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Original article

Rhodamine B-based lipase activity assay with natural substrates—development and application

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Summary For the various application fields of lipases in food, an accurate and reliable prediction of their performance is needed. This is not provided by the current artificial substrate-based assays, because natural substrates should be applied. The fluorescent dye Rhodamine B (RB) enables the tracking of released fatty acids in real time and therefore offers the possibility to assess lipase activity towards any substrate. A high-throughput assay including RB was established, validated and applied to a range of five fats/oils and two isolated lipids for a range of seven lipases. The results show that lipase reactions under different conditions are not comparable. Besides the substrate and the lipase, the milieu of the reaction has a decisive impact. For the prediction of lipase reactions in foods, further aspects such as the accessibility of substrates need to be taken into consideration.

Keywords Baking, enzyme, lipase activity, Rhodamine B, substrate specificity.

Introduction

Lipases are considered as the third largest group of commercial enzymes following peptidases and glycosylases (Hasan *et al.*, 2006). They catalyse the hydrolysis of lipids and release free fatty acids (FFA) and polar lipids. There is a wide variety of possible industrial applications for lipases, including the synthesis of surfactants and detergents as well as the use in fats, oils, dairy products and baking processes (Ramrakhiani & Chand, 2011). Although lipases react with a wide range of substrates, their activity depends on the substrate. The preference of one substrate over another, the lipase substrate specificity, is of importance for the possible applications of the lipase. While the preference for certain fatty acids (fatty acid substrate specificity, FASS) is used to modify sensory properties, the preference for the specific alcohol moieties (AMSS) is decisive for the technological properties of the resulting product. As an example, FASS can be used to modify aromas and flavours of cheeses (Broel *et al.*, 2022). For baking applications, the AMSS of lipids is decisive (Melis *et al.*, 2017; Stemler, 2023): while lipases with suitable reaction patterns can improve the baking quality of bread and cakes, the release of different alcohol moieties does not affect the properties of baked goods. For bread, an AMSS for monogalactosyl diacylglycerides and *N*-acyl glycerophosphatidyl ethanolamine was shown to be beneficial (Schaffarczyk *et al.*, 2016). For cakes, depending on the recipe, the release of monogalactosyl diacylglycerides and lysophospholipids led to softer products with improved storability (Stemler, 2023). Common assays used for the determination of lipase activity are therefore not suited for the prediction of their performance in foodstuff, as shown for the reactivity towards *p*-nitrophenyl palmitate and the application in bread baking (Gerits *et al.*, 2014). Instead, the reaction of lipases towards natural substrates and their AMSS for functional lipid classes needs to be determined.

Many high-throughput assays rely on synthetic substrates with chromogenic alcohol moieties such as *p*-nitrophenol or resorufin (Stoytcheva *et al.*, 2012). For the analysis of AMSS, these synthetic alcohol moieties are not an option. Following classical protocols to determine lipase activity, one possible approach is to quantify released FFA from natural substrates with different alcohol moieties titrimetrically, by the use of chromatography or to rely on radiolabeled substrates (Sahonero-Canavesi *et al.*, 2016). However, these methods are not suitable for a quick screening of lipases in the food industry due to the large amount of

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time and laboratory equipment required. Instead, an assay that is as easy to perform as the *p*-nitrophenyl assay is needed. This new assay should be both sensitive and repeatable. It should include unmodified natural substrates and be applicable in high-throughput techniques.

Our aim was therefore to develop an assay for the determination of lipase activities towards natural substrates, which can be modified for the analysis of lipase AMSS for the prediction of lipase performance in foodstuff. The novelty of our study therefore lies in the development of a highly confident method for lipase activity testing under food-relevant *in vitro* conditions.

For this purpose, the fluorescent dye Rhodamine B (RB) was chosen. It was first presented as a tool to detect lipase activity qualitatively in media in 1987 (Kouker & Jaeger, 1987) and first steps towards its use in quantitative assays were undertaken by Zottig *et al.* (2016) and by van Gaelen *et al.* (2021). Rhodamine B can be used to detect released FA independently of the alcohol moiety. In the study of van Gaelen *et al.*, olive oil and soybean oil were used as substrates. However, their approach had certain limitations: their procedure was not validated, *for example*, in terms of repeatability and tested for only one lipase.

We hypothesise that the RB lipase activity assay presents a reliable approach for the characterisation of lipase activities towards further substrates and can possibly be used for the analysis of lipase AMSS and therefore the prediction of lipase reactions in foodstuff.

Materials and methods

Reagents

All reagents were of analytical grade or higher. Fats and oils were of commercial quality and bought at a local supermarket. Glyceryl trioleate (TG 18:1/18:1/18:1), oleic acid (C18:1), linoleic acid (C18:2), α-linolenic acid (C18:3) and lauric acid (C12:0) were from Merck KGaA (Darmstadt, Germany) and 1,2-di-(9Z-octadecenoyl)-sn-glycero-3-phosphocholine (DOPC, PC 18:1/18:1) was from Avanti Polar Lipids (Birmingham, AL, USA). Rhodamine B was from Thermo Fisher Scientific (Schwerte, Germany). The lipases used were previously characterised concerning their reactivity in the *p*-nitrophenyl assay and towards emulsions of butter, margarine, rapeseed oil and wheat germ oil (release of FFA as determined by gas chromatography) (Stemler & Scherf, 2022a), as well as by the use of several commercially available assays (Stemler & Scherf, 2022b). They were from ABEnzymes (Darmstadt, Germany), DSM (Heerlen, The Netherlands), Kerry Group (Tralee, Ireland) and Novozymes (Bagsværd, Denmark) and kindly donated by ABEnzymes, DSM, Kuchenmeister (Soest, Germany) and Novozymes. In accordance with previous publications, the lipases are named A, E, G, J, K, M and O.

Optimisation of the procedure

The assay was based on Zottig *et al.* (2016) and van Gaelen *et al.* (2021) and modified in order to optimise its sensitivity, repeatability and comparability. The first set-up corresponded to the procedure described by van Gaelen *et al.*: A buffer solution (1.36 g potassium dihydrogenphosphate and 10 g gum arabic in 200 mL distilled water, pH 7.0) was used to dissolve lipases (10 mg/mL) and to emulsify the substrates (25 mg/mL). For the emulsification, a dispersing tool (Ultra-Turrax, IKA, Staufen, Germany) with a stator diameter of 8 mm was applied for 10 min at 12500 rpm. RB (10 mg/L) was added directly to the substrate solution. In black 96-well plates, 150 µL lipase solution and 75 µL substrate solution were briefly mixed and their fluorescence (λ_{ex} 350 nm and λ_{em} 580 nm) was recorded every 5 min for 4 h using a multiplate reader (Infinite 2000 pro, Tecan Group, Männedorf, Switzerland).

Based on this procedure, the parameters (i) measurement wavelength, (ii) workflow, (iii) emulsifier, (iv) inclusion of a calibration curve and (v) applicability to further substrates were optimised.

First, a wavelength check for both excitation (230–570 nm) and emission (360–850 nm) wavelengths was performed.

Second, the workflow was modified by preparing the substrate solutions without RB and adding 10 µL of a RB stock solution (0.1 mg/mL in ethanol) directly before measurement to each well.

Third, the potential use of the emulsifier Triton X-100, a commonly applied emulsifier for lipase activity measurements (Stemler & Scherf, 2022a), was evaluated instead of gum arabic. Therefore, the buffer solution was prepared with 600 µL Triton X-100 instead of gum arabic and the assay performed as described above.

Fourth, to ensure the comparability of assays performed on several days and/or with different substrates, calibration curves were added to the procedure. Oleic acid (5 mg/mL to 25 mg/mL) was used as a calibration standard in buffer solution and in different substrate solutions. All calibrations were performed in triplicate per plate. For comparison, calibrations towards α-linolenic acid, linoleic acid and lauric acid were prepared.

Fifth, for the inclusion of spreadable fats like butter, melt emulsions as described in Stemler & Scherf (Stemler & Scherf, 2022a) were prepared: The amount of gum arabic in the buffer solution was increased to 20% (w:v) for the melt emulsions. The buffer was heated to 50 °C, then the substrate was added and stirred for 10 min to gently melt the solid fat before homogenising with a dispersing tool as described above. Then, the assay was performed as described above.

Application of the optimised procedure to a range of natural substrates

The optimised procedure included the following modifications compared with the previous method:

- Excitation wavelength of 355 nm (compared to 350 nm)
- RB addition directly before the measurement
- Calibration against oleic acid (1–25 mg/mL) dissolved in the corresponding substrate solution
- For melt emulsions, a higher concentration of gum arabic (20% w:v instead of 5% w:v) was applied.

All analyses were carried out six times. For each combination of lipase and substrate, a lipase blank (75 µL buffer, 150 µL lipase solution and 10 µL RB stock solution) and a substrate blank (75 µL substrate solution, 150 µL buffer and 10 µL RB stock solution) were included.

The procedure was then applied to analyse the reaction rates of seven baking lipases towards olive oil, wheat germ oil, flaxseed oil, coconut oil and butter.

Application of the optimised procedure to isolated lipids

As examples for isolated lipids, glyceryl trioleate as a glycerolipid and DOPC as a glycerophospholipid were used as substrates and the procedure carried out as described for natural substrates.

Statistical analysis

Microsoft Excel (Microsoft Corporation, Redmond, WA, USA) was used for data evaluation. Linear ranges of fluorescence intensities over time were identified manually and all had a correlation coefficient of $R \geq 0.9$. Lipase activities were calculated as nmol oleic acid equivalents released per min per mg of lipase (U).

Origin Pro 2023 (OriginLab Corporation, Northampton, MA) was used to perform a one-way analysis of variance (ANOVA) followed by Tukey's test ($p \leq 0.05$) to analyse statistically significant differences between the lipase activities towards one substrate.

Results

Optimisation of the procedure

The optimisation comprised the parts measuring wavelengths (i), workflow (ii), emulsifier (iii) and the introduction (iv) and transferability of calibration curves.

i Concerning the wavelengths, an increase of 10.4%–12.8% in fluorescence intensity at concentrations of 1–25 mg/mL oleic acid was observed when an excitation wavelength of 355 nm was used instead of 350 nm. Therefore, all further experiments were carried out using 355 nm as excitation wavelength. The emission wavelength was kept at 580 nm.

ii In the original workflow by van Gaelen *et al.* (2021), RB was added to the emulsified substrate before partitioning the solution in wells. In the new workflow as described in 2.2, RB was added directly before the analysis. This change helped to increase the sensitivity of the assay, because the signal intensity increased by 58.1% for an oleic acid concentration of 25 mg/mL (Fig. 1). Additionally, the linearity of the calibration curve was improved (correlation coefficients $R = 0.7329$ and $R = 0.9906$ for the old and the new workflow, respectively).

iii When Triton X-100 was used as an emulsifier instead of gum arabic, no linear relationship between oleic acid concentration and signal intensity could be established ($R = 0.1424$, Fig. 1).

iv Calibration curves of oleic acid in gum arabic buffer were prepared on six different days (Table 1). They had an average slope of 42.17 ± 2.38 units of fluorescence intensity per mg of oleic acid. The intercepts varied from 337.54 to 1349.16 units of fluorescence intensity. All correlation coefficients were at least 0.9840.

v The influence of olive oil, wheat germ oil, flaxseed oil, coconut oil, butter, triolein and DOPC on the sensitivity of the assay was analysed by preparing the substrate solutions with the respective fats and oils as substrates. The substrate solutions were spiked with different concentrations of oleic acid to obtain matrix-matched calibration curves. The resulting slopes of linear fits ranged from 34.41 to 43.72 with an average slope of 39.17 ± 2.56 and corresponding intercepts from 493.95 to 1768.32 with an average of 840.09 ± 394.79 (Table 1). All correlation coefficients were greater than 0.9500. Besides oleic acid, the responses of three further FFA in the assay were assessed (Fig. 1, Table 1). The linear fits to concentrations ranging from 5 to 25 mg/mL had similar slopes as the results for oleic acid described above (average of 39.43 ± 3.93), intercepts from 389.90 to 642.80 and correlation coefficients greater than 0.9660.

Application of the optimised procedure to a range of natural substrates

The lipase activities measured with natural substrates are depicted in Fig. 2. They ranged from 9.2 U (lipase M in wheat germ oil) to 196.5 U (lipase O in coconut oil).

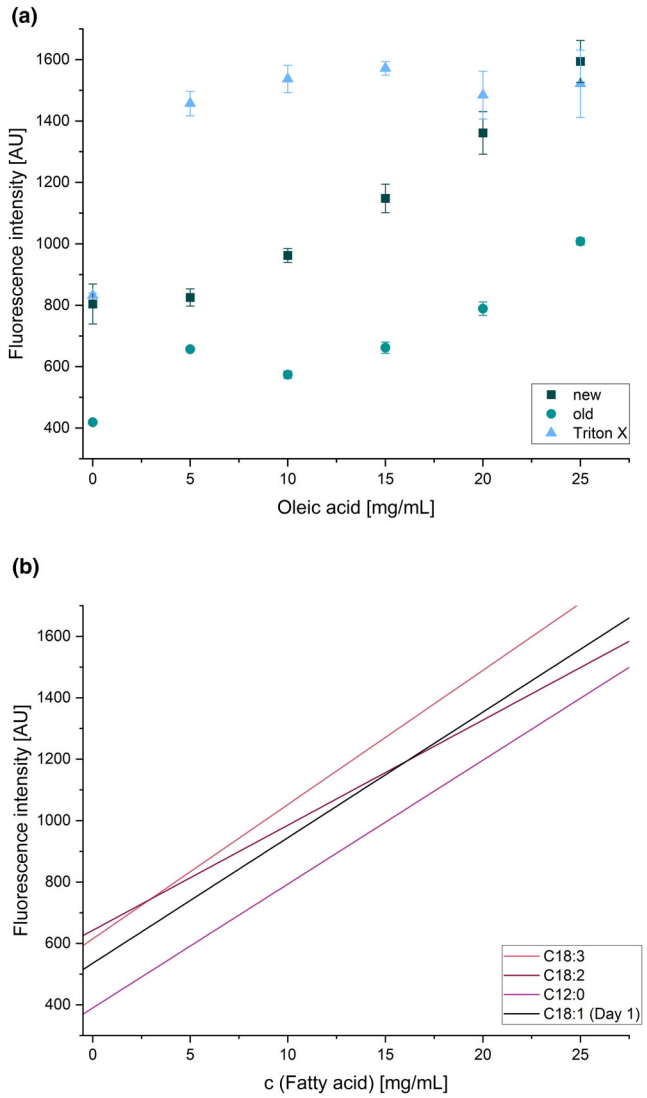


Figure 1 Fluorescence intensity of different concentrations of fatty acids with Rhodamine B. (a) Influence of the procedure (old: according to van Gaelen *et al.*; new: dye addition directly before the measurement; Triton X: replacement of gum arabic by Triton X-100). Values are shown as mean ± standard deviation (n = 3). (b) Calibration curves according to Table 1 depending on the fatty acid. For better visibility, values are shown as means without error bars.

Lipase activities depended both on the substrates and the individual lipases. The substrate causing the lowest average lipase activity was wheat germ oil (59.0 U); the substrate causing the highest average lipase activity was flaxseed oil (111.2 U).

For each substrate, the seven lipases led to individual activity patterns (Fig. 3): in olive oil, the most active lipases were lipase E (149.3 ± 11.6 U) and lipases O and M (107.3 ± 12.1 U and 100.3 ± 11.2 U, respectively). In wheat germ oil, the highest activities were measured for the lipases J (126.6 ± 15.1 U) and A (121.2 ± 5.5 U). In flaxseed oil, lipase J (158.0 ± 18.3 U) reacted fastest, followed by the lipases E (131.9 ± 5.0 U) and M (129.1 ± 7.3 U). In coconut oil, the activity of lipase O was highest (196.5 ± 9.9 U), while the second highest activity was only 88.9 ± 6.6 U (lipase J). In butter, lipase O (93.8 ± 1.1 U) had again a high activity. The second fastest reaction rate in butter was achieved with lipase G (90.2 ± 10.7 U).

Comparing the average activities of the lipases towards all substrates, lipase O had the highest average activity (108.2 U) whilst lipase K reacted with only an average activity of 30.7 U.

Application of the optimised procedure to isolated lipids

Compared with the application to natural lipids, the average lipase activities towards triolein and DOPC were lower (34.5 and 7.1 U, respectively) (Fig. 2). The fastest reaction rates were measured for lipase J (104.5 ± 9.6 U towards triolein and 16.8 ± 1.7 U towards DOPC). In triolein, the second fastest reaction rate was for lipase O (61.5 ± 5.1 U) while in DOPC, there were no significant differences between the reaction rates of the remaining lipases (average activity 5.4 ± 0.1 U).

Discussion

The modifications to the original workflow by van Gaelen *et al.* (2021) led to an increase in sensitivity, reproducibility and comparability of the procedure. This was achieved by adjusting the excitation wavelength and the addition of the dye directly before measuring. The detection of FFA by RB supposedly relies on complex formation between both components (Kouker & Jaeger, 1987). Slight photofading occurs for RB alone in aqueous solutions (Zhu *et al.*, 2012). The loss of sensitivity when adding the dye early in the procedure might be linked to this process. However, further research is needed to clarify the exact mechanism.

To improve the comparability of this assay with other lipase activity assays such as the *p*-nitrophenyl assay (Glogauer *et al.*, 2011; Stemler & Scherf, 2022a), we explored the application of Triton X-100 as an emulsifier. Triton X-100 is a nonionic surfactant consisting of a polyethylene oxide chain coupled to an aromatic group. Its structure therefore differs from gum arabic, which mainly contains polysaccharides. The use of Triton X-100 interfered with the detection of FFA. It possibly affects the abovementioned formation of FFA-RB-complexes. The overall high fluorescence intensity when Triton X-100 was used hints at competing complexes of RB and the emulsifier.

Table 1 Calibration data for oleic acid in buffer, measured at six consecutive days, oleic acid in different matrices and the most abundant free fatty acids of wheat germ oil, flaxseed oil and coconut oil in the corresponding matrices. All analyses were carried out in triplicate

	Free Fatty Acid		Slope	Intercept	Correlation Coefficient
Buffer		*Measurement time*			
	C18:1	Day 1	40.90	535.48	0.9840
	C18:1	Day 2	44.10	1349.16	0.9668
	C18:1	Day 3	39.91	919.93	0.9937
	C18:1	Day 4	44.35	605.89	0.9925
	C18:1	Day 5	38.82	1593.46	0.9903
	C18:1	Day 6	44.91	337.54	0.9906
	Average		42.17 ± 2.38	850.01 ± 450.59	0.9863 ± 0.0092
Different matrices		*Matrix*			
	C18:1	Olive oil	38.27	575.74	0.9914
	C18:1	Wheat germ oil	40.87	846.17	0.9780
	C18:1	Flaxseed oil	34.41	747.50	0.9504
	C18:1	Coconut oil	39.01	493.95	0.9392
	C18:1	Butter	42.73	677.67	0.9514
	C18:1	Triolein	43.72	771.27	0.9956
	C18:1	DOPC	39.73	1768.32	0.9943
	Average		39.82 ± 2.86	840.09 ± 394.79	0.9715 ± 0.0221
Different free fatty acids		*Matrix*			
	C18:3	Wheat germ oil	43.72	615.02	0.9664
	C18:2	Flaxseed oil	34.23	642.80	0.9721
	C12:0	Coconut oil	40.34	389.90	0.9992
	Average		39.43 ± 3.93	549.24 ± 113.24	0.9792 ± 0.0143

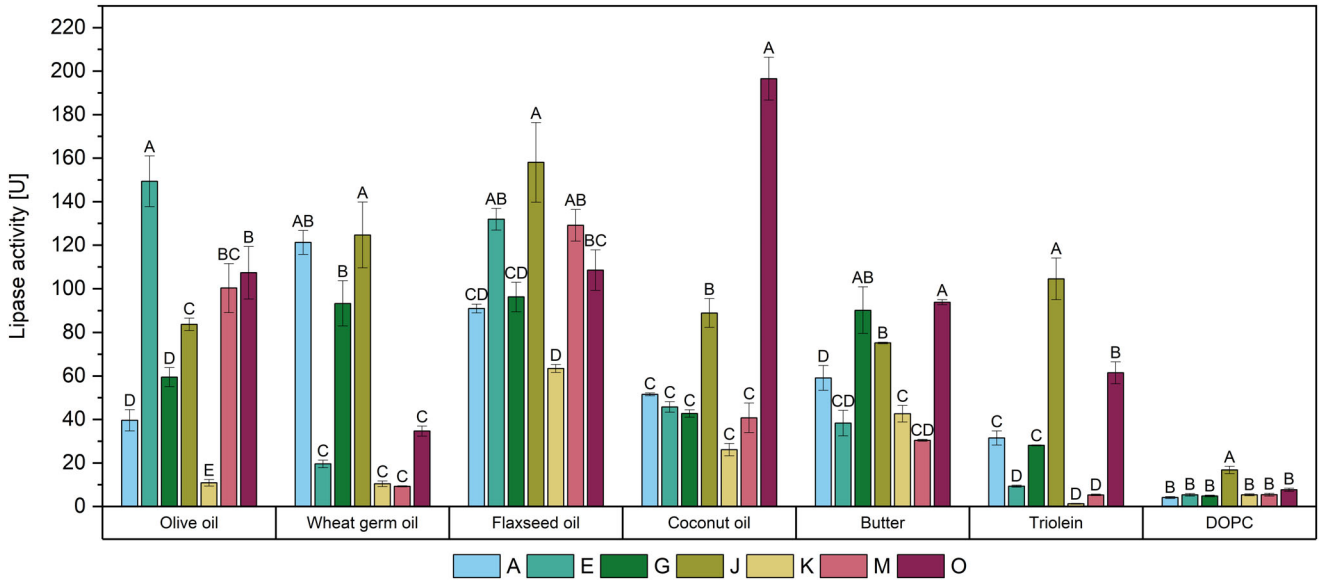
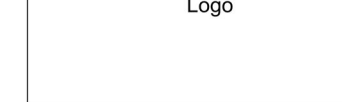
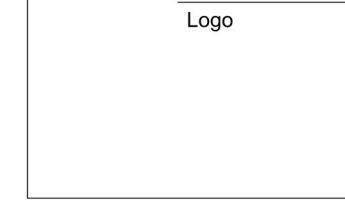


Figure 2 Activity of the lipases A, E, G, J, K, M and O towards different substrates. All activities are given as mean ± standard deviation ($n = 6$). Different capital letters indicate significant differences between the lipase activities towards one substrate according to ANOVA with Tukey's test ($p \leq 0.05$).

Zottig *et al*. already showed that the response of the oleic acid-RB complexes was reproducible in a pH range from 5 to 10 and at temperatures up to 65 °C (Zottig *et al*., 2016). However, they had not included further FA, further natural substrates or data on reproducibility. These limitations were met with the current study.

Activity determined in …		Lipases in decreasing order of activity from left to right							Literature
Emulsions as model systems	Rapeseed oil	O	A	M	E	G	J	K	Stemler & Scherf 2022a
	Margarine	E	M	A	K	O	G	J	
	Butter	O	A	K	G	M	J	E	
	Wheat germ oil	O	M	K	G	E	A	J	
	Logo								
Batters, doughs and cakes	Basic cake batter	O	G	E	M	J	A	K	Stemler 2023
	Basic cake	K	J	G	O	E	A	M	
	Pound cake batter	M	O	K	E	A	G	J	
	Pound cake	K	O	G	M	A	E	J	
	Brioche dough	A	O	K	M	J	G	E	
	Brioche	E	A	O	G	M	K	J	
	Logo								
Assays with artificial substrates	*p*-Nitrophenyl assay	J	G	A	M	E	O	K	Stemler & Scherf 2022a
	MAK 046	G	J	A	M	K	E	O	Stemler & Scherf 2022b
	MAK 047	J	G	O	A	M	E	K	
	MAK 048	J	G	M	A	E	O	K	
	Logo								
Rhodamine B assay	Olive oil	E	O	M	J	G	A	K	This paper
	Wheat germ oil	J	A	G	O	E	K	M	
	Flaxseed oil	J	E	M	O	G	A	K	
	Coconut oil	O	J	A	E	G	M	K	
	Butter	O	G	J	A	K	E	M	
	Triolein	J	O	A	G	E	M	K	
	DOPC	J	O	M	E	K	G	A	
	Logo								

DOPC - 1,2-di-(9Z-octadecenoyl)-sn-glycero-3-phosphocholine
MAK 046-048 – commercial lipase activity assay kits by Merck KGaA (Darmstadt, Germany)

Figure 3 Order of lipase activity for the lipases A, E, G, J, K, M and O as determined in different approaches. Logos are a graphical representation of the frequency of a lipase at a certain position within one approach.

By comparing calibration curves of oleic acid under different circumstances (consecutive days, different matrices), we showed that the slope of the curves varied by a maximum of 5.6% (consecutive days) to 6.5% (different matrices). The intercepts, however, depended on the exact set-up and were most likely linked to the exact amount of RB. For the calculation of lipase activities, the intercept of the calibration curves is not needed, as the increase in signal intensity over time is measured. All calibration curves showed strong linear correlations. This included the matrix butter, for which a melt emulsion was prepared, and the glycerophospholipid DOPC.

The same can be concluded for further FA that were assessed in the assay. The chosen FA were the main FA of the different matrices: C18:3 for wheat germ oil (52%), C18:2 for flaxseed oil (58%) and C12:0 for coconut oil (47%), while C18:1 is the main FA in olive oil (76%) (Belitz *et al.*, 2007). The corresponding slopes varied by a maximum of 10.0%, confirming an adequate comparability between the different FA. The release of different FA from different matrices therefore results in the same increase of signal intensity.

To sum up, the optimised procedure can be applied to different substrates and enables a comparison of lipase activities independent of the chosen substrate and released FA. Compared to the *p*-nitrophenyl assay, the advantages of the RB assay are that it is based on natural substrates and allows the inclusion of lipids with different alcohol moieties. It enables the determination of lipase reactions towards lipids belonging to different classes, whereas the *p*-nitrophenyl assay relies on a synthetic alcohol moiety without relevance to natural lipids.

Concerning the application of the assay to natural substrates, the results present the first comparative characterisation of the activities of a range of lipases towards different substrates. Individual patterns of lipase activities were determined for each lipase and each substrate (Fig. 3). Both the lipase and the substrate have an impact on the lipase activity. Even if lipases were designed for similar applications, their activities within an assay may differ, *for example*, by up to 45% (Frauenlob *et al.*, 2018). Based on our previous results for the same lipases (Stemler & Scherf, 2022b, 2022a; Stemler, 2023; Stemler *et al.*, 2023), differences between the lipases were expected in the RB assay. The substrates differed in their composition, *for example*, their FA distribution. Absolute lipase activities greatly depend on the surface of fat micelles where the hydrolysis reaction takes place (Sarda & Desnuelle, 1958). The substrate composition can affect the micelle surface and thereby also the lipase activity. Additionally, it might lead to different activities taking into consideration the FASS of the lipases. However, even if olive oil contains 76% of oleic acid randomly distributed along the triglyceride backbones (Belitz *et al.*, 2007) and triolein contains 100% of oleic acid, the patterns were not comparable. Lipase E was most active in olive oil, but less active than the lipases J, O, A and G when reacting with triolein.

Similar discrepancies apply to all approaches to determine lipase activities, even if the same substrates were used at different reaction conditions (as for butter and wheat germ oil in Stemler & Scherf (2022a)). In the case of reactivity towards wheat germ oil, *e.g.*, the RB assay showed the fastest reaction rates for the lipases J, A and G while the activity pattern as measured in terms of FFA after overnight incubation in a Triton X-100 emulsion resulted in the lipases O, M and K as most active ones.

In cake samples, lipase K had a rather high activity, while lipase J had a rather low activity (Stemler, 2023). By contrast, the activity trends of the two lipases were inversed in the RB assay (lipase K had the lowest activity and lipase J the highest activity towards 4 of the 7 substrates). In terms of AMSS, a high AMSS for the lipases A, G and J towards glycerophospholipids like DOPC was shown in pound cake (Stemler *et al.*, 2023). For lipase J, this AMSS could be confirmed by the RB assay, whereas for the lipases G and A, no AMSS for DOPC was found. A prediction of lipase AMSS and thereby their suitability for the use in cakes was therefore not achieved.

The hypothesis, that the RB assay can be used for the analysis of lipase reactions towards natural substrates, was thus met. However, the assumption that it could therefore also be used for the accurate prediction of lipase reactions in foodstuff was not proven. Instead, the prediction of lipase reactions remains challenging and even the use of the same substrates albeit at different conditions did not lead to corresponding activities. The surroundings of the substrates and their individual accessibility for hydrolysis reactions in combination with the properties of the lipases themselves impact the resulting lipase performance. This new hypothesis matches similar findings from our group concerning lipase FASS which also depends on the accessibility of FA rather than on lipase-inherent properties (Stemler & Scherf, 2022a). Further research on lipase activity assays should therefore aim to increase the transferability of results between different approaches and the usefulness for lipase applications.

Author contributions

Charlotte Dorothea Stemler: Conceptualization; data curation; formal analysis; investigation; methodology; visualization; writing – original draft. **Kim Karolin Lorenz:** Investigation; validation; writing – review and editing. **Katharina Anne Scherf:** Conceptualization; funding acquisition; project administration; resources; supervision; writing – review and editing.

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Conflict of interest

The authors declare no competing financial interest.

Ethical approval

Ethics approval was not required for this research.

Data availability statement

Data will be made available upon request.

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The work by Kouker and Jaeger represents the first use of Rhodamine B to quantitatively detect lipase activity. They applied Rhodamine B on agar plates, growed Pseudomonas aeruginosa strains and identified lipas-producing strains. They used the diameter of the resulting fluorescent halos for quantitative activity determination.

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