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An optimised MALDI-TOF assay for phosphatidylcholine-specific phospholipase C

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The *Bacillus cereus* phosphatidylcholine-specific phospholipase C (PC-PLC*Bc*) is an enzyme that catalyses the hydrolysis of phosphatidylcholines into phosphocholine and 1,2-diacylglycerols. PC-PLC*Bc* has found applications in both the food industry and in medicinal chemistry. Herein, we report our work in the development and optimisation of a matrix assisted laser desorption ionisation time-of-flight (MALDI-TOF) mass spectrometry-based assay to monitor PC-PLC*Bc* activity. The use of one-phase and two-phase reaction systems to assess the inhibition of PC-PLC*Bc* with different structural classes of inhibitors was compared. We also highlighted the advantage of our assay over the commonly used commercially available Amplex Red assay. This method will also be applicable to work on the activity and inhibition of other phospholipases.

Introduction

Phospholipases are a class of enzymes that catalyse the hydrolysis of phospholipids.1 Phospholipases are ubiquitously present in almost all organisms and they are involved in a variety of important biological functions.2 For example, in microbes, phospholipases are involved in assisting the acquisition of nutrients,3,4,5 whilst in eukaryotes, phospholipases help mediate the cell’s signal transmission cascade.6,7 Due to the biological roles that phospholipases play, many phospholipases are current inhibition targets for the development of new therapeutics.8,9 Phospholipases have also found applications in industry.10,11 For example, they are commonly used in the food industry to produce functional foods.12 In addition, owing to the ability for phospholipases to catalyse transesterification, transacylation and transphosphatidylation reactions, there is also growing interest in the application of phospholipases in industrial biocatalysis such as in the production of biodiesel.13,14,15,16



Fig. 1 PC-PLC*Bc* is a zinc-dependent enzyme that catalyses the hydrolysis of phosphatidylcholines (PC) into phosphocholine and 1,2-diacylglycerols (DAG).

One such enzyme is phosphatidylcholine-specific phospholipase C (PC-PLC). PC-PLC catalyses the hydrolysis of phosphatidylcholines (PC) (and to a lesser extent, phosphatidylethanolamines) into phosphocholine and 1,2-diacylglycerols (DAG) (Fig. 1).17 The most studied PC-PLC to date is from *Bacillus cereus* (PC-PLC*Bc*).18,19 It is a monomeric enzyme with a tri-metallic zinc centre at the active site.20,21 In *B. cereus*, PC-PLC*Bc* is translated as a preproprotein, which subsequently is processed into its mature form (Table S1 and Fig. S1).22,23 Mature PC-PLC*Bc* is secreted by the bacteria as an exoenzyme.24 It plays a vital role in the bacteria’s phosphate retrieval mechanism under growth limiting conditions, thereby hinting at a possibility of targeting PC-PLC*Bc* for the development of new antimicrobial agents.24 PC-PLC*Bc* has also emerged as an important model enzyme in medicinal chemistry for the evaluation of novel PC-PLC inhibitors as potential anticancer agents.18,19 This is because unregulated phospholipid metabolism and enhanced PC-PLC activity have been linked to tumour progression in humans.25,26 However, the exact identity of the human PC-PLC homolog as well as its encoding gene are not known.27,28 Besides its potential application in medicinal chemistry, the application of PC-PLC*Bc* in the food industry has also gained traction in recent years. In particular, the potential of utilising PC-PLC*Bc* in the degumming of vegetable oils has been actively investigated.29,30

The development and optimisation of PC-PLC as an industrial biocatalyst and the design of PC-PLC inhibitors in medicinal chemistry rely on efficient, high-throughput and accurate assays to measure the catalytic activity of PC-PLC. There already exist a number of assays to monitor PC-PLC activity. One of the most applied methods is the commercially available Amplex Red assay.31,32,33 This assay relies on the use of a fluorogenic probe 10-acetyl-3,7-dihydroxyphenoxazine (Amplex Red) to detect hydrogen peroxide, which is formed via the conversion of phosphocholine (a product of PC-PLC-catalysed reaction) to choline by alkaline phosphatase and the subsequent oxidation of choline by choline oxidase. Hydrogen peroxide reacts with Amplex Red in the presence of horseradish peroxidase to generate the highly fluorescent product, resorufin. However, the Amplex Red assay is not ideal as the detection of PC-PLC activity is indirect and is dependent on multiple enzymatic and chemical conversion steps. Other assays that rely on alkaline phosphatase have also been reported.34 Assays that allow the direct detection of PC-PLC activity have also been reported. For example, α-naphthylphosphorylcholine (α-NPPC) was used as a non-native substrate of PC-PLC.35 It gives *p*-nitrophenol as a product, which can be monitored spectrophotometrically at a wavelength of 410 nm. However, results obtained with non-native substrates may not reflect the catalytic properties of the enzyme with its native substrate. In addition, the stability and colorimetric properties of *p*-nitrophenol are pH dependent, which limits the applicability and negatively affects the accuracy of the assay. Assays that allow direct detection of the turnover of native PC-PLC substrate have also been developed.36 For example, Murakami and co-workers reported a liquid chromatography-mass spectrometry (LC-MS) assay that enables the separation and quantification of different PC-PLC substrates and products with limited throughput.36

Laser desorption ionisation (LDI) is a technique that uses high power laser beams to ionise analyte molecules.37 Typically, it is applied to analytes that have been co-crystallised with a suitable matrix, i.e. matrix assisted laser desorption ionisation (MALDI),37,38 although, in some cases, the laser can also be applied directly to the analyte without the matrix.37,39 The mass-to-charge (m/z) profiles of these ions can then be analysed using a detector such as a time-of-flight (TOF) analyser.37 Lee and co-workers reported the use of matrix-free LDI-TOF mass spectrometry to detect PC-PLC activity.40 However, the method requires the use of specialist graphene oxide/carbon nanotube double-layer films, which limits the general applicability of the technique. As conventional MALDI-TOF mass spectrometry using common organic matrices like 2,5-dihydroxybenzoic acid (DHB) has been applied to study lipids,41 we reasoned that conventional MALDI-TOF mass spectrometry could be applied as a rapid, convenient and cost-effective way to study PC-PLC*Bc* activity. Herein, we report our work in the development and application of MALDI-TOF mass spectrometry to follow and quantify the kinetics of PC-PLC*Bc* with its native substrate 2-oleoyl-1-palmitoyl-sn-glycero-3-phosphocholine (16:0/18:1-PC (34:1-PC)). The optimisation and application of this technique to study the inhibition of PC-PLC*Bc* with different structural classes of compounds is also discussed.

Results and discussion

Quantification of pure lipids by MALDI-TOF mass spectrometry

We first evaluated the feasibility of using MALDI-TOF mass spectrometry to quantify the concentration of lipids. In our assay, 34:1-PC was used as a substrate of PC-PLC*BC*, which forms phosphocholine and 1-palmitoyl-2-oleoyl-sn-glycerol (34:1-DAG) as products. Hence, pure samples of 34:1-PC and 34:1-DAG were first analysed individually using MALDI-TOF mass spectrometry. 2,5-Dihydroxy benzoic acid (DHB), a well-known MALDI matrix for lipid analysis, was used as the matrix. The MALDI mass spectra showed the molecules gave sharp and intense peak(s) (Fig. S2) with good signal-to-noise ratio (Table S2). The mass spectrum of 34:1-PC showed two peaks. One has a m/z ratio of 760.74, which corresponds to the [M+H]+ ion, and the other peak has a m/z ratio of 782.73, which corresponds to [M+Na]+ (Fig. S2a). In contrast, the mass spectrum of 34:1-DAG only showed one peak with a m/z ratio of 617.56, which corresponds to [M+Na]+ (Fig. S2b). Interestingly, when analysing a sample containing 34:1-PC and 34:1-DAG at a 1:1 ratio, the resulting mass spectrum was found to be dominated by the peaks that correspond to 34:1-PC (Fig. S3). This is likely because 34:1-PC has a better ionisation efficiency than 34:1-DAG. This provided a challenge for the quantification of the turnover of 34:1-PC to 34:1-DAG in the PC-PLC*Bc* assay, as the peak area ratio of the two species cannot be simply translated into concentration ratio. It was therefore decided to use an internal standard for quantification instead. 1,2-Dilauroyl-sn-glycerol (24:0-DAG) was chosen as an internal standard for the purpose of quantifying the product formation of the PC-PLC*Bc*-catalysed hydrolysis reaction. 24:0-DAG gave one peak in the MALDI-TOF mass spectrum with a m/z ratio 479.26 ([M+Na]+) (Fig. S2c). There are two reasons why 24:0-DAG was used as an internal standard. Firstly, it co-crystallises with the matrix to a similar extent as 34:1-DAG over different locations of the target spot, thereby negating region-dependent non-uniformity. Secondly, it has similar ionisation efficiency as 34:1-DAG owing to their similar molecular structures. These enabled the quantification of 34:1-DAG over a large concentration range (from 0 to 2.5 mM), as illustrated by a consistently linear concentration correlation curve with 24:0-DAG (at a fixed 500 μM concentration) (Fig. S4). In addition, we also showed that the presence of the large 34:1-PC peaks (at 2 mM concentration) in the mass spectra do not affect the relative peak intensity ratios between 34:1-DAG and 24:0-DAG (Fig. S5).

Enzyme kinetics of PC-PLC*Bc*

In our study, we used a two-phase reaction system to carry out the enzymatic reactions.42 The system includes an aqueous layer of PC-PLC*Bc* in buffer (50 mM 3,3-dimethylglutaric acid (DMG), 0.1 mM ZnSO4, pH 7.3) and an organic layer of lipids in chloroform. Through shaking (at 37 °C and 100 rpm), the PC-PLC-catalysed hydrolysis reaction was allowed to occur in the emulsion of the two phases. Reactions were stopped by removing the substrate from the enzyme by extracting the chloroform (lower) phase. MALDI-TOF analysis of the extracted chloroform phase gave us information about the extent of product formation during the reaction. We first conducted a time course analysis with 50 nM PC-PLC*Bc* and 2 mM 34:1-PC, with 500 μM 24:0-DAG used as internal standard. We found that there was circa 10% turnover after 2.5 minutes, and complete hydrolysis of the substrate was observed after approximately 50 minutes (Fig. 2). We next determined the kinetic parameters for the reaction by analysing the initial reaction rates with varying substrate concentrations (Fig. S6). The Michaelis constant (*K*M) was determined to be 2.15 ± 0.30 mM, the maximum velocity (Vmax) was found to be 0.12 ± 0.01 mM min-1 and the catalytic efficiency (kcat/*K*M) was 19.77 ± 2.6 s-1 mM-1 (Table S3). These results are consistent with previously reported PC-PLC*Bc* kinetic parameters.43

Inhibition studies of PC-PLC*Bc*

As alluded above, PC-PLC*Bc* is a model enzyme system for the development of new human PC-PLC inhibitors as potential anticancer agents. It is therefore important that our assay can be applied to study PC-PLC*Bc* inhibition in an efficient and accurate manner. PC-PLC*Bc* is a metalloenzyme, and it is known that metal-chelating agents such as ethylenediaminetetraacetic acid (EDTA) may deactivate the enzyme.44 We therefore first tested the ability of our assay to monitor EDTA inhibition of PC-PLC*Bc* (Table 1). The reaction was carried out in the presence of varying concentrations of EDTA. Product (34:1-DAG) formation was monitored using MALDI-TOF, as described above. A plot of the percentage activity of the enzyme as a function of the logarithm of the EDTA concentration using non-linear curve fitting gave us an IC50 of 4.07 ± 0.28 mM (Table 1 and Fig. S7), establishing that our assay can be used to monitor PC-PLC inhibition.

One of the most well studied inhibitors of PC-PLC is tricyclodecan-9-yl-xanthogenate (D609) (Table 1).45 By using D609 and its structural analogues that contain a non-ionic dithiocarbonate headgroup, we applied our assay to validate the mode of binding of D609 to PC-PLC*Bc*. We found that D609 inhibits PC-PLC*Bc* with an IC50 value of 0.20 ± 0.04 mM (Table 1 and Fig. S8). Our studies also showed that D609 analogues in which the xanthate moiety was replaced by a S-methyl xanthate group did not inhibit PC-PLC*Bc* (Analogues 1 and 2, Table 1). To further support our results, we measured the binding of D609 and its analogues by intrinsic tryptophan fluorescence spectroscopy.46 We found that D609 binds PC-PLC*Bc* with a dissociation constant (*K*D) value of 0.21 ± 0.01 mM (Table 1 and Fig. S9), confirming the results that we obtained with the MALDI-TOF assay. Furthermore, in agreement with the MALDI-TOF results, D609 analogues 1 and 2 did not bind to PC-PLC*Bc*. This is further confirmed by molecular modelling studies (Table S4 and Fig. S10), which showed that the S-methyl xanthate moiety of D609 analogues 1 and 2 may actually form unfavourable clashes with the Zn2+ metal ions and nearby anionic acidic residues including aspartic acids (Asp55 and Asp132) in PC-PLC*Bc*, which may explain the lack of inhibitory activities. Overall, our data therefore supports previous proposals that the ability for D609 to chelate the active site zinc ion is essential for its binding to PC-PLC.



Fig. 2 (a) Time-dependent MALDI-TOF mass spectra of the PC-PLC*Bc*-catalysed hydrolysis of 34:1-PC in a two-phase reaction system. The aqueous phase (100 µL) contains 50 nM PC-PLC*Bc* in 50 mM DMG, 0.1 mM ZnSO4 at pH 7.3, and the organic phase (100 µL) contains 2 mM 34:1-PC as substrate and 500 μM 24:0-DAG as internal standard. The mixture was mixed by shaking at 100 rpm at 37 °C. (b) Corresponding time course plot showing the formation of 34:1-DAG over time. An exponential growth line is fitted to aid visualisation.

One- and two-phase reaction systems to study PC-PLC*Bc* inhibition

We have recently reported a novel structural series of PC-PLC inhibitors that contain the 2-morpholinobenzoic acid scaffold (Table 1).47,48 Interestingly, when using the aforementioned two-phase system in the presence of varying concentrations of inhibitors containing the 2-morpholinobenzoic acid scaffold, no or very weak inhibition was observed (Table 1 and Fig. S11-S15) even though these compounds were found to bind PC-PLC*Bc* with micromolar binding affinity (Table 1 and Fig. S16-S20). As these compounds were relatively hydrophobic in nature, we suspected that these compounds may partition from the aqueous phase into the organic phase. By using PC\_350 (see Table 1 for structure) as an example, 1H nuclear magnetic resonance (NMR) studies using a CDCl3 and aqueous buffer (50 mM 3,3-dimethylglutaric acid (DMG), 0.1 mM ZnSO4 at pH 7.3) in D2O mixture showed that around 80% of the compound was partitioned into the organic phase (Fig. S21), hence they were unable to inhibit PC-PLC*Bc*, which is present in the aqueous phase. Our results highlight that it is vitally important to consider the nature of the inhibitor when conducting PC-PLC inhibition assays, especially when a two-phase reaction system is being used.

Table 1 IC50 and *K*D values of the compounds tested against PC-PLC*Bc* in this study.

|  |  |  |  |
| --- | --- | --- | --- |
| Structure | Two-phase system  IC50 / mM | One-phase system  IC50 / mM | *K*D / mM |
| EDTA | 4.07 ± 0.28 | 3.43 ± 0.52 | No binding |
|  | 0.20 ± 0.04 | 0.17 ± 0.02 | 0.21 ± 0.01 |
|  | No inhibition | No inhibition | No binding |
|  | No inhibition | No inhibition | No binding |
|  | 1.15 ± 0.18 | 0.36 ± 0.08 | 0.40 ± 0.01 |
|  | 1.20 ± 0.12 | 0.43 ± 0.10 | 0.41 ± 0.10 |
|  | 1.64 ± 0.25 | 0.70 ± 0.12 | 0.63 ± 0.04 |
|  | No inhibition | 1.97 ± 0.15 | 0.83 ± 0.16 |
|  | No inhibition | 2.40 ± 0.22 | 0.90 ± 0.04 |

This difficulty to study inhibition by these aromatic inhibitors thus prompted us to develop a single-phase reaction system. As the phospholipid substrate was supplied in chloroform, the lipid was first dried using nitrogen gas and resuspended in the aqueous reaction buffer. The reaction was carried out by incubating the resuspended lipid with a solution of the enzyme in the same buffer. The reaction was quenched by the addition of a 1:1 solution of acetonitrile and methanol, followed by extraction of the lipids into chloroform and analysing the product formation using MALDI-TOF mass spectrometry. This method gave us a similar time-course curve for the hydrolysis reaction as the two-phase system (Fig. S22). It also gave nearly identical kinetic parameters (Fig. S23 and Table S3), thus giving us confidence in this assay. We next applied this method to perform the inhibition assays. The IC50’s obtained for D609 and EDTA were 0.17 ± 0.02 mM and 3.43 ± 0.52 mM, respectively (Table 1 and Fig. S24 and S25), which are almost identical to the values from the two-phase system. We also managed to successfully measure the IC50 values of the 2-morpholinobenzoic acid derivatives (Table 1 and Fig. S26-S30). The IC50 values are in agreement with the *K*D values that were obtained by intrinsic tryptophan fluorescence (Table 1), therefore providing validation for this assay.

Comparison with the Amplex Red assay

Amongst the five morpholinobenzoic acid derivatives that we tested in this study, the strongest inhibitors were found to be for PC\_350 and PC\_332 followed by PC\_358. The weakest inhibitors were for PC\_325 and PC\_326. This is interesting as our results contradicted previous inhibition studies using the Amplex Red assay, which showed that PC\_325 and PC\_326 were the strongest inhibitors. As the Amplex Red assay is an enzyme-coupled assay that involves three downstream enzymes for detection (alkaline phosphatase, choline oxidase and horseradish peroxidase), we suspected that the 2-morpholinobenzoic acid derivatives may affect these downstream enzymes. We therefore conducted the Amplex Red assay with phosphocholine in the absence of PC-PLC. As expected, the Amplex Red assay gave a strong signal for the formation of the fluorescent product resorufin, which was monitored fluorometrically with an excitation wavelength of 560 nm and emission wavelength range of 580-600nm, with maximum emission occurring at 590 nm (Fig. S31). Interestingly, we found that the 2-morpholinobenzoic acid derivatives reduced the fluorescence signal of the Amplex Red assay (Fig. S30). Molecular modelling (Table S4 and Fig. S32) as well as careful analyses of the structure of the 2-morpholinobenzoic acid derivatives showed that they may act as a substrate/inhibitor of horseradish peroxidase.49 Indeed, in the presence of the 2-morpholinobenzoic acid derivatives, we found that the ability of horseradish peroxidase to catalyse the reaction between hydrogen peroxide and Amplex Red was significantly reduced (Fig. S33). Our results highlight the advantage of our MALDI-TOF assay, which allow direct observation of the PC-PLC-catalysed hydrolysis reactions and avoiding downstream enzymes that may affect the accuracy of the measurements.

Conclusions

We have developed an efficient MALDI-TOF mass spectrometry-based assay for the study of PC-PLC*Bc* activity and inhibition. Using 34:1-PC as a substrate, the formation of the reaction product 34:1-DAG can be readily and accurately quantified by comparing with an internal standard 24:0-DAG. Previous studies have shown that PC-PLC*Bc* activities may be monitored by using a one-phase aqueous-based reaction system or with a two-phase aqueous/organic reaction system.42 By using our MALDI-TOF assay, we showed that although both reaction systems give similar kinetic parameters for PC-PLC*Bc*, inhibition study of hydrophobic compounds using the two-phase reaction system may give false negative results as the compounds were found to be partitioned from the aqueous environment into the organic phase. Finally, we have also compared our results with those obtained from the commercially available Amplex Red assay, which is widely used to study PC-PLC*Bc* activity and inhibition. Our MALDI-TOF assay is superior due to its versatility, allowing direct monitoring of reaction product formation. In contrast, as the readout of the Amplex Red assay is dependent on several downstream enzymatic and chemical reactions, care must be taken to ensure the accuracy especially with inhibition studies. We envisage the assay will aid in the application of PC-PLC*Bc* in the food industry as it allows rapid analysis of enzyme kinetics. It will also allow development of new PC-PLC*Bc* inhibitors, which may have potential applications as antimicrobial and anticancer agents. More generally, this MALDI-TOF mass spectrometry-based method can be readily applied to study the kinetics and inhibition of phospholipases, a class of enzyme that have found an increasing number of applications in industry and in medicinal chemistry.

Conflicts of interest

There are no conflicts to declare.

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Notes and references

1. D. C. Wilton, in *Biochemistry of Lipids, Lipoproteins and Membranes*, ed. D. E. Vance and J. E. Vance, Elsevier, Amsterdam, 5th edn, 2007, ch. 11, pp. 305–329.
2. M. Waite, in *Handbook of Lipid Research 5. The Phospholipases*, Springer-Verlag, Boston MA, 1987, ch. 11, pp. 243–281.
3. L. A. Hinkel and M. J. Wargo, in *Health Consequences of Microbial Interactions with Hydrocarbons, Oils, and Lipids*, ed. H. Goldfine, Springer, Cham, 2020, pp. 181-203.
4. Y. Ishibashi, K. Aoki, N. Okino, M. Hayashi and M. Ito, *Sci. Rep.*, 2019, **9**, 16357.
5. P. H. Guddal, T. Johansen, K. Schulstad and C. Little, *J. Bacteriol.*, 1989, **171**, 5702–5706.
6. J. V. Bonventre, *J. Am. Soc. Nephrol.*, 1992, **3**, 128–150.
7. X. Wang, *Plant Physiol.*, 1999, **120**, 645-652.
8. P. Huang and M. A. Frohman, *Expert Opin. Ther. Targets*, 2007, **11**, 707–716.
9. B. S. Cummings, *Biochem. Pharmacol.*, 2007, **74**, 949–959.
10. L. De Maria, J. Vind, K. M. Oxenbøll, A. Svendsen and S. Patkar, *Appl. Microbiol. Biotechnol.*, 2007, **74**, 290–300.
11. S. Cerminati, L. Paoletti, A. Aguirre, S. Peirú, H. G. Menzella and M. Eugenia Castelli, *Appl. Microbiol. Biotechnol.*, 2019, **103**, 2571–2582.
12. V. Casado, D. Martín, C. Torres and G. Reglero, in *Lipases and Phospholipases. Methods in Molecular Biology (Methods and Protocols)*, ed. G. Sandoval, Humana Press, 2012, vol. 861, pp 495–523.
13. Y. C. Sharma, M. Yadav and S. N. Upadhyay, *Biofuel Bioprod. Bioref.*, 2019, **13**, 174–191.
14. S. Cesarini, F. I. J. Pastor, P. M. Nielsen and P. Diaz, *Sustainability*, 2015, **7**, 7884–7903.
15. S. Cesarini, R. F. Haller, P. Diaz and P. M. Nielsen, *Biotechnol. Biofuels*, 2014, **7**, 29.
16. Y. Li, W. Du and D. Liu, *Biochem. Eng. J.*, 2015, **94**, 45–49.
17. R. G. Sheikhnejad and P. N. Srivastava, *J. Biol. Chem.*, 1986, **261**, 7544–7549.
18. Y. Lyu, L. Ye, J. Xu, X. Yang, W. Chen and H. Yu, *Biotechnol. Lett.*, 2016, **38**, 23–31.
19. P. J. Hergenrother and S. F. Martin, in *Bioorganic Chemistry of Biological Signal Transduction*, ed. H. Waldmann, Springer, Berlin, 2001, pp. 131–167.
20. E. Hough, L. K. Hansen, B. Birknes, K. Jynge, S. Hansen, A. Hordvik, C. Little, E. Dodson, and Z. Derewenda, *Nature*, 1989, **338**, 357–360.
21. A. P. Benfield, N. M. Goodey, L. T. Phillips, S. F. Martin, *Arch. Biochem. Biophys.*, 2007, **460**, 41–47.
22. T. Johansen, T. Holm, P. H. Guddal, K. Sletten, F. B. Haugli and C. Little, *Gene*, 1988, **65**, 293–304.
23. C. A. Tan, M. J. Hehir and M. F. Roberts, *Prot. Exp. Purif.*, 1997, **10**, 365–372.
24. P. H. Guddal, T. Johansen, K. Schulstad and C. Little, *J. Bacteriol.*, 1989, **171**, 5702–5706.
25. F. Podo, L. Paris, S. Cecchetti, F. Spadaro, L. Abalsamo, C. Ramoni, A. Ricci, M. E. Pisanu, F. Sardanelli, R. Canese and E. Iorio, *Front. Oncol.*, 2016, **6**, 171.
26. F. Spadaro, C. Ramoni, D. Mezzanzanica, S. Miotti, P. Alberti, S. Cecchetti, E. Iorio, V. Dolo, S. Canevari and F. Podo, *Cancer Res.*, 2008, **68**, 6541–6549.
27. M. A. Clark, R. G. L. Shorr and J. S. Bomalaski, *Biochem. Biophys. Res. Commun.*, 1986, **140**, 114–119.
28. Identification of the Elusive Mammalian Enzyme Phosphatidylcholine-Specific Phospholipase C, https://apps.dtic.mil/dtic/tr/fulltext/u2/a611640.pdf, (accessed October 2020).
29. C. Elena, S. Cerminati, P. Ravasi, R. Rasia, S. Peiru, H. G. Menzella and M. Eugenia Castelli, *Process Biochem.*, 2017, **54**, 67–72.
30. P. Ravasi, M. Braia, F. Eberhardt, C. Elena, S. Cerminati, S. Peirú, M. Eugenia Castelli and H. G. Menzella, *J. Bacteriol.*, 2015, **216**, 142–148.
31. M. Zhou, C. Zhang, and R. P. Haugland, *Proc. SPIE Int. Soc. Opt. Eng.*, 2000, **3926**, 166.
32. M. Zhou, Z. Diwu, N. Panchuk-Voloshina and R. P. Haugland, *Anal. Biochem.*, 1997, **253**, 162–168.
33. J. G. Mohanty, J. S. Jaffe, E. S. Schulman and D. G. Raible, *J. Immunol. Methods*, 1997, **202**, 133–141.
34. M. A. Durban and U. T. Bornscheuer, *Eur. J. Lipid Sci. Technol.*, 2007, **109**, 469–473.
35. A. Flieger, S. Gong, M. Faigle and B. Neumeister, *Enzyme Microb. Technol.*, 2000, **26**, 451–458.
36. C. Murakami, S. Mizuno, S. Kado and F. Sakane, *Anal. Biochem.*, 2017, **526**, 43–49.
37. A. Mandal, M. Singha, P. S. Addy and A. Basak, *Mass Spectrom. Rev.*, 2019, **38**, 3–21.
38. I-C. Lu, C. Lee, Y.-T. Lee and C.-K. Ni, *Annu. Rev. Anal. Chem.*, 2015, **8**, 21–39.
39. D. S. Peterson, *Mass Spectrom. Rev.*, 2007, **26**, 19–34.
40. J. Lee, Y.-K. Kim and D.-H. Min, *J. Am. Chem. Soc.*, 2010, **132**, 14714–14717.
41. T. W. Jaskolla, K. Onischke and J. Schiller, *Rapid Commun. Mass Spectrom.*, 2014, **28**, 1353–1363.
42. R. Haftendorn and R. Ulbrich-Hofmann, *Anal. Biochem.*, 2002, **306**, 144–147.
43. M. Y. El-Sayed, C. D. DeBose, L. A. Coury and M. F. Roberts, *Biochim. Biophys. Acta*, 1985, **837**, 325–335.
44. E. Randell, H. Mulye, S. Mookerjea and A. Nagpurkar, *Biochim. Biophys. Acta*, 1992, **1124**, 273–278.
45. A. González-Roura, J. Casas and A. Llebaria, *Lipids*, 2002, **37**, 401–406.
46. A. Yammine, J. Gao and A. H. Kwan, *Bio-protocol*, 2019, **9**, e3253.
47. C. Eurtivong, L. I. Pilkington, M. van Rensburg, R. M. White, H. K. Brar, S. Rees, E. K. Paulin, C. S. Xu, N. Sharma, I. K. H. Leung, E. Leung, D. Barker and J. Reynisson, *Eur. J. Med. Chem.*, 2020, **187**, 111919.
48. L. I. Pilkington, K. Sparrow, S. W. P. Rees, E. K. Paulin, M. van Rensburg, C. S. Xu, R. J. Langley, I. K. H. Leung, J. Reynisson, E. Leung, D. Barker, *Eur. J. Med. Chem.*, 2020, **191**, 112162.
49. N. C. Veitch, *Phytochemistry*, 2004, **65**, 249–259.