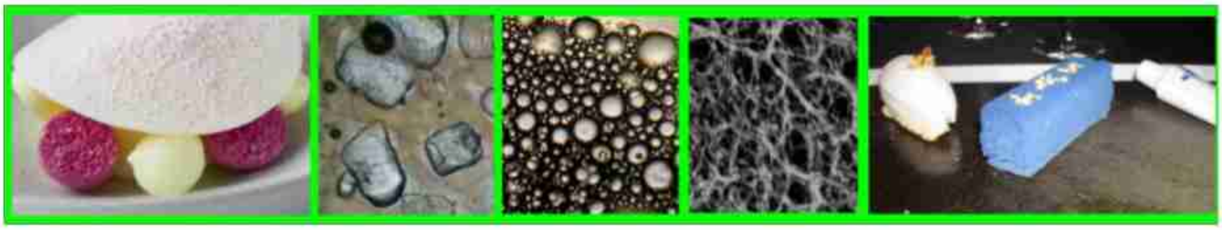

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Monitoring the bioactive compounds in culinary transformation of soymilk: An in situ quantitative NMR study

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ABSTRACT

Many products made from soybean are consumed in Asia. Soymilk, tofu and yuba are obtained by individuals at home. Changes of bioactive compounds during culinary process were rarely reported. In this study, the analytical method called "in situ quantitative nuclear magnetic resonance spectroscopy" (*is*q ^{1}H NMR) was applied to the quantitative determination of the variation in taste and functional molecules that are sucrose, lysine, arginine and valine during various culinary transformations of soymilk, involving fresh, thermal process, vacuum evaporation, tofu and yuba. Results suggest that thermal process at 100 ˚C increased the amount of free sucrose but significantly decreased the amount of lysine. Sweetness of soymilk could be simply enhanced. Evaporation of soymilk caused a higher reduction of lysine than thermal processing at 100 °C, indicating that complexation and Maillard reactions of lysine could be enhanced. The arginine content also decreased, albeit in less quantities. The changes of molecular conformation could also be monitored. In particular, resonances associated with the side-chain of valine were observed when most proteins were unfolded at high temperature. In mild acidic condition using glucono-delta-lactone as a coagulant, the amount of lysine was maintained higher than thermal process and evaporation while sucrose content was reduced. Tofu containing of lysine and sucrose could have a sweetness taste. However, there was no free sucrose in yuba. Understanding the change of taste molecules in soymilk during culinary transformation could lead to a better formulate soymilk products, thus this would be benefit to culinary application at home and industrial.

Key words: Molecular gastronomy, Soymilk, Sucrose, Amino, In situ quantitative ^{1}H NMR

INTRODUCTION

Food products prepared from soy (*Glycine max* (L.) Merr.) such as soymilk (obtained by hot water extraction from soybean), tofu (by protein aggregation of soymilk) and yuba (film on top of soymilk) are consumed all around the world, especially in Asia[1]. Because such products are reported to have nutritional advantages (soybean is rich in proteins and phytoestrogen[2]; soy products are free of lactose, cholesterol and gluten[3]), they have been extensively studied[4-5]. The protein composition, lipid, minerals, vitamins and organic acids in soymilk were initially reviewed by García and co-workers[6]. Volatile compounds that presented the characteristic of soymilk were analyzed using solid-phase microextraction-gas chromatography. Commercial soymilks could contain a total of 30 volatile compounds, hexanal, benzaldehyde, and pentanal being major volatile[7]. In 2003, when fermentation by lactic acid bacteria (LAB) and bifidobacteria was applied to produce the fermented soymilk, reduction of stachyose and raffinose together with the increment of sucrose, fructose, glucose and galactose were observed[8]. Proteins in soymilk played an important role in texture of tofu because of their cross link functionality. Soy proteins mainly contain glycinin and β-conglycinin in which glycinin had bigger molecular weight than β-conglycinin. Selective denaturation of these proteins by two-step heating that was treating at 75 °C for 5 min followed by 95 °C for 5 min therefore resulted in a more stable tofu than by one-step heating process. According to the two-step processing temperature of soymilk, the resultant tofu gained higher Young's modulus and decreased the syneresis rate[9]. The mixture of water, oil and proteins in soymilk represented the oil-in-water emulsion that stabilized by proteins. However, formation of a thin film on the soymilk surface could be observed during heat treatment. These yuba generally contained 57 % protein and 24 % oil but the study of yuba formation mechanism revealed that protein particles especially β-conglycinin were main functional molecule[10]. Yuba could be formed without oil but not β-conglycinin. Understanding the change of bioactive compounds during culinary transformation could lead to a precise recipe or innovation in food industry.

Chemistry techniques based on extraction, fractionation, chromatography and spectroscopy such as high-performance liquid chromatography (HPLC) and capillary electrophoresis (CE)[11] have been regularly used to determine the content in proteins, saccharides, isoflavones and vitamins in soymilk and soybean related products. Nuclear magnetic resonance (NMR) spectroscopy has been introduced in food analysis from 1990s[12], not only for structure identification but also for quantification (q NMR) because spectra of all components with concentrations higher than the detection threshold (around 0.1 mg in 1 g of fresh product) can be detected at high resolution[13]. Recently, a new non-destructive and fast analytical method called "*in situ* quantitative nuclear magnetic resonance spectroscopy" (isq ^{1}H NMR) was applied to the quantification of bioactive compounds in plant and animal tissues with no preliminary preparation of samples[14].

Both q NMR and isq NMR were applied to a lot of food systems, such as solutions, pigments extracts (for example, solutions containing chlorophylls, their derivatives, and carotenoids, extracted from immature pods of *Phaseolus vulgaris* L.). The q ^{1}H NMR gives higher precision for chlorophyll a, a', b, b', their derivatives and carotenoids than other methods[15]. The content of saccharides in aqueous extraction from onion (*Allium cepa* L.) bulbs was also studied, revealing rapidly the content in 3 saccharides, 17 amino acids and 5 organic acids[14]. During methodological comparisons of isq ^{1}H NMR and other methods based on extraction, it was shown that a higher concentration of glucose and sucrose in carrot root was observed by isq ^{1}H NMR method compared with other methods[16-17]. Therefore, isq NMR is useful for the quantitative determination of the bioactive compounds in food matrix. Although soymilk and other related products were good nutrition resource, their bitter taste prevented them from consumer acceptance. Therefore, the aim of this work was to follow the changes of bioactive compounds which were responsible for taste and nutrient like saccharides and amino acids in soymilk after the culinary transformations such as thermal process, evaporation, coagulation (tofu) and film formation (yuba) by using the isq ^{1}H NMR method. Sucrose was the main saccharide in soymilk and it also represented the importance of sweet taste and energy providing molecule. Three amino acids that are arginine (Arg), lysine (Lys) and valine (Val) were monitored as Arg and Val contributed to a bitter taste while Lys contributed to a sweet taste of soymilk. Moreover, Lys and Val are essential amino acid while Arg and Lys were important for building muscle as they involved in the protein synthesis.

MATERIALS AND METHODS

All samples, hardware, and solutions used at each step were precisely weighed using a balance with precision 0.00001 g (Mettler Toledo AG 153).

Chemicals

Deuterated water (D_2O, 99.9 %), 3-(trimethylsilyl)propionic-2,2,3,3-d4 acid, sodium salt (TSP; 98 %) were from Sigma-Aldrich. Ethylacetate (99.8 %) was from *Chromanorm*. Methanol (99.9 %) and chloroform (99.0 %) were from *Carlo Erba*. Sucrose, for biochemistry and microbiology was from *Merck*. L-Arginine (98 %), L-;ysine (98 %) and L-valine (98 %) were from *Sigma*.

Sample preparation

Commercial soymilks ("*Bio boisson au soja, nature*") were purchased daily from the local grocery store. Experiments were carried out within 3 days after the box was opened and stored at 4 ˚C. In order to analysis, sample was mixed with D_2O at 7:1 ratio (w/w).

Commercial soymilk was mixed with D_2O. For thermal processing, the commercial soymilk was heated at 75 °C or 100 °C for 60 min using electrical hot plate (IKA RCT Classic) controlled with a thermocouple and stirred magnetically (spinning rate 1000 rpm). After cooling, liquid samples were mixed with D_2O.

A variation of water content in different samples (fresh and thermal process) may lead to the difficulty in comparison of the obtained result. Therefore, dehydration of soymilk samples involving fresh, thermal process, vacuum evaporation, tofu, and yuba was considered. For evaporated soymilk, the commercial soymilk was transferred into a 250 mL round-bottom flask and vacuumed for 6 hrs. A water bath temperature was set at 60 ˚C. Evaporated soymilk was cooled down to room temperature before being frozen at -20 °C. For making soft tofu, 60 mg of glucono-δ-lactone (GDL) were added in 20 g of commercial soymilk. The solution was heated in a water bath at 95 ˚C for 60 min, before storage at room temperature; soft tofu was formed during this final storage, then cleaned under running distilled water until water was clear. For yuba, the same process as producing a heated soymilk was applied but temperature was kept at 85-90 ˚C without stirring for 3 hours. After this thermal processing step, a soft film on the top surface was carefully removed and rinsed by distilled water until washing water was clear. Samples were frozen (-20 ˚C) overnight and freeze-dried in lyophilizer machine for 2 days.

The lyophilisate resultant was dissolved in D_2O at a ratio of 1:35 (w/w). Solution samples of about precisely 700 mg were loaded into a NMR tube (quartz, 5 mm diameter). A capillary tube containing a known concentration of TSP was put in a tube before ^1H NMR acquisition. All analyses were done in triplicate.

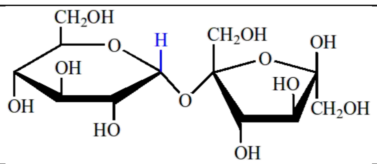
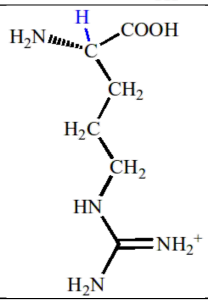
q ^1H NMR

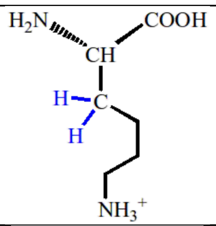
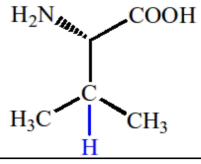
A superconducting Ultrashield 300 MHz (7.05 T) 54 mm magnet NMR spectrometer BZH 30/300/70 E *Bruker Biospin* (Germany) was used. All ^1H NMR spectra were recorded at a temperature of 21 ˚C in order to avoid different mutarotation equilibrium for the saccharides present in the samples[17] . For each spectrum, 64 scans of 32 k data points were recorded with a spectral width of 6172.8 Hz, an acquisition time of 5.3 s, and a recycle delay of 25 s per scan to allow complete relaxation and absolute quantification. The analysis of each sample was performed using D_2O as an internal lock. The acquisition procedure (shimming, gain signal and Fourier transformation using XWIN NMR 3.5 software) were automated.

Identification and quantification

The major components in soymilk samples were identified and quantified by q ^1H NMR. The identification was carried out by comparison with reference data from standard compounds and literature. The main saccharide was sucrose (Suc) and selected amino was arginine (Arg), lysine (Lys) and valine (Val). When TSP was used as reference for identification, the best results for quantitative determination were obtained using: the doublet at 5.42 ppm (H_1) for Suc, the triplet at 3.22 ppm ($H_{1\alpha}$) for Arg, the multiplet at 1.77-1.85 ppm ($2H_\beta$) for Lys, and the multiplet at 2.27 ppm (H_β) for Val as indicated in table 1. The identified compounds were quantified by reference to the peak area of the known concentration of TSP in capillary tube. The area of the interesting resonances in all spectra was integrated and autocorrected three times using *NMR Notebook 2.50* software. The area of the TSP resonance was used as a fixed reference of area equal to 1.

Table 1 The chemical shifts (ppm) and multiplicity of protons (highlight in blue) used for the quantitative determination of target compounds.

Compounds	Selected proton in structure	Chemical shift of selected proton (ppm)
Suc		5.42 (d)
Arg		3.22 (t)

Lys		1.77-1.85 (m)
Val		2.27 (m)

RESULTS

Changes in bioactive compounds during soymilk processing were analyzed by isq ^{1}H NMR. Samples from all kinds were used in the experiments including fresh soymilk immediately after opening of the box. A typical ^{1}H NMR spectrum of a soymilk sample was recorded by using the TSP as reference to a chemical shift at 0 ppm. Amino acids region appeared at 0.8 – 3 ppm, saccharide region appeared at 3 – 5.6 ppm and water molecules appeared around 4.8 ppm. Suc is the major saccharide in soymilk as well as in other soymilk products. Charged amino acids Arg and Lys were present in low amount (0.006 – 0.085 %) in fresh soymilk (Figure 1). We investigated these compounds in soymilk samples after culinary transformation using both liquid samples (Figure 1) and dried samples (Figure 2).

The amount of selected compounds in thermal processed soymilk is shown in Figure 1. Val concentration is higher than Suc, Arg and Lys in all samples. After thermal processing, Suc content increased from 0.23 to 0.54 %, Lys decreased at higher temperature, but Val increased from 0.82 to 1.94 % by heating at 100 ˚C.

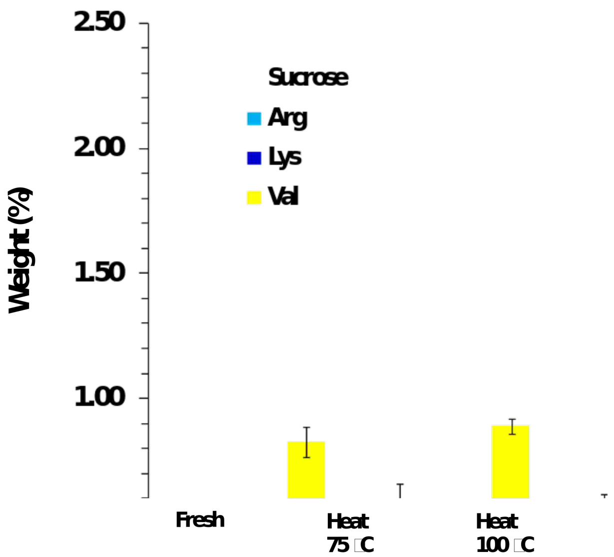


Figure 1 The amount of Suc, Arg, Lys and Val in fresh soymilk and after thermal processing. The percentages of bioactive compounds were calculated from the weight of the liquid samples.

Results from samples that were lyophilized are shown in Figure 2. Thermal processing increased Suc content from 5.57 to 7.06 %, Val content from 10.96 to 14.35 % but decreased the content in basic amino acid Arg and Lys. By evaporation, the amount of Suc and Val remained similar to the fresh soymilk, while basic amino acids Arg and Lys decreased to 0.27 % and 0.18 %, respectively, which was even lower than obtained from heated soymilk. In soft tofu, the content in Suc, Arg and Lys was much lower than the original soymilk by decreasing from 5.57 to 2.23 %, 1.50 to 0.32 %, and 0.79 to 0.34 %, respectively. Interestingly, in yuba, the Suc content was undetectable while the amount of Arg and Lys was similar to other treatments. Val concentration at 2.22 % was still the major content in soymilk product.

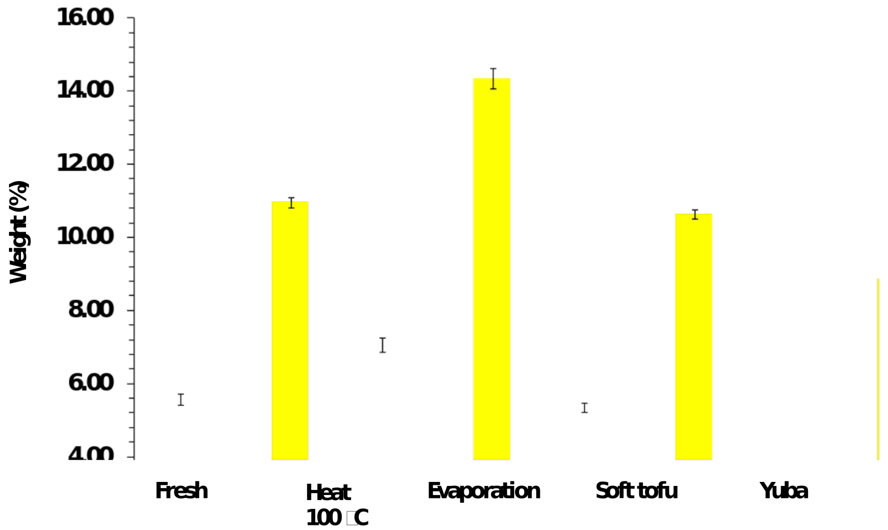


Figure 2 The amount of Suc, Arg, Lys and Val in soymilk, after culinary transformations. All samples were lyophilized for 2 days before dissolution in D_2O. The percentages of bioactive compounds were calculated from the weight of the dried samples.

DISCUSSION

See separate file, from the same authors.

DECLARATION OF INTEREST

The manuscript has not been submitted elsewhere. This study was funded by the Junior Research Fellowship Program 2011 from the French Ambassy, Bangkok, and partial supported from the Kasetsart University Research and Development Institute (KURDI).

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DISCUSSION

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Changes of Suc content during culinary transformation
Culinary transformations trigger changes in physical and chemical properties. Various chemical changes could occur during process but saccharides and proteins appear the main ones. Therefore, Suc, Arg, Lys and Val were selected for monitoring the chemical changes from the culinary transformation of soymilk. The result clearly revealed that Suc concentration increased after heating. This corresponds to the report that the amount of saccharides significantly increased after heating in yuba production[19]. The mechanisms for sucrose increase could relate to the interaction of Suc and other components. Soybean contained a sucrose binding protein (Suc transporter protein) as well as glycinin and lecithin that were able to bind sugar[20-21a, 18b]. In fresh soymilk, the doublet at 5.42 ppm (H_1) of Suc might be hindered due to Suc-protein binding. When proteins were denatured by thermal processing, free Suc molecules could be enhanced. Suc content was much reduced in tofu whereas coagulation was occurred. The protein network in this acidic coagulation tofu may squeeze out Suc. However some Suc could be trapped in tofu but not in yuba. Yuba on top of soymilk surface could be formed at the temperature of 65-75 ˚C when proteins connect into a network together with oil droplet. Reasons that no Suc was observed in yuba could be related to the structure of protein-lipid network where Suc (a hydrophilic molecule) could not bind. Therefore it could be removed by rinsing process during sample preparation before lyophilized. These suggested that a sweetness of soymilk could be simply enhanced by boiling of soymilk. Sweetness from sugar was lost when soymilk was processed into yuba.

Changes of amino content during culinary transformation
Two major proteins (glycinin and β-conglycinin) which represented 80 % of the total protein are present in soymilk. Glycinin is a plant globulin containing Arg (6.41 %), Lys (4.98 %) and Val (6.05 %), among other amino residues. The β-Conglycinin is composed of three protein subunits containing Arg (6.97 %), Lys (5.05 %) and Val (5.53 %), and other amino residues. Combination of these two proteins leaded to the total amount of Arg, Val and Lys at 6.65 %, 5.83 % and 5.01 %, respectively. In this study, the isq ^{1}H NMR detected only the content of free amino acid (AA). The observation in fresh soymilk revealed that ratio of Arg was higher than Lys which coincidentally corresponds to the level of amino residue content in total proteins. For free Val, the content of 10.96 % is much greater than Val residue in protein

(expectation of 5.83 %). High Val content suggests the uncorrelation between the amount of free Val and Val residue in protein.

In the thermal treatment, the amount of Arg and Lys in soymilk was reduced as compared to their content in the fresh soymilk. Extensive reduction of Lys was observed when temperature was increased from 75 ˚C to 100 ˚C. The opposite was found for the Val concentration, which increased significantly especially at 100 ˚C. The changes of these amino concentrations in the thermal treatment might be difficultly affected by hydrolysis because soymilk was heated in a non-acidic condition. Both Arg and Lys are basic amino residue which might be prone to oxidation and Maillard reaction in a presence of sugar especially xylose[22], causing a decrease in their concentration. The dramatic decrease of Lys indicates the high reactive property of Lys. Our finding based on the isq ^{1}H NMR method corresponded to the previous report on heat treatment and Lys stability[23-25]. Significant reduction of Lys in soybean meal after heat treatment by autoclave for a long time was reported[23]. Heat treatment of amino acid especially Lys in a presence of reducing sugar potentially converted reactive Lys to unreactive Lys. If excessive heat treatment was further applied, the amino content would be reduced by Maillard reaction[24]. Mottram D.S. reviewed that thermal treatment generated pathways for compound conversion especially for amino acid via Maillard reaction[25]. Maillard reaction products from soy produced the volatile molecules like furanmethanol and 4-hydroxy-2,5-dimethyl-3(2H)-furanone, while some generated compounds reacts with other compounds in food matrix yielding more complex molecules[25]. Extensive reduction of Lys over Arg in soymilk at 100 ˚C indicates that Lys is reactive to the reaction rather easier than Arg which corresponds to other reports[26]. Evaporation even produced lower amount of Arg and Lys. These indicate that a drastic Maillard reaction could also occur in a process using vacuum at low temperature. Therefore these results suggest that both Lys and Arg in soymilk were very sensitive to the culinary involving thermal process. Decreasing of Arg in soymilk from thermal process especially by evaporation could consequently reduce a bitterness of soymilk.

Val signal was significantly increased especially after thermal process at 100 ˚C. This might not correlate with Val concentration since some experiment reported that Val concentration was unaffected by heat in the heated soybean products[24]. This might due to the high sensitivity of the isq ^{1}H NMR method over chromatography technique which compounds were analyzed regardless of structural conformation. Since soymilk contained amino acids in both free and peptide form, they could be affected differently by heat. Dynamics of free Val could be monitored efficiently by NMR technique[27]. The chemical shift of Val side chain especially at H_{β} is known for the torsional dependence as described by Karplus parameter. The isopropyl group of Val side-chain generally preferred *trans* conformation but non staggered rotamers may presence from some free Val in fresh soymilk. Hence, increasing of Val signal at 100 ˚C in this study suggests that some restraint Val side-chain might rotate freely. The conformational change of Val side-chain might consequently lead to a bigger area at 2.27 ppm in the obtained spectrum. Taste was perceived by a mechanism that the taste molecule bound to receptor hence the binding was a molecular conformational dependence process[28]. While boiling soymilk usually provided less bitterness, conformational change of Val side-chain might therefore contribute in the process.

Physical properties of tofu and yuba were dramatically changed from soymilk when liquid becomes soft gel (tofu) and thin film (yuba). These physical change affected the concentration of Suc and amino. In tofu, a 60 % reduction of Suc was observed as related to a fresh soymilk while yuba contained none of Suc. Only few Suc was trapped during formation of protein network in tofu but it could not be trapped in yuba which generated from a formation between protein and lipid. Unfortunately, higher level of Lys was found in tofu and yuba as compare with thermal treatment at 100 ˚C. This corresponds to the report that unreactive Lys could be hydrolyzed to Lys in a presence of glucono-delta-lactone[29]. While yuba seems to composed of nonpolar compounds like protein and fat, there was less amount of *trans* Val. These suggest that natural sweetness of tofu could be contributed by Suc and Lys. Yuba tastes differently from tofu as it expected for a less sweetness because it contained no Suc and low *trans* Val. In this study, interactions and behavior of Suc and some amino in various treatment of soymilk were demonstrated, however many questions have been arisen including changing of these bioactive compounds in other food matrix.