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ORIGINAL PAPER

Effect of seven baking lipases on the lipid class composition of three different cakes

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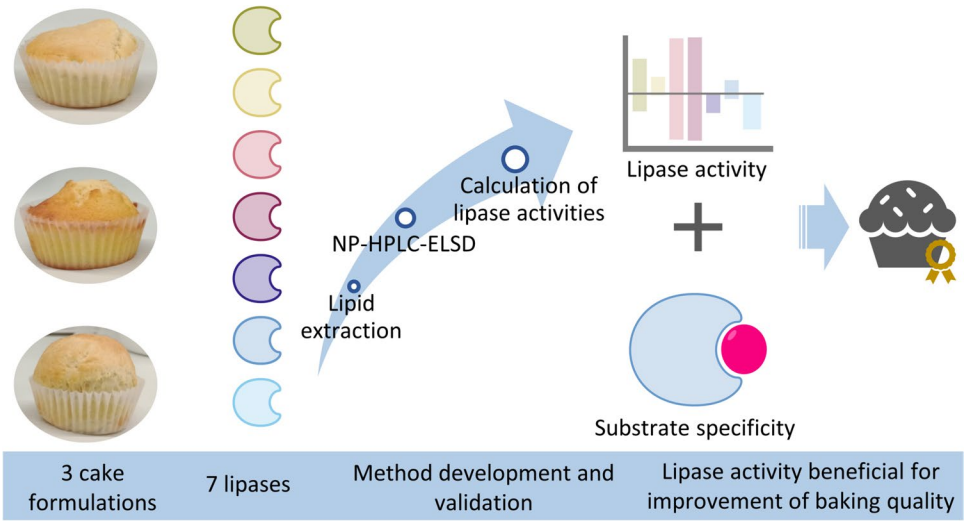
Abstract

Lipases are effective clean-label improvers for the baking quality of cake. Insights into lipase activities in different cake formulations in combination with the effect on batter/dough and baking quality are needed to further reveal the underlying mechanisms. Therefore, a method using normal phase high-performance liquid chromatography coupled to an evaporative light scattering detector was adapted and validated for the five most abundant lipid classes in three common cake recipes, namely triacylglycerols, diacylglycerols, monoacylglycerols, glycerophosphocholine and lysoglycerophosphocholine. The method revealed total changes of 0.2 mg/g to 186.5 mg/g in lipid class content per dry weight after lipase treatments. Comparative investigations on batter/dough and products of basic cake, pound cake and brioche without or with addition of seven lipases showed that the substrate specificity of lipases is the decisive factor for their effectiveness regarding improved dough and product quality. A high lipase activity only supported already matching substrate specificities.

Graphical abstract



Keywords HPLC-ELSD · Lipase activity · Lipids · Method development · Substrate specificity

Introduction

Cakes are sweet bakery goods consisting of flour, sugar, eggs, fat or oil and leavening agents. They are consumed all over the world and the variations range from, e.g., Japanese Mochi to Indian Mawa cakes, sponge cakes from Mexico, the fat cakes of South Africa and to German cheesecake. For all cake formulations, first a batter (liquid-like) or a dough (kneadable) is prepared by mixing all ingredients, which is then baked or cooked. Several lipids are introduced by the ingredients, mainly the fat component. Besides, eggs

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and wheat flour also contribute to the lipid spectrum. Lipids are an important factor for the baking quality of cakes, even if their share might be below 2%, depending on the exact formulation [1, 2]. They impact especially the texture, volume and shelf-life of cakes [2]. The functionality of lipids in cakes can be further improved using lipases, as recently demonstrated for three different cake formulations, namely an eggless basic cake, a traditional pound cake and a yeast-based brioche [3, 4]. The lipases led to a lighter and more liquid-like product with reduced stickiness. The lipase-induced effects depended on the recipe, as they were greatest in basic cake, less in pound cake and little to no effects occurred in brioche. Besides, various lipases affected the macroscopic level of batters and doughs to a different extent [4]. Similarly, both the recipe and the lipase were also decisive for effects in the baked products. There, the use of lipases led to an improvement of the parameters cake density, firmness, resilience and cohesiveness [3].

However, the underlying mechanisms for lipase effects on the baking quality of cakes are not fully understood yet. In bread it is known that the mode of action of the lipases can be attributed to four main mechanisms: first the interaction with gas cells, leading to higher product volumes [5], second the enhancement of gluten aggregation [6], third the interactions with starch causing their functionality as "softeners" by impairing retrogradation [7] and last the promotion of lipid oxidation related to a reduction of dough stickiness [8]. In a range of studies conducted by Gerits et al. and Schaffarczyk et al., the effect of lipases was found to be both dependent on the concentration and the lipase substrate specificity [9–13]. While the hydrolysis of non-polar lipids does not impact the baking quality, the release of lysoglycerogly-colipids is beneficial. Synergistic effects were reported for the simultaneous occurrence of digalactosyldiacylglycerol, monogalactosylmonoacylglycerol and *N*-acyl lysophosphoglyceroethanolamine [14].

A lipidomics mass spectrometry-based approach revealed possible key compounds for the effectiveness of lipases in cakes: in eggless recipes like basic cake, lysoglycerogly-colipids are decisive to improve the baking quality whereas in pound cake, lysoglycerophospholipids are more important [15]. These key compounds, however, are not sufficient to explain differences between the efficiency of different lipases in various recipes. Further investigations on the activity of lipases in cakes could offer a possible explanation. However, both commercially available assays [3] and approaches using naturally occurring substrates [16, 17] do not lead to reliable results for actual lipase activity in complex matrixes. Instead, they showed that lipase activity cannot be transferred to new reaction surroundings [17]. Therefore, lipase activity needs to be determined in actual cake formulations to complement the knowledge on lipase functionality in cakes.

The analysis of the lipid composition of cakes before and after treatment with lipases represents one possible approach to derive lipase activity from changes to the lipid composition. Normal phase high-performance liquid chromatography (NP-HPLC) coupled to an evaporative light scattering detector (ELSD) is the method of choice to analyze lipids from food samples [18]. Different lipid classes are separated according to their number and type of functional groups. It has already been applied to analyze the lipid composition of wheat flour [18], bread and dough [9, 14] as well as low-fat sponge cake [1]. Although mass spectrometry-based methods might capture more lipid classes than the chosen setup, the use of NP-HPLC-ELSD is less expensive and easier in terms of instrument maintenance and data evaluation.

By analyzing the lipid composition of different cake formulations before and after treatment with a range of lipases with known effects on baking quality, we can identify prerequisites for an improvement of baking quality by lipases in cakes. We hypothesize that, as described for bread, the effect is both dose-dependent and based on lipase substrate specificity. These insights are needed for the targeted use of lipases as baking improvers. The approach also enables the determination of possible residual lipase activity in cakes after baking. If residual activity was present, it could affect the admission of lipases as clean-label baking improvers in the European Union, where enzymes are only exempt from declaration if they have no functional effect in the final product [19].

Our aim was therefore to adapt and validate an NP-HPLC-ELSD method to capture the effects of lipases on the lipid composition of different cakes with known baking quality [3, 4] without and with lipase addition as well as to cakes during storage. The novelty of our work lies in the analysis of lipase-induced effects on the lipid composition of three different cake recipes combined with known functionality in batter/dough-making and baking which has not been analyzed before.

Materials and methods

Reagents, ingredients and lipases

All reagents were of analytical grade or higher. Based on the ingredients of the cake formulations, we expected the glycerolipids triacylglycerols (TG), diacylglycerols (DG) and monoacylglycerols (MG), the glycerophospholipids glycerophosphocholine (PC), lysoglycerophosphocholine (LPC), glycerophosphoethanolamine (PE), lysoglycerophosphoethanolamine (LPE), phosphatidic acid (PA), glycerophosphoinositol (PI), glycerophosphoserine (PS), sphingomyelin (SM) and the ceramides lactosylceramide (LacCer) and glucosylceramide (GluCer) [18, 20–23].

Further, glyceroglycolipids as well as *N*-acyl phosphoglyceroethanolamine and their corresponding lyso-lipids from wheat flour were expected, but not included due to a lack of commercially available standards and their suspected low occurrence.

The lipid standards 1-oleoyl-*rac*-glycerol (MG 18:1), a commercially available MG mixture (MG 18:1, MG 18:0 and MG 16:0), 1,2-dioleoyl-*sn*-glycerol (DG 18:1/0:0/18:1), a commercially available TG mixture (TG 8:0_8:0_8:0, TG 10:0_10:0_10:0, TG 12:0_12:0_12:0, TG 14:0_14:0_14:0, TG 16:0_16:0_16:0) and cholesterol were from Merck (Darmstadt, Germany). Further standards were from Avanti Polar Lipids (Alabaster, AL, United States) and consisted of glucosylceramide (GluCer), PI, LPI and PS from soy, LPC, LPE, PA, PC and PE from egg, SM from milk, and D-lactosyl-β-1,1'-*N*-palmitoyl-D-erythrosphingosine (LacCer d18:1/16:0).

For lipid extraction and measurement, formic acid, dichloromethane, methanol, n-heptane and hydrochloric acid were from VWR International (Darmstadt, Germany); n-butanol was from Carl Roth (Karlsruhe, Germany), 2-propanol was from Honeywell (Charlotte, NC, USA) and triethylamine was from Thermo Fisher Scientific (Darmstadt, Germany). Water-saturated n-butanol was prepared by stirring 1 L of 80 mmol/L HCl with 2.5 L of n-butanol and removing the lower, aqueous phase with a separating funnel [12].

The ingredients used for cake preparation were of commercial quality and correspond to the ones described in Stemler et al. [15]. The seven lipases used were chosen based on previous studies [3, 4, 15, 16] and named A, E, G, J, K, M and O accordingly. Their activity towards different *p*-nitrophenyl esters [16], in commercially available lipase activity assay kits [3] and towards different naturally occurring substrates [16, 17] was known. Their effect on the quality of cake dough/batter and corresponding baked products was described by our group [3, 4] together with lipidomic insights on key compounds [15].

Method development

The expected lipids cover a wide range of functional groups, polarities and concentrations in the cake formulations. We therefore had to adapt an existing NP-HPLC-ELSD method in a way that all lipids could be captured within a single run. The HPLC method was based on that by Kotapati and Bates [24] with several modifications.

Lipid standards were dissolved at a concentration of 50 µg/mL in dichloromethane:methanol 1:1 (v:v) and in n-heptane (TG). Additionally, a mixture of all standards (50 µg/mL each) was prepared. 2-Propanol, n-heptane and methanol were used as solvents for HPLC in combination with different aqueous phases: First an aqueous buffer with 25 mmol/L of formic acid and triethylamine, second a buffer with 7.5 mmol/L of formic acid and triethylamine and last purified water without further additives.

Table 1 HPLC conditions for the optimized gradient

Time [min]	Flow rate [mL/min]	A [%]	B [%]	C [%]	D [%]
0.00	0.6	10.0	90.0	0.0	0.0
3.00	0.6	10.0	90.0	0.0	0.0
5.00	0.6	10.0	89.5	0.5	0.0
7.00	0.6	46.0	53.0	0.5	0.5
15.00	0.6	79.6	14.6	3.3	2.5
17.00	0.7	88.0	5.0	4.0	3.0
18.00	0.7	88.0	5.0	4.0	3.0
24.00	0.7	74.0	2.5	14.5	9.0
27.00	0.7	74.0	2.5	14.5	9.0
31.00	0.7	60.0	0.0	25.0	15.0
39.00	0.7	65.0	0.0	35.0	0.0
44.00	0.7	65.0	0.0	35.0	0.0
49.00	0.7	100.0	0.0	0.0	0.0
59.00	0.7	100.0	0.0	0.0	0.0
69.00	0.6	10.0	90.0	0.0	0.0
79.00	0.6	10.0	90.0	0.0	0.0

Solvents: A: 2-propanol, B: n-heptane, C: methanol, D: water

Table 2 ELSD gain settings and resulting signal amplification in the optimized method

Time [min]	Gain	Resulting signal amplification
0.00–5.62	1	1
5.62–10.00	5	16
10.00–19.50	9	256
19.50–24.50	5	16
24.50–79.00	9	256

The HPLC was a modular Nexera LC-40 DXS NP-HPLC-ELSD system by Shimadzu (Kyoto, Japan), equipped with an ELSD-LT III (nebulizer gas was N_2 at a pressure of 348 kPa and 40 °C). Separation was achieved on a YMC-Pack PVA-SIL-NP column (250 × 4.6 mm, 5 µm pore size) with matching pre-column (10 × 4.0 mm, 5 µm pore size) by YMC Co. Ltd. (Kyoto, Japan) at 35 °C. An injection volume of 10 µL was used. The conditions for the final method are given in Tables 1 and 2. By varying the gain settings, all lipid classes could be captured within a single run.

Method validation

The method validation comprised calibration, determination of limits of detection (LOD) and quantitation (LOQ)

and repeatability using both multiple injections and multiple extractions. The calibration was performed for the lipid classes which were identified in the samples, namely MG, DG, TG, PC and LPC. The ranges were adapted to the content in the samples, with ranges of 6–75 µg/mL (MG, single standard), 75–175 µg/mL (MG, mixture), 32–320 µg/mL (DG), 1200–12000 µg/mL (TG), 20–250 µg/mL (PC) and 20–200 µg/mL (LPC). The calibration was injected three times. Contrary to other detectors, the ELSD leads to sigmoidal relationships between signal intensity and analyte concentration [25]. Different fits for calibrations are commonly compared to find the most suitable approximation. The chosen fits correspond to fits used previously [26], but the choice of best fit remains case-specific.

LOD and LOQ were determined for the lipids using two different approaches: First the calculation was based on the sensitivity of the calibration as suggested by Ramos et al. [26] for non-linear fits using the formulas

$$LOD = \frac{3.3 \times \sigma}{A} \; LOQ = \frac{10 \times \sigma}{A}$$

with σ being the standard deviation of the peak heights of the lowest calibration point in mV and *A* the sensitivity of the calibration curve (its slope) at the point of the lowest concentration (method a).

Method b was the calculation based on the signal-to-noise ratio using the formulas

$$LOD = 3 \times \frac{S}{N} \; LOQ = 10 \times \frac{S}{N}$$

with $\frac{S}{N}$ being the signal-to-noise ratio.

For repeatability, three different samples were injected three times each and the resulting areas and lipid content were compared. Additionally, a similar comparison was done for three samples with three extractions each.

Cake preparation

Cake preparation was done as previously established [3, 4, 15]. In brief, the three different cake formulations basic cake (250 g wheat flour, 200 mL water, 100 g butter, 50 g powdered sugar, 15 g baking powder and 2.5 g salt), pound cake (200 g wheat flour, 200 g butter, 200 g powdered sugar, 200 g pasteurized egg, 2 g salt and 0.6 g baking powder) and brioche (500 g wheat flour, 125 mL water, 125 g butter, 50 g powdered sugar, 50 g pasteurized egg, 35 g fresh yeast and 5 g salt) were prepared in triplicate. The lipases were added according to the manufacturers' instructions at batter-based dosages of 400 mg/kg (lipase A), 500 mg/kg (lipase E), 300 mg/kg (lipases G and K), 70 mg/kg (lipase J) and 200 mg/kg (lipases M and O) in basic cake and pound cake and 30% of those dosages flour-based in brioche. Batters and doughs were incubated for 1 h after lipase addition and then either directly frozen (batter/dough samples) or baked at 180 °C for 12 min and frozen after cooling. Samples without lipase addition served as controls. Additional basic cake samples to analyze the effect of different storage times were stored at 22 °C in closed ziplock bags and frozen after defined storage times (0 h, 24 h, 48 h and 96 h). All frozen samples were lyophilized, milled and stored at − 20 °C until further analysis.

Lipid extraction

Lipid extraction was performed according to Schaffarczyk et al. [12] with minor modifications. The frozen samples (100 mg) were shaken vigorously with 1 mL of water-saturated 1-butanol. The mixture was further shaken at 22 °C at 30 rpm on an orbital shaker for 16 h. After centrifugation (15 min, 22 °C, 7100 g), the supernatant was filtered (0.45 µm) and dried in a vacuum centrifuge for 6 h at 40 °C and 800 Pa (Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany). Dried extracts were stored at − 20 °C until further analysis. Directly before measurement, the extracted lipids were dissolved in 2-propanol:methanol 1:1 (v:v) at a concentration of 10 mg/mL.

Calculation of lipase activities and statistical analysis

The total content of all lipid classes in the samples was calculated to determine lipase activities. Then, the difference between lipase-treated samples and the corresponding control samples was determined. These changes are given as total change in [mg/g] of dry sample and as percentage values based on the change compared to the composition of the control sample. If a lipid class was not identified in the control sample but in lipase-treated samples, the LOD (method a) was used as an approximation to calculate percentage changes.

Lipase activities, means and standard deviations of triplicate determinations as well as LOD and LOQ were calculated using Microsoft Excel (Microsoft Office Professional Plus 2019, Microsoft Corporation, Redmond, WA, USA). Two-sided Dunnett's tests ($p \leq 0.05$) in IBM SPSS Statistics 29 (International Business Machines Corporation, Armonk, NY, USA) were used to see whether lipase-treated samples differed to the control for each lipid class. Origin Pro 2023 (OriginLab Corporation, Northampton, MA, United States) was used for calibrations and analysis of variance (ANOVA) with Tukey's test ($p \leq 0.05$) to determine whether differences in storage time were significant for each lipid class.

Results and discussion

Method development

An optimized HPLC-ELSD method with a quarternary mobile phase gradient was used to analyze lipid classes of cakes with and without lipase addition. Pure water was applied as aqueous phase instead of a formic acid/triethylamine buffer to reduce noise. The concomitant loss of sensitivity was met by increasing the gain. Lipid standard substances for method development included MG, DG, TG, cholesterol, GluCer, LacCer, PA, PC, LPC, PE, LPE, PI, LPI, PS and SM. The method was optimized based on the standards identified in the samples, namely MG, DG, TG, PC and LPC. Figures S1–S6 show exemplary chromatograms of batter/dough and cake samples with and without lipase addition. In the final method, all standards were eluted within 35 min (Fig. 1). The total method runtime including column cleaning and re-equilibration was 79 min.

From the 15 different lipid classes included in the first setup, 5 were identified and quantitated in the samples (Figure S7). These most abundant lipid classes provide a sufficient approximation to estimate lipase activity. To capture all minor abundant lipids, an additional separation and enrichment of polar lipids would be necessary. The introduction of pure water instead of buffer smoothed the base line and avoided previously reported challenges in the quantitation of polar lipids [12]. The concomitant loss of sensitivity was met by adjusting the gain settings. Additionally, further improvements of ELSD technology to use varying gain settings throughout one run made it possible to use a single run for both high-abundant lipids like TG and low-abundant ones like LPC, whereas other studies reported the necessity of several runs for the same sample [12].

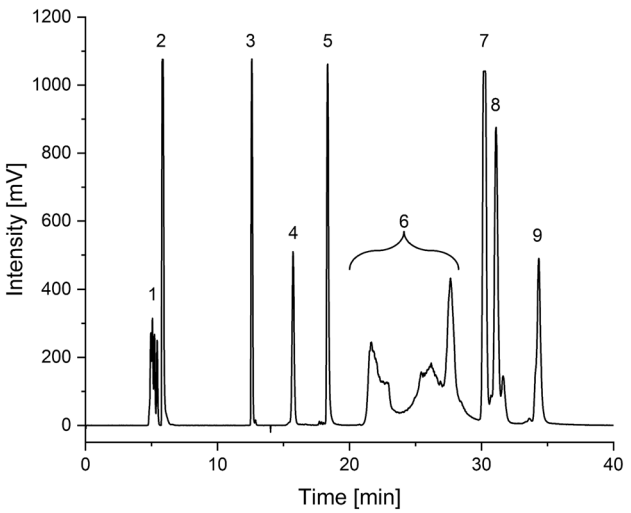

Fig. 1 Chromatogram of a mixture of lipid standard substances measured with the HPLC-ELSD method described in Table 1. 1: triacylglycerols, 2: diacylglycerols, 3: monoacylglycerols, 4: glucosylceramide, 5: lactosylceramide, 6: glycerophosphoethanolamine, lysoglycerophosphoethanolamine, phosphatidic acid, glycerophosphoinositol, lysoglycerophosphoinositol, 7: glycerophosphocholine, 8: sphingomyelin, 9: lyosglycerophosphocholine

Method validation

For calibration, three different fits were tested: linear, polynomial (2nd grade) and allometric. All determination coefficients were at least 0.9599 irrespective of the type of fit. For all lipid classes besides PC, the polynomial fit led to the highest determination coefficient (Table S1, Figure S8). For better comparability, the polynomial fit was thus chosen for all lipid classes.

The LOD values calculated based on the standard deviation of the lowest point of the calibration (method a) ranged from less than 0.000 µg to 0.014 µg with an average of 0.005 µg (Table 3). They were lower than the LODs determined with the signal-to-noise ratio approach (method b), which were from 0.001 µg to 0.058 µg with an average of 0.019 µg. The corresponding LOQs ranged from 0.001 µg to 0.044 µg and from 0.002 µg to 0.194 µg for methods a and b, respectively. Taking into consideration an average injection volume of 10 µL, this corresponds to concentrations of 0.1 µg/mL to 19.4 µg/mL. The largest differences between the methods were for PC (0.001 µg for method a and 0.033 µg for method b) and LPC (0.001 µg for method a and 0.058 µg for method b). Both methods represent only approximations for an exact determination of LOD and LOQ. For method a, the calibration also applied to the samples was used. Especially for highly abundant lipid classes like TG, this leads to discrepancies between the lowest point of the calibration and LOD/LOQ. However, it has not yet been analyzed in how far this affects the resulting values. As they lie in similar ranges as the ones determined with the common procedure relying on the signal-to-noise ratio, they seem reasonable. Concerning method b, it is based on a linear relationship between analyte concentrations and resulting signal intensities. This is not the case for our ELSD method, as shown with the different calibration approaches.

The LOD and LOQ values were all below 0.06 µg and therefore in a comparable range as reported by other studies, e.g., 0.02–0.03 µg [22, 27]. The concentrations of lipids in the sample extracts ranged from 16 µg/mL to 9420 µg/mL. The method was thus suitable.

Concerning the repeatability, the maximum standard deviation for three consecutive injections of the same sample was 8.6% for PC in basic cake (data not shown). This was most likely due to its small area overall, because the standard deviation was only 0.4% in pound cake with a considerably higher amount of PC. For TG, the maximum standard

Table 3 Lipid classes, retention times, calibration data, limit of detection (LOD) and limit of quantitation (LOQ) for all lipid classes included in the method validation

Lipid class	Abbreviation	Retention time [min]	Calibration range [µg]	LOD [µg]		LOQ [µg]	
				a	b	a	b
Triacylglycerides	TG	4.7–5.6	12–120	0.014	0.002	0.044	0.006
Diacylglycerides	DG	5.8	0.32–3.20	0.007	0.001	0.022	0.002
Monoacylglycerides	MG	12.6	0.06–0.75	0.000*	0.014	0.001	0.046
			0.75–1.75	0.004	0.003	0.012	0.011
Glycerophospho-cholines	PC	30.2	0.20–2.50	0.001	0.033	0.003	0.110
Lysoglycerophos-phocholines	LPC	34.3	0.20–2.00	0.001	0.058	0.001	0.194

Calibration range refers to the total lipid amount injected on column. LOD and LOQ of method a are calculated based on the standard deviation of the lowest point of the corresponding calibration, LOD and LOQ of method b according to the signal-to-noise ratio

*0.0002 µg

deviation was 0.5% in basic cake, for DG it was 1.1% in basic cake, for MG it was 5.4% in brioche and for LPC it was 5.1% in brioche. The maximum standard deviations per lipid class for three separate extractions of the same sample were in a similar range as for the multiple injections (data not shown). They also depended on the lipid class and content and were between 0.1% (TG in pound cake) and 13.6% (MG in pound cake). The method was therefore also repeatable, both in terms of extraction and injection.

Identified lipid classes and composition in basic cake, pound cake and brioche without lipase

The adapted HPLC-ELSD method was first applied to assess the lipid composition of control cake samples without lipase addition. As the response of the ELSD is affected by functional groups and fatty acid composition (chain length and number of double bonds), the results can only be compared within samples of similar composition [18].

The lipid classes TG, DG, MG, PC and LPC were identified in all recipes. Further lipids which were expected in the samples due to the ingredients used, namely cholesterol, GluCer, LacCer, PA, PE, LPE, PI, LPI, PS and SM, were not found. The five identified lipid classes were the main lipid classes based on the ingredients, e.g., TG as the main component of milk fat and PC as the dominant glycerophospholipid in eggs and wheat flour [18, 20, 23]. Further minor lipid classes including glyceroglycolipids, PA, PE and SM were identified in similar cake samples by mass spectrometry [15], which means that they were most likely present, but in quantities too low for the sensitivity of the chosen method. Other unidentified substances from the samples (Figures S1–S6) were eluted between 20 and 22 min runtime. These substances were most likely lipids originating from wheat as concluded by comparing the profile of a lipid extract from common wheat flour (Figure S7) to the chromatograms.

The lipid extracts from the control samples consisted to more than 95% of TG for all recipes (Fig. 2). The further lipid composition depended on the recipe (basic cake, pound cake or brioche) and whether the sample was baked or not. For basic cake, more lipid classes were identified in the baked product. While batter contained only 2.2% of DG and 0.3% of MG besides TG, the baked product also had 0.2% of PC and 0.9% of LPC. Pound cake preparations consisted of 2.1% and 1.9% of DG, 0.2% and 0.2% of MG as well as 2.5% and 2.3% of PC besides TG in batter and cake, respectively. After baking, 0.3% of LPC were additionally present.

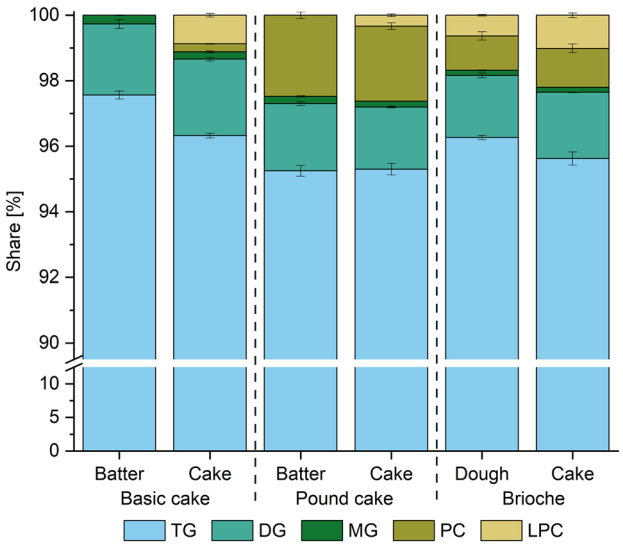

Fig. 2 Lipid composition of control samples of basic cake, pound cake and brioche. Data are given as means ± standard deviation (n = 3). *TG* triacylglycerols, *DG* diacylglycerols, *MG* monoacylglycerols, *PC* glycerophosphocholine, *LPC* lysoglycerophosphocholine

In brioche, all lipid classes were identified in both dough and baked product. Baking increased the share of glycerophospholipids (1.0% compared to 0.6% of LPC before baking and 1.2% instead of 1.0% of PC before baking). Baking thus increased the number of detectable polar lipids. This phenomenon was described before by Janssen et al.: Baking of wheat flour dough enhanced the extractability of polar lipids like LPC while impairing the recovery of non-polar lipids like TG [28]. The possible influence of varying extractability was therefore considered in the following discussion of lipase activities and the results from batter/dough samples were not considered as a preliminary stage of the results for the corresponding cakes.

Lipase activities

The lipases used in this study were previously characterized in terms of their activity with different methods. However, their absolute activity and their relative activity compared to one other were dependent on the approach [17].

If lipases were active in the cakes as defined for the scope of this work, they catalyzed at least the hydrolysis of either TG to DG, DG to MG, MG to glycerol, PC to LPC or further degraded LPC to a significant extent. Only significant changes to lipid composition (two-sided Dunnett's tests, $p \leq 0.05$) when comparing lipase-treated to control samples were considered as true lipase activity. When discussing the percentage changes, the composition of the control was considered, as small total changes may relate to large percentage changes when lipid classes with low abundance are concerned.

Basic cake

In basic cake, more lipases were found to be active in batter than in the baked product (Fig. 3, Table S2). In basic cake batter, all lipases except lipase K led to significant changes of lipid composition. The highest total change was caused by lipases O, E and G with a change of 395.2 mg/g, 330.5 mg/g and 328.8 mg/g, respectively. This was mostly due to their hydrolysis of TG, corresponding to 51.0%, 42.1% and 42.0% of the total TG present in the control sample by lipases O, E and G, respectively. For DG, the highest effect was induced by the lipases A, O, G and E with an increase by 97% for each lipase. Overall, lipase O had the highest influence on the share of TG, DG and MG.

Concerning the quality of basic cake batter, the lipases A, G and J led to an improved machinability by reducing the stickiness of the batter while rendering it more liquid-like [4]. This did not match the findings for lipase activity. Total lipase activity and the lipid classes TG, DG and MG did not seem to be decisive for the improvement of cake batter quality. Lipidomics analysis of lipase substrate specificity patterns also did not reveal key compounds responsible for the macroscopic effects, probably due to poor extractability of target compounds [15].

In baked basic cake, the lipases G, J, and A significantly altered the share of TG, DG, MG and LPC. The corresponding total changes were 50.0 mg/g, 34.6 mg/g and 24.4 mg/g,

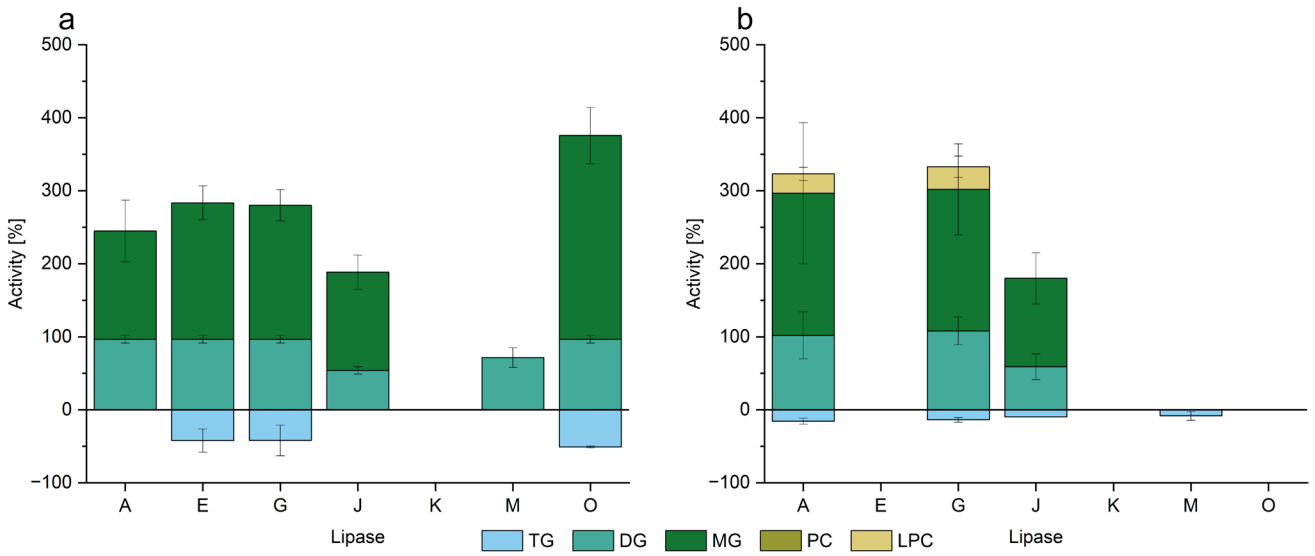

Fig. 3 Lipase activity in basic cake batter (**a**) and baked basic cake (**b**). All values are given as means ± standard deviation (n = 3) and as percentage change of the share of a lipid class compared to the control sample. Values greater than 0 stand for the release of lipids from a lipid class and the increase of its share, values lower than 0 for the hydrolysis and concomitant loss of lipids from the lipid class. *TG* triacylglycerols, *DG* diacylglycerols, *MG* monoacylglycerols, *PC* glycerophosphocholine, *LPC* lysoglycerophosphocholine

respectively, and thus considerably lower than in basic cake batter. The maximum increases in hydrolysis products ranged from 31% (LPC by lipase G) to 195% (MG by lipase A). Besides the lipases A, G, and J, lipase M caused a loss of 8% of TG, but did not significantly affect further lipid classes.

In terms of baking quality, all lipases acted as softeners and eased chewing of the cakes [3]. The three lipases A, G and J were again especially effective and were also the only lipases with a long-term softening effect during the 96 h of storage [3]. In the baked product, this matched the trends for total lipase activity, which was also greatest for the lipases A, G and J. The previous analysis of possible key compounds also revealed glyceroglycolipids as decisive factor for the mode of action of these three lipases in baked basic cake [15]. The combination of both results therefore strengthens the assumption of a combined dose-dependent and substrate specificity-dependent mode of action.

Pound cake

In pound cake, more lipases also led to significant changes of lipid composition in batter (all 7) than in the baked product (5) (Fig. 4, Table S3). The total changes were slightly higher than in basic cake in both batter and baked product. When comparing between different formulations, lipase activity therefore does not seem to be decisive for baking quality, as the effects in pound cake were lower compared to basic cake [3, 4].

In pound cake batter, the shares of all identified lipid classes were significantly affected by at least two lipases. The reaction pattern of lipase O was visibly different from the other lipases, as it interacted only with the glycerolipids TG, DG and MG and not the glycerophospholipids PC and LPC. Still, due to the high share of glycerolipids in the total lipid composition, lipase O caused the highest total change (186.5 mg/g), followed by lipases E (171.4 mg/g) and A (113.4 mg/g). Consequently, lipase O resulted in the highest changes to the shares of TG (17.2%), DG (180.3%) and MG (185.3%). The most affected lipid class was LPC with increases by 502.3% (lipase A), 443.1% (lipase G) and 427.7% (lipase J). For further glycerophospholipids, the changes in LPC by the lipases A, G, and J were matched with a loss of PC by 72.6%, 73.6% and 74.0%, respectively.

Regarding the quality of pound cake batter, the lipases A, G, J and K led to a liquefaction compared to the control [4]. This could already be partly traced back to the substrate specificity of lipases towards PC and PE [15]. The new results here now show that the impact on the liquefaction is not due to a greater lipase activity. A greater activity compared to the other lipases towards the target compound PC proves the necessary substrate specificity towards PC determined via mass spectrometry [15]. The visibly different reaction pattern for lipase O compared to the other lipases matches its classification as a TG-lipase [15]. TG-lipases therefore do not seem to be suitable for use as baking improvers in cakes.

For baked pound cake, however, no lipase activity was detected for lipase O. Additionally, also lipase K was not active. The reaction patterns of the lipases A, G and J were similar and included effects on DG, MG, PC and LPC while the lipases E and M were only active towards PC and LPC.

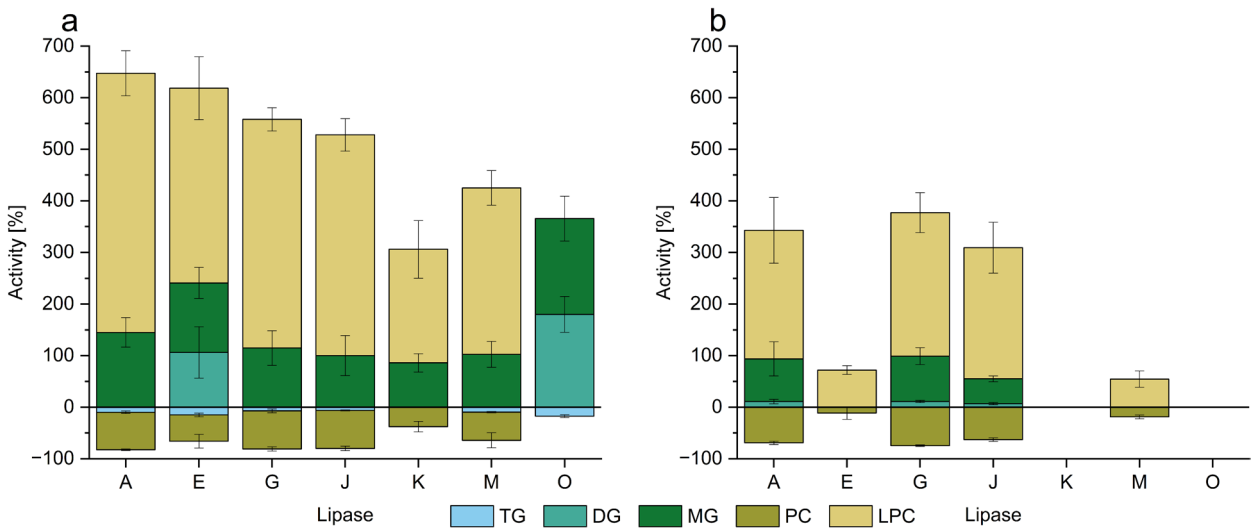

Fig. 4 Lipase activity in pound cake batter (a) and baked pound cake (b). All values are given as means ± standard deviation (n = 3) and as percentage change of the share of a lipid class compared to the control sample. Values greater than 0 stand for the release of lipids from a lipid class and the increase of its share, values lower than 0 for the hydrolysis and concomitant loss of lipids from the lipid class. *TG* triacylglycerols, *DG* diacylglycerols, *MG* monoacylglycerols, *PC* glycerophosphocholine, *LPC* lysoglycerophosphocholine

As for basic cake, the total changes were lower in the baked product than in the corresponding batter with a maximum of 53.0 mg/g by lipase G, followed by 38.9 mg/g for lipase J and 26.7 mg/g for lipase A. Their effects on the share of PC were similar to the ones in pound cake batter (74.5%, 69.0% and 62.9% for G, A and J, respectively), but met by increases of 277.8%, 248.8% and 253.7% instead of increases greater than 400% for LPC in batter.

In terms of baking quality, the lipases A, G and J and, to a lesser extent, also the lipase E led to better storage properties via softening of the products and reduced resilience and cohesiveness, resulting in better chewability [3]. Similar as for pound cake batter, lipidomics also revealed a substrate specificity towards PC and PE as important factor for the influence on the product quality of pound cake [15]. In the case of baked pound cake, the total change of lipid composition matched the effect on the baking quality. The activity was based on the effect on the share of PC and LPC, where they also had the greatest effects. Therefore, the apparent correlation between improvement of baking quality and lipase activity in the baked product contrary to the batter might be reinforced by the extraction: As discussed above, baking favors the extraction of polar compounds. PC and LPC are both polar and were already proven to be related to baking quality in earlier experiments [15].

Brioche

Contrary to the findings for basic cake and pound cake, baking increased the number of active lipases in brioche (Fig. 5, Table S4). While the lipases K and M were not active in brioche dough, all lipases led to significant changes of the lipid composition in baked brioche. Both the total changes and the effects on the percentage share were lower than in basic cake and pound cake: The maximum total change was 93.6 mg/g for lipase E in brioche dough and only 4.8 mg/g for lipase G in baked brioche while the greatest change of percentage share was by 40.6%, also for lipase G towards PC in brioche dough. Total changes to lipid composition in the dough corresponded to only 24 and 50% of the changes in basic cake and pound cake batter, respectively. Compared to baked basic cake and baked pound cake, the changes were below 10%. Lipase activity was therefore considerably lower in brioche than in the other formulations. This might be partly due to the lower dosage of lipases in brioche with only 30% on flour-base instead of batter-base, leading to an effective concentration of 17% compared to basic cake and pound cake.

In brioche dough, the lipases A, E, G, and J all led to a significant loss of PC, however without causing an increase in the share of LPC. Lipase O, on the other hand, caused a decrease of the share of LPC compared to the control sample (by 22.9%). In the sample treated with lipase E, more TG (11.7%) were detected than in the control sample. The total changes ranged from 3.9 mg/g for lipase O to 93.6 mg/g for lipase E.

In baked brioche, none of the lipases were active towards TG nor released LPC. The lipases A, E, G, J and M all hydrolyzed PC to a maximum extent of 33.9% (lipase J). The share of DG was significantly decreased

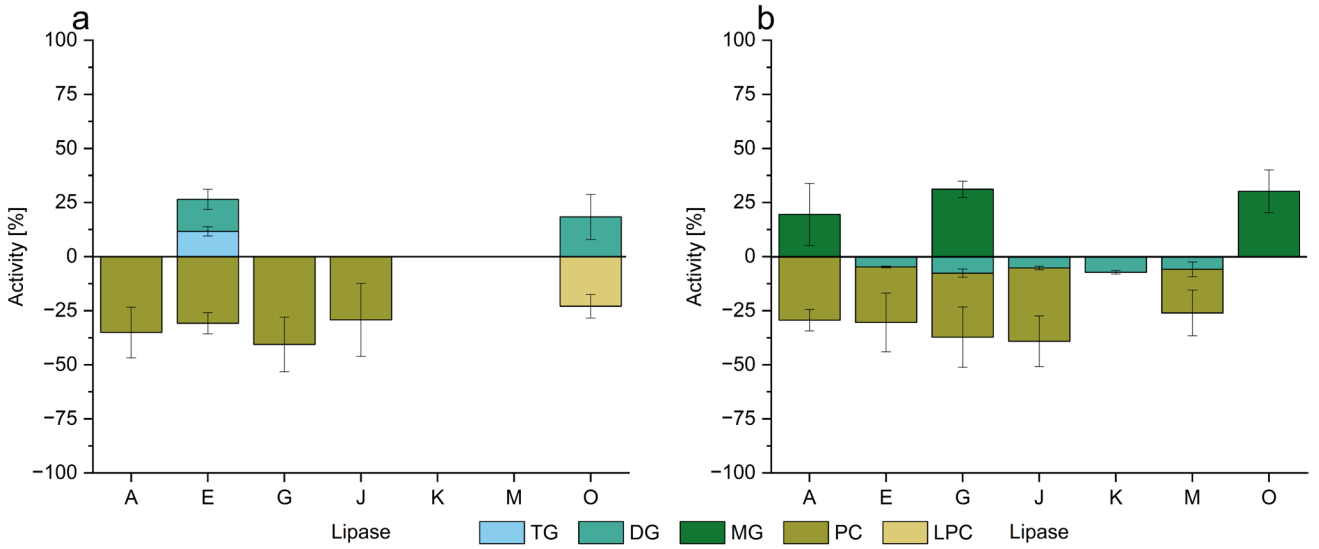

Fig. 5 Lipase activity in brioche dough (**a**) and baked brioche (**b**) (n = 3). All values are given as means ± standard deviation (n = 3) and as percentage change of the share of a lipid class compared to the control sample. Values greater than 0 stand for the release of lipids from a lipid class and the increase of its share, values lower than 0 for the hydrolysis and concomitant loss of lipids from the lipid class. *TG* triacylglycerols, *DG* diacylglycerols, *MG* monoacylglycerols, *PC* glycerophosphocholine, *LPC* lysoglycerophosphocholine

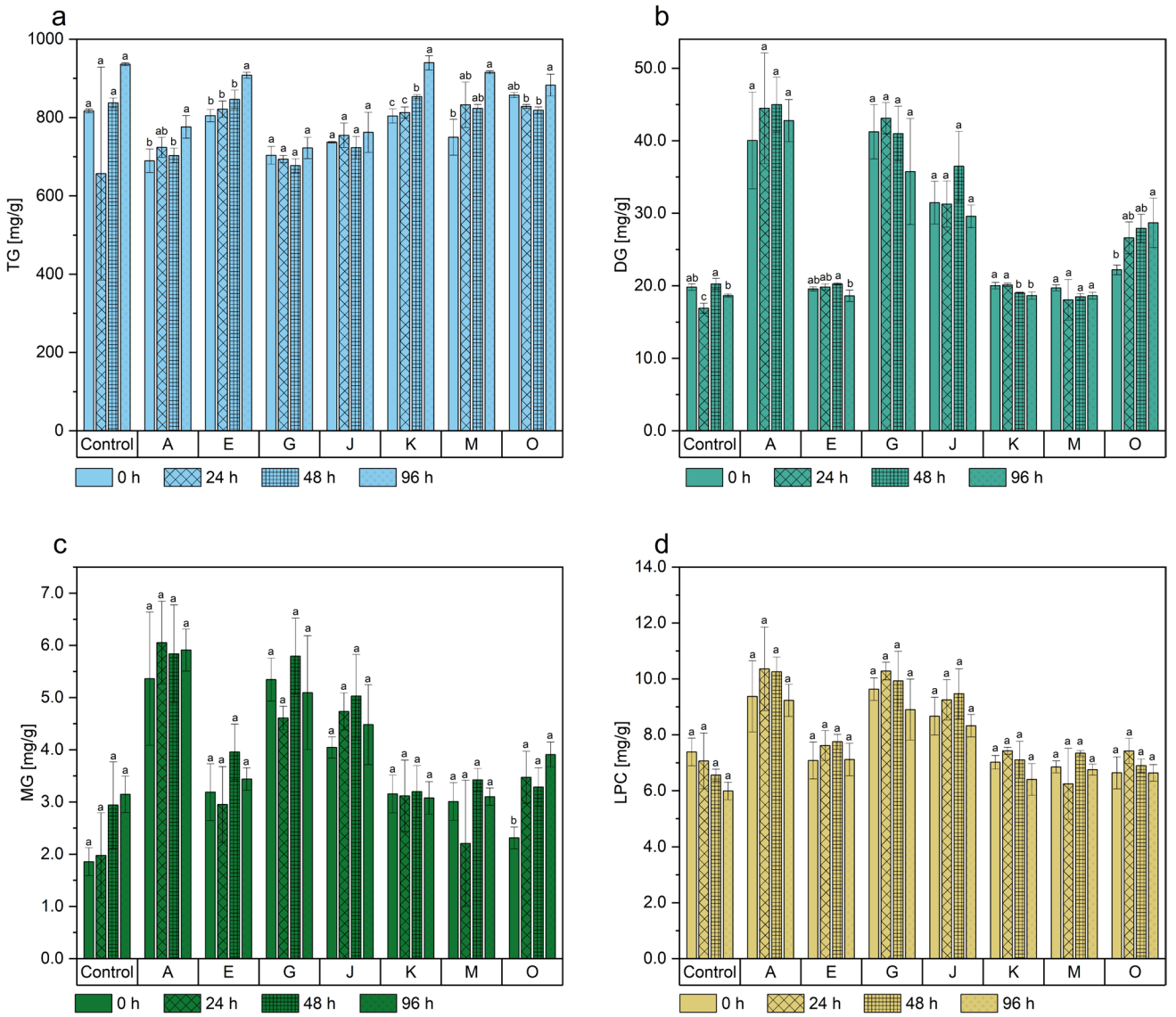

Fig. 6 Content of the lipid classes TG (**a**), DG (**b**), MG (**c**) and LPC (**d**) in basic cake after storage times of 0 h, 24 h, 48 h and 96 h. Data are given as means ± standard deviation (n = 3). Different letters indicate statistically significant differences compared to other storage times within each treatment (ANOVA with Tukey's test, $p \leq 0.05$). *TG* triacylglycerols, *DG* diacylglycerols, *MG* monoacylglycerols, *PC* glycerophosphocholine, *LPC* lysoglycerophosphocholine

by the lipases E, G, J, K and M with a maximum of 7.7% (lipase G). However, the share of MG only increased for the lipases A, G and O with a maximum of 31.2% (also lipase G). The lipases led to total changes from 0.4 mg/g (lipase O) to 4.8 mg/g (lipase G). The three lipases leading to the highest total changes in baked brioche (E, G and J) also slightly eased the chewability of baked products [3]. Although the lower lipase dosage was suggested by the manufacturer and the mass spectrometry analysis revealed unsuitable reaction patterns for the lipases in brioche [15], based on the current results higher dosages of lipases in brioche might improve their effectiveness.

To sum up the findings on lipase activity in cakes, a high activity can contribute to a positive effect on the baking quality of cakes, but a high activity without suitable substrate specificity will not lead to favorable effects.

Effect of storage time on the lipid composition of basic cake

Possible residual lipase activities in bakery products could affect their status as clean-label improvers in the European Union [19]. From the three formulations, basic cake was

chosen for further analysis, because lipase addition caused the greatest softening effect during storage.

To detect possible a residual activity of lipases during storage, the lipid composition of baked basic cake samples was assessed directly after baking and after storage times of 24 h, 48 h and 96 h (Fig. 6, Table S2). Residual activity was considered to be present if the content of a lipid class changed significantly during storage compared to the content determined directly after baking (ANOVA with Tukey's test, $p \leq 0.05$). To include all lipases in the analysis, only the lipid classes TG, DG, MG and LPC were taken into consideration.

Only small changes in lipid class content occurred during storage. Concerning TG, a further decrease was expected in case of residual activity. However, the content of TG significantly increased for the cakes treated with lipases A, E, K and M compared to the values directly after baking. For DG, both an increase (due to hydrolysis of TG) and a decrease (due to hydrolysis to MG) would point to residual activity. Both cases were found: a significant decrease of the content of DG for lipase K and a significant increase for lipase O. Concerning MG, their content was significantly increased in samples containing lipase O during the first day of storage (by 70%, corresponding to a total increase from 2.3 mg/g to 3.9 mg/g). The level of LPC remained constant in all samples during the complete storage time.

The results revealed some contradictory trends, e.g., a significant decrease of DG within the first 24 h of storage in the control sample or significant increases in TG (lipases A, E, K and M). These findings hint at further mechanisms affecting the lipid composition during storage besides lipase activity. In light of the importance of lipid extractability as seen for calculated lipase activities, the complexation of lipids in bakery goods needs to be further discussed. Exposed fatty acid chains can enter the internal cavities of amylose helices and thereby form starch-lipid complexes [29]. These complexes naturally occur in starch and are formed or re-formed during baking. When a lipid is included in a starch-lipid complex, its extraction is hindered. During baking, the lipids involved can be exchanged and thereby alter the composition of extractable lipids, as shown for straight dough bread by Janssen et al. [28]. Further changes in the fraction of complexed lipids would therefore offer an explanation for changes in lipid composition during storage. However, this mechanism only applies to exposed fatty acids, as occurring in lipids with only one fatty acid attached (e.g. MG or LPC). It does therefore not explain the changes to TG or DG. Further research is needed to clarify this point.

Concerning hints at true residual lipase activity, an increase of 70% of MG was observed in the samples with lipase O. Its original impact on the level of MG was quadrupled. It must be noted that a similar trend was also visible in the control sample (63% increase of MG), however, this change was not significant due to high variations of the measurement. To clearly trace the change in the samples treated with lipase O back to lipase activity and to exclude the discussed complexation procedures as possible cause, additional lipase activity measurements as done for, e.g., amylases in wheat bread [30] are necessary.

Conclusions

To summarize our findings, we successfully adapted and validated a reliable method for the quantitation of the major five lipid classes from three different cake formulations. The method was applied to assess the lipase activity of seven lipases in both batter/dough and baked products. The different extractability of different lipids before and after baking influenced the results, hinting that despite the range of already available lipid extraction techniques, further improvements are needed. In combination with previously published data on the effect of lipases on the baking quality of batters/doughs and cakes [3, 4], we complemented the knowledge on prerequisites for lipases as effective improvers of baking quality in cakes. Although lipase activity was not decisive for the macroscopic effects, a high lipase activity supported already matching substrate specificities. Our hypothesis that the effectiveness of lipases is both dose-dependent and dependent on substrate specificity [15] was thereby proven. Additionally, we performed first trials to investigate possible residual activity of lipases in bakery goods. In this regard, further investigations are needed to better understand lipid behavior in stored bakery goods.

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Data availability The data that support the findings of this study are available from the authors upon reasonable request.

Declarations

Conflict of interest The authors declare no competing financial interest.

Ethical approval This article does not contain any studies with human or animal subjects.

Informed consent Not applicable.

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