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Fatty Acid Increment during Senescence of Stored Cabbage: A Metabolomic Approach

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Abstract Freshness marker identification is an effort to create a simple system to indicate the freshness level of fresh produces. Freshness is generally related to the degree of senescence that reflects the membrane's lipid turnover, such as phospholipids and fatty acids in the fresh produces. In this study, a targeted analysis of phospholipids and fatty acids in stored cabbage for different duration was conducted by using liquid chromatography-tandem mass spectrometry (LC-MS/MS) for the purpose of identifying the freshness marker metabolites. Cabbage was used due to the specific characteristic as a multy-layer vegetable. Cabbage samples were freshly harvested and immediately stored for 2 and 4 days at 20 ºC. Lipids for the first layer of cabbage were extracted with methyl tert-butyl ether and subsequently transferred to a LC-MS/MS triple quadrupole linear ion trap system. The multiple reaction monitoring (MRM) program for 240 phospholipids and fatty acids was prepared and utilized for a highly sensitive detection of lipids. Peak area of MRM chromatogram for each detected lipid was calculated statistically analyzed using principal component analysis. The distributions of lipids were separated clearly according to storage duration where phophatidyletanolamines were decreased and accumulation of fatty acid 18:1 was significantly observed during storage. Further study is needed to confirm the accumulation of fatty acid 18:1 could be a potential freshness marker metabolite of stored cabbage through the quantitative determination. Moreover, the selection of another type of fresh produce as the sample is needed to enrich the data for discovery universal freshness marker.

Keywords Freshness Marker, Lipid Oxidation, Metabolomics, Multi-Layered Vegetable, Phospholipid

1. Introduction

Currently, consumers are increasingly paying attention to freshness in buying fruits and vegetables (fresh produces) for consumption where the color change and degree of wilting of the produce are commonly used to determine freshness. This kind of evaluation, on the other hand, would be subjective. As a result, there is an undeniable need for an objective assessment of the freshness of fresh produces [1].

Early detection of deterioration's symptom can be recommended as one of the key elements for freshness assessment. The fall of freshness occurs concurrently with the rise of deterioration. One characteristic that reflects how much fresh product has matured is senescence [2,3]. Fresh vegetable aging is a tricky and extremely constrained procedure. Senescence symptoms in plants include the deterioration of proteins, carotenes, and chlorophyll as well as the breakdown of cellular membranes and moisture loss [4–7]. However, in general, cellular membrane breakdown can be used to detect senescence [8]. The primary mechanism of membrane deterioration is lipid decomposition, which is demonstrated by an increase in peroxidized lipids [9] and a decrease in phospholipids [10].

In biological membranes and signal transduction cascades in plants, phospholipids play a crucial role.

Recent advances in plant phospholipid research have led to the widespread acceptance that most phospholipids, even those that are components of biological membranes and respond to environmental stress during storage, have some degree of regulatory functions [11]. The primary constituents of plant cellular membranes are phospholipids, which have a variety of compositions as a result of the mix of hydrophilic (head groups) and hydrophobic (fatty acid) parts [12]. The physicochemical characteristics of the membrane itself are influenced by the phospholipid content of the plant cellular membrane, which varies depending on the environmental conditions [13]. Therefore, the observation of phospholipids changes during senescence in storage could provide the objective indication of the degree of freshness in fruits and vegetables. However, there is only a little information on the analysis of the compositional changes of phospholipids of stored fruits and vegetables especially at the molecular level.

In this study, we have comprehensively analyzed the targeted lipid such as phospholipids and fatty acids of cabbage as a representative sample for multi-layered vegetable that is stored for different durations by utilization of a triple quadrupole linear ion trap system (QTRAP) mass spectrometry (MS). The method presented here uses a simple sample preparation to identify a number of lipids in stored cabbage using a multiple monitoring reaction (MRM) algorithm that allows to obtain a highly sensitive MS/MS lipid spectrum. The sensitive detection of compositional variation of phospholipids and fatty acids could be useful to identify potential freshness marker metabolites of stored vegetables and fruits.

2. Materials and methods

2.1. Plant Material, Sample Preparation and Stored Condition

The fresh cabbages were provided by the professional farmer in the local area (Gifu University) and immediately brought to the laboratory. After cleaning and sorting, the cabbages were stored for 2 and 4 days at 20 ℃. Fresh cabbages (0 day treatment) were used as control. A 100 mg of the first layer of cabbage was collected by cock borer apparatus of 5 mm diameter and put into 2 mL sampling tube with a piece of zirconia ball. For further analysis, samples were immerged in liquid nitrogen for 1 minutes before being stored at -80 ℃.

2.2. Lipid Extraction

The lipid was extracted using the methyl tert-butyl ether technique [14]. The frozen sample was mixed with 300 L of methanol that contained 0.05 percent BHT, and then it was crushed in an analytical cell destroyer for around 60 seconds. The slurry was then added with 100 l of a mixture (1:1) of 0.1 mol mL-1 phosphatidylcholine (PC 17:0/17:0) and phophatidyletanolamine (PE 17:0/17:0) as an internal standard. The mixture was then centrifuged using a refrigerator for 10 minutes at 12000 rpm. Then, 3 mL of methyl tert-butyl ether was added to 500 l of supernatant in a 10 mL test tube. For 60 minutes, the mixed solution was incubated at a constant temperature of 25 ℃. To separate the sample's protein component, 400 L of potassium chloride solution at 1% was then added and carefully mixed. After that, the solution was centrifuged for 5 minutes at room temperature with a speed of 4000 rpm. 2,5 mL of the solution's upper phase was then collected in a different testing tube. The solution's solvent was subsequently eliminated using a vacuum-centrifugal evaporator. The resulting residues were then dissolved in 500 L of acetonitrile/IPA/20 mm ammonium acetate combination solution (6/4/1). The fluid was then filtered using a 0.2 m PTFE filter membrane. Utilizing a liquid chromatography mass spectrometer (LC MS), a 5 µl aliquot of this solution was examined.

2.3. LC-MS/MS Analysis

Lipids were identified employing a LC MS series that consists of a high-performance liquid chromatography system tandem with a triple-quadrupole mass spectrometer (Q-TRAP 4500 AB-Sciex). A 150 mm in length, 2.0 mm i.d and 3.0 µm o.d. of reverse phase chromatographic column was used as separation column. The acidic mixture of 20 mM ammonium acetate/Methanol/Isopropanol (7/3/1) as solvent A and the acidic mixture of 20 mM ammonium acetate/Isopropanol/Acetonitrile (1/6/4) as B solvent were used as the mobile phases with binary gradient elution with the increasing of solvent B from 2 % to 90 % in 20 minutes. Lipids were ionized in positive mode and were detected using the multiplexing multiple reaction monitoring. Each sample injection was tracked on 240 MRM transitions for targeted phospholipids and fatty acids. To get a robust multiplexing data of the 240 MRM transitions, the determination of each sample was employed in four replications with two injections.

Marker View™ software 1.2.1 was employed to process data from peaks of detected lipids. Peaks in lipids were extracted using the specific value according to Zainal [15]. The area of IS and sample weights were compared to normalize the data. A principal component analysis with discriminant analysis using Pareto scaling and none weighting parameters was performed to see if there were any differences in characteristics between the sample groups.

3. Results and Discussion

Crude lipid extract of the first layer of the cabbage was separated by a reverse phase (an octyl silica-based column, C8). In the reverse phase chromatography, lipid molecules have separated according to the different polarity of their

fatty acids. The C8 is suitable for fast analysis of samples containing the large differences in hydrophobicity. Figure 1 shows the MRM extracted ion chromatogram (XIC) of lipids in 0 day of cabbage sample (fresh sample). Lipids were distributed from the retention times of 6 min to 11 min. The narrow distribution of lipids in the samples indicates the hydrophobicity of each lipid could not differentiate clearly in the present study. However, since the MRM is highly selective (targeted) detection mode in LC-MS/MS that allows to fine tune an instrument to specifically look for specific MS/MS transition, the greater specificity, sensitivity, speed and quantification of analytes could be achieved.

Figure 2 indicates the distribution of lipids in crude lipid extract of fresh cabbage. By the assessment of LC-MS/MS, the relative abundance of 184 lipid species was identified, including phosphatidylcholine (PC) (45 species), phophatidylethanolamine (PE) (46 species), phosphatidylglicerol (PG) (37 species), phosphatidylserine (PS) (29 species), phosphatidylinositol (PI) (22 species) and fatty acid (FA) (5 species). Our result indicated PE and PC as the main phospholipid in the first layer of cabbage whereas these phospholipids are well known as main phospolipids in plants [12,16,17].

Principal component analysis supervised with discriminant analysis (PCA-DA) was used to investigate the changes in all detected lipids as a function of storage durations as described in Fig. 3. The score plots (A) and corresponding loading plots of lipids (B) of stored cabbage relating to storage duration at 20 ℃ were clustered into three groups. In Fig. 3A, the sample clusters were circled and positioned in various regions of the score plot to differentiate differences between groups as a function of storage duration, which was observed in first, second, and third at 0, 2, and 4 days, and increased with the increment of D1 scores. Subsequently, referring to fig 3.A, the corresponding loading plot of lipid signals which increases by the positive direction of D1, it was possible to choose a circled signal at the positive edge of the D1 axis of the loading plot, which was responsible for clustering the samples according to the increment of storage duration, i.e. the signal of FA 18:1 (Fig 3B). Furthermore, the changes of peak intensity of FA 18:1 were increased significantly during 4 days storage at 20 ℃ where the increment of peak area of FA 18: 1 was approximately 4 times compared to the fresh ones (Fig 3C). Moreover, it suggests the accumulation of FA 18:1 in cabbage that was stored at 20 ℃ also related to the degradation of PE. In Figure 3A, at the 0 day's region, it also circled a group of PE that was dominant in 0 day but decreased during storage. Since the notion of freshness marker is more represented by the accumulation of certain compounds than damage of certain compounds during storage of fresh fruits and vegetables [(1)], therefore, the accumulation of FA 18:1 would be focused in the case of cabbage.

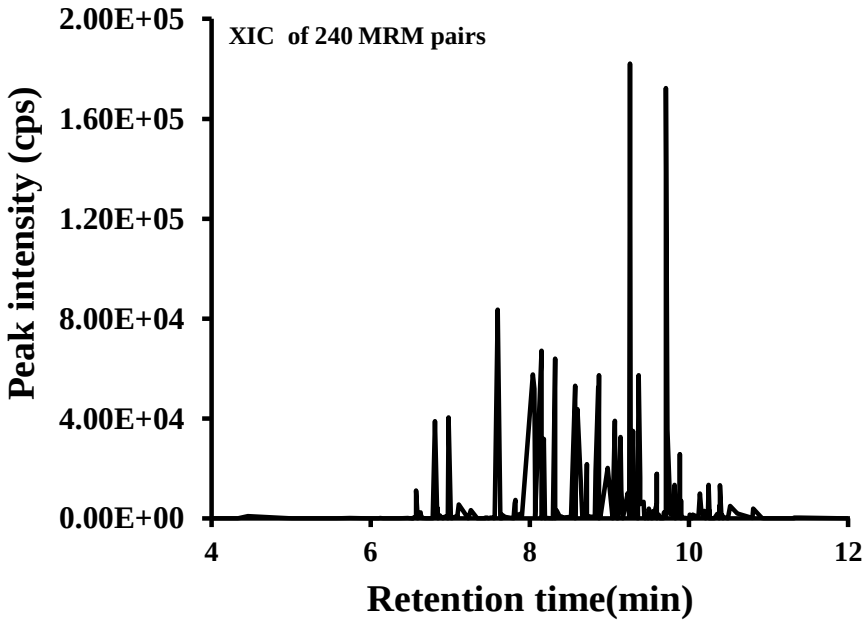


Figure 1. LC-MSMS chromatogram of lipids in fresh cabbage

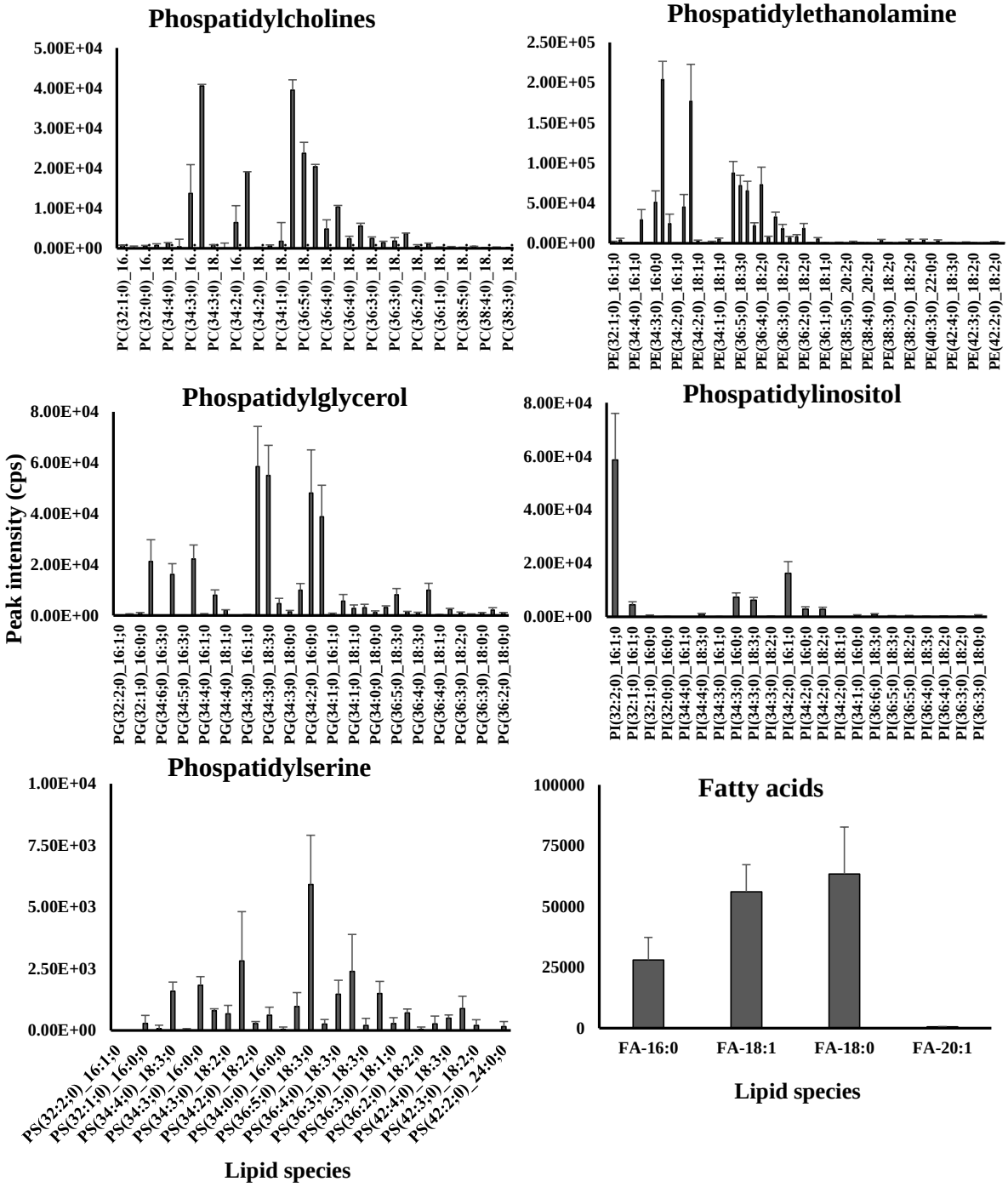
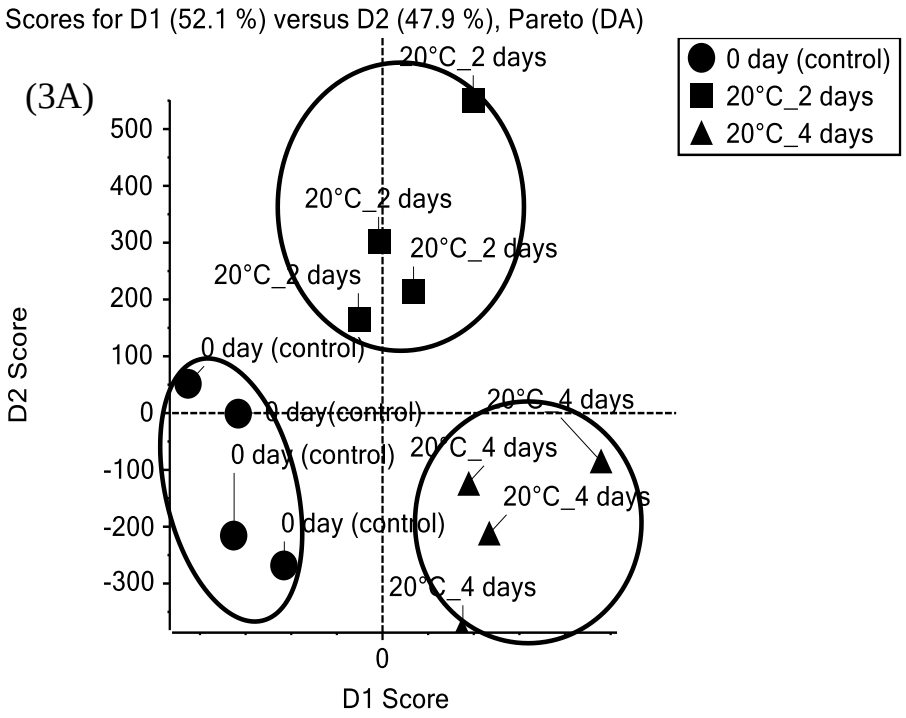
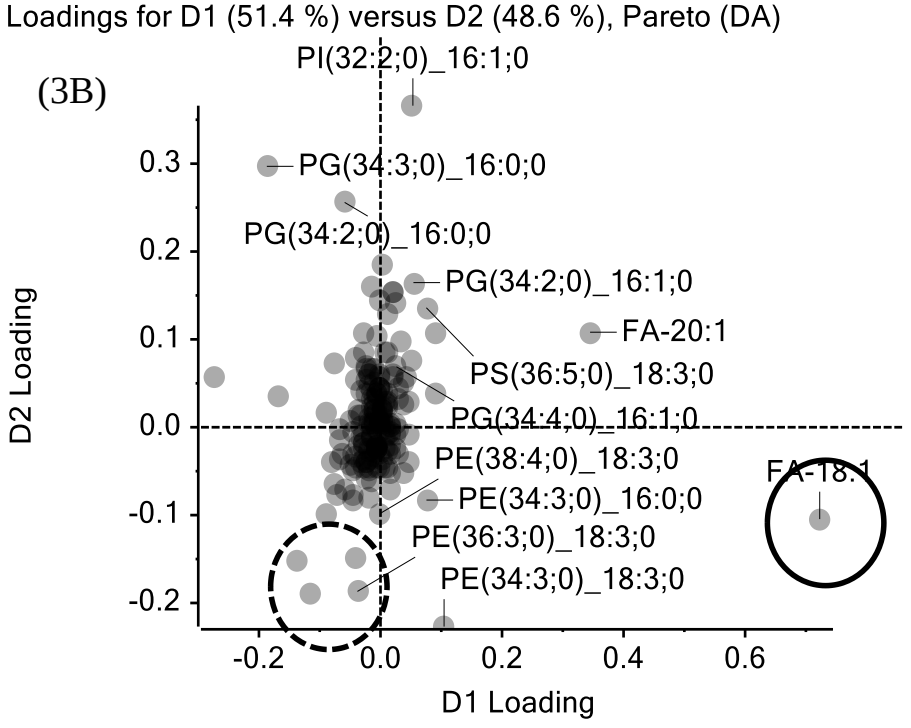


Figure 2. Lipid species in fresh cabbage


Scores for D1 (52.1 %) versus D2 (47.9 %), Pareto (DA)
(3A)
0 day (control)
20°C_2 days
20°C_4 days
D2 Score
D1 Score
500
400
300
200
100
0
-100
-200
-300
0


Loadings for D1 (51.4 %) versus D2 (48.6 %), Pareto (DA)
(3B)
PI(32:2;0)_16:1;0
PG(34:3;0)_16:0;0
PG(34:2;0)_16:0;0
PG(34:2;0)_16:1;0
FA-20:1
PS(36:5;0)_18:3;0
PG(34:4;0)_16:1;0
PE(38:4;0)_18:3;0
PE(34:3;0)_16:0;0
PE(36:3;0)_18:3;0
PE(34:3;0)_18:3;0
FA-18:1
D2 Loading
D1 Loading
0.3
0.2
0.1
0.0
-0.1
-0.2
-0.2
0.0
0.2
0.4
0.6

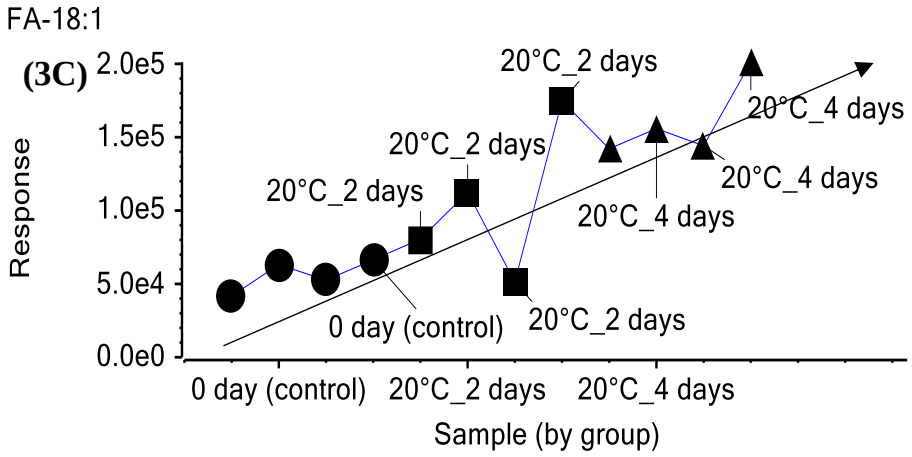


Figure 3. Lipid profile in cabbage stored at 20 ℃ for 0, 2days and 4 days. Score plot of lipids distribution in stored cabbage (3A), corresponding loading plot of lipid species in stored cabbage (3(B) where D1 axis is responsible for the storage periods and D2 axis is responsible for metabolites accumulation; and the changes of FA-18:1 in stored cabbage (3C)

Fatty acids that are formed from phospholipids, triglycerides, sphingolipids, or N-Acylethanolamines (NAEs) but are unattached to other molecules are generally referred to as free fatty acids (FFAs). FFAs have traditionally only been employed as the building blocks for complex lipids. According to recent studies [18,19] plants react immediately to biotic and abiotic stressors. Oleic acid, for instance, regulates nitric oxide synthesis in Arabidopsis and initiates nitric oxide-mediated defense signaling [20]. Therefore, we suggest that the FA 18:1 molecule could be represented as oleic acid based on this hunch. According to Kachroo & Kachroo [21] fatty acids, such as oleic acid, have also been demonstrated to be involved in stress reactions. The signaling enzyme phospholipase D, which prevents cell death, can be stimulated by oleic acid in order to perform its role [22]. Many research also strengthen this idea that fatty acids including oleic acid was turned over during senescence process of leaves of plants. Hence, in cabbage leaves may also develop this phenomenon [23,24]. In other words, when stored at 20 ℃ for a set period of time, cabbage may have a defense mechanism to prevent the death of membrane cells during senescence. Consequently, by observing the qualitative and quantitative change of oleic acid in cell membrane, we may be able to determine the freshness of fresh cabbage.

4. Conclusions

In this study, about 184 kinds of phospholipid and fatty acid species were detected in cabbage by spectrometric analysis. The statistical analysis by PCA-DA revealed that the accumulation of the compound that was marked as FA 18:1 which was proposed as oleic acid increased significantly when the cabbages were stored for 2 and 4 days at 20 ℃. The changes of specific fatty acid could be induced by the defense system of plant to recover the presence of death cell membrane. To our knowledge, this research is the first study that elaborates the application of analytical chemistry with the post-harvest study in development of the freshness technology enabling objective assessment of lipid. Furthermore, quantitative analysis of the oleic acid accumulation should be needed in further research for practical use.

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