Identification of a freshness marker metabolite in stored soybean sprouts by comprehensive mass-spectrometric analysis of carbonyl compounds

journal or publication title	Food Chemistry
volume	269
page range	588-594
year	2018-12-15
URL	http://id.nii.ac.jp/1578/00002737/

doi: 10.1016/j.foodchem.2018.07.036

Elsevier Editorial System(tm) for Food Chemistry

Manuscript Draft

Manuscript Number:

Title: Identification of freshness marker metabolite in stored soybean sprouts by comprehensive mass-spectrometric analysis of carbonyl compounds

Article Type: Research Article (max 7,500 words)

Keywords: Abscisic acid; Carbonyl compound; Freshness assessment; Metabolomics; Soybean sprouts

Corresponding Author: Professor Kohei Nakano, Ph.D

Corresponding Author's Institution: Gifu University

First Author: Daimon Syukri

Order of Authors: Daimon Syukri; Manasikan Thammawong, Ph.D; Hushna A Naznin, Ph.D; Shinichiro Kuroki, Ph.D; Mizuki Tsuta, Ph.D; Makoto Yoshida; Kohei Nakano, Ph.D

Abstract: The objective of this study was to identify metabolites that quantitatively indicate degrees of freshness of soybean sprouts. Self-cultivated soybean sprouts were stored at 5 °C, 10 °C or 20 °C, and respiratory CO2 production rates were monitored using a gas chromatography during storage. Carbonyl compounds (CCs) were analyzed comprehensively using mass-spectroscopic metabolomics analyses. CCs were derivatized using dansyl hydrazine (DH) and were then analyzed using high performance liquid chromatography-electrospray ionization tandem mass spectrometry (HPLC-MS/MS) with multiplexed multiple reactions monitoring (MRM). In the MRM chromatogram, 171 to 358 peaks were observed from stored soybean sprouts. Principle component analysis and discriminant analysis (PCA-DA) selected CC-DH derivative ion with m/z 512 at a retention time of 9.34 min as the most significant metabolite. Searching online metabolomics database and matching fragment patterns of product ion mass spectra of an authentic standard revealed abscisic acid is a freshness marker of soybean sprouts.

Identification of freshness marker metabolite in stored soybean sprouts by comprehensive mass-spectrometric analysis of carbonyl compounds

Daimon Syukri[a], Manasikan Thammawong[a], Hushna Ara Naznin[a], Shinichiro Kuroki[b], Mizuki Tsuta[c], Makoto Yoshida[d] and Kohei Nakano[a,*]

[a]*The United Graduate School of Agricultural Science, Gifu University, 1-1 Yanagido, Gifu, 501-1193, Japan.*

[b]*Graduate School of Agricultural Science, Kobe University,1-1 Rokkodai, Nada, Kobe, 657-8501, Japan*

[c]*Food Research Institute, National Agriculture and Food Research Organization, 2-1-12 Kannondai, Tsukuba, Ibaraki 305-8642, Japan*

[d]*Kanagawa Agricultural Technology Center, 1617 Kamiyoshizawa, Hiratsuka, Kanagawa 259-1204, Japan*

* Corresponding author at: The United Graduate School of Agricultural Science, Gifu University, 1-1 Yanagido, Gifu, 501-1193, Japan. Tel & Fax: +81-58-293-2996

E-mail address: knakano@gifu-u.ac.jp (K. Nakano)

ABSTRACT

The objective of this study was to identify metabolites that quantitatively indicate degrees of freshness of soybean sprouts. Self-cultivated soybean sprouts were stored at 5 °C, 10 °C or 20 °C, and respiratory CO_2 production rates were monitored using a gas chromatography during storage. Carbonyl compounds (CCs) were analyzed comprehensively using mass-spectroscopic metabolomics analyses. CCs were derivatized using dansyl hydrazine (DH) and were then analyzed using high performance liquid chromatography-electrospray ionization tandem mass spectrometry (HPLC-MS/MS) with multiplexed multiple reactions monitoring (MRM). In the MRM chromatogram, 171 to 358 peaks were observed from stored soybean sprouts. Principle component analysis and discriminant analysis (PCA-DA) selected CC-DH derivative ion with *m/z* 512 at a retention time of 9.34 min as the most significant metabolite. Searching online metabolomics database and matching fragment patterns of product ion mass spectra of an authentic standard revealed abscisic acid is a freshness marker of soybean sprouts.

Keywords: Abscisic acid, Carbonyl compound, Freshness assessment, Metabolomics, Soybean sprouts.

1. Introduction

Fresh fruits and vegetables are critical components of human diets and provide many health benefits (Slavin & Lioyd, 2012). Although freshness is associated with attractiveness and nutritional benefit, most consumers do not have direct access to fresh fruits and vegetables, and it can take several days for distribution of commercial harvests to consumers. Because substantial losses of nutritional value can occur during distribution, freshness of fruit and vegetable may facilitate consumer's decisions to purchase produce, and

could also be used to suggest appropriate postharvest techniques for farmers, distributors and retailers who intend to maintain freshness from farm to table.

Although freshness of fresh fruits and vegetables is widely assessed using observations of the color change and degree of wilting of produces, validated assessments of freshness are often required to avoid purchasing substandard produce, because visual assessment is limited to the skill of person. In particular, early reductions in freshness are often not visible (Nilsson, 2000).

Fruit and vegetable deterioration reflects increased prevalence of senescence (Paliyath & Droillard, 1992). Plant senescence is a complex and highly regulated process that is characterized by the degradation of chlorophyll, carotenoid, protein, and cell membrane and loss of moisture (Biswal, 1995; Zhou & Gan, 2009). Among these, cellular membrane integrity is an important indicator (Dörnenburg & Davies, 1999). In most cases, cell membrane degradation follows lipid decomposition and is indicated by increasing peroxidized lipid contents (Paliyath & Droillard, 1992), decreasing phospholipids (Lester & Whitaker, 1996) and unsaturated fatty acid levels (Lester, 2003). Numerous peroxidized lipids including subsequent production of carbonyl compounds (CCs) such as aldehydes, ketones, and carboxylic acids (Wills, 1980) are formed during lipid degradation. Therefore, specific CC accumulates during membrane lipid degradation as well as senescence could be a potential biomarker for quantitative freshness assessment of fruits and vegetables.

Recently, metabolomics using analytical instrumentations such as gas chromatography (GC) and liquid chromatography (LC) coupled with mass spectrometry (MS) has been introduced as a powerful approach to identify biomarkers in postharvest science (Singh, 2015). For instance, by means of GC-MS based metabolomics, 10 volatile compounds have been identified as potential markers of chilling injury of basil leaves (Cozzolino et al., 2016). Pentane and 2-ethylfuran have been detected as markers of quality

changes of stored wild rocket (Luca, Kjær & Edelenbos, 2017). In addition, Rudell, Mattheis and Hertog (2009) employed untargeted metabolic profiling to characterize metabolomic changes associated with superficial scald development in "Granny Smith" apple following 1-methylcyclopropene or diphenylamine treatment. In case of tomato fruits, LC-MS based metabolomics has been introduced to characterize metabolic changes during ripening (Moco et al., 2006). However, in the case of freshness biomarker identification, LC-MS based metabolomics is not yet done. Since, Tomono, Miyoshi and Ohshima (2015) developed and validated a method for detecting trace levels of CCs in mice plasma using HPLC/ESI-MS/MS with multiplexed multiple reactions monitoring after derivatization by dansyl hydrazine (DH), we applied this method for the establishment of CC profiles in fruits and vegetables to identify the freshness maker.

Soybean sprouts are a popular vegetable globally, and especially in Japan, Korea, China, and other Southeast Asian countries (Huang, Cai & Xu, 2014). Soybean sprouts are rich in nutrients and are available all year round, but are highly perishable due to high respiration rate (Snowdon, 2010). Therefore, a quantitative freshness assessment is necessary for postharvest management of soybean sprouts.

Brash, Charles, Wright and Bycroft (1995) indicated that rate of fruits and vegetables perishability is related to the cumulative respiratory CO_2 production during postharvest. Therefore, the cumulative postharvest CO_2 production can be used as a reference indicator for the degree of freshness. Hence, we compared CCs contents with cumulative CO_2 production during storage of soybean sprouts under various temperature conditions, and selected specific CC as a freshness biomarker. Subsequently, we identified the structure of the specific CC by using online metabolomics databases and confirmed by comparing fragmentation of mass spectra with the authentic standard.

2. Materials and methods

2.1. Plant material

Soybean sprouts were produced using *Glycine max*, cv. BS5012 seeds.

2.2.Reagents

Abscisic Acid (ABA), DH and *p*-Toluenesulfonic acid (*p*-TsOH) were purchased from Sigma-Aldrich (St. Louis, MO, USA). *p*-Benzyloxybenzaldehyde (*p*-BOBA), acetonitrile, methanol, chloroform and formic acid were obtained from Wako Pure Chemical Industries (Osaka, Japan). Butylated Hydroxytoluene (BHT) was purchased from Nacalai Tesque (Kyoto, Japan).

2.3.Cultivation and storage conditions

Ten g samples of soybean seeds were sterilized by dipping into water at 70 °C for 10 s and were then incubated (MIR-154-PJ Panasonic, Gunma, Japan) for 8 h in water at 20 °C to induce the germination. After soaking, the seeds were placed in 250 mL plastic cups, which were used as cultivation chambers, and were incubated under the dark condition at 20 °C with 70 % – 80 % relative humidity (RH). The seeds were watered with 100 mL of tap water twice daily at 10 am and 4 pm. After 4 days cultivated sprouts were harvested and selected on the basis of uniform hypocotyl lengths of 10 ± 2 cm. Samples were divided into three groups and each was stored in the incubators set at 5 °C, 10 °C and 20 °C with 70 % – 80 % RH and were collected periodically for measurements.

2.4.Measurement of respiration rate by a flow-through method

Rates of respiratory CO_2 production of stored soybean sprouts were measured by a flow-through method using on-line gas chromatography (GC) as described in Fahmy and Nakano (2014) with some modifications. Briefly, 40 g of soybean sprouts was placed into an acrylic chamber (2 L) equipped with gas inlet and outlet tubes. The chambers were closed and fresh air was flowed into the chamber from an air compressor through the inlet tubes at the flow rate of 6 L h^{-1}. The chambers were then placed in incubators at 5 °C, 10 °C and 20 °C. Inlet and outlet gas samples were injected automatically into a GC (GC-14A Shimadzu, Kyoto, Japan) alternately via a 0.5 mL sampling loop attached to a rotating stepping valve. CO_2 was separated using a Porapak Q column and detected by a thermal conductivity detector. Helium gas was used as a carrier gas. The chromatograms were analyzed using an integrator (C-R7A plus Shimadzu, Kyoto, Japan) based on a CO_2 standard curve. The results were expressed as percentage of total gas volume. The rate of CO_2 production was calculated from the differences in gas concentration between the inlet and outlet using Eq. (1) (Fonseca et al. 2002).

$$\left(\quad - \quad \right) 1 \qquad 1^{\,3} \qquad (1)$$

where is the respiration rate for CO_2 production (mmol kg^{-1} h^{-1}), and are volumetric concentration of CO_2 in inlet and outlet gas samples, respectively (%), W is the weight of the sample (kg), F is flow rate (L h^{-1}), P is the atmospheric pressure (= 101.3 kPa), R is the universal gas constant (= 8.314 L kPa K^{-1} mol^{-1}) and T is the absolute temperature (K).

CO_2 production rates of soybean sprouts were monitored every hour during storage and cumulative CO_2 production after harvest was calculated by integrating respiration rates throughout the storage duration using the trapezoidal rule.

148 *2.5.Sample preparation and CCs extraction*

149 Fleshly harvested soybean sprouts and those stored for 4, 8 and 12 d at 5 °C, 2, 4 and
150 6 d at 10 °C, and 0.5, 1 and 2 d at 20 °C were collected for determination of CCs contents.
151 Collected sprouts were then divided into cotyledon and hypocotyl parts and about 100 mg
152 samples of precisely weighted cotyledon and hypocotyl were placed in 2 ml self-standing
153 screw cap microtubes (Watson, Kobe, Japan) containing single zirconia ball of 5 mm
154 diameter. Samples were soaked into liquid nitrogen for 2 min and were finally stored at –80
155 °C for further analysis.

156 Frozen sprout samples were crushed in 200-µL aliquots of methanol containing
157 0.05% BHT using a bead crusher (Shake Master Neo BMS, Tokyo, Japan) at 1500 rpm for
158 180 s. Subsequently, 100-µL aliquots of 0.1 µmol mL^{-1} *p*-BOBA were added as an internal
159 standard. After adding 400-µL aliquots of chloroform, mixtures were homogenized again
160 using the bead crusher for 120 s, and mixtures were then centrifuged at 12000 rpm for 10 min
161 at 10 °C (1720 Kubota, Osaka, Japan). Finally, organic phases were collected and were
162 derivatized with DH.

163

164 *2.6.DH derivatization*

165 DH derivatization was performed as described by Tomono et al. (2015) with some
166 modifications. Briefly, 200-µL organic phases were mixed with 400-µL aliquots of
167 acetonitrile containing 200 µg of DH and 40 µg of *p*-TsOH using a water bath shaker
168 (Personal 11 Taitec, Saitama, Japan) at 75 rpm for 4 h in the dark at 30 °C. Mixtures were
169 then evaporated to be complete dried using a centrifugal evaporator (CV-2100 Eyela, Tokyo,
170 Japan) and residues were then dissolved in 500 µL of acetonitrile and filtered through 0.2-µm
171 membranes (RC15 Minisart Sartorius, Göttingen, Germany). Finally, 5-µL aliquots were
172 analyzed using LC/ESI-MS/MS.

173

174 *2.7. HPLC/ESI-MS/MS analysis*

175 CCs were determined using a HPLC series system with a high pressure gradient
176 pump, an autosampler, a column oven (Prominence HPLC 20A Shimadzu, Kyoto, Japan),
177 and a reverse phase chromatographic column (Unison UK-C8, 150 mm × 2.0 mm i.d., 3 μm
178 in particle size, Imtakt, Kyoto, Japan) coupled to a triple-quadrupole mass spectrometer (Q-
179 TRAP 4500 AB-Sciex, Framingham, MA, USA). Elution was performed in binary gradient
180 mode with mobile phases comprising 0.1 % formic acid in water (solvent A) and 0.1% formic
181 acid in acetonitrile (solvent B). DH-derivatized CCs (CC-DHs) were ionized using a Turbo-
182 V^{TM} ion source in positive mode and were detected using the multiplexing MRM of a specific
183 product ion with an *m/z* value of 236.1 by collision-induced dissociation. A total of 400
184 MRM transitions were monitored for all CC-DHs and a total of 100 channels were monitored
185 simultaneously for each sample injection. One channel from each injection was reserved for
186 monitoring of the transition of *p*-BOBA-DH as internal standard (IS) at *m/z* 460–236.1.
187 Determinations were performed in three replications with five injections for each sample to
188 complete multiplexing of the 400 MRM transitions. The detector conditions were as follows:
189 ion-spray voltage, 5500 V; source temperature, 300 °C; curtain gas, 206.8 kPa; collision gas,
190 62.1 kPa; ion source gas 1 (sheath gas), 344.7 kPa; ion source gas 2 (drying gas), 551.6 kPa.
191 Declustering potentials and collision energies were 100 V and 37 eV for *m/z* 275–374, 115 V
192 and 39 eV for *m/z* 375–474, 120 V and 43 eV for *m/z* 475–574, and 130 V and 50 eV for *m/z*
193 575–674, respectively. Nitrogen was used as a collision gas. To determine structures of
194 candidate freshness marker metabolites, the selected CC-DH ion was fragmented in the
195 product ion scanning detection mode.

196

197 *2.8.Data processing*

198 Data from peaks of CC derivatives were processed using Marker View™ software
199 1.2.1. (AB-Sciex, Framingham, MA, USA). CC-DH peaks were extracted using Gaussian
200 smoothing of 1.5 points, noise percentage of 50%, a baseline subtraction window of 8 min, a
201 peak splitting factor of 4 points, a retention time tolerance of 1 min, a minimum intensity of
202 1500 cps, a minimum peak with 2 points, and a minimal signal/noise ratio of 20. Peak areas
203 were normalized to that of the IS and to sample weights. Principal component analysis with
204 discriminant analysis (PCA-DA) was then performed using Pareto scaling and none
205 weighting in order to find out any differential features between sample groups. Selected
206 marker ion candidates were then identified using The Lipidmaps (www.lipidmaps.org), The
207 Human Metabolome Database (www.hmdb.ca), The METLIN Metabolomic Database
208 (https://metlin.scripps.edu), The ChemicalBook Database (www.chemicalbook.com) and
209 compared with a purchased authentic standard.
210

211 ## 3. Results and discussion

212 *3.1 Change of CO_2 production rate of soybean sprouts during storage*

213 Figure 1 shows the changes of CO_2 production rate of soybean sprouts during storage
214 at 5 °C, 10 °C, and 20 °C. CO_2 production rate of soybeans sprouts stored at 5 °C was almost
215 stable between 0.8 – 1.4 mmol kg^{-1} h^{-1} during storage, while it was slightly decreased from
216 2.4 to 1.5 mmol kg^{-1} h^{-1} at 10 °C. On the other hand, CO_2 production rate at 20 °C was
217 drastically decreased from 9 to 5 mmol kg^{-1} h^{-1} during storage. The rate of respiration is
218 affected by not only temperature but also the amount of the time elapsed after harvest.
219 According to Brash et al. (1995), the respiration rate of asparagus stored at 20 °C decreased
220 by over 50% in the first 24 hours after harvest, and the decrement was less significant with
221 decreasing of temperatures. Deterioration of fresh produce is primarily driven by the
222 product's own tissue metabolism and there is a tight linkage between metabolism and

perishability. Since CO_2 productions provide parallel measures of metabolic activity, the cumulative CO_2 production could be used as a reference of degree of freshness. In latter sections, we discuss the relationship between the cumulative CO_2 production and change of CCs in soybean sprouts during storage to identify the potential freshness marker.

3.2 Profile of CCs in soybean sprouts

Figure 2 demonstrates CC metabolites features in fresh soybean sprouts (A; cotyledons and B; hypocotyls). Detected CC-DHs were plotted in circles as a function of retention time (RT) and *m/z* value. The diameter of each circle represents peak area of detected CC-DH normalized to that of IS-DH at a RT of 11.34 min and to respective sample weight. Even in the fresh condition, about 171 of CC-DHs in cotyledons (A) and 228 of CC-DHs in hypocotyls (B) were detected. Most of these CC-DHs were distributed at the range of retention time from 3 min to 15 min and *m/z* from 350 to 600. Many of CCs in fresh soybean sprouts have a wide range of polarity and molecular weight that might be considered as secondary metabolites because there are numerous CCs classified as secondary metabolites including vitamins, isoflavones, flavonols, chalcones, and their derivatives which have carbonyl skeletons (Di Carlo, Mascolo, Izzo, & Capasso, 1999; Kim, Kim, Chung, Chi, Kim, & Chung, 2006; Gu et al., 2017). They likely react with DH forming CC-DH derivatives after extraction in polar and semi-polar solvent mixtures thus leading to the detection of numerous CC-DHs. Moreover, secondary metabolites in soybean sprouts vary in hypocotyls and cotyledons depending on the soybean variety (Plaza, Ancos & Cano, 2003; Youn, Kim, Lee & Kim, 2011). From our data, we can assume that, in soybean sprouts of *Glycine max*, cv. BS5012, higher number of CCs species are distributed in hypocotyls compared to cotyledons.

Figure 3 demonstrates the score plots (A) and corresponding loading plots of CC-DH signals (B) from hypocotyls of stored soybean sprouts relating to cumulative CO_2 production.

Four sample clusters were circled and positioned in different areas of the score plot to discriminate differences between groups as a function of cumulative CO_2 production, which was observed in first, second, third, and fourth clusters at 0, 87–110, 155–208, 274–299 mmol kg^{-1}, respectively, and increased with D1 scores (Fig. 3A). However, distributions of CC-DHs in cotyledons were not distinguished relating to the cumulative CO_2 production in PCA-DA score plots (data not shown). From corresponding loading plot of CC-DH signals from hypocotyls (Fig. 3B), increases in cumulative CO_2 production are explained by the positive direction of D1 where three signals were circled at the positive edge of D1 axis of the loading plot that responsible for clustering of the samples and had *m/z* of Q1_*m/z* of Q3_RT pairs of 364_236.1_8.82, 512_236.1_9.34, and 330_236.1_17.29.

Figure 4 demonstrates the correlation coefficient (*r*) of each detected CC-DH signal that was extracted by regression analysis between each of its normalized peak area and cumulative CO_2 production during storage under various temperatures. The three signals chosen previously from PCA-DA results indicate positive relationship with cumulative CO_2 production where signal of 512_236.1_9.34 has the highest *r* value (r = 0.84) (Fig. 4A). Moreover, accumulation of the CC-DH signal 512_236.1_9.34 shows an increasing trend with the increase of cumulative CO_2 production during storage under various temperatures in regression analysis (R^2 = 0.71) (Fig. 4B). Therefore, only the signal of 512_236.1_9.34 representing CC-DH metabolite with *m/z* 512 at RT of 9.34 min was finally selected as a freshness marker ion of soybean sprouts.

3.3 Structure elucidation of freshness marker ion

The enhance product ion (EPI) scan detection in Analyst® system of AB-SCIEX was performed against the selected ion of *m/z* 512 with RT of 9.34 min to identify the selected freshness marker ion. Figure 5 demonstrates the product ions mass spectra of the selected ion

of *m/z* 512 at RT of 9.34 min (upper part) and that of the standard abscisic acid (ABA)-DH (lower part). Initially, the losses of fragment ions with *m/z* of 18 mass units were observed in Fig. 5 (upper part). It likely reflects the loss of water molecules from protonated hydroxyl groups in allylic positions (Britton, 1996) and suggesting the presence of hydroxyl ions. Generally, a hydroxyl ion can be derived from fatty acids, carotenoids, flavonoids and their conjugates. Furthermore, since the selected marker ion is a protonated ion molecule, $[M+H]^+$, it comprises CC, DH and H^+ (hydrogen-adduct ion). Therefore, to calculate the molecular weight (MW) of the CC from this CC-DH derivative, *m/z* values of 1 for hydrogen-adduct ions and 263 for DH moieties were subtracted from the detected *m/z* value of 512, and an *m/z* value of 16 was added for the atomic mass of oxygen to form a carbonyl skeleton (Fig. S1). Based on these calculations, the MW of 264 mass units for the selected freshness marker metabolite was extracted. Using all the information obtained in our analysis, we have searched the online metabolomics databases for the corresponding names and structures of possible candidates. Five compounds were nominated as candidates, and are listed with their formulas, structures, and hydrophobicity (Log *P*) values in Table 1. These compounds are characterized as derivatives of fatty acids, flavonoids, and their conjugates. In addition, since a reverse phase chromatographic separation system was used for separating the complex CC-DHs in the samples, the resulting RTs reflect the polarities. Specifically, the RT of the selected CC-DH derivative was 9.34 min and was faster than that of *p*-BOBA-DH, which was detected at 11.34 min as an IS-DH derivative. Therefore, the selected freshness marker ion is more polar than *p*-BOBA-DH, and from the compounds listed with log *P* values in Table 1, only ABA has a lower log *P* value than *p*-BOBA. Thus, to confirm that ABA is the present freshness marker metabolite in soybean sprouts, we purchased an authentic ABA standard and conducted EPI detection against the ABA-DH derivative (Fig. 5, lower part), and indicated that fragmentation patterns of product ion mass spectra of ABA-DH exactly

298 matched those of the selected CC-DH. In addition, the RT of the ABA-DH derivative was
299 9.41 min, similar to that of the selected CC-DH. Finally we conclude that ABA is the
300 identified freshness marker metabolite for soybean sprouts.

301 ABA is a plant hormone that was discovered at least 50 years ago and has since been
302 shown to regulate many aspects of plant growth and development (Finkelstein, 2013). ABA
303 is a member of monocyclic monoterpene family and comprises the metabolic precursors
304 ketone and enolate (Duffield & Netting, 2001). Thus, ABA can be conjugated to DH through
305 its ketone group. To date, the best known functions of ABA are related to roles as a major
306 phytohormone that contributes to plant abiotic stress resistance. ABA is mainly induced by
307 moisture loss stress, chilling temperature and salt stress (Swamy & Smith, 1999; Lafuente &
308 Sala, 2002; Romero, Rodrigo, & Lafuente, 2013), and accumulates through the cleavage of a
309 C_{40} carotenoid precursor (Xiong & Zhu, 2003). According to Becker and Fricke (1996), fresh
310 fruits and vegetables lose their moisture through the transpiration during storage. Hence, the
311 presence of ABA in hypocotyls of soybean sprouts may have been induced by moisture loss.
312 Transpiration is associated with transport and evaporation of moisture from the skin, and with
313 convective mass transport of moisture to the atmosphere. Moreover, transpiration and
314 respiration have been correlated in previous study whereas CO_2 and heat from the associated
315 chemical reaction during respiration may accelerate transpiration in fresh produce.

316 As indicated in Fig. 4B, normalized areas of ABA signals at 512_236.1_9.34,
317 increased with cumulative CO_2 production. However, this correlation was moderate (R^2 =
318 0.71). As mentioned in the introduction part, senescence can be characterized by some
319 indications such as degradation of chlorophyll, protein and lipid as well as moisture loss.
320 Thus, we suggest that in combination with ABA levels, other prospective freshness marker
321 metabolites may give more valid assessments of freshness of soybean sprouts.

The present experiments did not show accumulation of specific lipid degradation derived CCs, potentially reflecting limitations of our analytical methods. Although DH derivatization-based LC-MS is satisfactory for profiling of CCs in biological samples, the formation of by-products (artifacts) and non-quantitative reactions can hamper accurate determinations (Moritz & Johansson, 2008; Xu, Zou, Liu, Zhang, & Ong, 2011; Qi, Liu, Wang, Cai, Yuan, & Feng, 2014). Moreover, plant materials such as soybean sprouts contain large varieties of metabolites with carbonyl skeletons that could lead to an abundance of DH derivative artifacts and non-quantitative reactions. Hence, the present conditions may impair detection efficacy for inherently low levels of lipid degradation derived CCs in test samples. These limitations warrant further optimization of the present method to minimize interference from complex biological matrices and to enhance detection selectivity.

4. Conclusion

Herein, the first use of HPLC–MS/MS based metabolomics approach to identify markers of freshness in stored soybean sprouts was reported. ABA was identified as a metabolite that can indicate the degree of soybean sprouts freshness. Although ABA has been associated previously with responses to abiotic stresses such as moisture loss, no studies suggest the use of ABA as a metabolite biomarker for freshness. Hence, the present data are the first to suggest the utility of ABA as a marker for freshness of soybean sprouts, particularly because ABA was absent in freshly harvested sprouts and accumulated during storage. However, these data advise further validation of using ABA as a marker of freshness in soybean sprouts, as well as in other fruits and vegetables.

Acknowledgement

This work was supported by JSPS KAKENHI (Grant Numbers JP16H02581). The authors wish to acknowledge Salada Cosmo. Co., ltd, Japan for providing the seed materials used for this research.

References

Becker, B. R., & Fricke, B. A. (1996). Transpiration and respiration of fruits and vegetables, new developments in refrigeration for food safety and quality. *International Institute of Refrigeration, Paris, France, and American Society of Agricultural Engineers, St. Joseph, Michigan*, 110–121.

Biswal, B. (1995). Carotenoid catabolism during leaf senescence and its control by light. *Journal of Photochemistry and Photobiology B: Biology*, 30, 3–13.

Brash, D. W., Charles, C. M., Wright, S., & Bycroft, B. L. (1995). Shelf-life of stored asparagus is strongly related to postharvest respiratory activity. *Postharvest Biology and Technology*, 5, 77–81.

Britton, G. (1996). Carotenoids. In Houghton., J. D., & Hendry, G. A. F. (Ed.), *Natural food colorants* (p. 225). London: Chapman & Hall.

Cozzolino, R., Pace, B., Cefola, M., Martignetti, A., Stocchero, M., Fratianni, F., Nazzaro, F., & De Giulio, B. (2016). Assessment of volatile profile as potential marker of chilling injury of basil leaves during postharvest storage. *Food Chemistry*, 213, 361–368.

Di Carlo, G., Mascolo, N., Izzo, A.A., & Capasso, F. (1999). Flavonoids: old and new aspects of a class of natural therapeutic drugs. *Life Sciences*, 65, 337–353.

Dörnenburg, H., & Davies, C. (1999). The relationship between lipid oxidation and antioxidant content in postharvest vegetables. *Food Reviews International*, 15, 435−453.

Duffield, P. H., & Netting, A.G. (2001). Methods for the quantitation of abscisic acid and its precursors from plant tissues. *Analytical Biochemistry*, 289(2), 251–259.

371 Fahmy, K., & Nakano, K. (2014). Optimal design of modified atmosphere packaging for
372 alleviating chilling injury in cucumber fruits. *Environmental Control in Biology*, 52(4),
373 233–240.
374 Finkelstein, R. (2013). Abscisic acid synthesis and response. *The Arabidopsis Book.*
375 *American Society of Plant Biologists*. e0166. doi: 10.1199/tab.0166.
376 Fonseca, S. C., Oliveira, F. A. R., & Brecht, J. K. (2002). Modelling respiration rate of fresh
377 fruits and vegetables for modified atmosphere packages: A review. *Journal of Food*
378 *Engineering*, 52, 99–119.
379 Gu, E., Kim, D. W., Jang, G., Song, H. S., Lee, J., Lee, S. B., Kim, B., Cho, Y., Lee, H., &
380 Kim, H. (2017). Modelling respiration rate of fresh fruits and vegetables for modified
381 atmosphere packages: A review Mass–based metabolomics analysis of soybean sprouts
382 during germination. *Food Chemistry*, 217, 311–319.
383 Huang, X., Cai, W., & Xu, B. (2014). Kinetic changes of nutrient and anti–oxidant capacities
384 of germinated soybean (*Glycine max* L.) and mung bean (*Vigna radiate* L.) with
385 germination time. *Food Chemistry*, 143, 268–276.
386 Kim, E. H., Kim, S. H., Chung, I. J., Chi, H. Y., Kim, J. A., & Chung, I. M. (2006). Analysis
387 of phenolic compounds and isoflavones in soybean seeds (*Glycine max* (L.) Merill) and
388 sprouts grown under different conditions. *European Food Research and Technology*,
389 222, 201–208.
390 Lafuente, M. T., & Sala, J. M. (2002). Abscisic acid levels and the influence of ethylene,
391 humidity and storage temperature on the incidence of postharvest rindstaning of
392 'Navelina' orange (*Citrus sinensis* L. Osbeck) fruit. *Postharvest Biology and*
393 *Technology*, 25, 49–57.

Lester, G. E. (2003). Oxidative stress affecting fruit senescence, in: Hodges, D. M. (Eds.), *Postharvest oxidative stress in horticultural crop*. (pp. 113–118). New York: Food Products Press.

Lester, G. E., & Whitaker, B. D. (1996). Gamma-ray-induced changes in hypodermal mesocarp tissue plasma membrane of pre- and post-storage muskmelon. *Physiologia Plantarum*, 98, 265–270.

Luca, A., Kjær, K., & Edelenbos, M. (2017). Volatile organic compounds as marker of quality changes during the storage of wild rocket. *Food Chemistry*, 232, 579-586.

Moco, S., Bino, R. J., Vorst, O., Verhoeven, H. A., de Groot, J., van Beek, T. A., Vervoort, J., & Ric de Vos, C. H. (2006). A liquid chromatography–mass spectrometry–based metabolome databases for tomato. *Plant Physiology*, 141, 1205–1218.

Moritz, T., & Johansson, A. I. (2008). Plant metabolomics. In: Griffiths, W. J. (Ed.), *Metabolomics, metabonomics and metabolite*. (pp. 254–272). London: The Royal Society.

Nilsson, T. (2000). Postharvest handling and storage of vegetables, in: Shewfelt, R. L., Brückner, B. (Eds.), *Fruits & vegetable quality: an integrated view*. (pp. 108–109). Pennsylvania: Technomic Publishing.

Paliyath, G., & Droillard, M. J. (1992). The mechanisms of membrane deterioration and disassembly during senescence. *Plant Physiology and Biochemisrty*, 30, 789–812.

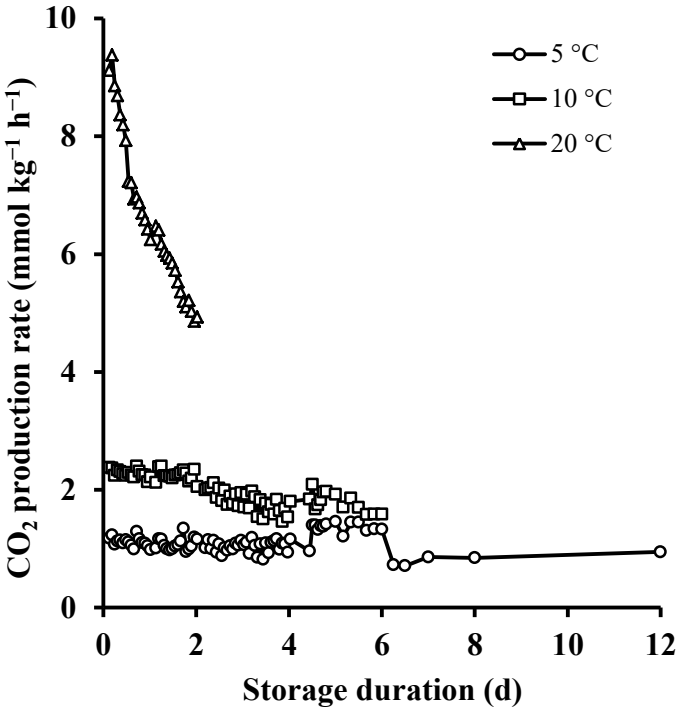
Plaza, L., de Ancos B., & Cano, P. M. (2003). Nutritional and health-related compounds in sprouts and seeds of soybean (*Glycine max*), Wheat (*Triticum aestivum* L.) and alfafa (*Medicago sativa*). *European Food Research* and *Technology* , 216, 138–144.

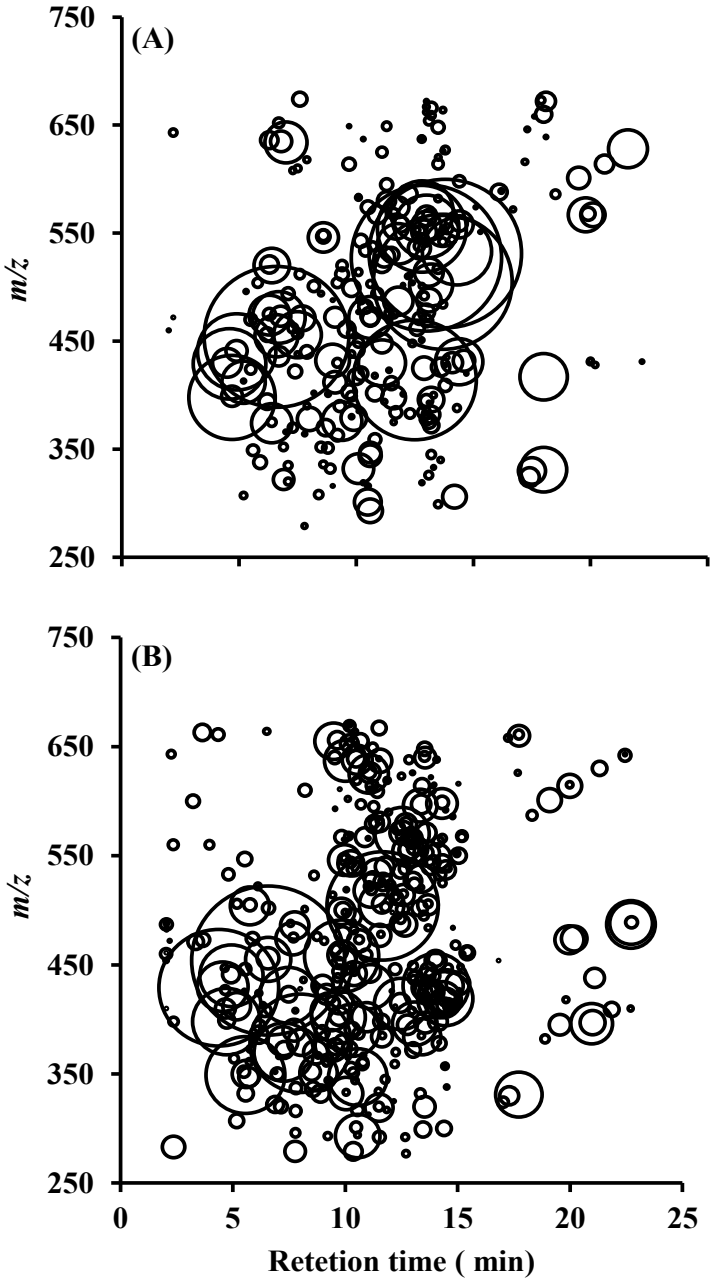
Qi, B., Liu, P., Wang, Q., Cai, W., Yuan, B., & Feng, Y. (2014). Derivatization for liquid chromatography-mass spectrometry. *Trends in Analytical Chemistry*, 59, 121–132

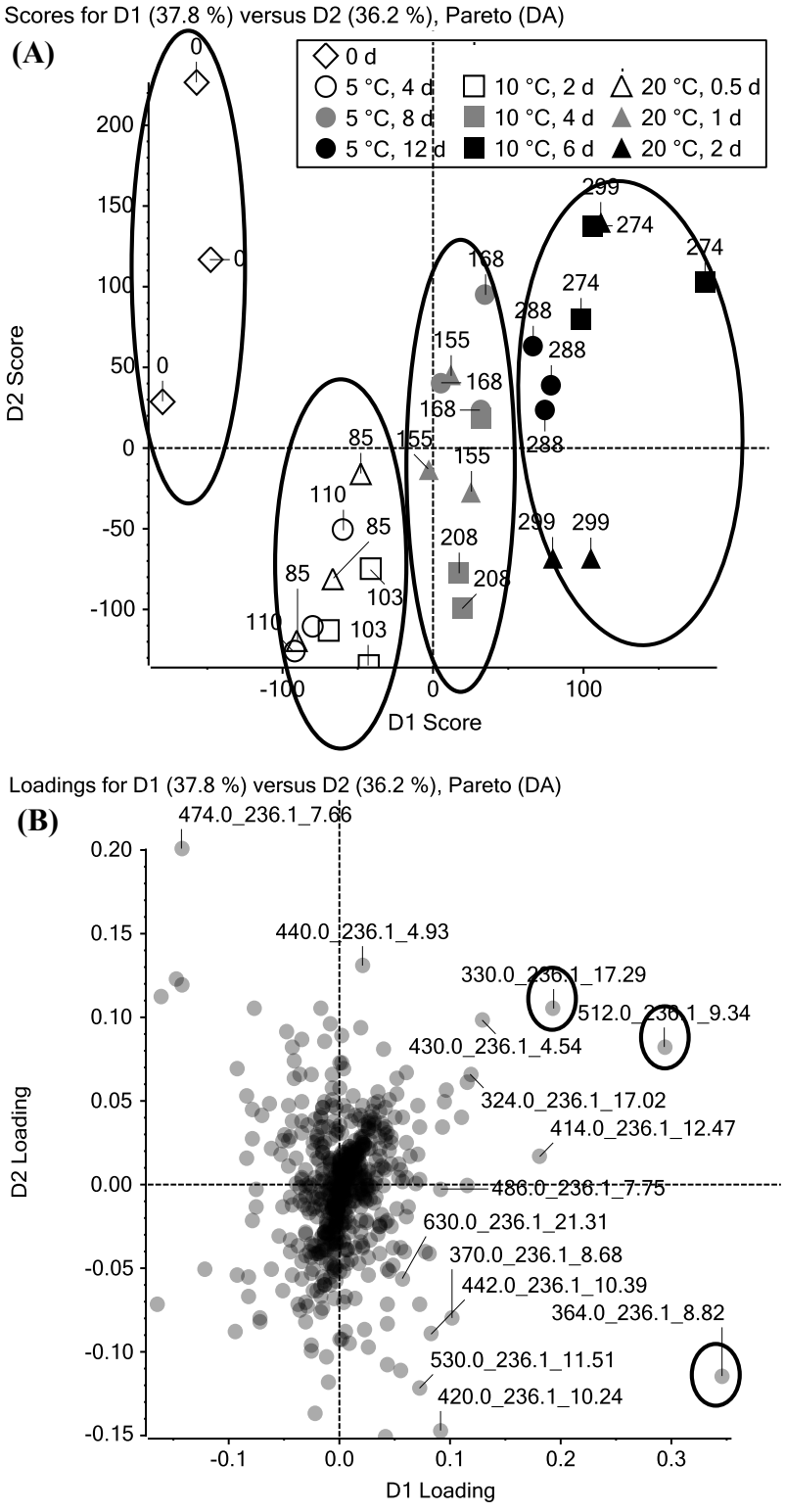
418 Romero, P., Rodrigo, M. J., & Lafuente, M. T. (2013). Differential expression of the Citrus
419 sinensis ABA perception system genes in the postharvest fruit dehydration. *Postharvest*
420 *Biology and Technology*, 76, 65–73.
421 Rudell, D. R., Mattheis, J. P., & Hertog, M. L. (2009). Metabolomic change precedes apple
422 superficial scald symptoms. *Journal of Agricultural and Food Chemistry*, 57(18), 8459–
423 8466.
424 Singh, S. P., 2015. Metabolomics tool for postharvest quality and safety of fresh produces, in:
425 Wills, R. B. H., Golding, J. (Eds.), A*dvances in Postharvest Fruit and Vegetable*
426 *Technology*. (pp. 285–308). Florida: CRC Press Taylor.
427 Slavin, J. L., & Lloyd, B. (2012). Health benefits of fruits and vegetables. *Advances in*
428 *Nutrition*, 3, 506–516.
429 Snowdon, A. L. (2010). *Post-harvest diseases and disorders of fruits and vegetables: Vol 2:*
430 *Vegetables*. London: Manson Publishing, (Chapter 4).
431 Swamy, P. M., & Smith, B. (1999). Role of abscisic acid in plant stress tolerance. *Current*
432 *Science*, 76, 1220–1227.
433 Tomono, S., Miyoshi, N., & Ohshima, H. (2015). Comprehensive analysis of the lipophilic
434 reactive carbonyls present in biological specimens by LC/ESI-MS/MS. *Journal of*
435 *Chromatography. B, Analytical Technologies in the Biomedical and Life*, 988, 149–156.
436 Wills, E. D. (1980). Studies of lipid peroxides formation in irradiation of synthetic diets and
437 the effect of storage after irradiation. *International Journal of Radiation Biology and*
438 *Related Studies in Physics, Chemistry, and Medicine*, 37(4), 383–401.
439 Xiong, L., & Zhu, J. (2003). Regulation of Abscisic Acid Biosynthesis1. *Plant Physiology*,
440 133, 29–36.

441 Xu, F., Zou, L., Liu, Y., Zhang, Z., & Ong, C. N. (2011). Enhancement of the capabilities of
442 liquid chromatography-mass spectrometry with derivatization: General principles and
443 applications. *Mass Spectrometry Reviews*, 30, 1143–1172.
444 Youn, J. E., Kim, H. S., Lee, K. A., & Kim., Y. H. (2011). Contents of minerals and vitamins
445 in soybean sprouts. *Korean Journal of Crop Science*, 56, 226–232.
446 Zhou, C., & Gan, S. (2009). Senescence, in: Pua, E. C., & Davey, M. R. (Eds.), *Plant*
447 *development biology-biotechnological perspectives*. Vol. 1. (pp. 152–153). Berlin:
448 Springer Verlag.
449
450 **Figures captions**
451 **Fig. 1** CO_2 production rates of soybean sprouts stored at various temperatures.
452 **Fig. 2** CC metabolites feature in fresh soybean sprouts; cotyledon (A) and hypocotyl (B).
453 **Fig. 3** Score scatter plot (A) and loading scatter plot (B) of the PCA-DA of CC-DHs in the
454 hypocotyls in stored soybean sprouts. Values beside data points in Fig. 3A indicate
455 cumulative CO_2 production during storage (mmol kg^{-1} FW). Values beside data points
456 in Fig. 3B indicate *m/z* of Q1_ *m/z* of Q3_RT pairs for each CC-DH signal.
Fig. 4 Relationship between each normalized peak area of CC-DH signal and cumulative CO_2 production during storage under various temperatures; (A) coefficient correlation of each CC-DH and *m/z* of CC-DHs. (B) Cumulative CO_2 production and linear regression of CC-DHs with *m/z* 512 at RT of 9.34 min and cumulative CO_2 production.
Fig. 5 Product ions mass spectra of protonated molecules ion with *m/z* of 512 at RT of 9.34 min in positive ion mode; (A) selected freshness marker ion and (B) authentic ABA standard-DH.

FIGURE 1


10
8
6
4
2
0
0
2
4
6
8
10
12
5 °C
10 °C
20 °C
CO2 production rate (mmol kg−1 h−1)
Storage duration (d)


(A)
(B)
m/z
750
650
550
450
350
250
0
5
10
15
20
25
Retetion time (min)


Scores for D1 (37.8 %) versus D2 (36.2 %), Pareto (DA)
(A)
0 d
5 °C, 4 d
5 °C, 8 d
5 °C, 12 d
10 °C, 2 d
10 °C, 4 d
10 °C, 6 d
20 °C, 0.5 d
20 °C, 1 d
20 °C, 2 d
D2 Score
D1 Score
Loadings for D1 (37.8 %) versus D2 (36.2 %), Pareto (DA)
(B)
474.0_236.1_7.66
440.0_236.1_4.93
330.0_236.1_17.29
512.0_236.1_9.34
430.0_236.1_4.54
324.0_236.1_17.02
414.0_236.1_12.47
486.0_236.1_7.75
630.0_236.1_21.31
370.0_236.1_8.68
442.0_236.1_10.39
364.0_236.1_8.82
530.0_236.1_11.51
420.0_236.1_10.24
D2 Loading
D1 Loading

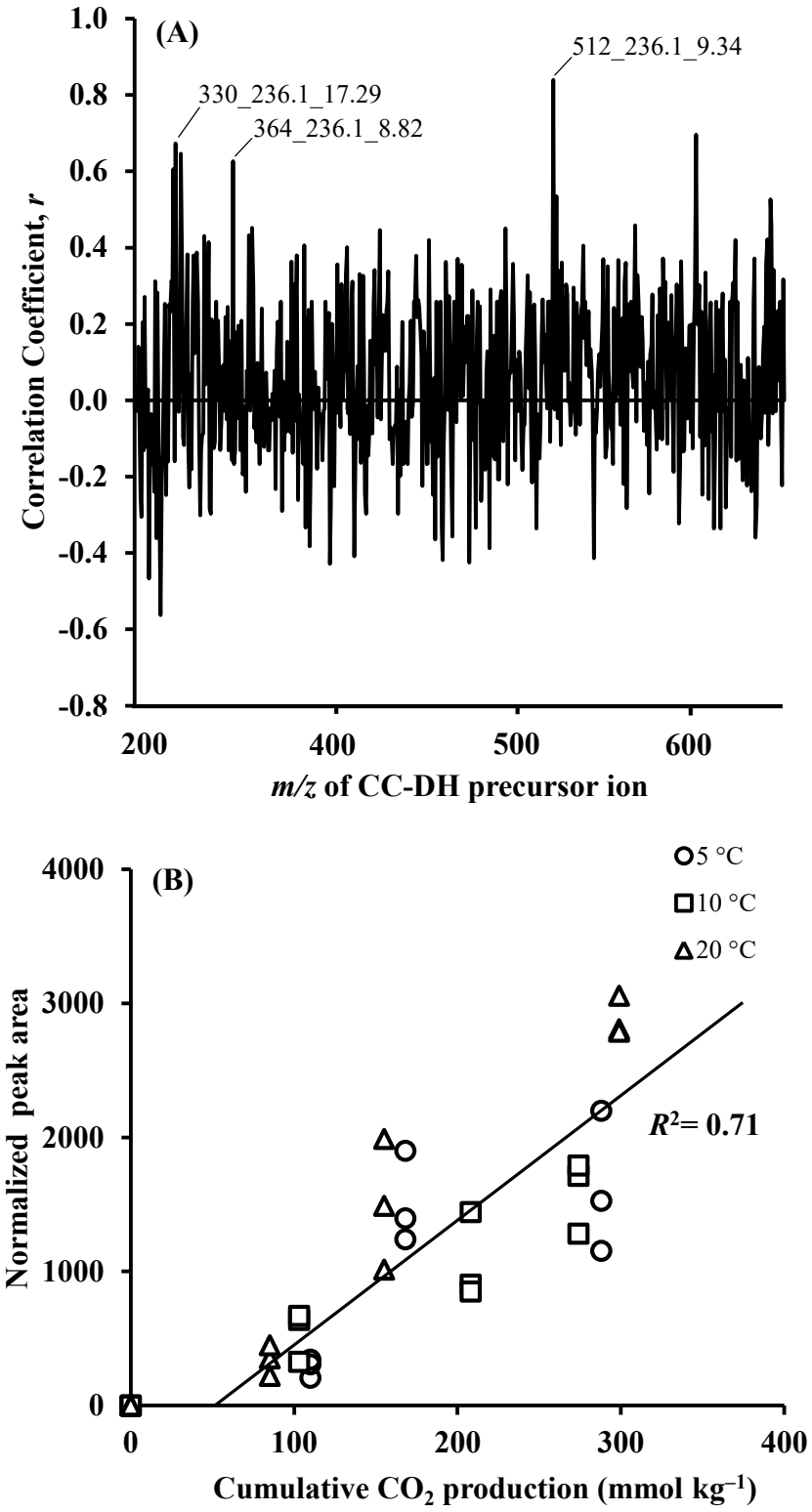

(A)
512_236.1_9.34
330_236.1_17.29
364_236.1_8.82
Correlation Coefficient, r
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.8
200
400
500
600
m/z of CC-DH precursor ion
(B)
5 °C
10 °C
20 °C
Normalized peak area
4000
3000
2000
1000
0
R2= 0.71
0
100
200
300
400
Cumulative CO2 production (mmol kg–1)

FIGURE 5

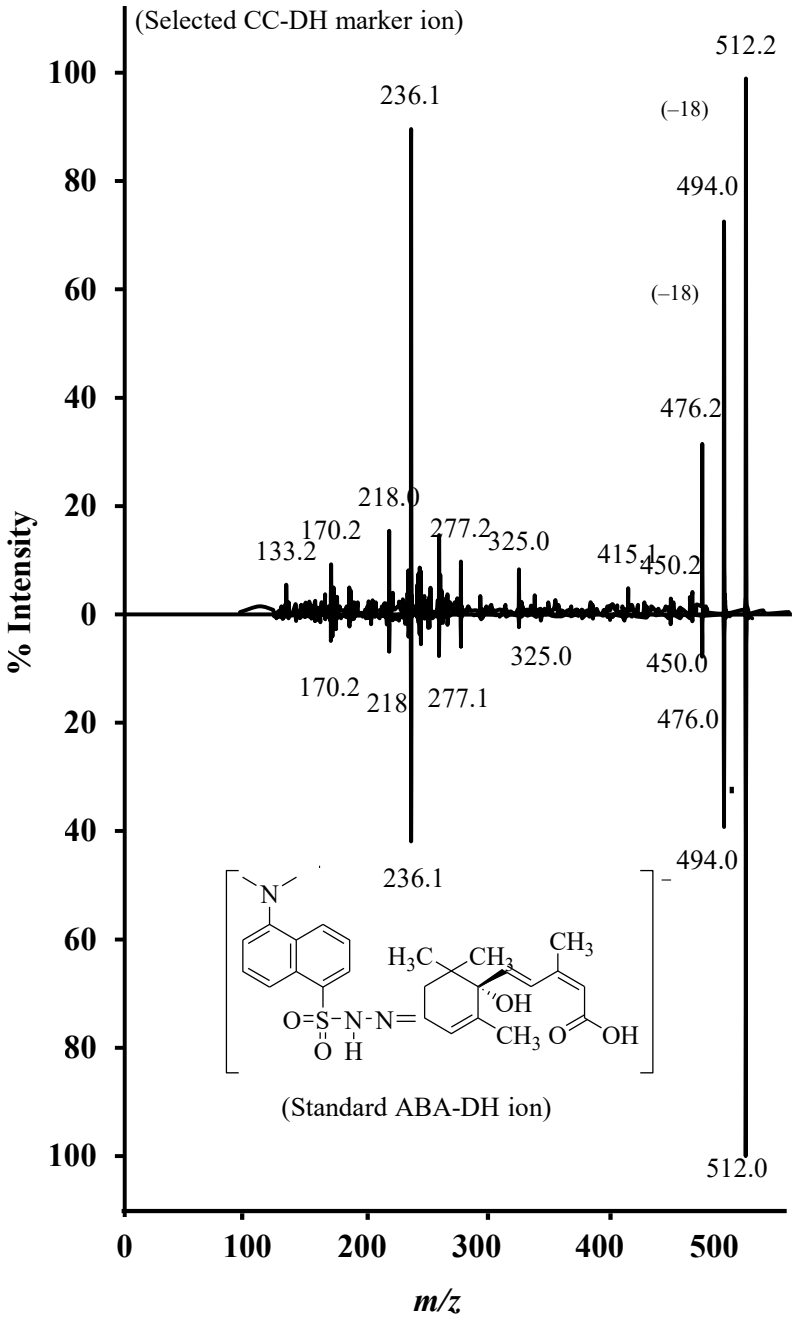
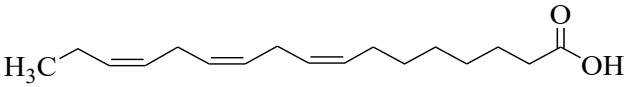
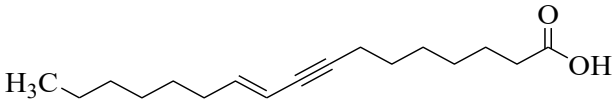
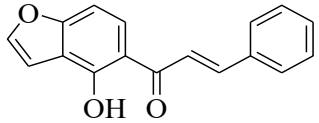
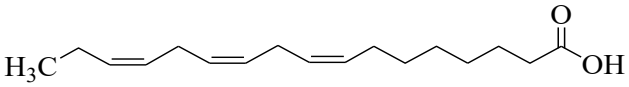
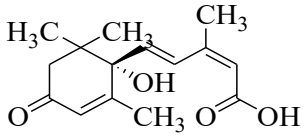
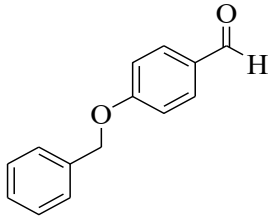

(Selected CC-DH marker ion)
512.2
236.1
(–18)
494.0
(–18)
476.2
218.0
170.2
277.2
325.0
133.2
415.1
450.2
325.0
450.0
170.2
218
277.1
476.0
494.0
236.1
(Standard ABA-DH ion)
512.0
% Intensity
m/z
100
80
60
40
20
0
20
40
60
80
100
0
100
200
300
400
500

Table 1 List of the name for the compound with MW of 264 that contain hydroxyl group (loss of *m/z*=18) and internal standard (RT of 11.4 min)

No	Name	Formula	Log P (Hydrophobicity)	Structure	On line databases
1	Norlinolenic acid	$C_{17}H_{28}O_2$	5.27		www.lipidmaps.org
2	10E-heptadecen-8-ynoic acid	$C_{17}H_{28}O_2$	4.94		www.lipidmaps.org
3	2'-Hydroxyfurano [2",3":4',3']chalcone	$C_{17}H_{12}O_3$	4.03		www.lipidmaps.org
4	all-trans-7-hydroxy hexadeca-2,4,8, 10-tetraenoic acid	$C_{16}H_{24}O_3$	3.91		www.lipidmaps.org
5	Abscisic acid	$C_{15}H_{20}O_4$	2.54		www.lipidmaps.org
6	*p*-BOBA (IS)	$C_{14}H_{12}O_2$	3.3		www.chemicalbook.com

Supplementary Material

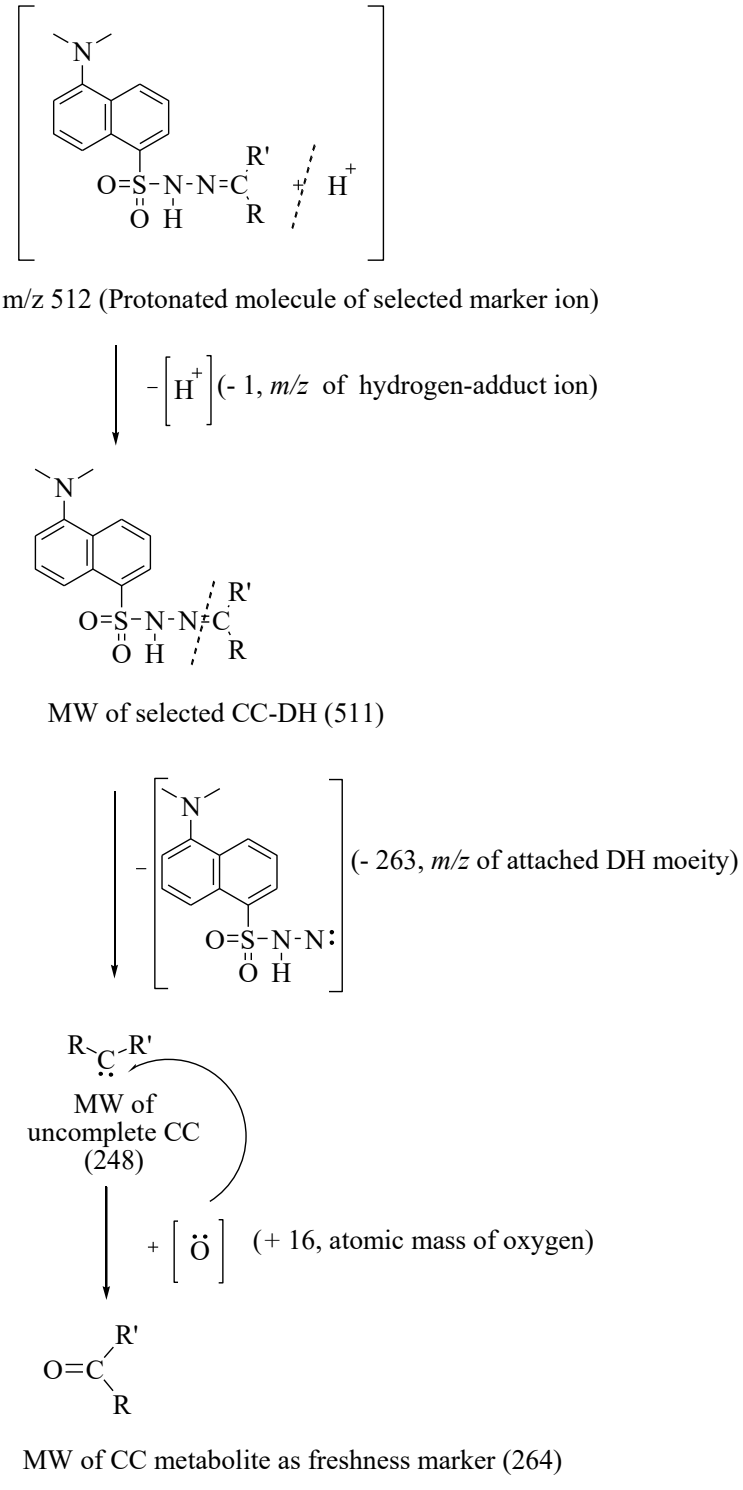


Fig. S1. Schematic of methods for determining the molecular weight of the CC-DH freshness marker metabolite of soybean sprouts with an *m/z* of 512 and a RT of 9.34 min.