RESEARCH PAPER

Hydrophilic interaction chromatography based solid-phase extraction and MALDI TOF mass spectrometry for revealing the influence of *Pseudomonas fluorescens* on phospholipids in salmon fillet

Qing Shen · Qi Yang · Hon-Yeung Cheung

Abstract Salmon is a popular food but it is easily susceptible to spoilage by contamination with microorganisms. In this study, a method using hydrophilic interaction chromatography (HILIC)-based solid-phase extraction (SPE) and matrix-assisted laser desorption and ionization time-of-flight/time-of-flight mass spectrometry was developed and applied to reveal the effect of *Pseudomonas fluorescens* on salmon fillet during the shelf-life period by measuring the changes in the levels of phosphatidylcholine and phosphatidylethanolamine. Fresh samples were inoculated with *P. fluorescens* (10^6 cfu g^{-1}) for 30 s, and lipids were extracted at 0, 24, 48, and 72 h. A homemade SPE cartridge packed with HILIC sorbent (silica derivatized with 1,2-dihydroxypropane) was used for matrix cleanup prior to analysis by mass spectrometry. In total, 30 phospholipids and 16 lysophospholipids were detected and elucidated. The results revealed that the content of phospholipids decreased significantly, whereas that of lysophospholipids increased initially, followed by a gradual reduction as the cold storage time increased. The contamination by *P. fluorescens* negatively affected the quality of fresh salmon without obvious physical changes, but it posed a potential threat to human health. This study suggests that the well-established method could be used for detecting phospholipids in salmon fillet and perhaps other foods as well.

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Introduction

Salmon, belonging to the family Salmonidae, is an economically important fish in contemporary daily life. Its habitats are all over the northern Pacific (both west and east) and the northern areas of Europe [1]. The fish has a flat side, sharp teeth, and an arched back, and is silver-gray [2]. Raw salmon is commonly served as a delicacy, which requires good storage in order to keep it fresh and safe for consumption [3]. As salmon contains abundant components of unsaturated fatty acids, which has the beneficial function of reducing the levels of blood fat and cholesterol, lowering the risk of blood-related diseases such as cardiovascular disease, it is nutritive for the human body. Eating salmon regularly can be conducive to preventing blood-related disease and to reducing death from such health problems [4]. Moreover, in field of traditional Chinese medicine, salmon has the effects of enhancing immunity and reducing blood glucose levels.

Phospholipids are increasingly regarded as important nutrients with putative benefits to health from the biochemical, physiological, and nutritional viewpoints. The daily intake of phospholipids represents 1–10 % of total daily fat intake [5]. There is consistent experimental evidence indicating the positive effects of dietary phospholipids on lipid digestion, absorption, and transport, in inflammatory processes, and in signaling pathways [6]. Meanwhile, the well-known ω-3 fatty acids, such as docosahexaenoic acid and eicosapentaenoic acid, are

more easily incorporated in phospholipids, such as phosphatidylcholine (PC) and phosphatidylethanolamine (PE), rather than other lipid forms, making phospholipids one of the most efficient forms of ω-3 fatty acid supplying ingredients [7, 8]. However, as the most abundant membrane lipid in salmon muscle, phospholipids are easily deteriorated during storage, which is detrimental to both the freshness and the quality of salmon. Microbial contamination is normally responsible for the oxidation and hydrolysis of fish phospholipids, resulting in a range of by-products with unpleasant taste and smell [9]. *Pseudomonas fluorescens* is a common gram-negative spoilage bacterium widely found in nature (soil, water, plants, and animals). When a refrigerated fish is about to die, the number of *Pseudomonas* spp. in the microbial count becomes the largest, usually composing more than 50 % [10]. Furthermore, these organisms multiply very fast even at 4 °C, although the ideal growing temperatures for *Pseudomonas* spp. are around 25–30 °C [11]. It has been found that in the presence of *P. fluorescens*, the B-type phospholipase expressed can remove the acyl chains from phospholipids and the single acyl chain from lysophospholipids [12]. Hence, *P. fluorescens* has been endorsed as a representative specific spoilage organism (SSO) for studying its effect on the spoilage of salmon.

There are protocols available for the analysis of lipids. Common approaches include thin-layer chromatography, high-performance liquid chromatography, and a shotgun method coupled with mass spectrometry (MS) [13–16]. Among all the techniques available today, electrospray ionization MS is the most widely used protocol for lipid analysis [17]. There is growing evidence that matrix-assisted laser desorption and ionization (MALDI) time-of-flight (TOF) MS is a useful alternative method allowing the simultaneous recording of mass spectra of cells, crude tissue, or body fluid extracts or even intact tissue fillets in a few minutes [18, 19]. The resolution and mass accuracy achieved by MALDI-TOF/ MS analysis are normally informative, and the composition of phospholipids can be identified without previous separation [20]. The specific advantages of MALDI-TOF/MS are its fast, simple, and convenient performance often in combination with automation and the high sensitivity down to a few attomoles of the analyte [21].

The purpose of the present study was to develop an efficient method for revealing the effect of *P. fluorescens* contamination on salmon fillet. PC and PE were chosen as two target analytes, because they are abundant in the cell membrane and play a vital role in the texture and mouthfeel of salmon fillet. The lipid composition of salmon samples in different periods was evaluated using multivariate statistical analysis (principal component analysis, PCA). This study provides information for monitoring the spoilage of salmon fillets caused by

contamination with *P. fluorescens* throughout the shelf-life of salmon fillets.

Materials and methods

Chemicals and reagents

PC (14:0/14:0) and PE (15:0/15:0) were obtained from Avanti Polar Lipids. (Alabaster, AL, USA) and were prepared in MeOH–CHCl$_3$ (1:1, v/v) to a final concentration of 1 μg mL^{-1}. These internal standards were selected because they represent 1 % or less of the endogenous cellular lipid molecular species present as demonstrated by MS analysis without addition of these internal standards. 2,5-Dihydroxybenzoic acid (DHB) was purchased from Sigma-Aldrich (St Louis, MO, USA) and was dissolved in MeOH to a final concentration of 30 mg·mL^{-1} as a stock solution. High-performance liquid chromatography grade CHCl$_3$ and MeOH were purchased from Fisher Scientific (Waltham, MA, USA). Water with a resistivity of 18 MΩ·cm was purified using a Milli-Q system from Millipore (Billerica, MA, USA).

Sample preparation

Salmon samples were purchased from a TASTE supermarket (Hong Kong), where the quality of food was strictly controlled. The salmon samples were quickly transferred to the Microbiology Laboratory of City University of Hong Kong within 10 min. For sterilization, physical techniques, such as high temperature, high pressure, or ultraviolet rays, are not suitable for raw meat, since use of such techniques will cause peroxidation damage to the unsaturated bonds of lipids [22]. The salmon samples were sterilized in accordance with the method of Herbert et al. [23]. Briefly, the salmon were washed in a 5 % Na$_2$CO$_3$ solution to remove the outer slime, which contains most of the surface and adhering bacteria. The skin was washed in 2 % formalin solution and removed under aseptic conditions. Then, the underlying tissue was cut into blocks (rectangles and 8 g each) with sterilized scissors. The sterile blocks were stored at a chill temperature and could be used for further storage experiments either as sterile blocks or inoculated with pure cultures of bacteria.

Bacterial culture

P. fluorescens (ATCC 948) culture was prepared by a technician (Kenneth Lau) in the Microbiology Laboratory of City University of Hong Kong, and was stored in a refrigerator (–5 °C) after the culture container had been sealed up. The culture method for *P. fluorescens* was from ATCC: broth containing 3.0 g beef extract, 5.0 g peptone, and 1,000 mL

deionized water. Autoclaving was performed at 121 °C for 15 min and the growth temperature was 26.0 °C.

Lipid extraction

Phospholipids were extracted from salmon samples according to a modified version of the Bligh and Dyer method [24]. Briefly, 0.1 g salmon sample was accurately weighed, placed in a 5-mL polytetrafluoroethylene tube, and mixed with 3 mL of $CHCl_3$–MeOH (2:1, v/v) solution. After ultrasound-assisted extraction for 15 min, 1 mL water was added and the tube was centrifuged at $8,000g$ for 10 min in order to separate the solvent phase. Then, the lower organic phase was recovered and transferred to a new glass tube by pipette. The aqueous phase was re-extracted with 2 mL $CHCl_3$ another two times and was treated as described before. The collected organic phases were combined and evaporated under a nitrogen flow. Dried lipid extracts were dissolved in 1 mL $CHCl_3$–MeOH (2:1, v/v) and stored in the dark at –80 °C. Then 10 μL of internal standards was added to each salmon sample prior to extraction of phospholipids. To minimize the risk of oxidation of the polyunsaturated fatty acids or lipid hydrolysis during the isolation process, it is recommended that the extraction of lipids should always be performed at low temperature (4 °C) as soon as possible.

Solid-phase extraction procedure

Particles of silica derivatized with 1,2-dihydroxypropane (SiO_2/diol) were obtained by digging out the sorbent from a scrapped YMC Triart diol hydrophilic interaction chromatography column (4.6 mm×250 mm, 3 μm; YMC Europe, Dinslaken, Germany). SiO_2/diol composite (200 mg) was packed into a 1-mL syringe. Polypropylene materials were set as frits at both sides to hold the sorbent. Before use, the homemade solid-phase extraction (SPE) cartridge was preconditioned and activated by washing it with 5.0 mL of 60 % aqueous acetonitrile and pure acetonitrile, respectively. The crude extract was loaded on and passed through the cartridge at a flow rate of 0.2 mL·min⁻¹. Then, the cartridge was washed with acetonitrile to remove the matrix. After that, the analytes retained on the SPE sorbent were eluted with 1 mL of 50 % aqueous acetonitrile. The eluent was filtered through a 0.22-μm filter, and then it was subjected to MALDI-TOF/MS analysis.

MALDI method and data analysis

For MALDI analysis, 10 μL of DHB solution (1 μg·mL⁻¹) was freshly prepared and mixed with an equal volume of sample solution before deposition onto the MALDI target. Then, 5 μL of the mixture was applied onto a MALDI target plate. MS analysis was based on use of an Applied Biosystems 4800 MALDI-TOF/TOF mass spectrometer (AB Sciex, Foster City, CA, USA) equipped with a 200-Hz tripled-frequency Nd:YAG pulsed laser with 355-nm wavelength. Measurements were performed in positive ion mode at an accelerating potential of 20 kV. A delayed ion extraction time of 450 ns was selected according to the mass range under observation (m/z 450–1,000), allowing baseline isotopic mass resolution. Mass spectra were obtained by applying a laser energy adjusted up to 5–10 % above the threshold irradiation according to the manufacturer's nominal scale. An integrated video imaging system (approximately ×25 magnification) allowed direct observation of the sample spots under investigation. External mass calibration was achieved using the mixture of phospholipid standards described above.

MS data acquisition was performed by 4000 Series Explorer, version 3.5.2. Data analysis was performed by the supplied instrument software, Data Explorer version 4.9 (Applied Biosystems). Lipid characterization was performed by comparing accurate mass measurements with the LIPID MAPS prediction tool (http://www.lipidmaps.org/tools/index.html) and confirmation by MS/MS, and representative MS/MS spectra were obtained (see the electronic supplementary material).

Results and discussion

SPE optimization

To evaluate the performance of the SiO_2/diol-based SPE cartridge, and to obtain extracts with high recovery and a low matrix effect from the salmon samples, several factors were evaluated to establish the optimum conditions for the SPE procedure, including loading flow rate and breakthrough volume, composition of the washing solvent, and composition and volume of the elution solvent. A standard solution containing PC (14:0/14:0) and PE (15:0/15:0) each at a concentration of 1,000 ng·mL⁻¹ in acetonitrile was used.

Loading flow rate and breakthrough volume

The sample loading flow rate is an important factor, because slow flow will waste time, whereas fast flow can cause less adsorption and retention of analytes onto the SiO_2/diol sorbent in the column. The effect of sample loading flow rate on the abundance of phospholipids was evaluated by loading 1 mL standard solution (PC 14:0/14:0 and PE 15:0/15:0 at a concentration of 1,000 ng·mL⁻¹) into the SiO_2/diol SPE cartridge at flow rates in the range from 0.1 to 1.0 mL·min⁻¹. As shown in Fig. 1a, the highest abundance of PC and PE signals was obtained at 0.5 mL·mL⁻¹, which was adopted for subsequent
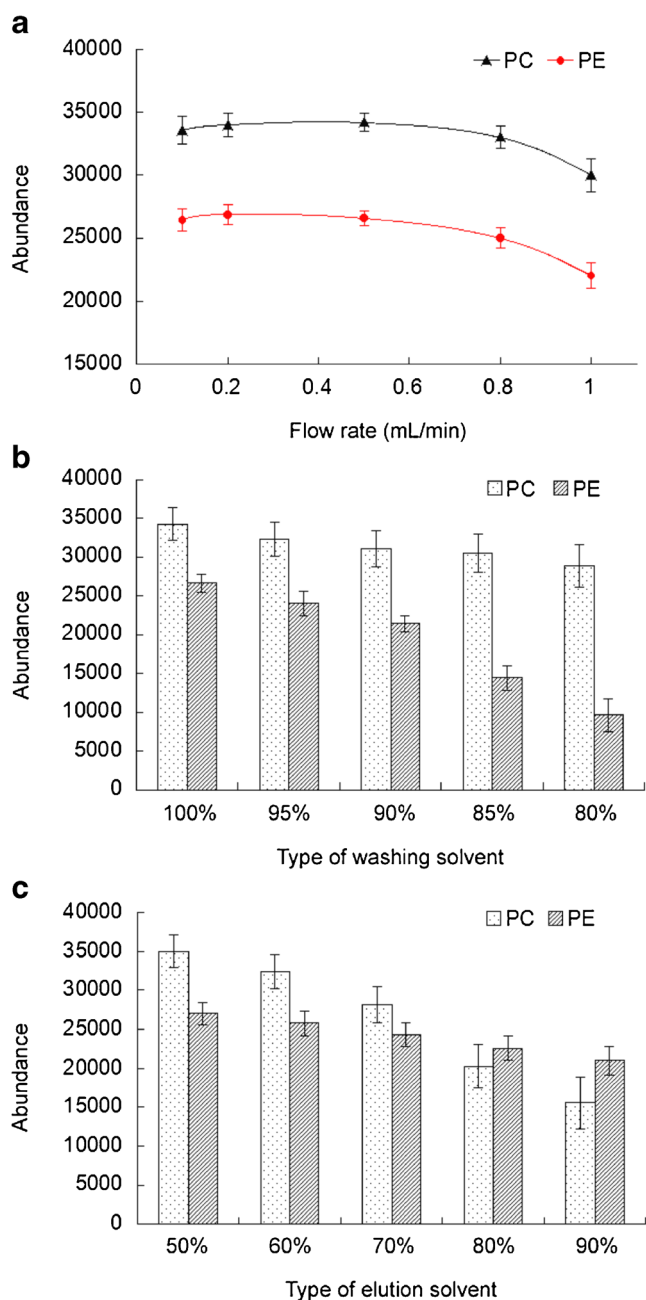
Fig. 1 **a** Effect of the sample loading flow rate on the abundance of phospholipids extracted by silica derivatized with 1,2-dihydroxypropane (SiO$_2$/diol)-based solid-phase extraction (SPE) (n=3). **b** Effect of the type of washing solvent on the abundance of phospholipids extracted by SiO$_2$/diol-based SPE (n=3). **c** Effect of the type of elution solvent on the abundance of phospholipids extracted by SiO$_2$/diol-based SPE (n=3). *PC* phosphatidylcholine, *PE* phosphatidylethanolamine

experiments. Higher loading rates decreased the abundance values, which was attributed to the reduced time of interaction between the phospholipids and the SiO$_2$/diol binding sites.

The breakthrough volume depends on the nature of the sorbent material and the type and concentration of the sample constituents. The effect of breakthrough volume

(0.1, 0.2, 0.4, 0.6, 0.8, and 1 mL), containing PC (14:0/14:0) and PE (15:0/15:0) at a concentration of 1,000 ng·mL^{-1}, was investigated (data not shown). The results showed that the abundance of standard analytes slightly decreased when sample volumes were increased to 0.8 mL, which seemed to be the tolerated volume for breakthrough. Considering the analytical time and complexity of a real sample, 0.5 mL was used as the optimum breakthrough volume.

Composition of the washing solvent

Nonspecific interactions between the matrix and the SiO$_2$/diol particles can be minimized using acetonitrile or its aqueous solution in the washing step. To evaluate this effect, the SPE cartridges, preloaded with standard solutions, were subjected to 2 mL of washing solvents of acetonitrile and aqueous acetonitrile (80, 85, 90, and 95 %). Figure 1b shows the effect of the types of elution solvents on the abundance of phospholipids extracted by SiO$_2$/diol-based SPE. The retention of phospholipids in the SiO$_2$/diol particle was higher with concentrated acetonitrile in the washing solvent. With the increase of water content in the washing solvent, the abundance of PC decreased gradually, whereas that of PE dropped dramatically. This indicated that the retention strength between the SiO$_2$/diol particle and PC was stronger than that between SiO$_2$/diol and PE, which agreed well with the findings of previously published studies [25]. Finally, 2 mL acetonitrile was used as washing solvent.

Composition and volume of the elution solvent

To desorb specifically the phospholipids in the cartridge, the SPE was followed by an elution step with an appropriate amount of eluent. Aqueous acetonitrile was used with a concentration ranging from 50 to 90 %, and the results of this study are shown in Fig. 1c. As expected, the abundance of both PC and PE decreased as the percentage of acetonitrile increased in the elution solvent. The function of SiO$_2$/diol particles in the SPE cartridge will be negatively affected if the water content in the elution solvent is higher than 50 %. Consequently, 50 % aqueous acetonitrile was selected as the optimum elution (disperser) solvent. Besides, the effect of the volume of eluent ranging from 1 to 5 mL on the elution efficiency was studied. It was found that 2 mL of 50 % acetonitrile was good enough to recover all the phospholipids.

The effect of incubation parameters

The method of bacterial inoculation was optimized on the basis of the inoculation time and the concentration of SSO

solution used. The bacterial content in fresh salmon fillet was $10^2–10^3$ cfu g^{-1}. With this low number, the impact of the original bacteria could be disregarded if the *P. fluorescens* used for inoculation is dominant as long as its concentration is more than two orders of magnitude greater than the original total bacterial count [26]. On that basis, the prepared sterile salmon fillets were soaked in highly concentrated SSO solution (10^6 cfu g^{-1}), and were removed in sequence every 10 s. The relation between the inoculation time and the total bacterial count in salmon fillet is depicted in Fig. 2a. As shown, the concentration of SSO increased with the increase of the inoculation time, and it reached 10^5 cfu g^{-1} after inoculation for 30 s. Then, the total bacterial count became stable and no further increase was observed even when the inoculation time was prolonged. Therefore, 30 s was enough for the inoculation of fresh salmon fillets with *P. fluorescens*.

The effect of SSO solutions, with concentrations ranging from 0 to 10^8 cfu g-^{1}, was also investigated, and the results are depicted in Fig. 2b. It indicates that highly concentrated SSO solutions, with concentrations such as 10^8 and 10^7 cfu g^{-1}, were not suitable for storage experiments, because heavily contaminated salmon fillets tended to undergo rapid spoilage. On the other hand, a bioburden lower than 10^5 cfu g^{-1} was also inappropriate, as the bacterial levels were too low to for bacteria to attach to the salmon fillets, and this would lead to inhibition of growth of *P. fluorescens* by other microorganisms. Therefore, 10^6 cfu g^{-1} was selected as the ideal inoculation concentration of SSO.

Lipid characterization and determination

DHB was applied to the lipid extractions since it is readily soluble in organic solvents, enhancing the homogeneity of crystallization of the sample and matrix mixture, and leading to reproducible MALDI-TOF/MS measurements and well-resolved mass spectra [27]. An isotopic correction was performed to ensure that minor peaks did not result from the isotopic distribution of the adjacent and more dominant lipid peaks. The mass spectra of different stages of salmon contaminated with *P. fluorescens* are depicted in Fig. 3 in a clear and systematic manner. On the basis of MS/MS data and the literature, and with the help of the database LIPID MAPS, the peaks observed in the m/z range 750−850 were identified as phospholipids, which were the major lipid groups detected in this experiment. For determination of each lipid species, the unnaturally occurring internal standards PC 14:0/14:0 (m/z 678.8) and PE 15:0/15:0 (m/z 664.9) were used to quantify PC and PE, respectively. With use of this method, at least 48 molecular species were identified, including 31 phospholipids and 17 lysophospholipids. Table 1 lists the molecular species and the abundance of phospholipid and lysophospholipid classes from fresh salmon fillet. According to the results, potassium/sodium-adducted PC species were the main ions
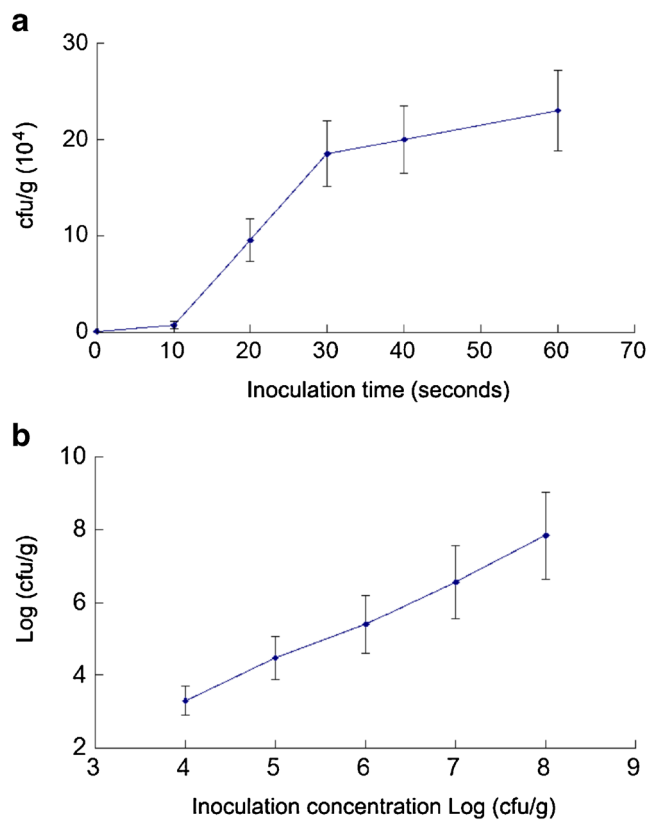

Fig. 2 a *Pseudomonas fluorescens* (cfu/g) in salmon fillet inoculated for different times. b *P. fluorescens* (cfu/g) in salmon fillet inoculated with different specific spoilage organism concentrations

detected, and PE species were also detected with minor abundance. Some peaks were composed of more than one lipid species. For instance, the peak at m/z 542 resulted from the overlap between the [lipophosphatidylcholine (LPC) 18:2+Na]$^+$ and [LPC20:5+H]$^+$ peaks, the peak at m/z 760 resulted from the overlap between the [PC34:1+H]$^+$ and [PE36:5+Na]$^+$ peaks, and so on. In addition, *sn*-2/polyunsaturated phospholipids accounted for a large part of the total lipid content. For example, ions at m/z 828, m/z 830, and m/z 878 contained docosahexaenoic acyl chains since the ion of the acyl chain (22:6) could be detected in their product ion scanning. Meanwhile, some phospholipids were identified as highly unsaturated, where both *sn*-1 and *sn*-2 moieties were the eicosapentaenoic acyl chain or the eicosapentaenoic acyl chain, such as the ions at m/z 826, m/z 852, and m/z 878, corresponding to PC 20:5/20:5, PC 20:5/22:6, and PC 22:6/22:6, respectively. It is well known that eicosapentaenoic acid and docosahexaenoic acid are regarded as important protective agents for health promotion and disease prevention.tgroup

Influence of *P. fluorescens* on salmon

Salmon fillets that were not inoculated with *P. fluorescens* were studied and used as a control. In general, the
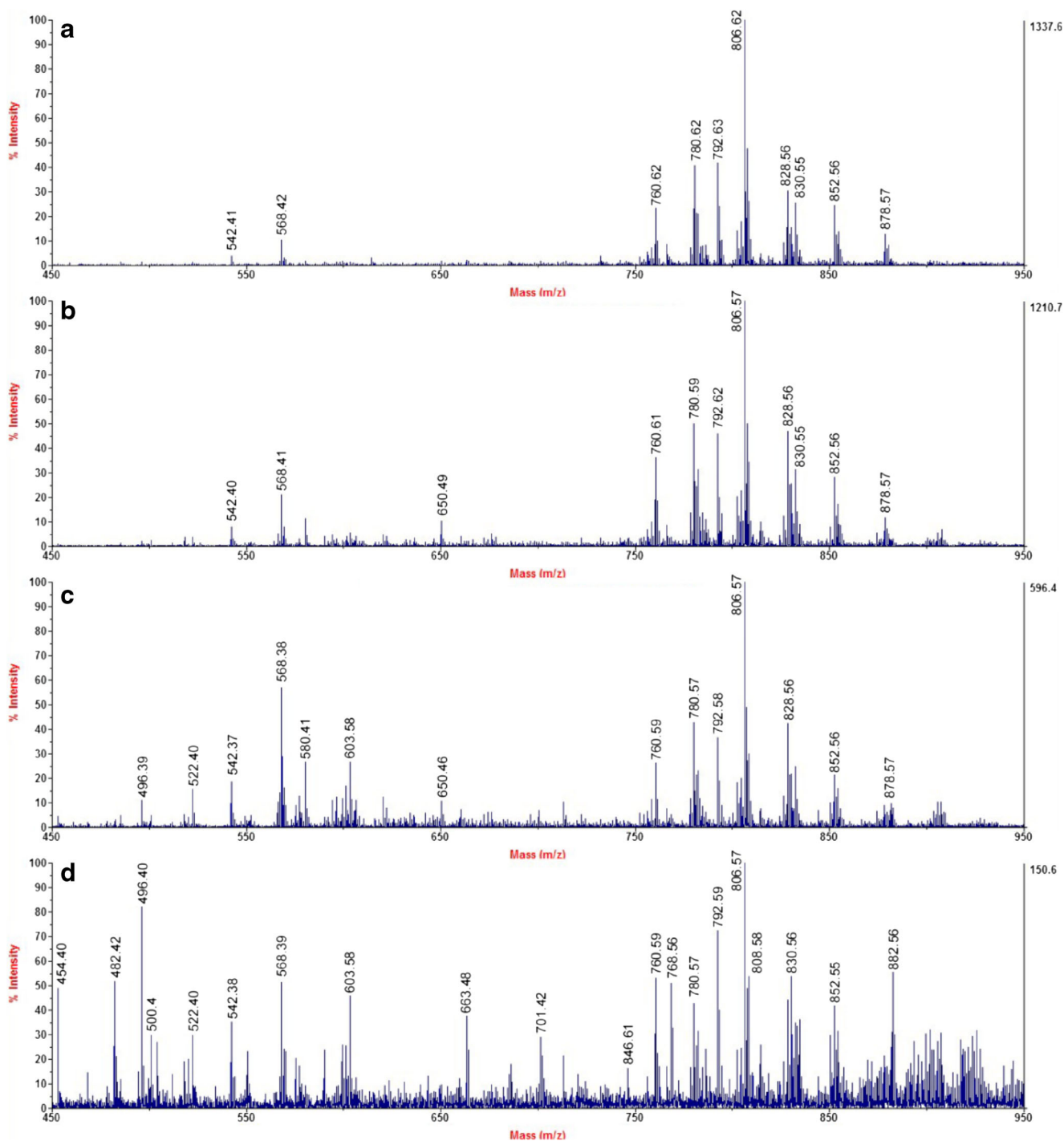
Fig. 3 Mass spectra of salmon fillets inoculated with *P. fluorescens* at 0 h (**a**), 24 h (**b**), 48 h (**c**), and 72 h (**d**)

contents of phospholipids were stable in 72 h, and the spectra of salmon fillets at different stages were generally similar (see Fig. 3a). The results indicated that the original microorganisms did not affect the quality of salmon fillets when they were stored at 4 °C.

Dynamic changes in the concentrations of phospholipids from contaminated salmon fillets at time points of 0, 24, 48, and 72 h are illustrated in Fig. 4. Initially, the ions of phospholipids in the mass range from m/z 700 to m/z 900 were dominant in the spectrum (Fig. 3a). The ion at m/z 806 was the most abundant, corresponding to the overlap between $[PC38:6+H]^+$ and $[PE38:4+K]^+$, followed by ions at m/z 780 and m/z 792. Besides, lysophospholipids were also observed in minor amounts,

**Table 1** Attribution and the relative amount of the main phospholipid ions in salmon fillet samples

Observed m/z	Probable attribution	Abundance (%)			
		Initial	24 h	48 h	72 h
Lysophospholipids					
482.6	[LPE18:0+H]$^+$	-	-	-	3.55
496.6	[LPC16:0+H]$^+$	-	-	1.35	3.61
504.4	[LPE20:3+H]$^+$; [LPE18:0+Na]$^+$	-	-	-	1.43
518.5	[LPC18:3+H]$^+$	-	-	-	1.32
520.6	[LPC18:2+H]$^+$	-	-	-	1.22
522.6	[LPC18:1+H]$^+$	-	0.42	1.69	1.34
542.6	[LPC20:5+H]$^+$	0.55	0.90	1.98	3.06
550.5	[LPC20:1+H]$^+$; [LPE22:5+Na]$^+$	-	-	-	1.27
566.6	[LPE24:0+H]$^+$	-	0.86	1.90	-
568.6	[LPC22:6+H]$^+$	1.32	2.91	4.81	3.71
570.5	[LPC22:5+H]$^+$	-	-	1.14	-
580.5	[LPC22:0+H]$^+$	-	1.48	2.62	-
590.5	[LPC22:6+Na]$^+$; [LPC20:0+K]$^+$	-	0.42	0.82	1.09
592.5	[LPC22:5+Na]$^+$	-	0.40	0.55	-
594.5	[LPC22:4+Na]$^+$	-	0.62	1.53	-
596.5	[LPC22:3+Na]$^+$	-	-	1.70	-
Phospholipids					
754.8	[PC34:4+H]$^+$	0.79	0.70	0.69	-
756.5	[PC34:3+H]$^+$	1.05	1.19	1.19	0.92
758.5	[PC34:2+H]$^+$	1.04	1.48	0.92	-
760.6	[PC34:1+H]$^+$; [PE36:5+Na]$^+$	4.50	4.38	4.04	3.62
766.5	[PE38:5+H]$^+$; [PE36:2+Na]$^+$	1.77	1.30	1.29	1.30
768.5	[PE38:4+H]$^+$; [PE36:1+Na]$^+$	2.32	0.43	1.18	4.67
778.6	[PC36:6+H]$^+$; [PC34:3+Na]$^+$	-	2.03	2.98	1.01
780.6	[PC36:5+H]$^+$; [PC34:2+Na]$^+$	7.90	6.44	5.61	2.78
782.6	[PC36:4+H]$^+$; [PC34:1+Na]$^+$; [PE36:2+K]$^+$	3.76	3.16	2.88	2.56
784.6	[PC36:3+H]$^+$; [PC34:0+Na]$^+$; [PE36:1+K]$^+$	1.46	1.58	1.34	0.95
786.6	[PC36:2+H]$^+$; [PE38:6+Na]$^+$; [PE36:0+K]$^+$	1.64	1.81	1.73	1.94
792.5	[PE40:6+H]$^+$; [PE38:3+Na]$^+$; [PC34:4+K]$^+$	8.50	6.35	5.54	5.49
794.5	[PE40:5+H]$^+$; PE38:2+Na]$^+$; [PC34:3+K]$^+$	1.50	1.07	0.94	-
800.6	[PC36:6+Na]$^+$; [PE38:7v+K]$^+$	0.97	0.78	0.69	-
802.6	[PC38:8+H]$^+$; [PC36:5+Na]$^+$; [PE38:6+K]$^+$	2.78	2.84	3.16	1.60
804.7	[PC38:7+H]$^+$; [PC36:4+Na]$^+$; [PE38:5+K]$^+$	3.42	2.89	2.85	1.32
806.7	[PC38:6+H]$^+$; [PE38:4+K]$^+$	20.95	14.66	11.85	7.66
808.7	[PC38:5+H]$^+$; [PC36:2+Na]$^+$; [PE38:3+K]$^+$	2.21	2.54	2.04	3.69
810.6	[PC38:4+H]$^+$; [PC36:1+Na]$^+$; [PE38:2+K]$^+$	-	0.71	-	0.84
814.6	[PC38:2+H]$^+$; [PE40:6+Na]$^+$;	1.33	1.62	1.36	2.56
818.6	[PC38:0+H]$^+$; [PE42:7+H]$^+$; [PE40:4+Na]$^+$	1.07	0.71	0.53	1.08

Table 1 (continued)

Observed m/z	Probable attribution	Abundance (%)			
		Initial	24 h	48 h	72 h
824.6	[PC38:8+Na]$^+$; [PE42:5+H]$^+$	0.79	0.68	0.92	1.43
826.5	[PC40:10+H]$^+$; [PC38:7+Na]$^+$; [PE42:3+H]$^+$	2.33	2.03	2.39	1.72
828.6	[PC40:9+H]$^+$; [PC38:6+Na]$^+$; [PE42:2+H]$^+$	5.71	6.85	6.44	5.14
830.6	[PC40:8+H]$^+$; [PC38:5+Na]$^+$; [PE42:1+H]$^+$	2.57	2.77	2.97	3.95
832.6	[PC40:7+H]$^+$; [PC38:4+Na]$^+$; [PE40:5+H]$^+$	5.41	4.72	3.49	2.57
834.6	[PC40:6+H]$^+$; [PC38:3+Na]$^+$; [PE40:4+H]$^+$	0.62	1.06	-	2.43
850.5	[PC40:5+H]$^+$; [PC38:2+Na]$^+$; [PE40:3+H]$^+$	-	1.16	1.76	3.35
852.6	[PC42:11+H]$^+$; [PC40:8+Na]$^+$; [PE42:9+K]$^+$	5.22	4.26	2.94	2.86
854.6	[PC42:10+H]$^+$; [PC40:7+Na]$^+$; [PE42:8+K]$^+$	2.00	2.52	1.67	1.95
878.6	[PC44:12+H]$^+$; [PC42:9+Na]$^+$; [PE44:10+K]$^+$	2.83	1.90	1.28	1.14

LPC lipophosphatidylcholine, *LPE* lipophosphatidylethanolamine, *PC* phosphatidylcholine, *PE* phosphatidylethanolamine

including m/z 542 and m/z 568, interpreted as [LPC20:5+H]$^+$ and [LPC20:5+H]$^+$, respectively. Lysophospholipids are biologically active cell membrane lipid derivatives that are involved in a variety of important processes, including cell proliferation, cell migration, angiogenesis, and inflammation [28]. In healthy cell membranes, lysophospholipids constitute between 0.5 and 6 % of the total lipid weight. At elevated concentrations, however, these lipids have been linked to a number of disease states, including atherosclerosis, inflammation, and hyperlipidemia [29]. After the salmon fillets had been stored for up to 24 h, it can be seen from Fig. 4 that the content of phospholipids was slightly reduced (blue line), whereas that of lysophospholipids increased. As shown in the spectrum (Fig. 3b), the peaks became more significant and diverse in the range from m/z 450 to m/z 600. Meanwhile, a new peak at m/z 580, corresponding to [LPC22:0+H]$^+$, was noted in a significant amount. This indicated that *P. fluorescens* started playing a role in salmon spoilage. At the third stage (48 h), the level of phospholipids was even lower, whereas that of lysophospholipids was even higher (yellow line, Fig. 4). The identities of the phospholipids in the range from m/z 700 to m/z 900 were unchanged, whereas the intensities of those phospholipids decreased from
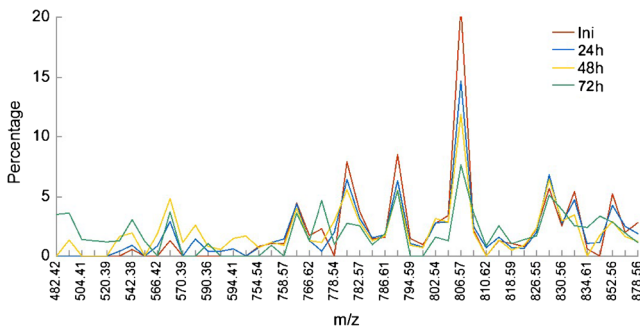
Fig. 4 Dynamic changes of phospholipid species extracted from salmon fillets contaminated with *P. fluorescens* with storage for different times (0, 24, 48, and 72 h). *Ini* initial

1,210.7 to 596.4, as shown in Fig. 3c. In contrast, more lysophospholipid peaks were observed, such as *m/z* 496 ([LPC16:0+H]$^+$), *m/z* 522 ([LPC18:1+H]$^+$) and *m/z* 580 ([LPC22:0+H]$^+$), and the intensities of *m/z* 542 and *m/z* 568 were greatly increased. The results implied that the number of *P. fluorescens* multiplied after 48 h cultivation, and the effect of phospholipase secreted by *P. fluorescens* became more significant. After 72 h, the intensity of not only phospholipids but also lysophospholipids was further reduced to 150.6 (Fig. 3d). This phenomenon could be tentatively explained by the phospholipids being biodegraded to lysophospholipids by phospholipase, and the lysophospholipids being further biodegraded by microbiological and enzymatic deterioration. The biodegraded products made the signal noise very significant and obvious (Fig. 3d). In summary, the quality of salmon fillet was easily influenced in a short time (24 h) if it was contaminated with *P. fluorescens*. Although the appearance as observed by the naked eye was unchanged, the phospholipids in salmon fillet had already

started the process of degradation, and the spoiled fish could be unsafe for eating.

Statistical analysis

To evaluate the difference between the salmon fillet samples at different time points after inoculation with *P. fluorescens*, PCA was applied to normalize the relative amounts of 48 identified lipid species and 24 salmon samples (six for each time point) in a reduced-dimension plot. The results of the PCA study showed that two principal components (PCs) were good enough to account for 72.0 cumulative percent (cum %) of the total variance and were considered to be significant. The score plot of the first two PCs demonstrated the variation of the time-dependent stages among these samples, where PC1 explained 50.7 cum % of the variance in the initial data set and PC2 explained 21.3 cum %. In Fig. 5a, PC1 is plotted against PC2, and the distribution of the samples implied that the interspecies variation was well controlled since almost all the samples at the same stage could be well grouped and located at different sites in the plot. This indicated that the conditions of the tested salmon fillets at each stage were different from each other. To find out which was the most influential lipid species, the coefficient that defines the weight of the original variable in the PC was investigated by a loading plot. Figure 5b showed the loadings for the variables in the first two PCs. Most of the lipid species were clustered at the central zero line, whereas the rest were dispersed far away from the center. The species with higher loading values and maximum variance in the data were most influenced by *P. fluorescens* during the storage. For example, the ion at *m/z* 806 in the right corner was the most noticeable species, and its content decreased dramatically as the storage time increased.

Fig. 5 a Principal component (*PC*) analysis of the identified phospholipid species of salmon fillets at different storage times. **b** Loading plot for the identified phospholipid species of salmon fillets at different storage times
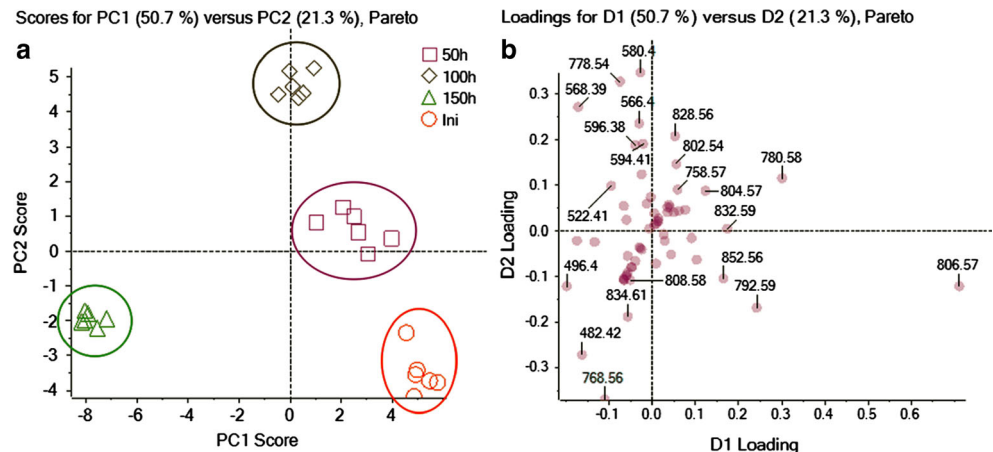
Conclusion

Exposure of salmon to *P. fluorescens* can occur through multiple routes, such as soil and water, during shipping. A good protocol for sample preparation and fast analysis of the samples is critical. A detection method was established in order to study the influence of *P. fluorescens* on salmon fillets. The method described in this study was optimized and successfully applied to reveal the dynamic changes of the contents of PC and PE in contaminated salmon fillets. It was found that salmon fillet was easily rotted once it had been contaminated with *P. fluorescens* without externally visible changes even at 4 °C, since the phospholipids were already degraded by phospholipases. To our knowledge, this is the first report on the effect of *P. fluorescens* on salmon fillets investigated by measuring the dynamic changes of each lipid species. Further studies aiming at selecting and optimizing an appropriate method to inhibit the activity of *P. fluorescens* for extending the shelf-life are being pursued.

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