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Key Odorants of Oscypek, a Traditional Polish Ewe's Milk Cheese

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ABSTRACT: The unique flavor of Oscypek, a Polish ewe's milk smoked cheese, is described as slightly sour, piquant, salted, and smoked. In this paper with the application of gas chromatography−olfactometry (GC-O) and combination of aroma extract dilution analysis (AEDA) 20 potent odorants of this cheese have been identified within the flavor dilution factor (FD) range of 4−2048. Among them, 2-methoxyphenol, 2-methoxy-4-methylphenol, 4-methylphenol, and butanoic acid showed the highest FD factors. Quantification results based on labeled standard addition followed by calculation of odor activity values (OAV) of 13 compounds with the highest FD factors revealed that 11 compounds were present at concentrations above their odor threshold values and therefore mostly contribute to the overall aroma of smoked ewe's milk cheese. Six of those compounds were represented by phenolic derivatives, with the highest OAV for 2-methoxyphenol (1280). Analysis of key odorants of an unsmoked cheese sample showed that the smoking process had a fundamental influence on Oscypek aroma formation.

KEYWORDS: smoked ewe's milk cheese, key odorants, phenols, AEDA

INTRODUCTION

Oscypek cheese is a brine-matured and smoked cheese made exclusively in the Podhale region of Polish Tatra Mountain. It is made from raw ewe's milk according to a traditional mountain recipe, which has been lately (2008) approved by the PDO (protected designation of origin) Regulatory Board. Oscypek has the shape of the spindle with a characteristic pattern imprinted by a carved wooden form in which Oscypek is shaped. The unique flavor of Oscypek, described as slightly sour, piquant, salted, and smoked, is conditioned by many factors such as milk flavor of a special breed of sheep; use of natural microflora; and traditional technology of production, which includes hand processing, application of wooden tools, and natural preservation methods such as brining and smoking in shepherds' huts. It can be assumed that at least some of these factors will influence Oscypek flavor, as it has been previously reported that animal feeding, origin of milk, or cheese-making method determine the cheese variety aroma.[1−3] The production of Oscypek has been described in detail in our previous paper.[4]

The increasing demand for traditional artisan foods labeled as PDO products such as Oscypek requires their full chemical, microbial, and sensory characterization to enable their differentiation from similar foods without this label.[5] Flavor is one of the key characteristics that determines cheese quality.[6,7] Although there are research papers describing the volatile profiles of different smoked cheeses[8,9] including Oscypek cheese,[4,10] only with the application of olfactometric tools are we able to identify the key odorants responsible for the specific aroma. There are a number of papers describing potent odorants of some type of cheeses: Cheddar, Emmentaler, Grana Padano, Camembert, or Gorgonzola.[11−16] However, to the best of our knowledge there are no data on smoked cheeses. In this paper with the application of gas chromatography−olfactometry (GC-O) and combination of aroma extract dilution analysis (AEDA),[17] the most potent odorants of ewe's milk smoked cheese have been described. Afterward, to complete sniffing results, odor activity values (OAVs) have been calculated with quantitative measurements and the correlation of odor threshold values. To describe the role of the natural smoking process in the development of the Oscypek cheese flavor, all analyses have been performed on both smoked and unsmoked cheeses.

MATERIALS AND METHODS

Cheese Samples. Smoked and unsmoked Oscypek cheeses with PDO certificate were obtained in the Podhale region directly from shepherds' huts called "Bacówka u Kazka", located in Leśnica. All 36 gathered samples (25 smoked and 11 unsmoked) were stored under vacuum and frozen until analyzed.

Chemical Standards. Solvents, such as diethyl ether, methylene chloride, and sodium sulfate, were obtained from Sigma-Aldrich (Poznań, Poland). The following reference aroma compounds were also purchased from Sigma-Aldrich: 2,3-butanedione, acetic acid, butanoic acid, 3-methylbutanoic acid, 2,3-dimethylpyrazine, 3-(methylthio)propanal, ethyl 4-methylpentanoate, dimethyl trisulfide, 2-acetylpyrazine, phenylacetaldehyde, 4-hydroxy-2,5-dimethyl-3(2*H*) furanone, 4-methylphenol, 2-methoxyphenol, 2,6-dimethylphenol, 3-hydroxy-2-methylpyran-4-one, 2,4-dimethylphenol, 3-ethylphenol, 2-methoxy-4-methylphenol, [$^2H_3$]-acetic acid, [$^{13}C_2$]-butanoic acid, and [$^2H_3$-phenol]. The following seven compounds were purchased from aromaLAB AG (Freising, Germany): [$^{13}C_4$]-2,3-butanedione, [$^2H_2$]-3-methylbutanoic acid, [$^2H_3$]-3-(methylthio)propanal, [$^2H_3$]-2-acetylpyrazine, [$^2H_3$]-2-methoxyphenol, [$^2H_3$]-3-hydroxy-2-methylpyran-4-one, and [$^2H_3$]-2-methoxy-4-methylphenol. The purity of solvents and reference standards was no lower than 99 and 97%, respectively.

Isolation Method. For both unsmoked and smoked Oscypek, the whole cheese without peeling of the skin was cut into 1 cm cubes, frozen in liquid nitrogen, and ground to obtained a homogenized sample (700 g). In the next step, 100 g of ground cheese was transferred into an Erlenmeyer flask and extracted separately with two solvents of different polarities, diethyl ether (200 mL) and methylene chloride (200 mL), for

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2 h each. Both fractions were filtered and combined prior to distillation, which was performed using a solvent-assisted flavor evaporation method (SAFE) described by Engel et al.[18] During this procedure, the temperature of the water bath was held at 40 °C, and the pressure was reduced using an Edwards RV5 rotary vane pump (<300 mTorr). Distillate with volatile flavor compounds was collected in a flask cooled with liquid nitrogen. After 30 min of distillation, the solution was dried over anhydrous Na_2SO_4, and the fraction was concentrated to about 400 μL using a Kuderna Danish concentrator.

Gas Chromatography−Olfactometry (GC-O). GC-O was performed on an HP 5890 chromatograph using the following capillary columns: SPB-5 (30 m × 0.53 mm × 1.5 μm) and Supelcowax 10 (30 m × 0.53 mm × 1 μm; Supelco, Bellefonte, PA). The GC was equipped with a Y splitter dividing effluent between an olfactometry port with humidified air as a makeup flow and a flame ionization detector. The operating conditions were as follows for the SPB-5 column: initial oven temperature, 40 °C (1 min), raised at 6 °C/min to 180 °C and at 20 °C/min to 280 °C. Operating conditions for the Supelcowax 10 column were as follows: initial oven temperature, 40 °C (2 min), raised to 240 °C at 8 °C/min rate, held for 2 min isothermally. For all peaks and flavor notes, retention indices were calculated to compare results obtained by GC-MS with literature data. Retention indices were calculated for each compound using a homologous series of C_7−C_{24} *n*-alkanes.[19]

Gas Chromatography−Mass Spectrometry (GC-MS). Compound identification was performed using two instruments: a Hewlett-Packard HP 7890A GC coupled to a 5975C MS (Agilent Technologies) with a Supelcowax10 column (30 m × 0.25 mm × 0.25 μm). Operating conditions for GC-MS were as follows: helium flow, 32.2 cm/s; oven conditions were the same as for GC-O. Mass spectra were recorded in electron impact mode (70 eV) in a scan range of m/z 33−350. Additionally, to confirm the identities of the compounds, samples were run on GC×GC-TOF-MS (Pegasus IV, Leco) running in both one- and two-dimension modes. The GC was equipped with a DB-5 column (25 m × 0.200 mm × 0.33 μm) and a Supelcowax 10 (1.3 m × 0.1 mm × 0.1 μm) as a second column. For one-dimensional analysis the secondary oven was kept at a temperature that was 30 °C higher than the first oven, for which a temperature program was used from 40 °C (1 min) at 5 °C/min to 220 °C and kept for 5 min. Mass spectra were collected at a rate of 30 scans/s, and the detector voltage was 1750 V. For two-dimensional analysis the temperature of the second oven was kept 5 °C higher than first oven. Modulation time was optimized and set at 7 s, and mass spectra were collected at a rate 200 scans/s. Identification of volatiles was performed in two ways, depending on the availability of standard compounds: full identification comprising comparison of mass spectra, retention indices (RI), and odor notes on two columns of different polarities was performed when the reference standard of the investigated compound was available. In some cases the MS signal of the analyte was too weak to facilitate mass spectra comparison. In these cases RI and odor notes of the compounds were compared to a reference standard. When standards were not available, tentative identification was performed on the basis of the comparison of the mass spectrum of a compound with a NIST 05 library match and comparison of retention indices with that available in the literature. Also, the odor characteristics for an analyzed compound were compared with literature data and used in tentative identification.

AEDA. The flavor dilution factor (FD) of each of the odorants was determined by an AEDA.[17] The flavor extract (2 μL) was injected into a GC column. Odor-active regions were detected by GC−effluent sniffing (GC-O), and three panelists determined the description of the volatiles. The extract was than stepwise diluted by addition of diethyl ether, and each sample of the dilution series was analyzed until no odor was perceivable at the sniffing port. Retention data of the compounds were expressed as RI on both columns.

Quantitation by Stable Isotope Dilution Assays (SIDA). For quantification stock internal standards of the respective labeled isotopes were prepared in diethyl ether and added to the cheese sample in a concentration similar to that of the relevant analyte present. The suspension was stirred, and volatiles were isolated as described before for the SAFE. Distillates were analyzed by GC×GC-TOFMS monitoring the intensities of the respective ions given in Table 1. For all compounds response factors were calculated in the standard mixture of labeled and unlabeled compound in a known concentration of 500 ppb. The concentrations in the sample were calculated from the peak area of the analyte and its corresponding internal labeled standard obtained for selected ions.[20] The calculation was done using Chroma TOF software (version 3.34).

Sensory Evaluation. Sensory analyses were evaluated by a 10 member panel experienced in descriptive analysis. Aroma profile analyses were performed by orthonasally scoring eight odor qualities on a 5 cm linear scale anchored on either side for the intensity of attributes as "none" and "very strong". The odor descriptors were chosen according to a published paper.[21] The 20 g cheese samples in 1 cm^3 cubes were presented to the panelists in 50 mL glass containers. The results from linear scale were converted into numerical values for data analysis.

■ RESULTS AND DISCUSSION

Odor Active Compounds in Smoked and Unsmoked Oscypek Cheese. The smoked Oscypek cheese exhibited an

Table 1. Quantification Ions and Response Factors Used in the Stable Isotope Dilution Assays

	compound	quant ion[a]	labeled isotope	quant ion	R_f[b]
1	2,3-butanedione	86	[$^{13}C_4$]-2,3-butanedione	90	0.56
2	acetic acid	60	[$^2H_3$]-acetic acid	63	1.19
3	butanoic acid	88	[$^{13}C_2$]-butanoic acid	90	0.83
4	3-methylbutanoic acid	87	[$^2H_2$]-3-methylbutanoic acid	89	0.66
5	3-(methylthio)propanal	104	[$^2H_3$]-3-(methylthio)propanal	107	0.32
6	2-acetylpyrazine	122	[$^2H_3$]-2-acetylpyrazine	125	1.2
7	4-methylphenol	107	[$^2H_3$]-2-methoxyphenol	127	0.96
8	2-methoxyphenol	124	[$^2H_3$]-2-methoxyphenol	127	0.83
9	2,6-dimethylphenol	122	[$^2H_3$-phenol]	97	0.65
10	3-hydroxy-2-methylpyran-4-one	126	[$^2H_3$]-3-hydroxy-2-methylpyran-4-one	129	0.76
11	2,4-dimethylphenol	122	[$^2H_3$-phenol]	97	1.15
12	3-ethylphenol	122	[$^2H_3$]-2-methoxy-4-methylphenol	97	1.11
13	2-methoxy-4-methylphenol	123	[$^2H_3$]-2-methoxy-4-methylphenol	126	1.89

[a] Ions used for quantification. [b] Response factor between analyzed compound and its internal standard (labeled isotope).

overall smoked, pungent, butyric, and buttery flavor and the extract of Oscypek obtained by using the SAFE method represented this typical aroma when sniffed on a strip of filter paper. In the case of unsmoked cheese, the main odor attributors were described as milky, buttery, butyric, and brined (see Figure 1). Application of GC-O together with AEDA revealed 20 odor-active compounds in smoked cheese and 8 in the unsmoked sample. The intensities of the odor notes expressed as FD factors ranged between 4 and 2048 in smoked cheese and between 4 and 512 in unsmoked cheese (Table2). For the unsmoked cheese the highest FD factors were obtained for four compounds with odor notes described as sweaty (×2), buttery, and cooked potato, which by the application of GC-MS were identified as a butanoic acid, 3-methylbutanoic acid, 2,3-butanedione, and methional, respectively. Odorants with somewhat lower FD factors were acetic acid with a vinegar note, maltol with a bread-like odor, and an unknown compound with a smell described as rancid. In the data published on key odorants of cheese products, those compounds are often mentioned as main aroma components. Kubickova and Grosch in their work on Camembert cheese identified acetic and butanoic acid, diacetyl, methional, and also 3-methylbutanoic acid as major aroma constituents.[15] Methional was also identified in Swiss Gruyere, Parmiggiano Regiano, and Emmentaler cheeses[16,22,23] and diacetyl in Cheddar cheese.[14] Those compounds are often associated with biochemical reactions that occur during curding and ripening between major milk constituents such as lipids, amino acids, or carbohydrates. Free fatty acids, which are formed either by lipolysis of milk fat[24] or from the deamination of amino acids, as metabolic products of lactose metabolism or even lipid oxidation,[25] are known to have a major impact on the flavor of Italian cheeses such as Fiore Sardo, giving them a characteristic piquant aroma. Diacetyl (2,3-butanedione), which originates from lactose and citrate metabolism, is responsible for buttery odor notes in a number of cheeses. Methional can derive either from enzymatic or nonenzymatic breakdown of methionine, which has been reported as the main constituent of casein.[26] Branched-chain fatty acids such as 2- and 3-methylbutanoic acid are known to be characteristic impact compounds of goat and sheep's milk cheeses, giving them sweaty and rancid notes.[27] They are derived from isoleucine and leucine, respectively.[28]

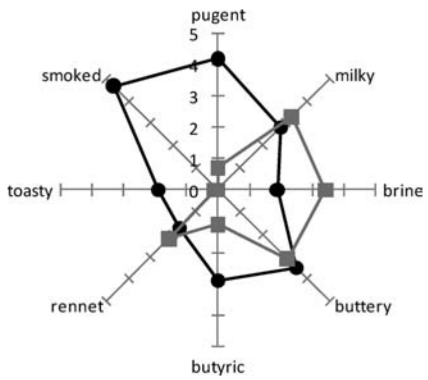

Figure 1. Orthonasal aroma profiles of unsmoked (■) and smoked (●) Oscypek cheese.

For the smoked Oscypek the aroma profile changed substantially, and the smoky, pungent, and butyric notes were scored highest by the panelists (Figure 1). At the same time a new note was detected: toasted. As compared to unsmoked cheese,

Table 2. Key Odorants Identified in Smoked (S) and Unsmoked (US) Oscypek Cheese by Aroma Extract Dilution Analysis

			RI[a]		FD[b]	
	compound	odor[c]	DB-5	Supelcowax 10	US	S
1	2,3-butanedione[d]	butter	589	970	256	256
2	acetic acid[d]	sour	603	1470	64	512
3	unknown	rancid	611	832	64	32
4	butanoic acid[d]	sweaty	820	1605	512	1024
5	3-methylbutanoic acid[d]	sweaty, rancid	877	1650	128	128
6	unknown	fruity	880		nd	32
7	2,3-dimethylpyrazine[c]	roasted	890	1240	nd	16
8	3-(methylthio)propanal[d]	cooked potatoes	909	1414	256	512
9	ethyl 4-methylpentanoate[d]	fruity	920	1173	nd	32
10	dimethyl trisulfide[c]	sulfury	978	1378	nd	16
11	2-acetylpyrazine[d]	roasted	1022	1615	nd	128
12	phenylacetaldehyde[d]	flowery	1053	1642	4	4
13	4-hydroxy-2,5-dimethyl-3(2*H*) furanone[c]	cotton candy, caramel	1076	2015	nd	128
14	4-methylphenol [d]	burnt, phenolic, fecal	1084	2063	nd	1024
15	2-methoxyphenol[d]	smoked	1089	1836	nd	2048
16	2,6-dimethylphenol[d]	burnt, smoked	1110	1976	nd	512
17	3-hydroxy-2-methylpyran-4-one[d]	roasted bread-like	1118	1985	64	256
18	2,4-dimethylphenol[d]	plastic, phenolic	1158	2051	nd	256
19	3-ethylphenol[d]	soap, plastic	1175	2198	nd	512
20	2-methoxy-4-methylphenol[d]	smoked	1203	1920	nd	2048

[a] Retention indices. [b] Flavor dilution factor on DB-5 column. [c] Odor percieved at the sniffing port. [d] Compounds identified by comparison with reference compounds on the basis of the following criteria: retention index (RI), mass spectra obtained by MS(EI), and odor quality at the sniffing port. [c] The MS signal was too weak for an unequivocal interpretation. The compound was identified on the basis of the remaining criteria detailed in footnote *d*.

buttery, milky, and rennet odors were perceived at the same level, and brine somewhat lower. Furthermore, GC-O analysis allowed identification of a larger number of potent odorants[20] in smoked cheese, including six phenolic compounds with flavors described as smoked (2-methoxyphenol and 2-methoxy-4-methylphenol), phenolic, and burnt wood or plastic (4-methylphenol, 2,4-dimethylphenol, 2,6-dimethylphenol, 4-ethylphenol). Because they were not identified in the unsmoked sample, their presence could be explained by employment of the smoking process. Additionally, in the smoked cheese extract a high FD has been found for acetylpyrazine and Furaneol (value = 128) with roasted and caramel notes. Those compounds are mostly associated with thermal degradations of carbohydrates and are reported in processed foods such as popcorn or cocoa powder,[29,30] but they were also reported as constituents of Emmentaler cheese.[16] To obtain more precise data on the source of key odorants in the smoked cheese, 13 compounds with the highest FD factors (>128) were quantified by the means of SIDAs developed and described by Schieberle and Grosch.[20] Results from AEDA for smoked Oscypek cheese have also been presented as a gas chromatogram and an aromagram sketch in Figure 2.

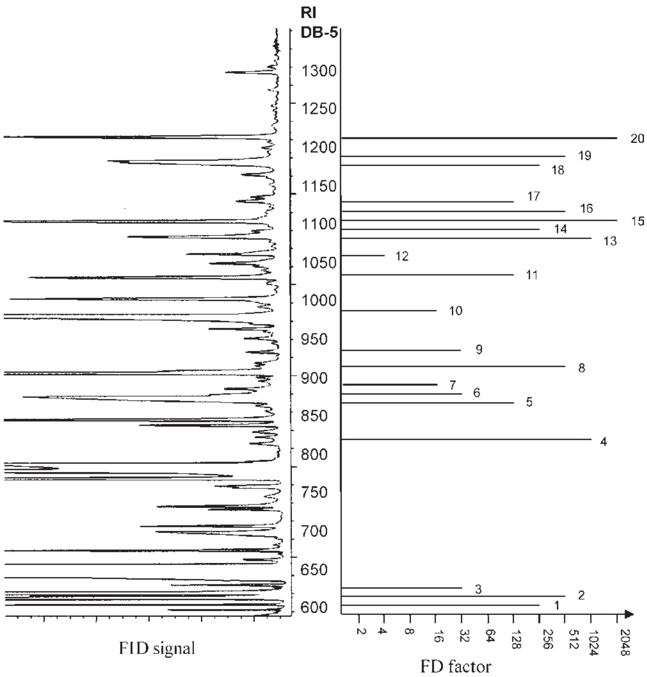

Figure 2. Chromatogram and aromagram of volatiles isolated from smoked Oscypek cheese with their dilution factors (FD) and retention indices (RI) on a DB-5 column. Numbers refer to Table 2.

Quantification and Calculation of OAVs for Key Odorants in Unsmoked and Smoked Ewe's Milk Cheese. Compounds can be a potent odorants of food aroma only when they are produced in foods in a concentration higher than their odor threshold value.[31] Therefore, for Oscypek cheese OAVs for main key odorants were calculated as a ratio of concentration to odor threshold.[31] Quantitative data were obtained using isotope-labeled internal standards, specified under Materials and Methods and listed in Table 1. Odor threshold values for each odorant were obtained from the literature.[32] The results of the quantitative measurement are summarized in Table 3. Quantification of 13 compounds with the highest FD factors (<256) followed by calculation of their OAVs revealed 11 compounds in smoked Oscypek in concentrations higher than their threshold values (OAV > 1); therefore, they contribute most to the overall aroma of smoked Oscypek. The most abundant odorant was without doubt guaiacol (2-methoxyphenol), with the highest OAV of 1280 as a consequence of its very low odor threshold (0.84 μg/kg) and high concentration in Oscypek cheese (1075 μg/kg). The results show that guaiacol and five other phenolic compounds compose the most important group of volatiles responsible for the special, characteristic smoked aroma of Oscypek cheese. Phenols were already recognized as the main contributors of the typical smoked flavor of fish such as salmon[33] or herring,[34] and they have been known for many years to be present in smoked meat;[35,36] however, to our best knowledge, this is the first time they have been quantified in smoked cheese. Because they were not detected in unsmoked cheese, their presence in Oscypek is clearly caused by the smoking process and wood smoke through

Table 3. Concentration, Odor Thresholds (OT), and Odor Activity Values (OAV) of Key Odorants of Smoked (S) and Unsmoked (US) Oscypek Cheese

			S		US	
	compound	OT[a] (μg/kg)	concentration[b] (μg/kg)	OAV[c]	concentration (μg/kg)	OAV
1	2,3-butanedione	3	396	132	837	279
2	acetic acid	99000	258145	2.6	4176	<1
3	butanoic acid	2400	21643	9	387	<1
4	3-methylbutanoic acid	490	6558	13.4	5489	11.2
5	3-(methylthio)propanal	0.43	1.2	2.8	0.598	1.39
6	2-acetylpyrazine	62	7.6	<1	nd[d]	nd
7	4-methylphenol	3.9	998	256	nd	nd
8	2-methoxyphenol	0.84	1075	1280	nd	nd
9	2,6-dimethylphenol	0.8	22	27.5	nd	nd
10	3-hydroxy-2-methylpyran-4-one	35000	2093	<1	nd	nd
11	2,4-dimethylphenol	3	63	21	nd	nd
12	3-ethylphenol	0.85	36	42	nd	nd
13	2-methoxy-4-methylphenol	21	1979	94	nd	nd

[a] Odor thresholds in water.[32] [b] Mean values based on three replicates with RSD value ≤10%. [c] Odor activity values calculated by dividing the concentration of analyte by its odor threshold value. [d] nd, not detected.

thermal degradation and depolymerization/oxidation of lignin as suggested by Guillén.[37] Another aroma active compound was diacetyl (2,3-butanedione), with a high OAV in both unsmoked and smoked cheeses. Somewhat lower odor activities have been found for 3-methylbutanoic acid and methional, identified in both smoked and unsmoked cheeses. The last two compounds, considered as potent odorants of smoked Oscypek cheese, were the fatty acids, such as acetic and butanoic acid. Although they have considerably higher odor threshold values, their concentrations of 258 and 21 ppm, respectively, still exceed it. Compounds determined in Oscypek cheese should be considered as potential key odorants. Definitive conclusions should be obtained through recombination experiments in a cheese-like matrix.

The results indicate that although the preparation of Oscypek cheese includes several treatments comprising acidification, coagulation, scalding, brining, and smoking, on the basis of performed analyses it has been shown that the overall aroma of Oscypek, ewe's milk smoked cheese, is mostly affected by the smoking process. During this process six key aroma active phenolic compounds were formed, including the most important, guaiacol (2-methoxyphenol), with an OAV of >1200.

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