Improvement of soybean product flavor and quality as affected by extraction of soybean oil bodies based on a soymilk model system

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ABSTRACT

Oil bodies (OBs) are lipid-storing organelles of plant seeds; they provide energy for seed germination and seedling growth by oxidative degradation as substrate. Soybean OBs are assumed to be involved in the enzymatic oxidation reaction of volatile flavor formation of soymilk. In order to establish a soymilk model system with soybean OBs as the enzymatic oxidation substrate to study the formation mechanism of soymilk flavor, the effects of extraction times and extraction pH on the composition, particle size and volatile flavor of soybean OBs extracted from raw soymilk were studied. The results showed that most of the extrinsic proteins on the surface of the OBs were removed with increasing extraction times. When the extraction pH increased from 7.0 to 11.0, the relative content of neutral lipids in OBs increased by 6.22%, and the relative content of protein and phospholipids in OBs decreased by 3.23% and 2.88%, respectively. The particle sizes of the OBs gradually decreased from 424 nm to 334 nm. The concentrations of volatile flavor components also gradually decreased from 129.29 μg/L to 52.75 μg/L. The integrity and stability of the OBs were compromised when the extraction pH was ≥12.0. The pH 11.0-OBs were the appropriate substrate for the soymilk model system because of their high purity and stability, better emulsion dispersion stability, and weaker flavor. These results are of great significance for exploring the formation mechanism of soymilk flavor in soymilk model systems to further improve the flavor of soymilk.

Introduction

Soybean is an important grain and oil crop. Soybean contains more than 20% oil contents, which are stored in soybean oil bodies (OBs). Soybean OBs have three main constituents: neutral lipids (mainly triacylglycerols), oleosins, and phospholipids. During soybean seed germination and seedling growth, lipids in OBs can be mobilized by phospholipase, lipase or lipoxygenase (LOX) and degraded to supply energy for their own growth. In the process of soybean grinding, large numbers of OBs are released from soybean seed cells and are present in the soymilk suspension. The beany flavor of soymilk is unacceptable to many western consumers. Numerous studies on the flavor of soybean products have confirmed that LOX is the key enzyme in the formation reaction of soymilk off-flavor. Moreover, multiple studies have focused on exploring the mechanism of volatile flavor generation in soybean products, and examining the use of free polyunsaturated fatty acids as the substrates and LOX, or its isoenzymes, as the catalysts. In addition, a large number of studies have been carried out to investigate the effects of soybean cultivars, cultivating field, storage time period, on the generation of off-flavor. However, studies using lipid storage organelles, i.e., soybean OBs, as the enzymatic substrate have not been reported.
Intact OBs can be regarded as a type of natural emulsified oil droplet, where the inner lipids are protected by the biofilm composed of a phospholipid monolayer and intrinsic protein molecules.\textsuperscript{[12]} The unique structure of the phospholipid layer of OBs promotes their dispersion in an aqueous phase and generates a natural oil-in-water (O/W) emulsification system.\textsuperscript{[1]} Therefore, OBs extracted from soybean seeds are used as a natural emulsifier and are widely applied in foods such as sauces, dressings, toppings, and beverages.\textsuperscript{[13,14]} As consumer demand for natural, healthy and vegetarian increases, animal-based emulsifiers such as milk casein and egg yolk are being replaced by plant-based OBs in the food industry.\textsuperscript{[15]} Therefore, OBs have significant market development potential in the food industry.

In OBs extraction, some protein components are normally absorbed on the surface of the OBs; however, the protein amount and composition of the extracted OBs have great effects on OBs properties, which affects the OBs utilization.\textsuperscript{[16]} Therefore, it is very important to reduce the protein adsorbed by OBs in OBs extraction. A certain purity of soybean OBs has been extracted from soymilk by using certain concentrations of urea,\textsuperscript{[17]} sodium chloride,\textsuperscript{[18]} or sucrose,\textsuperscript{[15]} combined with heating and adjusting the pH.\textsuperscript{[18,19]} However, some overtreatments, such as high concentrations of salt, excessive heating and higher alkaline pH values (pH > 11.0), can damage the structure of OBs or affect the stability of OBs suspension. To ensure the purity and stability of the extracted OBs, the selection of a suitable extraction method is key.

The objective of this study was to explore a suitable extraction method for intact, pure and low-flavor OBs, in order to provide an ideal enzymatic substrate for the soymilk model system and to study the formation mechanism of soymilk flavor. The effects of different extraction times and pH conditions on the structural properties of soybean OBs extracted by aqueous extraction with sucrose were studied. The particle size, lipids and volatile flavor components of the soybean OBs under different extraction pH conditions were analyzed by a Zetasizer Nano ZS analyzer, soxhlet extraction system and headspace-solid phase microextraction gas chromatography-mass spectrometry, respectively. Furthermore, the physical stability of the OBs emulsion was also investigated. The results of this study should aid the extraction and purification of other plant seed OBs in the future.
Materials and methods

Materials

Soybeans were obtained from the Nenjiang fumin Agricultural and Sideline Products Co., Ltd (Heihe, China). Sucrose of analytical purity was purchased from the Sinopac Chemical Reagent Co., Ltd (Zhenjiang, China). Analytically pure acrylamide, N,N'-methylene diacrylamide, bromophenol blue, β-mercaptoethanol, tricarboxylic aminomethane (Tris), glycine, tetramethylenediamine (TEMED), and Coomassie Brilliant Blue G-250 were purchased from Sigma (Shanghai, China). The protein markers (10–250 kDa) were purchased from Bio-Rad (Shanghai, China).

Preparation of raw soymilk

An aliquot of 50 g soybean was soaked in 300 mL deionized (DI) water and refrigerated at 4°C for 18 h (Figure 1). The soaked soybean was ground in DI water (precooled in 4°C, seed/DI water, 1/9, w/w) using a Waring blender MJ-60BE01B (Midea, China) for 2 min. The raw soymilk was harvested by filtering through four layers of gauze to remove all soybean particulate. Eighteen milliliters of the soymilk was immediately taken for the measurement of flavor volatile components according to the method described below, and the remaining soymilk was stored in an ice water bath for extracting OBs within 30 min.

Extraction of soybean oil bodies

Soybean OBs were extracted according to the method described by Zhao et al. The raw soymilk was divided into seven equal parts, according to the quality. Sucrose powder was added to each part at a concentration of 25% (w/w) and mixed well. The pH value of each part was simultaneously adjusted to 6.0, 7.0, 8.0, 9.0, 10.0, 11.0, or 12.0 with 0.1 and 0.01 M NaOH or HCl solutions. The mixtures were stirred evenly at 4°C for 15 min and centrifuged (19,000 g_{av}, for 30 min at 4°C) by a high-speed refrigerated centrifuge CR21N (Hitachi, Japan). The floating upper layer was collected and dispersed in DI water with the corresponding extraction pH value (OBs/DI water, 1/10, w/w) at 4°C. The procedure was repeated two more times. The floating fractions were collected and named pH 6.0-, 7.0-, 8.0-, 9.0-, 10.0-, 11.0-, and 12.0-OBs, respectively. One gram of the first and second extracted OBs at pH 7.0 was retained to study the effect of extraction times. OBs emulsions (10%, w/w) were prepared by mixing the OBs extracted above with DI water, and the pH of them was adjusted to 7.0 with 0.01 M NaOH and 0.1 M HCl. Then, the protein concentrations of the OBs emulsions were determined by the micro-Kjeldahl method. OBs emulsions were diluted appropriately, and equal volumes (0.5 mL) of OBs emulsions and SDS-PAGE sample buffer were mixed to achieve a protein concentration of 2.0 mg/mL to characterize the MW of the protein.

Observation of oil bodies

The pH 7.0-, 8.0-, 9.0-, 10.0-, and 11.0-OBs were diluted 100 times and 500 times with sodium phosphate buffer solutions (at the same pH as the extraction of OBs) and adjusted to their original pH values with 0.1 M and 0.01 M NaOH or HCl solutions for microscopic observation and particle size scanning. One drop of the diluted OBs emulsion was transferred onto the glass slide and covered with a coverslip. The glass slide was observed at a magnification of 400 × using optical microscopy CX31RTSF, (Olympus Corporation, Tokyo, Japan). Z-average diameter and size distribution by intensity were analyzed via dynamic light scattering measurements using a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK), with a milk module. The scanning time was 60 s, and the temperature was 25°C.
**Measurement of oil bodies stability**

Five milliliters of freshly extracted pH 7.0-, 9.0-, and 11.0-OBs emulsion (10%, w/w) were added to a 10 mL plastic centrifuge tube. The tube was capped and stored at room temperature, with restricted lighting for different time periods up to 7 days. The stabilities of the OBs emulsions were tested by observing their stratification or flocculation.

**Analysis of lipids in oil bodies extracted by organic solvents**

*Based on the method described by Cao et al.*[19] OBs were freeze-dried and weighed. The lyophilized OBs were extracted with acetone (1:10, w/v) at 4°C to remove neutral lipids. After stirring for 30 min, the homogenate was filtered through a layer of filter paper, and the residual was recovered. This extraction was repeated four more times. The filtered organic phase was pooled, concentrated at 45°C under vacuum and then vacuum dried at 60°C to a constant weight. The content of neutral lipids was calculated based on the weight.

The lyophilized OBs were transferred to a flat bottom-flask (total weight as \( m_1 \)) and mixed with chloroform–methanol solution (2:1, v/v) at 1:50 (w/v). The total lipids were extracted by refluxing extraction at 60°C for 8 h three times. Then, the pooled organic phases were filtered through a G3 sand funnel (total weight as \( m_2 \)). The filtrate was evaporated at 45°C in a vacuum rotary evaporator for organic solvent removal and then vacuum dried at 60°C to a constant weight. The content of phospholipids in the OBs was calculated as: phospholipids content = total lipids content – neutral lipids content. Subsequently, the flask and funnel used for extraction were dried at 105°C to constant weights as \( M_{101} \) and \( M_{102} \), respectively. The content of oleosins in the OBs was calculated as:

\[
\text{oleosin content} = \frac{\left( M_{101} + M_{102} \right) - \left( m_1 + m_2 \right)}{m_{OBs}} \times 100\% \quad (1)
\]

where \( m_{OBs} \) represents the weight of corresponding OBs.

**Analysis of volatile flavor components by gas chromatography-mass spectrometry**

The volatile flavor components were measured according to the method by Jung et al.[20] Briefly, the pH 7.0-, 8.0-, 9.0-, 10.0-, and 11.0-OBs were diluted 20 times with DI water and their pH values were adjusted to 7.0 (similar to the pH value of soymilk 6.8–7.0). The pHs-OBs emulsions were sealed and filled with nitrogen and stored in an ice water bath before measurement. The pH-OBs emulsion (4.5 mL) and DI water (1.5 mL) were added to a 15 mL glass headspace (HS) vial. The vial was fitted with a septum cap. Prior to HS-SPME analysis, 2-methyl-3-heptanone (1 μL of 0.5025 mg/mL in anhydrous methanol) internal standard was added via syringe. The CAR–PDMS SPME fiber (85 μm) was exposed to the HS above samples (6 mL) containing an internal standard in a 15 mL HS vial for 30 min at 40°C with stirring (magnetic stir bar, 120 r/min). SPME fibers were desorbed into a splitless injector at 260°C for 7 min on a combined 3800/1200 L GC-MS system (Varian Inc., USA) equipped with a DB-WAX column (30 m × 0.25 mm i.d., 0.25 μm df). The GC oven temperature program was as follows: 40°C (3 min hold), increase to 100°C at 6°C/min, increase to 230°C at 10°C/min (7 min hold). Scanned data acquisitions were made in the positive ionization (EI) mode over the mass range of 33–350m/z. The quantification of the volatile flavoring components was conducted using the method described by Yuan et al.[21] The standard curve was established by plotting response factors against the concentration of standard volatile compounds with DI water (6.0 mL) as the solvent.

**Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)**

Samples used for SDS-PAGE were prepared as described in the *Extraction of soybean oil bodies* section, and 10 μL of β-mercaptoethanol were added and heated in a boiling water bath for 3 min. The mixture was centrifuged at 12,000 g, for 10 min. Ten microliters of each sample supernatant was loaded into a sample well by microsyringe. SDS-PAGE was performed according to the method by Chen et al.[22]
with 5% stacking gel and 12.5% separating gel. SDS-PAGE was performed at 15 mA until completed.

After electrophoresis, the gel was fixed with a solution of 100 mM ammonium acetate dissolved in methyl alcohol/acetic acid (5/1, v/v) for 2 h. After fixing, gel was stained with 0.025% (w/v) Coomassie Blue G-250 in 10% (v/v) acetic acid for 2 h and destained by 10% (v/v) acetic acid.

**Statistical analysis**

All experiments and analyses were conducted in triplicate, and data are expressed as the mean value ± standard deviation (S.D.). Two-way analysis of variance (ANOVA) was performed by IBM-SPSS (ver. 20.0, IBM, USA), followed by assessment of differences by the least significance difference method (LSD) at \( P < .05 \).

**Results and discussion**

**Effect of extraction times on proteins of oil bodies**

Previous studies have shown that there are two types of integral proteins in soybean OBs, oleosin and caleosin. Oleosin is the major protein of OBs integral proteins and has three isoforms, with molecular weights of 24, 18, and 16 kDa. Caleosin is the minor integral protein, with a molecular weight of 30 kDa. The integral proteins were determined by SDS–PAGE. Nine bands were resolved by SDS–PAGE (Figure 2), which showed that in addition to intrinsic proteins, there were other soybean proteins present in OBs extracted at pH 7.0. The proteins adsorbed on the surface of extracted OBs are extrinsic proteins. Figure 2 shows that OBs extracted three times contained very few extrinsic proteins, and oleosin and caleosin were dominant. OBs extracted once and twice contained not only intrinsic but also extrinsic proteins (β-conglycinin (\( \alpha + \alpha’ \) and \( \beta \)), glycinin (acidic and basic peptides), γ-conglycinin, LOX). The same extrinsic proteins also existed in the OBs extracted by Fu et al. [24]

![Figure 2](image-url)  
**Figure 2.** SDS–PAGE pattern of proteins in oil bodies extracted at pH 7.0. Lane 1, markers; Lanes 2–3, protein of oil bodies obtained by extraction once, twice, and three times, respectively.
According to the electrophoresis pattern, it can be seen that multiple aqueous washes extractions removed most of the extrinsic proteins and increased the specific gravity of oleosins, indicating that there was a weak interaction between extrinsic proteins and OBs. LOX is the key enzyme that catalyzes the oxidation of lipids, and it can subsequently generate a large amount of volatile flavor components. Therefore, the elimination of LOX is necessary to improve flavor quality. From Figure 2, LOX in the extracted soybean OBs was completely removed after three extractions.

**Effect of extraction pH on proteins of oil bodies**

As shown in Figure 3, the protein bands resolved by SDS-PAGE gradually became lighter and smaller with increasing pH. Compared with the results of Chen et al.\(^{[22]}\) the OBs extracted in our study adsorbed more proteins at the same extraction pH. This was because the soymilk used in the study by Chen and Ono was heated, which resulted in the denaturation of some proteins and changes in the original characteristics of the soymilk. Compared with intrinsic proteins, the reduction of extrinsic proteins was more severe in our study, due to the weak binding force between extrinsic proteins and OBs. When the extraction pH was < 8.0, some extrinsic proteins remained on the OBs surface, in addition to the OBs integral proteins. When the pH was 6.0, OBs adsorbed more extrinsic proteins, which was inconsistent with the goal of our study to obtain pure OBs. When the extraction pH was > 8.0, LOX, β-conglycinin, γ-conglycinin, and glycinin were almost removed from the OBs surface, and most of the 16 kDa oleosin was released from the OBs. This phenomenon was consistent with the research results by Cao et al.\(^{[19]}\) The reason might be that the positively charged amino acid residues of N- and C-terminus were deprotonated by the alkaline pH, which decreased the number of salt bridges between the oleosins and phospholipids. As a result, part of the oleosin molecules could not securely attach to the OBs. The phenomena of demulsification and oil precipitation occurred in pH 12.0-OBs, indicating that the OBs were damaged under strong alkali conditions. At pH 11.0 extraction, most of the 30 kDa calceosin was also released from the OBs, and only the OBs integral oleosins remained on the OBs surface. It has been reported that half of α′ and α subunits of β-conglycinin were disulfide (SS) linked with 30 kDa calceosin, which might be the reason that some 30 kDa calceosin released from the integral OBs.\(^{[25]}\) These results revealed that high

![Figure 3. SDS–PAGE pattern of oil bodies proteins obtained after extracting three times at different pH values. Lane 1, marker; Lanes 2–7, protein of pH 6.0, 7.0, 8.0, 9.0, 10.0, and 11.0. OBs: oil bodies.](image-url)
alkaline pH extraction not only removed all contaminated proteins but also a portion of the intrinsic proteins.

**Effect of extraction pH on the lipids and proteins in oil bodies**

Intact OBs in soymilk are emulsified oil droplets with neutral lipids inside. They are surrounded by an outer layer of protein-phospholipid membrane.\(^{[12]}\) As shown in Table 1, the relative contents of neutral lipids increased from 85.56% to 91.78%, while the relative contents of phospholipid and protein decreased from 5.84% to 2.96% and from 8.41% to 5.18% (dry basis) respectively, with increasing extraction pH. The changes in relative protein contents are consistent with those shown in **Figure 3**. The relative content of proteins in our study was basically consistent with the data of Zhao et al.\(^{[16]}\) and Cao et al.\(^{[19]}\). The decrease of the relative proteins content in the extracted OBs was related to the removal of their extrinsic proteins. The reduction in phospholipid contents might be because the dissociation of both a large amount of extrinsic proteins and few oleosins from OBs under alkaline extraction conditions could alter the spherulitic structure of the phospholipids that integrate into the oleosins, effectively transporting some of the phospholipids out of the OBs. In addition, strong alkali conditions can disrupt the molecular structures of phospholipids, such as the hydrolysis of the polar head group, resulting in a reduction in the relative content of phospholipids. The relative content of neutral lipids increased with the decreased protein-phospholipid outer membrane of the OBs. According to Zhao et al.\(^{[16]}\) the changes in composition and amounts of extracted OBs at different pHs influenced their properties.

**Effect of extraction pH on the morphology and particle size of oil bodies**

Soybean seed OBs have smaller particle sizes with diameters of 0.2–0.5 μm, than those of other seeds, due to the lower oil and interfacial protein ratio.\(^{[22,23]}\) Particle size analysis revealed that the average particle sizes of pH 7.0-, 8.0-, 9.0-, 10.0-, and 11.0-OBs were 424, 392, 351, 340, and 334 nm, respectively. The particle size of the OBs decreased with increasing extraction pH, which was in agreement with the results by Zhao et al.\(^{[16]}\). In fact, the particle size of these extracted OBs was positively correlated with the amounts of extrinsic proteins of the OBs. By optical microscope, some regular, small, sphere-shaped, and intact oil droplets were observed in pH 7.0-, 9.0-, and 11.0-OBs emulsions (**Figure 4 (a-c)**). The observed diameter of oil droplets became smaller with increasing extraction pH, and there were few large oil droplets in pH 7.0-OBs emulsions, as shown in **Figure 4(a)**. The particle size distributions of pH 7.0-, 9.0-, and 11.0-OBs are shown in **Figure 4(d-f)**. All OBs showed a monomodal distribution, and the pH 7.0-OBs had a narrower distribution and shifted to a larger particle size compared with that of pH 9.0-OBs and pH 11.0-OBs. The data of particle size distribution, SDS-PAGE and microscopic morphology of pH 7.0-OBs suggested that slight aggregation of the pH 7.0-OBs occurred due to the noncovalent interactions or disulfide bond between storage proteins bound to OBs. In addition, no large-sized aggregation formed in pH 7.0-OBs due to the electrostatic repulsion and steric hindrance among oleosins.

| pH of extraction | proteins | neutral lipids | phospholipids |
|------------------|----------|----------------|---------------|
| 7                | 8.41 ± 0.25 \(^{a}\) | 85.56 ± 1.13 \(^{a}\) | 5.84 ± 0.17 \(^{a}\) |
| 8                | 7.12 ± 0.19 \(^{b}\) | 87.70 ± 1.05 \(^{b}\) | 5.01 ± 0.12 \(^{b}\) |
| 9                | 6.02 ± 0.09 \(^{c}\) | 89.28 ± 0.98 \(^{c}\) | 4.59 ± 0.11 \(^{b}\) |
| 10               | 5.54 ± 0.11 \(^{d}\) | 90.37 ± 1.17 \(^{c}\) | 3.93 ± 0.08 \(^{c}\) |
| 11               | 5.18 ± 0.10 \(^{d}\) | 91.78 ± 1.20 \(^{d}\) | 2.96 ± 0.09 \(^{d}\) |

\(^{1}\)Data are expressed as mean ± SD.

Different letters within each column represent significant differences (\(p < 0.05\)) in relative contents.
From the above results, the extraction pH greatly affected the protein composition of the extracted soybean OBs, which, in turn, greatly affects the OBs properties and utilization. Figure 4 (g) illustrates the physical stability of the pH 7.0-, 9.0-, and 11.0-OBs emulsions during storage. After two days of storage, stratification first appeared in the pH 7.0-OBs emulsion. The dispersion stability of the pH 11.0-OBs emulsion remained stable for four days. The emulsion storage experiment showed that the emulsion dispersion stability improved with increasing extraction pH. The same conclusion was obtained in the study of oxidation stability of pHs-OBs emulsion by Zhao et al.\textsuperscript{[16]} The reason may be that the endogenous enzymes contained in the extrinsic proteins of the pH 7.0-OBs had adverse effects on the stability of the OBs emulsion by hydrolysis or oxidation of the pH 7.0-OBs. The pH 11.0-OBs contained almost no extrinsic protein, mainly intrinsic oleosins, which had good hydrophilicity and emulsification.\textsuperscript{[26]} Therefore, the pH 11.0-OBs emulsion owned good stability.
Table 2. Contents of volatile components in soymilk and oil bodies extracted under different pH conditions (μg/L).

| volatile components | soymilk | pH 7 | pH 8 | pH 9 | pH 10 | pH 11 |
|---------------------|---------|------|------|------|-------|-------|
| pentanal            | 13.21 ± 0.50 a | 1.54 ± 0.07 b | 1.34 ± 0.04 b | 0.98 ± 0.03 c | 0.85 ± 0.11 c | 0.77 ± 0.04 c |
| 1-penten-3-one      | 4.71 ± 0.22 a  | 0.55 ± 0.02 b | 0.11 ± 0.01 c | 0.00 d   | 0.00 d  | 0.00 d  |
| hexanal             | 691.84 ± 9.56 a | 78.33 ± 2.49 b | 65.80 ± 2.46 b | 59.76 ± 2.47 b | 43.73 ± 2.87 c | 38.50 ± 2.48 c |
| (E)-2-pentenal      | 20.15 ± 2.24 a | 6.70 ± 0.32 b | 2.73 ± 0.33 c | 2.03 ± 0.21 c | 0.00 d  | 0.00 d  |
| heptanal            | 9.94 ± 0.37 a  | 2.20 ± 0.08 b | 1.50 ± 0.04 c | 1.36 ± 0.06 c | 1.06 ± 0.08 c | 0.98 ± 0.06 d |
| (E)-2-hexenal       | 76.96 ± 5.03 a | 8.50 ± 0.33 b | 6.04 ± 0.10 c | 3.13 ± 0.28 d | 1.99 ± 0.24 e | 1.88 ± 0.13 e |
| 2-pentyl-Furan      | 1.36 ± 0.12 a  | 0.55 ± 0.05 b | 0.52 ± 0.02 c | 0.51 ± 0.21 b | 0.45 ± 0.05 c | 0.42 ± 0.02 c |
| 3-octanone          | 44.42 ± 3.82 a | 1.54 ± 0.02 b | 0.74 ± 0.02 c | 0.48 ± 0.03 c | 0.10 ± 0.02 d | 0.00 d  |
| 1-pentanol          | 15.7 ± 0.41 a  | 2.57 ± 0.06 b | 1.24 ± 0.07 c | 0.90 ± 0.14 c | 0.33 ± 0.03 d | 0.28 ± 0.01 d |
| octanal             | 4.82 ± 0.62 a  | 1.28 ± 0.05 b | 1.04 ± 0.04 c | 0.67 ± 0.11 c | 0.40 ± 0.05 c | 0.33 ± 0.03 c |
| 1-octen-3-one       | 16.14 ± 0.29 a | 0.51 ± 0.02 b | 0.36 ± 0.03 c | 0.34 ± 0.04 c | 0.08 ± 0.01 d | 0.00 d  |
| (E)-2-heptenal      | 33.88 ± 1.40 a | 2.61 ± 0.13 b | 0.54 ± 0.04 c | 0.22 ± 0.04 c | 0.00 d  | 0.00 d  |
| 1-hexanol           | 314.55 ± 9.20 a | 5.82 ± 0.17 b | 1.53 ± 0.04 c | 1.16 ± 0.23 c | 0.53 ± 0.04 d | 0.51 ± 0.03 d |
| nonanal             | 11.52 ± 1.45 a | 4.24 ± 0.04 b | 4.10 ± 0.08 b | 4.06 ± 0.17 b | 3.58 ± 0.24 d | 3.47 ± 0.28 c |
| (E)-2-octenal       | 14.53 ± 0.89 a | 4.06 ± 0.04 b | 3.96 ± 0.04 b | 3.36 ± 0.28 b | 2.14 ± 0.20 c | 1.70 ± 0.21 c |
| 1-octen-3-ol        | 168.32 ± 3.23 a | 7.69 ± 0.05 b | 7.10 ± 0.06 b | 6.29 ± 0.21 c | 4.16 ± 0.52 d | 3.50 ± 0.27 d |
| (E)-2-nonenal       | 4.70 ± 0.37 a  | 0.57 ± 0.01 b | 0.52 ± 0.05 b | 0.52 ± 0.05 b | 0.44 ± 0.04 c | 0.40 ± 0.02 c |
| total               | 1446.53 ± 20.67 a | 129.29 ± 1.95 b | 99.22 ± 2.03 c | 85.81 ± 1.97 d | 59.89 ± 3.31 e | 52.75 ± 2.31 e |

1Data are expressed as mean ± SD.

Effect of extraction pH on volatile flavor components of oil bodies

Headspace solid-phase microextraction gas chromatography is a simple and accurate detection method. It is often used to analyze volatile components in various matrices, such as cheese, fruit juices and spices. A total of 17 volatile components were identified in pHs-OBs emulsions by the method described in the Analysis of volatile flavor components by gas chromatography-mass spectrometry section. As shown in Table 2, compared with the prepared soymilk, the concentration of a volatile flavor component measured in the pHs-OBs emulsions or the total concentration of the volatile flavor components measured in the pHs-OBs emulsions was less than that of the prepared soymilk. For example, the total concentration of volatile flavor components in the pH 7.0-OBs emulsion was only 8.93% that of soymilk. This indicated that no enzymatic oxidation to generate volatile components occurred in the extracted OBs due to the removal of its binding lipoxigenase. The volatile flavor components measured in the extracted OBs should be from the volatile flavor components generated by soymilk. So the flavor characteristics of pHs-OBs emulsions and soymilk had similarities. Hexanal was the most abundant component, which was consistent with the findings of Kobayashi et al. According to the conclusions of Yuan et al., aldehydes, alcohols, ketones and furans were the main contributors to soymilk flavor and flavor characteristics of soymilk were the result of a combination of many compounds, these were also confirmed in our current study. Table 2 shows that the concentration of volatile flavor components in the pHs-OBs emulsions gradually decreased with increasing extraction pH. Among the pH 7.0-, 8.0-, 9.0-, 10.0-, and 11.0-OBs emulsions, the concentration of volatile flavor components measured in the pH 7.0-OBs emulsion and pH 11.0-OBs emulsion were the highest and the lowest at 129.29 μg/L and 52.75 μg/L, respectively. The concentration of volatile components in the pH 7.0-OBs emulsion was only 76.54 μg/L more than that in the pH 11.0-OBs emulsion. Active enzymes that facilitate hydrolysis and oxidation of lipids in the pHs-OBs were not observed in the protein bands of the pHs-OBs, as shown in Figure 3, and the concentration of the volatile components measured in the pHs-OBs emulsion was very limited (Table 2). Thus, the difference in the concentration of the volatile components among the pH 7.0-, 8.0-, 9.0-, 10.0-, and 11.0-OBs emulsions was not caused by lipid oxidation reactions but by the adsorption of residual volatile flavor components of soymilk by OBs. Therefore, compared with the pH 7.0-, 8.0-, 9.0-, and 10.0-OBs, the protein of the pH 11.0-OBs was removed largely by high alkaline aqueous washing, and the concentration of adsorbed volatile flavor components was minimal. It can be seen that the concentration of
volatile components in the extracted OBs was positively correlated with the content of extrinsic protein combined with them.

Conclusion

In the extraction of OBs from soymilk without pH adjustments, increasing the extraction times was beneficial to the removal of extrinsic proteins from the extracted OBs. Increasing the extraction pH increased the purity of the extracted OBs and reduced the particle size and volatile components concentration. The pH 11.0-OBs emulsion (10%, w/w) had good dispersibility and remained stable at room temperature for 5 days. The pH 11.0-OBs contained only intrinsic protein, and the morphology and integrity of the OBs were maintained. These results indicate that the pH 11.0-OBs exhibited better stability and emulsification in the model system, which provides support for research on the formation mechanism of soymilk flavor. Thus, a simple and efficient extraction method that ensures the purity and stability of the extracted soybean OBs was confirmed herein. In the production and application of soybean OBs, the balance between extraction yield and the purity of OBs should be considered. This study also provides insight for OBs utilization and reducing off-flavors of soybean products.

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References

[1] Iwanaga, D.; Gray, D. A.; Fisk, I. D.; Decker, E. A.; Weiss, J.; McClements, D. J. Extraction and Characterization of Oil Bodies from Soy Beans: A Natural Source of Pre-Emulsified Soybean Oil. J. Agric. Food Chem. 2007, 55, 8711–8716. DOI: 10.1021/jf071008w.
[2] Schmidt, M. A.; Herman, E. M. Suppression of Soybean Oleosin Produces Micro-oil Bodies that Aggregate into Oil body/ER Complexes. Mol. Plant. 2008, 1, 910–924. DOI: 10.1093/mp/ssn049.
[3] Hildebrand, D. F.; Kito, M. Role of Lipoxigenases in Soybean Seed Protein Quality. J. Agric. Food Chem. 1984, 32, 815–819. DOI: 10.1021/jf00124a029.
[4] Nishiba, Y.; Furuta, S.; Hajika, M.; Irita, K.; Suda, I. Hexanal Accumulation and DETBA Value in Homogenate of Soybean Seeds Lacking Two or Three Lipoxigenase Isozymes. J. Agric. Food Chem. 1995, 43, 738–741. DOI: 10.1021/jf00051a033.
[5] Zhuang, H.; Hildebrand, D. F.; Andersen, R. A.; Hamilton-Kemp, T. R. Effects of Polysaturated Free Fatty Acids and Esterified Linoleoyl Derivatives on Oxygen Consumption and C6 Aldehyde Formation with Soybean Seed Homogenates. J. Agric. Food Chem. 2007, 55, 8711–8716. DOI: 10.1021/jf071008w.
[6] Kaneko, S.; Kumazawa, K.; Nishimura, O. Studies on the Key Aroma Compounds in Soy Milk Made from Three Different Soybean Cultivars. J. Agric. Food Chem. 2011, 59, 12204–12209. DOI: 10.1021/jf202942h.
[7] Shi, X.; Li, J.; Wang, S.; Zhang, L.; Qiu, L.; Han, T.; Wang, Q.; Chang, S. K.; Guo, S. Flavor Characteristic Analysis of Soymilk Prepared by Different Soybean Cultivars and Establishment of Evaluation Method of Soybean Cultivars Suitable for Soymilk Processing. Food Chem. 2015, 185, 422–429. DOI: 10.1016/j.foodchem.2015.04.011.
[8] Min, S.; Yu, Y.; Yoo, S.; Martin, S. S. Effect of Soybean Varieties and Growing Locations on the Flavor of Soymilk. J. Food Sci. 2005, 70, C1–C11. DOI: 10.1111/j.1365-2621.2005.tb09009.x.
[9] Achouri, A.; Boye, J. I.; Zamani, Y. Soybean Variety and Storage Effects on Soymilk Flavour and Quality. Int. J. Food Sci. Tech. 2008, 43, 82–90. DOI: 10.1111/j.1365-2621.2006.01393.x.
[10] Lv, Y.; Song, H.; Li, X.; Wu, L.; Guo, S. Influence of Blanching and Grinding Process with Hot Water on Beany and Non-Beany Flavor in Soymilk. J. Food Sci. 2011, 76, 520–525. DOI: 10.1111/j.1750-3841.2010.01947.x.
[11] Giri, S. K.; Mangaraj, S. Processing Influences on Composition and Quality Attributes of Soymilk and Its Powder. Food Eng. Rev. 2012, 4, 149–164. DOI: 10.1007/s12393-012-9053-0.
[12] Huang, A. H. Oil Bodies and Oleosins in Seeds. Annu. Rev. Plant Phys.. 1992, 43, 177–200. DOI: 10.1146/annurev. pp.43.060192.001141.
[13] Karkani, O. A.; Nenadis, N.; Nikiforidis, C. V.; Kiosseoglou, V. Effect of Recovery Methods on the Oxidative and Physical Stability of Oil Body Emulsions. Food Chem. 2013, 139, 640–648. DOI: 10.1016/j.foodchem.2012.12.055.

[14] Sukhnot, R.; Guo, S. W.; Xing, J. Y.; Hu, Q.; Wang, R. C.; Shi, X. D.; Nishinari, K.; Fang, Y. P.; Guo, S. T. Changes in Physicochemical Properties and Stability of Peanut Oil Body Emulsions by Applying Gum Arabic. LWT Food Sci. Technol. 2016, 68, 432–438. DOI: 10.1016/j.lwt.2015.12.055.

[15] Ishii, T.; Matsumiya, K.; Nambu, Y.; Samoto, M.; Yanagisawa, M.; Matsumura, Y. Interfacial and Emulsifying Properties of Crude and Purified Soybean Oil Bodies. Food Struct. 2017, 12, 64–72. DOI: 10.1016/j.foodstr.2016.12.005.

[16] Zhao, L.; Chen, Y.; Yan, Z.; Kong, X.; Hua, Y. Physicochemical and Rheological Properties and Oxidative Stability of Oil Bodies Recovered from Soybean Aqueous Extract at Different pHs. Food Hydrocolloid. 2016, 61, 685–694. DOI: 10.1016/j.foodhydrocolloid.2016.06.032.

[17] Chen, B.; McClements, D. J.; Gray, D. A.; Decker, E. A. Physical and Oxidative Stability of Pre-emulsified Oil Bodies Extracted from Soybeans. Food Chem. 2012, 132, 1514–1520. DOI: 10.1016/j.foodchem.2011.11.144.

[18] Wu, N.; Huang, X.; Yang, X.; Guo, J.; Zheng, E.; Yin, S.; Zhu, J.; Qi, J.; He, X.; Zhang, J. Stabilization of Soybean OBs Emulsions Using i-carrageenan: Effects of Salt, Thermal Treatment and Freeze-thaw Cycling. Food Hydrocolloid. 2012, 28, 110–120. DOI: 10.1016/j.foodhydrocolloid.2011.12.005.

[19] Cao, Y.; Zhao, L.; Ying, Y.; Kong, X.; Hua, Y.; Chen, Y. The Characterization of Soybean OBs Integral Oleosin Isoforms and the Effects of Alkaline pH on Them. Food Chem. 2015, 177, 288–294. DOI: 10.1016/j.foodchem.2015.01.052.

[20] Jung, D.; Ebeler, S. E. Headspace Solid-Phase Microextraction Method for the Study of the Volatility of Selected Flavor Compounds. J. Agric. Food Chem. 2013, 51, 200–205. DOI: 10.1021/jf020651+.  

[21] Yuan, S. H.; Chang, S. K. Selected Odor Compounds in Cooked Soymilk as Affected by Soybean Materials and Direct Steam Injection. J. Food Sci. 2007, 72, 481–486. DOI: 10.1111/j.1750-3841.2007.00461.x.

[22] Chen, Y.; Ono, T. Simple Extraction Method of Non-allergenic Intact Soybean Oil Bodies that are Thermally Stable in an Aqueous Medium. J. Agric. Food Chem. 2010, 58, 7402–7407. DOI: 10.1021/jf1006159.

[23] Lin, L.; Tai, S. S.; Peng, C.; Tzen, J. T. Stereolosin, a Sterol-binding Dehydrogenase in Seed Oil Bodies. Plant Physiol. 2002, 128, 1200–1211. DOI: 10.1104/pp.010928.

[24] Fu, L.; He, Z.; Zeng, M.; Qin, F.; Chen, J. Effects of Preheat Treatments on the Composition, Rheological Properties, and Physical Stability of Soybean Oil Bodies. J. Food Sci. 2020, 85, 3150–3159. DOI: 10.1111/1750-3841.15411.

[25] Wadahama, H.; Iwasaki, K.; Matussaki, M.; Nishizawa, K.; Ishimoto, M.; Arisaka, F.; Takagi, K.; Urade, R. Accumulation of β-conglycinin in Soybean Cotyledon through the Formation of Disulfide Bonds between α- and α-subunits. Plant Physiol. 2012, 158, 1395–1405. DOI: 10.1104/pp.111.189621.

[26] Chen, Y.; Zhao, L.; Cao, Y.; Kong, X.; Hua, Y. Oleosins (24 and 18 Kda) are Hydrolyzed Not Only in Extracted Soybean Oil Bodies but Also in Soybean Germination. J. Agric. Food Chem. 2014, 62, 956–965. DOI: 10.1021/jf405382w.

[27] Achiour, A.; Boye, J. I.; Zamani, Y. Identification of Volatile Compounds in Soymilk Using Solid-phase Microextraction-gas Chromatography. Food Chem. 2006, 99, 759–766. DOI: 10.1016/j.foodchem.2005.09.001.

[28] Kobayashi, A.; Tsuda, Y.; Hirata, N.; Kubota, K.; Kitamura, K. Aroma Constituents of soybean[G Lycine Max (L.) Merrill] Milk Lacking Lipoxygenase Isozymes. J. Agric. Food Chem. 1995, 43, 2449–2452. DOI: 10.1021/jf00057a025.

[29] Yuan, S.; Chang, S. K. Selected Odor Compounds in Soymilk as Affected by Chemical Composition and Lipoxygenases in Five Soybean Materials. J. Agric. Food Chem. 2007, 55, 426–431. DOI: 10.1021/jf062274x.

[30] Meynier, A.; Rampon, V.; Dalgallarrondo, M.; Genot, C. Hexanal and T-2-hexenal Form Covalent Bonds with Whey Proteins and Sodium Caseinate in Aqueous Solution. Int. Dairy J. 2004, 14, 681–690. DOI: 10.1016/j.idairyj.2004.01.003.