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Effect of germination time on antioxidative activity and composition of yellow pea soluble free and polar soluble bound phenolic compounds

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ABSTRACT

This research aims to study antioxidative activities of polar solvent extractable phenolic compounds from yellow pea with different germination time against oil-in-water emulsion oxidation. After germination (0, 2, 4, and 6 days), soluble free and polar soluble bound phenolic compounds were extracted and their antioxidative activity was evaluated using stripped soybean oil (SSO)-in-water emulsions. Liquid chromatography coupled with electrospray ionization quadrupole time-of-flight mass spectrometry (LC-ESI-QTOF-MS) and size-exclusion chromatography with multiangle-light-scattering and refractive-index detection (SEC-MALS-RI) were employed to analyze the phenolic composition and molar mass, respectively. Antioxidative activities of soluble free phenolic compounds increased in SSO-in-water emulsion system, while that of polar soluble bound phenolic compounds decreased with germination. On the basis of chemometric analysis, pratensein (**2**), phloridzin (**4**), quercetin (**9**), sayanedine (**12**), hesperetin (**13**), glyzaglabrin (**14**), and pinocembrin (**15**) were speculated as the pivotal phenolic compounds responsible for the hydrogen donating capacity. Additionally, decreased molecular weight of soluble bound phenolic compounds was accompanied with the reduction of antioxidative activity in SSO-in-water emulsions indicating the moieties of polar soluble bound phenolic compounds also have an important impact on the antioxidative activity of phenolic compounds.

KEYWORDS: natural antioxidant; lipid oxidation; emulsion; molar mass; dual effect

1. Introduction

Edible pulse seeds are high in antioxidant potential, dietary fiber, resistant starch, protein, vitamins, and minerals with a gluten-free status.[1] Germination has been regarded as an effective process to improve nutrient digestibility and accessibility, as well as phenolic compounds of pulse seeds.[2] An overwhelming amount of publications have reported that germination can improve the quantity of total phenolic compounds, which is considered an indicator of antioxidant potential.[3–6] However, the effect of germination on the quality of phenolic compounds, particularly their antioxidative activity, is inconsistent.

The antioxidative activity of phenolic compounds is believed to be associated with phenolic composition. Different phenolic compounds have different antioxidative potential, e.g. chlorogenic acid > quercetin > gallic acid > *trans*-resveratrol > rutin > caffeic acid based on 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging capacity.[7] During germination, a new class of phenolic compounds can be biosynthesized through the Shikimate pathway, while existing phenolic compounds can be either depleted by free radicals or as the precursors of phytochemicals.[8] Germination of pulse seeds would either cause the increase or decrease the amount of specific phenolic compounds. Gan et al. reported that after 5 days of germination, caffeic acid, ferulic acid, and *p*-coumaric acid in black mung bean increased from 2.51 to 6.72, 2.10 to 5.57, and 1.50 to 22.0 mg/100 g dry basis (d.b.), respectively. On the contrary, Kim et al.[5] reported that after 4 days of germination, veratric acid, protocatechuic acid, and *p*-coumaric acid in soybean decreased from 2.17 to 0.53, 85.57 to 57.36, and 2.96 to 0.39 μg/g, respectively.

In comparison to the individual phenolic compound, the moieties of phenolic compounds

play a preponderant role in phenolic antioxidative activity.[8] Phenolic compounds can be classified as either extractable or non-extractable based on their solubility in different solvents, e.g. ferulic acid is extractable when bound to glucoside; however, it becomes non-extractable after covalently bound to arabinoxylans.[9] Most extractable phenolic compounds are studied with polar solvent and named as polar solvent extractable phenolic compounds, while non-polar soluble bound phenolic compounds have not been widely concerned and were regularly discarded with the defatting process.[10,11] Polar solvent extractable phenolic compounds can be further divided into soluble free and polar soluble bound phenolic compounds depending on the absence or the presence of strong polar moieties. Polar solvent extractable phenolic compounds are of great antioxidative activity after extraction.[12] Soluble free phenolic compounds displayed better antioxidative activity than polar soluble bound phenolic compounds in the *in vitro* system.[13,14] However, the real food system is more complicated than the *in vitro* system.

Our recent research found that germination increases the total phenolic content of yellow pea, lentil, and chickpea. However, the antioxidant activities of these phenolic compounds showed different variations using *in vitro* assays.[15] Furthermore, we found only the polar soluble bound phenolics from chickpea can effectively prevent oil-in-water emulsion oxidation.[15] We then focused on the elucidation of how germination impacts the structural properties of both free and polar soluble bond phenolic compounds in germinated chickpea, and proposed a dual-effect antioxidant mechanism to describe the enhanced antioxidative efficacy of soluble bond phenolics in emulsions.[16] In this research, a mixed variety of yellow pea was employed to further our understanding of how the structural and composition

changes phenolic compounds during seeds germination could impact their antioxidative activity in real food system. The aims of this study were to (i) evaluate the antioxidative activities of phenolic compounds extracted from germinated yellow pea mixture in stripped soybean oil (SSO)-in-water emulsions oxidation, (ii) analyze the composition and molecular weight of phenolic compounds with electrospray ionization quadrupole time-of-flight mass spectrometry (LC-ESI-QTOF-MS), and size-exclusion chromatography with multiangle-light-scattering and refractive-index detection (SEC-LC-MALS-RI), respectively, and (iii) unravel the mechanism by which germination affects antioxidative activity of phenolic compounds from germinated yellow pea.

2. Materials and Methods

2.1. Materials and Chemicals

Yellow pea (*Pisum sativum* L.) from the 2017 crop year was gifted from JM Grain, Viterra Inc, and AGT Food and Ingredients. The manufactures have noted that yellow pea is the mixture of different cultivars collecting from farmers. Commercial soybean oil was purchased locally. Ammonium thiocyanate, hexanal, and hexanol were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). All other chemicals and solvents were purchased from VWR International.

2.2. Germination of Yellow Pea

Yellow peas from three different sources were mixed thoroughly before germination to better represent the species. The method of yellow pea germination was adapted from Xu and co-workers without modification.[15]

2.3. Extraction of Phenolic Compounds

Extraction of phenolic compounds from germinated yellow pea was performed as described by Xu and co-workers.[16] Briefly, 12 g of ground yellow pea flour was defatted with 60 mL of n-hexane. After shaking for 15 min under nitrogen, the solvent layer was discarded. A duplication was applied to ensure the complete defatting. After evaporating the solvent, the defatted meal was mixed with 60 mL of acetone/H_2O (7/3, v/v), purged with N_2, and then shaken for 1 h. After centrifugation of the resulting slurry for 5 min at 2,000×*g*, the supernatant was collected. The extraction was triplicated, and supernatants were combined. The crude phenolic solution was then prepared by evaporating the acetone from the supernatants at 40 °C with rotary evaporation (RE 111 Buchi, Flawil, Switzerland), and the pH value of the solution was adjusted to 3.0.

Soluble free phenolic compounds were extracted from the crude phenolic solution by partitioning three times with ethyl ether/ethyl acetate (1/1, v/v). The supernatants were combined, and the solvent was removed with rotary evaporation. Remaining solvent traces were removed with a nitrogen purge. The dried samples were redissolved using 15 mL methanol and the solution was transferred into storage vials. The vials were stored at 4 °C. Soluble free phenolic compounds extracted from yellow pea after 0, 2, 4, and 6 days germination were labeled as YF0, YF2, YF4, and YF6, respectively.

Polar soluble bound phenolic compounds were collected by evaporating any remaining organic solvent from the subnatant of the abovementioned ethyl ether/ethyl acetate solution. The remaining extract was transferred to storage vials using 50 mL water as a solvent and stored at 4 °C. Polar soluble bound phenolic compounds extracted from yellow pea after 0, 2, 4, and 6 days germination were labeled as YB0, YB2, YB4, and YB6, respectively.

2.4. Preparation of Stripped Soybean Oil-in-Water Emulsions

Stripped soybean oil (SSO) and SSO-in-water emulsions were prepared according to Xu et al without modification[15]. The final emulsion consisted of 2.0 wt % SSO and 0.2 wt% Tween 20, and the pH was adjusted to 7.0.

Yellow pea extracts were added into freshly prepared SSO-in-water emulsions at a fixed concentration of 200 μg gallic acid equivalence (GAE)/g oil to study the antioxidative activity. A 0.5 mL aliquot of mixture was transferred into a 20 mL vial which was then sealed with an aluminum cap. These vials were stored in an incubator (Heratherm Incubator, ThermoFisher Scientific, Waltham, MA, USA) at 25 °C and covered with aluminum foil for oxidative stability study.

2.5. Measurement of Emulsion Oxidation

Lipid hydroperoxides were determined using a method adapted from Xu et al.[15] The concentration of hydroperoxides was calculated from a cumene hydroperoxide standard curve. Hexanal and hexanol were determined directly from the sealed vials during incubation. A 50/30 mm DVB/Carboxen/PDMS solid-phase microextraction (SPME) fiber needle (57298U, Supelco, Bellefonte, USA) was employed to absorb volatiles in the headspace of GC vials. The analysis was performed with Agilent Technologies 7890B GC system and 5977A mass detector equipped with a PAL RSI 120 auto-sampler. Both SPME and GC-MS parameters were adapted from the method of Xu et al. Selected ion monitoring (SIM) was used for the detection of hexanal (*m/z*: 72 and 82) and hexanol (*m/z*: 55, 56, and 69). Identification of hexanal and hexanol were carried out by comparing GC retention time with those of reference standard compounds. The concentration of hexanal and hexanol (μmol/L

emulsion) were determined on the basis of the calibration curves. Limit of detection and quantification (LOD and LOQ, respectively) were calculated based on ratio of signal to noise (3 times and 10 times, respectively). Seven replicates of 2 mL fresh SSO-in-water emulsion were performed to evaluate the ratio of signal to noise. Finally, the LOD and LOQ of hexanal were 0.87 and 2.90 μmol/L, while those of hexanol were 0.39 and 1.30 μmol/L, respectively.

2.6. SEC-LC-MALS-RI Analysis

Polar soluble bound phenolic compounds collected in **Section 2.3** were concentrated by reducing the volume by 90%, and the resulting solutions were filtered through a 0.2 μm polytetrafluoroethylene (PTFE) disposable membrane filter prior to SEC-MALS-RI analysis. SEC-MALS-RI analysis was carried out using a Yarra 3 μm SEC-4000, 300 × 7.8 mm SEC column, an Agilent G1315 C DAD detector, an Agilent 1362 A RI detector, and a DAWN HELEOS II MALS detector. ASTRA 7.1.2.5 software was used for data analysis. Deionized water was used as the eluent for the SEC-MALS-RI analysis. SEC conditions were set as follows: injection volume of 10 μL, mobile phase flow rate of 0.4 mL/min, and a column temperature of 30 °C. Specific-refractive-index increments (dn/dc) of sample solutions with concentrations in the range of 0.8−1.5 mg/mL were determined using an Agilent 1362 A RI detector. The dn/dc values of YB0, YB2, YB4, and YB6 were 0.134, 0.109, 0.096, and 0.087 mL/g, respectively.

2.7. Hydrolysis of Polar Soluble Bound Phenolic Compounds

Thirty milliliters of polar soluble bound phenolic compounds were concentrated into 3 mL by freeze-drying; 200 μL of concentrated polar soluble bound phenolic compounds was hydrolyzed using 2 mL sodium hydroxide (3 M), purged with nitrogen, and shaken for 1 h.

After adjusting pH to 3.0, 3 mL ethyl ether/ethyl acetate (1/1, v/v) was added to extract the liberated phenolic compounds. The extraction was repeated thrice, and supernatants were collected. The residues were further hydrolyzed using 3 mL hydrochloric acid (6 M) in a 95 °C water bath, purged with nitrogen for 20 min. Aliquots of 3 mL of ethyl ether/ethyl acetate (1/1, v/v) were added to extract the phenolic compounds. The combined ethyl ether/ethyl acetate solvents were taken to dryness using a stream of nitrogen. Acetonitrile (500 μL) was added to each sample, and the samples were stored at −80 °C for further analysis.

2.8. LC-ESI-QTOF-MS Analysis

Both soluble free phenolic compounds and hydrolyzed polar soluble bound phenolic compounds extracted from germinated yellow pea were characterized using LC-ESI-QTOF-MS according to the method described by Kadam and Lele[17] with minor modifications. An Agilent 1290 series liquid chromatography system utilizing a Kinetex C18 (2.6 μm, 150 × 4.6 mm) column was used to separate phenolic compounds. The mobile phase comprised of water (solvent A) and acetonitrile (solvent B) with the following gradients: 0−5 min (A: 95%, B: 5%), 30−40 min (A: 0%, B: 100%), and 41−45 min (A: 95%, B: 5%). The flow rate was 0.5 mL/min, and the column temperature was 30 °C. The injection volume was 20 μL with a total run time of 45 min. A diode-array detector (DAD) with a working range from 190 to 600 nm was employed to observe the UV absorption of the separated yellow pea extracts. An Agilent G6540 UHD Accurate QTOF-MS was utilized to analyze the chromatographed yellow pea extracts. Sample ionization was achieved using an electrospray ionization (ESI) interface in negative-ion mode. The gas and vaporizer temperatures were set

to 300 °C, with a drying-gas flow rate of 7 L/min. The nebulizer (N_2) was set at 50 psi, the fragmentor voltage was set to 200 V, the skimmer voltage was set at 65 V, the octopole RF voltage was set at 750 V, and the capillary voltage was set at 4000 V. The collision energy was set as 0, 10, 25, 30, and 40 eV. The Agilent high-resolution mass spectrometer was operated for data acquisition using Data Dependent Acquisition mode (Auto MS/MS) combining a TOF scan (*m/z* 100-1000) followed by dependent TOF-MS scans (*m/z* 50-1000). Data analysis was performed using MassHunter Qualitative Analysis software B.05.00 (Agilent Technologies). The identification of the detected compounds proceeded by the generation of candidate molecular formulas with mass-accuracy limits of 5 ppm and MS scores >80. Agilent Personal Compound Database Library (PCDL) version B.05.01 build 92 was employed to create the custom database. For the retrieval of phenolic-compound chemical structures, the following databases were consulted: Agilent METLIN Metabolomics database, HMDB (http://www.hmdb.ca/), and Phenol-Explorer (http://www.phenol-explorer.eu).

2.9. Statistical Analysis

The experiments were performed at least twice, and data were expressed as means ± standard deviations of duplicate or triplicate from each of two independent experiments. Data were statistically analyzed using SAS version 9.4 (SAS Institute Inc.). One-way analysis of variance (ANOVA) was conducted, and the significant difference was defined at $p < 0.05$ by Tukey's test. The relationships between germination time and phenolic compounds of yellow pea extracts were analyzed by principal-component analysis (PCA) and constellation plot-cluster analysis (CPCA) based on the counts of each phenolic compounds using JMP Pro

14.0.0 (SAS Institute Inc.).

3. Results and Discussion

3.1. Antioxidative Activity of Germinated Yellow Pea Extracts on Emulsion Oxidation

Although *in vitro* assays have certain advantages in estimating antioxidative activity of phenolic compounds[15], their role in preventing lipid oxidation in a real food system is required to confirm the real antioxidative activity. In this study, SSO-in-water emulsions in absence of endogenous antioxidants were prepared as a model food system. The antioxidative activity of both soluble free and polar soluble bound phenolic compounds (200 μg GAE/g oil) in raw and germinated yellow pea against emulsion oxidation was assessed by measuring the formation of lipid hydroperoxides (LOOH) and hexanal during storage.

Figure 1 inserted here

With the addition of soluble free phenolic compounds extracted from yellow pea of variable germination time (i.e., YF0, YF2, and YF4), the primary oxidation products LOOH (**Figure 1A**) increased exponentially after the second day of incubation, sharing a similar pattern with the control emulsions. Nevertheless, YF6 can retard the emulsion oxidation by suppressing the generation of LOOH for 3 days. Likewise, YF6 retarded the formation of hexanal in emulsions for 3 days, 1 days longer than other soluble free phenolic compounds (**Figure 1B**). This finding from the mixed sources of yellow pea was corroborated with our previous study on the sole source of the samples and suggested that the soluble free phenolic compounds from raw and short time germinated yellow pea had little or no retardation effect on preventing emulsion oxidation, while the antioxidative activity of soluble free phenolic compounds was improved with germination particularly after 6 days .[15]

Interestingly, with the addition of polar soluble bound phenolic compounds YB0, YB2, YB4, and YB6, the formation of LOOH in emulsions increased exponentially after 11, 7, 4, and 6 days of storage, respectively (**Figure 2A**). In terms of secondary oxidation products, it was unexpected that hexanal had not increased significantly, except in YB4 (**Figure 2B**). Meanwhile, an exponential increase of hexanol was observed after 11, 10, and 13 days storage, with the addition of YB2, YB4, and YB6, respectively. While with the addition of YB0, hexanol had not boosted yet in the SSO-in-water emulsions after 17 days (**Figure 2C**). Hexanal is widely used as a secondary oxidation marker for the oxidation of high linoleic oil system. A significant increase of hexanal was observed in SSO-in-water emulsions in the presence of soluble free phenolic compounds during storage (**Figure 1B**). However, hexanal did not increase significantly in the presence of polar soluble bound phenolic compounds during storage, despite the degradation of LOOH. Alternatively, the substantial increase of hexanol was observed during storage which also correlated well with the development of LOOH. Interestingly, emulsions with the addition of soluble free phenolic compounds did not have any variation in terms of hexanol even when hexanal had boosted. As a consequence, we selected hexanol as an alternative oxidation marker only for polar soluble bound phenolic compounds to track the development of secondary oxidation products. Hexanol could be formed when antioxidants donate electron to fatty acid decomposition products that have a free radical. Reducing groups on the moieties of polar soluble bound phenolic compound may be responsible for this pathway.[10] On the contrary, soluble free phenolic compounds without moieties did not have a variation of hexanol. Moreover, since hexanol has a higher aroma threshold (2,500 ppb) than hexanal (22.8 ppb),[18] pushing the pathway from hexanal to

hexanol could be a strategy to minimize flavor changes.

Figure 2 inserted here

According to the formation of hexanol and LOOH, germination time was inversely proportional to the antioxidative activity of polar soluble bound phenolic compounds in SSO-in-water emulsions at the first 4 days. The decreased electron transfer capability (DPPH) and hydrogen atom transfer capability (ORAC) of polar soluble bound phenolic compounds following yellow pea germination may be responsible for it.[15] However, a contradictory phenomenon was observed in terms of the antioxidative activity of yellow pea extracts in *in vitro* assays and in emulsions, i.e., in comparison with polar soluble phenolic compounds, soluble free phenolic compounds had greater antioxidative activity in *in vitro* assays;[15] however, the opposite was revealed in SSO-in-water emulsions. The difference between polar soluble phenolic compounds and soluble free phenolic compounds, mainly phenolic composition and molecular weight, may be related to this contradictory phenomenon.

3.2. Compositional Changes of Phenolic Compounds during Yellow Pea Germination

The main differences between soluble free phenolic compounds and polar soluble bound phenolic compounds in general are (i) the chemical profiles of phenolic compounds and (ii) the moieties the polar soluble phenolic compounds attached. Therefore, the composition of phenolic compounds, including soluble free and polar soluble bound phenolic compounds, were investigated using LC-QTOF-MS. In addition, the molar mass of polar soluble bound phenolic compounds was measured with SEC-LC-MALS-RI.

3.2.1. Effect of Germination on Phenolic Composition of Yellow Pea Extracts

Sixteen phenolic compounds, including 1 phenolic acid, 2 chalcones, and 13 flavonoids, were identified in yellow pea extracts based on their *m/z* values in conjunction with product ions (**Table 1**). In fact, more than 16 compounds may present in yellow pea extracts as indicated by the UV spectrum; the phenolic compounds reported here are restricted to the availability of databases and the fragments we observed (**Supporting Information**). Significant variations of individual phenolic compounds were identified in both soluble free and polar soluble bound forms during yellow pea germination.

Table 1 inserted here

Phenolic acids widely exist in all kinds of pulse seeds. Shikimate pathway and acetate pathway are two main metabolic pathways that are responsible for the biosynthesis of phenolic acids, that can be classified as benzoic acid and cinnamic acid.[19] *p*-Salicylic acid (**10**) in the soluble free phenolic compounds reduced significantly, while it remained consistently in polar soluble bound phenolic compounds during yellow pea germination (**Table 2**). *p*-Salicylic acid (**10**) is a benzoic acid that has been reported in wheat, barley, sorghum,[20] and pulse seeds.[21] It is a key phytohormone for inducing the expression of the gene encoding phenylalanine ammonia-lyase (PAL).[22]

Table 2 inserted here

The precursor of chalcones is coumaric acid that can be synthesized by PAL from phenylalanine.[23] Chalconaringenin (**1**) and phloridzin (**4**), a glucoside of phloretin, are two chalcones identified in the germinated yellow pea extracts with LC-QTOF-MS. Both chalconaringenin (**1**) and phloridzin (**4**) increased in the forms of soluble free and polar soluble bound phenolic compounds during yellow pea germination (**Table 2**). Chalcones such

as phloretin and chalconaringenin (**1**) can spontaneously cyclize to flavanones. Flavanones can be further modified into isoflavanone, flavone, flavonol, flavanol, and anthocyanidins by all kinds of enzymes.[24] These flavonoids are closely related to the color, fragrance, and taste of the pulse seeds.[23] Totally, 1 flavanonol, 2 flavonols, 2 flavones, 2 flavanones, 3 isoflavans, and 3 isoflavones were identified and listed in **Table 1**.

3.2.2. Chemometric Analysis of Phenolic Compounds

In the present study, constellation plot-cluster analysis (CPCA) and principal component analysis (PCA) were performed to statistically analyze the impact of germination time on the compositional changes of phenolic compounds in yellow pea.

According to the results of constellation plot-cluster analysis (CPCA), phenolic compounds were separated into 6 groups at the distance 3.939 on account of the variation in the forms of soluble free and polar soluble bound phenolic compounds during yellow pea germination (**Figure 3 & Table 2**). Cluster I, which involved pratensein (**2**), epigallocatechin (**5**), arachidoside (**7**), *p*-salicylic acid (**10**), tangeretin (**11**), and sayanedine (**12**), located at the bottom of Y-axis. The amounts of these phenolic compounds were higher in polar soluble bound phenolic compounds than these in soluble free phenolic compounds. Nevertheless, their concentration decreased over the course of germination. Cluster II was kaempferol (**8**) that decreased in both soluble free and polar soluble bound phenolic compounds. Cluster III, involving chalconaringenin (**1**), (2*S*,3*S*,4*R*)-3,4,4',7-tetrahydroxyflavan (**3**), and aromadendrin (**6**), presented both in soluble free and soluble bound forms and had a significant increase in soluble bound form during yellow pea germination. Hesperetin (**13**), the only phenolic compound located in Cluster IV, raised sharply in soluble free phenolic compounds, while

increased subtly in polar soluble bound phenolic compounds during germination. Genkwanin (**16**) presented in Cluster V in soluble free form and had a significant decrease during germination. Cluster VI, involving phloridzin (**4**), quercetin (**9**), glyzaglabrin (**14**), and pinocembrin (**15**), located at the top of the Y axis, which was low in polar soluble bound form while had an increment in soluble free form during germination. Researchers have studied the variation of individual phenolic compounds in germinated pulse seeds. Wu et al.[25] reported an increase of soluble free pratensein (**2**) in germinated chickpea. López-Amorós et al.[26] declared that soluble quercetin (**9**) was increased from 0 to 291−311 μg/100 g in germinated beans. Epigallocatechin (**5**), kaempferol (**8**), and pinocembrin (**15**) had been identified in the seed coat of pea.[27,28] However, none of these studies separately evaluated the antioxidative activities of polar solvent extractable phenolic compounds (i.e., soluble free and polar soluble bound) in food systems.

Figure 3 inserted here

Principal component analysis (PCA) was performed to further analyze the relationship between phenolic clusters and germination treatment. The principal component 1 (PC1) and principal component 2 (PC2) explained 48.2% and 30.3% of the total variance in the data set, respectively (**Figure 4**). Soluble free phenolic compounds and polar soluble bound phenolic compounds can be well differentiated based on PC1 and PC2. Polar soluble bound phenolic compounds located in the second and third quadrant, while soluble free phenolic compounds located in the first, third, and fourth quadrants.

Figure 4 inserted here

Soluble free phenolic compounds varied from negative regions to positive regions of

PC1 and PC2 with 6 days of germination. Phenolic compounds involving in Cluster V played important roles in the soluble free phenolic compounds from raw yellow pea (YF0), while Cluster IV and Cluster VI was charged for YF6 (**Figure 4**). In accordance to the greater antioxidative activities of YF6 than that of YF0 in SSO-in-water emulsions (**Figure 1**), it could be speculated that phenolic compounds in Cluster IV and Cluster VI have better antioxidative activities than those in Cluster V. Unlike soluble free form, polar soluble bound phenolic compounds in raw yellow pea varied from the second quadrant to zero point after 6 days of germination (**Figure 4A**). By combining the CPCA with the loading plot (**Figure 4B**), it was deduced that Cluster I and II exerted remarkable impacts on polar soluble bound phenolic compounds. As shown in **Figure 2**, antioxidative activity of polar soluble bound phenolic compounds decreased in SSO-in-emulsion system during germination. The decrease of polar soluble bound phenolic compounds, such as pratensein (**2**), epigallocatechin (**5**), kaempferol (**8**), and sayanedine (**12**), in Cluster I and II were accounted for the reduced antioxidative activities.

Comprehensively, soluble free and polar soluble bound phenolic compounds shared the same variation trend on PC1, from negative to positive (**Figure 4A**) upon germination. Nevertheless, soluble free phenolic compounds altered from negative to positive on PC2, where polar soluble bound phenolic compounds varied oppositely following germination. Two pivotal phenolic compounds, pratensein (**2**) and sayanedine (**12**) in Cluster I (**Figure 3**) increased in soluble free phenolic extracts, while decreased in polar soluble bound phenolic extracts, which may account for the opposite antioxidative performances of soluble free and polar soluble bound phenolic compounds in SSO-in-water emulsions (**Figure 1&2**). Senthil

et al.[29] reported that pratensein (**2**) required lower energy for both hydrogen atom and electron transfer mechanisms than prunetin, genistein, and calycosin, which resulted in the better antioxidative activity. In addition, sayanedine (**12**) has been identified in peas as a plant growth regulator, although its antioxidative activity has not been studied individually.[30]

3.2.3. Effect of Germination on the Molar Mass of Polar Soluble Bound Phenolic Compounds in Yellow Pea

Opposite to our *in vitro* assays, the antioxidative activity of polar soluble bound phenolic compounds in raw yellow pea was greater than that of soluble free phenolic compounds in SSO-in-water emulsions (**Figure 1 & 2**).[15] The phenolic composition of yellow pea extracts may partly explain their antioxidative activity. According to our previous study, molar mass difference between soluble bound and soluble free phenolic compounds might be another factor that affects their antioxidative activity.[16] Therefore, molar mass of major polar soluble bound phenolic compounds in germinated yellow pea was investigated by means of SEC-MALS-RI (**Figure 5**).

Figure 5 inserted here

Polar soluble bound phenolic compounds were separated with size exclusion chromatography based on the size of molecules. The major peak eluted out at 27-32 min were believed to have larger molecular weight than the following substances. Concentrations of the phenolic compounds, quantified by UV and RI detectors, are the fundamental for the calculation of molar mass. The resonance of chemical bonds, such as hydroxyl group and phenyl ring, is responsible for the UV absorbance. The remarkable increase of UV signal

(260 nm) from 0 to 4 days of germination implied that composition of polar soluble bound phenolic compounds changed substantially. The increased area of eluting peaks indicated that the type of polar soluble bound phenolic compounds increased. This is most probably attributed to the biosynthesis of polar soluble bound phenolic compounds originating from soluble free phenolic compounds and/or the decomposition of non-extractable phenolic compounds over the course of yellow pea germination. RI detector is responsible for the concentration changes of solutes based on the refractive index d*n*/d*c*. As displayed in **Figure 5**, the major changes of RI value were on the peak eluting at 27-32 min. Area of RI peaks increased tremendously over the course of germination, which meant the concentration of the solutes had a great increment during germination. The increase of total phenolic content (TPC) during yellow pea germination corresponded with the increase of RI peak areas.

Multiangle-laser-light-scattering (MALS) detector is responsible for the molecular shape and absolute molar mass. With the detection of the molecules scattering light, coupled with the variation of molecule concentration determined by the RI detector, the instrument can calculate the absolute molar mass of molecules. **Figure 5 and Table 3** depicted the molar mass of major polar soluble bound phenolic compounds during germination. Opposite to our previous finding of chickpea[16], the germination decreased the molecular weight of soluble bound phenolic compounds in yellow pea. Number average molecular weight (Mn) of major polar soluble bound phenolic compounds in yellow pea dropped from 6722 ± 195 to 5271 ± 199 g/mol after 4 days of germination. Similarly, the weight average molecular weight (Mw) of major polar soluble bound phenolic compounds in yellow pea declined from 7110 ± 69 to 6266 ± 14 g/mol after same germination time. However, both Mn and Mw of polar soluble

419 bound phenolic compounds in yellow pea after 6 days of germination were statistically larger
420 than those in the shorter germination periods. In addition, germination had no impact on the
421 shapes of polar soluble bound phenolic compounds as indicated by Mw/Mn. By correlating
422 the molar mass to the antioxidative activity of polar soluble bound phenolic compounds in
423 SSO-in-water emulsions, a similar variation trend was observed that both decreased from 0 to
424 4 days of germination and reversed after 6 days of germination. This finding was
425 corroborated with our previous research that molecular weight of polar soluble bound
426 phenolic compounds is associated with their antioxidative activity in SSO-in-water
427 emulsions.[16]

428 **Table 3 inserted here**

429 **3.3. Insight into the Antioxidative Activity of Polar Solvent Extractable Phenolic**
430 **Compounds in Germinated Yellow Pea**

431 The current findings reinforce the concept that a protective or a dual antioxidant effect
432 derived from moieties might contribute to the enhanced antioxidative activity of polar soluble
433 bound phenolic compounds as compared to the phenolic compounds free of moiety. Since
434 free radicals gradually generate from emulsions during storage, large amounts of phenolic
435 compounds can scavenge free radicals immediately on the first 2 days. Then soluble free
436 phenolic compounds lose their antioxidative capability in the aerobic environment, while
437 polar soluble bound phenolic compounds are protected by their moieties against oxygen.
438 Oxidized polar soluble bound phenolic compounds can even be regenerated by moieties of
439 polysaccharides or proteins that may carry reducing power. The schematic diagram for the
440 speculated antioxidative mechanism of soluble phenolic compounds was exhibited in our

441 previous research.[16] Curcio et al.[31] and Xie et al.[32] grafted phenolic compounds on
442 polysaccharides, such as gallic acid conjugated chitosan. The stability of phenolic compounds
443 was improved due to the protective effect of polysaccharides. In addition, a synergistic theory
444 that soluble polysaccharides donate hydrogen from their activated reducing ends to these
445 oxidized phenolic compounds was reported by Xu et al.[10] An adamant evidence is that polar
446 soluble bound phenolic compounds have been shown to exhibit higher antioxidant activity
447 than their hydrolyzed counterparts.[33,34] Synergistic effect between polymers and phenolic
448 compounds has also been proposed to explain improved antioxidative activity of caffeic acid
449 and polysaccharides from *Echinacea purpurea*.[35]
450 An interesting finding on the opposite antioxidant efficacy of phenolic compounds from
451 germinated chickpea and yellow pea against emulsion oxidation may relate to their different
452 molecular weight changes during germination. Larger molecular weight of moieties may
453 exert greater protection effect and synergistic effect with the phenolic compounds attached.
454 Thus, polar soluble phenolic compounds extracted from ungerminated yellow pea had higher
455 antioxidative activity than that from germinated yellow pea (2 and 4 days). Nevertheless, the
456 increased molecular weight of polar soluble bound phenolic compounds after 6 days of
457 germination (**Figure 5 and Table 3**) did not follow such speculation in a review of their
458 antioxidative activity in emulsions since it was still inferior to those extracted from the raw
459 yellow pea. This indicates that molecular weight of polar soluble bound phenolic compounds
460 is not a sole factor to regulate the antioxidative activity; the composition of phenolic
461 compounds also matters. Numerous studies have found that phenolic composition and
462 concentration are crucial to their antioxidative activity.[7–9] With the germination, the improved

antioxidative activities of soluble free phenolic compounds in yellow pea against SSO-in-water emulsions oxidation is accompanied by the increased amounts of crucial phenolic compounds such as pratensein (**2**), phloridzin (**4**), quercetin (**9**), sayanedine (**12**), hesperetin (**13**), glyzaglabrin (**14**), and pinocembrin (**15**) (in Cluster I, IV, and VI). It is plausible that the composition of phenolic compounds is also responsible for the antioxidative activity of polar soluble bound compounds in SSO-in-water emulsions. The decrease of pivotal phenolic compounds, e.g., pratensein (**2**) and sayanedine (**12**) in Cluster I, may be responsible for their attenuation of antioxidative activity. Such phenolic composition changes after 6 days germination in yellow pea overruns the protective or the dual antioxidant effect stemming from the increased molecular weight of moieties, which explains its relatively poorer antioxidative activity. The current research in conjunction with our previous findings indicate that both phenolic composition and the nature of the moieties phenolic compounds attached are critical to determine their antioxidative activity against lipid oxidation in emulsions.

4. Conclusion

With the 6 days of germination, soluble free phenolic compounds and polar soluble bound phenolic compounds extracted from yellow pea had an opposite variation of antioxidative activity in SSO-in-water emulsion systems. The phenolic compositions of both types of phenolic compounds are undoubtedly contributed to their antioxidative activity. Surprisingly, molecular weight is positively related to the antioxidative activity of polar soluble bound phenolic compounds. Synergistic effect between polar soluble bound phenolic compounds and their moieties is proposed to explain the variation of antioxidative activity following

germination. This research shed new light on the development of antioxidants with effectiveness, safety, and sustainability. Our future research would focus on the critical fractions of polar soluble bound phenolic compounds separated by SEC. With the composition and structure analysis of these fractions, mimic polar soluble bound phenolic compounds would be synthesized to verify the synergistic effect between phenolic compounds and their moieties.

Acknowledgment

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The authors declare no competing financial interest.

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554 Table 1 Phenolic compounds profiles of yellow pea extracts identified by LC-ESI-QTOF-MS

Peak No.	RT (min)	collision energy (ev)	observed *m/z* $[M-H]^-$	calculated *m/z* $[M-H]^-$	molecular formula	diff. (ppm)	product ions	proposed compounds	class
1	15.32	10	271.0619	271.0612	$C_{15}H_{12}O_5$	-2.70	109.0166, 125.0098	chalconaringenin	chalcone
2	16.03	25	299.0584	299.0561	$C_{16}H_{12}O_6$	-7.62	131.0348, 147.0276, 159.0263, 201.0320, 241.0616	pratensein	isoflavone
3	16.89	25	273.0777	273.0768	$C_{15}H_{14}O_5$	-3.16	109.0166, 121.0151, 137.0082	(2*s*,3*s*,4*r*)-3,4,4',7-tetrahydroxyflavan	flavan
4	17.31	10	435.1314	435.1297	$C_{21}H_{24}O_{10}$	-3.96	273.0768, 167.0350	phloridzin	chalcone
5	17.44	25	305.0676	305.0667	$C_{15}H_{14}O_7$	-3.17	110.9956, 125.0094, 281.0104	epigallocatechin	flavan
6	17.65	25	287.0577	287.0561	$C_{15}H_{12}O_6$	-5.6	107.0016, 121.0154, 150.9858	aromadendrin	flavanonol
7	18.80	30	303.0869	303.0874	$C_{16}H_{16}O_6$	1.85	161.0429, 217.0263, 245.0175	arachidoside	flavan
8	19.42	10	285.0413	285.0405	$C_{15}H_{10}O_6$	-4.71	108.0090, 117.0205, 131.0507, 227.0081	kaempferol	flavonol
9	21.16	10	301.0365	301.0354	$C_{15}H_{10}O_7$	-3.75	109.0174, 134.9938, 216.9664	quercetin	flavonol
10	22.56	10	137.0245	137.0244	$C_7H_6O_3$	-0.68	93.0346, 65.0397	*p*-salicylic acid	phenolic acid
11	23.40	10	372.5577	372.5562	$C_{20}H_{20}O_7$	-4.03	255.0656, 135.0081	tangeretin	flavone
12	24.78	30	297.0777	297.0768	$C_{17}H_{14}O_5$	-2.99	116.9952, 130.9928, 199.0765, 225.0545, 239.0354	sayanedine	isoflavone
13	26.90	10	301.0734	301.0718	$C_{16}H_{14}O_6$	-5.29	286.0483, 242.0585	hesperetin	flavanone
14	27.06	30	297.0395	297.0405	$C_{16}H_{10}O_6$	3.10	183.0129, 255.0294	glyzaglabrin	isoflavone
15	31.40	10	255.0671	255.0663	$C_{15}H_{12}O_4$	-2.62	213.0557	pinocembrin	flavanone
16	33.47	10	283.0633	283.0612	$C_{16}H_{12}O_5$	-7.38	268.0377	genkwanin	flavone

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556 RT, retention time; Diff., difference between calculated *m/z* and observed *m/z*.

Table 2 Dynamic changes of proposed phenolic compounds during yellow pea germination [a]

Peak No.	proposed compounds	class	YF0	YF2	YF4	YF6	YB0	YB2	YB4	YB6
1	**c**halconaringenin	chalcone	0.13±0.01 ab	0.61±0.04 c	1.44±0.24 d	0.49±0.05 bc	0.05±0.00 a	0.25±0.02 abc	0.57±0.01 c	1.42±0.15 d
2	pratensein	isoflavone	0.05±0.00 a	0.16±0.02 a	0.30±0.11 a	3.00±0.11 d	1.37±0.02 c	0.65±0.13 b	0.43±0.03 a	0.17±0.02 a
3	(2*S*,3*S*,4*R*)-3,4,4',7-tetrahydroxyflavan	flavan	0.01±0.00 a	0.22±0.06 a	1.32±0.28 b	2.22±0.58 b	N.A.	0.36±0.02 a	1.31±0.14 b	1.83±0.05 b
4	phloridzin	chalcone	0.01±0.00 a	0.14±0.01 a	1.88±0.56 b	3.42±0.26 b	0.01±0.00 a	0.01±0.00 a	0.12±0.02 a	0.14±0.00 a
5	epigallocatechin	flavan	N.A.	0.02±0.00 a	0.27±0.04 b	0.36±0.03 bc	1.11±0.09 e	0.65±0.12 d	0.51±0.01 cd	0.42±0.02 bc
6	aromadendrin	flavanonol	1.35±0.2 cd	1.33±0.05 cd	1.21±0.01 bcd	0.66±0.02 a	0.91±0.03 ab	1.07±0.09 bc	1.40±0.01 d	1.43±0.00 d
7	arachidoside	flavan	0.02±0.01 a	0.14±0.01 ab	0.36±0.04 c	0.59±0.00 d	0.83±0.05 e	0.96±0.08 e	0.86±0.10 e	0.3±0.04 bc
8	kaempferol	flavonol	0.94±0.03 b	0.12±0.00 a	0.01±0.00 a	0.01±0.01 a	2.55±0.05 e	1.86±0.06 d	1.36±0.17 c	0.74±0.03 b
9	quercetin	flavonol	0.10±0.00 a	0.49±0.04 c	0.68±0.03 d	1.09±0.04 e	0.11±0.01 a	0.11±0.02 a	0.22±0.02 b	0.30±0.01 b
10	*p*-salicylic acid	phenolic acid	1.59±0.08 d	0.11±0.02 a	0.04±0.01 a	0.04±0.01 a	0.60±0.01 b	0.83±0.01 c	0.56±0.04 b	0.54±0.03 b
11	tangeretin	flavone	0.01±0.01 a	0.02±0.00 a	0.03±0.00 a	0.04±0.01 a	0.68±0.05 c	0.29±0.08 ab	0.51±0.15 bc	0.60±0.13 c
12	sayanedine	isoflavone	N.A.	0.02±0.00 a	0.14±0.03 a	0.32±0.00 b	1.46±0.18 d	1.22±0.04 d	0.78±0.04 c	0.32±0.02 b
13	hesperetin	flavanone	0.05±0.00 a	2.83±0.10 b	7.89±1.09 c	12.86±0.79 d	0.01±0.00 a	0.01±0.00 a	0.05±0.00 a	0.06±0.00 a
14	glyzaglabrin	isoflavone	1.27±0.22 b	1.65±0.23 bc	2.07±0.31 c	3.74±0.20 d	0.01±0.00 a	N.A.	0.01±0.00 a	N.A.
15	pinocembrin	flavanone	0.41±0.02 b	0.36±0.09 b	0.82±0.04 c	3.22±0.06 d	0.01±0.00 a	N.A.	N.A.	N.A.
16	genkwanin	flavone	6.29±0.99 c	2.75±0.68 b	1.42±0.24 ab	0.14±0.03 a	N.A.	N.A.	N.A.	0.01±0.00 a

[a]YF and YB denote soluble free phenolic compounds and polar soluble bound phenolic compounds extracted from germinated yellow pea, respectively, followed by the different germination times (0, 2, 4, or 6 days). The amounts of phenolic compounds were expressed by absolute area of extracted ion chromatography (EIC) with the unit $\times 10^6$ counts; N.A., absolute area was below 10,000 counts. Different letters indicate statistically significant intraspecies differences ($p<0.05$).

564
565
566
567 Table 3 Molar mass of soluble bound phenolic compound extracted from germinated yellow pea [a]
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germination time (days)	Mn (g/mol)	Mw (g/mol)	Mw/Mn
0	6722±195 bc	7110±69 b	1.06
2	6095±117 b	6879±65 ab	1.13
4	5271±199 a	6266±14 a	1.19
6	7353±112 c	8574±546 c	1.17

569 [a]Data points represent means ± standard deviations. Different letters indicate statistically significant intraspecies differences (p<0.05)

Figure captions

Figure 1. Formation of lipid hydroperoxide (A) and hexanal (B) in SSO-in-water emulsion system, without (control) and with the addition of 200 μg GAE/g oil soluble free phenolic compounds extracted from yellow pea. Data points represent means ± standard deviations.

Figure 2. Formation of lipid hydroperoxide (A), hexanal (B), and hexanol (C) in SSO-in-water emulsion system, without (control) and with the addition of 200 μg GAE/g oil polar soluble bound phenolic compounds extracted from yellow pea. Data points represent means ± standard deviations.

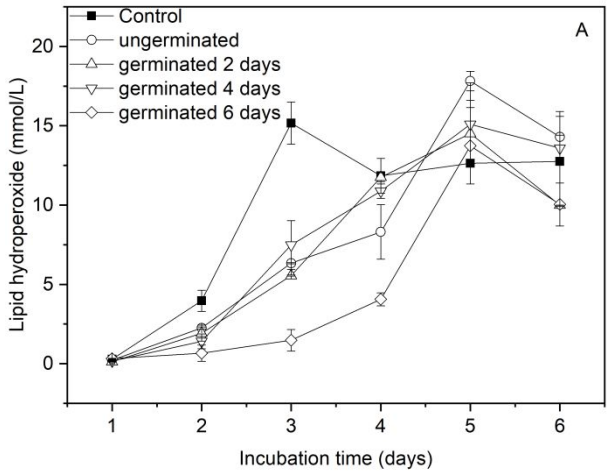
Figure 3. Constellation plot-cluster analysis (CPCA) representing the relation between eighteen phenolic compounds. Six clusters were separated based on the distance 3.939 with the different symbols: cluster I +, II ◇, III ○, IV Y, V △, and VI ×. Numbers indicate the different phenolic compounds: chalconaringenin (**1**), pratensein (**2**), (2*S*,3*S*,4*R*)-3,4,4',7-tetrahydroxyflavan (**3**), phloridzin (**4**), epigallocatechin (**5**), aromadendrin (**6**), arachidoside (**7**), kaempferol (**8**), quercetin (**9**), *p*-salicylic acid (**10**), tangeretin (**11**), sayanedine (**12**), hesperetin (**13**), glyzaglabrin (**14**), pinocembrin (**15**), genkwanin (**16**).

Figure 4. Principal component analysis (PCA) (A) score plot and (B) loading plot of phenolic compounds extracted from yellow pea during the period of germination. YF and YB denoting soluble free and polar soluble bound phenolic compounds after 0, 2, 4, and 6 days of germination; numbers indicate the different phenolic compounds: chalconaringenin (**1**), pratensein (**2**), (2*S*,3*S*,4*R*)-3,4,4',7-tetrahydroxyflavan (**3**), phloridzin (**4**), epigallocatechin (**5**), aromadendrin (**6**), arachidoside (**7**), kaempferol (**8**), quercetin (**9**), *p*-salicylic acid (**10**), tangeretin (**11**), sayanedine (**12**), hesperetin (**13**), glyzaglabrin (**14**), pinocembrin (**15**), genkwanin (**16**).

Figure 5. Physical characteristics of polar soluble bound phenolic compounds dynamically changed during yellow pea germination.

27

28 Figure 1



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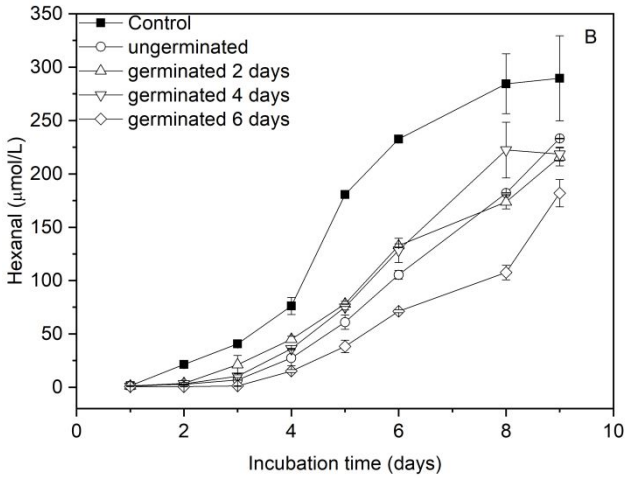

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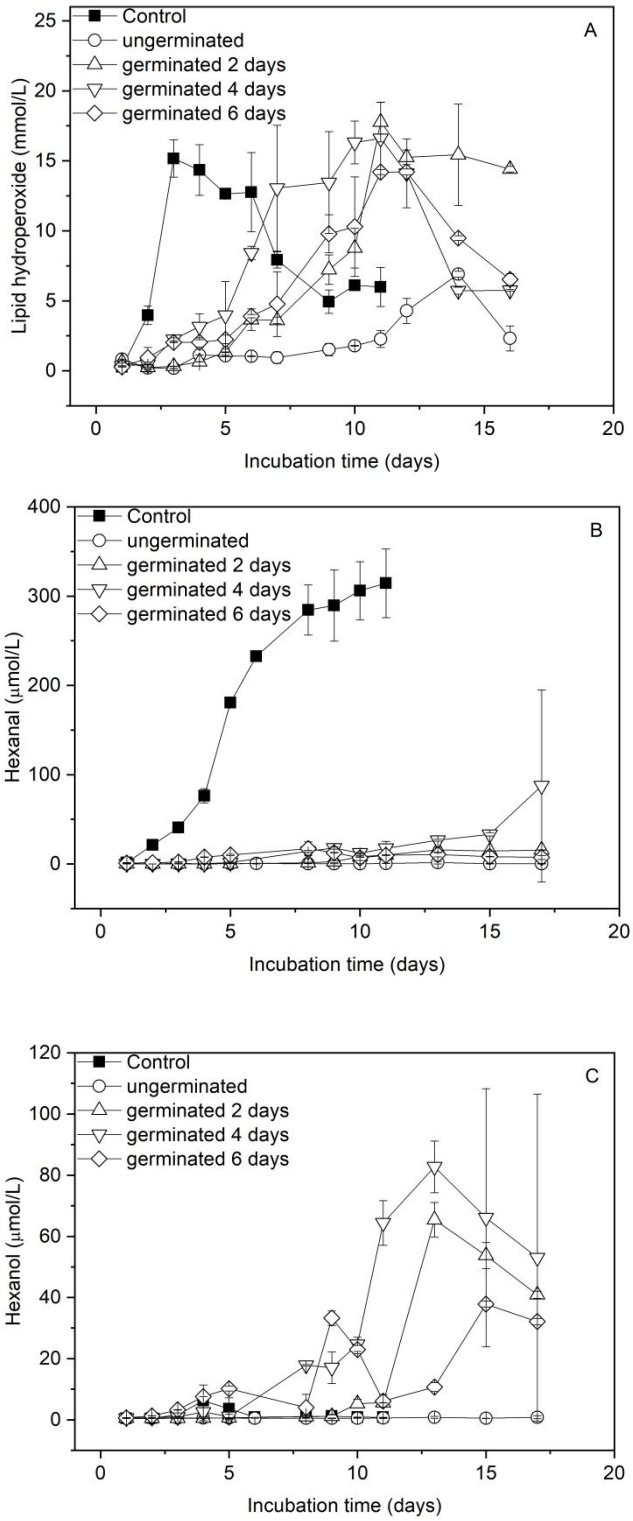
Figure 2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Control
ungerminated
germinated 2 days
germinated 4 days
germinated 6 days
Lipid hydroperoxide (mmol/L)
Incubation time (days)
B
Hexanal (μmol/L)
Incubation time (days)
C
Hexanol (μmol/L)
Incubation time (days)

Figure 3

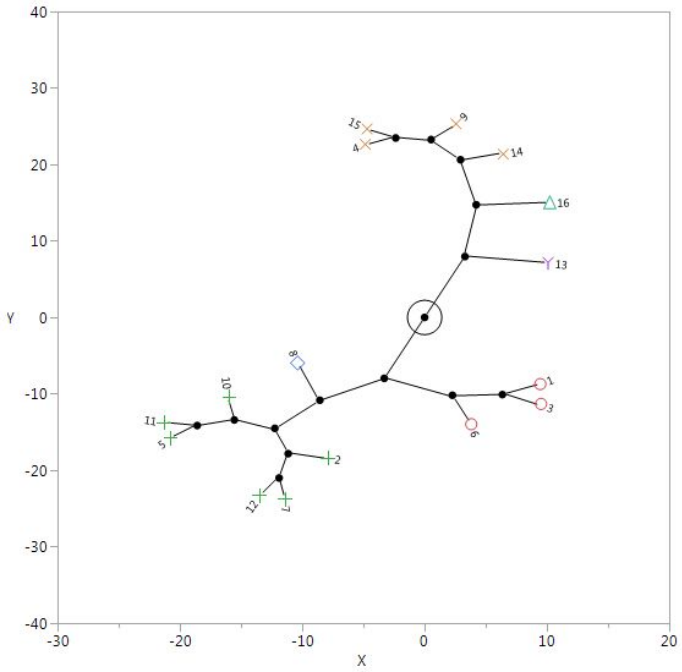
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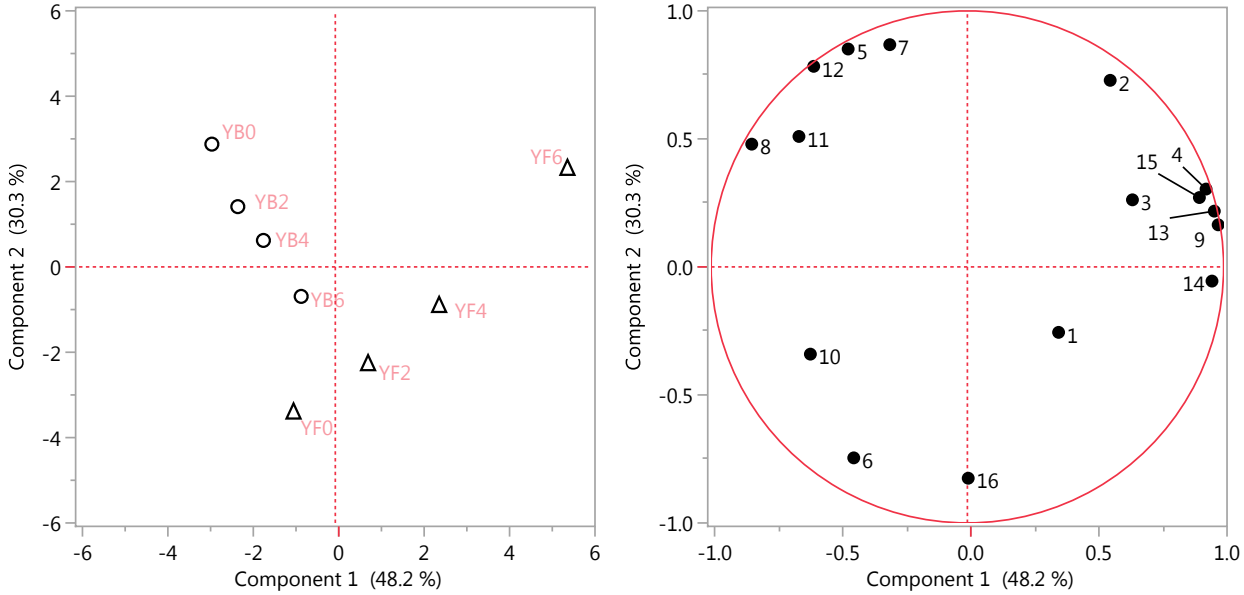
Figure 4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YB2
YB4
YB6
YF0
YF2
YF4
YF6
Component 2 (30.3 %)
Component 1 (48.2 %)
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2
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Component 2 (30.3 %)
Component 1 (48.2 %)

Figure 5

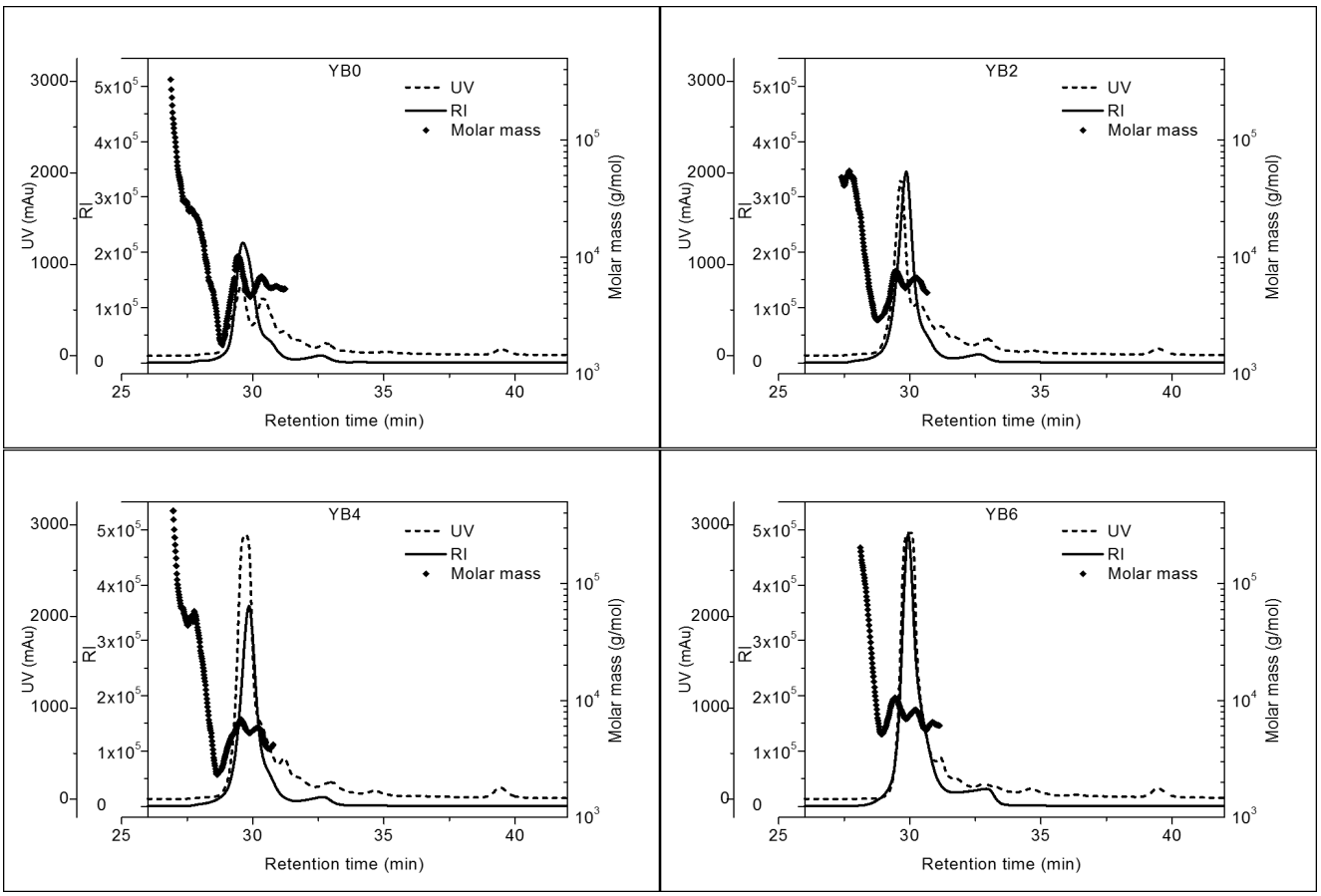
YB0
YB2
YB4
YB6
UV
RI
Molar mass
UV (mAu)
RI
Molar mass (g/mol)
Retention time (min)

Table of contents entry