted at the sniff port during chromatography were recorded for each observed odorant by pushing a button on a switch connected to a Rainin Instruments A/D converter (Varian). The square signal was registered and recorded by Rainin Dynamax software using a Macintosh computer. Odor assessors verbally described the odors as they emitted from the sniff port, and the verbal odor descriptions and corresponding retention times were recorded manually by an assistant. In this way, an individual aromagram was generated, and the area under each odor peak was obtained using the Rainin HPLC integration software.

Aromagrams for the same brine sample were generated by eight different assessors. The eight individual aromagrams were summed to create one aromagram. The summed areas are referred to here as surface of nasal impact frequency (SNIF) values. Further details of the detection frequency olfactometry technique have been previously published (4,5).

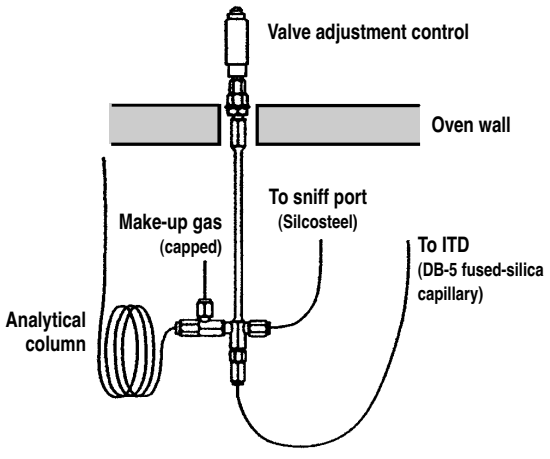

Figure 1. Connections of the analytical column, the ITD transfer line, and the olfactometry detector (sniff port) transfer line to the variable split valve. No make-up gas was used to flush the dead volume in the valve. The valve adjustment screw was set to the 100% open position.

Recombination studies

Finally, once key suspect odorants were identified by SPME–GC–MS–OD and their concentrations in brine estimated, samples of a base brine solution (6500-ppm lactic acid, 500-ppm acetic acid, and 8% sodium chloride in distilled water) were spiked with various combinations and levels of the suspect odorants and evaluated by a sensory panel to determine how closely the aroma of the synthetic brine samples matched the aroma of the authentic brine sample.

Results and Discussion

A comparison of typical chromatograms from P&T, SPE, and SPME with the brine sample analyzed by P&T, SPE, and SPME is illustrated in Figures 2, 3, and 4, respectively. These results dramatically emphasize how the selection of the sample preparation technique influences the amounts and types of chemicals extracted and detected by an analytical procedure. Using P&T, it was possible to detect a greater number of volatiles in the brine than either SPE or SPME. Most of the volatiles detected by P&T and not by SPE or SPME were early-eluting fusel oil components. Preliminary olfactometry studies of volatiles extracted by P&T showed that the fusel oil fraction contributed few significant olfactometry properties, and no single compound was detected in P&T chromatograms that had odor characteristics similar to the fermented brine samples. In general, P&T generated more chromatographic peaks, but few had significant intense odor characteristics. In this case, more peaks did not result in a better understanding of which chemicals were responsible for the odor of fermented cucumbers.

Because P&T was apparently not extracting the impact odor chemical or chemicals, additional sample preparation techniques were investigated. SPE with C_{18} cartridges extracted fewer compounds and did a poor job extracting fusel oil components. However, olfactometry experiments showed that SPE extracted more compounds with strong aromas than P&T, and the odor characteristics of two chemicals (*cis*- and *trans*-4-hexenoic acids) were observed to strongly match the odor of brine samples. One problem observed with SPE was that degradation of nonvolatile brine components (e.g., chlorophyll and other plant pigments) tended to elute as broad peaks at the end of the chromatographic runs. This problem intensified as more samples were analyzed by SPE. These "dirty" injections eventually resulted in column fouling.

Initially, two types of SPME fibers were investigated: 75-µm Carboxen–PDMS and 70-µm Carbowax–DVB Stable Flex. Both fibers were selected for study because a previously published work (8) indicated their appropriateness for extracting organic acids—compounds that SPE studies had shown to be important odorants in the brine. The two fibers performed nearly

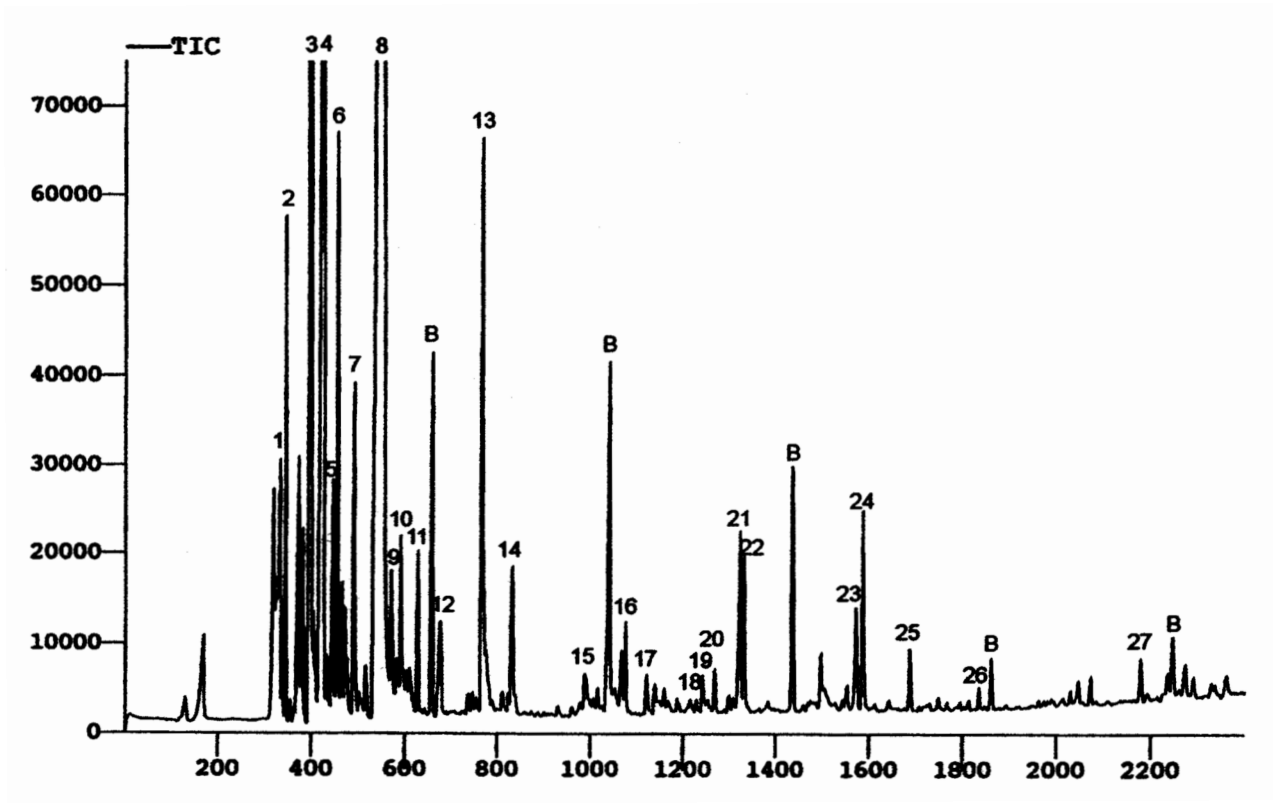

Figure 2. Chromatogram of tankyard brine sample using P&T. Peak identities are indicated in Table I.

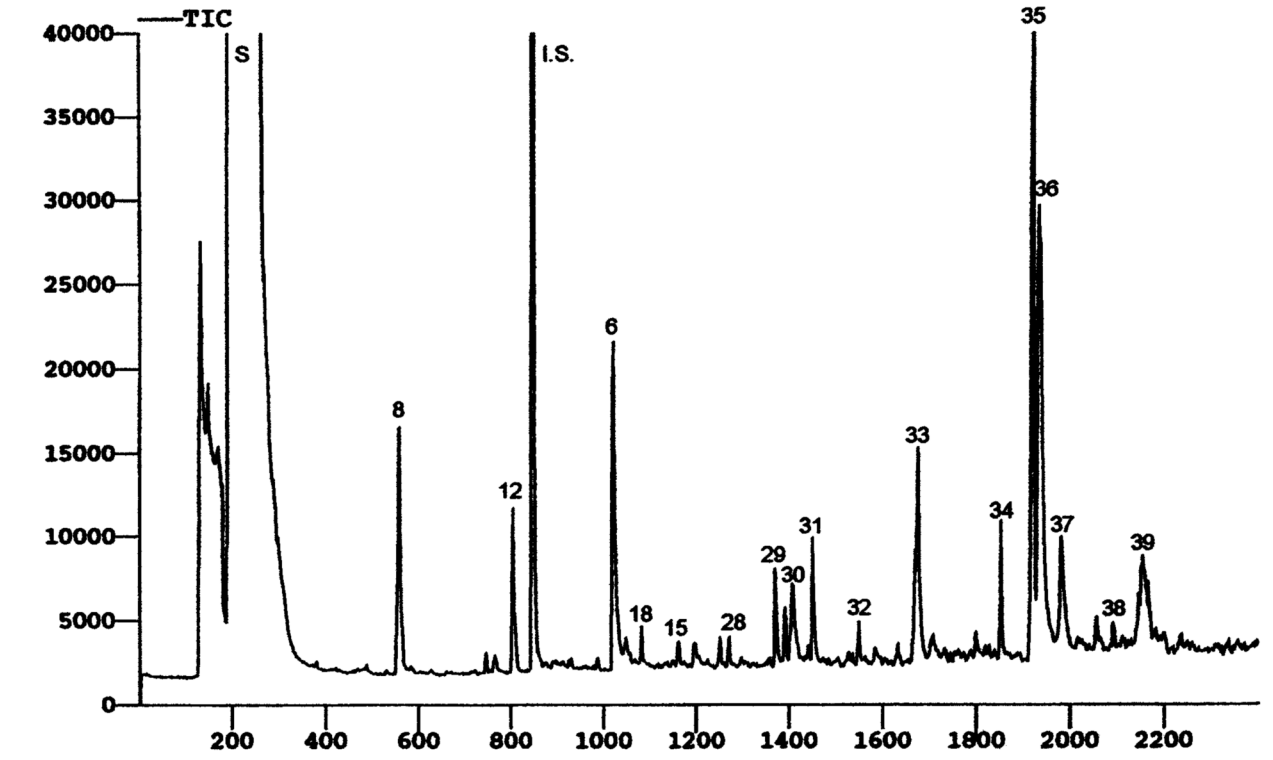

Figure 3. Chromatogram of tankyard brine sample using SPE. Peak identities are indicated in Table I.

equally well, with both extracting detectable levels of organic acids even when present at low parts-per-billion levels. The Carboxen–PDMS fiber demonstrated superior sensitivity for a few of the more volatile compounds compared with the Carbowax–DVB fiber, and it extracted significantly more compounds than SPE. Therefore, SPME using the Carboxen–PDMS fiber was ultimately selected for olfactometry detection frequency experiments.

It is interesting to note that some flavor chemists criticize SPME for not extracting a truly representative sample of the headspace gas above a food sample. These critics point out that static headspace and possibly solvent extraction techniques do a better job of extracting a profile of volatiles that is consistent with what people actually detect when a food product is smelled. Although it is true that for some applications SPME demonstrates a bias for extracting higher boiling compounds over lower boiling point compounds (9), the higher boiling polar components are sometimes the odor impact compounds of interest, and techniques such as static headspace and solvent extraction are not sensitive enough to detect them. P&T techniques in which the aroma chemicals are concentrated on an adsorbant (e.g., Tenax) are also biased in that stripping the liquid sample or flushing the sample surface with a gas causes the most volatile components to be enriched (compared with the less volatile components) and the composition will not be representative of the gas phase at equilibrium as it is perceived by the nose. Every sample preparation technique has its limitations and biases. However, if these limitations and biases are understood, they can be used as an advantage rather than a liability. For example, in the research presented here, the selectivity and bias of SPME is a benefit rather than a detriment in performing flavor research studies.

Table I lists the chemicals identified in the P&T, SPE, and SPME chromatograms of the brine sample and the sample preparation method used. Compounds that were previously observed in brine samples by Zhou and McFeeters (2) are also indicated in the table.

Of the significant odor-active compounds observed, only *cis*- and *trans*-4-hexenoic acids and phenyl ethyl alcohol (or phenyl acetaldehyde, an oxidation product of phenyl ethyl alcohol) were present in all brine samples tested (it should be noted that phenyl acetaldehyde has a floral, lilac, hyacinth aroma). Therefore, these highly odiferous chemicals are likely to be key impact odor components of fermented cucumbers.

The identity of *trans*-4-hexenoic acid was confirmed by MS matching and retention time matching of the suspect peak with a pure authentic *trans*-4-hexenoic acid standard (experimental sample from McCormick Flavors, Hunt Valley, MD). A pure sample of *cis*-4-hexenoic acid was not commercially available. However, chromatographing standard solutions of *cis*- and *trans*-2-hexenoic acids and *cis*- and *trans*-3-hexenoic acids on the FFAP column showed that the unidentified peak eluting immediately after *trans*-4-hexenoic acid was likely *cis*-4-hexenoic acid. Furthermore, the mass spectrum of this peak closely matched that of the *trans*-4-hexenoic acid.

Quantitation of eight brine samples (two from four different pickle processing plants) by SPE using 2-ethylhexyl acetate as internal standard showed that these important odorants were present in the following concentration ranges: *trans*-4-hexenoic acid, 5–114 ppm; phenyl ethyl alcohol, 2–30 ppm; and

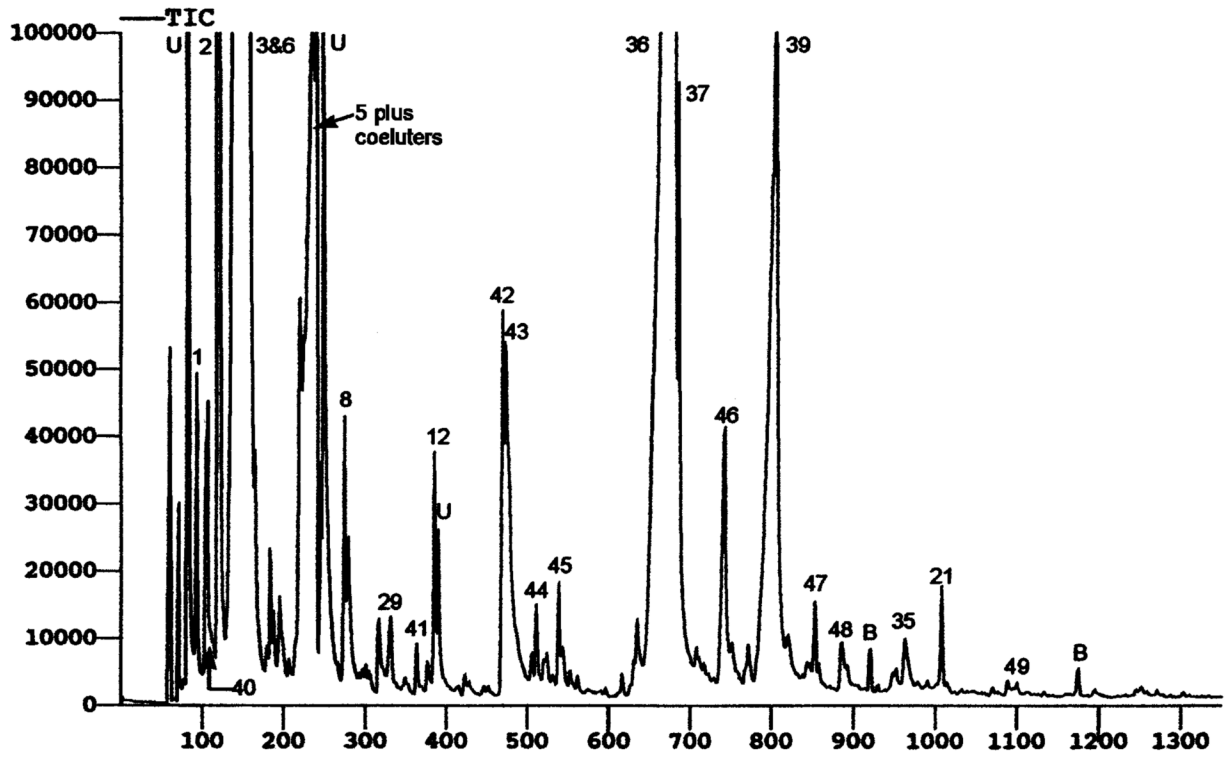

Figure 4. Chromatogram of tankyard brine sample using SPME (75-µm Carboxen–PDMS). Peak identities are indicated in Table I.

Table I. Peak Identification for Chemicals in Brine Analyzed by Various GC–MS Sample Preparation Techniques

Peak number*	Compound	Analytical technique
1	Acetone	P&T, SPME
2	Isopropanol	P&T
3	Ethyl acetate	P&T, SPME, Z
4	Isobutyl alcohol	P&T
5	*n*-Butyl alcohol	P&T
6	Acetic acid	P&T, SPME, Z
7	2-Pentanol	P&T, Z
8	Isoamyl alcohol	all
9	1-Pentene	P&T
10	1-Pentanol	P&T
11	Ethyl butyrate	P&T, Z
12	2,3-Butanediol	P&T, SPME, SPE
13	1-Hexanol	P&T, Z
14	Dihydro-4,5-dimethyl-2[^{3}H]-furanone‡	P&T
15	Benzaldehyde	P&T, SPE, Z
16	Octanal	P&T, Z
17	Dichlorobenzene	P&T
18	2-Ethyl-1-hexanol	P&T, SPE, Z
19	Decanal	P&T, Z
20	Linalool oxide‡	P&T
21	Linalool	P&T, SPME, Z
22	Undecyl aldehyde	P&T
23	α-Terpineol	P&T, SPME
24	Dodecyl aldehyde	P&T
25	Isothiocyanato cyclohexane‡	P&T
26	Tetradecanal	P&T
27	Geranyl acetone	P&T
28	5-Methyl-2-furancarboxaldehyde‡	SPE
29	Butyric acid	SPE, SPME
30	Phenylacetaldehyde	SPE
31	Hexanoic acid	SPE
32	3-(Methylthio)-1-propanol‡	SPE
33	Acetamide‡	SPE
34	Benzyl alcohol	SPE
35	Phenyl ethyl alcohol	SPE, SPME
36	*trans*-4-Hexenoic acid	SPE, SPME
37	*cis*-4-Hexenoic acid	SPE, SPME
38	Phenol	SPE
39	*trans*-2,4-Hexadienoic acid	SPE,SPME
40	Dimethyl sulfide	SPME
41	Hexanal	SPME, Z
42	2-Methyl-1-pentene	SPME
43	2-Heptanol	SPME, Z
44	5-Hepten-2-one‡	SPME
45	2,4-Hexadienal	SPME
46	Nonanal	SPME, Z
47	*cis*-2,4-Hexadienoic acid	SPME
48	2,6-Nonadienal	SPME, Z
49	2-Dodecen-1-al‡	SPME
B	Artifact (not from sample)	
U	Unknown	
S	SPE eluting solvent (methanol)	SPE
IS	Internal standard (4-methyl-2-pentanone)	SPE

* Peak numbers apply to all figures.
† Z refers to previously published results (2) based on P&T–GC–MS with an HP-5 capillary column.
‡ Tentative identification based on MS.

and phenyl acetaldehyde, 0–20 ppm.

Linalool was observed in only 5% of the brine samples tested and therefore is not likely to be a major contributor to the characteristic brine aroma, as was previously implicated (2). The reason for this discrepancy may be that previous studies analyzed cucumbers fermented in only 2% salt solution, whereas samples analyzed here all contained salt levels in the 8–10% range. Variations in salt levels in the brine could impact the composition of the microflora and therefore the types of metabolites and odorants produced.

Olfactometry detection frequency results

Detection frequency results are presented in Figure 5. The larger the SNIF value, the greater the odor impact of the chemical. The seven odorants with the largest SNIF values were (from highest to lowest) *trans*-4-hexenoic acid with a peak number of 36, *cis*-4-hexenoic acid at peak 37, 2-heptanol at peak 43, *cis*-2,4-hexadienoic acid at peak 47 (a tentative identification), phenyl ethyl alcohol at peak 35, 2,6-nonadienal at peak 48, and 2-dodecen-1-al at peak 49 (also a tentative identification). Based on SNIF values, *trans*-4-hexenoic acid was by far the most powerful odorant observed in the brine sample. The *trans*-4-hexenoic acid and *cis*-4-hexenoic acid were detected by all panelists and were the only odorants characterized as definitely similar to the aroma of the brine sample. One other odorant that was observed in many samples of brine but not in the one studied here was phenyl acetaldehyde.

Prior to conducting detection frequency experiments, panelists were given a sample of the brine to smell and asked to determine if any odorants emitted from the sniff port during GC analysis were similar to the odor of the brine.

Without repeating the sniffing at several dilution levels as in the more commonly used olfactometry methods of CHARM, AEDA, and OSME (10,11), the detection frequency approach can be used to determine odor impact chemicals with just a few injections and by untrained panelists. Pollien et al. (4) reported that with the detection frequency method, interpanel reproducibility was comparable with intrapanel repeatability although no training of the panelists was required, contrary to intensity methods. In other words, two independent panels were able

to generate similar aromagrams of a given product.

Recombination study results

The results of recombination studies (Table II) confirmed detection frequency results that indicated *trans*-4-hexenoic acid was the major odor impact chemical in brine. Although the odor of the synthetic *trans*-4-hexenoic acid solutions was close to that of the authentic brine sample, panelists indicated that sensory matching was not perfect. Contributions from *cis*-4-hexenoic acid and several other odorants were not evaluated because of the unavailability of pure standards. One or more of these chemicals is likely to be responsible for the sweet, floral, green note that is lacking in solutions spiked with only *trans*-4-hexenoic acid. Addition of the rose floral note from phenyl ethyl alcohol to the synthetic *trans*-4-hexenoic acid solution provided a somewhat closer match to the aroma of authentic brine samples.

Conclusion

For the first time, the key odor chemicals in fermented cucumber brine have been identified. To our knowledge, the primary impact odorants *trans*-4-hexenoic acid and *cis*-4-hexenoic acid have never been reported in the literature. SPME

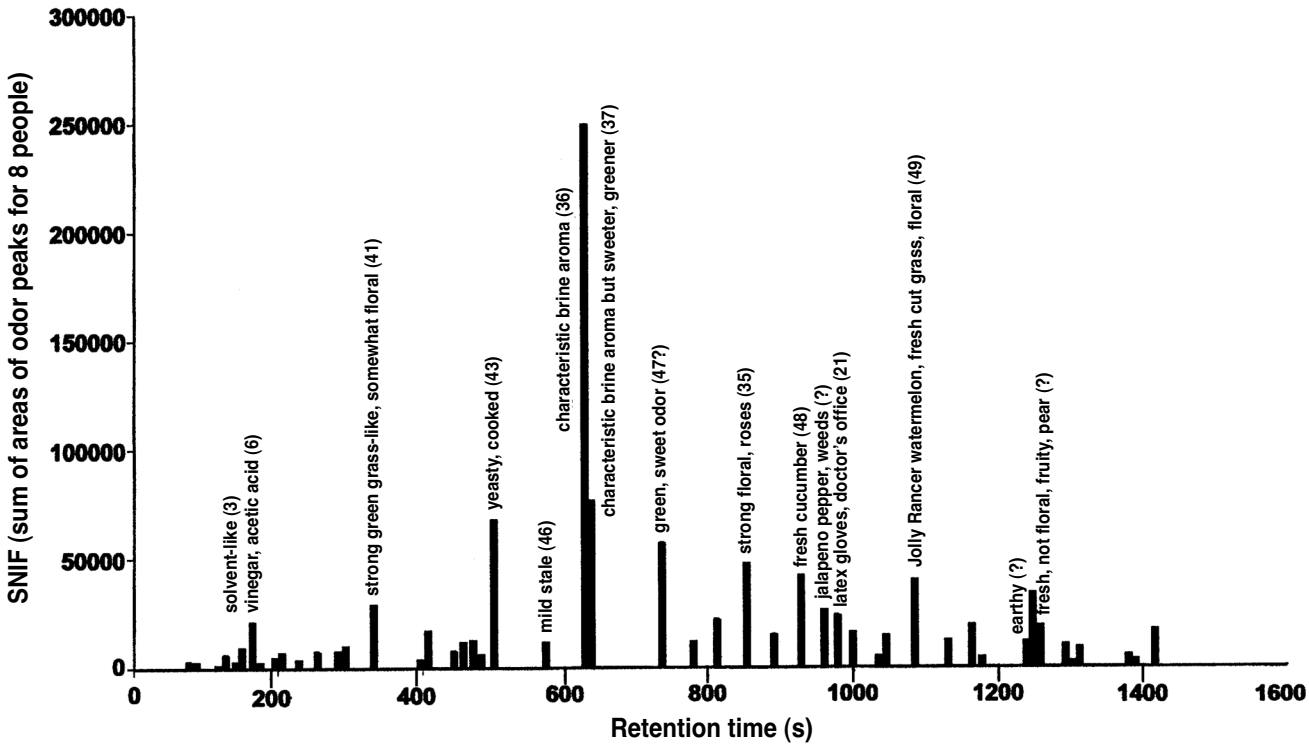

Figure 5. Summed aromagram of olfactometry detection frequency results. Aromagrams for the same brine sample were generated by 8 different assessors. The 8 individual aromagrams were summed to create the aromagram above.

Table II. Recombination Study

Sample	*trans*-4-Hexenoic acid (ppm in base*)	Phenyl ethyl alcohol (ppm in base)	Odor match score	Comment
A	0	0	0	slight vinegar, acetic acid odor
B	2	0	4	mild fermentation aroma
C	10	0	6.3	good fermentation aroma but lacking sweet green notes
D	25	0	7	better match than C but still lacking sweet green notes
E	25	0.5	7	similar odor to D
F	25	10	7.7	somewhat closer match than D and E but missing a sour green note
G	25	40	5	too floral

* Base solvent composed of 6500-ppm lactic acid, 500-ppm acetic acid, and 8% sodium chloride in distilled water.
† Average odor match scores of 3 panelists. Match score of 0 is no comparison to typical brine odor; match score of 10 is perfect match to typical brine odor.

proved to be superior to P&T and SPE for extracting these key odorants from brine. The detection frequency olfactometry method was a rapid, efficient, and objective way to identify volatiles and semivolatiles that were most important to the characteristic odor of brine. Agreement between detection frequency results and recombination studies further substantiated that *trans*-4-hexenoic acid is the key impact odorant in fermented cucumber brine.

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