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# Effect of Storage Conditions on Physical Properties, Lipid Oxidation, Isoflavones and Antioxidant Capacity of Flour Prepared from Soy Milk By-Product

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**Abstract:** During the production of soy milk, a by-product is produced, which is typically treated as a waste material. This by-product has significant levels of dietary fibre, proteins, isoflavones and antioxidant capacity. As such, it has been recommended as an effective functional ingredient when milled to a flour after drying at 100 °C. The shelf-life stability of this dried by-product is relatively unknown when stored under different storage conditions (2 °C, 20 °C and 40 °C) and initial moisture content (9%, 12% and 14%), both packaged and exposed to immediate environments. This study investigated the impact of storage over ten weeks on the physical properties, lipid oxidation, antioxidant capacity and stability of isoflavones of this functional ingredient. The results showed that exposure significantly affected the stability of flour, especially on moisture content, water activity, isoflavone concentration and lipid oxidation. Different initial moisture contents significantly affected the initial and final colour, alongside final moisture and water activity when stored covered at 2 °C and 20 °C. Different storage temperatures were found to affect the moisture content, water activity, lipid oxidation, conversion of isoflavones and antioxidant capacity, with all tested initial moisture contents. Of note, higher conversion rates of malonyl isoflavones to β-glucosides were observed at high temperatures and longer times, while a minimum change in aglycone content occurred. In conclusion, the stability of this flour is least influenced by the initial moisture content but is more affected by high storage temperature and unpackaged conditions.

**Keywords:** shelf life; soy milk by-product; valorisation; isoflavones; okara; functional foods; waster utilisation



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## 1. Introduction

Soy milk by-product (SMB), also known as okara, is often regarded as an industrial waste product. A large volume of this material is produced around the world. For instance, over 3.5 million tonnes of SMB is produced annually in Japan and China; however, utilisation of this by-product is limited [1]. This by-product is a rich source of nutrient components, such as dietary fibre and protein. Additionally, it contains a significant number of bioactive compounds, with the ability to function as an antioxidant [2]. As such, SMB could be beneficial to human health, leading to research focusing on valorising this by-product through utilisation as a functional ingredient in foods and nutraceuticals [3].

There is increasing consumption of soy as food and its inclusion as part of a healthy diet [4]. SMB has been shown to contain many bioactive compounds, including isoflavones, a sub-class of flavonoids, with known benefits to human health [5]. Isoflavones are found in the three main aglycone forms: daidzein, genistein and glycitein. Each isoflavone can be glycated (β-glucoside), which results in daidzin, genistin and glycitin. These can be further esterified with the addition of acetyl and malonyl groups attached to the glucose [6].

These functional groups can be removed during processing and storage, leading to changes in the concentration of other forms [7]. The glycoside form of each has been reported to have the highest concentration in many soy products; however, traditional fermented soy foods, such as miso and tempeh, contain mainly aglycones [8]. These aglycones have been reported to have higher bioavailability in human digestion [9].

One major barrier that has limited the utilisation of this by-product is the high moisture content (MC), which remains after separation from soy milk during the production process (≈MC 86%). If the crude (wet) SMB is left in this state, rapid spoilage and degradation occur due to microorganisms, enzymes and oxidation. It has been reported that it is possible to effectively dry this by-product by many methods, stabilising it for further application, with authors also demonstrating that bioactive compounds and antioxidant capacity can be maintained or even improved during processing [10–13]. More specifically, Guimarães et al. [14] reported that SMB dried by a fan-forced oven had good retention of macronutrients and isoflavones, which was higher compared to microwave and freeze-drying. Of particular importance to the current study is the drying of crude SMB at 100 °C in a fan-forced oven. This optimised drying condition was shown to have no negative impact on bioactive compounds or the antioxidant capacity. Subsequently, milling produced a flour (SMB100), resulting in a functional ingredient that has been shown to be high in dietary fibre, proteins, isoflavones and soy saponins, with significant antioxidant capacity. Furthermore, the flour was quantified for its physical qualities and ability to be substituted in a basic bread formulation [15,16]. Studies in this area of research have reported the potential benefit of this by-product; however, studies on the shelf-life stability of these dried by-products over extended storage are scarce.

The development of a food item not only relies on how it performs after manufacturing but also on how it will behave during storage. This is particularly significant when the food ingredient is considered to have functionality; however, research in this area for SMB is limited. Azanza and Gascon [17] demonstrated that dried okara obtained from tofu producers had a shelf life of up to six months when stored in vacuum-sealed, triple-layer packaging at 30 °C. Their study used rancid aroma as the end point of the study and determined that as the temperature increased, the shelf life decreased. While macronutrients were analysed at the start and completion of storage, bioactive compounds were not assessed. Sengupta et al. [18] studied the microbial count of okara stored for 28 days and showed that it increased during storage, after drying in both vacuum and microwave ovens or stored wet at 4 °C. Voss et al. [19] studied SMB stored wet at −18 °C and 4 °C and compared these to samples partially dehydrated to a MC of 45%, at 80 °C and 200 °C during a 15-day trial. They observed differences through time and storage conditions for macronutrients, isoflavones, antioxidant capacity and microbial load. Deterioration in the quality of dried food products, such as soy and wheat flours, has been reported to be from a range of factors, including oxidation of fatty acids, humidity, temperature, light, microbial contamination and enzymatic activity [20].

It is vitally important to understand how this by-product is affected by different storage conditions, not only on the physical characteristics, such as colour, moisture content and water activity, but also any changes in the concentration of bioactive compounds and antioxidant capacity over extended periods. As such, this study demonstrated the effect of exposure to storage conditions over time on the MC, water activity ($a_w$), colour, lipid oxidation, isoflavone content and antioxidant capacity, alongside changes due to the starting MC.

## 2. Materials and Methods

### 2.1. Materials

Soybeans of medium size and clear hilum were purchased from Australian Wheatgrass (Riverstone, NSW, Australia). Analytical- and HPLC-grade chemicals were purchased from Merck (Rahway, NJ, USA), including p-anisidine, glacial acetic acid, ammonium acetate, methanol (MeOH), acetonitrile, 2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonic

acid) (ABTS), 2,9-dimethyl-1,10-phenanthroline (neocuproine), Trolox, copper (II) chloride, potassium persulfate ($K_2S_2O_8$), daidzin, daidzein, genistin and genistein. Malonyl genistin and malonyl daidzin were purchased from Millenium Science (Mulgrave, VIC, Australia).

### 2.2. Methods

#### 2.2.1. Sample Preparation and Drying

SMB was produced using a benchtop soy milk machine following a previously described process [16]. After visual inspection and soaking of the soybean in DI water (12 h, 22 ± 1 °C), the soy milk was produced as follows. Soybeans were processed through a soy milk machine at a water-to-bean ratio of 10:1 ($v/w$), pasteurised at a final temperature of 95 °C for 1 min, then separation of the soluble milk solution and the insoluble by-product through muslin cloth occurred. This was followed by rinsing with extra water and pressing to remove residual soy milk. Drying the SMB was carried out in a convection oven at 100 °C, as described in a previous study [15], to a final moisture content (MC) of 9%, 12% and 14% (SMB9, SMB12 and SMB14), then milled in a Perten 3100 Laboratory Mill (Perten Instruments, Stockholm, Sweden). The flours were milled through a 0.8 mm mesh, then stored at room temperature in a sealed container for 2 days to allow the flour moisture content to equilibrate. The moisture content for all samples in this study was determined by a Unibloc Infrared (IR) Moisture Analyser (MOC 63u, Shimadzu, Kyoto, Japan), which returned percentage MC, while the $a_w$ was measured with a Rotronic Hygropalm water activity meter (Rotronic Instruments, Bukit Merah Central, Singapore).

#### 2.2.2. Storage Conditions

After two days of storage, each flour was retested and the initial MC was determined by IR moisture balance, while the initial $a_w$ and colour were recorded. Samples of each starting material were stored at −18 °C for testing lipid oxidation, antioxidant capacity and isoflavone content. Following this, each flour (SMB9, SMB12 and SMB14) was divided into six sample groups for storage, both covered (C) and uncovered (UC), at temperatures of 2 °C, 20 °C and 40 °C. Figure 1 shows the experimental design for the storage conditions. The covered samples ($n$ = 3/condition/week) were stored in heat-sealed individual bags made of dual-layer polyethylene plastic film, while the exposed/uncovered samples ($n$ = 3/condition) were stored in bio-plastic (polylactic acid) open containers. Samples stored at 2 °C were stored in a refrigerator, 20 °C in a temperature-controlled laboratory, while samples stored at 40 °C were stored in a laboratory oven. Samples stored in each different condition were removed weekly over a 10-week period and tested for MC, $a_w$ and colour, then stored at −18 °C in sealed containers for further testing of lipid oxidation, antioxidant capacity and isoflavone content.

#### 2.2.3. Colour

Colour was determined by the CIE Lab colour space, which is a common method used for the measurement of colour in food [21]. Changes in colour were noted and compared to week 0. Values were measured by a handheld Minolta Chroma Meter (CR-400 Chroma Meter, Konica Minolta Sensing, Sakai, Osaka, Japan) to yield values of L, a* and b*. These values were then used to determine the total colour change (ΔE) by the formula: $\Delta E = \sqrt{\Delta L^2 + \Delta a^2 + \Delta b^2}$ [22]. Changes in lightness were determined by comparison to the initial mean measurement for each flour.

#### 2.2.4. Lipid Oxidation

To determine any secondary oxidation of lipids, p-anisidine values (pAV) were measured, with slight modification of the method described by Xie et al. [23]. In brief, 6 g samples of SMB flours stored for 0, 6, 10 and 20 weeks for each storage temperature were suspended in 25 mL of hexane in 50 mL vortex tubes. Samples were placed in an ultrasonic bath (Soniclean, 220 V, 50 Hz and 250 W, Soniclean Pty Ltd., Thebarton, SA, Australia) set at 30 °C and 250 W for 30 min, mixed by vortex every 5 min, then centrifuged at 2900× $g$

relative centrifugal force (RCF) for 30 min. After this, two 2.5 mL aliquots of extract were pipetted into glass test tubes (A1 and A2), followed by the addition of 0.5 mL of 0.5% (*w*/*v*) p-anisidine in glacial acetic acid to A2 samples. All tubes were covered with parafilm, mixed via vortex and kept in the dark for 8 min. Tubes were then centrifuged for 2 min at 2900× *g* RCF, then measured at 350 nm in a Cary 50 Bio UV Spectrophotometer (Varian Australia Pty. Ltd., Mulgrave, VIC, Australia). Lipid oxidation of flour was determined from the defined equation: pAV = 25(1.2(A2 − A1))/mass of sample.

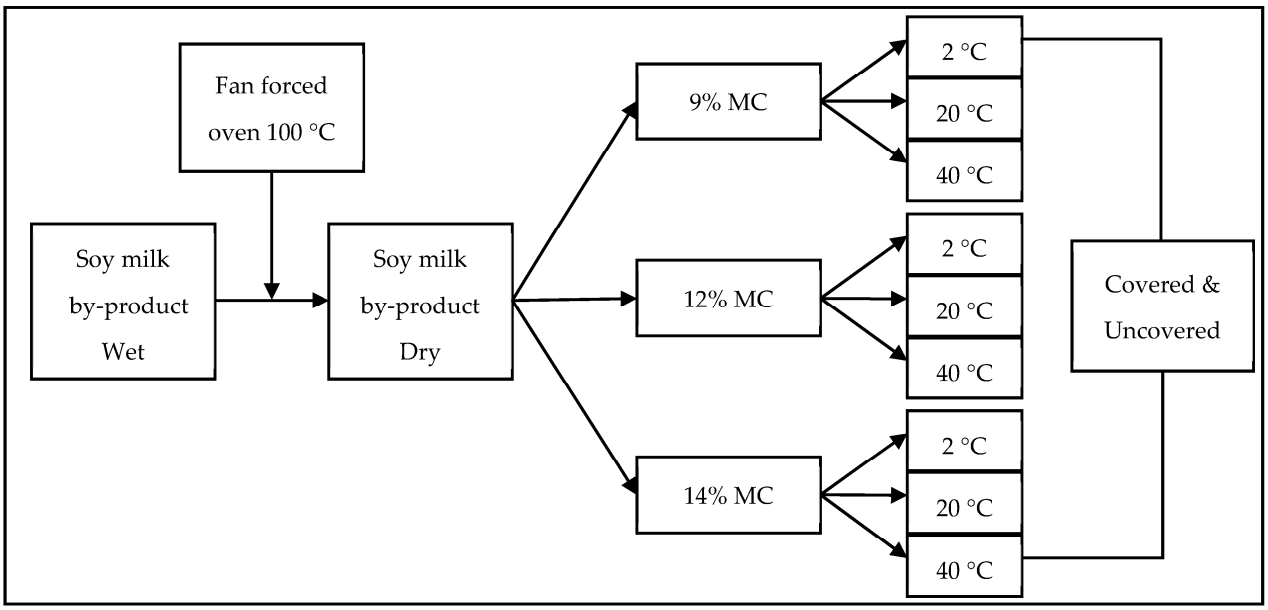


**Figure 1.** Experimental design for drying and storage of SMB100 for 10 weeks at 3 initial moisture contents (MC) and storage temperatures, both covered and exposed (uncovered). Samples were $n = 3$ for each condition.

#### 2.2.5. Extraction of Bioactive Compounds

SMB flours were extracted in 50% aqueous MeOH at a solvent:sample ratio of 20:1 (*v*/*w*) using an ultrasonic bath set at 30 °C and 150 W for 1 h with agitation using a vortex mixer every 5 min. After extraction, all samples were centrifuged at 2900× *g* RCF for 20 min to collect the supernatant, which was subsequently stored at −18 °C for further analysis of isoflavone content and antioxidant capacity.

#### 2.2.6. Isoflavone Content by High-Performance Liquid Chromatography (HPLC)

Isoflavone aglycones genistein and daidzein, alongside the β-glucoside and malonyl forms, were determined by HPLC. Reverse-phase HPLC with a photodiode array detector (Shimadzu 20 series, UV-Vis detector (Shimadzu Scientific, Rydalmere, NSW, Australia)) was used to determine the composition of these isoflavones following a previous method [24], with modifications. Extracts were filtered through a 0.45 µm syringe filter into HPLC vials. A reverse-phase HPLC column (Agilent Raptor C18 column, 4.6 × 150 mm, with 5 µm particles (Restek Corporation, Bellefonte, PA, USA)) was used under a column temperature of 30 °C, auto-injection was applied with a 25 µL injection volume and absorption was measured at 254 nm. The mobile phase consisted of 0.2% aqueous formic acid solution (solvent A) and 100% acetonitrile (solvent B). A solvent gradient was used, with an increasing concentration of solvent B, as follows: 5 min 0% of solvent B, increasing to 25% by 15 min, 40% after 25 min and 60% by 35 min, and decreasing to 10% at 40 min and 0% at 45 min at a constant flowrate of 0.7 mL/min. Standard curves were developed for all isoflavones using the same procedure, for a concentration range of 1.56–125 µg/mL. The linear equation of the standard curves was used to determine the concentration of each isoflavone adjusted for dilution and MC, reported as µg/g dry weight (µg/g DW) of flour.

The retention time of analytes was daidzin 22.1 min, genistin 24.5 min, malonyl-daidzin 24.9 min, malonyl-genistin 27.0 min, daidzein 29.5 min and genistein 32.3 min. Figure 2 shows the HPLC chromatogram of SMB100 flour for all tested isoflavones.

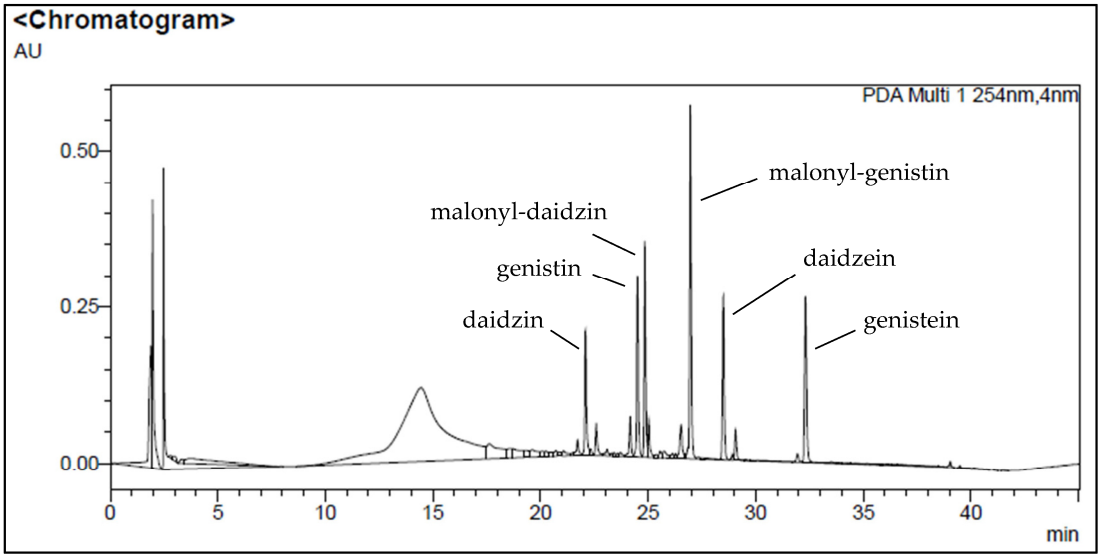


**Figure 2.** HPLC chromatogram of isoflavones in SMB100 after 10 weeks of storage. The retention time of analytes: daidzin 22.1 min, genistin 24.5 min, malonyl-daidzin 24.9 min, malonyl-genistin 27.0 min, daidzein 29.5 min and genistein 32.3 min.

#### 2.2.7. Antioxidant Capacity

The antioxidant activity of SMB100 was determined by the ABTS and CUPRAC assays. Scavenging capacity by ABTS was measured as previously [15] described, with minor modifications. Stock reagent was produced by combining 10 mL each of aqueous ABTS (7.4 mM) and $K_2S_2O_8$ (2.6 mM), developed for 16 h at room temperature in the dark, then stored at −18 °C until required. Working solution were prepared daily by diluting the ABTS stock reagent with MeOH to achieve an absorbance of 1.1 ± 0.02 at 734 nm. The final assay was completed by combining 0.15 mL of extract with 2.85 mL of ABTS working reagent, allowing the solutions to react for 2 h in a dark room and measuring absorbance at 734 nm on a Varian Cary 50 spectrophotometer (Varian Australia Pty. Ltd., Mulgrave, VIC, Australia). Trolox was used to develop a standard curve, and the results were expressed as millimole Trolox equivalents per gram of sample (mmol TE/g).

The metal chelating capacity of SMB was determined using the Cupric Reducing Antioxidant Capacity (CUPRAC) assay described by Apak et al. [25], with some modifications. Initially, 1 mL aliquots of aqueous copper (II) chloride (10 mM), ammonium acetate buffer (pH 7) and aqueous neocuproine (7.5 mM) solutions were mixed with 1.1 mL of extract then reacted for 1.5 h. Following this, the absorbance was measured at 450 nm on a Varian Cary 50 spectrophotometer. Trolox was used to develop a standard curve and the results were expressed as millimole Trolox equivalents per gram of sample (mmol TE/g).

#### 2.2.8. Statistical Analysis

All experiments were performed in triplicate ($n$ = 3) and results were determined as the means ± standard deviations (SD) using Microsoft® Excel 365. One-way ANOVA (analysis of variance) was performed on each dataset to determine statistical significance, and the means were compared by Dunnett's method to compared with the control, while others were compared by all-pairs Tukey HSD to determine the connecting letters, reported using JMP® Pro Statistical Software 16.2.0 (SAS Institute Inc, Cary, NC, USA). Statistically significant differences between results were determined by $p < 0.05$ throughout the study.

## 3. Results and Discussion

### *3.1. Change in Moisture Content at Different Weekly Storage Conditions*

It is essential to understand the change in moisture content of SMB from the initial MC when stored under different conditions. The results showed that the MC of SMB9 and SMB12 stored at 2 °C and covered became statistically similar ($p > 0.05$) from week 5 onwards (Figure 3a). However, at an initial MC of 14%, no similarity ($p < 0.05$) was observed at any stage. When flour was stored uncovered in these conditions (Figure 3b), the MC became similar at week 1; however, it only stabilised at week 4 until the completion of the storage time. Figure 3c reveals that the MC of all samples stored covered at 20 °C became similar at week 3; however, the similarity of MC was only maintained for SMB9 and 12 throughout, showing significant ($p < 0.05$) differences from SMB14. The same trend was not observed in uncovered samples (Figure 3d) stored at this temperature, as the initial MC stabilised and remained similar from week 4 onwards. Regarding the samples stored at 40 °C, both covered (Figure 3e) and uncovered (Figure 3f) samples showed inter-conditional similarity in their MC after one week of storage and at the completion of the trial period.

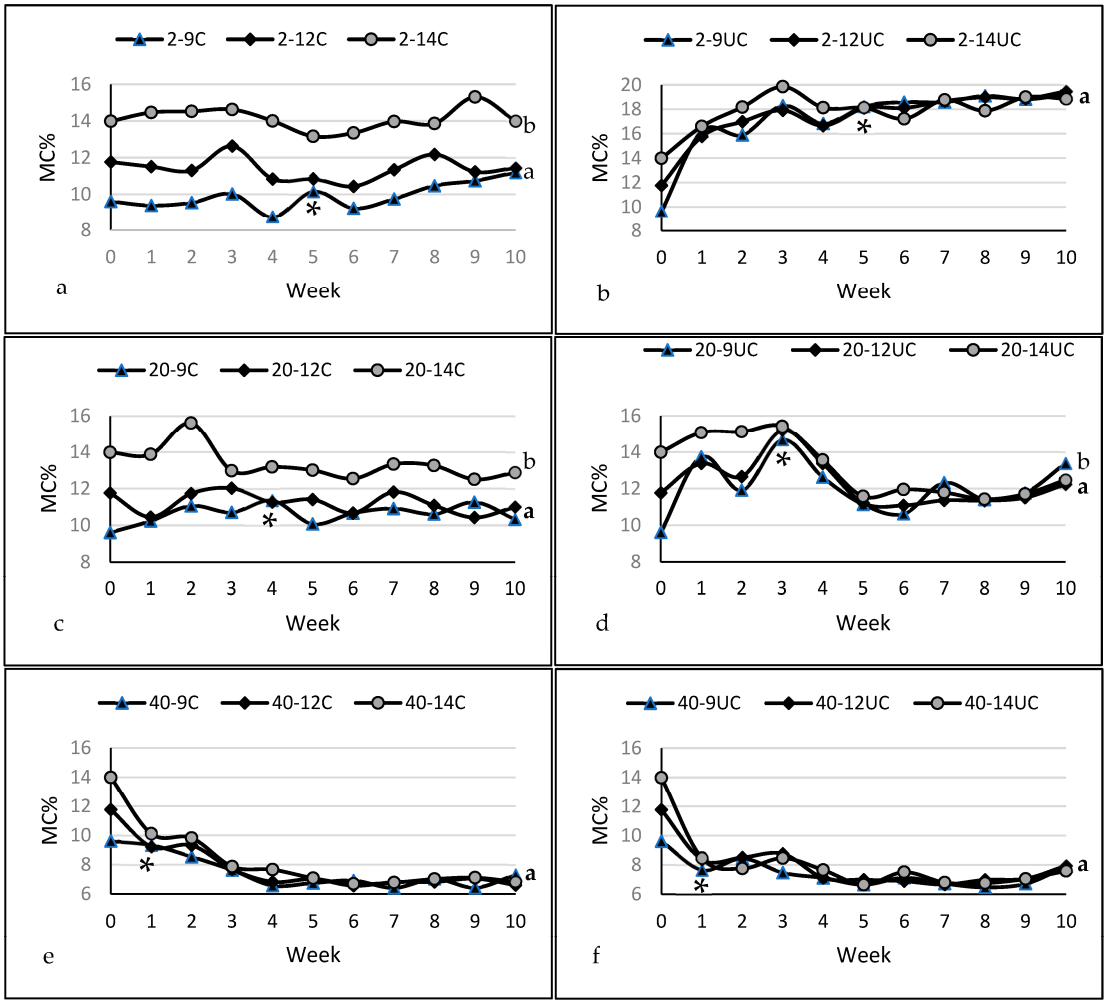


**Figure 3.** The mean ($n = 3$) changes in percentage moisture content (MC%) over 10 weeks of storage of SMB flour. Different connecting letters show significantly ($p < 0.05$) different moisture contents after 10 weeks of storage, while lines marked with an asterisk (*) show when similarity ($p > 0.05$) in MC was reached. Initial moisture contents of 9%, 12% and 14% were stored both covered (C) and uncovered (UC) at temperatures of 2 °C ((**a**) 2 °C covered, 2-9C, 2-12C, 2-14C and (**b**) 2 °C uncovered, 2-9UC, 2-12UC, 2-14UC), 20 °C ((**c**) 20 °C covered 20-9C, 20-12C, 20-14C and (**d**) 20 °C uncovered 20-9UC, 20-12UC, 20-14UC) and 40 °C ((**e**) 40 °C covered 40-9C, 40-12C, 40-14C and (**f**) 40 °C uncovered 40-9UC, 40-12UC, 40-14UC).

When comparing covered and uncovered storage, with samples at each temperature, the storage of uncovered SMB resulted in greater differences from the initial MC when stored at 2 °C and 20 °C. Conversely, covered SMB12 and SMB14 at these temperatures were able to maintain MC, at levels independent ($p < 0.05$) of each other. When stored at 40 °C uncovered, a rapid decrease in MC was observed regardless of the initial moisture content. When stored covered, at this temperature, this loss in moisture was slowed but still significant. While the loss of moisture has not been previously tracked for SMB, Agrahar-Murugkar and Jha [26] observed similar results in sprouted soybean flour. At ambient conditions, flour stored uncovered increased in MC at a fast rate and led to a higher final MC over 75 days of storage. They also reported different MCs for a range of packaging materials and when stored under ambient and accelerated storage conditions.

Water loss can occur in food as a result of convective heat exchange, radiation energy differences and evaporation on the surface of the food. This results in the evaporation of water when the food is directly exposed to storage conditions or stored in semi-porous materials. The extent of water loss can be limited by the air velocity, shape and thickness of the boundary surface and the heat transfer coefficient [27]. Moisture will move into and out of the food matrix as it tries to reach equilibrium with the storage environment, and it has been described by absorption and desorption isotherms. The transfer of moisture can be influenced by the vapor pressure of endogenous water and dissolved solids, alongside storage temperature and relative humidity [28]. The absorption and desorption of moisture may be responsible for the fluctuation in MC, as can be seen in Figure 3.

### *3.2. Change in Moisture Content and Water Activity of SMB between the Initial and 10-Week Storage at Different Conditions*

The results (Table 1) present data comparing the moisture content and water activity of SMB in week 10, with week 0 as a control. The initial MC for SMB9 was 9.61% with an $a_w$ of 0.45, SMB12 started the trial with an 11.76% MC and activity of 0.57, while SMB14 had an MC of 13.99% and a 0.67 $a_w$. At the completion of the storage period of 10 weeks, there was a significant change in both the MC and $a_w$ for most conditions tested. Changes were dependent on the storage temperature and relative humidity (RH%), initial moisture content and whether stored covered or exposed. With storage at 2 °C and a mean RH% of 89.0 (±10.4), the MC of SMB9 flour significantly increased for both covered and exposed flour, with increases of 16.5% and 99.9%, respectively. Only the uncovered samples for SMB12 and SMB14 significantly increased, with changes of 66.2% and 34.8%, respectively. A similar trend was observed when assessing $a_w$ for these same samples, with final measurements of 0.52 (covered) and 0.84 (uncovered) for SMB9 and 0.82 and 0.78 for SMB12 and SMB14 (uncovered). These results highlight that SMB flour stored unexposed, at cold temperatures with a high RH%, can maintain a moisture content above 11%, while sustaining an $a_w$ that does not support the growth of microorganisms. However, if flour is exposed to this combination of conditions for extended periods, it will extensively increase the MC and $a_w$, resulting in a potential loss of quality.

When stored at 20 °C with a 62.4 RH%, the MC of SMB14, for both SMB14 covered and uncovered, observed a significant reduction, with changes of 7.8% and 10.8%, while SMB9 significantly increased by 39.2%. No change in MC was observed for SMB12. Significant changes in $a_w$ were observed for all conditions, with SMB9 increasing to 0.53 for covered and 0.62 for uncovered, while SMB12 decreased to 0.50 for covered and increased to 0.61 for exposed samples. The $a_w$ of SMB14 at 20 °C was 0.54 (covered) and 0.61 (uncovered), which was a significant decrease in comparison to the initial reading. Jointly, these results suggest that SMB100 stored under these storage conditions, regardless of the starting MC, $a_w$, or exposure to the immediate environment, can be considered safe, as all $a_w$ observed limited the growth of microorganisms. Similar MC changes were observed by Lancelot et al. [29] in wheat flour stored in permeable paper bags for 30 months at 20 °C, with an initial water content of 13.5%, which dropped to 12.5% at the completion of the trial period.

**Table 1.** Percentage of (a) moisture content (MC%) and (b) water activity ($a_w$) of SMB9, SMB12 and SMB14 flours stored at 2 °C, 20 °C and 40 °C after 10 weeks.

| | | SMB 9% | SMB 12% | SMB 14% |
|---|---|---|---|---|
| Week 10 MC% (a) | Initial MC% | 9.61 ± 0.15 | 11.76 ± 0.75 | 13.99 ± 0.13 |
| | 2 °C covered | 11.20 ± 0.45 * | 11.45 ± 0.46 | 14.00 ± 0.09 |
| | 2 °C uncovered | 19.21 ± 0.39 * | 19.47 ± 0.05 * | 18.85 ± 0.18 * |
| | 20 °C covered | 10.34 ± 0.20 | 11.01 ± 0.32 | 12.89 ± 0.66 * |
| | 20 °C uncovered | 13.39 ± 0.78 * | 12.24 ± 0.44 | 12.47 ± 0.26 * |
| | 40 °C covered | 7.32 ± 0.27 * | 6.61 ± 0.52 * | 6.83 ± 0.18 * |
| | 40 °C uncovered | 7.88 ± 0.09 * | 7.94 ± 0.27 * | 7.59 ± 0.34 * |
| Week 10 $a_w$ (b) | Initial $a_w$ | 0.45 ± 0.03 | 0.57 ± 0.03 | 0.67 ± 0.05 |
| | 2 °C covered | 0.52 ± 0.003 * | 0.58 ± 0.004 | 0.66 ± 0.003 |
| | 2 °C uncovered | 0.84 ± 0.004 * | 0.82 ± 0.006 * | 0.78 ± 0.006 * |
| | 20 °C covered | 0.53 ± 0.005 * | 0.50 ± 0.005 * | 0.54 ± 0.005 * |
| | 20 °C uncovered | 0.62 ± 0.004 * | 0.61 ± 0.003 * | 0.61 ± 0.004 * |
| | 40 °C covered | 0.18 ± 0.004 * | 0.26 ± 0.01 * | 0.29 ± 0.005 * |
| | 40 °C uncovered | 0.35 ± 0.001 * | 0.37 ± 0.01 * | 0.41 ± 0.03 * |

Data in the same column marked with an asterisk (*) are statistically different ($p < 0.05$) from the initial moisture content (MC%) and water activity ($a_w$) by Dunnett's control, after 10 weeks of storage.

Under the storage condition of 40 °C and 18.8 RH%, both the MC and $a_w$ were significantly reduced for all samples over a period of 10 weeks of storage. Covered samples were reduced by 23.8%, 43.4% and 51.2%, while uncovered flour recorded changes of 14.5%, 35.3% and 45.7%, for SMB9, SMB12 and SMB14, respectively. Similarly, the $a_w$ values of these samples were significantly reduced, showing a range of activities from 0.18 to 0.29 for the SMB9 to SMB14 covered samples and 0.35–0.41 $a_w$ for uncovered. After ten weeks of storage, it was observed that an initial moisture content resulted in a lower final $a_w$ at this storage temperature. For SMB stored at a higher ambient temperature and low RH%, the observed results were to be expected, as these conditions are indicative of solar drying methods. Agrahar-Murugkar and Jha [26] observed a final moisture content of 7.1% for sprouted soybeans for soy flour using sun drying. Their study reported an average temperature of 35 °C, with a maximum of 45 °C. Forsido et al. [30] observed that $a_w$ was reduced to 0.18 when storing flour mixtures for 90 days at 45 °C and an RH% of 15.5 in a woven plastic packaging material, while the same flour stored at a lower temperature did not experience this reduction in $a_w$.

Food matrices during storage will absorb or release moisture according to the relative humidity of the surrounding environment and will move towards an equilibrium relative humidity, accordingly. At equilibrium, there is a known mathematical relationship with the $a_w$ of the foods. Food stored in an environment that has a high relative humidity will increase in MC and $a_w$ to achieve equilibrium, while the opposite is also true [31]. This was observed in the results (Table 1) for SMB flour stored at low (40 °C, 18.8 RH) and high (2 °C, 89.0) relative humidity.

The $a_w$ value has been used as a tool to predict the shelf-life stability of food items. It indicates the available moisture for the action of microorganisms and enzymes and is determined as the ratio of the vapour pressure of water in the food item compared to pure water [32]. According to [33], all biological growth of microorganisms will cease at an $a_w$ of 0.62; as such, SMB14 may be susceptible to growth of yeasts and moulds at the initial MC of 13.99%. Meanwhile, others reported that an $a_w$ of 0.62–0.67 is sufficient to retard degradation of wheat flour [34]. For the current study, a low MC and $a_w$ are preferable for the minimisation of microorganic growth; however, this may show negative effects for other quality points, such as bulk density. It has also been reported that an $a_w$ of 0.2 and below can increase lipid oxidation [30,35].

Flours are typically considered to be shelf-stable commodities; as such, they are stored and transported under ambient conditions. During this study, the stability of MC and $a_w$ was evident for samples at 20 °C when either enclosed in poly-plastic bags or exposed to

surrounding conditions, regardless of the initial MC. As such, a starting MC of between 12% and 14% may be considered as optimal.

### 3.3. Changes in the Colour of SMB between the Initial and 10-Week Storage at Different Conditions

Changes in colour, including the lightness index (L*) and total colour change, of SMB with different initial moisture contents after 10 weeks of storage, across the range of conditions, are shown in Table 2a. The initial L* value for SMB9 was 88.63, while after the 10 weeks of storage, only covered samples stored at 20 °C and 40 °C were determined to be significantly different, at 87.37 and 87.60, respectively. SMB flour with an initial moisture content of 12% showed a wider distribution in L*, with all samples returning significantly lower values when compared to week 0 at 90.43. The values ranged from least change at 89.00 for 20 °C covered, to the greatest change at 87.00, for samples stored at 40 °C covered. Samples from SMB14, which started with an L* index of 89.20, showed significant changes for all temperatures when covered, but only at 40 °C when uncovered, recording values from 88.00 to 86.70.

**Table 2.** Changes in lightness (a) (L*) and total colour change (b) (ΔE) of 9%, 12% and 14% SMB flours stored at 2 °C, 20 °C and 40 °C after 10 weeks.

| | | SMB 9% | SMB 12% | SMB 14% |
|---|---|---|---|---|
| Week 10 L* (a) | Week 0 | 88.63 ± 0.31 | 90.43 ± 0.21 | 89.20 ± 0.50 |
| | 2 °C covered | 88.80 ± 0.08 | 87.67 ± 0.29 * | 88.00 ± 0.42 * |
| | 2 °C uncovered | 88.73 ± 0.45 | 88.60 ± 0.37 * | 88.33 ± 0.12 |
| | 20 °C covered | 87.37 ± 0.31 * | 87.83 ± 0.50 * | 87.83 ± 0.12 * |
| | 20 °C uncovered | 88.47 ± 0.17 | 89.00 ± 0.43 * | 88.57 ± 0.17 |
| | 40 °C covered | 87.60 ± 0.29 * | 87.00 ± 0.25 * | 86.70 ± 0.29 * |
| | 40 °C uncovered | 88.70 ± 0.08 | 88.80 ± 0.37 * | 88.23 ± 0.33 * |
| Week 10 ΔE (b) | Week 0 | 0 | 0 | 0 |
| | 2 °C covered | $2.14 \pm 0.10^{d}$ | $2.95 \pm 0.25^{cd}$ | $1.23 \pm 0.45^{ab}$ |
| | 2 °C uncovered | $1.27 \pm 0.02^{bc}$ | $1.84 \pm 0.39^{ab}$ | $2.21 \pm 0.11^{c}$ |
| | 20 °C covered | $1.35 \pm 0.17^{c}$ | $2.88 \pm 0.45^{bcd}$ | $1.84 \pm 0.07^{bc}$ |
| | 20 °C uncovered | $0.99 \pm 0.12^{b}$ | $1.49 \pm 0.41^{a}$ | $0.79 \pm 0.04^{a}$ |
| | 40 °C covered | $1.24 \pm 0.07^{bc}$ | $3.56 \pm 0.21^{d}$ | $2.58 \pm 0.27^{c}$ |
| | 40 °C uncovered | $0.34 \pm 0.08^{a}$ | $2.09 \pm 0.07^{abc}$ | $1.33 \pm 0.23^{ab}$ |

Data are means ($n$ = 3) ± SD for lightness (L*) and total colour change (ΔE). Asterisk (*) in the same column are statistically different ($p < 0.05$) from the initial colour (week 0) by Dunnett's control, after 10 weeks. Superscript letters in the sample column show differences by Tukey's HSD test.

Colour is an important characteristic in food and can be used as an indicator of many quality points, including acceptability [36] and as a predictor of oxidation [37] and phytochemical content [38]. Lightness is an achromatic measurement, between black and white, with values from 0 to 100, with the range of greyscale between them. It denotes the lightness of a defined chroma [39]. These results showed minor darkening of SMB100 flour when stored at these conditions, with similar results observed by Sopiwnyk et al. [40] for a range of pulse flours.

Total colour change was calculated from the L*, a* and b* values from the initial and ten-week-stored samples. From the results (Table 2b), the least total change for SMB9 was observed for samples stored at 40 °C uncovered, at ΔE 0.34, which was significantly different from other storage conditions. The greatest effect for this initial MC% was observed in covered samples stored at 2 °C, with a change of 2.14. As with the results for L*, an initial MC of 12% had the greatest effect on ΔE, with the lowest change at an ΔE value of 1.49, 1.84 and 2.09, for uncovered samples at 20 °C, 2 °C and 40 °C, respectively. The greatest changes were from covered samples at 40 °C and 20 °C, with values of 3.56 and 2.88. The change in colour for the SMB14 samples was least evident for 20 °C covered, 2 °C covered and 40 °C uncovered, with ΔE of 0.79, 1.23 and 1.33, respectively. The greatest

difference in colour change for this condition was for 2 °C uncovered and 40 °C covered, calculated to be 2.21 and 2.58.

Total colour change has been used to compare many foods, including drying of lemon [41], apple, banana, carrot, potato [42] and pumpkin [38]. Grobelna et al. [43] has noted that when ΔE values < 1, no change can be detected, 1–2, only discernible by an experienced observer, 2–3.5, possible detection by consumers, while 3.5–5, a clear difference in colour can be observed. With storage of flour at ambient conditions (20 °C) showing colour changes of less than 3.5, storage at this temperature, whether covered or exposed, will not negatively influence the perceivable colour of SMB100 flour.

### *3.4. Lipid Oxidation during Storage*

The effect of storage temperature on the oxidative stability of lipids in SMB by pAV value/g of flour exposed to the immediate storage environment is presented in Table 3. Minimal secondary oxidation was observed for the samples at weeks 0 and 6, stored at all temperatures. No pAV was observable for samples stored at 2 °C, while 20 °C and 40 °C returned values of 0.3 and 0.2, respectively, but were not significantly different. After 10 weeks of storage, samples stored at 2 °C showed some mild oxidation, with a mean value of 1.2, while a slightly lower value (0.7) was observed at week 20. This decrease could be due to the volatility and instability of aldehydes and ketones at that low temperature [44]. For 20 °C, similar pAV values were recorded at 10 and 20 weeks. Meanwhile, for samples stored at 40 °C, the pAV value at 10 weeks was consistent with other temperature treatments; however, at 20 weeks, pAV significantly increased to 2.4, a trend that was not observed for flour stored at 2 °C and 20 °C. This increase in pAV at 40 °C is consistent with a previous study, where the pAV of soybean flour stored at 45 °C increased after 12 weeks of storage [45]. It was reported that a pAV of <1 can be considered in freshness, while values of 1–5 show low oxidation [46]. In any event, the lipids in the SMB100 flour used in this study were, therefore, found to be stable under the storage conditions analysed.

**Table 3.** The changes of p-anisidine values of flour stored under different conditions.

| | | Week 0 | Week 6 | Week 10 | Week 20 |
|---|---|---|---|---|---|
| **p-anisidine (pAV/g)** | 2 °C | 0.2 ± 0.1 | nd | 1.2 ± 0.2 $^{a}$ | 0.7 ± 0.1 $^{a}$ |
| | 20 °C | 0.2 ± 0.1 | 0.3 ± 0.1 $^{a}$ | 0.7 ± 0.2 $^{ab}$ | 0.5 ± 0.2 $^{a}$ |
| | 40 °C | 0.2 ± 0.1 | 0.2 ± 0.1 $^{a}$ | 0.6 ± 0.4 $^{b}$ | 2.4 ± 0.5 $^{b}$ |

Data are mean ($n$ = 6) ± SD for each treatment. Different superscript connecting letters in the same column are statistically different ($p$ < 0.05) by Tukey's HSD test.

Oxidation of fats and fatty acids results in the degradation of essential fatty acids to compounds with low-molecular mass, which have been associated with negative aromas. These compounds can affect the nutritional, sensory and storage potential of foods. Primary oxidation products of fatty acids results in the production of hydroperoxides. Further (secondary) oxidation will result in the breakdown of these peroxides to produce alcohols, ketones, free fatty acids and aldehydes [47,48]. Oxidation of unsaturated fatty acids, such as linolenic acid, have been reported to be the driver of the negative aroma in soy flour [49].

### *3.5. Changes of Major Isoflavones during Storage*

As discussed, the MC of SMB100 flour stored under ambient conditions (20 °C) stabilised at approximately 12%; as such, the assessment of isoflavones during storage was determined from samples with an initial MC of 12%. All results for isoflavones were determined from the dry weight (DW) of flour to allow comparison across each storage condition, as MC changed across storage conditions. The total isoflavone DW content of flour at week 0 was 1686 μg/g of flour. During storage, there was an insignificant change in the total isoflavone content for most conditions at both weeks 5 and 10, with results ranging from 1683 to 1766 μg/g. The only condition that significantly affected the total

isoflavones content was 40 °C uncovered after 5 weeks of storage, with a concentration of 1514 µg/g. It was interesting to note that after 10 weeks of storage, no significant decrease in isoflavone content was observed for this storage condition, with a total isoflavone content of 1638 µg/g. As isoflavones are degraded to other forms under stressors, the conversion of malonyl to acetyl isoflavones may explain a loss of this extent, as these were not measured as part of this study.

Individual isoflavone content (DW) of flour stored across the range of conditions at weeks 5 and 10 has been presented in Figure 4a (week 5) and Figure 4b (week 10). Malonyl isoflavones were the predominant forms at weeks 0 and 5, with concentrations ranging from 346.6 µg/g (40 °C UC) to 422.5 µg/g (week 0) for malonyl daidzin and 362.5 µg/g (40 °C UC) to 446.4 µg/g (week 0) for malonyl genistin. This prevalence was also observed for most conditions at week 10; however, samples stored at 40 °C covered were noted to be of similar malonyl concentration to that of daidzin and genistin.

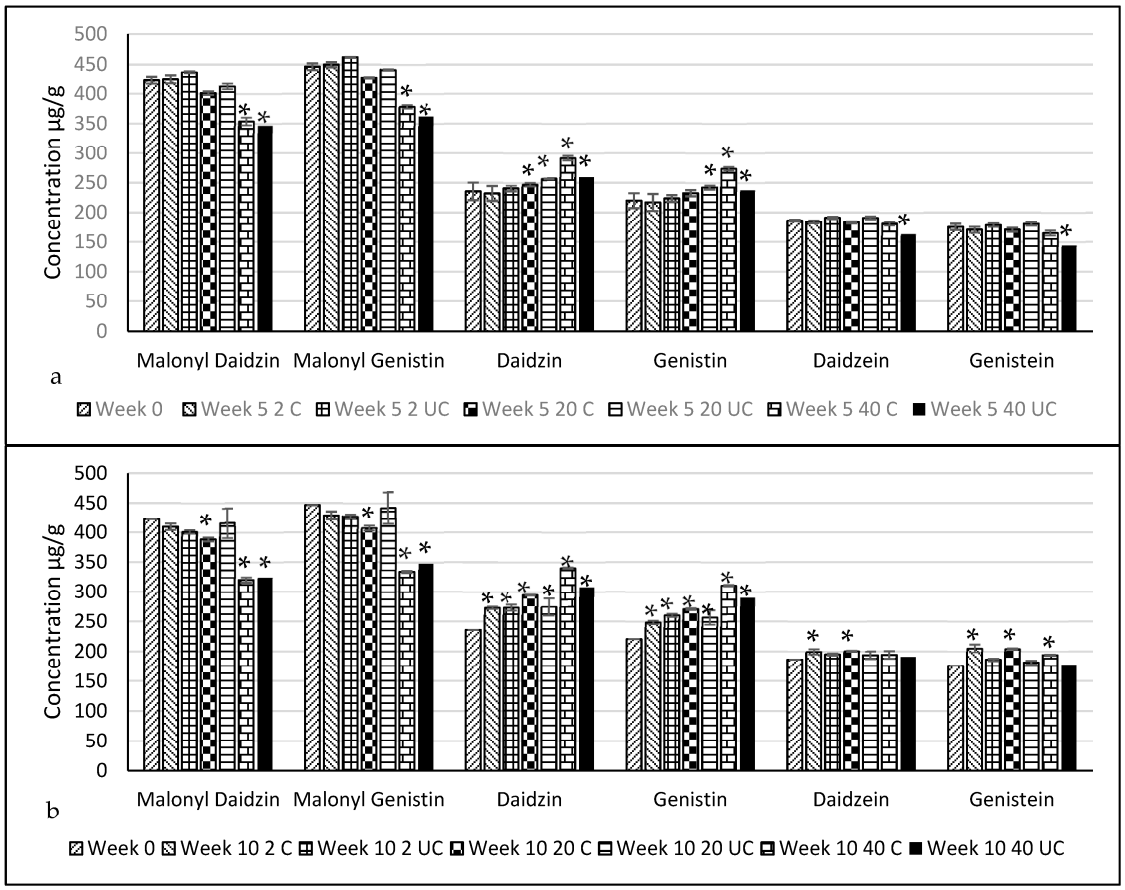


**Figure 4.** The effect of storage on the concentration of isoflavone (µg/g flour, DW) for six major isoflavones in SMB100 with an initial MC of 12% at week 5 (**a**) and week 10 (**b**) compared to week 0 as a control, stored at 2 °C, 20 °C and 40 °C, both covered (C) and uncovered (UC). Data are mean ($n$ = 3) ± standard deviation, and * shows a significant ($p < 0.05$) difference to the initial content for individual isoflavones.

Significant reductions in malonyl isoflavones were observed at week 5 for samples stored at 40 °C, when compared to the control, with conversions of 24.2% (C) and 23.2% (UC) for malonyl daidzin and 25.3% (C) and 22.0% (UC) for malonyl genistin. Samples stored at 20 °C UC were also significantly reduced to a lesser degree, with a 1.65% and 1.1% reduction in malonyl daidzin and malonyl genistin. The high content of malonyl isoflavones has been reported by Jackson et al. [50] for SMB, while β-glucosides were also found to dominate by Jankowiak [51].

After 5 weeks of storage, flour stored at 20 °C and 40 °C showed significant increases in daidzin and genistin, with the greatest increase observed in 40 °C covered samples.

Increases of 23.9% and 24.5% were determined, to a final concentration of 291.9 μg/g and 274.0 μg/g for daidzin and genistin, respectively. Significant increases in these isoflavones after ten weeks of storage were observed for all test conditions, with changes ranging from 16.1% to 44.5% (273.4–340.4 μg/g) for daidzin and 21.1% to 49.5% (284.6–311.0 μg/g) for genistin. Samples stored at 2 °C had the lowest changes, with samples at 40 °C covered having the greatest change for both β-glycosidic isoflavones.

Regarding the conversion of isoflavones to their aglycone forms, no significant ($p > 0.05$) increase under any condition was observed after 5 weeks of storage. However, flour stored at 40 °C uncovered was observed to have significantly reduced in concentration for both daidzein and genistein. The initial content of daidzein was determined to be 186.1 μg/g, which was reduced to 163.6 μg/g, a decrease of 12.1%. For genistein, the initial content was 175.5 μg/g, which was reduced by 17.7% to 144.3 μg/g. These results highlight that isoflavones aglycones can be broken down to form other products when stored in unfavourable conditions [52], such as exposure to high temperatures. For this sample condition after 10 weeks of storage, daidzein showed an increase of 26.7 μg/g and 32.9 μg/g for genistein from the week 5 data, which showed similarity to week 0. Furthermore, covered samples stored at 2 °C and 20 °C after week 10 observed significant increases for daidzein, with increases of 7.2% and 7.7%. Covered samples at all temperatures increased in concentration of genistein, at rates of 16.9% (2 °C), 16.5% (20 °C) and 10.2% (40 °C).

Kinetic modelling for the conversion of malonyl isoflavones to their glycosides and aglycones has been discussed in both model systems [53] and pilot-scale processing [54]. Conversion from malonyl isoflavones to their glucosides is through decarboxylation to form the acetyl group, followed by de-esterification releasing the glucoside, and is known to happen during heating when producing soy milk and the related SMB [50]. This has also been observed during extended storage of soybean flour, as the rate of degradation of malonyl isoflavones during simulated accelerated storage trials shows that this reaction can be catalysed by heat [55]. This was observed during the current study, as the development of daidzin and genistin was greater for samples stored at 40 °C. Conversely, the removal of the glucose unit to form the aglycone has been reported to be enzymatically catalysed by β-glucosidase, which is endogenous to soybeans, soy milk and SMB [56,57]. Specifically, β-glucosidase has been isolated from SMB and shown to be stable below 70 °C. As both the processing to produce the SMB and drying conditions are above this stable temperature, it can be assumed that the action of this would be significantly reduced. This can explain the limited conversion of isoflavones to aglycones. The increase in both daidzein and genistein across all temperatures indicates that this reaction is not significantly associated with temperature, as observed in the other forms. However, degradation of both aglycones was observed at 40 °C after week 5 of storage, and it is reasonable to assume that this process may have continued concurrently. The results of this study are in agreement with others, as a similar trend was observed by Mayakrishnan et al. [55] and Pinto et al. [7] on soy flour stored across a range of temperatures. Further to this, Pinto et al. [7] also reported that another variety of soy flour tested during their study, stored at a high temperature (42 °C), increased in aglycone content.

### 3.6. Changes in the Antioxidant Capacity of SMB12 during Storage

As shown above (Table 1), when stored at ambient conditions, SMB100 MC will stabilise at ≈12%; as such, SMB12 was used to determine the effects of storage temperature, time and uncovered storage on antioxidant capacity. Samples stored across the range of conditions were analysed at weeks 5 and 10 by ABTS and CUPRAC assays and compared to the initial antioxidant capacity. The results of the tests were adjusted for MC content and are presented in Figure 5. After 5 and 10 weeks of storage, no statistical change in ABTS was observed across all conditions when compared to an initial antioxidant capacity of 13.8 ± 0.2 mM TE/g. When assessing the antioxidant capacity by the CUPRAC assay at week 5 (Figure 5a), samples stored at 40 °C uncovered with a capacity of 3.68 ± 0.03 mM TE/g were significantly different from week 0 at 7.29 ± 1.8 mM TE/g of

flour. However, after 10 weeks (Figure 5b) of storage, flours stored at 2 °C, both covered and uncovered, were significantly affected, with antioxidant capacities of 4.37 ± 0.7 and 4.49 ± 0.6, respectively. Interestingly, at week 10, samples stored at 40 °C UC were not observed to be different from the initial antioxidant capacity. Changes in bioactive compound forms and the release of bound compounds may explain the increase in antioxidant capacity compared to week 5 [58]. Mayakrishnan et al. [55] determined that the polyphenolic content of soybean flour increased during storage after 2 weeks, with the highest increase for samples stored at 45 °C, and increased over 48 weeks of storage. It was hypothesised that the increase was due to depolymerisation of phenolics and the release of phenolic acids from other structures. While their study did not assess the antioxidant capacity, it has been established that phenolic compounds are the main contributors to antioxidant activity [59]. Conversely, Pinto et al. [7] reported a loss in total phenolic content and antioxidant capacity (β-carotene bleaching method) during long storage of soybean flour and protein isolates, reporting a significant correlation between these factors. Their study also determined that there was no correlation between isoflavone content and a reduction in antioxidant capacity, as total isoflavones did not decrease. However, a positive correlation existed between β-glucosides content and antioxidant activity by the ferric reduction (FRAP) and free radical (DPPH) assays when drying soybean [60]. They concluded that the antioxidant mechanisms of isoflavones had greater affinity as an electron donor [60]. In the present study, the significant drop in both CUPRAC antioxidants and total isoflavones at week 5 for flour stored at 40 °C uncovered, with recovery in week 10, may point to a correlation. More investigation is required, as this was not seen for samples stored at 2 °C.

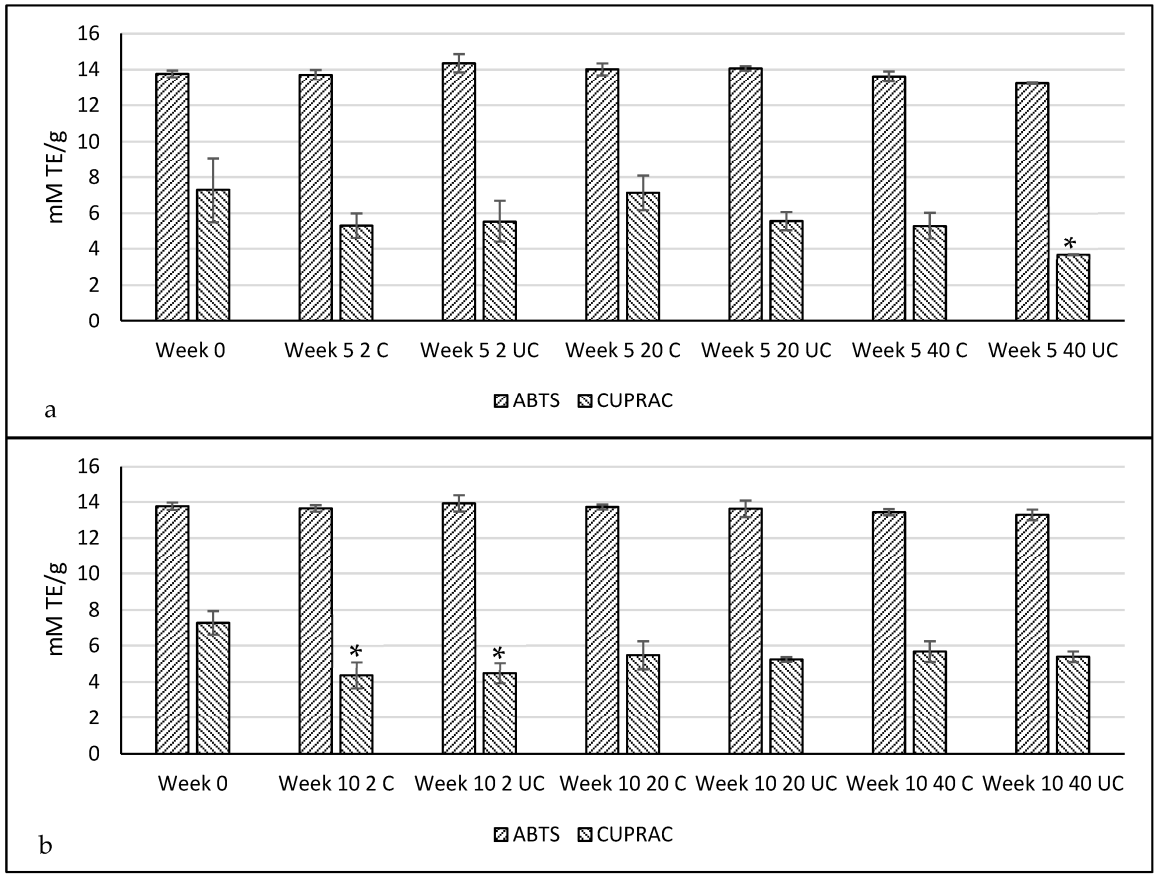


**Figure 5.** Antioxidant capacity of flour with an initial moisture content of 12%, stored for 5 weeks (**a**) and 10 weeks (**b**), compared to week 0 as control, stored at 2 °C, 20 °C and 40 °C, covered (C) and uncovered (UC). Data are mean ($n$ = 3) ± standard deviation, and * shows a significant ($p < 0.05$) difference to the initial content for each antioxidant assay.

## 4. Conclusions

During storage, shelf-stable food items may experience dynamic changes in conditions from a range of influencing factors. This study is the first shelf stability test for flour produced from dried SMB, with a focus on the functional characteristics of antioxidant capacity and isoflavone content, while tracking the changes in MC and $a_w$. In general, storage temperature influenced MC, $a_w$, colour and the conversion of malonyl isoflavones to their β-glucosides of flour prepared from a soybean by-product. Similar trends were observed for uncovered samples. Of note, there was an observable degradation of aglycones at week 5 when stored at 40 °C uncovered. The initial MC had less influence on all factors. However, it was possible to see that when stored at 20 °C covered, both 12% MC and 14% MC were able to sustain their MC independently, while $a_w$ remained acceptable for shelf stability.

Prolonged storage at 2 °C with a high RH% was the least preferred storage condition, as the $a_w$ increased to levels where mould growth was possible and metal chelation antioxidant capacity was significantly reduced. It was evident that SMB100 flour showed shelf stability when stored under ambient conditions, such as those reported for 20 °C in this study. Additionally, the functional properties of this adjunct flour have been shown not only to be able to be maintained, but that malonyl isoflavones can be de-esterified to form higher proportions of β-glucosides.

The storage time of 10 weeks can be seen as a limitation of this study, and extensive storage beyond this time may show negative changes, particularly under extended exposure to specific conditions. Future studies may be needed to determine the effects of long-term storage under ambient conditions. During this study, it was also noted that the antioxidant capacity of soy, its by-products and individual isoflavones remains a topic of debate. Further studies are needed to determine how antioxidant-rich fractions of SMB can influence the shelf life of processed foods.

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