Evaluation of Fat-Derived Aroma Compounds in Blue Cheese by Dynamic Headspace GC/Olfactometry–MS

Michael Qian[a,*], Cara Nelson[b], and Scott Bloomer[b]

[a]Department of Food Science and Technology, Oregon State University, Corvallis, Oregon 97331, and
[b]Strategic Technology Development Section, Land O'Lakes, Inc., Arden Hills, Minnesota 55126

ABSTRACT: The aroma compounds in Blue cheese were studied with a dynamic headspace–GC/olfactometry (GC/O-DH) technique and evaluated by both aroma extract dilution analysis (AEDA) and the Osme technique to identify and assign importance to aroma compounds. AEDA aroma dilution values were obtained by sequential reduction of sample size, and Osme values were obtained based on aroma intensity and duration. The most important compounds contributing to Blue cheese aroma were diacetyl, 2-methylpropanal, 3-methylbutanal, ethyl butanoate, ethyl hexanoate, methional, dimethyl trisulfide, 2-heptanone, and 2-nonanone. Compounds central to the characteristic Blue cheese aroma and typically derived from milkfat included 2-heptanone, 2-nonanone, butanoic acid, hexanoic acid, and ethyl esters. Since the methyl ketones contributed the characteristic Blue cheese aroma, a flavor concentrate enriched in methyl ketones to impart characteristic Blue cheese aroma was synthesized using *Penicillium roqueforti* spores in a model system. A fivefold enhancement in methyl ketone production was obtained by providing aerobic conditions. The addition of sucrose and serine, compounds known to affect mold spore metabolism, did not enhance methyl ketone production but caused generation of an uncharacteristic fruity, floral aroma.

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KEY WORDS: AEDA, biocatalyst, Blue cheese aroma, dynamic headspace GC/olfactometry, FA, flavor dilution value, methyl ketones, Osme.

Aroma compounds in foods are typically identified by GC/olfactometry (GC/O) techniques, which are based on the sniffing by the researcher of GC effluents of aroma extracts. The relative aroma importance of aroma compounds can be assigned by several methods, including Charm analysis (1), aroma extract dilution analysis (AEDA) (2), or Osme analysis (3). Both Charm analysis and AEDA are based on the sequential dilution of an aroma concentrate. In Charm analysis, both the sensory character and the aroma duration of compounds eluting from a GC column are recorded, and the importance of individual aroma peaks to the overall aroma profile is determined by the time–intensity combination. In AEDA, an aroma concentrate is sequentially diluted and analyzed by GC/O. The aroma profile of sequential dilutions is recorded, and the maximal dilution value at which the aroma compound is detectable from the GC effluents is used to calculate the flavor dilution (FD) value. AEDA has been used to study the important aroma compounds in several cheeses, including Cheddar (4,5), Camembert (6), Emmentaler (7), and Grana Padano cheeses (8).

Because the AEDA technique is based on sequential dilution analysis, it is extremely labor-intensive and time-consuming. Fast analysis is highly desirable in an industrial research setting. An alternative method was described by McDaniel *et al*. (3) as the Osme technique. In the Osme technique, an aroma extract is injected into a GC, and the aroma description and intensity of each peak are recorded. Higher Osme numbers are assigned to the more intense aromas, which are considered to be more important. Since this technique is simple and fast, it was evaluated in this study and compared with the AEDA technique for rapid identification of the important aroma compounds in Blue cheese.

Aroma extracts for GC characterization can be obtained by several methods, including solvent extraction, static headspace sampling, and dynamic headspace sampling. In certain circumstances, the headspace techniques are preferred because they are milder than solvent extraction and provide solvent-free aroma isolates that are more representative of the aroma composition perceived in food. In addition, they are simple and reliable and can be highly automated. Large amounts of analytes can be effectively concentrated and injected onto the GC column. Dynamic headspace techniques have been broadly applied to identify or quantify volatile compounds and have only recently been combined with GC/O and adapted to the study of the relative potency of aroma compounds (9).

Here, a rapid, dynamic headspace GC/Osme technique was applied to the study of aroma compounds derived from lipids in Blue cheese. The important aroma compounds were identified by FD values of AEDA and compared with Osme values. The production of some of the compounds that contribute the characteristic Blue cheese aroma was evaluated using *Penicillium roqueforti* spores as biocatalysts in a model system.

MATERIALS AND METHODS

Cheese sample. A crumbled Blue cheese (Blue Moon Cheese, Thorp, WI) was used for this study. The sample was carefully

*To whom correspondence should be addressed at Department of Food Science and Technology, 100 Wiegand Hall, Oregon State University, Corvallis, Oregon 97331. E-mail: Michael.qian@orst.edu

selected by several experienced cheese experts at Land O'Lakes, Inc. (St. Paul, MN) and judged to be "typical." The sample was frozen at −20°C and used within 1 mon.

Dynamic headspace (purge-trap) instrument. A purge-trap system (O.I. Analytical, College Station, TX) consisting of a sample concentrator, a multiple purge module, and a multiple heating controller was used in this study. Helium was used for the purge gas as well as the desorption gas. The purge gas flow rate was controlled by the purge-trap sample concentrator. The desorption gas was introduced from the gas chromatograph as an auxiliary gas. The communication between the purge trap sample concentrator and the GC–MS was achieved by using the Handshake Pigtail connector (part #252213; O.I. Analytical). The desorption gas was routed to the inlet of the GC–MS, and the desorption signal from the purge trap sample concentrator triggered the start of each chromatographic analysis in the GC–MS system.

Dynamic headspace sampling. Blue cheese samples were placed into 25-mL frit sample spargers (O.I. Analytical) that were loaded onto the dynamic headspace (purge-trap) instrument. Samples were heated to 50°C with a heating mantle and equilibrated for 10 min at 50°C. The volatile aroma compounds were purged from the sample with helium for 60 min at 50°C at a purge gas flow rate of 40 mL/min and transferred to the sample concentrator through a 60-inch heated silicosteel line. In the sample concentrator, the analytes were retained in a Tenax trap (14 cm, trap #7; O.I. Analytical) held at 20°C during purging. After the sample purging process was completed, the trap was dry purged for 5 min with helium to remove condensed water. The aroma analytes were next desorbed from the trap by heating to 250°C for 4 min and then transferred to the injection port of the gas chromatograph through a silicosteel line maintained at 90°C. The trap was baked at 260°C for 20 min after each sample run. An HP-FFAP column (Hewlett-Packard, Palo Alto, CA) (30 m, 0.32 mm i.d., 0.5 μm film thickness) was used for chromatographic resolution. The injection port temperature was set at 240°C with a split ratio of 10:1. The GC oven temperature program was held at 35°C for 4 min, heated from 35 to 130°C at 2°C/min, 130 to 250°C at 4°C/min, and held at 230°C for 5 min.

GC/O–MS. An olfactometry device was installed directly at the interface of a GC–MS system. A column splitter at the end of the GC column directed part of the effluent to the MS through a deactivated fused-silica column (50 cm, 0.1 mm i.d.) and the remainder of the effluent to the olfactometry device. A column flow rate of 2 mL/min of helium was used for chromatographic separation, and 0.8 mL/min of column flow was directed to the mass spectrometer while 1.2 mL/min of column flow was directed to the olfactometer. The sniffing port of the olfactometer was heated with a heating tape to eliminate condensation. A gentle stream of humidified air was mixed with column effluent gas in the olfactometry device to prevent drying of olfactory mucous membranes. The MS temperature was set at 280°C. The mass scan range was from 29 to 300 mass units.

AEDA analysis. A series of sequentially reduced samples of Blue cheese (10.28 to 0.01 g, each sample half the weight of the previous sample) was subjected to dynamic headspace extraction and chromatographic analysis. The aroma compounds detected at the smallest sample size (0.01 g) were assigned an FD value of 1024, whereas the aroma compounds detected at the highest sample size (10.28 g) were assigned an FD value of 1. All compounds were identified by mass spectra, aroma characteristics, and retention index.

OSME analysis. Osme analysis was performed in duplicate by one well-trained flavor chemist. Aroma attributes and intensities were recorded, and the Osme value was assigned using a 10-point scale with 10 representing extremely strong.

Production of enzyme-modified butterfat. Enzyme-modified butterfat was prepared according to Tomasini *et al.* with slight modifications (10). Emulsifying salts [monosodium phosphate (3 g), disodium phosphate (0.75 g), and citric acid (0.3 g)] were dissolved in 275 mL water. Butter (454 g) was melted, and 8.25 g lecithin (Emulpar NP-1; Lucas Meyer, Decatur, IL) and 56 g nonfat dry milk (Land O'Lakes) were vigorously mixed into the melted butter. The emulsifying salts slurry and the melted butter slurry were combined and heated to 77°C and held for 5 min. After cooling to 43°C, 484 mg of Lipase AY-30 (EC 3.1.1.3, from *Candida rugosa;* Amano Enzyme USA Co. Ltd., Lombard, IL) was added to the slurry and incubated for 30 min at 43°C with continuous overhead stirring. This slurry was then allowed to cool to room temperature (21°C) and stirred for 18 h. The resulting enzyme-modified butterfat was then heated to 80°C and held for 5 min to inactivate lipase.

Blue cheese flavor concentrate model system. Pure dried, powdered *P. roqueforti* conidia (Chr. Hansen, Inc., Milwaukee, WI) were used to synthesize a Blue cheese flavor concentrate according to Kosikowski and Jolly (11) with minor modifications. Substrate for Blue cheese flavor production consisted of enzyme-modified butterfat (46 g), cottage cheese whey powder (87 g), and NaCl (5.5 g) slurried in water (361 g) and homogenized. Blue mold conidia (20 mg) were hydrated in water (2 mL) for 60 min and added to the slurry. The mixture was incubated at 25°C for 48 h in a closed system. To determine the effects of aeration on Blue cheese flavor development, an identical slurry was prepared and sparged with humidified air during incubation ("aerobic incubation"). To determine the effects of added compounds reported to enhance development of Blue cheese flavor, sucrose (0.5 g) and L-serine (0.5) were added to a third slurry and incubated with aeration. After incubation, Blue cheese flavor concentrates were stored frozen (−20°C) until analyzed. Dynamic headspace sampling of 0.1-g samples and GC analysis were carried out as described above, and the MS was used as the detector. Peak areas were used for qualitative comparison.

RESULTS AND DISCUSSION

Eighteen compounds were found to contribute to Blue cheese aroma by both AEDA and Osme techniques; 17 were identified by MS, retention index, and aroma character (Table 1). The sum of aroma contributions of such a large number of

TABLE 1
Comparison of Blue Cheese Aroma Determined by AEDA (FD) and Osme Technique[a]

Compound	Retention index (FFAP column)	Aroma description	AEDA FD value	Osme value
Diacetyl	987	Buttery	1024	9
?	1349	Baked	512	9
Methional	1480	Baked potato	512	9
Butanoic acid	1651	Sweaty, cheesy	512	4
2-Heptanone	1186	Blue cheese	256	10
3-Methyl-butanoic acid	1688	Sweaty, cheesy	256	4
Dimethyltrisulfide	1383	Cabbage	128	6
2-Nonanone	1410	Blue cheese	128	8
Ethyl hexanoate	1225	Fruity	64	4
2-Undecanone	1616	Musty	64	3
Phenylacetaldehyde	1668	Floral	64	2
Ethyl butanoate	1039	Fruity, banana	32	5
Hexanoic acid	1869	Sweaty, cheese	32	3
3-Methylbutanol	1208	Malty	16	4
2-Heptanol	1325	Fruity	8	3
2-Hexanone	1086	Fruity	2	3
Acetoin	1294	Malty	1	3
2-Pentanone	983	Malty, fruity	1	3

[a]AEDA, aroma extract dilution analysis; FD, flavor dilution.

discrete compounds is the reason for the very complex nature of Blue cheese aroma. When detected alone at the GC/O port, only two methyl ketones (2-heptanone and 2-nonanone) contributed the aroma characteristic of Blue cheese (Table 1). Six of the remaining compounds were fruity, three were malty, and three were sweaty/cheesy.

When analyzed by AEDA, several compounds in Blue cheese were found to have high FD values (Table 1). Diacetyl, methional, butanoic acid, 2-heptanone, dimethyltrisulfide, 2-nonanone, 3-methylbutanoic acid, and an unknown compound were clearly detectable at high dilutions (FD ≥ 128), so they are probably very important to Blue cheese aroma. These eight compounds contributed six distinct aroma notes to Blue cheese. Interestingly, the two methyl ketones so distinctive of Blue cheese aroma did not have the highest FD values. Ethyl hexanoate, 2-undecanone, phenylacetaldehyde, ethyl butanoate, and hexanoic acid had FD values of 32–64, so they probably have some impact on the Blue cheese aroma profile and may contribute especially to the background aromas. Although detectable, the contributions of 2-heptanol, 2-hexanone, acetoin, and 2-pentanone to Blue cheese aroma are probably relatively small.

In the Osme technique, sequential dilution is not used. Instead, the aroma intensity of individual peaks is assigned by the sniffer. High-intensity aroma compounds could have bigger impacts on the aroma of the food, thus are more important (3). The highest Osme values (6–10) were assigned to six of the eight compounds with highest FD values, with the highest value being assigned to a methyl ketone typical of Blue cheese, 2-heptanone. 2-Nonanone was also among those assigned a very high Osme value (8). In addition, ethyl butanoate, 3-methylbutanaol, ethyl hexanoate, butanoic acid, and 3-methylbutanoic acids were assigned intermediate Osme values (4–5) by the Osme technique and may contribute to background aroma. The remaining compounds were detected, but their contribution to Blue cheese aroma is suspected to be quite small.

Good agreement between the FD values of AEDA and the Osme values was obtained (Table 1). Aroma compounds with high Osme values also showed high FD values. Since Osme value is based on perceived intensity while AEDA is based on threshold aroma detection, we suspected that compounds with high FD values may not have high Osme values. However, this was not the case, and the Osme technique effectively selected important aroma compounds in Blue cheese with reduced analysis times. However, FD values offered a higher level of discrimination for compounds believed to make only minor contributions to flavor (FD values 1–16). The dynamic headspace method used in this experiment only recovers the compounds with high and intermediate volatilities. Compounds with low volatility (high boiling point) or high polarity will not be recovered with this technique. Other techniques such as solvent extraction-high vacuum distillation could be used to recover those compounds.

Cheese is a very complicated physical, biochemical, and microbiological matrix. Cheese flavor is developed in this system through a series of chemical and biochemical reactions during the cheese-aging process. The volatile compounds in cheese are mainly derived from three major metabolic pathways: lipid catabolism, lactose catabolism, and protein catabolism. Lipid catabolism produces aldehydes, ketones, FA, lactones, and esters. Lactose catabolism produces organic acids, alcohols, aldehydes, and dicarbonyls. Protein catabolism generates peptides, amino acids, Strecker aldehydes, and sulfur compounds.

Compounds generated by lipid metabolism predominated among the aroma compounds identified in Blue cheese. Methyl ketones central to the aroma of Blue cheese are produced by β-oxidation of FFA and are normally dominant in mold-ripened cheeses. The methyl ketones, 2-heptanone and 2-nonanone, were two of the most important aroma compounds in this cheese, contributing the characteristic Blue cheese aroma. Although 2-nonanone predominates in many soft mold-ripened cheeses (13), both AEDA and Osme results suggest that 2-heptanone was more important than 2-nonanone in the experimental Blue cheese. 2-Pentanone, 2-hexanone, and 2-undecanone also contributed to the aroma of this cheese. In Camembert cheese, which is also mold-ripened, methyl ketones are the most abundant aroma compounds, with concentrations on the order of 25 to 60 μmol/100 g of fat (14). The major methyl ketones in Camembert cheese are also 2-heptanone and 2-nonanone. Ketones are also important contributors to the aroma and flavor of non-mold-ripened cheeses, and were found to be the major components in the headspace of Parmigiano Reggiano cheese (15). Methyl ketones with chainlength from C3 to C15 and ethyl ketones with chainlength from C5 to C8 have been identified in Parmesan cheese. Methyl ketones can be reduced to 2-alkyl alcohols; we found that 2-heptanol was present in

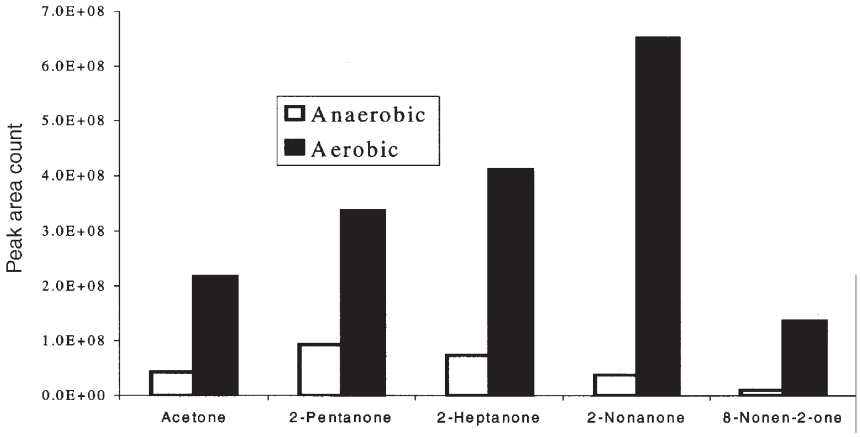

FIG. 1. Effect of aerobic condition on methyl ketone production.

Blue cheese aroma, and that it contributed a weak fruity component to the aroma of this cheese.

Most FFA in cheese are derived from lipolysis of milkfat, although proteolysis and lactose fermentation during cheese ripening also can contribute certain FA. Esterases and lipases hydrolyze milkfat TAG to FFA. Butanoic and hexanoic acids mainly come from TG hydrolysis by milk and microbial lipases during cheese aging. A small proportion of butanoic acids can be produced from lactose fermentation by microorganisms. Butanoic and hexanoic acids were the major straight -chain FFA contributing to the Blue cheese aroma in this study. They contributed sweaty, cheesy, lipolyzed aroma notes. Branched-chain FFA can be generated by hydrolysis from milkfat, or by microbial amino acid degradation to generate branched-chain FA such as 2-methylpropanoic and 3-methylbutanoic acids. We found that 3-methylbutanoic acid contributed a sweaty, cheesy component to Blue cheese aroma. 3-Methylbutanoic acid has been reported to be important to several varieties of cheeses (5,6,8) and has a cheesy, rancid aroma.

Esters are common constituents of cheese flavor. Esters are formed from the esterification of an alcohol and a FFA by chemical or enzymatic pathways. One such enzyme is carboxyl esterase, which is present in most of the microorganisms involved in cheese ripening. Ethyl butanoate and ethyl hexanoate were present in Blue cheese but had low FD and Osme values, so their contribution to the aroma was not central. Methyl, ethyl, propyl, butyl, and isobutyl esters of even-numbered carbon-chain acids from C_2 to C_{16} have been found in Parmesan cheese (15,16). High concentrations of ethyl esters contribute to the important sweet, estery, fruity notes of Parmesan cheese (16).

Diacetyl was identified as very important to this cheese, with the highest FD value and a very high Osme value. Diacetyl is produced by microorganisms mainly from lactose and citrate. Diacetyl and acetaldehyde are present in large quantities in fresh cheeses; lactic acid bacteria, especially *Lactococcus lactis* ssp. *lactis* biovar *diacetylactis,* are producers of diacetyl and acetoin (17). As cheese ages, most diacetyl undergoes chemical reactions to generate other compounds that contribute to the desirable aroma of matured cheese. Diacetyl can be reduced to acetoin, which we also detected in Blue cheese, and the latter can be further reduced to 2,3-butanediol (18), which does not have a flavor impact.

Methional and dimethyltrisulfide are important aromas in many types of cheeses (5–7) and were also identified as important contributors to this cheese. Methional is generated by Strecker degradation of methionine and contributes a baked potato-like aroma. Dimethyltrisulfide is generated from methanethiol and contributes a cabbage-like or onion-like aroma note. Phenylacetaldehyde, an important aroma compound in Camembert cheese (6), can be formed by Strecker degradation of phenylalanine, and contributes a floral aroma to Blue cheese.

Since the methyl ketones were identified as being the aroma components central to Blue cheese aroma and flavor, production of methyl ketones by biocatalysts has attracted much interest. Flavor concentrates generated in this manner are generally not enriched in all flavor components but contain large amounts of specific target aroma molecules. To demonstrate the utility of the analytical methods discussed here in a practical context of industrial relevance, we investigated methyl ketone production by *P. roqueforti* spores. Industrial research demands analytical methods that are rapid and robust; as Blue cheese aroma compounds are of significant utility and application in the food industry, methyl ketones are an important class of biosynthetic target compounds. Production of Blue cheese concentrates from lipolyzed dairy fat is preferred if the FFA level is kept low enough to prevent inhibition of spore metabolism (10). Acetone, 2-pentanone, 2-heptanone, 2-nonanone, and 8-nonen-2-one were the major ketones produced by the mold spores (Fig. 1). In flavor concentrates incubated under anaerobic conditions, 2-pentanone predominated, and the concentrations of 2-heptanone and 2-nonanone decreased as methyl ketone chain lengths increased.

The production of methyl ketones by *P. roqueforti* spores was dramatically different under aerated conditions, and was enhanced fivefold relative to anaerobic incubation (Fig. 1). Methyl ketone concentrations increased as chain lengths increased, and 2-nonanone became most abundant. The addition of sucrose and

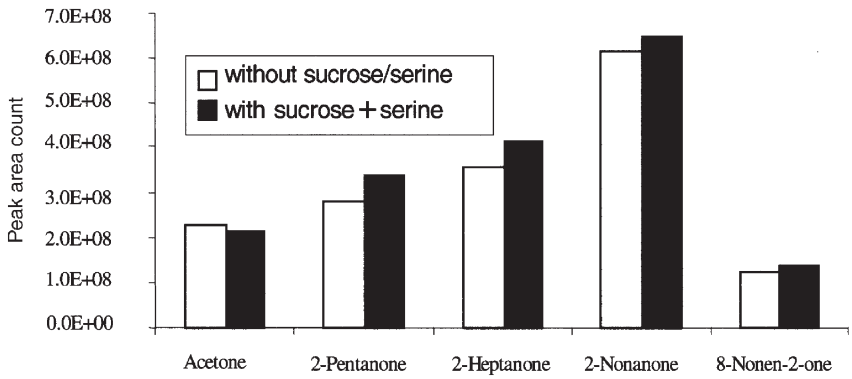

FIG. 2. Effect of sucrose and serine on methyl ketone production.

serine, compounds known to affect mold spore metabolism (12), did not enhance methyl ketone production (Fig. 2).

Penicillium roqueforti, P. camemberti, and *Geotrichum candidum* are important molds used to produce methyl ketones. These molds oxidize FA to keto acids by β-oxidation *via* β-ketoacyl-CoA. The β-ketoacids are then rapidly decarboxylated by β-keto-acyl-decarboxylase (12) to methyl ketones. The β-oxidation pathway of FFA generally produces methyl ketones with one carbon less than the parent FA. However, the parent FA may not be the sole source for 2-heptanone and 2-nonanone because their concentrations in cheeses are extremely high, whereas the concentrations of octanoic and decanoic acids are relatively low in milkfat. A study of the oxidation of ^{14}C-labeled palmitic and lauric acids by *P. roqueforti* spores revealed the formation of 2-heptanone and 2-nonanone from the labeled FFA (19). Furthermore, addition of oleic acid in a milk-based medium causes an increase in production of 2-heptanone and 2-nonanone by *P. camemberti*, and addition of lauric acid does not increase the production of 2-undecanone (20). These studies suggested that successive β-oxidation cycles of long-chain FA are involved in the generation of short-chain methyl ketones. The β-oxidation metabolic pathway of FA is metabolically very important since it allows microorganisms to detoxify FFA in the media (10). Both the mycelium and spores are able to metabolize FA to methyl ketones. The mycelium of *P. camemberti* uses individual FA more rapidly than that of *P. roqueforti* but is less resistant to FFA inhibition (10).

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