Volatile Compounds in Crumb of Whole-Meal Wheat Bread Fermented with Different Yeast Levels and Fermentation Temperatures

Nor Qhairul Izzreen M. N.,[1,2,†] Mikael A. Petersen,[1] and Åse S. Hansen[1]

ABSTRACT

The influence of fermentation temperatures (8, 16, and 32°C) and yeast levels (2, 4, and 6%) on the formation of volatile compounds in the crumb of whole-meal wheat bread was investigated. Volatile compounds were extracted by dynamic headspace extraction and analyzed by gas chromatography–mass spectrometry. Results were evaluated with multivariate data analysis and ANOVA. Bread fermented at a high temperature (32°C) had higher peak areas of the Maillard reaction products 2-furancarboxaldehyde, 2-acetylfuran, 2-methylpyrazine, and phenylacetaldehyde compared with bread fermented at lower fermentation temperatures. Bread fermented at low temperatures (8 and 16°C) was characterized by having higher peak areas of the fermentation products 3-methylbutanal, 2-methylbutanal, ethyl acetate, ethyl hexanoate, ethyl propanoate, and 3-methylbutanol. Fermentation of bread with 6% yeast resulted in a higher peak area of the important fermentation product 2-phenylethanol. It also reduced the peak areas of important lipid oxidation products. The peak area of 2,3-butanedione was also relatively higher in bread fermented with 6% yeast compared with lower yeast levels; however, an interaction was seen between the high yeast level and all three fermentation temperatures. In contrast, fermentation with a low yeast level (2%) resulted in bread with relatively higher peak areas of 2- and 3-methylbutanal, as well as (*E*)-2-nonenal and (*E,E*)-2,4-decadienal, which are important lipid oxidation compounds in bread.

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Consumption of whole grain products is recommended in several countries owing to its health benefits (Slavin et al. 2013). Epidemiological studies have reported that consumption of whole grain products may reduce various chronic diseases; for example, it may lower blood pressure (Tighe et al. 2010), reduce total and LDL cholesterol, lower the risk of type 2 diabetes, reduce obesity (Ye et al. 2012), and reduce the risk of several types of cancer (Chatenoud et al. 1998). AACC International has defined whole grain products as consisting of all the anatomical parts from the grain whose principal components, such as starchy endosperm, germ, and bran, are present in the same relative proportions as they exist in the intact grain (Slavin et al. 2013).

Whole grain products are healthy because they provide nutrients such as dietary fiber, B vitamins, vitamin E, several minerals, and phytochemicals (Slavin et al. 2013). Wheat bread is widely consumed around the world, and it should therefore be possible to promote the intake of whole grain in bread. In a survey of British adults regarding the consumption of whole grain products, over 40% of the whole grain products came from varieties of bread (Lang and Jebb 2003). To increase the consumption of healthy whole grain bread, the bread must be of high quality with good aroma so it can be accepted by consumers. Whole grain bread has been described as dry and bitter (Lang and Jebb 2003) and the volume as smaller compared with bread made from low-extraction flour (Pomeranz et al. 1977). Therefore, it is important to find the optimal fermentation conditions needed to get the best quality from whole grain bread.

Fermentation time and temperature were reported to influence the formation of aroma compounds in wheat bread crumb (Gassenmeier and Schieberle 1995; Czerny and Schieberle 2002; Birch et al. 2013b), wheat sourdough bread (Gobbetti et al. 1995; Hansen and Hansen 1996), and French bread crumb (Frasse et al. 1992).

Volatile compounds in wheat bread mainly consist of aldehydes, alcohols, ketones, pyrazines, furans, esters, pyrolines, hydrocarbons, and lactones (Birch et al. 2014). Among alcohols, 2-methylpropanol and 2- and 3-methylbutanol were found to be the most abundant compounds in whole wheat bread (Jensen et al. 2011), wheat bread (Birch et al. 2013a, 2013b), and sourdough wheat bread (Gobbetti et al. 1995; Hansen and Hansen 1996). Schieberle and Grosch (1991) found 2-phenylethanol, (*E*)-2-nonenal, and (*E,E*)-2,4-decadienal to be the most important aroma compounds in wheat bread crumb, and Birch et al. (2013b) found 3-methylbutanal, 3-methyl-1-butanol, 2,3-butandione, and (*E*)-2-nonenal to be the most aroma-active compounds in wheat bread crumb.

The volatile compounds 2-methylpropanol, 2- or 3-methylbutanol, 2,3-butanedione, 3-hydroxy-2-butanone, and 2-phenylethanol are mostly formed by the metabolism of yeast (Frasse et al. 1992, 1993), whereas (*E*)-2-nonenal and (*E,E*)-2,4-decadienal are formed by oxidation of flour lipids (Frasse et al. 1992, 1993; Czerny and Schieberle 2002).

Generation of volatile compounds in wheat bread can be affected by yeast level, yeast strain, and fermentation conditions (Birch et al. 2013a, 2013b). The yeast level influences the content of the fermentation products 2-methyl-1-propanol, 3-methylbutanol, 2-phenylethanol, phenylacetaldehyde, 2,3-butanedione, and 3-hydroxy-2-butanone in wheat bread (Birch et al. 2013b) and in sourdough wheat bread (Gobbetti et al. 1995). By using different commercial yeast strains in wheat bread production, the amounts of 3-methylbutanol and 2-phenylethanol can also be varied depending on the yeast strain used (Birch et al. 2013a). Meanwhile, a higher content of lipid oxidation products was found in French dough made without yeast compared with dough fermented with yeast (Frasse et al. 1993).

The effect of fermentation temperature on wheat bread fermented with liquid preferment (flour, water, and yeast) has been investigated by Gassenmeier and Schieberle (1995). They found a significantly higher concentration of 3-methyl-1-butanol and 2-phenylethanol in wheat bread fermented at 35°C compared with fermentation at 25, 30, and 40°C. Birch et al. (2013b) found a higher content of the esters ethyl acetate and ethyl hexanoate in bread fermented at 5°C compared with bread fermented at 15 and 35°C. They also found a higher content of lipid oxidation products in bread fermented at a high fermentation temperature.

Czerny and Schieberle (2002) found that the content of most lipid oxidation products was influenced by the extraction rate of the flour. Specifically, the content was higher in whole-meal wheat flour and the corresponding sourdoughs compared with low-extraction flour. Gobbetti et al. (1995) reported that fermentation of sourdough at 35°C can reduce the content of all volatile compounds identified in

[†] Corresponding author. Phone: +60 88320000, ext. 8573.
E-mail: qhairul@ums.edu.my

[1] Department of Food Science, Faculty of Sciences, University of Copenhagen, Rolighedsvej 26, DK-1958 Frederiksberg C, Denmark.
[2] Faculty of Food Science and Nutrition, University Malaysia Sabah, Jalan UMS, 88400 Kota Kinabalu, Sabah, Malaysia.

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the corresponding sourdough bread compared with sourdough fermentation at 25°C.

Despite several investigations carried out to elucidate the influence of fermentation conditions on flavor formation in wheat bread, the influence of fermentation conditions on the formation of important volatile compounds in whole wheat bread has scarcely been investigated. Therefore, the aim of this work is to investigate whether the levels of volatile compounds in whole-meal bread are influenced by yeast concentrations (2, 4, and 6% of flour) and dough fermentation temperatures (8, 16, and 32°C) to find optimal fermentation conditions for producing good quality whole-meal bread.

MATERIALS AND METHODS

Experimental Design. Bread was made with whole-meal wheat flour (*Triticum aestivum* L.) with three levels of yeast corresponding to 2, 4, and 6% of the flour. The dough was fermented at three different temperatures: 8, 16, and 32°C. The combination of different yeast levels and fermentation temperatures required nine treatments. Bread with the same treatment was made twice (duplicated) on two consecutive days, which resulted in 18 bread samples in total. The analysis of volatile compounds was carried out in triplicate using a total of 54 samples. The bread samples were named according to yeast level (2, 4, or 6), fermentation temperature (8, 16, or 32), treatment duplicate (a or b), and analysis triplicate (1, 2, or 3).

Wheat Grain and Whole-Meal Wheat Flour. Wheat grains of the Øland wheat variety (organically grown) were purchased from Aurion A/S Milling and Baking Company (Hjørring, Denmark). The grains were milled on a Brabender Quadrumat Junior laboratory roller mill (Duisburg, Germany). The particle sizes of the meal were as follows: 27% of the flour had a particle size less than 75 µm, 37% between 75 and 160 µm, 16% between 160 and 250 µm, 8% between 250 and 500 µm, and 8% between 500 and 1,000 µm. Only 4% had a particle size bigger than 1,000 µm.

The moisture content of the flour was measured on the day of baking (HOH-Express, Pfeuffer, Kitzingen, Germany) and varied from 10.7 to 10.9%. The wet gluten content in the sifted flour with particle size <160 µm was 27.9% (Glutomatic 2100, Perten, Hägersten, Sweden) (AACCI Approved Method 38-12.01). The falling number of the whole-meal flour was 230 (Falling Number 1500, Perten) (method no 107/1, ICC 1995).

Yeast. Commercial compressed fresh baker's yeast *Saccharomyses cerevisiae*, Skærtoft Mølle, an organically produced baker's yeast from Agrano, Germany, was used. The yeast was taken from the same batch for all bakings.

Breadmaking. Whole-meal flour (300 g, adjusted to 14% moisture content), 192 of mL water (30°C), 4.2 g of sucrose, 4.2 g of NaCl, and 6, 12, or 18 g of yeast (corresponding to 2, 4, and 6% of the flour, respectively), were mixed in a bread-making machine (XBM 5, Fovea A/S, Hagan, Norway) and set to knead for 19 min. After kneading, 400 g of dough from each batch was transferred to a baking tin and then placed in an incubator at 8, 16, or 32°C for fermentation. The fermentation was terminated when the dough reached optimal dough height. Preliminary tests were carried out to find the optimal fermentation time and fermentation temperature for each yeast concentration by monitoring the optimal dough height by a web camera during fermentation (Table I). The optimal dough height was defined as the height of dough just before collapse. The dough was baked at 200°C for 17 min to a center temperature of 99°C in a convection oven (Conmatic line, Hounö Brønnum, Herlev, Denmark). After baking, the loaves were cooled at ambient temperature for 15 min and then taken out of the baking tin and further cooled on a grate at the same temperature for another hour. The loaves were then packed in aluminum foil and a plastic bag and kept frozen at –18°C for two weeks until analyzed.

TABLE I
Fermentation Times for the Doughs to Reach Optimal Height According to Fermentation Temperature and Yeast Concentration

Fermentation Temperature (°C)	Yeast Concentration (% of Flour)		
	2	4	6
8	20 h 15 min	8 h 45 min	4 h 50 min
16	7 h 30 min	4 h	3 h
32	2 h	1 h 40 min	1 h 10 min

Dynamic Headspace Extraction. Bread crumb (15 g) was weighed, cut (10 × 10 × 10 mm), and transferred to a 500 mL purge glass flask (7.5 cm diameter) for dynamic headspace extraction. The purge flask containing the sample was closed by a purge head connected to a Tenax trap (250 mg Tenax TA, 60/80 mesh, Buchem BV, Apeldoorn, the Netherlands). The flask was placed in a water bath on a stirrer plate (220–250 rpm) at 40°C for 10 min to equilibrate. Afterward, it was purged with nitrogen (150 mL/min) for 1 h. The Tenax TA trap was removed from the purge head and further purged directly with nitrogen (50 mL/min) for 10 min to remove excess water from the trap. The Tenax TA traps were closed with caps and kept at 5°C for 1–3 days before analysis by gas chromatography–mass spectrometry (GC-MS).

GC-MS. The trapped volatiles were desorbed with an automatic thermal desorption unit (ATD 350, Perkin Elmer, Waltham, MA, U.S.A.). Primary desorption was carried out by heating the trap to 250°C with a 50 mL/min flow of the carrier gas hydrogen for 15 min. The stripped volatiles were retrapped in a Tenax TA cold trap (30 mg, 5°C), which was subsequently heated at 300°C for 4 min (secondary desorption, outlet split 1:10). This allowed a rapid transfer of volatiles to the GC-MS (7890A GC system interfaced with a 5975C VL Mass Selective Detector (MSD) with a triple-axis detector from Agilent Technologies, Palo Alto, CA, U.S.A.) through a heated (225°C) transfer line with hydrogen as the carrier gas (1 mL/min). The column temperature was kept at 40°C for 10 min, increased at 8°C/min to 240°C, and kept isothermal for 5 min. The GC-MS was equipped with a mass spectrometer operating in electron ionization mode at 70eV and scanned at mass/charge (*m/z*) range between 15 and 300.

Compound Identification. Volatile compounds were identified by matching their mass spectra with those of a commercial database (Wiley275.L, G1035A, Agilent Technologies). A software program, GCD Plus ChemStation G1701EA (version E.01.00.237, Agilent Technologies), was used for data analysis. The retention indices of all identified volatiles were calculated as the retention time of the volatile normalized to the retention times of an adjacently eluting series of *n*-hydrocarbons (C_6–C_{22}) and compared with the values reported previously in published literature and online databases (www.flavornet.org, www.leffingwell.com, and www.chemspider.com). All identified compounds were relatively quantified as peak areas in the total ion chromatogram (TIC). The odor threshold and odor descriptions of each volatile compound were also referenced from the abovementioned databases unless otherwise stated (Table II).

Data Analysis. Discriminant partial least squares regressions (dPLS) were computed with LatentiX software (version 2.00, LatentiX Aps, Frederiksberg, Denmark) by correlating the peak areas of volatile compounds (*X* matrix) to a dummy matrix (*Y* matrix) describing the experimental design. All data were autoscaled, and the models were validated with full cross-validation. The statistical analyses were done on the peak areas of the volatile compounds. One-way and two-way analyses of variance (ANOVA) were computed with JMP software (version 7.0, SAS Institute, Cary, NC, U.S.A.) to test the significance of differences between the mean of two factors as model effects: fermentation temperature and yeast concentration. When ANOVA showed significant differences, Tukey's test was computed to enable multiple comparisons of the levels of volatile compounds (95% significance level) according to the two factors.

RESULTS AND DISCUSSION

Although different treatments of fermentation temperatures and yeast levels were used for preparation of the different breads, fully risen doughs with similar height were obtained. This resulted in divergent fermentation times for each bread treatment (Table I). No statistical correlation, however, was found between fermentation time and the peak areas of the volatile compounds identified in the crumb of whole-meal wheat bread.

dPLS analysis was used instead of principal component analysis (PCA) because dPLS was able to capture the level of differences between bread samples and volatile compounds with regard to

TABLE II
Volatile Compounds Identified in the Crumb of Whole-Meal Wheat Bread[u]

Volatile Compound	Kovats Retention Index			Origin	Odor Description[w,x,y]	OT (μg/L)[x,y,z]
	Calculated	Ref.	Literature[v,w]			
Aldehydes						
Hexanal	1,089	1,089	1,084	LO	Fishy, grassy	4.5
Heptanal	1,192	1,192	1,174	LO	Dry fish	8
Octanal	1,303	1,306	1,280	LO	Fatty, pungent	0.58
Nonanal	1,403	1,404	1,402	LO	Green, fatty	1.1
Pentanal	980	983	986	LO	Malt, green	n/a
Decanal	1,514	1,512	1,510	LO	Orange peel-like	0.1
2-Methylbutanal	914		912	M,F	Nutty	3
3-Methylbutanal	917		912	M,F	Almond, nutty	0.2
(*E*)-2-Heptenal	1,340		1,334	n/a	Green-like	n/a
(*E*)-2-Nonenal	1,553		1,558	LO	Green	0.08
(*E,E*)-2,4-Decadienal	1,729		1,710	LO	Fatty	0.07
Benzaldehyde	1,541	1,541		LO,M,F	Almond	350
Phenylacetaldehyde	1,662		1,623	M,F	Honey-like	4
Alcohols						
Propanol	1,055		1,037	F	Plastic, musty	6,600
Pentanol	1,273	1,274	1,255	LO	Fruity	4,000
Hexanol	1,375	1,372	1,379	LO	Green, grassy	5.6
Butanol	1,165	1,166	1,145	UD	Solvent-like	459
Heptanol	1,471		1,451	LO	Fresh, nutty	5.4
Octanol	1,574	1,573	1,553	LO	Fatty, green	125
1-Penten-3-ol	1,178		1,162	LO	Burnt, meaty	358
(*Z*)-2-Pentenol	1,341		1,317	LO	Green, plastic	n/a
(*Z*)-3-Hexenol	1,398		1,378	n/a	n/a	n/a
1-Octen-3-ol	1,464		1,445	LO	Fishy, grassy	1.5
2-Ethyl-hexanol	1,505		1,504	n/a	Green rose	25,482
(*Z*)-3-Nonenol	1,701		1,682	LO	n/a	n/a
2-Phenylethanol	1,935		1,915	F	Honey, rosy	564
2-Methylpropanol	1,112	1,110	1,099	F	Solvent-like	6,505
3-Methylbutanol	1,226		1,205	F	Balsamic	4
3-Methyl-2-butanol	1,143		1,124	n/a	n/a	n/a
Esters						
Ethyl acetate	897	895	898	F	Fruity, orange	5–5,000
Ethyl hexanoate	1,251		1,229	F	Fruity	2.2
Ethyl octanoate	1,448		1,448	F	Sweet, apple	19.3
Ethyl benzoate	1,690		1,687	F	Flowery, musty	55
Ethyl phenylacetate	1,724		1,724	F	Honey, rosy	156
Ethyl propionate	959		950	F	Fruity, solvent	10
Ketones						
2,3-Butanedione	986		986	F	Creamy, cheesy	0.06
3-Hydroxy-2-butanone	1,301		1,303	F	Butterscotch	8,000
2,3-Pentanedione	1,074		1,071	M	Cream, butter	0.65
2-Heptanone	1,190	1,192		LO	Soapy	140
3-Octen-2-one	1,423		1,388	LO	Rose	n/a
6-Methyl-5-hepten-2-one	1,354	1,354	1,365	n/a	Sweet, fruity	68
Furans						
2-Ethylfuran	952	953	960	LO	Rubber-like	2.3
2-Pentylfuran	1,247	1,246	1,240	LO,M	Green bean	5.8
3-Furancarboxaldehyde	1,443		1,426	M	n/a	n/a
2-Furancarboxaldehyde	1,478		1,457	M	Bread, almond	3,000
2-Acetylfuran	1,522		1,511	M	Balsamic	80,000
Other compounds						
2-Methylpyrazine	1,286		1,281	M	Roasty, popcorn	60,000
1*H*-Pyrrole	1,531		1,530	M	Nutty	n/a
2-Acetylthiazole	1,670		1,674	M	Roasted, popcorn-like	10
αβ-Farnesene	1,679		1,648	n/a	Citrus, green	n/a
3-Ethyl-2-methyl-1,3-hexadiene	1,430		1,421	n/a	n/a	n/a

[u] Ref. = reference compounds; OT = odor threshold in water; LO = lipid oxidation; M = Maillard reaction; F = fermentation; UD = undetermined; and n/a = not available.
[v] www.chemspider.com.
[w] www.flavornet.org.
[x] Giri et al. (2010).
[y] Buttery et al. (1999).
[z] Leffingwell and Associates (2014), www.leffingwell.com.

different treatments of yeast concentration (2, 4, or 6% of flour) and fermentation temperatures (8, 16, or 32°C) separately. Whereas PCA is usually applied without the consideration of the correlation between bread samples and fermentation treatments, dPLS is based on the correlation. Therefore, because fermentation treatment was specified, the dPLS analysis was more efficient in generating an overview compared with PCA.

Volatile Compounds in Whole-Meal Wheat Bread. A total of 51 volatile compounds were identified in the samples of whole-meal wheat bread crumb fermented at different yeast concentrations (2, 4, and 6%) and temperatures (8, 16, and 32°C). The volatile compounds comprised 16 alcohols, 13 aldehydes, six ketones, six esters, five furans, and five other compounds (Table II).

PLS score plots of the different bread samples were calculated based on all identified volatile compounds to get an overview of the effects of the fermentation temperatures (Fig. 1) and yeast levels (Fig. 2). With few exceptions, the duplicates (a and b) of the same fermentation treatments and the analysis of the triplicates (1, 2, and 3) were placed close to each other, which indicates good reproducibility for the fermentation treatments and analytical method.

Because two factors were examined in this study (fermentation temperature and yeast level), a two-way ANOVA was calculated for every volatile compound identified (Table III). It was striking that the peak areas of 41 compounds showed a nonsignificant interaction between fermentation temperature and yeast level, indicating that the effects of yeast levels on the peak areas of the volatile compounds were not influenced by the fermentation temperature. Meanwhile, 27 compounds were significantly influenced by yeast level, and 24 compounds were significantly influenced by fermentation temperature. Decanal, heptanol, octanol, (*Z*)-2-pentenol, ethyl phenylacetate, and 3-octen-2-one were not significantly influenced by yeast levels or fermentation temperatures.

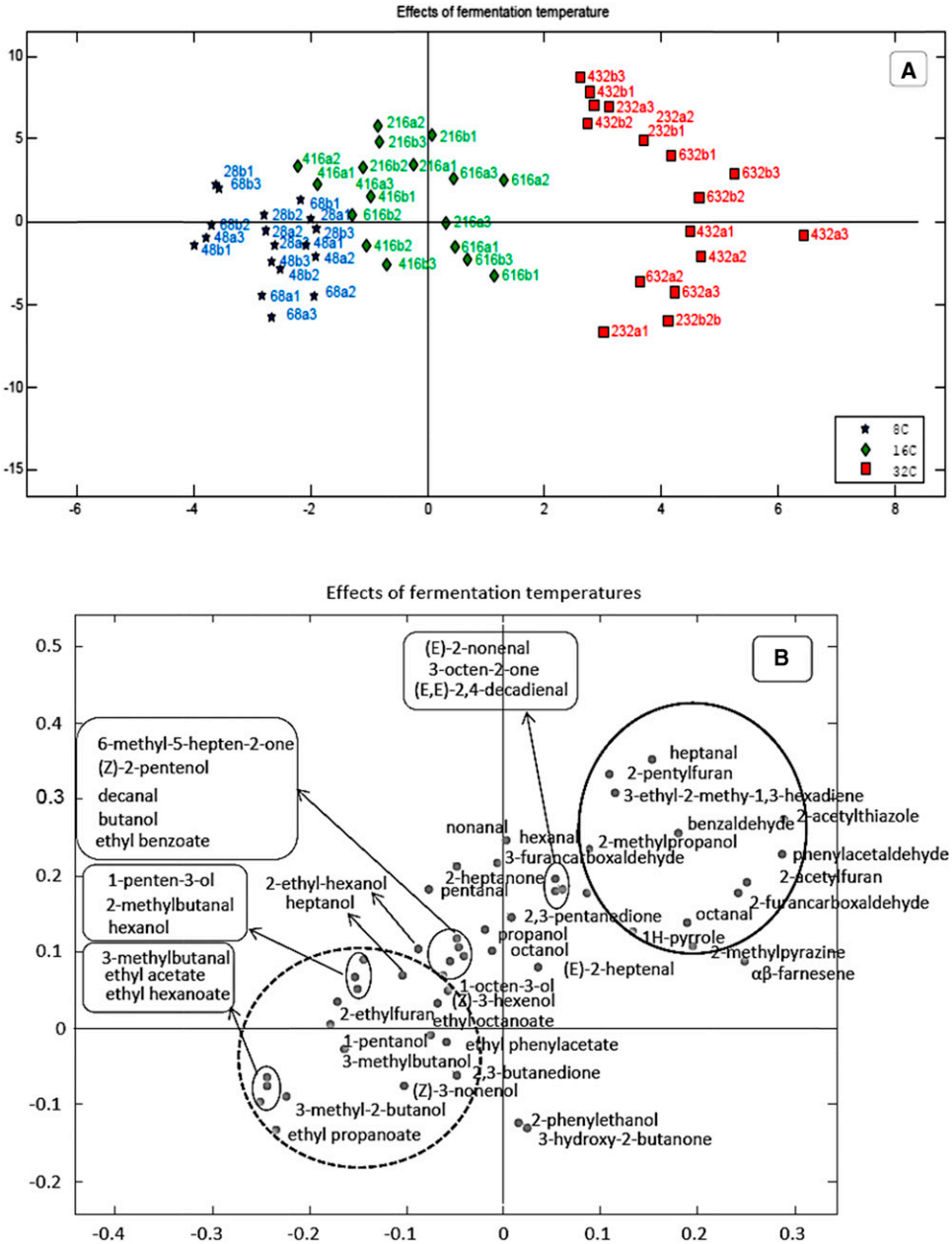

Fig. 1. PLS score plot (**A**) and loading plot (**B**) showing the effect of fermentation temperature on the formation of volatile compounds in the crumb of whole-meal wheat bread. The shapes (star, diamond, and square) in the score plot show samples fermented at 8, 16, and 32°C, respectively. The line patterns (square dot and solid line ellipses) in the loading plot show volatile compounds found in bread fermented at a low and high temperatures, respectively. The sample codes consist of yeast level (2, 4, and 6%), fermentation temperature (8, 16, and 32°C), treatment duplicate (a and b), and analysis triplicate (1, 2, and 3).

Because 10 compounds showed a significant interaction between the two factors, one-way ANOVA was calculated to analyze the differences resulting from the combined effect of the two factors (Table III). This analysis revealed that there were significant differences in the mean peak areas for all compounds analyzed.

Outlying Samples. Samples with the codes 632a1 and 616a3 were considered to be outliers based on residual variance, hoteling, and the chromatogram (TIC) pattern that deviated from the other samples with the same treatment. Therefore, these were not included in the analysis.

Effect of Fermentation Temperature. The dPLS plots shown in Figure 1 demonstrate a clear effect of fermentation temperature. Samples fermented at 8°C are placed to the left in the score plot, whereas samples with higher fermentation temperatures are placed further to the right. The two-way ANOVA analysis shows that 24 volatile compounds were significantly influenced by fermentation temperature (Table III). The total explained variance by both dPLS components 1 and 2 is 57%.

Bread Fermented at High Temperature. At the highest fermentation temperature (32°C), samples were characterized by having high peak areas of phenylacetaldehyde, 2-furancarboxaldehyde, 2-acetylfuran, and 2-methylpyrazine (Table III; solid line ellipse, Fig. 1B). The peak area of 2-acetylthiazole was also high in samples fermented at 32°C; however, interaction was seen with different yeast levels (Table III). Benzaldehyde showed no significant differences when bread was fermented at 16 and 32°C.

Most of the compounds associated with fermentation at a high temperature are related to Maillard reactions. Phenylacetaldehyde and benzaldehyde can be formed through Maillard reactions and from amino acid phenylalanine through the Ehrlich pathway (Perpète and Collin 2000). 2-Furancarboxaldehyde, 2-acetylfuran, and 2-methylpyrazine are well-known Maillard reaction products,

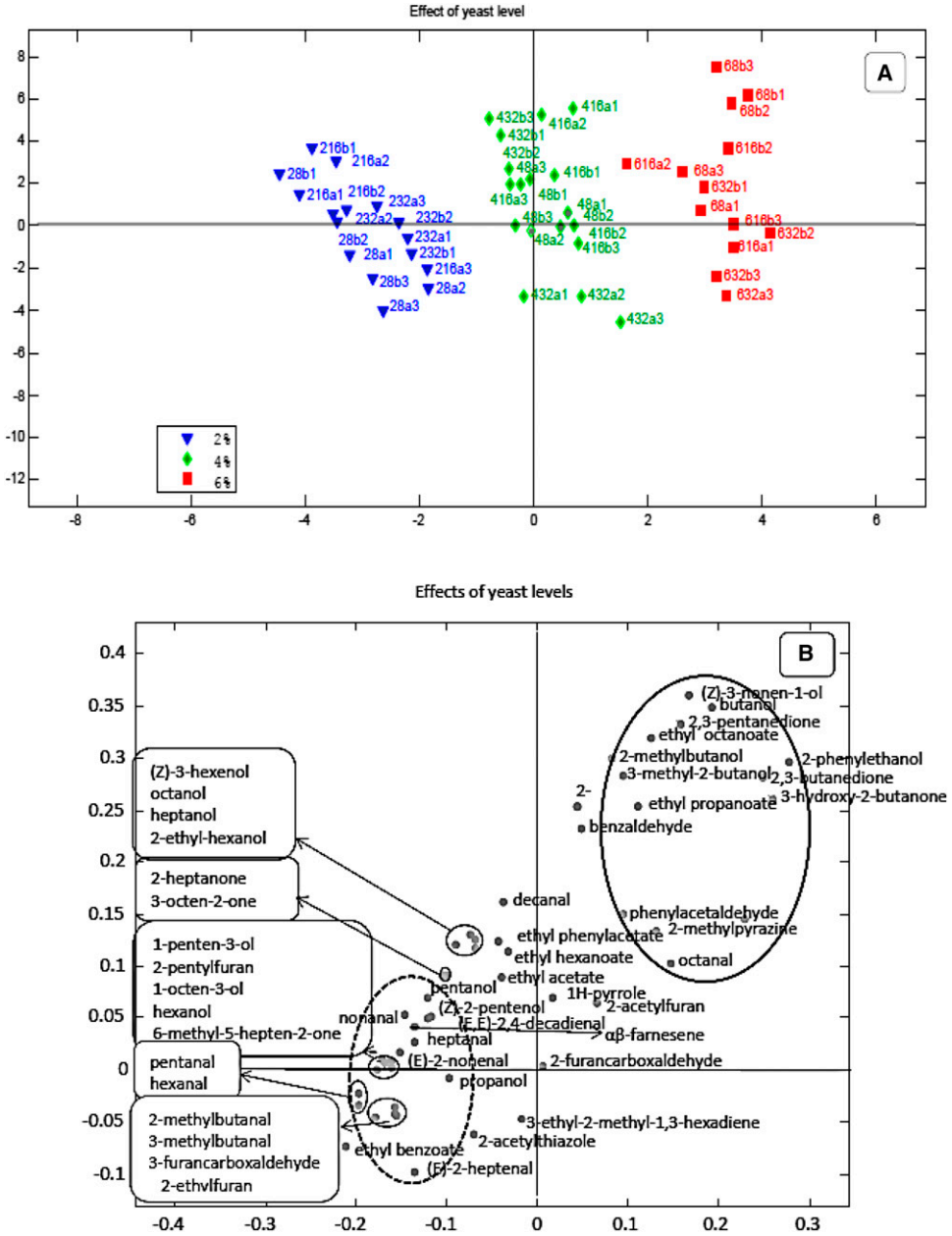

Fig. 2. PLS score plot (**A**) and loading plot (**B**) showing the effect of yeast level on the formation of volatile compounds in the crumb of whole-meal wheat bread. The shapes (triangle, diamond, and square) in the score plot show samples fermented at 2, 4, and 6% yeast, respectively. The line patterns (square dot and solid line ellipses) in the loading plot show volatile compounds found in bread fermented with low and high yeast levels, respectively. The sample codes consist of yeast level (2, 4, and 6%), fermentation temperature (8, 16, and 32°C), treatment duplicate (a and b), and analysis triplicate (1, 2, and 3).

whereas phenylacetaldehyde and benzaldehyde are formed through Maillard reactions and yeast fermentation. Therefore, it seems that some precursors for the Maillard reaction might be formed during fermentation of dough at a high temperature. Except for phenylacetaldehyde, the odor threshold values of these compounds were relatively high (Table II); therefore, each of them probably plays a minor role in bread crumb aroma. However, they also might have an influence together.

Bread Fermented at a Low Temperature. Breads fermented at a low temperature (8°C) are placed to the left of the score plot (star shape, Fig. 1A). When the score plot is compared with the corresponding loading plot, these breads are shown to be characterized by having high peak areas of 3-methylbutanal and ethyl acetate (Table III; square dot ellipse, Fig. 1B). On the other hand, the levels of several volatile compounds in bread fermented at 8 and 16°C did not differ significantly and were characterized by having high peak areas of 2-methylbutanal, pentanol, hexanol, 1-penten-3-ol, (*Z*)-3-hexenol, 3-methylbutanol, 1-octen-3-ol, (*Z*)-3-nonen-1-ol, and 2-ethylfuran (Table III). Seven out of 16 alcohols were identified in samples fermented at a low temperature. A low fermentation temperature resulted in longer fermentation time, which is in accordance with the observations of Maeda et al. (2009), who found high amounts of alcohols in wheat bread when the fermentation time was increased. Longer fermentation time allows the dough protease to generate more

TABLE III
Mean Peak Area (×10⁶) and Standard Deviation of Compounds in Whole Wheat Bread Crumb Influenced by Yeast Concentration and Fermentation Temperature[z]

	Aroma Compound Peak Area Value in Bread Crumb Samples (Mean)									Two-Way ANOVA								
	2% Yeast			4% Yeast			6% Yeast			YC×FT	YC (%)				FT (°C)			
Aroma Compound	**8°C**	**16°C**	**32°C**	**8°C**	**16°C**	**32°C**	**8°C**	**16°C**	**32°C**	***P***	***P***	**2**	**4**	**6**	***P***	**8**	**16**	**32**
Aldehydes																		
Hexanal	17.0	25.2	26.6	16.3	21.5	20.0	12.5	13.2	15.3	ns	<0.0001	a	a	b	0.0038	b	a	a
Heptanal	1.35	2.09	2.41	1.26	1.85	1.98	1.94	1.65	1.29	ns	0.0065	a	ab	b	0.0009	b	a	a
Decanal	0.10	0.10	0.09	0.07	0.09	0.08	0.10	0.08	0.07	ns	ns				ns			
Octanal	0.44	0.57	0.52	0.59	0.88	0.93	0.75	0.70	1.21	ns	0.0007	b	b	a	0.0125	b	ab	a
Nonanal	0.86	1.12	0.95	0.75	1.02	0.82	0.73	0.71	0.57	ns	0.0104	a	ab	b	ns			
Pentanal	2.74	3.18	3.27	2.60	3.29	2.10	1.94	2.32	1.10	ns	<0.0001	a	b	c	0.0010	b	a	b
2-Methylbutanal	9.6	9.8	9.5	9.0	5.8	5.4	8.4	4.6	2.6	ns	0.0002	a	b	b	0.0074	a	ab	b
3-Methylbutanal	15.7	13.8	9.9	14.2	8.5	5.7	12.6	5.6	2.7	ns	<0.0001	a	b	b	<0.0001	a	b	c
(*E*)-2-Heptenal	0.17	0.35	0.22	0.16	0.15	0.17	0.14	0.41	0.31	ns	0.0168	a	ab	b	ns			
(*E*)-2-Nonenal	0.29	0.29	0.27	0.13	0.20	0.23	0.17	0.16	0.17	ns	0.0002	a	b	b	ns			
(*E,E*)-2,4-Decadienal	0.7	0.72	0.47	0.20	0.48	0.49	0.33	0.42	0.37	ns	0.0157	a	b	b	ns			
Benzaldehyde	2.1	2.6	2.7	2.2	3.2	3.3	2.4	3.3	3.0	ns	ns				0.0024	b	a	a
Phenylacetaldehyde	1.2	1.3	1.7	1.1	1.3	2.1	1.3	1.7	2.5	ns	0.0259	b	ab	a	<0.0001	b	b	a
Alcohols																		
Propanol	10.4	7.5	9.2	7.8	8.5	9.3	7.2	7.9	5.3	ns	0.0207	a	ab	b	ns			
Pentanol	5.4	5.9	4.7	5.2	5.4	4.1	5.6	4.3	3.4	ns	ns				<0.0001	a	a	b
Hexanol	27	28	26	26	27	23	26	24	22	ns	0.0057	a	ab	b	0.0464	a	a	b
Butanol	0.9d	1.8bc	1.7bc	1.8bc	1.8bc	1.5c	3.1a	2.1b	2.2b	<0.0001								
Heptanol	1.0	1.0	0.9	0.9	1.0	0.9	1.0	0.8	0.8	ns	ns				ns			
Octanol	0.09	0.09	0.08	0.08	0.09	0.08	0.09	0.07	0.07	ns	ns				ns			
1-Penten-3-ol	13.0	14.7	12.2	11.6	12.1	8.7	11.7	9.2	6.8	ns	0.0007	a	b	b	0.0068	a	a	b
(*Z*)-2-Pentenol	2.5	2.6	2.1	2.1	2.3	2.0	2.3	1.9	1.9	ns	ns				ns			
(*Z*)-3-Hexenol	0.66	0.72	0.57	0.61	0.72	0.57	0.66	0.62	0.55	ns	ns				0.0385	ab	a	b
1-Octen-3-ol	3.6	4.1	2.6	3.3	3.8	2.8	2.8	2.8	2.1	ns	0.0189	a	ab	b	0.0055	a	a	b
2-Ethyl-hexanol	0.20d	0.41a	0.25cd	0.28bc	0.36ab	0.20d	0.30bc	0.29bc	0.21d	<0.0001								
(*Z*)-3-Nonenol	0.20	0.17	0.12	0.20	0.27	0.19	0.34	0.31	0.19	ns	0.0002	b	b	a	0.0032	a	a	b
2-Phenylethanol	6.2	4.0	3.3	10.1	8.7	8.9	17	13	17	ns	<0.0001	c	b	a	ns			
3-Methylbutanol	135	136	129	144	154	129	156	155	125	ns	ns				0.0004	a	a	b
2-Methylpropanol	54	64	63	61	66	67	65	68	73	ns	ns				0.0130	b	ab	a
3-Methyl-2-butanol	0.4cd	0.5bc	0.3d	0.6ab	0.5b	0.3d	0.7a	0.5bc	0.4cd	<0.0001								
Esters																		
Ethyl acetate	28	22	17	32	21	12	33	16	5.9	ns	ns				<0.0001	a	b	c
Ethyl propanoate	0.5cd	0.4cde	0.3de	1.0b	0.9b	0.3de	1.9a	0.7bc	0.2e	<0.0001								
Ethyl hexanoate	1.1	1.2	0.4	1.7	1.7	0.5	1.1	1.2	0.4	ns	0.0003	b	a	b	<0.0001	a	a	b
Ethyl octanoate	0.7cd	0.7cd	0.4d	0.7cd	1.1b	0.7c	0.9bc	1.5a	0.6cd	<0.0001								
Ethyl benzoate	2.0	1.7	1.5	1.5	1.5	1.5	1.3	1.2	1.0	ns	<0.0001	a	a	b	ns			
Ethyl phenylacetate	0.4	0.3	0.2	0.3	0.3	0.3	0.3	0.3	0.3	ns	ns				ns			
Ketones																		
2,3-Butanedione	15d	18cd	21c	27b	27b	20c	31a	28ab	28ab	<0.0001								
3-Hydroxy-2-butanone	26	24	20	30	34	30	37	39	40	ns	<0.0001	c	b	a	ns			
2,3-Pentanedione	1.5d	2.2cd	3.0bc	1.9cd	4.1ab	3.1bc	4.1ab	4.7a	2.9bcd	0.0004								
2-Heptanone	1.2	1.6	1.5	1.4	1.2	1.2	1.2	0.9	0.8	ns	0.0078	a	ab	b	ns			
3-Octen-2-one	0.14	0.15	0.13	0.10	0.13	0.13	0.12	0.14	0.09	ns	ns				ns			
6-Methyl-5-hepten-2-one	0.40bc	0.60a	0.40bc	0.44b	0.5ab	0.40bc	0.44b	0.30c	0.20d	<0.0001								
Furans																		
2-Ethylfuran	0.70	0.67	0.43	0.48	0.36	0.26	0.47	0.29	0.17	ns	<0.0001	a	b	b	0.0012	a	a	b
2-Pentylfuran	4.8	7.1	8.2	4.8	6.5	6.1	3.8	4.4	3.3	ns	0.003	a	a	b	0.0457	b	a	a
3-Furancarboxaldehye	1.2	1.1	1.1	0.9	1.0	1.1	0.7	0.9	0.9	ns	0.039	a	a	b	ns			
2- Furancarboxaldehye	2.6	1.7	3.4	1.5	1.8	3.7	2.0	2.0	3.3	ns	ns				<0.0001	b	b	a
2-Acetylfuran	0.2	0.2	0.4	0.2	0.2	0.5	0.2	0.2	0.4	ns	ns				<0.0001	b	b	a
Other compounds																		
2-Methylpyrazine	0.3	0.3	0.5	0.3	0.3	0.8	0.7	0.7	0.9	ns	0.0054	b	ab	a	0.003	b	b	a
1H-Pyrrole	0.20ab	0.09c	0.23a	0.1bc	0.07d	0.23a	0.20ab	0.09c	0.23a	<0.0001								
2-Acetylthiazole	0.08b	0.08b	0.1ab	0.04c	0.08bc	0.12a	0.01d	0.07bc	0.12a	<0.0001								
αβ-Farnesene	2.1	1.8	1.3	1.9	1.9	1.6	1.4	1.4	1.0	ns	0.0389	a	ab	b	ns			
3-Ethyl-2-methyl-1,3-hexadiene	0.6	0.8	0.9	0.5	0.7	0.7	0.5	0.6	0.6	ns	0.0116	a	ab	b	0.0368	b	a	a

[z] The results of two-way ANOVA are shown for the two factors: yeast concentration (YC) and fermentation temperature (FT) and their possible interaction (YC×FT). If a significant interaction effect was found between YC×FT, the two-way ANOVA was not performed on the main effects (YC and FT separately but one-way ANOVA on the combination of both effects). Hence, the interaction effect is then the most important effect. Different letters in the same row indicate significant differences (95% probability). When the interaction YC×FT is significant, the *P* value is the value for the combination effect. All compounds were identified by comparing with mass spectra and Kovats retention index by using a series of hydrocarbons and comparing with those reported previously in the published literature and several authentic databases. ns = not significant.

free amino acids that act as a precursor to produce alcohols via Ehrlich pathways (Maloney and Foy 2003; Hazelwood et al. 2008).

Also, high peak areas of the esters ethyl acetate, ethyl hexanoate, and ethyl propanoate were formed in bread fermented at a low temperature. The peak area of 3-methylbutanal was almost two times higher in samples fermented at a low temperature, which might be sensory important based on the low odor threshold value (Buttery et al. 1999). 3-Methylbutanal can be formed by catabolism of the amino acid leucine from the flour by yeast via an Ehrlich pathway (Perpète and Collin 2000) and through Strecker degradation, but the latter pathway is generally low at a low temperature.

A high peak area of ethyl acetate in bread fermented at a low temperature is in accordance with the observations of Birch et al. (2013b), who found the highest content of ethyl acetate in bread fermented at 5°C compared with 15 and 35°C. Ethyl acetate was also found in Altamura bread (Bianchi et al. 2008) and in whole wheat bread (Jensen et al. 2011). Generally, esters are characterized as having a fruity, sweet odor (Lee and Noble 2003), and they can be formed enzymatically by yeast fermentation from the available alcohols and acids (Lilly et al. 2000). The reported odor threshold values of ethyl acetate vary considerably (Table II), making the sensory importance of this compound difficult to evaluate.

The presence of 1-octen-3-ol and some other volatile compounds that are scattered in the middle of the loading plot (Fig. 1B) was also found in various wheat bread (Frasse et al. 1993; Jensen et al. 2011; Birch et al. 2013a, 2013b). Starr et al. (2015) used the same wheat variety in their work on volatile compounds in wheat varieties, but they did not find 1-octen-3-ol in their samples. On the other hand, these compounds were reported to have occurred in whole wheat bread (Harris et al. 1986) and wheat grains (Kaminski et al. 1974) owing to fungal contamination. *Saccharomyces cerevisiae* is common yeast for food contamination (Pitt and Hocking 1997) that produces these metabolites during amino acid catabolism (Hazelwood et al. 2008).

Effect of Yeast Concentration. The dPLS plots shown in Figure 2A demonstrate a clear effect of yeast concentration, because samples fermented with 2% yeast are placed to the left in the score plot, and samples with higher yeast concentrations yeast are placed further to the right. The peak areas of 27 volatile compounds were significantly influenced by yeast levels (Table III). Duplicates of bread samples (432a, 432b), especially for bread fermented with high yeast levels, are separated from each other in the score plot, indicating differences in the breadmaking procedure. However, the analytical triplicates of each sample are placed close together, suggesting good reproducibility for the method. The total explained variance by both dPLS components 1 and 2 is 56.5%.

Bread Fermented with a High Yeast Level. Bread fermented with the highest yeast level (6%) (square shape, Fig. 2A) was characterized by having higher peak areas of 2-phenylethanol and 3-hydroxy-2-butanone compared with bread fermented with lower yeast levels. The peak areas of phenylacetaldehyde and 2-methylpyrazine show a nonsignificant difference between samples fermented with 4 and 6% yeast (Table III). Relatively high peak areas of 2,3-butanedione, 2,3-pentanedione, butanol, 3-methyl-2-butanol, and ethyl propanoate were observed, but interactions were seen between yeast levels and fermentation temperatures. Most importantly, the peak area of the lipid oxidation indicator hexanal was reduced when the doughs were fermented with the highest yeast level (solid line ellipse, Fig. 2B). Additionally, aldehydes may be reduced to their corresponding alcohols by the yeast during fermentation (Gardner 1996).

2-Phenylethanol is one of the most potent odorants in wheat bread crumb (Frasse et al. 1992; Gassenmeier and Schieberle 1995) and sourdough rye bread crumb (Hansen et al. 1989). 2-Phenylethanol is one of the important fermentation compounds in bread; it has a flowery note (Gassenmeier and Schieberle 1995) and can be formed from catabolism of the amino acid phenylalanine. Birch et al. (2013a) and Frasse et al. (1992) found that the content of 2-phenylethanol was the highest in yeast-fermented bread crumb. Also, Hansen and Hansen (1996) found that sourdough bread made with added yeast had a higher content of 2-phenylethanol compared with sourdough bread without the addition of yeast to the sourdough. Nevertheless, Birch et al. (2013a) found variation in the content of 2-phenylethanol in wheat bread depending on the type of yeast used.

This finding is partly in accordance with the results of Birch et al. (2013b), because they found the highest levels of 2-phenylethanol and phenylacetaldehyde in wheat bread fermented with 6% yeast compared with lower yeast levels. 2,3-Butanedione is formed from oxidative decarboxylation of 2-acetolactate, whereas 3-hydroxy-2-butanone is formed from decarboxylation of 2-acetolactate. 2,3-Butanedione can be further reduced to 3-hydroxy-2-butanone by yeast (Rothe and Stockel 1978). This explains the high peak areas of 3-hydroxy-2-butanone that occurred when a higher amount of yeast was used compared with a low level of yeast. However, compared with 3-hydroxy-2-butanone, 2,3-butanedione has been found to be an important aroma compound in bread owing to a high peak area and low odor threshold value (Birch et al. 2013b).

Bread Fermented with a Low Yeast Level. When breads fermented with 2% yeast, which were placed at the left side of the score plot, were compared with the volatile compounds in the corresponding loading plot (square dot ellipse, Fig. 2B), they were characterized by having relatively higher peak areas of important lipid oxidation products (*E*)-2-nonenal and (*E,E*)-2,4-decadienal, as well as 2- and 3-methylbutanal (Table III). However, the peak area of the lipid oxidation indicator hexanal was not significantly different between bread fermented with 2 and 4% yeast (Table III).

Grosch and Schieberle (1997) regarded (*E,E*)-2,4-decadienal and (*E*)-2-nonenal as key aroma compounds in wheat bread crumb. These provide fatty notes to the bread flavor and are generated by oxidation of linoleic acid (Adams et al. 2011). 2-Ethylfuran has been characterized as having a pungent odor (Giri et al. 2010) and a sweet, burnt odor (Fors 1983). It can be formed by the oxidation of linoleic acids (Adams et al. 2011). Although the peak area of 2-ethylfuran was low in our sample, it might be sensory important owing to the low odor threshold value (Table II).

The occurrence of high peak areas of lipid oxidation products in bread fermented with a low yeast level is in accordance with the results of Frasse et al. (1993), who found that the content of lipid oxidation products was the highest in wheat dough made without yeast compared with dough fermented with yeast. It seems that yeast is able to reduce the lipid oxidation products in dough. The peak areas of the lipid oxidation products, however, were still considered low owing to the use of newly milled whole wheat grains in this experiment. A high peak area of 3-methylbutanal was found in bread fermented with a low yeast amount. Similarly, the content of 3-methylbutanal was also high in French bread crumb fermented with a low yeast level compared with bread fermented with a high yeast level (Gassenmeier and Schieberle 1995). In addition, Frasse et al. (1992) found a higher content of 3-methylbutanal in the crumb of wheat bread made with deactivated yeast compared with bread fermented with yeast.

CONCLUSIONS

A high fermentation temperature resulted in relatively high peak areas of the Maillard products 2-furancarboxaldehyde, 2-acetylfuran, and 2-methylpyrazine, as well as phenylacetaldehyde, compared with lower fermentation temperatures. Bread fermented with a high yeast level (6%) had a higher peak area of the important bread flavor compound 2-phenylethanol. Bread fermented with a low yeast level (2%) had higher peak areas of the main lipid oxidation products hexanal, (*E*)-2-nonenal, and (*E,E*)-2,4-decadienal. Although the peak areas of the lipid oxidation products were relatively high in the doughs fermented with a low yeast level, the overall level might be considered low in the newly milled flour used in this experiment. Bread fermented at low temperatures (8 and

16°C) was characterized by having relatively higher peak areas of the fermentation products 3-methylbutanal, 2-methylbutanal, ethyl acetate, ethyl hexanoate, ethyl propanoate, ethyl octanoate, and 3-methylbutanol compared with bread fermented at a high fermentation temperature. A high yeast level (6%) was able to reduce the peak areas of important lipid oxidation products (*E*)-2-nonenal and (*E,E*)-2,4-decadienal, compounds that give fatty notes and rancidity to the bread. Additionally, bread fermented at a low temperature had high peak areas of the esters ethyl acetate, ethyl hexanoate, and ethyl propanoate, which have sweet and fruity odors. Fermenting bread at a low temperature with a high yeast level can be recommended for fermentation of whole-meal wheat bread because it might reduce the off-flavor of lipid oxidation products and increase the malty, sweet, and fruity odors in whole-meal wheat bread.

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