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B-Group Vitamin and Mineral Contents of Soybeans during Kinema Production

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Abstract: Concentrations of several B-group vitamins, determined by high-performance liquid chromatography (HPLC), and minerals, determined by inductively coupled plasma atomic emission spectroscopy (ICP-AES), in soybeans during different kinema production stages were compared. After soaking soybeans in water, thiamine (B_1) content decreased, whereas riboflavin (B_2) content remained unchanged. Cooking had no influence on the B_1 content, but it enhanced the level of B_2 and niacin (B_3). Incubation of beans at 37°C for 48 h, when mixed with *Bacillus subtilis*, caused an increase in concentration of both B_1 and B_2. Vitamin B_1 levels decreased when either *Enterococcus faecium* accompanied *B subtilis* or the temperature was elevated for 18 h fermentation. Traditionally prepared kinema contained 8 mg B_1, 12 mg B_2, 45 mg B_3, 683 mg Ca, 4 mg Cu, 18 mg Fe, 494 mg Mg, 10 mg Mn, 1257 mg P, 2077 mg K, 13 mg Zn and $<0{\cdot}5$ mg of Cd, Cr, Pb, Ni and Na per kg dry matter. While the vitamin B_1 content was significantly ($P < 0{\cdot}05$) higher, the contents of vitamins B_2 and B_3 were significantly ($P < 0{\cdot}05$) lower in raw soybeans than those in kinema. Mineral concentrations were 3·1–8·3 times higher in raw soybeans than in kinema. © 1998 Society of Chemical Industry.

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Key words: soybean; kinema; B-vitamins; minerals; *Bacillus subtilis*

INTRODUCTION

Kinema is a meat analogue consumed by most of the inhabitants of the Darjeeling hills of West Bengal and Sikkim in India, Nepal and some parts of Bhutan. Traditionally, locally grown yellow-seeded soybeans are washed, soaked in water overnight, cooked by boiling until softened, crushed to grits, wrapped in fern leaves and sackcloth, and left to ferment for 1–3 days in a warm place (35–25°C). The resulting kinema is fried briefly in oil, cooked with vegetables, salt and spices to a thick curry and eaten as a side dish with boiled rice (Tamang *et al* 1988).

Bacillus subtilis, the most predominant microorganism in kinema, is responsible for its production. *Enterococcus faecium*, which occurs as an opportunist in all market samples tested, has no detectable influence on the growth of *B subtilis*, proteolytic activity, ammonia production or the final pH of fermentation. The sensory score of kinema produced in the presence of *B subtilis* and *E faecium* is significantly lower than that produced by the *B subtilis* alone (Sarkar *et al* 1993, 1994; Sarkar and Tamang 1994). The fermentation process and also kinema organoleptic acceptability are improved further by incubating sterilised beans at 45°C in the presence of *B subtilis*, leading to a more desirable product within a much shorter period (18 h), compared to the traditional fermentation process (Sarkar and Tamang 1995).

Estimation of dietary B-group vitamin and mineral intake presents a challenge, since these are often present in food in trace quantities. Vitamin and mineral conservation in foods is important to prevent marginal deficiencies. Public, industry and regulatory agencies have increased their awareness of the nutritional quality of food. The growth of the soy food industry and the

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advent of nutritional labelling requirements have necessitated the quantitation of vitamins and minerals in soy products.

Micro-organisms differ in their ability to synthesise vitamins. While some are unable to synthesise any of the vitamins needed, others may be able to synthesise some or all. Therefore, vitamin contents of various fermented foods may be greater than in the unfermented substrates (Wang 1986).

High-performance liquid chromatography (HPLC) is a useful alternative to chemical and microbiological assays because of increased specificity, sensitivity and reduced analysis time. In addition, several vitamins can be analysed simultaneously and contact with air, light and high temperatures can be minimised. The HPLC methods for B-group vitamin analysis have been reviewed extensively by Finglas and Faulks (1987).

Although kinema is a common dietary component (daily average per capita intake of approximately 50 g wet weight) for about 80% of the inhabitants of these regions, B-vitamin and mineral profiles of this food have not been investigated to date. This information is required to answer the following questions: what is the likely contribution of vitamin and mineral daily intake from kinema, and how does processing effect their levels? The answers will help in preparing experimental or household diets. Hence, the objective of the present study was to determine the influence of processing on these parameters during kinema production.

EXPERIMENTAL

All the chemicals used were of highest purity grade and the water used was deionised (conductivity $<0{\cdot}05$ μS cm^{-1}).

Bacteria

Bacillus subtilis DK-W1 (MTCC*1747 (*Microbial Type Culture Collection, Institute of Microbial Technology, Chandigarh, India)) and *Enterococcus faecium* DK-C1, used in this investigation, were representative of isolates from commercial kinema (Sarkar *et al* 1994).

Preparation of kinema

Seeds of soybean (*Glycine max* (L) Merrill cultivar 'local yellow'), purchased from seven retail outlets of a Gangtok town market in Sikkim, were mixed, cleaned, washed thoroughly and soaked in water (water : bean, 5 : 1 w/w) for 16 h at 22–25°C. After decanting the water, beans were mixed with fresh water (water : bean, 2 : 1 w/w), autoclaved at 121°C for 15 min and cooled to about 50°C. The beans were drained, transferred to a sterile polyethylene bag and pestled from outside the bag, so that about two-thirds of the beans were dehulled and crushed to give grits of mainly half-cotyledons.

The bacillus and enterococcus inocula were prepared by streaking plate count agar (Difco Laboratories, Detroit, MI, USA) and all-purpose Tween (APT) agar (Difco) slants, respectively, incubating those at 37°C for 18 and 24 h, respectively, introducing 5 ml of sterile water onto the cultures, scraping off the growths into tubes and agitating them for 30 s on a Vortex-Genie (Scientific Instruments Co, Springfield, MA, USA). Cell numbers were determined using a Neubauer's counting chamber and a phase contrast microscope. These suspensions were used as inocula, with concentrations of bacillus and enterococcus cells being 10^{6-7} and 10^{5-6}, respectively, per gram of sterilised beans. The beans were then distributed in approximately 50 g (wet weight) amounts in sterile 250-ml Erlenmeyer flasks plugged with cotton wool and incubated at 95% relative humidity in an environmental chamber. The process variables included: beans (unfermented or fermented) in the presence of *B subtilis* or *B subtilis* plus *E faecium*, and incubation at either 37°C for 48 h or 45°C for 18 h.

Preparation of samples and determination of vitamins

The entire procedure was conducted under dimmed incandescent light to avoid exposure to UV rays. Raw soybeans were ground to a fine powder. Soaked, cooked and fermented soybeans were blended for 1 min using a Bamix (Switzerland) Model 122 blender to a smooth paste. Moisture content was determined by drying approximately 10 g of homogenous powder and 5 g of homogenous paste samples at 105°C to constant weights.

Weighed (2–3 g) powder or paste samples were taken in 50 ml screw-cap teflon centrifuge tubes (Nalgene), mixed with 20 ml of 0·1 M hydrochloric acid (Ajax Chemicals, Sydney, Australia), and digested by heating at 121°C for 30 min. The pH of the cooled sample was adjusted to 4·0–4·5 using 2·5 M sodium acetate (Ajax), mixed with 1·0 ml takadiastase (Sigma Chemical Co, St Louis, MO, USA) solution (100 mg takadiastase in 1·0 ml of 2·5 M sodium acetate) and incubated in a shaking water-bath at 45°C for 25 min. The solution was then mixed with 2·0 ml of 45% (w/v) trichloroacetic acid (BDH Chemicals Ltd, Poole, UK), and heated for 5 min in a water-bath at 55°C. The medium was centrifuged (Centra-8R; International Equipment Co, Needham Heights, MA, USA) at 2500*g* for 10 min at 15°C, and filtered through a 0·45-μm filter (Millipore Corp, Milford, USA). The volume was made up to 50 ml with water and an aliquot was placed in a 5-ml amber glass vial for HPLC analysis (APFAN 1994).

The chromatographic system consisted of a programmable solvent module (No 126; Beckman Instrument, San Ramon, CA, USA), a Waters (Millipore) Model 510 pump for post-column reagent (for thiamine), a Waters μBondapak C_{18} (10-μm irregular particle size) reverse-

phase analytical column (150 mm × 3·9 mm) with a Waters C_{18} μBondapak guard column, a fluorescence detector (F1000; Hitachi Instruments, Danbury, CT, USA) and a C-R6A Chromatopac (Shimadzu Corp, Tokyo, Japan) integrator. For niacin determination, a normal-phase Hypersil (Shandon Scientific, Cheshire, UK) amine (3-μm spherical particle size) column (150 mm × 4·6 mm) and a Waters Lambda-Max 480 UV detector were used. A 20-μl sample was applied to the column with a Waters WISP710B automatic sample injector. The flow rate was constant at 1·0 ml min^{-1}. Analytes were separated using a binary gradient. Eluant A contained 0·005 M sodium salt of hexane sulphonic acid, adjusted to pH 3·4 with filtered and degassed 10% v/v phosphoric acid (Ajax), and eluant B was 95% v/v methanol (Merck, Dermstadt, Germany) in water. Post-column derivatisation of thiamine was achieved by continuously mixing the column eluate with the oxidising agent (freshly prepared 1% w/v potassium ferricyanide (Ajax) in 15% w/v sodium hydroxide (Ajax) and filtered through a 0·45-μm filter (Millipore)) at 0·7 ml min^{-1} at ambient temperature. The excitation and emission wavelengths for fluorometric detection of thiochrome (a fluorescent derivative of thiamine) and riboflavin were 375 and 430 nm and 430 and 530 nm, respectively (Wehling and Wetzel 1984). Niacin was detected at 260 nm. The run time was maintained for 15 min.

Identification of the peaks of interest was determined by comparison with retention times of external standards (thiamine hydrochloride (Sigma), riboflavin (BDH) and niacin (Sigma)) and by spiking samples with standards. The purity of the peaks was established by destroying the riboflavin ($Na_2S_2O_4$ reduction) or by preventing the oxidation of thiamine to thiochrome (Soliman 1981).

Calibration for each vitamin, using the external standards, had a correlation coefficient for linear regression of at least 0·9. Calibration graphs were prepared from the concentrations of the external standards of vitamins in the free form vs peak heights. Vitamin concentrations were quantitated by reference to these calibration graphs. Recovery efficiency was determined using samples spiked with the standard vitamin amounts.

Preparation of samples and determination of minerals

The procedure followed was based on a previously described method (APFAN 1994). Weighed quantities (2–4 g) of ground (made after drying in a hot air oven at 105°C for 24 h) or blended homogenous samples were placed in platinum crucibles, charred on a hot plate and then incinerated in a muffle furnace for 16 h at 500°C. After cooling, the ashes were mixed with 4 ml of 20% v/v distilled nitric acid (BDH) and again placed on the hot plate, evaporated to dryness and heated for 2 h in the muffle furnace to complete the digestion. Samples were allowed to cool at room temperature, made up to 15 ml with 2% v/v nitric acid and analysed using a Philips (Industrial and Electro-acoustic Equipment Division, Lelyweg 1, The Netherlands) Model PV8050 inductively coupled plasma atomic emission spectrometer (ICP-AES). Argon gas (99·997% pure) was obtained from BOC Gases, Australia. The operating conditions were as follows: power, 1·2 kW; radio frequency, 50 Mhz; observation height, 13 mm; analytical wavelengths of 315·8, 324·7, 238·2, 766·4, 279·5, 257·6, 589·5, 178·3, 213·8, 226·5, 267·7, 220·3 and 231·6 mm for calcium, copper, iron, potassium, magnesium, manganese, sodium, phosphorus, zinc, cadmium, chromium, lead and nickel, respectively.

Certified standard reference materials (SRMs) 1566a oyster tissues, obtained from the National Institute of Standards, US Department of Commerce (Washington, DC, USA), and IAEA-153 milk powder and V-8 rye flour, obtained from the International Atomic Energy Agency (Analytical Quality Control Services, Agency's laboratories Seibersdorf, Vienna, Austria) were used for method validation.

Statistical analysis

The data were analysed by determining standard deviation (SD), standard error of measurements (SEM) and analysis of variance (ANOVA) (Snedecor and Cochran 1989).

RESULTS AND DISCUSSION

Vitamins

Following HPLC analysis in the present study, 82% of thiamine, 84% of riboflavin and 75% of niacin were recovered. The SDs for standard recoveries were 2%.

B-group vitamin contents of differently processed soybeans during kinema production are shown in Table 1. Soaking of beans led to a significant decrease in thiamine content, but there was no change in riboflavin content. The remarkable decrease in thiamine content may be due to increased thiaminase activity or complex formation. Cooking had no significant influence on thiamine content, although riboflavin and niacin levels increased. The increase in riboflavin contents after cooking may result from a more complete extraction of coenzyme forms, eg FMN and FAD. B-group vitamin content of kinema was generally higher than in unfermented beans, although only significantly so for riboflavin. Thiamine content improved significantly in kinema prepared at 37°C for 48 h with *B subtilis* alone. However, the effect of fermentation on thiamine level is unclear (Ohta 1986). During oilseed and grain fermentations, thiamine levels can either decrease or remain constant (Roelofsen and Talens 1964; van Veen and Steinkraus 1970), whereas thiamine content of peanut

TABLE 1
Moisture and B-group vitamin contents of soybeans processed under various conditions

Sample[a]	*Components*[b]			
	Moisture ($g\ kg^{-1}$)	*Vitamins* ($mg\ kg^{-1}$ *dry matter*)		
		Thiamine (B_1)	*Riboflavin* (B_2)	*Niacin* (B_3)
Raw beans	$120{\cdot}9^{b}$ (0·2)	$16{\cdot}8^{a}$ (0·4)	$3{\cdot}4^{c}$ (0·2)	ND[c]
Soaked beans	$748{\cdot}8^{a}$ (3·6)	$6{\cdot}9^{bc}$ (0·3)	$2{\cdot}9^{c}$ (0·2)	ND
Cooked beans	$752{\cdot}3^{a}$ (0·9)	$5{\cdot}8^{c}$ (0·3)	$6{\cdot}8^{b}$ (0·5)	$36{\cdot}4^{a}$ (4·8)
Kinema (Bs, 37°C, 48 h)	$757{\cdot}9^{a}$ (2·2)	$8{\cdot}4^{b}$ (0·6)	$11{\cdot}6^{a}$(0·3)	$44{\cdot}8^{a}$ (5·6)
Kinema (Bs + Ef, 37°C, 48 h)	$743{\cdot}6^{a}$ (4·2)	$6{\cdot}4^{c}$ (0·4)	$9{\cdot}8^{a}$(0·8)	$25{\cdot}8^{a}$ (3·7)
Kinema (Bs, 45°C, 18 h)	$749{\cdot}0^{a}$ (0·7)	$6{\cdot}3^{c}$ (0·4)	$11{\cdot}9^{a}$ (0·5)	$42{\cdot}0^{a}$ (2·8)

[a] Bs, *Bacillus subtilis*; Ef, *Enterococcus faecium.*
[b] Values are the means of three replicate sets, with standard error of measurements in parentheses. Means with similar superscripts, within columns, are not significantly different ($P \leq 0{\cdot}05$).
[c] ND, not detected (detection limit, 14·7 mg kg^{-1}).

flour increases significantly during mould fermentation (Quinn *et al* 1975). Rajalakshmi and Vanaja (1967) reported increased thiamine level in idli and khaman after bacterial fermentation. During tempe production, thiamine content in soybeans is reduced by approximately 50%, whereas all other B-group vitamin levels increase significantly (Steinkraus *et al* 1960; Shurtleff and Aoyagi 1979; Murata 1985).

There is general agreement that riboflavin content in natto is higher than that of soybeans. A significant increase in riboflavin content during kinema production is consistent with previous reports on other fermentations (Roelofsen and Talens 1964; Wang and Hesseltine 1966; Murata *et al* 1967; Rajalakshmi and Vanaja 1967; Van Veen and Steinkraus 1970; Quinn *et al* 1975).

The present study found no significant change in niacin levels during kinema production, as was shown previously by Quinn *et al* (1975) in peanut flour fermentation. However, an increase in niacin content occurs during tempe production (Roelofsen and Talens 1964; Wang and Hesseltine 1966; Murata *et al* 1967; Van Veen and Steinkraus 1970).

Bacillus fermentation (at 37°C, 48 h) enhanced thiamine, riboflavin and niacin levels by 45, 71 and 23%, respectively. This shows that *B subtilis* has a great synthetic capacity for all these vitamins. These levels declined by 31, 18 and 74%, respectively, in the presence of *E faecium*, indicating that this bacterium uses readily available vitamins for growth and metabolism. *Enterococcus* requires vitamin-rich media for growth and would be expected to decrease vitamin levels in foods when it is present during fermentations. Since thiamine is susceptible to heat, kinema prepared at 45°C led to a 33% decrease in levels of their vitamin compared to that prepared at 37°C. Riboflavin and niacin are heat-stable, and no significant change in their concentrations during fermentation at the elevated temperature were noted.

In terms of overall B-group vitamin content, *Bacillus*-fermented kinema produced at 37°C for 48 h was superior to that prepared by other fermentation conditions. Increase in these vitamins as a result of fermentation has important nutritional implications in communities where this food contributes to the formation of the only side dish of rice.

Minerals

The results obtained from the SRMs were in good agreement with the certified values (t-values at $P < 0{\cdot}05$ being 2·09–2·31), and the recoveries ranged from 84 to 110% (SD 2·1–4·6). Means values (g kg^{-1}) of mineral contents in raw soybeans and kinema are expressed on dry weight basis (Table 2). Mineral content of soybean seeds of the 'local yellow' cultivar compared favourably with other varieties (Abdullah and Baldwin 1984; Iskander 1987; Van der Riet *et al* 1987). Slight variations were probably due to mineral contents of soybean seeds being affected by genetic factors, agricultural methods, soil composition, fertilizer usage and climate (Iskander 1987). Kinema contained significantly lower levels of minerals than found in raw beans. Potassium, the most abundant mineral in raw beans, showed the largest reduction (8·3 times) during kinema production. In the kinema production process, soaking water as well as cooking water are discarded, and this may be responsible for the six-fold depletion in mineral levels. However, kinema mineral content is influenced by the mineral content of the water used for soaking soybean seeds. Since, in practice, spring water is used for soaking mineral content of traditional kinema may not be less than that observed and may even be higher in some instances than in raw beans. Indeed, the levels of most minerals in tempe prepared with tap water is higher than corresponding values in raw soybeans (Van der Riet *et al* 1987). Calcium, iron, phosphorus and pot-

TABLE 2
Mineral contents of raw and processed soybeans

Minerals[a]	*Contents (mg kg^{-1} dry matter)*[b]	
	Raw beans	*Kinema*[c]
Calcium	2273 (42)	683 (16)
Copper	14·4 (0·2)	4·2 (0·1)
Iron	96·2 (5·2)	18·0 (0·7)
Magnesium	2443 (114)	494 (10)
Manganese	29·4 (0·2)	9·6 (0·3)
Phosphorus	5892 (172)	1257 (31)
Potassium	17 317 (662)	2077 (55)
Zinc	42·1 (0·4)	12·7 (0·5)

[a] Cadmium, chromium, lead, nickel and sodium were not detected (detection limit, 0·5 mg kg^{-1} dry matter).
[b] Values within rows are statistically different ($P < 0·05$) means, with standard error of measurements in parentheses, of three replicate sets.
[c] Prepared using the optimised traditional method (*Bacillus subtilis*, 37°C, 48 h).

assium concentrations in kinema are similar to those reported in natto (Ohta 1986). Importantly, levels of toxic elements such as cadmium, nickel and lead were below the detection limit. Despite such large losses in minerals during processing, kinema still contains appreciable quantities of calcium, magnesium, phosphorus and potassium.

The results indicate that kinema is a valuable source of B-group vitamins and minerals, particularly where intake from other sources is marginal. The data provided in this study might be the basis of formulating a balanced diet and improving nutritional status by food fortification to prevent micronutrient deficiencies.

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