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Lipidomic Profiling to Assess the Freshness of Stored Cabbage

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Abstract

The quantitative freshness assessment method for vegetables is desired to upgrade the quality management system in agricultural distribution chain. Since lipid has broad diversity in species and plays an important role in the biological metabolism of plants, there is a possibility that lipid profile indicates the freshness of harvested vegetables. The aims of this study were to clarify the lipidomic alteration of stored cabbage and identify lipid molecules indicative of freshness. Cabbage leaves stored at 5 °C, 10 °C, and 20 °C were sampled periodically for lipidomic analysis by liquid chromatography-tandem mass spectrometry. The cumulative respiratory CO_2 production was determined using a flow-through method via gas chromatography. A total of 74 lipid species had a significant correlation with cumulative CO_2 production. Hierarchical cluster analysis (HCA) clustered them into three main groups. A partial least squares regression (PLSR) model established the relationship between the abundance of lipid species and the cumulative CO_2 production. Four lipid molecules were selected as potential freshness markers. The PLSR model with the selected markers had a better performance in predicting the cumulative CO_2 production than that by ascorbic acid which is conventionally used as a quality indicator of fresh produce. Our results show that lipidomic profiling could be viable for assessing the freshness of whole cabbage.

Keywords Cabbage · Cumulative CO_2 production · Freshness marker · Lipidomics · Mass spectrometry

Introduction

Vegetables contain essential nutrients for life and health as well as beneficial phytochemicals such as phenolics, ascorbic acid, carotenoids, and flavonoids (Slavin and Lloyd 2012). These compounds quickly degrade during the postharvest stage because of continued biological activity (Paliyath et al. 2012). Because postharvest methods like low-temperature storage and controlled atmosphere can reduce biological activity, they are commonly used for quality maintenance of vegetables throughout the distribution chain (Tonutti and Bonghi, 2014). Despite the progress of postharvest technology, producing freshness is not optimal because the focus is on reducing transportation time and efficiency. Refocusing the supply chain on improving the quality of fresh produce will be a more effective solution.

Recently, consumers' perception has been changed from quantity to quality. Therefore, the personnel involved in food distribution should consider product quality. The degree of freshness has been used to express the overall quality of fresh produce, especially in vegetables. Freshness is usually judged by appearance such as skin color and degree of wilting (Péneau et al. 2006, 2007). However, such an assessment is entirely subjective and therefore inadequate. A freshness assessment providing quantitative results should be developed. To date, in the research field of post-harvest technology, the effectiveness of developed storage methods and distribution framework has been quantitatively evaluated by measuring multiple quality factors that decrease with time, such as ascorbic acid, sugar content, moisture content, and texture (Barth and Zhuang 1996; Medina et al. 2012; Hasperué et al. 2016). However, since these assessment methods are based on relative evaluation to the values at the time of harvest, it is difficult to apply them to the freshness

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assessment in the actual distribution process, where the values at the time of harvest cannot be obtained retrospectively.

Several trials for developing the freshness assessment method based on physical and physiological changes during senescence have been conducted. For instance, chlorophyll fluorescence properties, which reflect the quality of the photosynthesis system in the chloroplast, were related to storage duration and temperature. These properties were found to be one of the freshness indicators in spinach leaves and broccoli heads (Qiu et al. 2017). Light-emitting diode (LED)-induced fluorescence was also applied to measure firmness and soluble solid content, which decline with aging in apple fruit. These freshness parameters could be predicted by the partial least squares regression (PLSR) model obtained from the fluorescence spectra excited by an LED light at 375 nm (Gao et al. 2016). Moreover, Raman spectroscopy applying a 532-nm laser for excitation was utilized to determine the carotenoids of citrus fruits as a freshness parameter (Nekvapil et al. 2018). The distribution of luminance characteristics in images of cabbage was used for freshness assessment because it is highly correlated with the perceived glossiness and lightness of visual texture (Arce-Lopera et al. 2013). These methodologies are only available for a few products. For example, the chlorophyll fluorescence method can only be applied to fresh produce containing chlorophyll. Also, they are based on the measurement of a specific quality parameter such as firmness, pigment, and nutritional values. Freshness, however, must represent the difference in overall quality at harvest.

Because freshness is associated with the progress of senescence characterized by the loss of biomembrane integrity, focusing on biomembrane integrity is a better method for assessing freshness (Paliyath and Droillard 1992). Biomembrane is formed by a lipid bilayer that contains amphiphilic molecules. It plays essential roles, acting as a barrier and functioning in membrane trafficking (Van Meer et al. 2008). Furthermore, lipids act as an energy source for membrane biogenesis (Casares et al. 2019) and signaling molecules for transmitting information from outside (Xiong et al. 2002). Lipids have a broad diversity in species due to the variation in class and the number of carbon and double bound in acyl chain (Harayama and Riezman 2018). Reportedly, more than 10,000 lipid species exist in biomembrane (Sud et al. 2007). Membrane homeostasis is maintained by changing the composition of these species in response to environmental stress (Agmon and Stockwell 2017). Lipid profiles, therefore, provide useful information on biological status, including senescence.

Lipidomics is a subset of metabolomics focusing on lipids. It is useful not only for understanding the biological mechanism but also for finding biomarkers based on comprehensive lipid analysis. Mass spectrometry-based lipidomics is a powerful technique with high sensitivity, resolution, and accuracy in measuring thousands of lipid species. It has been utilized in food science such as food origin identification (Peršurić et al. 2018; Hammann et al. 2019), food safety assurance (Capriotti et al. 2015; Gorassini et al. 2017), and food adulteration detection (Wei et al. 2015; Righetti et al. 2018). Also, it has been applied to understand physiological responses during the ripening process (Sun et al. 2020) and chilling injury phenomena in stored fruits (Bustamante et al. 2018; Kong et al. 2018; Liu et al. 2020; Xu et al. 2021). Lipidomics is expected, therefore, to provide useful information on physiological reactions occurring in fresh produce during senescence. Furthermore, it will elucidate the specific lipid species that indicate the degree of freshness. However, no studies on lipidomic profiling regarding the freshness assessment of vegetables have been published to date.

This study aimed to investigate the lipidome alteration in whole cabbage during storage and identify freshness markers using liquid chromatography-tandem mass spectrometry (LC–MS/MS)-based lipidomics. The whole cabbage was selected as a sample material because it is one of the most common vegetables in the world, and the freshness is an important attribute due to its short shelf-life. Moreover, the quantitative freshness assessment method is strongly desired, especially in the fresh-cut fruits and vegetable industry. Raw cabbage is often stored at low temperatures for a long period, impacting the freshness and quality of the final fresh-cut product. For these reasons, whole cabbage stored at different temperatures and durations was used for the widely targeted lipidomics analysis to investigate the feasibility of the freshness assessment by lipidomic profiling in this study.

Materials and Methods

Plant Material and Storage Condition

Cabbage (cv. "Asashio") was harvested at a farmer's orchard in Ibaraki, Japan, in July 2020 and transported to the laboratory by same-day refrigerated delivery service. Immediately after delivery, the cabbages were selected on the basis of size uniformity and the lack of wound symptoms. A total of 66 selected cabbages were divided into two groups: sixty cabbages were used for lipid and ascorbic acid analysis, and six were used for respiration rate measurement. Cabbages for lipid and ascorbic acid analysis were put in foamed polystyrene boxes and stored in incubators at 5 °C, 10 °C, and 20 °C for 8, 4, and 3 weeks, respectively. The relative humidity (RH) inside the box was kept at 92–95% because it was closed with a lid to avoid direct wind from the incubator. The cabbages stored at 5 °C were sampled every 2 weeks and those at 10 °C and 20 °C were sampled every week. Each experiment unit was replicated five times using five different cabbage heads.

The first and second outer leaf was removed from sampling. The sample leaf disk was excised from the third outer leaf using a 3-mm-diameter cork borer. Subsequently, approximately 100 mg of disks was put into a 2-ml cryotube with two pieces of 5-mm-diameter zirconia balls. Then, it was rapidly frozen by liquid nitrogen and stored at − 80 ℃ until lipid analysis.

Measurement of Respiration Rate

The respiration rate of cabbage during storage was measured by a flow-through method using gas chromatography (GC) as described in Fahmy and Nakano (2014) with some modifications. First, approximately 500 g of cabbage was placed into an acrylic chamber (4.8 L) with a gas inlet and outlets tube. Two chambers were placed in each incubator set at 5 °C, 10 °C, and 20 °C for a duplicate test. The RH in the chamber was 92–95%. Fresh air was pumped into the chamber using an air compressor through the inlet tube at a flow rate of 100 ml min^{-1}. The gas flow rate was controlled using a mass flow controller (SEF-E40, Horiba, Japan). Then, the inlet and outlet gas samples were automatically injected into the GC (GC-2014, Shimadzu, Kyoto, Japan) via a 0.5-ml sampling loop attached to a rotating valve. CO_2 in the sample gas was separated using a Porapak Q column, and O_2 and N_2 were separated using a Molecular Sieve-13X column. Subsequently, these gases were detected using a thermal conductivity detector and analyzed using GC-Solution software (Shimadzu, Kyoto, Japan) on the basis of a standard external method. The rate of respiratory CO_2 production was calculated from differences between the outlet and inlet using Eq. (1) as described by (Fonseca et al. 2002).

$$R_{CO_2} = \frac{y_{CO_2}^{out} - y_{CO_2}^{in}}{100} \times \frac{F}{W} \times \frac{P}{RT} \times 10^3 \quad (1)$$

where R_{CO_2} is the respiratory CO_2 production of the sample (mmol kg^{-1} h^{-1}), y_{CO_2} is volumetric concentration in inlet and outlet (%), W is the weight of the sample (kg), F is the flow rate (ml h^{-1}), P is the atmospheric pressure (= 101.3 kPa), R is the universal gas constant (= 8.3141 J K^{-1} mol^{-1}), and T is the absolute temperature (K). The cumulative CO_2 production from the initial to the sampling for lipid analysis was obtained by numerical integration according to the trapezoidal rule using the respiration data and used as a reference of the degree of freshness.

Ascorbic Acid Analysis

Ascorbic acid (AsA) was measured using liquid chromatography method as described by Thammawong et al. (2019) with some modifications. Firstly, the frozen sample leaves were cryogenically ground in a bead crusher (ShakeMaster® NEO, Biomedical Science, Tokyo, Japan) using three of 3 mm diameter zirconia balls for 3 min at 1500 rpm. Then, 100 mg of sample powder was put into a 2-ml Eppendorf tube with 1.5 ml of 5% metaphosphoric acids. The mixture was vortexed for 1 min and subsequently centrifuged at 20,000 × g and 4 °C for 15 min (Model 1720, Kubota Corp., Tokyo, Japan). Afterward, 500 µl of supernatant was collected and 50 µl of Tris (2-carboxyethyl) phosphine hydrochloride was added to reduce dehydroascorbate to L-AsA. The mixture was continuously shaken using a block bath shaker (MyBL-100SC, As One, Osaka, Japan) set at 252 rpm and 25 °C for 25 min in a dark place.

A HPLC system (UltiMate 3000, Thermo Fisher Scientific, Massachusetts, USA) equipped with a polymer amino column (Asahipak NH2P-50 4E, 250 × 4.6 mm i.d., 5 µm in particle size, Shodex, Tokyo, Japan) was utilized to determine AsA concentration. Before measurement, the column was equilibrated by flowing 60 mM phosphoric acid at 0.5 ml min^{-1} for 2 h. A mixture of acetonitrile / 20 mM $NaH_2PO_4O_6$ + 30 mM H_3PO_4 (80/20, v/v) was used as a mobile phase and flowed at 1.0 ml min^{-1}. Just prior to injection, AsA extraction was diluted 10 times by Milli-Q water automatically using an in-needle mixing function of the autosampler, and then 10 µl of diluted sample was injected into the system. The column temperature was kept constant at 35 °C. The chromatograms were recorded at 244 nm using the UV detector. From the equation of the calibration curve plotted for the standard solutions, the concentration of AsA was estimated.

Comprehensive Lipid Analysis

LC–MS/MS grade methyl-tert-butyl ether (MTBE), isopropanol, acetonitrile, chloroform, methanol, and acetic acid were obtained from Fujifilm Wako Pure Chemical Corporation (Osaka, Japan). LC–MS/MS grade ammonium acetate was purchased from Sigma-Aldrich (St. Louis, MO, USA). Phosphatidylcholine (PC) 17:0/17:0 and phosphatidylethanolamine (PE) 17:0/17:0 were purchased from Avanti Polar Lipid, INC (USA) and used as internal standards.

Lipid was extracted using an MTBE method described by Matyash et al. (2008) with some modifications. Frozen sample leaf disks were cryogenically ground in a bead crusher for 1 min at 1500 rpm. Approximately fifty milligrams weighed sample powder was put into a 2-ml Eppendorf tube with 1000 µl of MTBE, 300 µl of methanol, and 50 µl of internal standard (20 µg ml^{-1} of each of PC 17:0/17:0 and PE 17:0/17:0 dissolved in a mixture of chloroform, methanol, and H_2O (6/4/0.5, v/v/v) and mixed thoroughly by a vortex mixer for 1 min). In dark conditions, the homogenates were shaken in a water bath set at 25 °C for 1 h at 100 rpm. Afterward, 250 µl of H_2O was added to induce phase separation and vortexed and then centrifuged for

5 min at 20,000 × g. Subsequently, 1000 µl of the upper layer was collected into a different tube and evaporated to dryness with a centrifugal evaporator for approximately 3 h at 30 °C. The residue was dissolved again in 500 µl of mobile phase B used for high-performance liquid chromatography (HPLC) separation (described below). The concentrate was filtrated through a 0.2-µm pore size membrane syringe filter (Minisart RC-4, Sartorius, Germany) and used for LC–MS/MS analysis.

Lipid analysis was conducted by liquid chromatography-mass spectrometry system via an HPLC system (Prominence, Shimadzu, Kyoto, Japan) and a linear ion trap triple-quadrupole mass spectrometer (QTRAP 4500, AB-Sciex, Framingham, MA, USA). Briefly, an autosampler injected 10 µl of sample extract into the system. Then, liquid chromatography separation was performed at 40 ℃ using a reverse-phase column (Cadenza CD-C18, 150 × 2.0 mm i.d., 3 µm in particle size, Imtakt, Kyoto, Japan) at a flow rate of 0.35 ml min^{-1}. Next, gradient elution was applied for preliminary separation via HPLC using the mobile phases of 20 mM ammonium acetate, isopropanol, and methanol (7/1/3, v/v/v) containing 0.1% acetic acid for A, and 20 mM ammonium acetate, isopropanol, and acetonitrile (1/7/3, v/v/v) containing 0.1% acetic acid for B. In the gradient program, the ratio of the mobile phase B was started at 40% for 1 min, increased to 80% B in 3 min, and then to 95% B in 4 min, followed by a linear gradient to 100% B in 4 min, maintained at 100% B for 14 min, and then decreased to 40% B and kept constant for 2 min to equilibrate the column for next injection.

The eluent from liquid chromatography was introduced to the mass spectrometer for further mass separation and detection. First, an electrospray ionization using a Turbo-V™ ion source was conducted at 300 °C with − 4.5 and 5.0 kV of spray voltage for negative and positive ionization mode, respectively. Other conditions were set as follows: ion source gas 1 (sheath gas), 50 psi, and ion source gas 2 (drying gas), 80 psi. Mass separation and detection were performed using multiple reaction monitoring (MRM) mode, where the transition from precursor ion (Q1) and product ion (Q3) of each target lipid, were set on the basis of the predicted fragmentation pattern (Wewer et al. 2011; Tarazona et al. 2015; Abhyankar et al. 2018). In this study, 1347 lipid species were targeted. To obtain enough number of data points in the MRM chromatogram and a high signal-to-noise ratio for accurate quantification, the mass spectrometry analysis was conducted separately in six batches containing approximately 200 transitions per batch. Each transition was performed with a dwell time of 5 and 4 ms for the positive and negative modes, respectively. The MRM transitions and setting parameters, such as the collision energy applied for each species, are shown in Table 1S–6S (Supplementary information). In fact, it is impossible to distinguish isomers such as stigmasterol and Δ^7-avenasterol in MRM analysis. However, in terms of these two isobaric sterols, listed features in Table S1 and S2 were annotated as stigmasterols, because they are the second most important sterols (Piironen et al. 2003).

The mass spectrometry analysis was conducted in five replicates from five different cabbage samples, and two injections were performed for each sample. The sequence of testing sample injection was randomized so as not to be biased by the sensitivity fluctuation of the mass spectrometer. Additionally, the pooled quality control (QC) sample, prepared by mixing a small aliquot of each testing sample, was injected every five samples for monitoring and correcting the drift of the sensitivity of the mass spectrometer.

Structural Confirmation of Selected Lipid Species by Product Ion Scanning

For each lipid species selected as an important molecule by the multivariate analysis described later, MRM information-dependent acquisition-enhanced product ion scan (MRM-IDA-EPI) was applied to get more structural information, increasing annotation accuracy. The MRM-IDA-EPI is a kind of auto MS/MS, where the product ion scanning is triggered when the signal intensity exceeds the setting level. In this study, 2000 cps was set as a triggering criterion, and the product ion spectra were acquired from 50 to 900 Da with dynamic fill time and a scan rate of 10,000 Da s^{-1}. The collision energy to get the fragment was appropriately adjusted depending on the analyte. The values of declustering potential (DP) and entrance potential (EP) were set at 120 and 10 V, respectively. The condition of the ion source was the same as the MRM acquisition mentioned above. Subsequently, the product ion spectra at the retention time corresponding to the target analyte were compared with the predicted spectra recorded in online databases, such as Human Metabolome Database (www.hmdb.ca) and Lipid Maps (www.lipidmaps.org). The specific *m/z* signals characterizing the target analyte in product ion spectra were annotated to validate structural matching.

Predata Processing and Statistical Analysis

The peak picking from the MRM chromatogram and the integration of the peak area were performed using MarkerView software (SCIEX, Framingham, MA, USA). The parameter settings for processing were as follows: Gaussian smoothing of five points, a baseline subtraction window of 1 min, noise percentage of 50%, a peak splitting factor of four points, a minimum required intensity of 1500 cps, a minimum peak width of four points, and a minimal

signal/noise of 300. Each peak area in tested samples was compensated by applying the QC-based-robust locally estimated scatterplot smoothing (LOESS) signal correction (QC-RLSC) using pooled QC sample data (Dunn et al. 2011). Subsequently, the corrected data obtained in positive and negative mode was normalized by PC 17:0/17:0 and PE 17:0/17:0, respectively, followed by sample weight. Normalization and QC-RLSC were conducted using in-house R scripts (Ver. 3.6.2, R Foundation for Statistical Computing).

Statistical analysis was conducted to evaluate the lipidome alteration depending on cumulative CO_2 production. First, a regression analysis was performed to investigate the linearity between the abundance of each detected lipid species and the cumulative CO_2 production. Then, the hypothesis test for the regression coefficient was done by R to eliminate the redundant data for further multivariate analysis with the probability threshold (p-value) less than 0.05.

Next, a heatmap based on HCA was built as an unsupervised multivariate analysis to visualize the data of the screened lipid in MetaboAnalyst 5.0 web interface (https://www.metaboanalyst.ca/). The metric Pearson correlation and Ward's linkage algorithms were used in HCA. Subsequently, PLSR as a supervised multivariate analysis was applied to elucidate the important lipid species strongly related to the increment or decrement of cumulative CO_2 productions using SOLO (Ver.8.9, Eigenvector Research Inc., Manson, USA). In PLSR, each normalized peak area of lipid species and the cumulative CO_2 productions were set as explanatory (X) and objective variables (Y), respectively. A total of 60 datasets was split into calibration datasets (34 observations) to build the PLSR model and validation datasets (26 observations) to test the robustness of the model.

Prior to the model development, both explanatory and objective variables were standardized by autoscaling (mean-centring and scaled to unit variance). Venetian blinds cross-validation was applied in building a PLSR model, where the maximum number of latent variables (LVs) and the number of data splits were set as 20 and 10, respectively. Then, the number of LVs was chosen on the basis of the lowest root-mean-square error of calibration (RMSEC). Afterwards, the root-mean-square error of cross-validation (RMSECV), R^2Y, and Q^2Y were utilized to evaluate model performance. A permutation test ($n = 100$) was also conducted to confirm model robustness. Finally, on the basis of the developed model, important lipid species were selected according to both variable importance in projection (VIP) scores and the p-value of the significance test of the correlation coefficient in univariate analysis.

Results and Discussion

The Differences of CO_2 Production Rate of Cabbage During Storage

Figure 1 presents the changes in CO_2 production rate in cabbage during storage at 5 °C, 10 °C, and 20 °C. The CO_2 production rate was suppressed more when cabbage was stored at lower temperatures. The changes at 5 °C and 10 °C were almost stable throughout the storage period. In the case of storage at 20 °C, the CO_2 production rate decreased drastically in the first 100 h and then became steady. Since the respiration rate strongly correlates with quality degradation, it has been used as a benchmark of the perishability or storability of fresh produce after harvesting in postharvest technology studies. According to Kader (2002), the respiration rate of fresh produce is classified into six levels, and cabbage is categorized in the moderate level of respiration rate. Brash et al. (1995) revealed that the cumulative respiratory CO_2 production after harvest of asparagus stored at various temperatures had a strong negative correlation with residual shelf-life at 20 °C and is suggested to be the same in other crops. The loss of ascorbic acid in broccoli, which is an important quality attribute, could also be predicted by the model as a function of the cumulative reparatory CO_2 production after harvest (Techavuthiporn et al. 2008). On this basis, Syukri et al. (2018) utilized the cumulative CO_2 production as a reference of the freshness degree and succeeded in identifying the freshness maker of soybean sprouts by the comprehensive analysis of carbonyl compounds using LC–MS/MS. Li et al. (2021) also demonstrated the capability of visible and near-infrared (Vis–NIR) spectroscopy in estimating the freshness of Japanese mustard spinach by

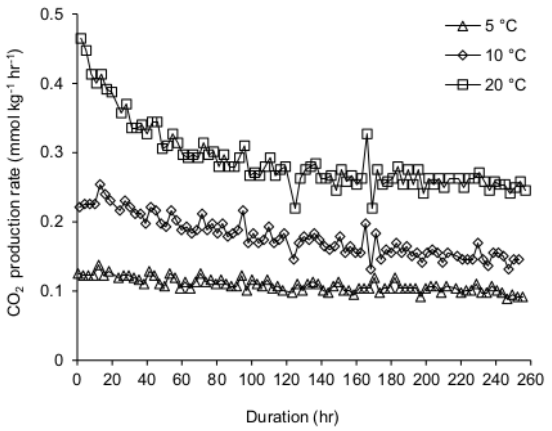

Fig. 1 Changes in the CO_2 production rates of cabbage stored at various temperatures

using it as a reference of freshness. Thus, cumulative CO_2 production from the beginning of the storage to sample collection was applied as a reference of the freshness degree of cabbage in later analysis and discussion in the present study.

Lipid Profile in Cabbage Stored at Different Cumulative CO_2 Productions

Lipids in cabbage samples stored at different temperatures and durations were comprehensively analyzed using LC–MS/MS. In total, approximately 600 peaks were detected. Only signals having less than 20% relative standard deviation (RSD) in peak area reproducibility across the QC sample were screened. Approximately 170 peaks fulfilled the above criteria and were then subjected to simple regression analysis to examine the linearity against the cumulative CO_2 production. According to the regression coefficient, a total of 74 lipid species including 13 species of phosphatidylcholines (PC), 17 species of phosphatidylethanolamines (PE), three species of phosphatidylglycerols (PG), three species of phosphatidylinositols (PI), two species of lysoglycerophospholipids (LGPL), seven species of monogalactosyldiacylglycerol (MGDG), three species of digalactosyldiacylglycerol (DGDG), eight species of sulfoquinovosyldiacylglycerols (SQDG), three species of sphingolipid (SM), three species of acylsterylglycosides (ASG), three species of diacylglycerol (DG), and nine species of triacylglycerol (TG) were significant. Significantly different species were subsequently analyzed further via multivariate analysis.

The profiles of screened lipid species were visualized as a heatmap and a dendrogram obtained by HCA shown in Fig. 2. According to the HCA, lipid species are clustered into three main groups. The first group had an upward trend, and the second and the third groups had a downward trend. Furthermore, the decreasing rate of the second group was faster than that of the third one.

The first group comprised the lipid molecules belonging to TG and ASG classes except for DG 18:3_18:3. TG is well known as a storage lipid and coalesces into lipid droplets in the cytoplasm. Generally, TG maintains homeostasis in the cell membrane. Considering the lipid metabolism pathway, the increment of TG observed in this study was implied that DG and/or other phospholipids and glycolipids converted

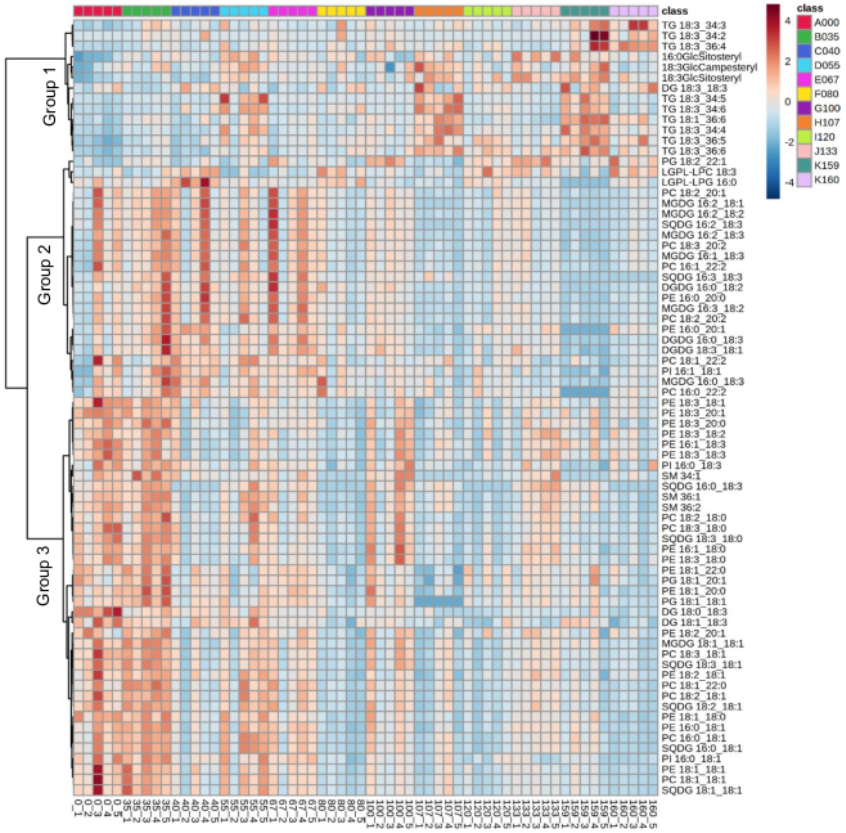

Fig. 2 Heatmap representing the alteration of relative response of lipid species in stored cabbage with the progress of the cumulative CO_2 production; 74 significant species are presented ($p < 0.05$ in the slop test of the simple regression analysis). The different colors at the top of the heatmap show the level of cumulative CO_2 production. A000 to K160 indicate the cumulative CO_2 production from 0 to 160 mmol $kg.^{-1}$

into TG during senescence. An increase in TG was also observed in Arabidopsis leaves during the aging process. Research suggests that it is caused by the detachment of FAs from glyceroglycolipid in the thylakoid membrane followed by TG formation in plastoglobuli (Kaup et al. 2002; Watanabe et al. 2013). Mueller et al. (2017) revealed that TG accumulation occurred when Arabidopsis leaves were exposed to heat stress; also, they suggested that the accumulation of TG could be a way to improve heat tolerance in plants. By contrast, Lin and Oliver (2008) mentioned that the conversion of galactolipids to TG caused metabolic disruption in crabapple leaves during senescence. In this study, the heatmap showed that the lipid molecules in group 1 had an upward trend with increasing cumulative CO_2 production. However, several TGs tended to increase at particular cumulative CO_2 production. The causes of this phenomenon are unclear and will be a subject for future work.

Sterols are natural organic compounds that have an essential function in all eukaryote cell membranes. They are also a membrane reinforcer in lipid rafts that contribute to maintaining membrane fluidity and permeability. Sterols interact with sphingolipids to influence membrane properties to aid in adapting to environmental changes (Dufourc 2008). Sterols are divided into free and conjugated form such as steryl esters (SE), steryl glycosides (SG), and acyl steryl glycosides (ASG). SG and ASG are secondary metabolites that are widely distributed in plants. SG is a hydroxyl group of C3 from sterol bound to sugar, synthesized by sterol glucosyltransferase (SGT). The sugar moiety from SG is acylated with FA in the C6 hydroxyl group to form ASG (Ferrer et al. 2017). In this study, several ASGs were detected in stored cabbage and increased with cumulative CO_2 production. Li et al. (2016) also reported that ASG increased, but SEs decreased during senescence in tobacco leaves. Takahashi et al. (2016) observed changes in ASG during cold acclimation in two types of plants: ASG content increased from 68.2 to 71.7% in oat, whereas it decreased from 14.7 to 7.7% in rye. Although different behavior was observed between them, these facts implied that cold acclimation and senescence were caused by alterations in physiological functions of the microdomains in the cell membrane.

The molecular species in the second group were mainly from the glyceroglycolipid class such as MGDG, DGDG, and SQDG. The third group was dominated by species from the glycerophospholipid class such as PC and PE. These classes are abundant in plants, mainly located in the biomembrane. MGDG, DGDG, and PC decreased during senescence in barley leaves (Wanner et al. 1991). Decreasing of MGDG was caused by the conversion to DG by galactolipid-galactolipid galactosyltransferase, followed by the conversion to PC. Then, PC enters the glycolysis pathway or it is hydrolyzed to PA (Wanner et al. 1991; Jia et al. 2013). This journey is almost in accordance with Li et al. (2016), showing MGDG, DGDG, SQDG, and several lipids in the PC and PE classes decreased in the early senescence of tobacco leaves. Watanabe et al. (2013) also stated that decreasing MGDG, DGDG, SQDG, and PG in thylakoid occurred during senescence in rosette leaves, and their alteration correlated with the degradation of chlorophyll.

Interestingly, in this work, the rate of decline in MGDG, DGDG, and SQDG was faster than in PC and PE classes. Previous research showed that phospholipase activity such as phospholipase D (PLD), PA phosphatase, and lipolytic acyl-hydrolase triggers the breakdown of membrane phospholipids (Brown et al. 1987). Particularly, Jia et al. (2013) mentioned that an increase of PLDδ activity stimulated the degradation of PC, MGDG, and DGDG during ABA-promoted senescence in Arabidopsis. MGDG and DGDG decreased more than PC under the increase in PLDδ activity. The plastidic lipids such as MGDG, DGDG, and SQDG degraded rapidly during ethylene-promoted senescence when compared with extraplastidic lipids such as PC, PE, and PI (Jia and Li 2015).

To date, lipid class-based analysis has been mainly conducted to investigate the behavior of lipid alteration in stress response. However, as shown in Fig. 2, a molecular-based analysis revealed that lipid molecules belonging to the same class grouped into different clusters. For example, DG 18:3_18:3 had an upward trend (Group 1), whereas DG 18:0_18:3 and 18:1_18:3 had a downward trend (Group 3). These results indicated that even in the same class of lipids, the hydrophobic tail has a significant impact on the stress response. However, there are few studies examining stress response and aging of plants at the lipid molecular level. The findings presented here by the lipidomics approach will provide useful information to elucidate the mechanism of lipid dynamics associated with plant senescence.

PLSR Analysis and Identification of Potential Freshness Markers in Cabbage

PLSR is a multivariate machine learning algorithm that allows datasets with more variables than samples to be modeled without resorting to prefiltering variables. Moreover, once optimized, a PLS model can be reduced to the form of a standard linear regression, from which inference about the importance of variables can be made (Mendez et al. 2019). For these reasons, PLSR has become the gold standard in metabolomics where a large member of metabolites interacting with each other in a biological system is simultaneously analyzed. It has been used for metabolic fingerprinting, profiling (Gao et al. 2020), and screening of biomarkers (Zhou et al. 2021). We performed PLSR to determine the relative response of 74 selected species and cumulative CO_2 production as explanatory (X) and predictor (Y) variables, respectively. As a result of the model

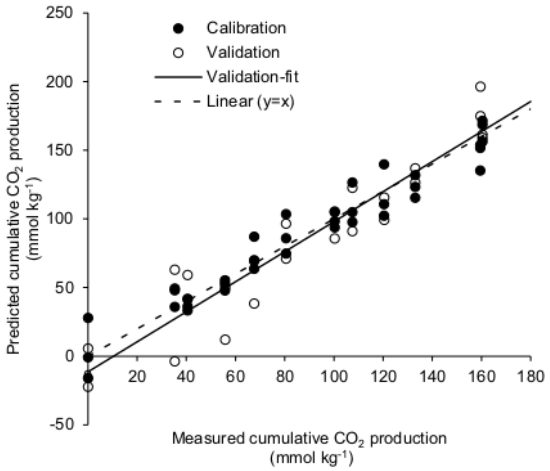

Fig. 3 PLSR plot for predicting the cumulative CO_2 production using 74 species with four latent variables

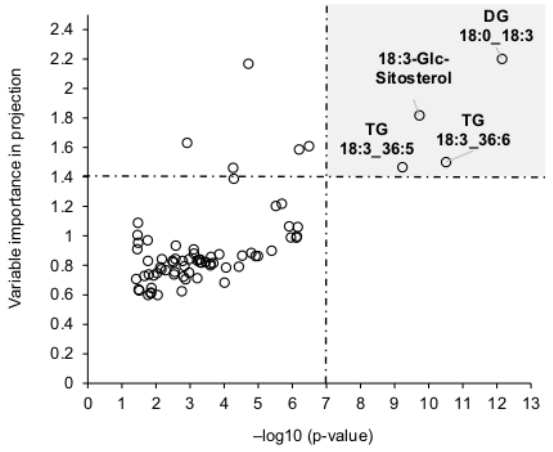

Fig. 4 Selection of the potential freshness marker based on the VIP scores and *p*-value of the test for correlation coefficient in a simple regression

development by internal cross-validation, the model with four LVs had the minimum RMSECV. Figure 3 shows the scatter plot of measured versus predicted cumulative CO_2 production using the developed model. The fraction of the sum of the square of all the Y's explained by the PLS model (R^2Y) and that of the total variation of the Y's that can be predicted by the PLS model (Q^2Y) were 0.93 and 0.82, respectively. These results demonstrate the strong explanation and prediction capabilities of the model. The permutation test showed that the unpermuted model was significantly different ($p < 0.05$) from the model created with randomly permuted samples. These results indicate that the developed PLS model was not overfitted and was reliable for predicting the cumulative CO_2 production using lipidome data. Additionally, the determination coefficient (R^2) in the external validation using the test dataset, which was not used for the model development, was 0.89. In other words, the fact that a highly accurate PLS model could be created successfully implies the existence of the specific lipid molecule that expresses the freshness of cabbage.

Since the VIP scores can rank variables in terms of their importance in the projection of the PLS model, it has often been used to elucidate the potential markers in metabolomics studies (Parijadi et al. 2018; Li et al. 2020). Thus, we used the VIP score for marker selection in this study. Additionally, to achieve a more reliable marker selection, the *p*-value of a hypothesis test for the regression coefficient in simple linear regression was considered. The potential freshness markers were selected based on the criteria both > 1.4 in VIP score and ≥ 7 in $-\log_{10}$ (*p*-value). Consequently, four lipid molecules were selected as indicated in Fig. 4.

All selected lipids were validated their annotation by matching the MS/MS spectrum obtained by post hoc MRM-IDA-EPI scans with putative fragment daughter ions of each lipid molecule (Fig. 5). Particularly, the annotation of TGs, which have three acyl chains, is impossible in MRM because it detects a target analyte on the basis of the combination of one parent ion (Q1) and one daughter ion (Q3), so it cannot cover the information on the third acyl chain in TG. However, because of the post-hoc MRM-IDA-EPI scanning, TG 18:3_36:6 could be annotated as TG 18:3_18:3_18:3 because only daughter ions resulting from linolenic acyl chain (18:3) was found in the MS/MS spectrum (Fig. 5a). In the case of TG 18:3_36:5, because only the signals resulting from linolenic (18:3) and linoleic (18:2) acyl chains were found, it was annotated as TG 18:3_18:2_18:3 (Fig. 5b). In the case of DG 18:0_18:3 and 18:3-Glc-sitosterol, specific daughter ions characterizing those compounds were observed in each MS/MS spectrum as shown in Fig. 5c and d.

Performance of Potential Freshness Markers in Predicting Cumulative CO_2 Production

In this study, we observed the alteration trend of lipid species to evaluate the freshness in whole cabbage. DG 18:0_18:3, 18:3-Glc-sitosterol, TG 18:3_18:3_18:3 and TG 18:3_18:2_18:3 were selected to be potential freshness markers in stored cabbage. The coefficient determinant (R^2) of the relationship between the cumulative CO_2 production during storage and each potential freshness marker ranged from 0.41 to 0.57 (Fig. 6). Based on the R^2 values, it is difficult to accurately evaluate the cabbage freshness by using

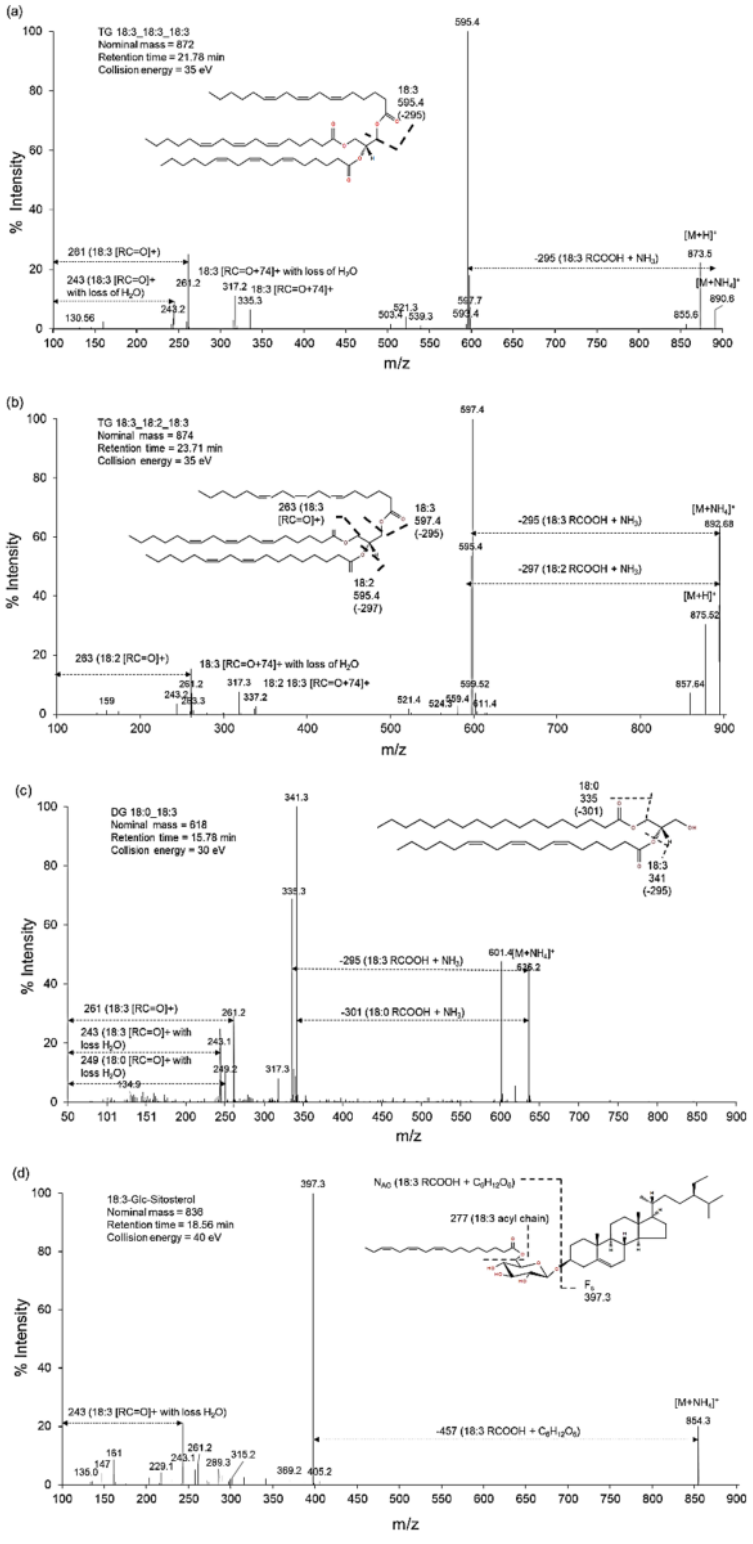

Fig. 5 Confirmation of potential freshness markers by product ion spectra

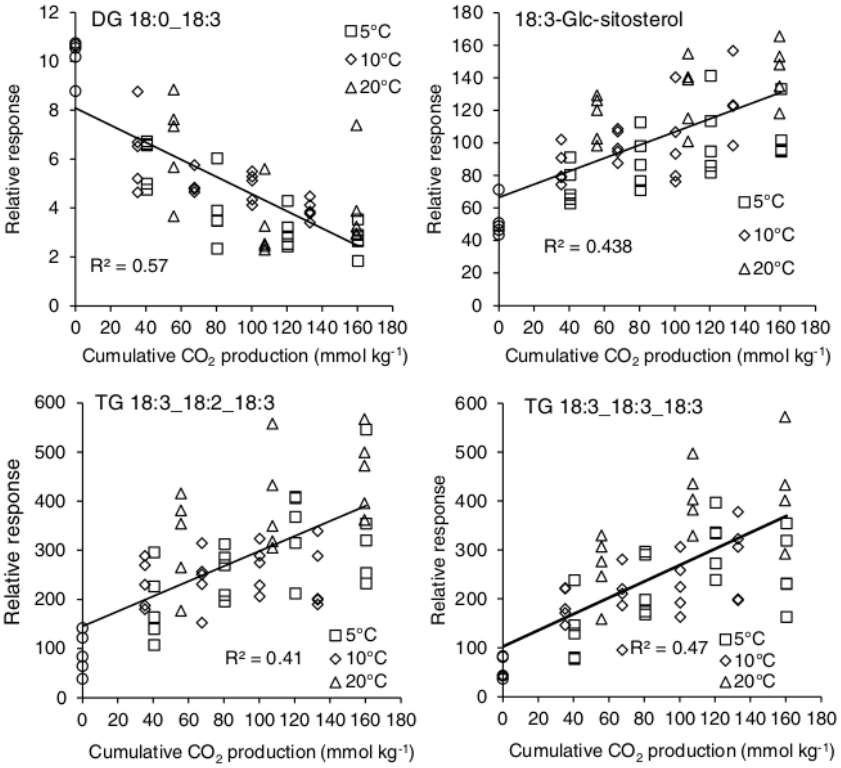

Fig. 6 Relationship between the relative response of potential marker and cumulative CO_2 production of stored cabbage

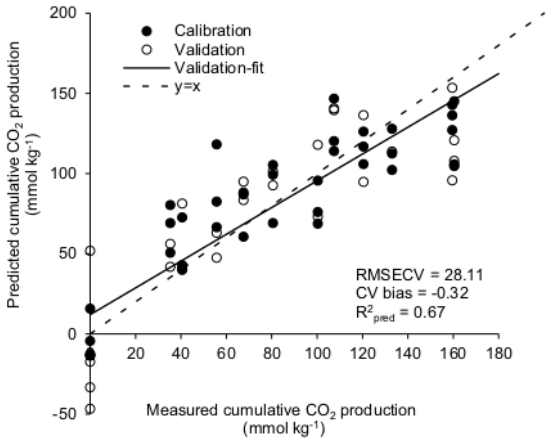

Fig. 7 PLSR plot for predicting cumulative CO_2 production. The PLSR plot was developed using four potential markers with two latent variables

a single marker. Hence, the PLSR model was rebuilt using the four selected potential markers, and its performance was evaluated. Figure 7 shows the measured and predicted cumulative CO_2 production by the PLSR model using four potential markers with two latent valuables. R^2Y and Q^2Y values were 0.70 and 0.66, respectively, indicating moderate predictability and high robustness of the model. The root-mean-square error of prediction (RMSEP) of this model was 30.2 mg CO_2 kg^{-1}, which corresponded with 10, 6, and 3.5 days of storage at 5 °C, 10 °C, and 20 °C, respectively. The determinant coefficient of prediction (R^2_{pred}) was 0.67. Considering that cabbage is stored at low temperature for a maximum of 4 months in the fresh-cut vegetable industry, this level of model error is considered to be acceptable in practical use.

AsA is one of the major quality attributes of fruits and vegetables after harvesting. Since AsA declines depending on storage duration and temperature, it has been employed to quantify the quality degradation of fruits and vegetables during storage (Saito et al. 2000; Yamauchi and Kusabe 2001; del Aguila et al. 2006; Galani et al. 2017). Hence, we compared the performance of selected four lipids and AsA in predicting the cumulative CO_2 production. AsA in stored cabbage decreased with an increase of the cumulative CO_2 production, as shown in Fig. 8a. Using the same method described earlier, PLSR was performed to build the predictive model using AsA as an explanatory value. Figure 8b shows the relationship between measured and predicted cumulative CO_2 production using the developed

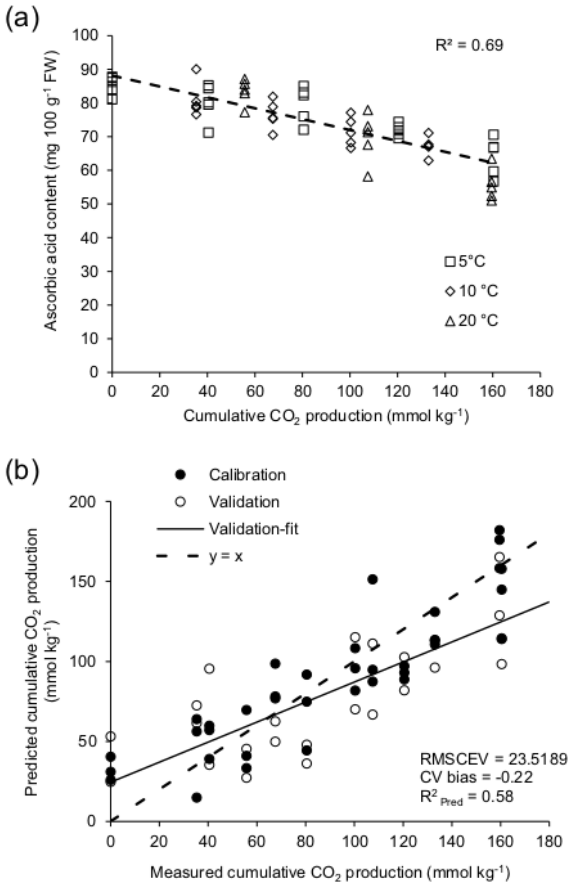

Fig. 8 The changes of AsA in stored cabbage with an increasing of cumulative CO_2 production (**a**), and the performance of PLSR model using AsA for predicting the cumulative CO_2 production (**b**)

PLSR model by AsA. The RMSEP and R^2_{pred} were 33.0 mg CO_2 kg^{-1} and 0.58, respectively. These values indicate that the model with four important lipid molecules can express the degree of freshness of cabbage more accurately than AsA, which has been used conventionally. In addition, the freshness assessment by AsA cannot be applicable for vegetables with very low AsA content, such as carrots, tomatoes and, fresh-cut potatoes, because it is difficult to detect minute changes of AsA (Howard et al. 1999; Mazurek and Pankiewicz 2012; Tudela et al. 2002). In some cases, it is stable even senescence occurs (del Aguila et al. 2006). On the other hand, the freshness assessment by lipid analysis has great potential to be applied for all vegetables because lipids are present universally in plant.

In this study, we succeeded in identifying four important lipid molecules that express the degree of freshness of cabbage. To verify the effectiveness of freshness assessment by lipidomic profiling, tests using other kinds of vegetables and considering stress factors other than temperature that fresh produce endure during distribution such as gas modification, vibration, and impact should be conducted in future research. In addition, rapid and simple lipid detection methods must be developed for the practical use of proposing freshness assessment methods for fruits and vegetables. Raman spectroscopy has recently been shown to be useful for lipid analysis (Uematsu and Shimizu 2021; Wieland et al. 2021), and its application to this freshness evaluation method is highly promising. In the future, the development of non-destructive and noninvasive measurement of lipid profiles in fruits and vegetables based on such spectroscopic methods is also required.

Conclusion

In this study, wide target lipidomics using a triple-quadrupole mass spectrometer were conducted to understand changes in the lipid profile alteration of cabbage during storage and to find out the specific lipid species that indicate the degree of freshness. Among a large number of detected

lipid species, 74 molecules were found to have a significant correlation with the cumulative respiratory CO_2 production during storage. Additionally, the PLSR model as a function of these lipid molecules could predict cumulative respiratory CO_2 production accurately. Based on the VIP score in PLSR and the *p*-value of the test for correlation coefficient in a simple regression, we succeeded in elucidating the four potential freshness markers that express the degree of freshness of cabbage. Moreover, the prediction model with these potential markers gave a more accurate result than the conventional AsA method in freshness assessment. To the best of our knowledge, this is the first attempt using the lipidomics approach for developing the freshness assessment method of vegetables.

Further research is needed to verify the availability of selected potential markers by testing the effect of not only temperature but also other stress factors such as controlled atmosphere and vibration/impact. Furthermore, the quantitative analysis of the selected potential freshness markers should be undertaken to develop a robust freshness measurement model. Then, it should be combined with non-destructive technology for the practical use of freshness assessment. Nevertheless, we believe that these preliminary findings will be a basic reference in postharvest science for further exploration of freshness assessment.

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Author Contribution Putri Wulandari Zainal: Investigation, Formal analysis, Software, Writing—original draft. Daimon Syukri: Methodology, Investigation. Khandra Fahmy: Writing—review & editing. Teppei Imaizumi: Formal analysis. Manasikan Thammawong: Formal analysis. Mizuki Tsuta: Software, Writing—review & editing. Masayasu Nagata: Writing—review & editing. Kohei Nakano: Conceptualization, Methodology, Supervision, Writing—review & editing, Funding acquisition.

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Data Availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics Approval This study does not involve usage of sample related to human participants or animals.

Consent to Participate Not applicable.

Conflict of Interest Putri Wulandari Zainal declares that she has no conflict of interest. Daimon Syukri declares that he has no conflict of interest. Khandra Fahmy declares that he has no conflict of interest. Teppei Imaizumi declares that he has no conflict of interest. Manasikan Thammawong declares that she has no conflict of interest. Mizuki Tsuta declares that he has no conflict of interest. Masayasu Nagata declares that he has no conflict of interest. Kohei Nakano declares that he has no conflict of interest.

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