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The physiochemical and nutritional properties of high endosperm lipids rice mutants under artificially accelerated ageing

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ABSTRACT

High lipids in rice endosperm can lower the digestibility of rice starch. However, as lipids tend to oxidize and hydrolyze, whether the high lipids rice is easily deteriorated during storage is unknown. In this study, artificially accelerated ageing was adopted as a fast way to investigate the changes of endosperm lipids and the physiochemical properties of high lipids rice mutants (i.e. ALK3 and RS4) during storage. Ageing treatment did not affect total lipids content, but NSL and γ-oryzanol in aged ALK3 (2.30%, 113.6 μg/g) and RS4 (2.10%, 72.2 μg/g) significantly decreased. Aged ALK3 had a lower pH of cooking solution (6.92) and lower *Tp* (64.04 °C) of the second endothermic peak in DSC compared to untreated ALK3 (7.04, 65.86 °C, respectively), suggesting the hydrolysis of lipids and damage of amylose-lipids complex. After ageing treatment, all varieties had lower proportion of unsaturated fatty acid in NSL and lower breakdown, indicating an inferior lipids quality and rice eating and cooking quality. However, no more serious quality deterioration was observed in high lipids rice compared to common rice. The high-lipid mutants had better nutritional quality in terms of higher γ-oryzanol content and lower initial digestion rate (IRR_{20}) even after storage.

1. Introduction

Rice is the staple food for over half of the population in the world, and mostly consumed as white rice. During the milling of rice, most nutrients i.e. oryzanol, tocopherol, dietary fiber and etc., which are concentrated majorly in the aleurone layer and embryo, are removed (Goufo & Trindade, 2014; Lillioja, Neal, Tapsell, & Jacobs Jr, 2013). The propensity of consuming highly processed foods brings increasing incidence of diet-related chronic diseases such as diabetes, obesity, and cardiovascular events (Butardo & Sreenivasulu, 2016). High consumption of white rice is linked to high risk of type 2 diabetes due to its high glycemic index (Hu, Pan, Malik, & Sun, 2012).

In recent years, healthier rice with reduced digestibility has attracted great attention. Lipid is an important nutrient in rice which surrounds the starch granules and can interact with amylose upon starch gelatinization or occur in native starch granules (Zhang et al., 2019). Lipids play an important role in starch physiochemical properties, including nutritional quality, although is present in very low amounts (Amagliani, O'Regan, Kelly, & O'Mahony, 2016). The presence of starch lipids can also hinder the contact of starch granule with digestive enzymes, and hence reduce the digestibility of rice starch granules (Ye et al., 2018). As high lipids rice has high resistant starch content (Zhang et al., 2019), enhancing the levels of endosperm lipids is a potential way to reduce the glycemic index of white rice.

From farm to table, storage is an essential process of rice to take into account. During storage, a number of physical, chemical, biological changes occurs in rice, which is termed as ageing (Tran et al., 2005). Rice can be easily deteriorated when exposed to high temperature and humidity during storage due to the absence of external protective husk. Aged white or brown rice has an inferior quality compared to fresh rice, such as decreased breakdown value, harder and less sticky texture (Zhou, Robards, Helliwell, & Blanchard, 2002; Zhou, Yang, Su, & Bu,

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2016). Lipids hydrolysis and oxidation is thought to be one of the key factors for rice quality deterioration (Zhou, Wang, Si, Blanchard, & Strappe, 2015). For high lipids rice mutants, whether the dramatically high content of endosperm lipids makes them go rancidity and deterioration more easily than other varieties is important to explore. As the rice analyzed in previous studies had low lipids content in endosperm, changes in lipids and its interaction with starch during ageing may not be significant and has not attracted enough attention. Therefore, rice high-lipids mutants were used in this study, which could provide a better perspective on ageing.

Storage studies on rice are usually time consuming (at least 3–6 months), therefore, artificially accelerated ageing as a rapid and efficient method was widely adopted in recent years (Devraj, Natarajan, Ramachandran, Manicakam, & Saravanan, 2020). There are mainly two kinds of artificially accelerated ageing methods: i) short-time heat treatment; and ii) high temperature and high humidity (HT-HH) treatment. The former one uses heating method (i.e. microwave) to induce the oxidation of thiol groups, the formation of disulfide bonds, and adhesion strength between cells, so as to mimic the ageing process of rice (Zhong et al., 2020; Devraj et al., 2020). However, in this process, temperature inside the rice kernel can reach higher than 80 °C, during which enzymes, i.e. peroxidase, are inactivated, in hence it cannot help to mimic the lipid oxidation during natural storage (Zhong et al., 2020). The HT-HH method provides a relatively moderate way (40–60 °C, 70–100% humidity) to mimic the decrease in seed vigor and changes in physiochemical properties, since temperature and humidity are regarded as the most crucial environmental factors affecting rice quality during storage (Wang, Wang, Jing, & Zhang, 2012; Likitwattanasade & Hongsprabhas, 2010; Biao et al., 2019). HT-HH method is more suitable to mimic the changes of lipids during storage.

In this study, a HT-HH method (45 °C, 90% humidity) was adopted to mimic the extreme weather in southeast and southern of China, under which rice fast goes deterioration (Wang, Hu, Mariga, Cao, & Yang, 2018). In this way, the changes of endosperm lipids and their interaction with starch in high lipids rice mutants during storage can be fastly investigated. This information might be helpful to understand the influences of high endosperm lipids on deterioration of rice quality during storage and provide some indications on suitable shelf life.

2. Materials and methods

2.1. Materials

Two rice mutants with high endosperm lipids (ALK3 and RS4) and one rice variety with low endosperm lipids (GZ93) were obtained from R7954 by gamma irradiation (300 Gy), as previously described (Zhang et al., 2019). The varieties were grown in the experimental farm of Zhejiang University, Hangzhou, China (120.2E, 30.3N), in the summer of 2019. After harvested, the rice was dried and de-hulled using a paddy husker (Satake Co., Tokyo, Japan). Then brown rice was polished by removing the outer layer (15% of total weight) using a rice whitener machine (Satake Corp., Tokyo, Japan).

The artificially accelerated ageing procedure was performed using the method reported by Jin et al. (2018). Briefly, white rice of three varieties was divided into two portions, one was treated at 45 °C and 90% humidity for 14 days to artificially age, the other one was kept at 4 °C in dark and regarded as untreated control. After treatment, all samples were freeze dried. Then part of each sample was grinded into flour by a milling machine (FOSS CT140, Höganäs, Sweden) and passed through a 150 μm sieve. All samples were stored at 4 °C until used.

D-Methionine, pepsin (P6887), α-amylase (A3176), pancreatin (P1750, 4 × USP specifications), amyloglucosidase (10113), sodium methoxide solution were purchased from Sigma–Aldrich (St Louis, MO, USA). n-propanol, n-hexane, methanol, formic acid, acetic acid, dichloromethane were of HPLC grade. All other chemicals were of analytical grade. FAME standard mixture was purchased from Supelco (CRM18918, Bellafonte, PA, USA). γ-oryzanol standard was purchased from FUJIFILM Wako Pure Chemical Corp (156–02831, Tokyo, Japan).

2.2. Lipids extraction

Lipids of aged and untreated rice flour were extracted by a two-step procedure as previously described (Zhang et al., 2019). Briefly, the rice flour was soaked in petroleum ether (60–90 °C) for 2 h, then refluxed for 6 h using a Soxhlet extractor (Hanon SOX406, Shandong China). The petroleum ether extracts were collected as non-starch lipids (NSL). The residues after NSL-removed were dried under room temperature and further extracted using *n*-propanol:water (3:1 v/v) at 37 °C for 24 h with continuous shaking at 200 rpm to obtain starch lipids (SL). The extracts were dried by rotary evaporation and re-dissolved in 1000 μL *n*-hexane for further analysis.

2.3. Compositions of rice samples

For aged and untreated rice flour, apparent amylose content and total starch were measured following the methods reported by Sun et al. (2018). Lipids content was measured using the acid hydrolysis method as described by Zhang et al. (2019). Protein content was measured by Dumas (Thermo Quest NA 2100 Nitrogen and Protein Analyser, Interscience, Breda, the Netherlands) using a protein-to-nitrogen conversion factor of 6.25 with D-Methionine as a standard.

2.4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Protein of aged and untreated rice flour was extracted using the method reported by Ohno, Tomatsu, Toeda, Ohisa (2007). Rice flour of 1 g was added to 20 mL of 10 mM sodium hydroxide containing 1% sodium dodecyl sulfate (SDS). The mixture was shaken at 250 rpm at room temperature for 1 h, then centrifuged at 10000 g for 15 min. The protein content in the extract was determined using BCA assay kit (Thermo Fisher Scientific, USA), and diluted to proper concentration. Then 10 μL of each sample was mixed with 10 μL 2 × concentrated loading buffer, 2 μL 10 × concentrated reducing agent (Thermo Fisher Scientific, Massachusetts, USA) or without reducing agent and heated at 70 °C for 10 min. Mixture of 10 μL was loaded onto a 12% Bis-Tris gel and run in MOPS buffer at 120 V for ~60 min. The gel was stained with Coomassie Brilliant Blue R-250 for 1 h and then destained with washing buffer (10% ethanol and 7.5% acetic acid in Milli-Q water) overnight with mildly shaking.

2.5. Acidity of cooking solution

For aged and untreated rice flour, the pH of cooking solution was measured to indicate the fat acidity according to the method reported by Tran et al. (2005) with some modifications. Briefly, 0.1 g rice flour was weighed into a 50 mL tube, and 10 mL distilled water was added, then boiled for 30 min after vortex for 2 min to fully disperse the starch. After cooled to room temperature, the mixtures were centrifuged at 4000 g for 20 min, and the pH of the supernatant was measured.

2.6. Fatty acid profile

The fatty acids in NSL/SL were converted into fatty acid methyl esters (FAME) according to AOAC official method No. 969.33 with some modifications (Martínez-Padilla et al., 2020). The re-dissolved NSL/SL (as described in 2.2) was added to 2 mL 0.5 mol/L sodium methoxide, incubated at 50 °C for 10 min. Then 0.1 mL acetic acid, 5 mL saturated sodium chloride, 3 mL *n*-hexane was added. After vortex and standing, the mixture was separated into two phases, then the hexane phase FAME was transferred into GC vials and analyzed by GC-FID. GC-FID analysis was performed on a 7890A GC system (Agilent, Santa Clara, CA, USA)

equipped with flame ionization detector using an Agilent HP-5MS column (30 m × 0.25 mm × 0.25 μm). Helium was used as the carrier gas at a flow rate of 1 mL/min. The oven temperature program was: 50 °C for 2 min, 20 °C/min to 150 °C and hold for 2 min, 10 °C/min to 180 °C and hold for 12 min, 5 °C/min to 215 °C and hold for 8 min, 10 °C/min to 270 °C. The temperature of the flame ionization detector was 270 °C. An injection of 1 μL with a split ratio of 10:1 was used.

2.7. γ-oryzanol content

γ-oryzanol of aged and untreated rice flour was extracted following the method described by Insuan, Chariyakornkul, Rungrote, and Wongpoomchai (2017) with some modifications. Briefly, 0.5 g rice flour was soaked in 5 mL dichloromethane and vortexed for 2 h. The extraction was repeated for three times, then all extracts were combined, dried under nitrogen flow and redissolved in 500 μL methanol. The content of γ-oryzanol was measured by a HPLC system equipped with a column Vydac C18 201 TP (250 × 4.6 mm, AllTech) with a guard column (Calvo-Castro et al., 2019). The mobile phase was methanol containing 1% formic acid at a flow rate of 1 mL/min, the fluorescence detection wavelength was set at 320 nm. For each measurement, 20 μL samples was injected.

2.8. Differential scanning calorimetry (DSC)

The thermal properties of the aged and untreated rice flour were measured using a differential scanning calorimeter (DSC Q20, TA,USA) according to Sun et al. (2018). Rice flour of 2.5 mg was weighed into an aluminium pan, then 7.5 μL deionized water was added. The pan was hermetically sealed and kept at room temperature for 12 h for equilibration. The samples were scanned from 30 to 130 °C at a rate of 4 °C/min. A hermetically sealed empty pan was used as a reference. The onset temperature (*To*), peak gelatinization temperature (*Tp*), and enthalpy changes (*ΔH*) were calculated by the instrument's software.

2.9. Thermogravimetric analysis (TGA)

Thermogravimetric property was measured using a Mettler-Toledo TGA/SDTA851E (Lv et al., 2019). The operation was performed from 30 °C to 500 °C at a heating rate of 10 °C/min under nitrogen. A sample mass of 6 mg was used for tests. The first derivative of the TG curve was obtained using Origin 9 software (OriginLab Inc., Northampton, MA, USA).

2.10. Pasting properties

The pasting properties of the aged and untreated rice flour were determined by a Rapid Visco Analyzer (RVA 3, Perton. Ltd, Australia) according to the methods described by Zhang et al. (2019). Briefly, 3 g rice flour was mixed with 25 g distilled water in the RVA sample can. The RVA program was set as: holding at 50 °C for 1 min, heated to 95 °C in 3.8 min and holding for 2.5 min, cooled to 50 °C in 3.8 min and holding for 1.4 min. The RVA paddle speed was 960 rpm in the first 10 s, then kept at 160 rpm. The peak viscosity (PKV), hot paste viscosity (HPV), cool paste viscosity (CPV) and their derivative parameters breakdown (BD, = PV–HPV), setback (SB, = CPV–PV) were recorded from the Thermocline for Windows software (Version 1.2).

2.11. In vitro digestion study

2.11.1. In vitro digestion procedure

Digestion property was studied using a standard protocol as reported by Minekus et al. (2014) with some modifications. Two g of aged and untreated rice grains were boiled in excessive water (20 mL) for 30 min to ensure full gelatinization. After cooking, the rice was immediately homogenized for 10 s using an Ultra Turrax T25 IKA homogenizer to avoid any retrogradation. This time for homogenizing was determined by comparing the particle size to human chewed particle size (Chen, Capuano, & Stieger, 2020). After blending, 5 g crushed samples was digested through three phases (oral, gastric and intestinal phase). All solutions used were pre-incubated at 37 °C.

During the simulated intestinal phase, totally 8 time points (0, 10, 20, 30, 45, 60, 90, 120 min) was chosen for sampling. At each time point, 0.1 mL of digesta was taken out, mixed with 0.4 mL ethanol and vortexed to stop the reaction. After centrifugation at 4000 *g* for 10 min at 4 °C, the digested starch content in the supernatant was determined by glucose analysis.

2.11.2. Determination of the digested starch

For glucose analysis, supernatant was first hydrolyzed by amyloglucosidase at 37 °C for 1 h, then boiled to inactivate the enzyme activity. The samples were centrifuged at 4000 *g* for 15 min. Supernatant of 0.1 mL was combined with 0.5 mL GOPOD reagent (GOPOD FORMAT, Megazyme International, Ireland Ltd., Bray, Ireland) and incubated at 40 °C for 20 min. The absorbance of the samples was measured at 510 nm.

The percentage of the starch hydrolysis was calculated by the following equation:

$$SH(\%) = S_h / S_i = G_p / S_i \times 0.9 \quad (1)$$

Where SH is the percentage of starch hydrolysis, S_h is the amount of hydrolyzed starch at different time points in intestinal phase, S_i is the initial amount of starch in the samples, G_p is the amount of glucose released due to starch hydrolysis. A conversion factor of 0.9 was used based on the molecular weight ratio of a starch monomer to glucose.

The experiment data were fitted to a first order model, as previous reported by Goñi, Garcia-Alonso, and Saura-Calixto (1997):

$$C_t = C_0 + C_\infty (1\text{- } e^{-kt}) \quad (2)$$

Where C_t, C_0 and C_∞ are the percentage of digested starch at time t, 0 and infinite time, respectively, and k is a pseudo-first order rate constant. Box Lucas model as the nonlinear curve fit model in OriginPro 9 (OriginLab corporation, Northampton, MA, USA) was used for estimating k and C∞ value.

Initial digestion rate in the first 20 min (IRR_{20}) was calculated by the following equation, as described by Kan, Oliviero, Verkerk, Fogliano, and Capuano (2020) with some modifications:

$$IRR_{20} = (C_{20}–C_0) / 20 \quad (3)$$

where C_{20} and C_0 are the percentage of digested starch at 20 min and 0 min.

2.12. Statistic analysis

All tests were triplicates. One-way analysis of variance (ANOVA) was conducted by the Duncan factorial scheme (significance level at $P < 0.05$) on means with SPSS 26 (SPSS Inc., Chicago, IL, USA). Independent *t*-tests were used to test the differences of pasting parameters between the untreated and aged groups by SPSS 26 (SPSS Inc., Chicago, IL, USA).

3. Results and discussions

3.1. Proximal compositions of untreated and aged samples

The content of starch, protein and lipids was measured to investigate the effect of artificially accelerated ageing on the composition of rice (Table 1). GZ93 had significantly higher total starch content than ALK3 and RS4. ALK3 and RS4 had significantly higher amylose, total lipids, NSL content and protein content compared to GZ93. Ageing treatment caused no significant change in total starch nor amylose content for all three rice varieties. Total lipids content remained unchanged after

Table 1
Proximate composition of untreated and aged rice samples.[a].

	Total starch (%)	Amylose (%)	Total lipids (%)	Non-starch lipids (%)	Protein content (%)
AU	72.84 ± 0.84^{c}	52.42 ± 0.48^{a}	2.40 ± 0.06^{a}	1.54 ± 0.03^{a}	8.55 ± 0.01^{a}
AA	73.24 ± 0.92^{bc}	52.51 ± 1.74^{a}	2.30 ± 0.04^{ab}	1.24 ± 0.01^{c}	8.22 ± 0.01^{b}
RU	75.46 ± 0.15^{c}	43.51 ± 1.31^{b}	2.29 ± 0.07^{ab}	1.33 ± 0.00^{b}	8.71 ± 0.01^{a}
RA	75.16 ± 0.99^{bc}	46.49 ± 0.64^{b}	2.10 ± 0.10^{b}	1.26 ± 0.01^{c}	8.70 ± 0.03^{a}
GU	79.34 ± 0.05^{a}	18.70 ± 0.66^{c}	0.86 ± 0.09^{c}	0.50 ± 0.01^{d}	7.94 ± 0.06^{c}
GA	81.59 ± 1.37^{a}	17.50 ± 1.91^{c}	0.96 ± 0.09^{c}	0.51 ± 0.02^{d}	7.36 ± 0.09^{d}

[a] The data were expressed as means ± SD, n = 3. Different letters in the column indicate significant differences among different samples at the 0.05 level. Abbreviations: AU, ALK3 untreated samples; AA, ALK3 aged samples; RU, RS4 untreated samples; RA, RS4 aged samples; GU, GZ93 untreated samples; GA, GZ93 aged samples.

ageing in all rice varieties, while NSL of ALK3 and RS4 significantly decreased. For protein content, ALK3 and GZ93 had a lower content after ageing, while RS4 remained unchanged.

The changes in starch content were insignificant during storage, demonstrating agreement with previous studies (Zhou et al., 2002; Zhou et al., 2015). Lipids are generally stable when exist in the intact spherosomes in the cells, but high temperature during storage may destroy the lipid membrane and enable the hydrolysis by lipases (Zhou et al., 2002). Shin, Yoon, Rhee, and Kwon (1986) found lipids content of brown rice unchanged when stored at 5 °C for 12 months, but significantly decreased when stored at 35 °C. Regarding the lipids in brown rice were mostly NSL (Choudhury & Juliano, 1980), the significant decreases of NSL in two high lipids rice might be due to their higher NSL content compared to common rice GZ93 (Table 1). Similarly, Ahmad et al. (2017) found the NSL content slightly decreased in white rice after 31 days of storage at 45 or 60 °C. For GZ93, the total lipids and NSL content was low, and thus the changes after ageing can be neglected.

3.2. Proteins profile

Proteins are quite sensitive to ageing and can be responsible for the changes in the surface properties of starch granules (Zhou, Robards, Helliwell, & Blanchard, 2010). The shifts of the sub-groups of proteins can be investigated through SDS-PAGE (Thanathornvarakul, Anuntagool, & Tananuwong, 2016). All rice varieties had proteins of 14, 22–24, 32, 48 kDa (Fig. 1a). After ageing, the content of 22–24 kDa and 32 kDa proteins decreased, but proteins with large molecule weight (>80 kDa) increased. When reducing agent was added to proteins samples, all samples showed higher content of 22–24 kDa and 32 kDa proteins (Fig. 1b). The differences in the proteins between aged and untreated samples seem disappeared. What's more, the 48 kDa protein of all samples almost disappeared. The aged samples still showed higher content of proteins with large molecules (>80 kDa) than untreated samples, but the difference was not as evident as that in non-reduced samples (Fig. 1).

For rice grain proteins, generally the 14 kDa protein is prolamin, 22 kDa protein is a basic subunit of glutelin, 24 kDa protein is globulin, and 32 kDa protein is an acidic subunit of glutelin, while 48 kDa protein is formed by a basic subunit and an acidic subunit of glutelin through disulfide linkage (Ohno & Ohisa, 2005). The ageing treatment may induce aggregation of subunits of glutelin into larger molecules through disulfide linkage, and when reducing agent was present, those aggregated proteins were cleaved into smaller ones (Ohno & Ohisa, 2005). Actually, other proteins besides glutelin also aggregated during ageing process, such as proteins between 32 kDa and 48 kDa, which were faintly dyed in aged samples (Fig. 1a). Increased proportion of high molecular weight protein (>225 kDa) in stored rice was also observed by Thanathornvarakul et al. (2016). The three rice varieties seem to have similar changes in protein profiles after ageing (Fig. 1a), indicating that high

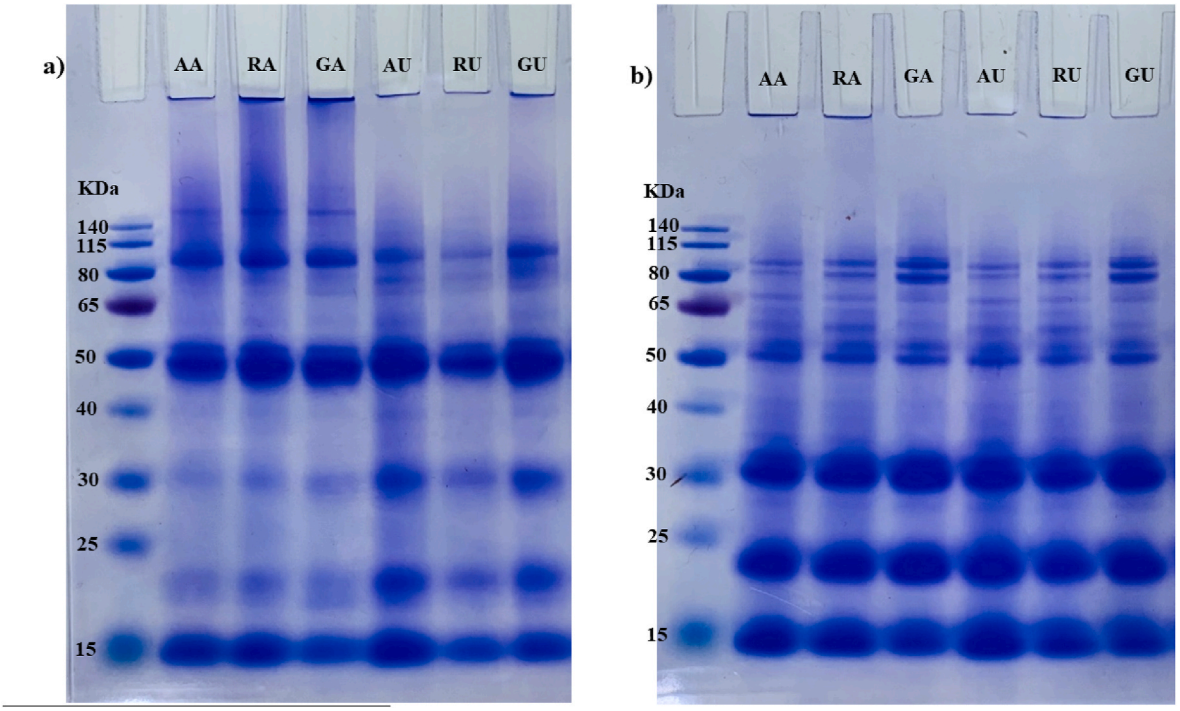

Fig. 1. SDS-PAGE patterns of alkaline extractable proteins from untreated and aged rice samples. (a) non-reducing agent buffer, (b) reducing agent buffer. Abbreviations: AU, ALK3 untreated samples; AA, ALK3 aged samples; RU, RS4 untreated samples; RA, RS4 aged samples; GU, GZ93 untreated samples; GA, GZ93 aged samples.

lipids rice mutants showed similar storage properties as common rice.

3.3. Fatty acid profile

During ageing, the changes in total fatty acid content can be reflected by pH of cooking solution (Tran et al., 2005). The pH of the cooking solution of aged ALK3 and RS4 was lower than that of the untreated samples, while GZ93 had no change (Table 2). The decrease of the pH of the cooking solution was linked to more fatty acid released by lipolysis of lipids (Tran et al., 2005). Park, Kim, Park, and Kim (2012) also observed higher fat acidity (lower pH) in the cooking solution of stored white rice.

Three rice varieties had similar fatty acid profile in NSL and SL (Fig. 2). In NSL, oleic acid had the largest proportion, followed by linoleic, palmitic, and stearic acids. In SL, linoleic and palmitic acids were the most two abundant fatty acids, followed by oleic, stearic and myristic acids. The ratio of UFA: SFA is an indicator for the nutritional value of the lipids (Zhang, Jin, et al., 2020). As shown in Table 2, the ratio of UFA:SFA in NSL was about two times higher than that in SL for all three rice varieties, in agreement with the results reported by Zhou, Blanchard, Helliwell, and Robards (2003).

Lipid peroxidation is considered as the main reason for deterioration of rice grains during storage, and the changes in fatty acid profile were usually observed during storage (Zhou et al., 2015). After ageing treatment, significant decreases in oleic, linoleic and linolenic acids were observed in NSL, while no significant changes were found in SL for all three rice varieties (Supplementary Table S1). This lead to a significant decrease in UFA:SFA ratio of NSL, while no changes in UFA:SFA ratio of SL (Table 2). These results suggested that SL was more stable than NSL, which are consistent with the results reported by Zhou et al. (2003). A lower ratio of UFA:SFA after rice storage was also reported by another study (Zhou et al., 2002), which found that oleic and linoleic acids in brown rice decreased after storage. In white rice stored at 37 °C for 4 and 7 months, a decrease of linoleic acid was also observed (Zhou et al., 2003). The decreased UFA:SFA ratio in NSL after ageing treatment for all rice varieties indicated the oxidation of unsaturated free fatty acid during ageing. However, after ageing treatment, ALK3 had a similar UFA:SFA ratio in NSL to that of GZ93, while RS4 had a slightly lower ratio, indicating the high lipids in high-lipids rice mutant did not lead to an excessive oxidation of lipids.

3.4. γ-oryzanol content

γ-oryzanol is a bioactive compound especially abundant in rice compared to other cereals, and is a potential antioxidant to screen UV radiation and to lower cholesterol in humans (Truong et al., 2017). As shown in Table 2, ALK3 and RS4 had significantly higher γ-oryzanol content (130.5 and 82.9 μg/g, respectively) compared to GZ93 (3.0 μg/g). After ageing treatment, γ-oryzanol content in ALK3 and RS4 significantly decreased, but was still 44 and 28 folds of that in GZ93, respectively.

γ-oryzanol is abundant in brown rice, but little in white rice. γ-oryzanol in brown rice was reported varying from 163 to 979 μg/g in different rice varieties, while in white rice it ranges from 6.11 to 120.7 μg/g (Goufo & Trindade, 2014). Compared to these reported values, the γ-oryzanol content in GZ93 white rice was quite low, while the content of γ-oryzanol in ALK3 and RS4 white rice was comparable to that in normal brown rice, even after ageing treatment, indicating a high nutritional quality. γ-oryzanol content was reported correlating to NSL content (Bergman & Xu, 2003). The decrease of γ-oryzanol in ALK3 and RS4 was in line with the decrease of NSL in the ageing process. The high content of γ-oryzanol in ALK3 and RS4 may also be a protective agent for lipids rancidity (Minatel et al., 2014), which can help to explain why neither ALK3 nor RS4 had a sharp decrease of UFA:SFA ratio in NSL compared to that of GZ93.

Regarding there was still significant decrease of γ-oryzanol in the two high lipids rice varieties after ageing, methods i.e. infrared radiation treatment or nano packaging can be used during storage to reduce the ageing of high lipids rice and get full use of the nutrients (Ding et al., 2015; Wang, Hu, Mariga, Cao, & Yang, 2018).

Table 2
Cooking solution acidity and lipid composition of untreated and aged rice samples.[a].

	pH of cooking solution	UFA: SFA in NSL	UFA: SFA in SL	γ-oryzanol (μg/g)
AU	7.04 ± 0.03[a]	4.1 ± 0.1[b]	1.4 ± 0.0[a]	130.5 ± 3.9[a]
AA	6.92 ± 0.02[c]	3.7 ± 0.0[c]	1.4 ± 0.1[a]	113.6 ± 2.4[b]
RU	7.02 ± 0.01[ab]	3.7 ± 0.1[c]	1.1 ± 0.0[b]	82.9 ± 1.2[c]
RA	6.98 ± 0.01[b]	3.3 ± 0.0[d]	1.2 ± 0.1[b]	72.2 ± 0.2[d]
GU	6.70 ± 0.03[d]	4.2 ± 0.1[a]	0.9 ± 0.0[c]	3.0 ± 0.1[e]
GA	6.74 ± 0.01[d]	3.6 ± 0.0[c]	1.0 ± 0.0[c]	2.6 ± 0.2[e]

[a] The data were expressed as means ± SD, n = 3. Different letters in the column indicate significant differences among different samples at the 0.05 level.
Abbreviations: AU, ALK3 untreated samples; AA, ALK3 aged samples; RU, RS4 untreated samples; RA, RS4 aged samples; GU, GZ93 untreated samples; GA, GZ93 aged samples; UFA:SFA, ratio of unsaturated fatty acid to saturated fatty acid; NSL, non-starch lipids; SL, starch lipids.

3.5. Thermal properties

DSC has been widely used to investigate the amount and heat stability of starch crystalline through enthalpy and temperatures for melting the starch crystalline structure (Ding, Zhang, Tan, Fu, & Huang, 2019). In this study, DSC was measured to study the interactions of lipids and amylose during ageing (Table 3). All three rice varieties had two typical endothermic peaks: the *Tp* of the first endothermic peak was at about 64–70 °C, the *Tp* of the second endothermic peak was at about 96–98 °C. The biphasic endotherm of rice flour with high lipids content was also reported by Zhang et al. (2019). The first endothermic peak indicates the energy required for melting of starch crystallites, while the second endotherm peak represents the consecutive melting of amylose-lipids complexes (Putseys, Lamberts, & Delcour, 2010). ALK3, which had the highest amylose and lipids content, showed the lowest first peak enthalpy and the highest second peak enthalpy. The existence of high lipids caused inadequate gelatinization of starch crystallites, thus the first peak did not completely represent the starch gelatinization (Zhang et al., 2019). The higher enthalpy of the second peak suggested a larger amount of lipids-starch complexes.

After ageing treatment, the enthalpy of the first peak in all rice varieties decreased (Table 3). This indicated that ageing may affect starch structure, thus less energy was needed for melting starch crystallites. The $\triangle H$ of the second peak in aged samples showed no differences from that of the untreated samples, suggesting no changes in the amount of lipids-starch complex during ageing treatment. However, there was a minor decrease in *To* and *Tp* of the second peak for ALK3, which meant the thermal stability of lipids-starch complex decreased. This may be due to the changes in NSL content and profile.

The effect of ageing on thermal properties of rice varied among different studies. Keawpeng and Venkatachalam (2015) and Zhou et al. (2010) observed *Tp* increased in white rice after storage, while Teo, Karim, Cheah, Norziah, and Seow (2000) found there were no changes in DSC parameters in rice flour before and after storage. Gu et al. (2019) found decreased $\triangle H$ in white rice after storage, which is consistent with results of present study. Lower $\triangle H$ meant less energy was needed for aged rice to gelatinize, which may because of the molecular degradation of starch (Gu et al., 2019). ALK3 seemed more sensitive to ageing, as the transition temperatures of two endothermal peaks all decreased after ageing (Table 3). The changes in the second endothermal peak of ALK3 suggested that lipids were involved in the gelatinization properties of rice during ageing.

TGA is also widely used to study thermal stability and decomposition

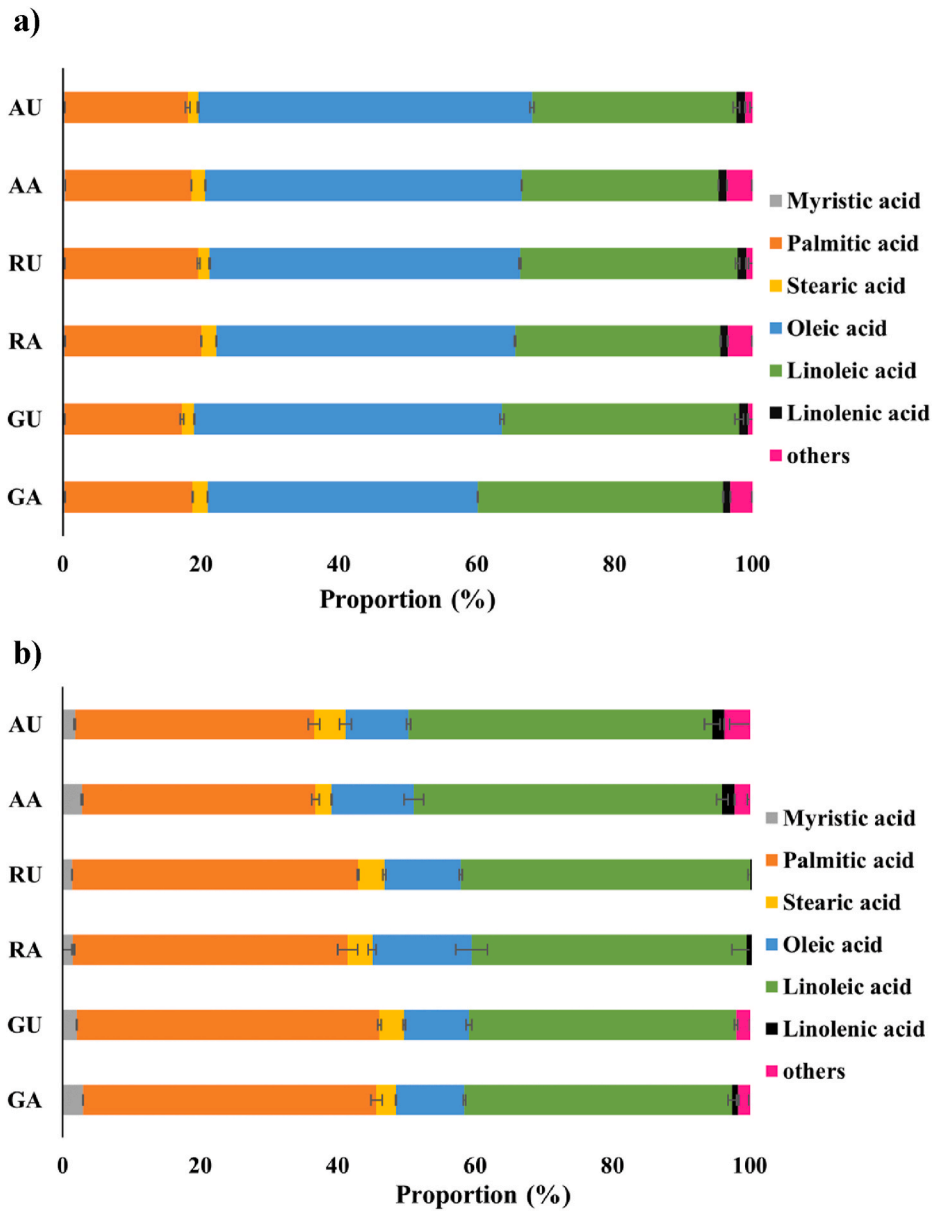

Fig. 2. Fatty acid profile of the untreated and aged samples in (a) NSL and (b) SL. Abbreviations: AU, ALK3 untreated samples; AA, ALK3 aged samples; RU, RS4 untreated samples; RA, RS4 aged samples; GU, GZ93 untreated samples; GA, GZ93 aged samples. Error bars represent standard deviation of the mean for triplicate tests.

of polysaccharides. The TG and DTG curves are shown in Fig. S1. During the thermal degradation process, three distinct weight loss process were shown on the curves. The first stage was mainly caused by the loss of residual water. The second mass loss represented the thermal decomposition of the starch, and was observed as a sharp and well-defined peak in the DTG curve (Fig. S1). The third weight loss corresponded to the oxidation of organic matter (Lv et al., 2019). The degradation behavior of untreated and aged rice flour had no obvious difference in TGA results, suggesting no changes in residual water content or starch structure. This result suggests that DSC was more sensitive to the changes in lipids and starch during rice ageing compared to TGA.

3.6. *Paste viscosity*

Pasting properties are regarded as one of the most sensitive indices to evaluate the physiochemical changes of rice during ageing process (Zhou et al., 2015). The pasting properties of three studied rice varieties were shown in Table 4. ALK3 and RS4 had similar pasting properties, while GZ93 had a much higher viscosity compared to ALK3 and RS4, which is consistent with a previous study (Zhang et al., 2019). After ageing treatment, peak viscosity of all three rice varieties decreased, while cold paste viscosity and setback of ALK3 increased. Breakdown viscosity, the widely used parameter in ageing studies, decreased in all three rice varieties after ageing treatment. This indicates the capacity of the starch granules to rupture after cooking was significantly reduced by ageing of the starch granules (Katekhong & Charoenrein, 2012; Zhou et al., 2003). The changes in pasting properties after ageing do not have consistent results among different studies. Lots of studies found that aged rice had higher final viscosity and setback, while lower breakdown compared to fresh rice (Keawpeng & Venkatachalam, 2015; Sowbhagya & Bhattacharya, 2001). This may be due to high proportions of short amylopectin chains produced in ageing process (Wu, Li, Bai, Yu, & Zhang, 2019). Haydon and Siebenmorgen (2017) observed an increase in breakdown and decrease in setback of brown rice after storage, which suggest the effect of ageing on pasting properties is depending of varietal different.

Table 3
Thermal properties of untreated and aged rice samples.[a].

	First endotherm peak			Second endotherm peak		
	To (°C)	*Tp* (°C)	△*H* (J/g)	*To* (°C)	*Tp* (°C)	△*H* (J/g)
AU	58.60 ± 0.27^{bc}	65.86 ± 0.18^{b}	1.82 ± 0.03^{d}	88.78 ± 0.07^{ab}	97.32 ± 0.01^{a}	4.42 ± 0.03^{a}
AA	55.38 ± 3.72^{c}	64.04 ± 0.96^{a}	1.22 ± 0.10^{e}	87.36 ± 0.34^{c}	96.39 ± 0.20^{b}	4.68 ± 0.11^{a}
RU	59.21 ± 0.24^{abc}	66.52 ± 0.20^{b}	2.42 ± 0.06^{c}	87.81 ± 0.10^{bc}	97.50 ± 0.09^{a}	3.70 ± 0.01^{b}
RA	56.31 ± 0.09^{c}	66.15 ± 0.27^{b}	1.80 ± 0.24^{d}	87.97 ± 0.41^{bc}	97.44 ± 0.11^{a}	3.43 ± 0.02^{b}
GU	64.48 ± 0.12^{a}	70.01 ± 0.23^{a}	6.62 ± 0.09^{a}	89.03 ± 0.46^{ab}	97.57 ± 0.23^{a}	1.19 ± 0.15^{c}
GA	62.45 ± 0.18^{ab}	69.19 ± 0.09^{a}	5.76 ± 0.12^{b}	89.97 ± 0.49^{a}	97.37 ± 0.23^{a}	1.16 ± 0.11^{c}

[a] The data were expressed as means ± SD, n = 3. Different letters in the column indicate significant differences among different samples at the 0.05 level.
Abbreviations: AU, ALK3 untreated samples; AA, ALK3 aged samples; RU, RS4 untreated samples; RA, RS4 aged samples; GU, GZ93 untreated samples; GA, GZ93 aged samples; *To*, onset temperature; *Tp*, peak temperature; △*H*, Enthalpy changes.

Table 4
Pasting properties of untreated and aged rice samples.[a].

	PKV (10^{-3} Pa · s)	HPV (10^{-3} Pa · s)	CPV (10^{-3} Pa · s)	SB (10^{-3} Pa · s)	BD (10^{-3} Pa · s)
AU	87 ± 2	74 ± 2	220 ± 5	146 ± 3	13 ± 0
AA	75 ± 1*	67 ± 2	264 ± 3*	197 ± 2*	9 ± 1*
RU	122 ± 2	104 ± 2	230 ± 3	126 ± 1	18 ± 1
RA	69 ± 1*	63 ± 1*	182 ± 2*	120 ± 2	7 ± 1*
GU	5246 ± 34	2836 ± 54	4669 ± 57	1833 ± 3	2411 ± 20
GA	4328 ± 43*	2159 ± 39*	3872 ± 27*	1713 ± 12	2169 ± 4*

[a] The data were expressed as means ± SD, n = 3. "*" indicates there were significant differences between the aged and untreated sample at the 0.05 level.
Abbreviations: AU, ALK3 untreated samples; AA, ALK3 aged samples; RU, RS4 untreated samples; RA, RS4 aged samples; GU, GZ93 untreated samples; GA, GZ93 aged samples; PKV, peak viscosity; HPV, hot paste viscosity; CPV, cool paste viscosity; SB, setback; BD, breakdown.

However, the oxidation of protein and lipids during ageing may affect the maximum viscosity of the amylogram and cause a decrease in starch viscosity (Park et al., 2012; Shi, Zhang, Wang, & Wu, 2021; Teo et al., 2000). During the oxidation process, the cross-linking between protein/lipids and amylose is strengthened, providing the granule a rigidity or strength and thereby restricting starch swelling (Shi et al., 2021). The lower peak viscosity and breakdown of aged rice in this study (Table 4) might be due to greater integrity of the starch granules in aged rice, and thus more resistant to swelling and harder to rupture. Also the aggregation of proteins during ageing (Fig. 1) may prevent the starch granules from swelling and breaking and resulted in lower paste viscosity (Table 4) (Sowbhagya & Bhattacharya, 2001). Breakdown value indicates the gel consistency, and is positively correlated with eating and cooking quality (ECQ) of rice (Pang et al., 2016). The lower breakdown of all three rice varieties after ageing treatment indicated an inferior ECQ of aged rice.

3.7. Digestion properties

Digestion data of all samples well fitted by a first-order equation ($R^2 > 0.95$). GZ93 had the highest digestion rate and extent as suggested by the highest C_∞, k and IRR_{20} value. RS4 had the intermediate digestion rate and extent, while ALK3 had the lowest (Fig. 3). No difference in digestion properties was shown between the untreated and aged samples for all three rice varieties. This indicates the ageing process did not significantly affect the digestion properties of the studied rice varieties. After ageing treatment, the high lipids varieties ALK3 and RS4 had similar digestion extent (C_∞), but significantly lower initial digestion rate (IRR_{20}) compared to GZ93.

Amylose content was regarded as the determinant of rice cooking and processing behavior (Li, Prakash, Nicholson, Fitzgerald, & Gilbert, 2016). Amylose content is negatively correlated to digestion rate, as it increases the chain packing density of starch structure and thus inhibits enzyme hydrolysis (Li et al., 2020). Amylose is also able to form complex with endosperm lipids and enhance the resistance to digestive enzymes (Zhang, Jin, et al., 2020). This is consistent with present study that low-amylose variety GZ93 had the highest digestion rate, while the high-lipids and high-amylose variety ALK3 and RS4 had lower digestion rate (Table 1, Fig. 3).

Azizi et al. (2019) found the changes in peptide subunit compositions could result in a reduced starch digestibility of rice stored for 6 months but this reduction was dependent on varieties. Zhou et al. (2016) found white rice stored at 37 °C had lower digestion rate and extent compared to rice stored at 4 °C, due to changes in grain structures during storage. However, Tulyathan and Leeharatanaluk (2007) found there was no difference in digestion properties between white rice stored at 30–35 °C for 8 months and the fresh rice. In this study, all rice varieties had no significant changes in digestion properties after ageing treatment, though there were changes in protein and lipids composition. This may be because there was no enormous changes in protein pattern after ageing (Fig. 1) as observed by the study of Azizi et al. (2019), in which there was new band of aggregated thiol groups that were able to affect

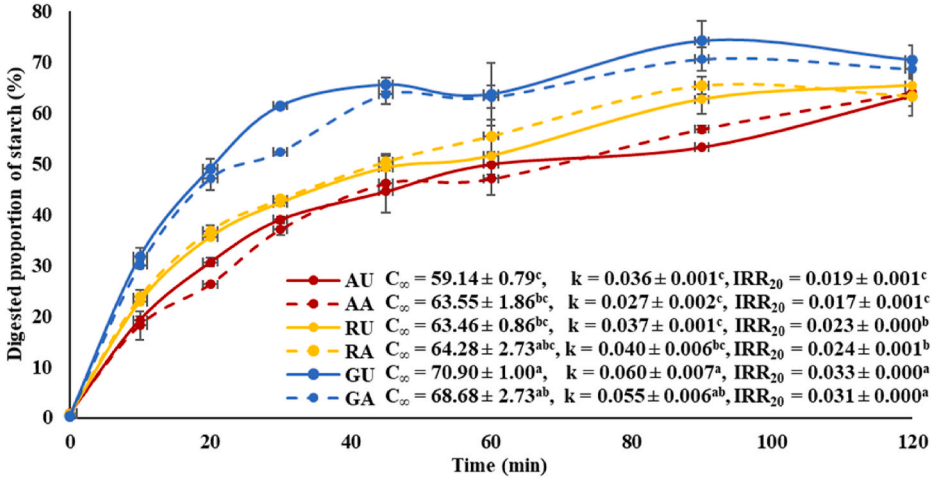

Fig. 3. *In vitro* starch hydrolysis profiles of the untreated and aged samples. Error bars represent standard deviation of the mean for triplicate digestions. AU, ALK3 untreated samples; AA, ALK3 aged samples; RU, RS4 untreated samples; RA, RS4 aged samples; GU, GZ93 untreated samples; GA, GZ93 aged samples. C∞, concentration of the percentage of digested starch at infinite time; k, digestibility rate constant; IRR20, initial digestion rate in first 20 min. The data are expressed as the means ± SD, n = 3. Different letters in the column indicate significant differences among different samples at the 0.05 level.

the digestion properties of the starch. These results may also suggest that the higher lipids in ALK3 and RS4 did not bring any negative effect on starch digestion properties during ageing: storage did not change the low digestibility of high lipids rice.

4. Conclusions

After artificially ageing treatment, all rice varieties presented typical changes happened during natural storage, such as protein aggregation and decrease in breakdown value. Total lipids, as the most interested components in these rice mutants, had a stable content in all rice varieties after ageing treatment. However, NSL and γ-oryzanol significantly decreased in ALK3 and RS4, while did not change in GZ93. ALK3 with the highest lipids content had a lower pH of cooking solution and lower *Tp* of the second endothermic peak in DSC, suggesting the hydrolysis of lipids and damage of amylose-lipids complex. After ageing treatment, all rice varieties had a lower UFA:SFA value in NSL and lower breakdown compared to fresh rice, indicating an inferior quality of lipids and rice ECQ. However, no more serious quality deterioration was observed in high lipids rice compared to common rice. These indicate the enhanced lipids in high-lipids rice did not accelerate the deterioration of ECQ or result in serious lipids rancidity and quality deterioration during ageing. Conversely, high lipids content contributes to a better nutritional quality in terms of high bioactive compound content, i.e., higher γ-oryzanol content, and lower IRR_{20} even after storage. High lipids rice mutants have potential to be developed into a healthier staple food.

In this study, a time-saving artificially accelerated ageing method was established, which can effectively mimic the changes of lipid during rice storage. However, different sets of the temperature and humidity to mimic different ageing degree of rice with different storage time need to be tried in future studies, which may give more accurate suggestions on shelf life of the high-lipids rice.

CRediT authorship contribution statement

Yi Shen: Conceptualization, Methodology, Writing – original draft, Data curation, Visualization. **Wanxin Gong:** Validation, Methodology, Investigation. **Yu Li:** Writing – review & editing. **Jiaming Deng:** Methodology. **Xiaoli Shu:** Conceptualization, Writing – review & editing. **Dianxing Wu:** Supervision. **Nicoletta Pellegrini:** Writing – review & editing. **Ning Zhang:** Writing – review & editing.

Declaration of competing interest

The authors have no affiliation with any organization with a direct or indirect financial interest in the subject matter discussed in the manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2021.112730.

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