ISOFLAVONE LEVELS AND THE EFFECT OF PROCESSING ON THE CONTENT OF ISOFLAVONES DURING THE PREPARATION OF SOYMILK AND TOFU

MOLAMMA P PRABHAKARAN
B.Sc (Tech), UDCT, Mumbai.

A THESIS SUBMITTED

FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

FOOD SCIENCE AND TECHNOLOGY PROGRAMME

DEPARTMENT OF CHEMISTRY

NATIONAL UNIVERSITY OF SINGAPORE

2005

ACKNOWLEDGMENTS

I would like to take this opportunity to express my sincere gratitude to my supervisors, Dr. Conrad O Perera and Dr. Suresh Valiyaveettil for introducing me to the field of phytochemicals and for placing outstanding working facilities at my disposal. I am deeply grateful to them for their support, encouragement, patient guidance and suggestions in bringing this thesis to completion. I am also thankful to Dr. Philip J Barlow for his continuous support and advice during this research tenure.

Thanks are also given to my colleagues in the Food Science & Technology programme, and especially to Dr. Lina Goh, Ms. Nang Sabei Myint and Ms. Mya Mya Khin, who have given me great help in my research work. I would like to thank Ms. Ravinder Kaur from Unicurd Food Company in helping to carry out different soy based preparations and for providing the samples. I appreciate the great help from Madam Lee Chooi Lan for her numerous acts of help in solving day to day laboratory problems. Special thanks also go to ADM Company, USA for providing me with a free gift of defatted soy flour during this research work. I am grateful to the National University of Singapore for providing me the research scholarship and funds to let me have this great opportunity to complete this research study.

I am extremely grateful to my family members and especially to my husband, Mr. Biju Nair for his substantial support with endless love, advice and encouragement in my life. Last but not least, many thanks to all those who have contributed in one-way or another in making this thesis possible.

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SUMMARY

Isoflavones are phytoestrogens, belonging to a group of phenolic compounds found in soybeans and soy foods. The parent isoflavones in soybeans are genistein, daidzein and glycitein, while their respective glucosides are genistin, daidzin and glycitin. Others include their corresponding acetyl and malonyl glucosides. These compounds have been associated with the decreased incidence of different types of cancers, cardiovascular diseases and osteoporosis. With the several health benefits associated with these compounds, this research work was set out to examine the effect of processing on the content and composition of isoflavones in different soy products during their manufacture as well as to study the content and composition of various soy based health products, supplements and infant formulas.

Initial investigations were aimed at choosing an ideal method for efficient extraction of isoflavones, followed by its quantification. An RP-HPLC method was developed and it was applied for the quantification of isoflavones in different soy based products, which proved successful. LC-MS using ESI interface, was further used for the peak identification studies.

Evaluation of the effect of different extraction methods and UHT heat treatments on isoflavones in the prepared soymilks was carried out. Samples were drawn at different points during the processing and were analyzed for their isoflavone concentrations. Results showed that hot grinding caused a higher extraction of isoflavones into the

soymilk than cold-grinding process. However, direct or indirect heating in the UHT process did not cause a difference in the concentration of isoflavones in the final soymilk obtained. Tofu was made by the coagulation of soymilk with salt or acid to produce a soy protein gel which traps water, soy lipids and other constituents in the matrix. Firm tofu was prepared using different coagulants and the quantification of isoflavones in the tofu and separated whey were carried out. This study further evaluated the yield and physical properties such as the moisture, texture and color of tofu prepared from different coagulants. Among the different coagulants studied, calcium sulfate was identified as the most suitable coagulant for tofu making in terms of its high yield, retention of maximum amount of isoflavones and in obtaining a firm, but smooth tofu. Selecting an appropriate processing condition can therefore result in retaining higher amounts of isoflavones in the soy products; soymilk or tofu, thus reducing their loss into the by-products of the process.

Analysis of isoflavones in different soy based health products and supplements were also carried out, and the possible isoflavone intake, calculated according to the recommended dosage levels showed a higher degree of variability from product to product. Similarly, soy based infant formulas had wide variations in their isoflavone levels. This research also highlights the need for expressing the concentration of isoflavones in a standardized manner, namely, in aglycone equivalent concentrations to minimize the differences in the molecular weights of the different isoflavones in different products.

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ABBREVIATIONS

BSA	Bovine serum albumin
CIE	Commission International de L'Eclairage
DAD	Diode array detector
ERβ	Estrogen receptor beta
ESI-MS	Electrospray ionization- mass spectrometry
GC-MS	Gas chromatography- mass spectrometry
GDL	Glucono-δ-lactone
HCl	Hydrochloric acid
HDL	High density lipoprotein
Hg	Mercury
HPLC	High performance liquid chromatography
HRT	Hormone replacement therapy
LDL	Low density lipoprotein
LMW	Low molecular weight
LOX	Lipoxygenase
MS	Mass spectrometry
NaOH	Sodium hydroxide
6OAcGlc	6"-*O*-acetyl glucoside
6OMalGlc	6"-*O*-malonyl glucoside
ODMA	*O*-desmethylangolensin
PDA	Photo diode array
RP- HPLC	Reversed phase- high performance liquid chromatography
SBIF	Soy based infant formula
SD	Standard deviation
SPI	Soy protein isolate
TFA	Trifluroacetic acid
TI	Trypsin inhibitor
TPA	Texture profile analysis
UHT	Ultra high temperature
UV	Ultra violet

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PART I

INTRODUCTION AND EXPERIMENTAL

CHAPTER 1

INTRODUCTION AND LITERATUR REVIEW

1.1 SOYBEAN

1.1.1 Origin of soybeans

Soybeans are believed to have originated in China 4000-5000 years ago. The first written record of the plant was contained in the book *Materia Medica* by the Chinese emperor Shen Nong in about 2838 B.C. **(Anonymous, 1993).** The soybean was considered one of the five sacred grains, along with rice, wheat, barley and millet essential to Chinese civilization. However large-scale production of soybean varieties did not occur until the 1920's. Since that time, world production of soybeans has increased by 400% **(Anonymous, 1993).** Much of the demand for soybeans in the United States, other developed countries and in many Asian countries derives from its popularity as a cooking oil source and as a base for margarine and other consumer products.

1.1.2 Agronomic characteristics

Botanically, soybean belongs to the family *Leguminosae*, subfamily *Papilionoideae* and the genus *Glycine*, L **(Liu, 1997)**. The cultivated form, called *Glycine max* (L.) Merrill, grows annually. The seeds are nearly spherical in shape with an average seed weight of 120-180mg. Soybean is well known for its variation in physical properties as well as in its chemical composition **(Liu, 1997)**.

1.1.3 Composition of soybean

An excellent source of good quality protein, the soybean consists of about 38 percent protein, which is about double the protein content of even the other protein-rich legumes; 18% oil, 15% soluble carbohydrates, 15% insoluble carbohydrates and 14% other components (moisture and ash). The average chemical composition of soybean seed on a dry weight basis is shown in Table 1.1 **(Liu, 1997)**.

Table 1.1: Average chemical composition of soybean seed (dry weight basis)

Whole soybeans	U .S. soybeans (%)	Japanese soybeans (%)
Crude protein	40.70	39.20
Crude fat	22.50	18.40
Carbohydrate	31.90	37.40
Ash (mineral)	4.90	5.00

Most plant sources are deficient in one or more of the essential amino acids. Soybean is no exception and is limiting in methionine, followed by cyst(e)ine and threonine **(Eggum and Beames, 1983)**. However, soy protein contains sufficient lysine, which is deficient in most cereal proteins. It is also unique because of the presence of isoflavones. Isoflavones have an extremely limited distribution in nature, while soybeans and soy foods can be considered as the major natural dietary sources of these compounds **(Coward et al., 1993)**. Increasing evidence has indicated that soybeans might have cancer-preventive properties by epidemiological **(Adlercreutz et al., 1986; Lee et al., 1991)**, animal **(Baggot et al., 1990)** and *in vitro* **(Adlercreutz et al., 1992; Wei et al., 1993)** studies. Results from these studies have suggested that the isoflavones might be the contributing factors in prevention of cancer. These compounds also possess estrogenic **(Miksicek, 1993)**, antioxidative **(Naim et al., 1976; Wei et al., 1993)** and antifungal

(Weidenborner et al., 1990) properties. Recently there has been much interest among clinicians and researchers, in the potential role of soybeans and soy foods in preventing and treating chronic diseases.

1.2 SOY FOODS

Soybeans have been incorporated into the popular human diet throughout Asian countries **(Wang and Murphy, 1996)**. These soy containing foods are traditionally divided into two groups: fermented and non-fermented. The non-fermented soy foods include fresh soybeans, soymilk, tofu, soybean sprouts and toasted soy protein flours. Miso, natto, soy sauce and tempeh are representatives of the fermented soy food group. A group of soy foods often referred to as 'soy-added second-generation soy foods' recently started appearing in the market, include the soy hot dog, soy bacon, tempeh burger, soy yogurt, soy Parmesan cheese, soy-Cheddar, soy-noodles etc **(Wang and Murphy, 1994)**. However, they contain considerable amounts of non-soy ingredients in them.

1.2.1 Soymilk

Soymilk is a colloidal solution that is obtained as a water extract from swelled and ground soybeans. It is a very popular beverage consumed in the Orient. It is especially important for people who are allergic to lactose in cow's milk and is an attractive alternative to cow's milk. It is sugar and cholesterol free **(Chinyere et al., 1997)** and is very low in saturated fats and hence a popular health food for the health-conscious.

1.2.1.1 Composition of soymilk

Soymilk and cow's milk have approximately the same protein content (3.5 – 4.0%). The main deficiency of soybean protein as compared with the protein content of cow's milk and human milk is that of the sulfur containing amino acids. The chemical composition of typical soymilk is presented in Table 1.2, along with those of cow's milk and human milk **(Chen, 1989)**.

Table 1.2: Composition of soymilk, cow's milk and human breast milk

Item/100g	Soymilk	Cow's milk	Human milk
Calorie	44.00	59.00	62.00
Water (g)	90.80	88.60	88.20
Protein	3.60	2.90	1.40
Fat	2.00	3.30	3.10
Carbohydrates	2.90	4.50	7.10
Ash	0.50	0.70	0.20
Saturated fatty acids (%)	40 - 48	60 - 70	55.30
Unsaturated fatty acids (%)	52 - 60	30 - 40	44.70
Cholesterol (mg)	0	9.24 - 9.90	9.30 - 18.60

Soymilk is traditionally and still commonly made by soaking the soybeans in excess water, draining, grinding with additional water, extracting the raw soymilk from the soy pulp residue (okara) and cooking the soymilk **(Liu, 1997)**. Figure 1.1 shows the traditional method of preparation of soymilk, and tofu from it.

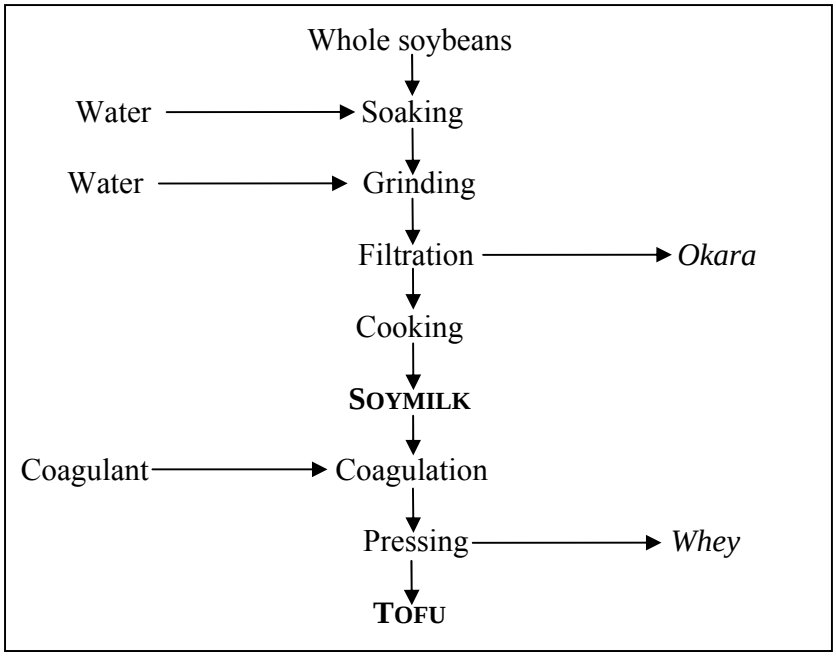


Figure 1.1: Schematic diagram for production of soymilk and tofu

1.2.1.2 Status of soymilk in Asian countries

Singapore imports more than 50,000 tons of soybeans annually, which are used mainly for the production of soymilk, tofu, soy sauce, and soybean oil **(Ang et al., 1985)**. There are many small backyard industries producing soymilk daily by the traditional method. The milk is usually sold freshly prepared in glasses or in take-away plastic bags and is consumed daily by persons in all age groups and in all economic strata. Large industrial productions of soymilk using modernized methods and sophisticated equipment resulted in soymilks packed in bottles, tetrapak, cartons and tear tab cans which are sterilized either by the conventional sterilization methods or by the ultra high temperature (UHT) treatment methods. However, in order to help the consumers enjoy quality soymilk and to prevent the production of a much diluted soymilk, many countries have established

soymilk quality standards. Table 1.3 shows the standards established for soymilk in some Asian countries **(Chen, 1989)**.

Table 1.3: Soymilk standards followed in Asian countries

Country	Product	Protein (%)	Fat	Total solids (%)
Singapore	soymilk	2.0		
	soy drink	<2.0		
Thailand	soymilk	2.0	1.0 (from soybean)	
Japan	soymilk	3.8		8.0
	soy drink	1.8		4.0
Taiwan	soymilk	3.4	2.0	
	soy drink	1.4	1.0	

In Singapore, the standard of identity for 'soymilk' specifies a minimum protein content of 2% (Soy foods association of America, 1996). Though higher protein soymilk has a slightly darker color, Singapore consumers prefer this slightly off-white color, associating it with a richer and creamier product.

1.2.1.3 Soymilk preparation methods

Preparation of soymilk in the Orient (traditional soymilk preparation method) basically involves the overnight soaking of whole soybeans in water which are then washed and ground with fresh water at a bean: water ratio of 1: 8 to 1:10. The slurry is filtered, whereby the okara is separated and the filtrate is boiled for a few minutes. This method of soymilk preparation is commonly used by both Chinese and Japanese people. The soymilk so obtained not only has a residual soybean flavor and aroma, but also a

characteristic aftertaste frequently described as “beany”, “painty”, “rancid” or even “bitter”. Lipoxygenase (LOX) catalyzes the hydroperoxidation of polyunsaturated lipids in the presence of molecular oxygen and the primary products are hydroperoxides. The initial products of LOX activity may be degraded into a variety of C-6 and C-9 products through the action of hydroperoxide lyases or isomerases. These volatile carbonyl compounds including the aldehydes, ketones and alcohols are partly responsible for the objectionable odor and off-flavors in soymilk. During the preparation of soymilk, soybean is ground with water and the LOX activity is greatly enhanced when the soybean is damaged or crushed. Therefore the inactivation of LOX is essential and it is usually carried out at a higher temperature (80-100°C) during the preparation of soymilk.

A number of new methods have been developed for soymilk production and a few of them have been commercialized. **Wilkens et al (1967)** developed the Cornell process, where unsoaked, dehulled soybeans were ground with hot water. The slurry was maintained at a temperature of 80-100°C to inactivate the LOX enzyme, further boiled in a steam jacketed kettle for 10min under stirring. After passing through a filter press, the resulting soymilk was formulated, bottled, sealed and sterilized at 121°C for 12min. Further to this, **Nelson et al (1976)** developed the Illinois process, where soaking of soybeans in water (optionally added with 0.5% $NaHCO_3$) was first carried out followed by blanching it in boiled water to inactivate the enzyme. This hydrated bean is further ground with cold water, slurry heated and homogenized. A very bland soymilk was obtained and the milk felt chalky in the mouth, which prevented its commercial success. A rapid hydration hydrothermal cooking process was developed by **Johnson et al (1981),**

where soybeans were ground into flour, made into a slurry in hot water and subjected to high pressure steam infusion (154°C for 30 sec). The slurry was adjusted for its solid content and centrifuged. A bland flavored soymilk with high yields of solids and protein is obtained by this method.

Soymilk is an ideal medium for bacterial growth and hence a thermal treatment is necessary to extend its shelf life. Heat processes are involved at several stages during soymilk preparation, including the pretreatment of beans and extraction to produce the soymilk, followed by either pasteurization or sterilization to increase its shelf life. Most commercial methods therefore employ single or multiple heat techniques to improve both milk quality and yield. By controlling the microbiology of the product and packaging it in appropriate containers, the shelf life of soymilk can be greatly extended and the product can be distributed over a wider area. Three basic types of heat treatments are usually carried out to extend the shelf life of soymilk (1) pasteurization (2) in-container sterilization (3) ultra-high temperature treatment. The details of heat treatment along with the shelf life for commercial soymilk, as explained by **Sizer et al (1989)** are shown in Table 1.4.

Table 1.4: Processes used for the extension of shelf life of soymilk

Treatment	Temperature	Time	Package	Shelf life
Pasteurization	75°C	15 sec	plastic bag	1 week (Refrig.)
Sterilization	121°C	20min	can glass bottle	2 years (Non refrig.)
UHT	140°C	2 sec	aseptic pouch	6 – 8months (Non refrig.)

Refrig. = refrigeration required; Non refrig.= refrigeration not required

UHT processing involves the use of high temperatures (135 - 150°C) for short time (a few seconds) to obtain a product, which is commercially sterile. The advantage of such higher temperature treatment for a few seconds is in obtaining sterilization with greatly reduced product sensory and nutritional damage. The benefits of UHT sterilization cannot be realized in conventional canning due to the long process times needed to achieve high temperature. Further an aseptic packaging technology marked the milestone for commercialization of soymilks. An aseptic packaging consists of sterilization of the packaging material, filling with a sterile product in a sterile environment and thereby preventing any re-contamination of the product **(Gosta, 1995)**. Additional advantages include low cost, light-weight, easy handling and stocking of products with longer shelf life. As a result, it is now used worldwide for the packaging of soymilks **(Shurtleff and Aoyagi, 1984; Sizer, 1989)**. There are two main types of UHT processing systems: a direct UHT system or an indirect UHT system. In the direct UHT system, the product comes in direct contact with the heating medium, followed by flash cooling in a vacuum

vessel and further indirect cooling to packaging temperature. The direct systems are divided into (1) steam injection systems or (2) steam infusion systems. In an indirect UHT system, heat is transferred from the heating media to the product through a partition. These are based on plate heat exchangers, tubular heat exchangers or scraped surface heat exchangers **(Gosta, 1995).** After UHT processing, the sterile soymilk is aseptically packaged.

Innovative technology developments by companies such as the Prosoya (Prosoya Inc., Canada) and TetraPak (TetraPak Co', USA) have resulted in the evolution of modern soymilk processing units with many additional advantages. Lately, Prosoya introduced the VS 30/40/200 system while TetraPak introduced the Tetra Ipps Soy4000B system for integrated soymilk processing and packaging needs. Every stage of soymilk production from soybean handling to UHT treatment and aseptic packaging was achieved by these systems. Soymilk with a reduced beany flavor and with high protein content was achieved by many of these continuous extraction and processing units. However, reports are not available on the evaluation of the isoflavone contents in soymilk prepared from these processing systems. The only studies being carried out were on the available lysine, thiamin and riboflavin content in soymilk during its thermal processing **(Kwok et al., 1998).** More interestingly, the fate of isoflavones on different types of extraction methods during such type of soymilk manufacturing is almost unknown. A similar understanding on the level of isoflavones during the different UHT treatment conditions also remains unknown.

1.2.1.4 Soy pulp or okara – the byproduct during soymilk making

The residue left from ground soybeans after extraction of water extractable fraction used to produce soymilk and tofu, is called soy pulp or okara **(Liu, 1997). Hackler et al (1963)** reported as obtaining 1.1 pounds of okara from every pound of soybeans processed into soymilk. Okara is a rich source of dietary fiber. It also contains a high quality of protein and appreciable amounts of oil. **Wang and Cavins (1989)** observed 30% of bean solids, 20% of bean protein and 11% of oil as being retained in okara. The summary of proximate composition of okara obtained from two different studies and as reported by **O'Toole (1997)** is shown in Table 1.5.

Table 1.5: Protein, fat, crude fiber and carbohydrates in okara expressed on a dry weight basis.

Protein %	Crude fat %	Crude fiber %	Carbohydrate %	Reference
18.20 - 32.20	6.90 - 22.20	9.10 - 18.60	-	Bourne et al., 1976
25.40 - 28.40	9.30 - 10.90	52.80 - 58.10	3.80 - 5.30	Riet et al., 1989

Bourne et al (1976) reported the proximate composition of soymilk residue (hulls included) from 30 cultivars with a mean moisture content of 76.8%. Crude fiber values reported by **Riet et al (1989)** was higher than those reported by **Bourne et al (1976),** but the reported soluble fiber levels of 12.6 -14.6% by **Riet et al (1989)** were similar to the levels for crude fiber that the latter reported (Table 1.5), and the. insoluble fiber amounts for 40.2 - 43.6% on a dry matter basis. Large quantities of okara produced annually by the soymilk and tofu industry pose a significant disposal problem. Okara contains crude fiber; namely cellulose, hemi-cellulose and lignin and have little starch or simple carbohydrates. Greater consumption of okara can thus cause diarrhea due to its high fiber

content. Its use as a human food is constrained by its high fiber content. However, it is a suitable dietary additive in biscuits and snacks because it reduces calorie intake and increases dietary fiber intake **(Khare et al., 1995).** A portion of the isoflavones is also fractionated into the okara during the preparation of soymilk.

1.2.2 Tofu

Tofu, also referred to as soybean curd, is a protein rich, bland tasting, non-fermented cheese-like product **(Shurtleff and Aoyagi, 1979a; Wang and Hesseltine, 1982)**. It is also consumed in significant amounts in Asian countries because of its inexpensive, high quality protein **(Koury and Hodges, 1968)**. By definition, it is water-extracted and salt- or acid- coagulated soy protein gel with water, soy lipids and other constituents trapped in its network **(Solomon et al., 2000)**. Tofu was even judged to be nutritionally equivalent to the protein derived from a mixture of eggs, fish and liver **(Muto et al**., **1963)**.

Tofu making process generally involves the preparation of soymilk (i.e, slurried soybeans), which is boiled, filtered and then treated at high temperature, with a coagulant that precipitates the soy proteins with the concomitant release of curds and whey; the curds are filtered off and moulded into shape under pressure. A large amount of original soybean mass is unavoidably lost into the okara during the first filtration step. While, tofu whey is a byproduct of relatively low nutritive value obtained during the preparation of pressed tofu. It also has a disadvantage of containing appreciable amounts of flatulence causing carbohydrates **(Liener, 1981; Rackis et al., 1981)**.

1.2.2.1 Types of tofu

Many different types of tofu have appeared in the market. Based on water content and textural properties, tofu is generally classified into soft, firm and extra firm tofu. Basically, these tofus are made in a similar fashion except for variations in the bean: water ratio, the type and concentration of coagulants and the amount of whey being pressed out.

Soft or silken tofu contains 88 – 90% moisture and approximately 6% protein. It has a soft cheese like texture, but is firm enough to retain its shape after slicing. Silken tofu is normally made from soymilk containing 10% solids. Relatively low concentrations of calcium sulfate or glucono-δ-lactone (GDL) are used as coagulants for commercial production of silken tofu. The coagulant and cold soymilk are mixed, run into a container and sealed. The container is then immersed in hot water (85-95°C) for about 45min to coagulate the soy protein. The resulting curd is cooled in the container by immersing in cold water, after which it is refrigerated.

Firm or extra firm tofus are mostly pressed tofus. There are two basic features in making pressed tofus. First, the coagulant is stirred into the hot soymilk rather quickly and vigorously. Second, the curds are broken and pressed. The heavier the weight or higher the pressure applied, the firmer the tofu. Therefore pressed tofu is ideal for use in pan frying, deep frying, freeze-drying and dicing into other foods or soups. Based on the methods of subsequent processing, tofu is also classified into plain tofu, frozen tofu,

dried-frozen tofu, deep-fried tofu, grilled tofu and fermented tofu. Their preparations involve either exposure to severe cold temperatures, further expulsion of water or even addition of seasoning ingredients and non soy components like spice powder or sweet and sour sauce. These are the many different types of tofu that have appeared in the market.

1.2.2.2 Tofu coagulants

Salts, acids and enzymes are the substances that have the ability to coagulate soy proteins. Coagulants commonly used for tofu making are (1) sulfate-type coagulants, including calcium sulfate and magnesium sulfate (2) nigari-type or chloride type coagulants, including natural nigari and calcium chloride (3) acidic coagulants including GDL, citrus juices, acetic acid and lactic acid. Coagulants are sometimes used in combinations in order to obtain a tofu of better quality. The most widely used tofu coagulant in the world is calcium sulfate, namely the di-hydrate form ($CaSO_4.2H_2O$). Calcium sulfate is relatively insoluble in water (3.0g/L); and thus it forms a colloidal suspension which, though difficult to mix uniformly in soymilk, works well in coagulation. It is well suited for modern mass production methods of tofu. In all coagulants consisting of calcium or magnesium salts, the positive double-bonded ions of calcium or magnesium are responsible for coagulating the soy proteins and hence they become part of the tofu and enhance its nutritional value. In terms of their isoflavone contents, different coagulants may have different ability to retain isoflavones in the tofu prepared, while few reports have discussed such an effect too.

1.2.2.3 Tofu gelation mechanism

Kohyama et al (1995) proposed a gelation mechanism of tofu. They suggested the gelation of tofu as a two-step process. The first step, being the protein denaturation by heat and the second being the hydrophobic coagulation accelerated by calcium ions. The heat denatured soy protein is negatively charged, and the calcium ions from calcium salt coagulants neutralize the net charge of the protein in the next step. Aggregation is induced due to the hydrophobic interaction of the neutralized protein molecules. Random aggregation further resulted in the formation of a gel. In addition to hydrophobic interactions, hydrogen bonds and charge-to-charge interactions might also be involved in forming the network.

1.2.2.4 Factors affecting the quality attributes of tofu

The textural properties of tofu play an important part in influencing its quality and consumer acceptability, especially because of its bland taste. These include the hardness, chewiness, brittleness, elasticity etc, according to definitions by **Bourne (1978),** which can also be experimentally measured by using a textural measuring instrument. Good texture of tofu means it should be coherent, smooth and firm but not hard and rubbery. However, the yields of tofu are an important attribute of economic importance. High protein varieties of soybean yielded tofu with a higher protein content, which had a firmer and springy texture than the lower protein varieties **(Schaefer and Love, 1992).**

Tofu making is a complex interaction of many factors and it is not uncommon to find conflicting results among reports. The quality and yield of tofu are influenced by the

quality of soymilk and its subsequent coagulation process, while the quality of soymilk depends on the variety of soybean used and the preparation conditions of soymilk. Coagulation being the most important step in tofu making, it depends on the concentration and temperature of soymilk, type and relative amounts of coagulant, method of mixing etc. The most difficult part of tofu making is in determining the exact concentration of coagulant to be added to the soymilk, since it greatly affects the quality and yield of tofu. Whey appearance can also be used as an indication of coagulant amounts. Whey becomes transparent with amber or pale yellow color, if proper amount of coagulant is used. But, if too much coagulant is added, whey becomes yellowish in color with bitter taste and the curds will have a coarse texture. At a low concentration of coagulant, interaction of protein molecules induced by the coagulant is not enough to form a firm gel, while at higher concentration, increased interaction lead to compaction of protein matrix, resulting in increased syneresis and loss of water, whey protein and other soluble solids. Studies by **Kao et al (2003)** found that tofus with homogenous and uniform network with the highest protein recovery are obtained if prepared with optimum coagulant concentrations. The temperature of soymilk affects the tofu quality and reports suggested **(Beddows and Wong, 1987; Wang and Hesseltine, 1982; Ohara et al., 1992)** an optimum temperature range of 70-85°C, because tofu produced below 70°C was soft and watery whereas above 85°C the tofu was hard and uneven, with considerable loss of bulk yield. Additionally, a smooth and firm tofu is obtained by pouring coagulants into soymilk without further mixing, because stronger mixing result in not only hard curds, but also low yields **(Wang and Hesseltine, 1982)**. A time period of 20-25min is required for the complete coagulation process to occur, after the addition of coagulant to

the soymilk. Other factors critical during the pressing and molding of curds are the curd temperature, pressure applied to curds and pressing time. Pressing should be such as to allow the association of protein coagula and thereby to increase tofu firmness.

1.2.2.5 Tofu wheys

Whey is the expelled liquid obtained during the preparation of a firm tofu. The soy protein literature considers 'tofu whey' as a waste stream that cannot be economically processed or may even be toxic **(Uzzan and Labuza, 2004).** Tofu whey contains a high % of oligosaccharide sugars. These flatulence causing factors being removed into the whey, the tofu does not cause intestinal gas. Tofu whey contains a smaller % of protein, which is mainly of low molecular weight (LMW) proteins with a molecular mass below 16kDa **(Kao et al., 2003)**. The LMW proteins get well dispersed in the pores of the homogeneous network structure with few being dispersed into the whey, if optimum coagulant concentrations are used. However, insufficient coagulant concentrations can result in the loss of LMW proteins into the tofu whey. A certain amount of isoflavones might also be lost into the whey. But there are few reports which have evaluated the amount of isoflavones being lost into the tofu wheys **(Wang and Murphy, 1996).**

1.2.3 Other soy based products

1.2.3.1 Soy supplements and health products

Extracted isoflavones are commercially available in the market as dietary supplements. Dietary supplements containing soy extracts are more acceptable in many countries and they are growing in numbers over the years. Available in the form of capsules and tablets,

they are widely commercialized as an alternative therapy for alleviating menopausal discomforts, and are also advertised for the prevention of menopause related diseases such as osteoporosis **(Penalvo et al., 2004)**. Moreover, the manufacturers of soy supplements are often competing to produce a concentrated pill of isoflavones. These manufacturers also label these products with high isoflavone contents and thus promoting their own individual products as the best. However consumers are often unaware of the consequences that may result from a self-induced mega dosing of these compounds **(Setchell et al., 1997)**. At high dosages, isoflavones may act as antagonists of estrogen **(Ren et al., 2001)**. Very disturbing is the fact that, many soy isoflavone supplements are flooded into the market with wide ranging claims and little regulation exits regarding their manufacture or efficacy **(Setchell et al., 2001)**. Moreover, the units of labeling the concentration of isoflavones done by these manufacturers are many times misleading. One of the major issues regarding isoflavones is the question regarding the safety of phytoestrogens. There is abundant evidence in animals that phytoestrogens may delay reproductive status, as for example infertility observed in captive cheetahs that were fed a high phytoestrogen diet. California quail is another example, which has reduced breeding owing to a diet rich in phytoestrogens **(Fitzpatrick, 2003).** There is a paucity of data to confirm that isoflavone supplements are as nutritionally effective as isoflavone-rich foods.

Additionally, there are the soy based health products, available as 'health supplements', intended to improve the health of women either for weight management or as instantaneous nutritional beverages. These products are commonly prepared from either

soy protein isolate or soy protein itself. Reports on the analysis of isoflavones in soy supplements sold in the United States **(Nurmi et al., 2002)** and isoflavone levels in oral / enteral diets from Brazil **(Genovese and Lajolo, 2002)** are available, while reports on the profile and content of isoflavones in soy supplements and health products from South East Asia are fewer. Soy isoflavone supplements and health products are plentiful in nutrition/ health shops in Singapore, Malaysia, Thailand and Indonesia. An analysis of isoflavone contents in various soy supplements and health products if carried out can be useful in evaluating the total amount of isoflavones being ingested after their consumption. A standard unit of expressing the concentration of isoflavones in the products might be even more appropriate, enabling a direct comparison of the total amount of isoflavones in different products available over-the-counter.

1.2.3.2 Soy based infant formulas

The development of soy based infant formulas (SBIFs) grew out of need for a non-milk based formula alternative for infants who had intolerance to lactose. These are another group of foods consumed to a greater extent by infants with allergies to cow's milk protein **(Badger et al., 2002).** Earlier, SBIFs were made of soy flour which compared with soy protein isolate (SPI) had a lower protein content and digestibility. SBIFs have several non protein components in it such as soy carbohydrates, fibers, protease inhibitors etc. By 1960s soy flour replaced SPI for SBIF making, where the used SPI had a high protein digestibility and or corrected amino acid score. Other recommended compounds and nutrients were added to SBIF to meet the requirements for the growth of infants. However, the recent concerns with SBIFs are with the phytochemicals found in them.

Isoflavones being bioactive phytoestrogens with hormonal and non-hormonal activities, may cause adverse effects in infants fed soy-based formulas. Studies by **Setchell et al (1997)** showed the isoflavone exposure in infants to be much higher than that of other age groups. However, no growth or developmental defects are related to this effect **(Merritt and Jenks, 2004).** Data on the composition of isoflavones in SBIFs are scant, while concerns are being expressed about the possibility of hormonal effects from exposure of infants to phytoestrogens from SBIFs. Thyroid abnormalities were documented in some case studies, where it was associated with ingestion of soy infant formula **(Pinchera et al., 1965).** For infants whose iodine intake is low or borderline or the thyroid function is compromised, there is potential for clinical concern. Hypothyroidism is also associated with infants fed soybean diets **(Fort et al., 1990).**

Reports are available on the isoflavone contents in SBIFs from the United States and Brazil **(Setchell et al., 1998, Genovese and Lajolo, 2002).** However, there are few reports, in which the SBIFs available in South East Asia have been analyzed quantitatively for their isoflavone content. The results of analysis on the isoflavone contents in these products if available could be further utilized in calculating the daily intake of isoflavones for infants of each age group. Though there are no deleterious effects reported with consumption of SBIFs, long term studies to assess the possible chronic effects of high isoflavone ingestion levels are necessary. Moreover, the isoflavones may adversely affect developmental processes influenced by sex steroids with potential consequences perhaps manifested only in puberty or adulthood **(Mendez et**

al., 2002). Hence it is most essential to evaluate the concentrations of isoflavones in SBIFs available in the market and the choice of recommending the formula with lesser amounts of these estrogenic compounds can be made possible.

1.3 ISOFLAVONES

Isoflavones belong to a group of compounds that share a basic structure consisting of two benzyl rings joined by a three-carbon bridge, which may or may not be closed in a pyran ring. The structure is generally simplified as C_6-C_3-C_6. This group of compounds is known as flavonoids, which include by far the largest and the most diverse range of plant phenolics. Besides isoflavones, other subclasses of flavonoids include the red and blue anthocyanin pigments, flavones, flavonols, aurones and chalcones **(Deshpande et al., 1984)**. Isoflavones differ from flavones (Figure 1.2) in that the benzyl ring B is joined at position 3 instead of position 2 **(Anderson and Garner, 2000)**.

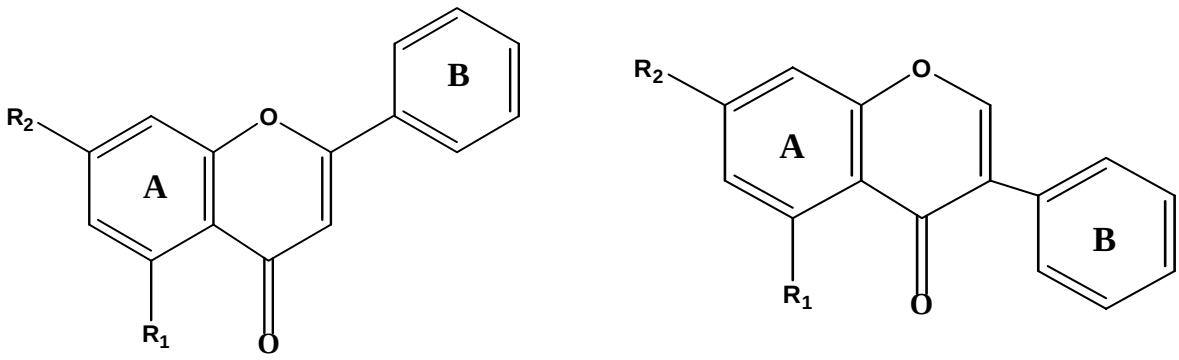
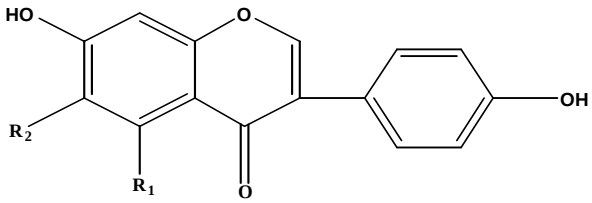


Figure 1.2: Structure of flavone versus isoflavone

1.3.1 Isomers, Structure and Occurrences

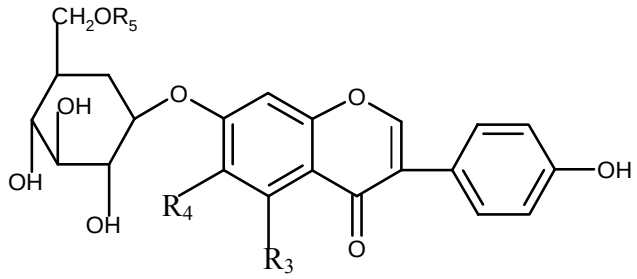
Soybeans contain three types of isoflavones (Figure 1.3), which are found to exist in four chemical forms **(Kudou et al., 1991; Barnes et al., 1994; Wang and Murphy, 1994).** They are the aglycones daidzein, genistein and glycitein; the glucosides daidzin, genistin, glycitin; the acetyl glucosides 6"-*O*-acetyldaidzin, 6"-*O*-acetylgenistin and 6"-*O*-acetylglycitin; the malonyl glucosides 6"-*O*-malonyldaidzin, 6"-*O*-malonylgenistin and 6"-*O*-malonylglycitin. Figure 1.3 shows the structure of 12 soy isoflavone conjugates.

Aglycones



R_1	R_2	Compounds
H	H	Daidzein
OH	H	Genistein
H	OCH_3	Glycitein

Glucosides



R_3	R_4	R_5	Compounds
H	H	H	Daidzin
OH	H	H	Genistin
H	OCH_3	H	Glycitin
H	H	$COCH_3$	6"-*O*-acetyldaidzin
OH	H	$COCH_3$	6"-*O*-acetylgenistin
H	OCH_3	$COCH_3$	6"-*O*-acetylglycitin
H	H	$COCH_2COOH$	6"-*O*-malonyldaidzin
OH	H	$COCH_2COOH$	6"-*O*-malonyl genistin
H	OCH_3	$COCH_2COOH$	6"-*O*-malonylglycitin

Figure 1.3: Chemical structures of 12 soy isoflavones

In soybean, isoflavones exist in the entire plant, including the seeds, leaves, stems, seedlings and roots. Though isoflavones are present in several species of legumes, most of the research has been done on soybean and soy foods, due to the widespread use of soybean in traditional and modern foods. In soybeans and foods derived from soy, isoflavones are found in different concentrations ranging from < 0.05mg to > 5.00mg/gm (**Coward et al., 1993; Barnes et al., 1994)**.

1.3.2 Soy and role of isoflavones in disease prevention

Apart from the potential cancer prevention effect, isoflavones have also been found to have other health benefits, including heart disease prevention, bone mass density increase to prevent osteoporosis and the reduction of postmenopausal syndromes in women (**Dixon and Ferreira, 2002**). Isoflavones may have benefit in decreasing the risk of cardiovascular diseases by reducing the level of total cholesterol as well as low-density lipoprotein cholesterol (**Carroll and Kurowska, 1995; Anderson et al., 1999)**. Structural similarities also exist between genistein and tamoxifen, a synthetic anti-estrogen that has been clinically tested as a chemo-preventive agent in women with high risk of breast cancer **(Gajdos and Jordan, 2002).** An early epidemiological study of Singapore Chinese women that included 420 healthy controls and 200 with histologically confirmed breast cancer indicated a direct correlation ship between soy consumption and reduced risk of cancer, and the effects appeared to be dietary rather than genetic **(Lee et al., 1992).** An inverse relationship between soy consumption and breast cancer risk was also obtained from the case-control studies carried among Japanese **(Hirose et al., 1995),** Asian American **(Wu et al., 1996)** and Chinese populations **(Dai et al., 2001)**. If it is true

that a regular consumption of isoflavones can help reduce the risk of certain diseases, then it is a good reason to educate people to consume isoflavone-rich foods. But, a confirmation of safety and efficacy is essential for the legal or public acceptance of isoflavone related health claims. It is also possible that soy substances other than isoflavones, such as the saponins, phytic acid, amino-acid composition or a protein-isoflavone interaction might be involved for their health benefits **(Ren et al., 2001)**.

1.3.2.1 Soy intake and heart disease

The cholesterol lowering effects of soy protein has been studied since 1967 **(Hodges et al., 1967)** and was followed by many others. A positive relationship between the starting cholesterol level of the subjects and a decrease in serum cholesterol following soy protein consumption was also established by **Potter et al (1993)**. Studies by **Anderson et al (1995)** using SPI and texturized soy protein were found to reduce cholesterol and the average amount of protein consumed was 47gm/day and a dose-response relationship between soy protein and serum cholesterol levels was also explained by these researchers. However, it is not well understood whether soy protein or isoflavones or a combination of these are responsible for the lowering of cholesterol **(Coward et al., 1998; Nurmi et al., 2002)**. Studies showed that an isoflavone containing soy product fed to rats resulted in a reduction in their cholesterol levels than those fed without isoflavones **(Anthony et al., 1996).** Apparently, the mechanisms associated with soy's beneficial effects on cardiovascular health are not fully understood. Suggested mechanisms of action include a reduction in low-density lipoprotein (LDL) cholesterol, an improvement in vascular reactivity and an inhibition of platelet aggregation **(Asahi et al., 1992;**

Williams and Clarkson, 1998). The preferential binding of isoflavones to estrogen receptor beta (ERβ) and the increasing recognition of the role of this receptor in the endothelial walls provide additional justification for increasing awareness of the heart-health effects of diets rich in these phytoestrogens **(Zhu et al., 2002)**.

The amount of isoflavones required for lowering the cholesterol can be achieved more easily by consumption of soy protein products, while the use of isolated soy protein products in the market represent an intermediate approach between diet and drugs. However, if isoflavones lower the cholesterol, then these concentrated forms of soy protein may not be needed, while incorporating reasonable amounts of soy foods (i.e., about two servings) into the diet should be sufficient to lower the cholesterol, because on a per protein basis, soy foods are 2 to 3 fold higher in isoflavones than many soy protein isolates **(Liu, 1997)**.

1.3.2.2 Soy intake and menopause

The consumption of soy foods has been proposed for the lower incidence of menopausal symptoms in Japanese women **(Lock, 1994).** Less than 20% of the Japanese women have hot flushes compared with 80% of the European women. This is partly attributed to the differences observed in their diet consisting of soy **(Murkies et al., 1998).** The estrogenic effect of isoflavones was an explanation for the above results **(Adlercreutz et al., 1992)**, though some conflicting reports also exist (**Woods et al., 1996**). The targeted intake of isoflavones from soy foods has been derived empirically because there are no guidelines for optimal levels of intake **(Setchell, 2000)**. Most of the studies suggested the

consumption of soy isoflavones, intact with soy protein so as to reduce hot flushes **(Germain et al., 2001**; **Knight et al., 2001).** This might be considered as a healthy alternative to hormone replacement therapy (HRT). More research is still required to understand the magnitude and significance of hormonal effects of soy consumption and to determine the extent to which soy foods can be a substitute for HRT. Many menopausal women are opting to take soy isoflavone supplements as an alternative to postmenopausal hormone therapy and for improving bone health. Though soy consumption appears to exert modest hormonal effects in both pre- and post menopausal women **(Kurzer, 2000)**, the effects are generally in the direction of providing positive health benefits. However, an exact definition on the dose and composition of isoflavone conjugates required for such type of disease alleviations has not been determined.

1.3.2.3 Soy intake and bone health

Osteoporosis is often associated with women in menopause. Studies by **Tham et al (1998)** suggest a diet rich in isoflavones as having a protective effect on bone mineral density. Similarity in structure also exists between soy isoflavones and ipriflavone, a drug used to inhibit bone resorption **(Valente et al., 1994)**. Ipriflavone is metabolized to daidzein, which itself is a soy isoflavone. **Erdman et al (1996)** found that the consumption of isolated soy protein containing isoflavones as significantly improving the bone mineral density in the lumbar spine relative to controls who were given casein/nonfat dry milk per day. The possible mechanisms of action to explain the beneficial effects of isoflavones on bone loss include preventing urinary calcium loss, beneficial effects on osteoblasts and influences on the secretion of calcitonin which

suppresses bone resorption **(Kurzer and Xu, 1997).** However, data supporting claims that isoflavone consumption improves bone health are not as strong as the data on lipid lowering and reduction of menopausal symptoms. More long term studies are required on bone density and fracture rates to determine safety, efficacy and appropriate dosages for these compounds.

Soybeans are also a rich source of calcium. Calcium from soy is well absorbed by human body, to the same extent as it is absorbed from dairy milk (**Heaney et al., 1991**). Reducing calcium excretion is important for optimizing bone calcium retention. The somewhat lower sulfur amino acid content of soy protein may help reduce calcium excretion in comparison to animal protein **(Pennington, 1998).**

1.3.2.4 Soy intake and breast health

Epidemiological data supporting an inverse relationship between soy isoflavones and breast health are probably weak at best. Some studies reported inverse associations between soy intake and breast cancer risk in Asian populations **(Lee et al., 1991)** while this was not observed for some other case control studies **(Horn-Ross et al., 2001)**. Soy isoflavones may be cancer preventive, if taken early in life. Effects in adulthood are less clear, and it is possible that they actually stimulate the growth of established breast cancer cells. According to **Peterson and Barnes (1991),** soy isoflavones can function as antiestrogens when administered in a high estrogen environment found pre-menopausally. While *in vitro* studies by **Zava and Duwe (1995)** in an estrogen-free environment showed that genistein stimulates the growth of estrogen-sensitive breast

cancer cells. Thus the time of life of exposure to phytoestrogens may actually determine whether the effects on carcinogenesis are beneficial. There is a particular concern about soy consumption in breast cancer patients taking tamoxifen because of the potential for isoflavones to interfere with the efficacy of this drug.

1.3.2.5 Major concerns about soy supplements and health products

Soy isoflavone supplements contain relatively high concentrations of isoflavones, mainly in the form of β-glycoside conjugates. When ingested, the conjugated isoflavones undergo hydrolysis by β–glucosidases in the jejunum and the aglycones, daidzein and genistein are released. Further metabolism occurs in the intestine resulting in the formation of specific metabolites. However, till today there is no evidence to support the absorption of conjugated forms of isoflavones. Moreover, it is the aglycones that are explained as having an affinity for estrogen receptors and other non-hormonal effects on the cell machinery. A study by **Izumi et al (2000)** conducted on Japanese women also reported isoflavone aglycones (fermented soybean extract) as more efficiently absorbed than the isoflavone glucosides (unfermented soybean extract). In Asian countries soy intake is a marker of traditional diet and lifestyle. Positive health effects between soy intake and health evolved mainly from the Asian population, which occurred after consumption of soy foods like soymilk and tofu rather than from soy supplements. Many questions however remain unanswered concerning the effects of isoflavone supplements for women.

1.3.3 Isoflavones as phytoestrogens

Phytoestrogens are phenolic compounds that are natural components of certain plant foods and are structurally similar to mammalian estrogens. Estrogens are hormones that our bodies make and are required for normal growth and development and to maintain good health. Estrogens are not only essential for the female reproductive system, but are also important for the bones, the heart and possibly the brain. Though phytoestrogens have estrogenic activity in humans, their activity is much lower than that of human estrogens. As a result phytoestrogens inhibit the activity of human estrogens and may provide desirable effects, for example in reducing the risk of breast cancer.

Isoflavones represent one of the classes of the so called phytoestrogens **(Setchell, 1998)**, are the bioactive non-nutrients that are strikingly similar in chemical structure to estradiol (Figure 1.4), the main female hormone. Superimposing the structure of isoflavones and estradiol, they become indistinguishable, and therefore fit beautifully into the pocket representing the binding domain of the estrogen receptor. Isoflavones therefore share many of the properties of endogenous estrogens and have the ability to behave as estrogen mimics **(Naftolin and Stanbury, 2002)**.

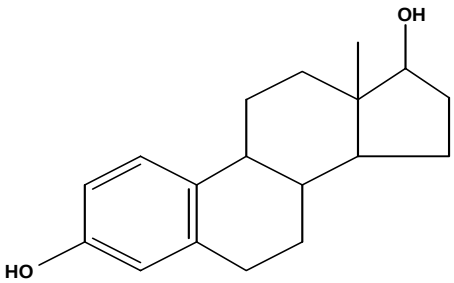


Figure 1.4: Structure of estradiol

Estrogens form complex with proteins called estrogen receptors (ERs). These complexes "dock" at sites within selected genes in the cell nucleus, switching those genes on or off. Cells have two types of estrogen receptors, α and β. Recent research has shown that human estrogen has a high binding affinity for the α-receptor while isoflavones have a high affinity for the β-receptor **(Kuiper et al., 1998)**. The predominance of ERβ in the cardiovascular system suggests that soy isoflavones may be partly responsible for the lower incidence of heart disease **(Kuiper et al., 1997; Cassidy and Faughnan, 2000)**

1.3.4 Absorption and metabolism

The definition for "bioavailability" is the "rate and extent to which the active ingredient or moiety is absorbed from a drug product and become available at the site of action" **(Uzzan and Labuza, 2004).** Thus the bioavailability of isoflavones is usually evaluated in terms of its plasma concentration and or urinary excretion.

Following oral ingestion, β–glucosidases, which are produced by intestinal bacteria, metabolize the glucosidic isoflavones to their corresponding aglycones. Before absorption occurs, however, intestinal bacteria may further metabolize the isoflavone aglycones to isoflavone metabolites, specifically genistein to p-ethyl phenol and daidzein to equol and or *O*-desmethylangolensin (ODMA), all of which may also be absorbed **(Heinonen et al., 1999)**. Following absorption, isoflavones undergo hepatic conjugation to glucuronic acid or sulphate **(Setchell 1998; Setchell and Cassidy, 1999)** to produce forms that are measured in biological fluids. Thus intestinal bacteria are central for the

absorption and metabolism of isoflavones. A clear pattern of relative bioavailability among the different aglycones or the corresponding conjugates is unknown. The main difficulty in finding a clear pattern seems to be the large variability among individuals concerning bioavailability and metabolites produced. Possible reasons might be the gut microflora, intestinal transit time, pH and redox potential **(Munro et al., 2003)**.

Furthermore, **Setchell et al (2002)** reported that isoflavone glucosides were not absorbed intact across the enterocyte of healthy adults, and the glucoside forms must be hydrolyzed for it to be absorbed. This suggests that consuming isoflavone aglycone-rich soy foods may be more effective in preventing chronic diseases. Improving the bioavailability of isoflavones from soy foods therefore requires both the enrichment of isoflavone aglycone prior to consumption and the modulation of intestinal microflora via the ingestion of viable bacteria. This knowledge also led to the development of aglycone-enriched products **(Park et al., 2001**; **Tsangalis et al., 2003)**. Human bioavailability of isoflavones from soy products was also evaluated by **Xu et al (1994),** who found daidzein as more bioavailable than genistein and according to them, approximately 85% of isoflavones degraded in the intestine when 12 adult women received single doses of isoflavones in soymilk as part of a liquid diet.

The level of isoflavonoids were found to be high in the urine and plasma of the Japanese and the Chinese **(Adlercreutz et al., 1991)**, whose traditional foodstuffs contained large amount of soy in the form of bean curd (tofu), soymilk, miso and tempeh. These groups are considered to have a low risk for development of breast and other hormone dependent

cancers. Among the isoflavones, genistein has been reported to be the most potent inhibitor of cancer cell growth **(Pandjaitan et al., 2000)**. However, 'isolated isoflavones' were found not to have the ability to lower the cholesterol in humans **(Dewell et al., 2002; Fitzpatrick, 2003)**. Long term effects of consuming high doses of extracted isoflavones or their safety and the efficacy are still unknown. Thus the natural soy protein matrix might be crucial in this respect.

1.3.5 Adverse effects of phytoestrogens

Contrary to health benefits, dietary soy intake has also been associated with increased bladder cancer incidence **(Divi et al., 1997)**, thyroid disorders **(Phillips et al., 1998; Ibarreta et al., 2001)**, breast cell hyperplasia **(Petrakis et al., 1996)** and dementia **(White et al., 1996)**. Some studies have raised concerns about potential adverse effects from soy-based formula intake in infants as a result of high circulating phytoestrogen concentrations **(Setchell et al., 1997)** and the potential for early and premature estrogenic effects in infants **(Irvine et al., 1995, Sheehan, 1998)** and its ability to modify sex steroid metabolism **(Fitzpatrick, 2003).** Research is still needed to evaluate the safety of phytoestrogens on human systems, to find out their beneficial and harmful doses, gender differences in response to phytoestrogens, differences in the chemical classes of phytoestrogens and the effects phytoestrogens may have with other drugs or dietary products.

Although it may be difficult for adults to consume sufficiently large quantities of isoflavones from normal dietary sources to cause the type of deleterious effects

previously experienced by several animal species **(Bennetts et al., 1946)**, there is a distinct possibility of risk associated with the use of these compounds as uncontrolled over-the-counter pharmacologic agents, because estrogens exhibit biphasic responses that are highly dose dependent. Thus certain regulations on soy isoflavone supplements sold in health or nutrition shops are urgently required. Or at least there is a need for necessary information to be made available to the physicians, so that the consumption of isoflavones can be regulated on the advice of a physician.

1.4 SOY ISOFLAVONES - ANALYSIS AND PROCESSING EFFECTS

1.4.1 Methods for extraction and analysis of soy isoflavones

Efficient extraction of the relatively polar isoflavones from foodstuffs requires the use of polar solvents **(Coward et al., 1993)**. Solvent extraction methods are commonly used for isoflavones. These methods can extract all the different isoflavone forms as such, and a detailed data about the conjugated forms and aglycones are obtained. But the main disadvantage of this method is the poor stability of the 'ester glucoside' standards in solution and that compromises the precision of analyses and limits its application as a reliable routine method. Moreover, it is very difficult to procure these standards. Efficiencies of different extraction solvents were tested by many researchers (**Wang et al., 1990; Barnes et al., 1994**) and it was found that 80% aqueous methanol (MeOH) was as equally superior to 80% acetonitrile-0.1% HCl, another commonly used extraction solvent. Extractions of isoflavones using 80% MeOH at various temperatures are also being studied by some researchers **(Coward et al., 1998)**. Extraction carried out at room temperature caused a slight conversion of the 6-*O*-malonyl glucoside (6OMalGlc)

conjugates to the β–glucoside conjugates, while if carried out at 80°C caused an extensive conversion of 6OMalGlc conjugates to β–glucoside conjugates **(Coward et al., 1998).** But if the extraction is carried out at 4°C, highest concentrations of 6OMalGlc conjugates are obtained. Though the composition of the individual β–glucoside conjugates extracted can be drastically altered by temperature, the total amount of isoflavones extracted remains constant. An enzyme hydrolysis **(Liggins et al., 1998)** or acid hydrolysis **(Mazur et al., 1996)** of the soybean extract is sometimes applied for the quantification of isoflavones.

Different analytical techniques including GC-MS **(Mazur et al., 1996; Liggins et al., 1998)**, electrochemical and fluorometric analysis **(Adlercreutz and Mazur, 1997)** have been applied for the determination of isoflavones in soybeans and soy foods. **Naim et al (1974)** prepared the trimethyl derivatives of genistein, daidzein etc and quantified them by gas-liquid chromatography, while **Shihabi et al (1994)** developed capillary electrophoresis techniques for the separation of isoflavones**. Mazur et al (1996)** modified the method by isotope dilution gas chromatography-mass spectrometry in a selected ion monitoring mode for quantitative measurement of isoflavones. Though several analytical methods for isoflavone analysis are utilized, high performance liquid chromatography (HPLC) technique is the most often applied method for separation and quantitative analysis of isoflavone from soybeans and soy products **(Wang and Murphy, 1994; Klump et al., 2001)**.

1.4.2 Concentration of isoflavones in soybeans and soy foods

High concentrations of isoflavones are found in raw soybeans. **Franke et al (1999)** found an average of 2mg/gm of total isoflavones in soybean seeds relative to dry weight, but the concentrations varied depending on a variety of factors such as genetic, environmental, harvesting and processing conditions **(Tsukamoto et al., 1995)**. The majority of isoflavones in soybeans are in the form of malonyl glucosides, while aglycones and acetyl glucosides are the minor components **(Kudou et al., 1991)**. Japanese soybeans have a higher ratio of 6"-*O*-malonyl isoflavones to glucosides than do American soybeans **(Wang and Murphy, 1994)**. Moreover, the effect of crop year on isoflavone concentrations seems to be more influential than that of its growth location **(Wang and Murphy, 1994)**.

Murphy et al (1999) carried out analysis of several institutional soy foods and found the level of isoflavones in soymilk, tempeh, tofu etc from the American market. They found a higher concentration of malonyl glucosides in the soybean seeds. While aglycones were the predominant forms in fermented soy products such as the tempeh and miso. Aglycones are formed by the action of β–glucosidases of the fermentation organisms. Soy sauce was found to be a poor source of isoflavones as the fermentation organisms probably degraded them. Further to this, **Franke et al (1999)** did a comparative study of isoflavone contents in a small group of soy foods from Hawaii and Singapore. They observed a mean total isoflavone levels in soy food groups within a range of 0.035 -7.50 mg/gm, with the lowest values observed in soymilk and highest values in soybean seeds. However, they found no consistent pattern when the isoflavone amounts were compared

between the same food groups from Hawaii and Singapore. No conclusion was therefore derived from this study. An understanding of the protein content of the products was not carried out by these researchers. Neither did they evaluate the relationship between the protein content nor isoflavone amounts in these products.

Traditionally, soy foods are consumed in high quantities in South East Asian countries **(Murphy et al., 1999)** and the most frequently consumed items being the soymilk and tofu. They are commonly served in food courts as well as in supermarkets and the consumption of these products continue to increase, since people are becoming more health conscious. However, there are few published reports that give quantitative data on the concentration of isoflavones in soy foods from Singapore, Malaysia, Thailand, Indonesia etc, while data available are mainly drawn after analysis of foods from USA, Brazil and Finland. Because of the ever increasing use of these soybean products, especially in the region, it was necessary to know the concentration of these biologically active compounds, isoflavones, in various commercial products consumed by the people of South East Asia. There are several ongoing clinical studies as well to explore the relationship between soy consumption and reduction in risk of many diseases **(Seow et al., 1998; Jakes et al., 2002)**. Moreover, to evaluate the potential of isoflavones as health-enhancing dietary compounds, their exact concentrations in typical soy foods need to be quantified. It is therefore crucial to determine the qualitative and quantitative composition of isoflavones in selected soy foods commonly consumed by the multiethnic population in the region.

1.4.3 Effects of processing on isoflavone levels

The chemistry of isoflavone conjugates altered during the commercial processing of soy into food products and further depended on the extent to which consumers cooked these soy foods **(Setchell, 1998)**. The original conjugation pattern of soy foods with predominating malonates changed as a function of treatment by heat and bacterial fermentation **(Kudou et al., 1991)**. Processing results in intra-conversions of isoflavones between the different forms, while cooking of soy products in water resulted in leaching of isoflavones into the cooking water **(Setchell et al., 1998; Grun et al., 2001)**.

In non-fermented soy foods, isoflavones are present almost exclusively as their glycosidic conjugates, whereas in fermented soy products, such as miso, soybean paste and tempeh, a large proportion of the isoflavones are in the unconjugated forms **(Coward et al., 1993; Wang and Murphy, 1994)**. Conjugation patterns of the isoflavonoids are significantly affected by the style and duration of cooking, as well as their interaction with other ingredients **(Setchell et al., 1997)**. Baking or frying of textured vegetable protein at 190°C and baking of soy flour in cookies does not alter the total isoflavone content, but there was a steady increase in β–glucoside conjugates at the expense of 6OMalGlc conjugates. Dry heat, as in toasting of soy flour causes the decarboxylation of malonyl glucosides and leads to the formation of substantial amounts of the acetyl glucosides. In general, moist heating causes conversion to β–glucoside conjugates and dry heat results in the formation of 6-*O*-acetyl glucoside conjugates. Thus the normal cooking conditions do not reduce the concentration of isoflavone in soy foods, since the thermal treatments

only alter the isoflavone conjugate profiles, while the total isoflavone concentrations do not change. But as the food is burned, a decrease in total isoflavones will result.

Process engineering of the common soy foods and ingredients are traditionally optimized for higher yield along with functionality and organoleptic properties, while isoflavone content has not been a major concern. Ultimately the processing of soybeans affects the nutritional content of the soy food products significantly. For example, textured soy protein is a unique product prepared by extrusion of soy isolates or soy concentrates. Alternatively, soy protein concentrates can be produced with an ethanol-washing step, resulting in an ingredient with almost no isoflavones **(Song et al., 1998)**. **Wang and Murphy (1996)** studied the mass balance of isoflavones during the manufacture of tempeh, tofu, soymilk and soy protein isolate. The main losses of isoflavones were related to the manufacturing steps such as: soaking (12%) and heat processing (49%) in tempeh production, coagulation (44%) in tofu processing, and alkaline extraction (53%) in soy protein isolate production. **Grun et al (2001)** studied the kinetics of tofu heating and found a decrease in the total isoflavone content because of leaching into cooking water, but the cooking water was not analyzed in their study. Therefore, the distribution of isoflavones in commercial soy food products is determined by the variety of soybeans, the storage conditions of raw materials and products, the processing conditions and dilution with non-soy ingredients.

Different types of extraction procedures are being followed by different manufacturers for the preparation of soymilk. The extraction procedure used for the preparation of

soymilk might have a significant influence towards the retention of isoflavones in the final product. Moreover, in large-scale production of soymilk, the continuous high-temperature, short time processes often substitute the normal low-temperature, long-time thermal processes. The advent of ultra high temperature (UHT) treatment processing and aseptic packaging has further contributed to the production of long-life soymilk packaged aseptically in paper-plastic cartons, which are more convenient for transportation, distribution and storage **(Gosta, 1995)**. However, an understanding on the concentration of these beneficial phytochemicals in the soymilk prepared by different extraction methods or processing conditions are nearly unknown. Therefore, an evaluation on the isoflavone contents in the soymilk obtained after a particular extraction method and UHT treatment process was considered essential during this study.

In a similar way, coagulation of soymilk is the most important step in the tofu-making process and different types of coagulants are used for its preparation. The thermal denaturation, aggregation and gelation properties of soy proteins during tofu making have been intensively investigated by many researchers **(Lakemond et al., 2002; Mujoo et al., 2003).** However, the loss of isoflavones might be significant when the soy beverage is coagulated to form tofu and the type of coagulant may have a different influence towards the retention of isoflavones in tofu and whey. But, little information is available on the effect of various coagulants on the level of isoflavones in tofu. An evaluation of the quality parameters, together with an understanding of isoflavone levels in tofu prepared from different coagulants, might be essential in knowing the nutritive quality of the obtained tofu. Moreover, the released whey has proteins associated with it and a certain

amount of isoflavones might be lost into it. But, there was no literature available which evaluated the retention of isoflavones in tofu or whey with regard to the coagulant used for the tofu preparation. A detailed study in this respect can provide information about the functional benefits and physical properties of tofu obtained after using different coagulants. The data on the loss of isoflavones into the whey while using different coagulants might also be useful in process optimization for the maximum retention of isoflavones into the tofu. Such an evaluation study was considered crucial during this research.

1.4.4 Methods for reporting the concentration of isoflavones

Some confusion exists on how the isoflavone content of a product shall be expressed. **Setchell and Cole (2003)** reported that the isoflavone content of products are occasionally expressed without indicating whether the stated amount refers to aglycone or glycoside value. This distinction is critical because the sugar moiety does not contribute to the biological activity and the aglycone present only 60% of the glycoside weight. Therefore, the total isoflavone content of a food sample is not the simple sum of the mass of the 12 isomers present in the food. The normalized concentrations of each isoflavone (daidzein, glycitein and genistein) need to be reported. Therefore the need for standardization of expressing the concentration of isoflavones in soy products in terms of aglycone equivalent amounts was considered important during this study. More confusion can be avoided if these are expressed in molar values, but this has not been the convention within the scientific community for food labeling **(Erdman et al., 2004)**.

1.5 OBJECTIVES OF THE STUDY

The objectives of the study are as follows:

- The initial objective of this study was to identify an extraction procedure for the efficient extraction of isoflavones from soy based products with minimum co-extractives. Further aim of the study was to develop a method for the quantification of isoflavones in different soy based samples using HPLC method. The second objective of this study was to understand if there is any relationship between the protein content and isoflavone amounts in soymilk and tofu samples. It was hypothesized that a higher amount of isoflavones are present in soymilk and tofu samples with higher protein content.

- The amount of isoflavones in commercially available soy based supplements, health products and infant formulas varied. An understanding of the actual amounts and composition of isoflavones in these commercially available products was also part of this study.

- The types of processing have had a significant influence on the level of isoflavones in soymilk and tofu. With the novel and cost-effective methods of making soymilk in industry, it is important to know the effect of processing conditions on the extractability of isoflavones into the soymilk. Therefore the third objective of this study was to evaluate the loss or improvement in the extraction of isoflavones into the soymilk by hot grinding of soybeans compared to cold grinding. Soymilks are subjected to UHT treatment processes for extending its shelf life. Understanding the fate of isoflavones in

soymilk obtained from a direct UHT process compared to an indirect UHT process was also part of this study.

- Different types of coagulants are used for the preparation of a firm tofu. The retention of isoflavones in the prepared tofu and wheys varied with the type of coagulant used for its preparation. Understanding the effect of different coagulants on the level of isoflavones in the tofu and whey was another objective of this study. Evaluating the quality parameters of the prepared tofu was also part of this study. This study was thus aimed at identifying the best coagulant that might result in a better quality tofu with a higher functional food value.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Chemicals

1) Acetonitrile, HPLC grade
2) Acetic acid, glacial
3) Sodium hydroxide
4) Methanol, HPLC grade
5) Calcium sulfate $CaSO_4.2H_2O$
6) Calcium chloride $CaCl_2.2H_2O$
7) Magnesium sulfate $MgSO_4.7H_2O$
8) Magnesium chloride $MgCl_2.6H_2O$
9) Calcium acetate $Ca(CH_3COO)_2$
10) Calcium lactate $(CH_3CHOHCOO)_2Ca.5H_2O$
11) Isoflavone standards

Acetic acid and sodium hydroxide pellets were purchased from Merck (Darmstadt, Germany), while all the other chemicals except isoflavone standards were purchased from Sigma Chemical Co. (St Louis, USA). Authentic standards of daidzin ($C_{21}H_{20}O_9$), genistin ($C_{21}H_{20}O_{10}$), glycitin ($C_{22}H_{22}O_{10}$), daidzein ($C_{15}H_{10}O_4$), genistein ($C_{15}H_{10}O_5$) and glycitein ($C_{16}H_{12}O_5$) of high purity were purchased from LC laboratories (Woburn, USA).

Bio-Rad protein assay dye reagent and bovine serum albumin standard were purchased from Bio-Rad Laboratories (Hercules, USA) and were used for the protein estimation in various soy based samples. HPLC-grade water was obtained from a Milli-Q purification system (Millipore Corp., Bedford, USA) and was used for preparing all solutions throughout this research.

2.1.2 Food materials

2.1.2.1 Commercially available brands of soymilk and tofu samples

Different brands of commercially available samples of soymilk and tofu were purchased from supermarkets, cold storages or retail outlets in Singapore, Malaysia, Indonesia and Thailand and they were analyzed for their moisture content, protein content and isoflavone concentrations. The commercially available soymilks collected from all these countries were mostly obtained in tetrapak® forms. Few branded soymilk products were found in Indonesia and Thailand, while a wider variety of branded soymilk products was found in Malaysia than in Singapore. Two varieties of tofu were obtained: the soft and the firm tofu types. Soymilk and tofu samples were stored at a temperature range of 2-7°C after their purchase and they were analyzed within a day of purchase.

2.1.2.2 Preparation of soymilk – Traditional method

Soymilk was prepared by traditional processes either to obtain soymilk (9^o brix) itself or to obtain soymilk (12^o brix) suitable for the preparation of tofu.

Soymilk was prepared in the pilot plant of a local soymilk processing industry by the traditional method. Identity preserved soybean seeds and particularly the 'Harovinton' cultivar was obtained from a local supplier and was used as the raw material for this study. Soybean seeds were soaked in water at ambient temperature for a period of 5 hr, rinsed and ground in water at a bean: water ratio of 1:4. The resultant slurry was cooked at 100°C for more than 15min and passed through a centrifugal separator to remove the soy residue (okara). The raw soymilk thus obtained had an approximate soluble solids content of 9^{o} brix as obtained by a refractometer (Abbe Model 3T, Atago, Japan). The okara from the traditional soymilk preparation method was also collected separately and was stored at -20°C until analyzed, in order to prevent any deterioration in its quality. Care was always taken to analyze every sample within a day or two.

Raw soymilk with approximately 12^{o} brix was prepared and used for the preparation of firm tofu. The prepared tofus were evaluated for their isoflavone concentrations to understand the effect of different coagulants towards the retention of isoflavones during a firm tofu making.

2.1.2.3 Preparation of soymilk – UHT processing system

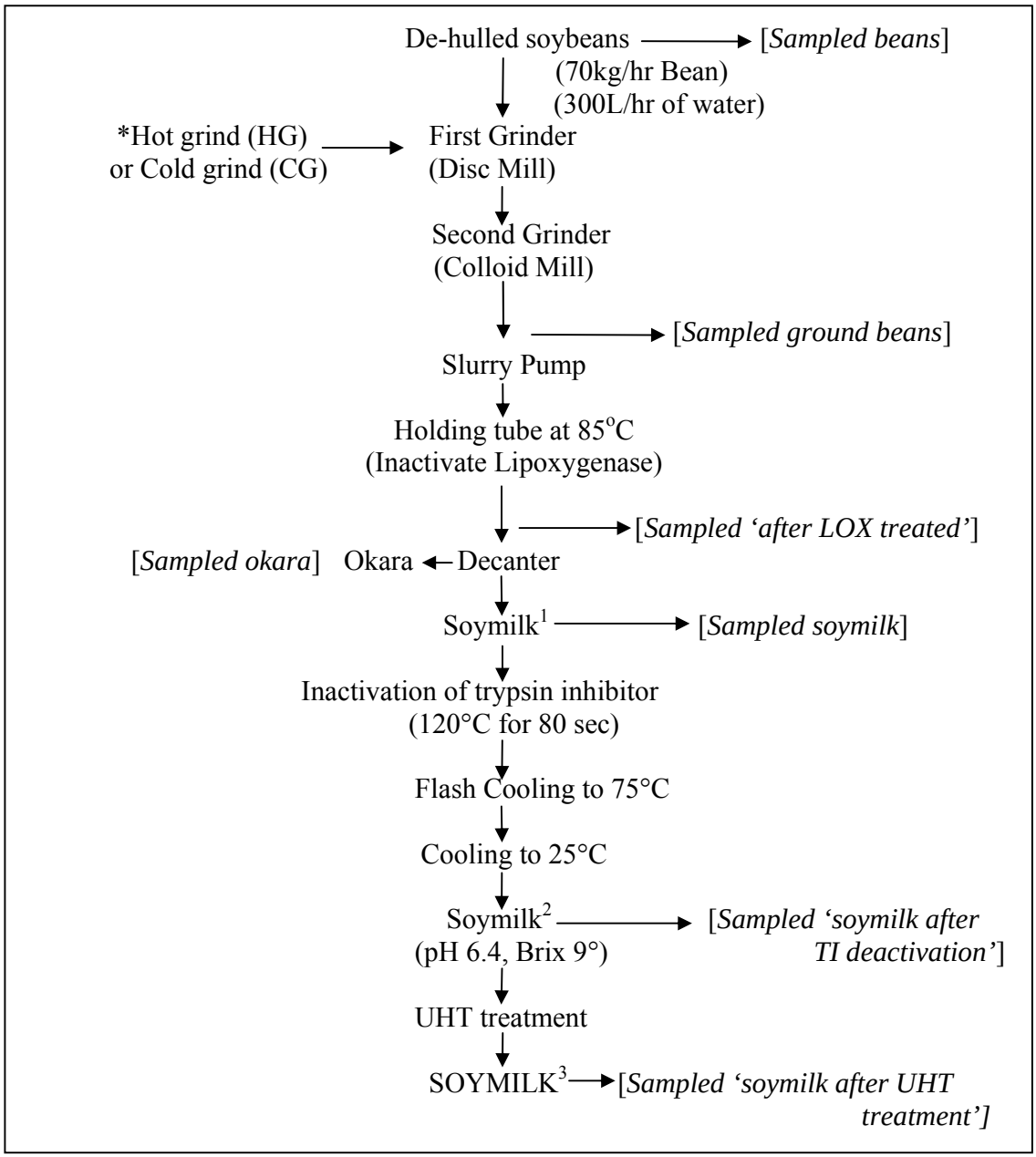
The preparation of soymilk by the UHT processing system was carried out in a pilot plant provided by a private firm in Singapore (name withheld). Sampling points at different stages of the manufacturing process is shown in Figure 2.1. Therefore a comparative study on the amounts of isoflavones in the final product obtained from two different extraction methods and from two different UHT treatment methods was made easier. The

analysis of samples at different stages of the process also helped in evaluating the comparative amounts of isoflavone glucosides and aglycones in each sample with respect to the treatments it has undergone.

Identity preserved 'Harovinton' soybean seeds were obtained from a local seed supplier who provided the same cultivar for all the trials. Dehulled soybeans were either hot ground or cold ground, using a disc mill (Fryma Grinder, FrymaKoruma AG, Switzerland) with water at 95°C or 45°C respectively. Soybeans were added at the rate of 70 kg/hr and water was added at the rate of 300 L/hr. The resulting slurry was passed through a colloid mill (Fryma Grinder, FrymaKoruma AG, Switzerland) and then through a holding tube at 85°C to inactivate the LOX enzyme. Further it was passed through a decanting centrifuge for separation of the insoluble residue (okara). The soymilk thus obtained (soymilk^{1}) was held at 120°C for 80 sec to inactivate approximately 80% of the Kunitz trypsin-inhibitor (TI) and there-by improving the soy protein digestibility **(Kwok et al., 1993),** flash-cooled to 75°C and further cooled to 25°C. The cooled soymilk (soymilk^{2}) was subjected to different ultra high temperature (UHT) treatment conditions: one was a direct UHT process and the other was an indirect UHT process. This was mainly to understand the influence of the type of UHT process on the level of isoflavones in the end product (soymilk^{3}). For the direct UHT system, steam was injected into the product and soymilk (soymilk^{2}) came in direct contact with the heating medium. The direct UHT process (Tetra Therm VTIS, TetraPak Co', USA) was carried out at a temperature of 143ºC for 10 sec. The indirect system (Tetra Therm FLEX, TetraPak Co', USA) was based on tubular heat exchangers whereby heat was transferred from the

heating medium to the product through a heat exchanger. The indirect UHT process was carried out at a temperature of 140ºC for 4 sec. These conditions were chosen from the normal industry practices used for UHT treatment of soymilk in the region.

Figure 2.1 shows the processing of soybeans to soymilk. The sampling points at different stages of the manufacturing process are also indicated. Soymilk preparations were carried out with replicate trials being further carried out to confirm the reproducibility of the results obtained. Every trial involved either a direct UHT process (injection of steam into the product at 143°C for 10 sec) or an indirect UHT process (using a heat exchanger at 140^{o}C for 4 sec). Two pilot runs were carried out on each of the trials, one involving hot grinding (grinding of dehulled soybeans with water at 95^{o}C) and the other involving cold grinding (grinding of dehulled soybeans with water at 45^{o}C).



*Hot grind process (HG) is grinding of beans with water at 95°C, while grinding of beans with water at 45°C is called Cold grind process (CG). *Sampling points are shown in italics.*

Figure 2.1: Flow diagram for the processing of soybeans to soymilk

2.1.2.4 Preparation of tofu

A single bigger batch of soymilk of 12^{o} brix was prepared according to the traditional soy milk making procedure *(index no: 2.1.2.2)* and it was used for the preparation of every coagulant-based tofu. This was done in order to avoid any changes in the soymilk composition and or concentration factors. Moreover, this enabled a comparative study of the nutritional (isoflavone amounts) and property differences (moisture, texture and color) of obtained tofu with respect to the different coagulants used for its preparation.

For the preparation of a tofu, a 500gm portion of soymilk was taken and heated to a temperature of 80°C under stirring. The time period of heating, and stirring speed were kept constant for every tofu preparation. Tofu was made by coagulating the soymilk using any one of the coagulants, calcium chloride, calcium sulfate, magnesium chloride, magnesium sulfate, calcium acetate or calcium lactate. Coagulants were used at two different concentration levels to better understand the level of coagulant required and possible effects if any, on isoflavone concentrations. The coagulant concentrations used were 0.4% and 0.5% based on the amount of soymilk used. These coagulant concentrations (0.4 % and 0.5%) were chosen based on a preliminary set of experiments on coagulant concentrations carried out prior to this study. Coagulant concentration of 0.10% was too less for coagulation to occur, while 0.60% of coagulant resulted in stronger binding and hence too compact network of tofu. Coagulation of soymilk by salts such as calcium sulfate and calcium acetate were also studied by **Kamel and de Man (1982)**, where they described the minimum concentration required for coagulation with calcium sulfate as 0.25% and with calcium acetate as 0.20%. Coagulant concentrations of

0.4 – 0.5% based on the amount of soymilk, were found to result in the coagulation of soymilk by the different coagulants of this study. Each coagulant was dissolved completely in 20ml of cold water and was used immediately. Calcium sulfate was not completely soluble in water, and hence saturated solutions of calcium sulfate obtained with the above concentrations were used. The hot soymilk and coagulant solution were poured simultaneously into a glass container ensuring good mixing without stirring. The soymilk-coagulant suspensions were allowed to stand undisturbed for a period of 20min to ensure that coagulation occurred. The curds thus formed were broken thoroughly and transferred into a specially designed mould (9 x 9 x 8cm) lined with cheesecloth. The mould had perforations on the sides and bottom. The whey was drained off naturally for 10min and the curd was pressed for 1hr using a pressure of 28.0 gm/cm^2. After pressing, tofu and whey were weighed separately. Tofu was transferred into a plastic bag and stored in a refrigerator until analyzed. Tofu was also prepared by coagulating soymilk with 20ml of a 5% glacial acetic acid solution in water

2.1.2.5 Soy supplements, health products and infant formulas

Soy isoflavone supplements, health products and infant formulas were purchased from the local stores or nutrition shops in Singapore, Malaysia, Indonesia and Thailand and they were analyzed for their isoflavone concentrations. Soy supplements obtained were either as tablets or capsules, and were advertised as providing relief for menopausal discomforts or as a remedy for women's problems. The type of product, their health implications and dosages are described in Table 2.1. Supplements, which stated that the source of phytoestrogens was from kudzu root, or clover, were not analyzed for their

isoflavone concentrations since the present study was conducted only on products prepared from soy. The soy based health products obtained were suitable for making shake mixes and the directions for their preparations were indicated on the labels. Ready-to-drink liquid products (RD) of this category were also obtained. All of them were advertised as helpful in weight-management in a healthy way. Soy based infant formulas (SBIFs) were mainly obtained in the powder form and were prescribed for children who are intolerant to lactose. Only five commercially available SBIFs were obtained during this study and they were also analyzed for their isoflavone concentrations.

Table 2.1: Identification of the different soy isoflavone supplements involved in the study

Product code and specific health effect of the products		Dosage/ day	[a]Product form
A	Hi-Soy isoflavone supplement for women	1	tablet
B	Women's soy isoflavones dietary supplement	2	capsule
C	Supplement for women	1	capsule
D	Natural soy isoflavones	2	capsule
E	Phytoestrogen veg capsules	4	capsule
F	To support breast health	2	capsule
G	Calcium with soy isoflavone for menopausal health	1	tablet
H	Calcium and isoflavone	4	tablet
I	Female support formula	2	tablet
J	Help relieves menopausal discomfort	1	tablet
K	For women over 40	2	capsule
L	Phytoestrogens from soy extract	4	capsule
M	Multivitamins together with isoflavones	1	tablet

[a]Products (A – M) type of formulation was soy isoflavone extract / concentrate.

Unless otherwise stated, '*soy isoflavone supplement*' refers to a product obtained as a capsule or tablet and '*soy based health product*' refers to a soy protein-based product obtained in the powder form throughout, in this particular study.

2.2 METHODOLOGIES ADOPTED

The various methods used to carry out the analytical work for the research were either used directly or modified from previously established methods.

2.2.1 Sampling procedure

2.2.1.1 Soybean seeds

Soybean seeds of 50 - 100gm were finely ground in a coffee grinder (Sumeet Multi Grinder, Inno Concepts Inc., GA,USA). The finely ground soybean flour was then mixed thoroughly before a representative sample was chosen for analysis.

2.2.1.2 Sampling of soymilk, tofu, whey and related products

Total contents of the commercially obtained sample of soymilk and tofu were homogenized separately, before a representative sample was chosen for analysis. The sample packaging was unwrapped only on the specific day of analysis, prior to which the products were stored in a refrigerator at 2-7°C. In general, every soymilk and tofu prepared was also thoroughly homogenized before an extraction or analysis was carried out on a respective sample. Wheys obtained from a single batch of tofu preparation were collected together and mixed well before sampling was done. Tofu and wheys obtained from a single preparation were always analyzed on the day of preparation.

Samples collected at different stages of a traditional or UHT soymilk preparation processes were homogenized thoroughly before an analysis was carried out. The preparations were carried out on a plant scale and the samples were brought to the lab and

immediately stored in a freezer at -20°C. Samples were thawed individually and homogenized well before a representative sample was chosen for extraction purpose.

2.2.1.3 Sampling of soy supplements, health products and infant formulas

Fifteen randomly selected tablets of a particular soy supplement were ground into fine powder to ensure a homogeneous mixture using a coffee grinder. In a similar way the contents of 15 capsules of a supplement were mixed together and a representative sample was always used for the extraction of isoflavones. This was done to minimize the effect of variation between single capsules, while the capsules themselves were discarded. For the soy based health products and SBIFs, the total content of the product was mixed well. Further a certain portion (50gm) of the sample was taken from different locations of the product container before a representative sample was chosen from this for the extraction purpose. The ready-to-drink (RD) products were freeze dried, ground well and used for analysis. Freeze drying was carried out using a freeze-dryer (Virtis Model no. BT4K XL, Gardiner, USA).

2.2.2 Moisture analysis

The moisture contents of soybean seed powders were measured as the loss in weight of 2gm ground sample after drying for 24hr at 105°C in an air oven to a constant weight (Memmert Universal Ovens Model U, Wisconsin Oven Corp., USA). Moisture analysis was carried out in triplicate and mean value was obtained.

Moisture content of samples such as the commercial soymilk and tofu products, prepared soymilk and related products of traditional and UHT soymilk making process as well as the prepared tofu samples were determined by vacuum oven-drying **(AOAC, 2002)**. Accurately weighed 5gm well-mixed test portion in a covered dish, which is previously dried at 98-100ºC, cooled in desiccator and weighed soon after reaching room temperature. The dish with contents was dried in a 70ºC vacuum oven (model no: 14-OD-2E, Sheldon Manufacturing Inc., Cornelius, USA) at 25mm Hg until a constant weight was reached. At the end of the drying, the loss in weight was obtained and the mean value of triplicate analysis was reported as the moisture content of respective soy based samples.

Moisture content of tofu wheys was obtained by drying 5gm of samples to a constant weight at 105°C in an air oven for 24hr **(AOAC, 2002)**. Mean value was obtained from the triplicate moisture analysis of each samples. Tofu whey being very watery with especially high water content, an air oven method was chosen for determining its moisture content instead of the vacuum oven method.

2.2.3 Protein assay

The protein content of commercially available and purchased soymilk and tofu products was determined during this study. This was carried out to establish a correlation between the protein content and isoflavone concentrations of the soymilk and tofu samples.

Different analytical methods such as Kjeldahl method and Dumas method are being utilized for protein determination. Kjeldahl method is the universally accepted method of choice for protein estimation especially with high precision and reproducibility. However, the method uses high temperature with hazardous chemicals and is very time consuming. Alternative methods are the dye-binding methods and the rapidity of these methods is well acceptable with good sensitivity, reproducibility and accuracy **(Boyer, 2000)**. The BioRad Protein Assay is a dye-binding assay in which a differential color change of a dye occurs in response to various concentrations of the protein. It is a very popular method used for the quantification of proteins, because it is simple, rapid, inexpensive and sensitive **(Bradford, 1976).** The dye, Coomassie® Brilliant Blue G-250 (CBBG) exists in three forms: red (λ max = 465nm), blue (λ max = 595nm) and green (λ max = 655nm). The absorbance maximum for an acidic solution of CBBG dye shifts from 465nm to 595nm when binding to protein occurs. Thus the assay can be monitored at 595nm by a spectrophotometer, which measures the CBBG-complex with the protein. The assay is useful since the extinction coefficient of a dye-albumin complex solution is constant over a 10-fold concentration range. Thus, Beer's law may be applied for accurate quantification of protein by selecting an appropriate ratio of dye volume to sample concentration. According to Beer's law, the relationship between the absorbance of a solution and the concentration of the absorbing species is given as $A = \varepsilon bc$, where A is the absorbance, ε= molar absorptivity, b= path length through solution (cm) and c= concentration of absorbing species in molarity. Beer's law is applicable only to dilute solutions, up to approximately 10mM for most analytes.

Moreover, the assay is compatible with most agents that interfere with other assays. The addition of 1 M NaOH was also suggested by **Stoscheck (1990)** to allow the solubilization of some proteins and reduce the protein-to-protein variation in color yield. However, the assay must be read quickly after its completion, because the proteins will precipitate in the reagent over time, affecting the linearity of the response. Bovine serum albumin (BSA) has long been the standard of choice for this assay. BioRad assay method was chosen for the quantification of protein in soy based samples during this study.

2.2.3.1 Measurement of protein in soy based samples

A calibration curve was prepared by using a series of standard solutions of BSA. Standard solutions of 0.25, 0.50, 0.75 and 1mg mL^{-1} of BSA in water were prepared. An aliquot of 0.1ml of each standard was transferred to clean and dry test-tubes and 5ml of BioRad dye reagent was added to this. Tubes were covered, shaken well and incubated for 30min at room temperature. The absorbance was measured by using a UV-VIS spectrophotometer (Shimadzu UV 1601, Kyoto, Japan) at 595nm. A calibration curve was plotted for the BSA standard. The absorbance of soy samples were also measured after proper dilutions, such as to fit within the standard calibration curve, while the slope of the curve was used to calculate the protein content in soymilk and tofu samples.

2.2.4 Yield of tofu

During the studies on the retention of isoflavones in tofu prepared using different coagulants, it was necessary to calculate the yield of tofu. The yield of tofu was calculated as the weight of fresh tofu obtained from a specified amount of the soymilk

used for its preparation. This was carried out in order to identify the coagulant, which gave a better yield of tofu compared with others.

2.2.5 Texture measurement

2.2.5.1 Principle of texture measurement

Texture is defined as the mechanical, geometrical and surface attributes of a product perceptible by means of mechanical, tactile and where appropriate, visual and auditory receptors **(Rosenthal, 1999).** The test which is more applicable for food industry in this respect, is the *Texture Profile Analysis* (TPA) created at General Foods in the mid- 1960s. The TPA test provides textural parameters which correlate well with the sensory evaluation parameters. During the TPA test, the food product is brought in contact with a plunger which via a pivotal motion (resembling the human jaw) caused the deformation of the food. A two-bite cycle is employed. After the 1st bite, the load is removed from the sample and allowed to relax somewhat. While the plunger pulled away from the sample surface, any tension due to stickiness can be observed. The 2nd bite compressed the sample again before allowing it to relax for a second time. The resistance during deformation of the food can be monitored throughout the two-bite cycle and a TPA curve can be obtained. Figure 2.2 shows a typical TPA curve from a two-bite test (Stable Micro Systems, UK).

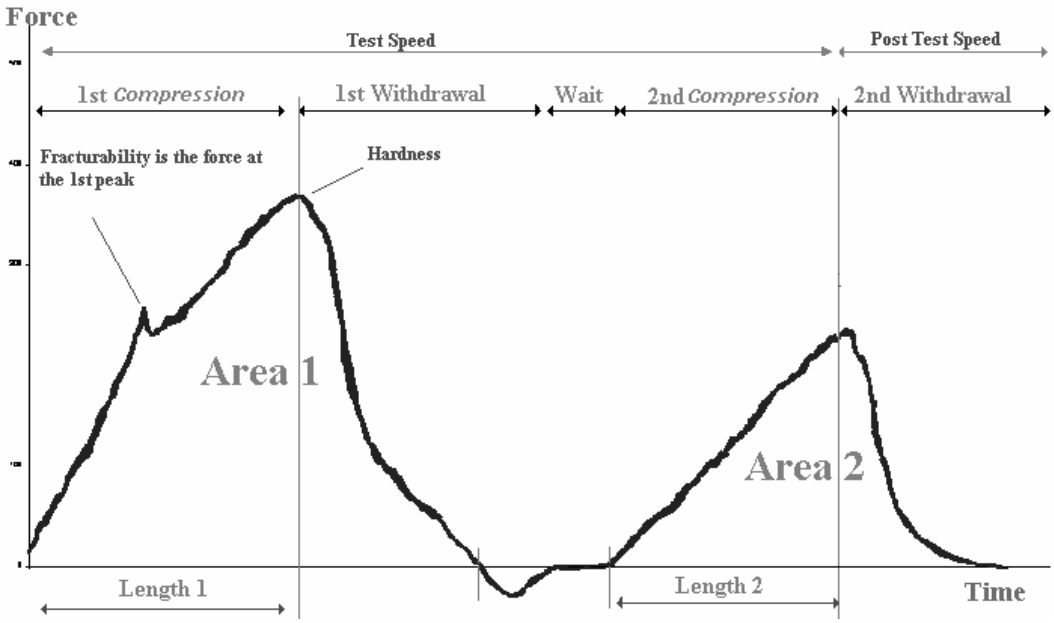


Figure 2.2: Texture profile analysis curve from 'two-bite test'

Different parameters measured from the TPA curve include the hardness, elasticity, cohesiveness, brittleness, chewiness, gumminess etc **(Bourne, 2002)**. Hardness is defined as the height of the peak force on first bite, which is the force necessary to attain a given deformation. Certain products fracture at the Fracturability point (Figure 2.2). Elasticity is the extent to which a compressed food returns to its original size when the load is removed. Also called springiness, it is the ratio of the distance of the detected height of the product on the 2nd compression to the original compression distance (Length 2/ Length 1). Cohesiveness is how well the product withstands a second deformation relative to how it behaved under the first deformation (Area 2/ Area 1). It is measured as the area of work during the second compression divided by the area of work during the first compression. Gumminess is the product of hardness and cohesiveness, while chewiness is the product of gumminess and springiness.

2.2.5.2 Method used for texture analysis of tofu

The texture of a prepared tofu was determined in order to evaluate the physical quality of the tofu obtained while using different coagulants for its preparation. Texture of tofu was determined by TPA using a TA.XT2i texture analyzer (Stable Micro Systems, Godalming, UK) equipped with a 5-kg load cell. A sample from the central part of tofu was always used for the texture evaluation. A 10mm diameter probe was used at a test speed of 2.0mm/sec for this purpose. Cylindrical samples (1.5cm dia x 1cm height) were prepared from the central portion of tofu with a stainless steel boring tube and a wire cutter. The samples were compressed to 50% deformation. Six replicate tests were carried out for every coagulant tofu and the average value was obtained. Hardness, cohesiveness and gumminess of individual tofu samples were determined from the TPA curve and a comparative study on the texture properties of tofu prepared by using different coagulants was made possible.

2.2.6 Color analysis

2.2.6.1 Principle of color analysis

Color is an appearance property attributable to the spectral distribution of light. It arises from the presence of light in greater intensities at some wavelengths than at others. In practice, it is limited to the band of the spectrum from 380 to 770nm, the part of the electromagnetic spectrum that is visible to the human eye. In 1931, the Commission International de L'Eclairage (CIE), worked in developing a method for systematically measuring color in relation to the wavelengths they contain. They adopted one set of

color-matching functions to define a *standard colorimetric observer*, whose color matching characteristics are representative of those of the human population having normal color vision. This system known as the CIE color model was developed based on the tristimulus theory of color perception. The CIE system specifies a color by three quantities, *X, Y,* and *Z* called *tristimulus values*. These values represent the amounts of three primary colors, red, green and violet, that are required for a standard observer to get a match. If each of the tristimulus values is divided by the sum of the three, the resulting values *x, y*, and *z,* called *chromaticity coordinates* can be obtained. Color identification can be carried out using the tristimulus photoelectric colorimeter, which is relatively simple, inexpensive, rugged and well adapted to routine tests.

However, the food industry more often uses Hunter L*, a*, b* so most communication of results within this industry is being done using this scale (Figure 2.3). In this coordinate system L* is a measure of the lightness of a sample, which is directly comparable to *Y* in the CIE system and ranges from 0 (black) to 100 (white). The Hunter a* value denoted redness (positive a*) or greenness (negative a*); the Hunter b* value measures yellowness (positive b*) or blueness (negative b*). The a* values are functions of *X* and *Y*, the b* values of *Z* and *Y*. The formulas for Hunter L*, a* and b* are calculated from square roots using CIE *XYZ* and thus Hunter and CIE values are inter-convertible by calculations **(Hunter, 1958).**

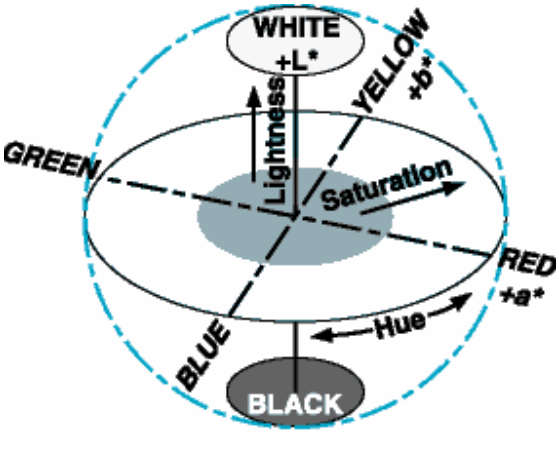


Figure 2.3: CIE L*, a*, b* color space

2.2.6.2 Color analysis of tofu

The effect of different coagulants towards the color of tofu was evaluated. During this study, the color of tofu, expressed in L*, a* and b* values, according to the CIE definition were measured using Minolta spectrophotometer model CM-3500d (Osaka, Japan). The instrument settings were prescribed for a solid sample of tofu, with the calibration mode set to reflectance. The lens position and target mask were set to medium, while the zero calibration and white calibration were carried out using a black box and white plate, respectively. The color measurements for each sample were replicated six times and the mean value was obtained.

2.2.7 pH measurement

The pH of the wheys was measured using a ThermoOrion model 410A pH meter (ThermoOrion, Beverly, MA, USA). Commercially prepared buffer solutions of pH 4.00 and 7.00 obtained from Merck (Darmstadt, Germany) were used to standardize the pH meter.

2.2.8 Isoflavone analysis

The initial objective of the study was to find an extraction procedure for the maximum extraction of isoflavones from soy based products with minimum co-extractives. The method so chosen shall be simple, rapid and reproducible. HPLC analysis was further chosen as an analytical tool for quantification of isoflavones in soy based foods, while LC-MS with electron spray ionization was utilized for peak identification studies of different soy isoflavone conjugates.

2.2.8.1 Method adopted for extraction of isoflavones from soy samples

Of the 12 soy isoflavones, there are six isoflavone glucoside esters. They are the acetyl and the malonyl isoflavone glucoside esters; which have either an acetyl or a malonyl group attached to the isoflavone glucoside at the 6"-*O*- position. The major isoflavones in soybean seeds are mainly the malonyl glucosides of daidzein and genistein **(Kudou et al., 1991)**. But processing of soybeans to soy products results in the conversion of malonyl glucosides to other forms. Further to this, the extraction techniques for the analysis of isoflavones can cause conversions of the ester glucosides to their respective glucosides or aglycone forms, depending on the conditions utilized.

Due to the effects (various temperature applications) of processing and sample extractions, the concentration of malonyl and acetyl isoflavones are affected, resulting in its conversion to other forms (aglycones and glucosides). Therefore, an understanding on the concentration of heat sensitive and unstable isoflavone forms especially during the

different stages of a soy food product processing is difficult. However, uniformity during quantification studies on isoflavones can be achieved if the ester glucosides can be converted to their respective glucosides during the sample extraction process. Moreover, much difficulty also exists in procuring the pure standards of isoflavone glucoside ester compounds. They are unstable in solution too. With all these technical difficulties, a detailed understanding of individual isoflavone conjugate concentrations was not an aim of this study. Thus the quantification of all the 12 soy isoflavone conjugates was not carried out but instead, a quantification of the total concentration of isoflavones in different soy based samples was considered as the major aim of the study. This can be achieved by converting the different isoflavone glucoside esters (i.e., malonyl and acetyl forms) to their respective glucosides. Further the total concentration of isoflavones can be obtained by quantification of the three isoflavone glucosides (daidzin, glycitin, genistin) and the three isoflavone parent aglycones (daidzein, glycitein, genistein). Thus a total of only six isoflavones need to be quantified for obtaining the total concentration of isoflavones in different soy samples.

Choosing a solvent that can result in efficient extraction of isoflavones from the soy products was the initial criterion of study. The various extraction solvents used by researchers include 70% ethanol, 80% methanol, 80% aqueous acetontirile - 0.1% HCl etc **(Wang and Murphy, 1996; Eldridge, 1982)**. While in accord with most previous studies, 80% aqueous methanol was found to be the optimum solvent for the extraction of isoflavones **(Coward et al., 1993; Setchell et al., 1987; Eldridge, 1982; Murphy, 1981)** and was chosen for this study**.** The temperature chosen for extraction was 65°C because

an optimized extraction of isoflavones can be obtained at a higher temperature. Maximum recovery of isoflavones from soy foods with 80% methanol sufficient for reproducible quantitative measurements was also obtained by tumbling the samples for 2hr **(Barnes et al., 1994)**. Also the extraction procedure if followed by saponification process can result in conversion of all the isoflavone glucoside esters to their corresponding isoflavone glucosides, leaving the 3 parent isoflavones intact. This approach will allow a direct comparison with stable and available, isoflavone aglycone and glucoside reference standards. Saponification using a mild alkali solution will neither destroy the isoflavone aglycones or the isoflavone glucosides. Hence an extraction followed by a mild saponification was chosen as the method for quantification of isoflavones during this research. This method was also established by the Association of Official Analytical Chemists in the year 2001, after a collaborative study was carried out in twelve different laboratories around the world following this particular procedure for the quantification of isoflavones in different soy based products **(Klump et al., 2001)**.

Since the samples for isoflavone extraction included a variety of soy based materials ranging from soymilks to soy supplements, the test portion used for the extraction purpose also differed. For the various product categories, the amount of test portions used for isoflavone extraction was optimized for the above established method of extraction process. A test portion of 0.50gm of soybean seed powder, 5.0gm soymilk, 5.0gm tofu, 0.1 – 0.5gm soy supplements, 1.0 – 3.0gm soy based health products and 1.0 – 3.0gm SBIFs were commonly used for the extraction purposes.

An accurately weighed amount of test sample was placed in a 250ml Erlenmeyer flask with ground-glass stopper **(Klump et al., 2001).** To this, 40ml of 80% aqueous methanol was added. The flask with its contents were shaken in a 65°C water bath for 2hr, cooled to room temperature, and 3ml of 2M NaOH was added. They were stirred well at room temperature for 10min and the reaction arrested by adding 1ml of glacial acetic acid. The contents were swirled and poured into a 50ml volumetric flask, diluted to mark with the extraction solution and mixed well. The contents were centrifuged for 10min at 5000rpm or 4332 xg (Eppendorf Centrifuge 5804, Hamburg, Germany). An aliquot of the supernatant was filtered through a 0.45µm non-pyrogenic filter unit (Schleicher & Schuell, Dassel, Germany) and analyzed by HPLC. Storage of isoflavone extracts for extended periods might lead to gradual changes in the composition of isoflavone conjugates **(Barnes et al., 1994)**. Hence during the quantitation studies, all samples were analyzed immediately after their extraction. Samples were extracted and analyzed at least in triplicate and otherwise, the extraction and analysis were carried out in six replicates (soybean seed powders).

2.2.8.2 Optimization of gradient profile for HPLC analysis of isoflavones.

Reversed phase HPLC technique (RP-HPLC) is the more commonly used method for analysis of phenolic compounds. The separation of soy isoflavones during this study were carried out on a YMC-pack ODS-AM 303 column (5µm, 25cm x 4.6mm id), purchased from Waters Corp., Milford, USA. Various solvents are being used for RP-HPLC analysis, while acetonitrile is the most extensively used solvent. Acetonitrile and water were used as the eluting agents for the separation of isoflavones during this study.

Aiming for simplicity and better reproducibility, a gradient HPLC method was developed to separate the three isoflavone glucosides and the three aglycones simultaneously. Though different compositions of acetonitrile and water (binary mobile phase) were tested, a satisfactory result was not obtained until a small concentration (0.1%) of acetic acid was added to the mobile phases. The gradient profile was further optimized in order to obtain a good resolution among the peaks. Glycitin co-eluted with daidzin and the optimization of the method required several adjustments including the optimization of flow rates to resolve the problem. The addition of polar modifiers such as acetic acid caused a decrease in the mobile phase surface tension and also caused an influence on the characteristics of the RP adsorbent surface. Some researchers **(Setchell et al., 1997; Setchell and Cole, 2003)** used a certain % of trifluroacetic acid (TFA) in the mobile phase for a better separation, while the handling of TFA was not considered easier and hence rejected for a long term research work, as in this study. HPLC analysis of isoflavones at different pHs by utilization of buffer solutions was another alternative choices of mobile phases **(Jones et al., 1989)**. However, utilization of buffer solution was also avoided in this study to prevent any clogging of the column and trouble shooting during the analysis. Results of analysis showed that a lower concentration of acetic acid significantly improved the chromatographic separation of the peaks, by an increased resolution and peak sharpness. Thus 0.1% of acetic acid in water and 0.1% acetic acid in acetonitrile were used as eluents for the HPLC analysis. The flow rate was another major factor of study. Flow rates of eluents were studied between 0.5 – 1.0ml/min until the optimization of the chromatographic conditions to enable a better separation of isoflavones was obtained at a solvent flow rate of 0.8ml/min.

The HPLC apparatus used for isoflavone analysis was a Waters separations module, equipped with an inline degasser (Waters In-Line Degasser AF), two liquid chromatograph pumps (Waters 515 HPLC pump) and a Waters 2996 photodiode array detector. The diode array detector was set from 200-350nm, and the eluting components were monitored at 260nm, while processing was carried out using the Waters 'Empower Software System' (Waters Corp., Milford, USA). A sample of 20 µL was loaded onto the column through an auto-sampler.

The details of the HPLC gradient profile developed for the isoflavone analysis are as follows. A linear HPLC gradient composed of (A) 0.1% glacial acetic acid in water and (B) 0.1% glacial acetic acid in acetonitrile. Following injection of 20µL of sample, 10% solvent B was run for 5min period and was increased from 10% to 35% over 50min, and finally held at 35% for 10min (Table 2.2). The detector wavelength was set to 260nm, while the system was allowed to equilibrate after a complete gradient run of a sample. A total time period of 80min was allowed for each analysis, as it was the optimized gradient profile developed for isoflavone analysis and it was used during this entire study.

Table 2.2: HPLC gradient developed for soy isoflavone analysis and separation

Start time (min)	End time (min)	Flow rate ml/min	Mobile phase composition at end time	
			%A	%B
0	5	0.8	90	10
5	50	0.8	65	35
50	60	0.8	65	35
60	65	0.8	90	10
65	85	0.8	90	10

The chromatographic conditions were thus optimized to obtain a good separation of isoflavone peaks. The identities of separated compounds in the extracts were also confirmed by comparison of retention times and co-chromatography with the authentic standards. Further to which, as required the purity and identity of peaks in different samples were confirmed by LC-MS.

2.2.8.3 Calibration curve for isoflavones standards

Six isoflavone standards purchased from LC laboratories (Woburn, MA, USA) were used for quantifying the amounts of isoflavones in soy based samples. Concentrations levels for isoflavones daidzin, glycitin and genistin were within the range of 2 - 50 ppm. While the aglycones, daidzein, glycitein and genistein were used within the range of 0.5 – 25 ppm. Isoflavone standards were prepared after separately dissolving them in methanol. Calibration curves were obtained for the six isoflavone standards with high linearity (r^2>0.995) by plotting the standard concentrations as a function of peak area obtained from HPLC analysis of 20µL injections. The concentrations of standard solutions for each isoflavone standards required for a calibration curve (Appendix A1 & A2) were chosen in such a way, as to cover the possible isoflavone contents in different soy based samples. The standard curves were further used to calculate the concentration of isoflavones in different soy based products. The standard stock solutions prepared were stored at less than 5°C to minimize any solvent evaporation and deterioration of the respective isoflavones.

2.2.8.4 Isoflavone structure confirmation by LC-MS

To confirm the correct identity of the peaks obtained by LC-UV, the standards and samples were analyzed by LC-MS. Analyses were performed on a Finnigan Quadrupole (MAT LCQ) ion trap mass spectrometer (Thermo Finnigan, San Jose, CA, USA) coupled with an electrospray ionization (ESI) source and equipped with a TSP 4000 HPLC system, which includes UV600LP PDA detector, P4000 quaternary pump and AS3000 auto-sampler. The ionization technique used for the structural elucidation of the isoflavones was electrospray ionization in the positive and negative mode. The isoflavones were separated by RP- HPLC on the YMC pack ODS AM-303 column itself, at a flow rate of 1ml/min using the developed gradient run (Table 2.2) as described earlier. Chromatogram (Appendix A3) was also obtained by LC-MS analysis, which was carried out using the same conditions used for the HPLC analysis. Selected peaks were further extracted using the +ve and -ve acquisition mode over an m/z range from 160-800 with a scan speed of 1 sec per scan. The spray voltage was set at 4.5kV and the capillary temperature was set at 250°C. The isoflavone glucosides and aglycones in the extract were identified by comparison with that of the retention time and mass spectral data of the standards. ESI-MS thus proved the most sensitive and selective method allowing a comprehensive identification of all the six isoflavones involved in the study.

2.2.8.5 Procedure for calculation and expression of isoflavone amounts

At present there is no consistency in the manner in which isoflavone levels in foods are expressed or even labeled. There are two different methods of reporting isoflavones, though some discrepancy exists in its expression. The detailed isoflavone conjugate

profile is obtained by some researchers **(Murphy et al., 1999; Walsh et al., 2003)** and their total concentration is expressed as the sum of the 12 isoflavone conjugates. Such explanations are however too complex with thorough descriptions of the entire isoflavone compositions including all of the individual conjugate forms. Considering the need to simplify this, it would be more preferable to express the isoflavone amounts as simply the total isoflavones in aglycone equivalents. Moreover, since the isoflavones are absorbed as the aglycone, the total concentration of isoflavones in food products should not be expressed as the arithmetic sum of the individual forms. The molecular weights of the glucosides are 1.6- 1.9 times greater than the aglycones and hence the total isoflavone content should only be expressed after adjusting for their molecular weight differences. Ideally molar concentrations could be used. But molar concentrations are the scientific units that are not familiar to common people. Hence expressing the isoflavone concentrations in µg/gm or mg/100gm of the soy based products might be appropriate.

The knowledge of the potentially maximum bioavailable level of isoflavones is more important and the various conjugate forms in which isoflavones exist are of little physiological or nutritional significance. Expressing the total concentration of isoflavones in 'aglycone equivalents' was therefore carried out in this study. This can be recommended as the universally acceptable method of choice for expressing the concentration of isoflavones. The method of expressing the isoflavone concentrations in aglycone equivalents and the steps involved in its calculations are described by the following equations.

The concentrations of isoflavone glucosides daidzin, glycitin and genistin were converted to aglycone equivalents, using the following equation **(Klump et al., 2001)**.

Cae = { MWa / MWg } x Cg ----------(1)

where Cae = isoflavone aglycone equivalents, µg/gm; MWa =molecular weight of aglycone; MWg = molecular weight of glucoside and Cg = concentration of glucosides i.e., daidzin, glycitin or genistin, µg/gm. The calculated aglycone conversion factors are shown in Table 2.3.

Table 2.3: Aglycone conversion factors

Isoflavone glucoside	MWa	MWg	$\frac{MWa}{MWg}$
Daidzin	254	416	0.611
Glycitin	284	446	0.637
Genistin	270	432	0.625

Total isoflavones in µg aglycone equivalents/gm of the sample were calculated by summing the concentrations of daidzein, glycitein and genistein and adding this total to the sum of aglycone equivalent concentrations of daidzin, glycitin and genistin.

T_1 = Ca (daidzein) + Ca (glycitein) + Ca (genistein) ----------(2)

T_2 = Cae (daidzin) + Cae (glycitin) + Cae (genistin) ---------- ---------- (3)

where T_1 = sum of the concentrations of aglycones and T_2 = sum of aglycone equivalent concentrations of glucosides.

Therefore

Total isoflavones, µg aglycone equivalents/gm = $T_1 + T_2$. ---------- (4)

2.2.9 Statistical analysis of data

The mean value obtained from triplicate moisture and protein analyses of soy based samples are presented throughout the study. The results of TPA and color analysis data were the obtained average value of six replication tests. And the mean values were compared for their statistical significance. Analysis of variance was carried out using SPSS version 12.0 (SPSS Inc., Chicago, USA). Probability of $p \leq 0.05$ was considered statistically significant. Isoflavone extraction experiments and analysis were performed in triplicate and the isoflavone values are reported as mean ± standard deviation as obtained. Analysis of variance was conducted to find the statistical significance between the treatments, wherever applicable in the study and it was determined at $p \leq 0.05$ levels.

PART II

RESULTS AND DISCUSSIONS

CHAPTER 3

METHOD APPLICATION FOR THE QUANTIFICATION OF ISOFLAVONES IN SOY BASED FOODS

The initial objective of the study was to develop a method for the efficient separation of isoflavones. After optimization of the method for better separation of isoflavones, the developed method was applied in determining the concentration of isoflavones in different soy based samples. Soymilk and tofu being the most widely consumed soy food in the region, they were chosen as the primary material of study. The quantification of isoflavones in these soy foods was carried out using the optimized HPLC method. The efficient separation of the six isoflavone peaks of a single run together with its reproducibility of analysis was evaluated for extending the application of the developed HPLC method throughout this research work. The identification and characterization of individual peaks was further carried out using LC-MS.

3.1 APPLICATION OF METHOD DEVELOPED FOR ISOFLAVONE ANALYSIS

Commercially available soymilk samples from Singapore were initially chosen for evaluation of the developed HPLC method of isoflavone analysis. Further to this many commercially available soymilk samples were collected from other South East Asian Countries such as Malaysia, Indonesia and Thailand. A collective database on the content of isoflavones in different commercially available soymilk samples was thus made possible.

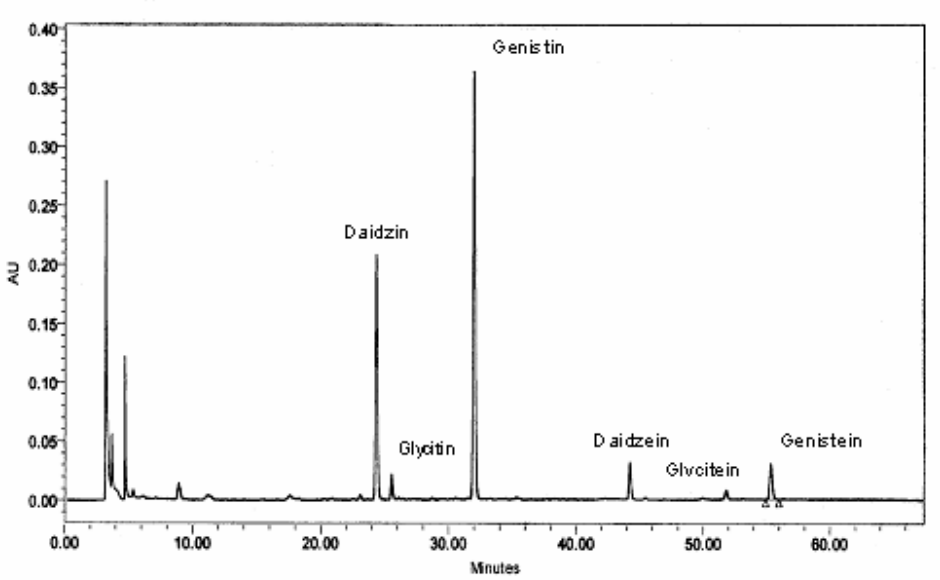


Figure 3.1: HPLC chromatogram of the six isoflavones in a sample of soymilk

The typical HPLC chromatogram of isoflavones obtained by reversed phase HPLC analysis of a soymilk sample is shown in Figure 3.1. The elution order and retention time (min) of isoflavones are as follows: daidzin 24.71±0.4; glycitin 25.94±0.5; genistin 31.61±0.6; daidzein 44.34±0.4; glycitein 45.82±0.3; genistein 55.47±0.44 minutes. The developed HPLC method also had additional advantages of not having many interfering peaks, which further decreased the complexity of sample analysis. The relative standard deviations (RSD) on repeated analysis of the same sample was determined and proved acceptable levels of accuracy and precision (<10% RSD). However, the time required for an analysis was lengthy (80min). But an effective separation of individual peaks with a longer time gap between the neighboring peaks served for an accurate quantification of the compounds. This also avoided any co-elution that might have occurred during a shorter run time. The developed and optimized HPLC method therefore proved

reproducible and repeatable with effective resolution of peaks providing a means for accurate quantification of isoflavones in different soy based samples.

The concentration of genistin, daidzin and glycitin followed a decreasing order in many of the soy food samples analyzed during this study. This is because, among all the isoflavones, genistein derivatives are present in higher proportions followed by daidzein and glycitein derivatives respectively. An alkaline hydrolysis caused the removal of the acylated groups (malonic acid and acetic acid), causing an increase in the area of the existing glucoside peaks. Aglycones were found in lesser amounts than the glucosides in many of the soy samples. Glycitein approached zero values in certain cases and was always present in least concentrations for every sample analyzed. The peaks obtained from isoflavone analysis in soymilk samples by HPLC method was further confirmed by LC-MS. It was this optimized and developed HPLC method that was further extrapolated for an LC-MS application and the selected peaks were detected in the +ve and –ve ionization modes. The results of LC-MS analysis further affirmed the suitability of the developed method for the quantification of isoflavones in various soy based samples.

The protein content of the soymilk and tofu samples was also determined in parallel for an understanding of its relationship to the isoflavone contents of the individual products. Further the protein content of the product was related to their isoflavones concentrations. This is the first set of studies which determined the level of isoflavones and protein content in the soymilk / tofu samples for an evaluation of its direct relationship.

3.2 RESULTS OF LC-MS ANALYSIS OF ISOFLAVONES

Electrospray ionization mass spectrometry (ESI-MS) provides mass spectrum with little or no fragmentation and this technique is suitable for the characterization of phenolic compounds in food products. The isoflavone aglycones, daidzein, glycitein and genistein and the isoflavone glucosides, daidzin, glycitin and genistin, were identified by comparison of the retention time and mass spectral data with those of their standards. Details of ions observed in positive and negative ion spectra of the isoflavones generated by ESI-MS are shown in Table 3.1.

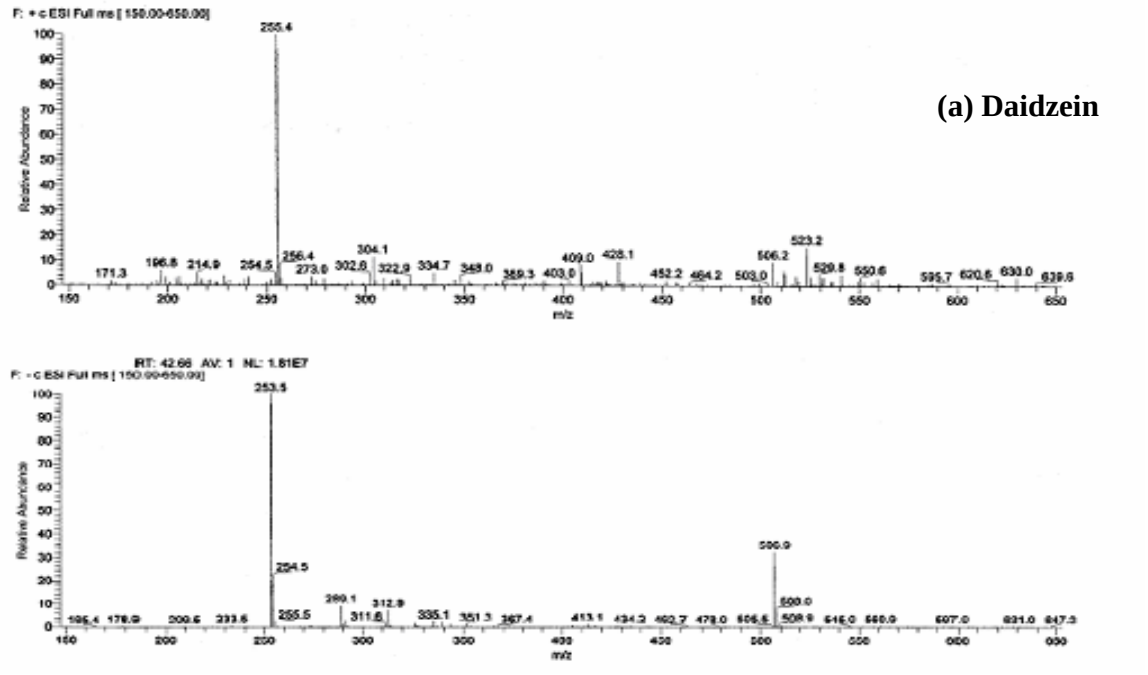
Table 3.1: Ions observed in positive and negative ion spectra of isoflavone glucosides and aglycones, generated by ESI – MS

Isoflavone	+ve ions	-ve ions
Daidzein	255.4 $[M+H]^+$	253.5 $[M-H]^-$
Glycitein	285.3 $[M+H]^+$	283.5 $[M-H]^-$
Genistein	271.4 $[M+H]^+$	269.5 $[M-H]^-$
Daidzin	255.3 $[M+H-Glc]^+$ 417.0 $[M+H]^+$	253.4 $[M-H-Glc]^-$ 415.4 $[M-H]^-$ 475 $[M-H+CH_3COOH]^-$
Glycitin	285.0 $[M+H-Glc]^+$ 447.2 $[M+H]^+$	283.1 $[M-H-Glc]^-$ 445.2 $[M-H]^-$ 505 $[M-H+CH_3COOH]^-$
Genistin	271.1 $[M+H-Glc]^+$ 433.1 $[M+H]^+$	269.1 $[M-H-Glc]^-$ 431.3 $[M-H]^-$ 491 $[M-H+CH_3COOH]^-$

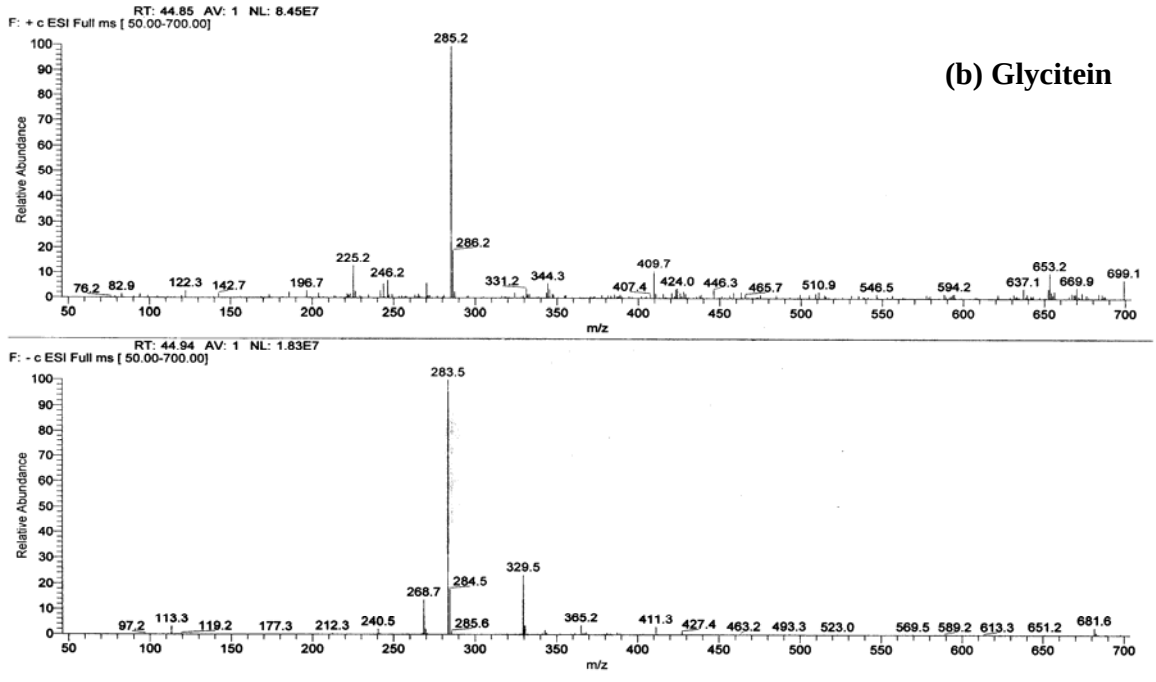
*Isoflavones were separated by 0.1% acetic acid in water and acetonitrile gradient profile on a YMC pack ODS column.

Using the ESI mode, the molecular ions $[M+H]^+$ and $[M-H]^-$ appeared as the most abundant ions in the positive and negative ion spectra respectively. Ion peaks at m/z

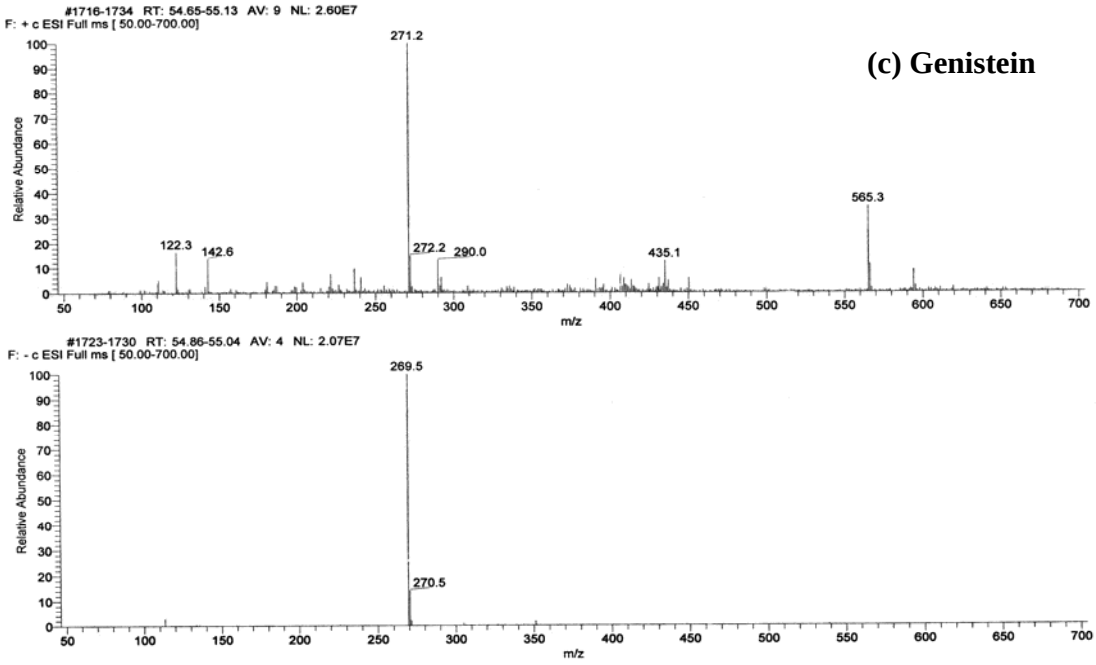
255.4, 285.2 and 271.2 are the positive molecular ions obtained for the three isoflavone aglycones of daidzein, glycitein and genistein respectively. Each isoflavone β-glucoside conjugate produced an $[M+H]^+$ ion and the corresponding aglycone fragment ion in the +ve acquisition mode. Figure 3.2 illustrates the MS spectra of soy isoflavone aglycones daidzein, glycitein, genistein and β-glucoside conjugates daidzin, glycitin, genistin respectively. Molecular ions $[M+H]^+$ of m/z 417.0, 447.2 and 433.0 and the specific fragments of $[M+H - glucosyl]^+$ at m/z 255, 285 and 271 for daidzin, glycitin and genistin are obtained respectively. For the isoflavone glucosides, they readily formed adducts with the acetate ions and these ions were the most abundant in negative ion mass spectra. Thus additionally, the acetate adduct $[M-H+CH_3COOH]^-$ ions were observed (Table 3.1). The molecular ions and the adduct ions generated by the ionspray interface provided an ideal method for detection of soy isoflavones. This confirms the application of ESI-MS for the identification of soy isoflavones.



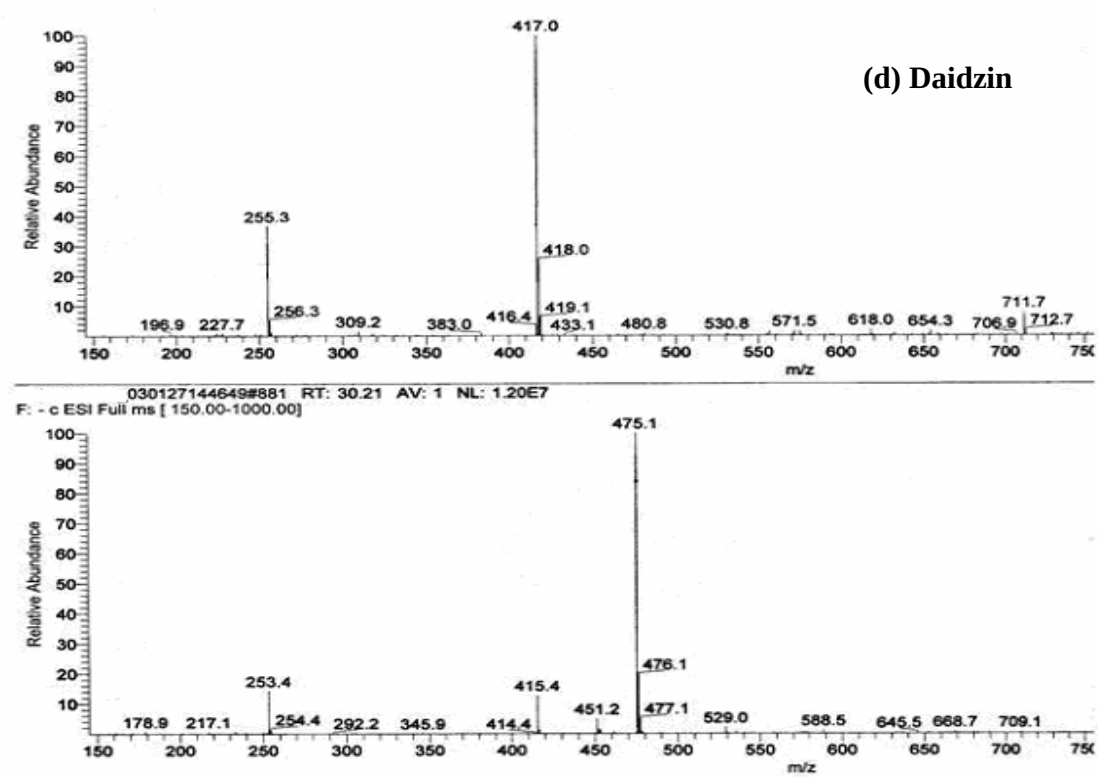
(a) Daidzein



(b) Glycitein



(c) Genistein



(d) Daidzin

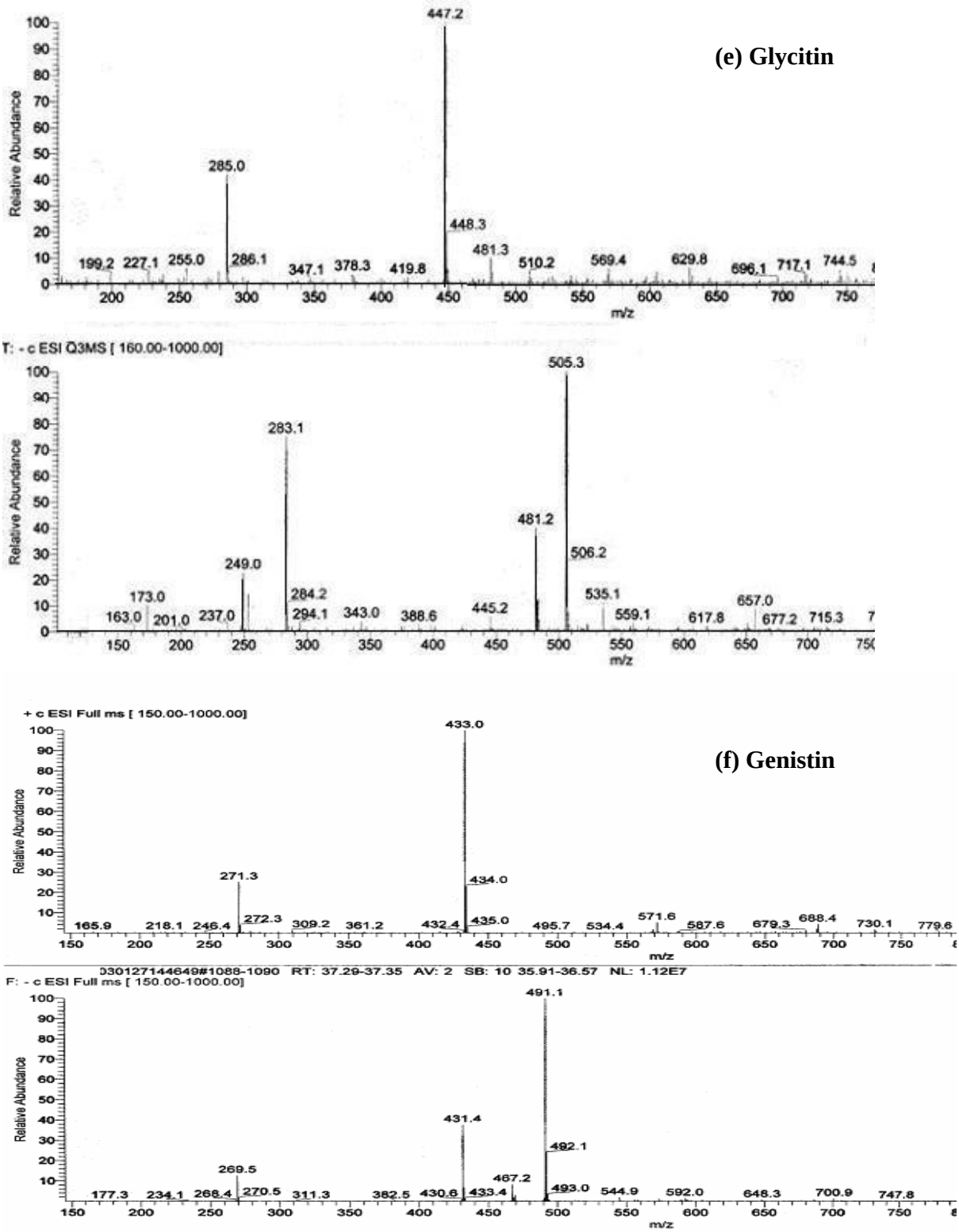


Figure 3.2: MS spectra of soy isoflavone aglycones (a) Daidzein (b) Glycitein (c) Genistein and their respective glucosides (d) Daidzin (e) Glycitin (f) Genistin

3.3 QUANTIFICATION OF ISOFLAVONES IN SOYMILK

Results of isoflavone analysis of soymilk samples from Singapore are presented collectively, while the results of isoflavone analysis in soymilk samples from other South East Asian countries are separately presented. A similar arrangement was also done while describing the isoflavone analysis results of tofu samples.

3.3.1 Isoflavone concentrations in soymilk samples from Singapore

Table 3.2 shows the results of isoflavone analysis of commercially available soymilk samples collected from Singapore market. The samples analyzed from Singapore had protein contents ranging from 2.0 – 4.2gm/100gm and the protein is derived almost exclusively from soy in all of them. Globally, there are two different starting materials used for the production of soymilk: whole soybeans and soy isolates. But in South East Asian countries, whole soybeans are used traditionally to prepare soymilk and, the protein content in the beans might also affect the final amount of protein in the soymilk prepared from it.

Table 3.2: Total isoflavone data (on wet weight basis) reported as μg aglycone equivalents per gram of the sample for commercially available soymilks from Singapore.

Milk type	M%	P%	Din	Glyin	Gin	Dein	Glyein	Gein	Total[A] (μg/gm)
Brand A	96.0	2.04	44.23	8.41	49.92	6.85	1.11	4.86	76.41±0.28
Brand B	90.6	2.85	69.77	11.58	76.88	3.63	0.11	1.71	103.52±0.83
Brand C	85.2	3.67	102.56	11.56	143.18	4.09	0.36	3.74	167.71±1.60
Brand D	82.6	3.70	115.07	24.81	142.37	1.32	0.09	0.95	177.46±0.97
Brand E	86.2	4.20	136.72	11.95	140.12	12.35	0.77	7.40	199.25±0.99

M% =% moisture, P% =% protein, where the protein content is calculated from the BioRad protein assay. Din =daidzin, Glyin =glycitin, Gin =genistin, Dein =daidzein, Glyein =glycitein, Gein =genistein. Total[A] = total isoflavone ±SD where SD is the standard deviation of four replicated samples and the mean values for total isoflavones are stated here.

The moisture content of the samples varied among one another and ranged from 82.60 - 96.00%, while the total isoflavone concentrations in soymilks ranged from 76.41 to199.25 μg/gm on a wet weight basis. Among the different brands of soymilk analyzed, 'Brand E' contained the highest amounts of daidzein and genistein. When soy foods are processed with water for a long residence time, the native soy β–glucosidase will become active generating more of aglycones as in soaking of soybeans prior to processing into soymilk. The soybeans used for preparing 'Brand E' soymilk might have had a longer soaking period than the other soymilk brands. Within the soymilk group, the items with higher protein content showed significantly higher isoflavone levels than the items with lower protein content (Figure 3.3). From the results of analysis of isoflavones in this soy food group, it appears that isoflavones are found at higher concentrations in high protein

containing soy foods. However commercial products being analyzed, the source of soybean used by different producers might be different and hence a comparison of isoflavone contents in them based on dry weight basis was not carried out.

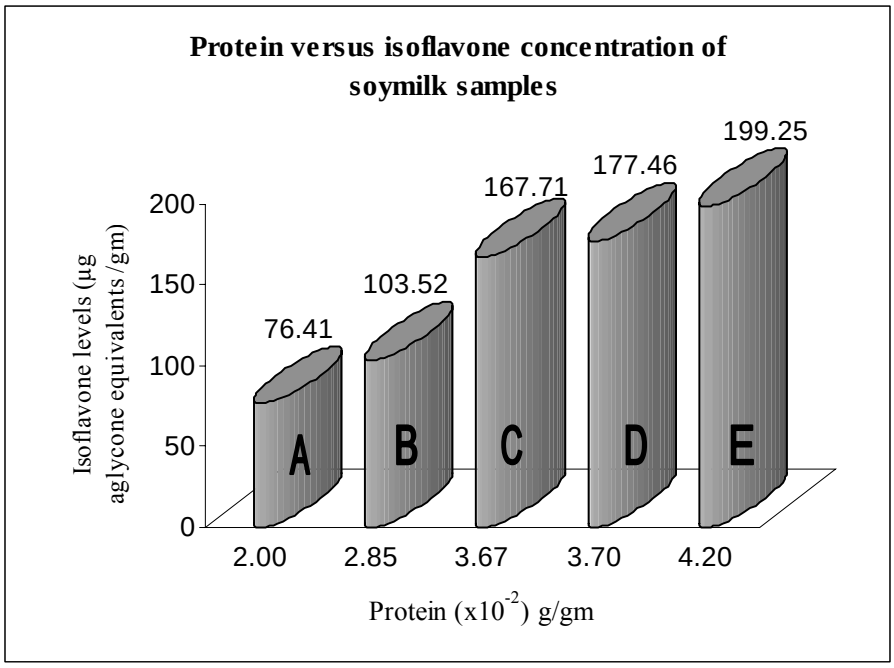


Figure 3.3: Correlation of protein and isoflavone levels for soymilk samples

3.3.2 Isoflavone concentrations in soymilks from other South East Asian countries

Table 3.3 shows the level of isoflavones in commercially available soymilk samples obtained from Malaysia, Indonesia and Thailand. Soymilks sold in Malaysia had protein contents much lower than those sold in Singapore. Soymilks with >2.5% protein content were rarely obtained from Malaysia.

Table 3.3: Total isoflavone data (on wet weight basis) reported as μg aglycone equivalents per gram of sample for the commercially available soymilks from Malaysia*, Indonesia[Ĭ] and Thailand[Ť].

Milk type	M%	P%	Din	Glyin	Gin	Dein	Glyein	Gein	Total[A] (μg/gm)
Brand M1*	86.0	2.50	54.63	9.36	82.94	3.33	2.26	2.81	99.60±0.28
Brand M2*	87.2	2.30	20.13	4.25	27.56	20.53	3.04	14.34	70.15±0.41
Brand M3*	88.0	2.20	33.71	6.59	49.61	2.06	2.19	1.09	61.16±0.10
Brand M4*	87.6	2.12	28.17	4.08	36.54	7.68	2.28	5.22	58.02±1.40
Brand M5*	88.2	2.00	47.76	4.52	ND	3.18	2.06	1.80	39.12±0.03
Brand M6*	88.2	2.00	22.68	4.19	28.79	1.20	ND	0.39	36.12±1.40
Brand M7[Ĭ]	89.0	3.80	45.67	6.74	62.71	6.61	2.31	5.92	86.24±0.22
Brand M8[Ĭ]	88.5	1.50	31.38	5.82	43.04	3.07	ND	2.08	54.94±0.20
Brand M9[Ť]	87.4	3.79	35.54	6.40	55.82	9.29	2.50	7.60	80.08±0.67
Brand M10[Ť]	86.4	3.83	59.98	9.44	100.98	ND	ND	ND	105.77±1.41

M% =% moisture, P% =% protein. Din =daidzin, Glyin =glycitin, Gin =genistin, Dein =daidzein, Glyein =glycitein, Gein =genistein. ND= not detected. Total[A] = total isoflavone ±SD, where SD is the standard deviation of four replicated samples and the mean values for total isoflavones are stated here.

If the soymilks had relatively similar moisture content with different protein contents, the trend of higher isoflavone levels with increase in protein content can be easily established. Though generally high concentrations of isoflavones were found in soymilks with higher protein contents, inconsistency of results were also found in some cases. Brand M7, a product of Indonesia, was found to contain a comparatively low concentration of isoflavones in spite of having higher protein content (3.80%). This might be an exception, where the soymilk is probably not prepared from whole soybean itself, but might be prepared from chemically treated (i.e., alcohol washed) soy protein concentrate or SPC. Aqueous alcohol extraction is commercially used for removing the

soluble carbohydrates from defatted soy flakes. Since aqueous alcohols are excellent sources for isoflavones, the process would lead to considerable loss of isoflavones. Therefore if an alcohol washed SPC is used for the preparation of soymilk, a product with less isoflavones are obtained.

The total concentration of isoflavones in the soymilks collected from different South East Asian countries ranged from 36.12 - 199.25 μg/gm, on a wet weight basis. Generally, soymilks were found to contain a higher concentration of glucosides, primarily genistin, followed by daidzin and glycitin respectively. Aglycones can be formed during the traditional method of preparation of soymilk, whereby the soaking of soybeans in water for a longer period of time might have been followed in some cases. 'Brand M2' a soymilk brand from Malaysia was the only exception (Table 3.3), where a higher amounts of aglycones were found with respect to its glucoside amounts, possibly due to drastic differences in processing conditions used for its preparation. Soaking of soybeans for a period of 16-24hr or a fermentation process are possible explanations for the higher amounts of aglycones in 'Brand M2' than the others. The variety of soybeans and the processing techniques applied to produce soymilk could be the other factors contributing to the variation of isoflavone conjugates in different brands of soymilk. However, it was apparent that higher concentrations of isoflavones are found in soymilks with higher amounts of protein.

3.4 QUANTIFICATION OF ISOFLAVONES IN TOFU

3.4.1 Isoflavone contents in commercially available tofu samples from Singapore

Many different varieties of tofu suitable for different cooking purposes were found in Singapore market. Various processing methods might be utilized in making different types of tofu. There are the aseptic style tofus, where commercial soymilks are coagulated in the package with glucono-δ-lactone and no whey is removed or tofu can be even momen or traditional style, where whey is discarded after the curd production **(Dwyer et al., 1994).** While if the coagulation was carried out using calcium salt, the metallic cation from the coagulant Ca^{2+}, reacts with proteins in soymilk and precipitate to form curds **(Shurtleff and Aoyagi, 1979b)**. Some protein associated isoflavones might be released into the whey as well, during a firm tofu preparation. Therefore in establishing a database on isoflavone concentrations in tofu, it is critical to understand the type of processing each type of tofu has undergone to properly interpret the results of isoflavone concentration and its distribution. The concentrations of isoflavones in different types of commercially available tofus collected from the Singapore market are shown in Table 3.4.

Table 3.4: Total isoflavone data (on wet weight basis) reported as μg aglycone equivalents per gram of the sample for the commercially available tofus from Singapore market.

Tofu type	M %	P %	Din	Glyin	Gin	Dein	Glyein	Gein	Total[A](μg/gm)
Silken tofu	85.9	5.28	131.73	15.40	109.28	2.44	ND	2.82	163.85±2.45[a]
Chinese tofu	89.6	6.76	129.92	16.95	109.53	1.94	0.05	2.28	162.91±2.29[a]
Organic tofu	88.6	6.53	139.77	16.14	120.67	4.26	0.04	2.50	177.90±1.81[a]
Braised tofu	67.4	9.69	109.36	19.75	123.82	6.04	0.25	4.82	167.91±6.59[a]
Deep-fried	74.4	11.70	217.32	26.42	205.96	10.88	0.49	7.84	297.55±4.54[b]
Taw kwa	74.4	9.67	236.66	23.98	229.06	5.50	0.21	3.68	312.42±2.33[bc]

M% =% moisture, P% =% protein, where protein content is calculated from the BioRad protein assay. Din =daidzin, Glyin =glycitin, Gin =genistin, Dein =daidzein, Glyein =glycitein, Gein =genistein. ND = not detected. Total[A] = total isoflavone ±SD, where SD is the standard deviation of four replicated samples and the mean values for total isoflavones are stated here. Like superscripts in the same column do not differ significantly at $p \leq 0.05$. All tofus analyzed were from the same manufacturer (Unicurd Food Co', Senoko, Singapore).

'Silken', 'Chinese' and 'Organic' tofu were the soft tofu varieties obtained from Singapore market. These tofus differed in their moisture and protein contents. Also the types of coagulants used for their preparation were different (as observed from labeling). However, the concentrations of isoflavones in these commercially available soft tofu varieties were nearly the same ($p \leq 0.05$). The minor differences in the isoflavone concentrations could be partly due to the variety of beans used for its preparation or the manner in which the tofu was prepared using different coagulants. Some coagulants might be even better and more efficient than the other in terms of retaining isoflavones in tofu from soymilk **(Jackson et al., 2002)**.

'Taw kwa' is a type of firm tofu while, deep-fried and braised tofu is prepared from 'taw kwa'. 'Deep-fried' tofu is usually made by frying the firm tofu in oil at a high temperature of 180-185ºC. For braised tofu making, the firm tofu is marinated with a sauce (sweet or spicy), whereby the pH will be reduced and it further undergoes a pasteurization procedure. These procedures caused the isoflavone levels in braised tofu to be much lower than that of 'taw kwa' and 'deep-fried' tofu. The presence of other non-soy components such like spice powder, sweet and sour sauce etc in the braised tofu greatly influenced the isoflavone content of the total food consumed. A linear trend in isoflavone levels with the protein content of tofus couldn't be established in this study (Figure 3.4). But it was apparent that higher concentrations of isoflavones were present in tofus which had higher protein content. Thus taw kwa and deep-fried tofu contained a much higher protein content than the soft tofu samples and they also contained higher isoflavone amounts than the soft tofu types.

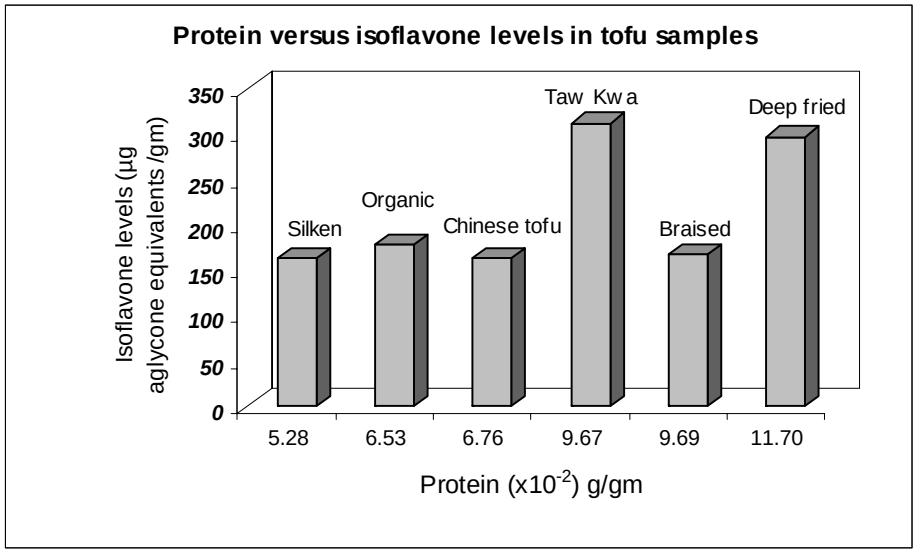


Figure 3.4: Protein-isoflavone relationship in tofu samples

Currently, it is not mandatory to label the concentration of isoflavones in soymilks or tofus. But protein contents are sometimes labeled on soy foods, which can serve as an indication of the comparative amounts of isoflavones in the available products found on the shelf. Every products purchased from Singapore indicated the approximate amount of protein levels on their labels. This can be very helpful for the consumers and nutritionists in identifying a product with more functional benefits.

3.4.2 Isoflavone concentrations in tofu samples from other South East Asian countries

A soft tofu from Malaysia was labeled as 'Tofu lembut' while a firm one was labeled as 'bean curd'. The tofu samples collected from Malaysia and Indonesia were either a soft tofu or a firm tofu, made from pure soymilk and were not added with other non soy components. Tofus are commonly sold in open streets of Indonesia without any packing, but commercially available tofu samples from local stores in Indonesia were purchased during this study. Table 3.5 details the level of isoflavones in tofu samples collected from other South East Asian countries.

Table 3.5: Total isoflavone data (on wet weight basis) reported as µg aglycone equivalents per gram of sample for the commercially available tofu samples from Malaysia*, Indonesia[Ĭ] and Thailand[Ť].

Tofu type	M%	P%	Din	Glyin	Gin	Dein	Glyein	Gein	Total[A] (µg/gm)
Soft tofu [Ť]	90.8	6.07	24.66	8.74	62.90	2.08	4.42	8.50	74.95±0.13
White tofu [Ĭ]	87.00	6.10	16.54	ND	9.85	32.92	6.35	40.33	95.87±0.19
Silk tofu [Ť]	91.00	6.70	31.31	9.99	50.92	22.49	5.70	21.39	106.91±0.09
Silk tofu [Ĭ]	91.20	6.70	39.21	8.35	58.32	21.41	5.19	26.54	118.87±0.63
Silk tofu*	90.20	7.10	76.70	13.43	98.43	7.61	4.48	4.92	133.95±1.19
Taufu lembut*	89.00	6.30	112.62	18.76	178.47	2.96	6.45	2.42	204.14±0.14
Sakura tofu[Ť]	83.73	8.14	13.63	6.88	34.71	51.19	ND	56.92	142.52±0.13
Organic tofu[Ť]	77.60	14.30	22.93	5.68	63.20	57.29	4.07	60.35	178.85±1.62
Tahu [Ĭ]	76.50	9.36	96.14	21.23	156.71	17.97	4.66	15.70	208.55±0.32
Firm tofu*	81.40	13.10	94.08	15.15	181.48	19.18	4.82	19.33	223.90±2.63
Selan Jaya [Ĭ]	81.50	13.80	92.90	18.03	139.85	45.65	7.00	21.59	229.91±2.75
Yellow tofu [Ĭ]	72.80	11.61	105.68	21.02	186.20	29.50	6.09	26.25	256.18±3.43
Bean curd*	75.00	12.90	247.19	21.4	309.05	10.62	ND	6.42	374.86±0.05

M% =% moisture, P% =% protein where protein content is calculated from the BioRad protein assay.
Din =daidzin, Glyin =glycitin, Gin =genistin, Dein =daidzein, Glyein =glycitein, Gein =genistein. ND = not detected. Total[A] = total isoflavone ± SD, where SD is the standard deviation of four replicated samples and the mean values for total isoflavones are stated here. Soft tofu [Ť], White tofu [Ĭ], Silk tofu [Ť], Silk tofu [Ĭ], Silk tofu*, Taufu lembut* are all soft tofu samples while Sakura tofu [Ť], Organic tofu [Ť], Tahu [Ĭ], Firm tofu*, Selan Jaya tofu [Ĭ], Yellow tofu [Ĭ], Bean curd* are all firm tofu samples

Generally a soft tofu contained less protein and less isoflavones amounts than the firm tofu. Firm tofu making, uses a soymilk of higher solid content than that required for soft tofu making. Moreover, wheys are pressed out during a firm tofu preparation and that makes the protein molecules to bind together and form a firm network, which is not in the case of a soft tofu. Hence the firm tofus if not added with non soy components are higher in protein and isoflavone contents than the soft tofu types. While most of the Singapore

tofu products are not mixed with any non-soy components, the Thai products are sometimes incorporated with seaweeds and other food substances, which ultimately reduced the total concentration of isoflavones in the product. For example, organic tofu from Thailand was a firm tofu, but was composed of other non soy components and hence the isoflavone concentration was comparatively low.

3.5 DISCUSSION ON THE RESULTS OF ISOFLAVONE ANALYSIS

The extraction procedure followed in this study used a mild saponification step that converted the isoflavone glucoside ester forms to corresponding isoflavone glucoside forms, leaving the three parent isoflavones intact. This approach allowed a direct comparison with the stable, readily available, isoflavone parent and glucoside reference standards. The optimum conditions for the extraction of isoflavones from food products were thoroughly investigated before a method of extraction was chosen. Solvent loss if any by evaporation, during the extraction procedure was also corrected in this extraction procedure by the final volume make up done towards the end of the extraction procedure *(index no: 2.2.8.1)*. This further helped in obtaining a high reproducibility of the sample analysis. However, one cannot exclude the possibility that an alternative solvent mixture may be optimal for different soy food matrices.

The isoflavones were well separated by the developed and optimized reversed-phase HPLC analysis method, permitting the analysis of both the glucosides and the aglycones in a single chromatographic run and a legitimate quantification was easily made possible. The procedure followed in this study for the analysis of isoflavones in soy foods was

simple, rapid, reproducible and less detrimental to column life. It also had a particular advantage over methods in which the isoflavone glucosides were calculated by the differences in concentration of isoflavone aglycones, determined using isocratic HPLC methods or between acid-hydrolyzed and unhydrolyzed samples **(Wang et al., 1990).** However, the method used in this study didn't give a detailed analysis pattern of every conjugates of soy isoflavones. Thus the malonyl and acetyl conjugate concentrations were not estimated individually, but the total concentration of isoflavones in the soy product was only determined during the complete study.

The present study therefore resulted in a comprehensive analysis of several brands of soymilk and tofu samples from South East Asian countries. No published reports are available on the collective data of isoflavone contents in soymilk and tofu samples from South East Asian Countries. **Franke and others (1999)** did a comparative study on the isoflavone levels in raw and cooked forms of 'taw kwa', raw tofu, 'fried-taw kwa' and dried soybean curd sticks. Cooking was carried out for 5min for all these soy foods by these researchers. In general, cooked products showed lesser isoflavone levels than the raw products probably because of the leaching of isoflavones into the water. Since there are many different types of tofu and their methods of preparation are different, the results reported by these authors were a little confusing. For example, raw 'taw kwa' and raw tofu showed nearly an equal amount of isoflavones (297µg/gm) in their study. However, it was not understood, how raw 'taw kwa' differed from raw tofu and why both showed equal amounts of isoflavones. In this study, we differentiated between the soft and firm tofu types available in the market for a better and general understanding on the protein

content and concentration of isoflavones. A guideline towards identifying the product of high nutritional value was hence made possible in a more convenient way.

The protein content of soymilk and tofu samples were always labeled for Singapore and Thailand products, while it was sometimes labeled for Malaysian products too. Indonesian products were usually not labeled with their protein contents, especially for tofu samples. Protein labeling can be used as a good indication of isoflavone contents in soymilk and tofu samples, if they are prepared from soybean itself and provided the protein content is entirely derived from it. When other food components are added to the soybean products, the overall isoflavone concentrations were lower. In a similar way, if soy protein isolate is used as a raw material instead of soybean for the preparation of soymilk or tofu, then the isoflavone contents in the soy food will be much lower.

A mass balance of isoflavones for the soy products or a direct comparison of isoflavones between the above listed foods cannot be made in this study, since the soybean variety used to produce each food is unknown and the total isoflavone content can vary depending on many factors including the variety, geographical location and growth year of the soybeans. Results of the isoflavone analysis of these commercially available products were similar to those reported by other researchers **(Wang and Murphy, 1994).** However, it was apparent that higher concentrations of isoflavones are present in those soymilk or tofu samples containing high protein content. There aren't any published reports which quantified the isoflavones and protein simultaneously to understand the co-relation between the protein content and isoflavone amounts in soy foods. This is the first

set of studies which analyzed wide brands of soymilk and tofu samples from the South East Asian Countries to obtain a generalized conclusion of their isoflavone and protein contents. Moreover, the concentration of isoflavones in these commercially available soy foods if labeled can serve as an indication of its functional value for the consumers. Levels expressed as total aglycone equivalents would also provide an indication of the potentially maximum bioavailable level of isoflavones in the food, because it is only the aglycones that are absorbed from the human intestine. It is even more logical to label the amount of isoflavones in mg aglycone equivalents per 100 gram of the soy food, for which the public will have a better grasp of understanding.

CHAPTER 4

EFFECT OF EXTRACTION METHODS AND UHT TREATMENT CONDITIONS ON THE LEVEL OF ISOFLAVONES DURING SOYMILK MANUFACTURE

With the advancement in process development and equipment design, different processing systems are being made available for the soymilk manufacturing industry. These production modules can result in continuous extraction of soymilk from soybeans. However, the fundamental consideration of what happens to the nutritional quality and functional ingredients of soymilk is lacking. Determining the fate of isoflavones, the principal phyto-nutrients in soymilk, during various stages of processing was therefore considered essential. The processing conditions used for its preparation are the most important parameters that ultimately determine the quality of soymilk obtained. A particular processing system might have its own advantages and disadvantages in terms of the nutritional quality of the soymilk obtained. One such soymilk processing system is studied here by evaluating the effect of different extraction methods and heat treatments on the level of isoflavones in the final soymilks obtained. The HPLC method developed for isoflavone analysis during this study was further utilized for the quantification of isoflavones in the raw material, intermediates, byproducts and final soy preparations of the particular soy processing system.

4.1 ISOFLAVONE LEVELS IN SOYMILK PREPARED BY HOT-GRIND VERSUS COLD-GRIND METHOD

The processing system used in this study for the preparation of soymilk had a particular advantage of grinding the soybeans directly with hot or cold water rather than a 'soak and grind process' used conventionally. The traditional soymilk making process, termed as the 'soak and grind process' involves the soaking of soybeans in water for a prescribed period of time (4-12hrs) followed by grinding them in fresh water. **Wang and Murphy (1996)** did a mass balance study during the preparation of soymilk, where soaked beans were found to contain a lesser concentration of isoflavones than the raw soybeans. The differences in their content of isoflavones were explained as due to the leaching of isoflavones into the soaking water. Such losses of isoflavones into the soaking water were avoided by using the direct grinding process utilized in this study.

There is an extensive literature available on the processing effects of isoflavones during the preparation of soymilk and tofu by the traditional process **(Coward et al., 1993; Jackson et al., 2002)**, but little information is available on the effect of extraction methods and ultra high temperature (UHT) treatment conditions on the level of isoflavones during soymilk manufacture. Moreover, the recent trend in utilization of these automated extraction and UHT processing modules for the preparation of soymilk makes it essential in understanding the nutritive contents of the end product obtained from such processing systems. The most common new extraction methods utilized for the preparation of soymilk are either a hot grinding or a cold grinding process, while the UHT treatment commonly applied to soymilks are either a direct or an indirect UHT

process. The processing module used in this study resulted in the direct grinding of soybeans with hot or cold water, while the sterilization of soymilk was achieved by either a direct or an indirect UHT treatment so as to obtain an aseptic style soymilk. Thus a comparison in the isoflavone concentrations between the hot grinding and cold grinding of soybeans, as well as between the direct and indirect UHT treatment processes was carried out in order to understand its effect on the concentration of isoflavones in the soymilk obtained.

The total concentration of isoflavones in soy samples were expressed in microgram aglycone equivalents per gram of the sample on a dry matter basis (μg/gm DM). Table 4.1 & 4.2 shows the results of isoflavone analysis in samples of the hot grind and cold grind process respectively, while the results of the duplicate trials are shown in Table 4.3 and 4.4.

The malonyl isoflavone forms are highly unstable to heat. The high-temperature heat processing of soymilk may cause changes in the concentrations of malonyl isoflavone conjugates, due to the conversion of the malonyl forms to their respective glucoside forms. However, a detailed investigation on the individual isoflavone conjugates profile was not carried out but it was only the total amount of isoflavones that was determined in this study to understand the effects of processing. Moreover, there is a difficulty in comparing the isoflavone levels in the soybeans with that of the soymilks obtained on a dry weight basis, because the composition of dry matter in the whole beans and that obtained for soymilk differ as a result of the removal of okara and injection or removal of

steam/water at different steps involved in the soymilk manufacturing system. Hence, a comparison of isoflavone levels in soymilks obtained from two different grinding procedures (Hot versus Cold) and after two different UHT treatment methods (Direct versus Indirect) were carried out to better understand the effects of processing on the isoflavone levels in the soymilks obtained. Isoflavone concentrations are reported as the means of three determinations with their standard deviations (SD). Test for analysis of variance were carried out to determine the significant differences between the isoflavone values in soymilk obtained by hot and cold grinding methods as well as between the two different UHT treatment processes. $P \leq 0.05$ was considered significant (SPSS version 12.0, SPSS Inc., Chicago, USA).

Table 4.1: Total isoflavone concentrations in µg aglycone equivalents per gram of the sample reported on a dry matter basis, in various samples of a hot grind process trial.

Sample names (HG process)	M%	Din	Gln	Gin	Dein	Glen	Gein	Total[A] (µg/gm)
Soybeans for indirect process	10.0	805.66	184.55	948.33	19.11	51.77	ND	1273.40±1.13[a]
Ground beans	86.4	1023.67	223.08	1006.76	ND	ND	ND	1396.79±3.52[b]
LOX treated	85.8	1016.17	210.49	990.77	8.69	0.70	10.84	1394.61±1.79[b]
Okara	73.0	625.66	143.70	638.22	19.51	1.66	15.18	909.05±0.54[c]
Soymilk[1]	90.0	1397.40	271.00	1288.40	19.50	0.75	14.60	1866.66±1.22[d]
Soymilk after TI deactivation	90.8	1374.23	286.84	1352.93	5.00	0.65	9.45	1883.05±0.35[d]
Soymilk after indirect UHT	90.4	1270.52	276.87	1322.08	6.66	ND	10.20	1795.81±1.10[d]
Soybeans for direct process	10.0	798.91	188.00	945.00	21.80	52.94	ND	1273.25±3.94[a]
Ground beans	84.5	1094.58	196.06	1037.87	9.48	ND	7.93	1459.75±6.18[b]
LOX treated	84.6	1114.93	198.63	1056.75	9.61	ND	8.37	1486.19±1.65[b]
Okara	73.8	746.06	3.88	762.40	13.12	4.92	9.61	962.46±1.00[c]
Soymilk[1]	90.0	1490.00	273.50	1493.60	11.50	0.20	9.30	2039.11±3.28[e]
Soymilk after TI deactivation	91.0	1624.88	275.88	1482.33	13.22	0.33	10.77	2119.31±0.67[e]
Soymilk after direct UHT	90.6	1313.83	251.06	1322.34	11.11	0.32	8.72	1809.28±0.38[d]

M % = moisture %. Din= daidzin; Gln= glycitin, Gin=genistin; Dein= daidzein; Glen= glycitein; Gein=genistein. ND = not detected.
Soymilk after TI deactivation = soymilk[2].
Soymilk after direct UHT = soymilk[3].
Total[A] = total isoflavones expressed in µg aglycone equivalents ± standard deviation.
Means with different letters in the same column are significantly different at $p<0.05$.

Table 4.2: Total isoflavone concentrations in µg aglycone equivalents per gram of the sample reported on a dry matter basis, in various samples of a cold grind process trial.

Sample names (CG process)	M%	Din	Gln	Gin	Dein	Glen	Gein	Total[A] (µg/gm)
Soybeans for indirect process	10.0	805.66	184.55	948.33	19.11	51.77	ND	1273.40±1.13[a]
Ground beans	86.2	350.00	100.72	424.63	390.57	72.24	269.13	1275.34±1.35[a]
LOX treated	85.0	418.80	95.13	464.60	269.73	57.33	201.86	1135.78±5.55[ab]
Okara	74.0	361.96	94.46	419.73	171.92	29.65	105.19	850.41±1.01[c]
Soymilk[1]	89.4	1158.86	216.50	1164.71	73.30	8.11	50.56	1705.88±2.68[d]
Soymilk after TI deactivation	90.4	1162.18	218.43	1150.72	ND	5.65	32.81	1606.89±0.57[d]
Soymilk after indirect UHT	90.8	1189.00	207.90	1188.30	72.28	7.71	54.78	1736.36±0.61[d]
Soybeans for direct process	10.0	798.91	188.00	945.00	21.80	52.94	ND	1273.25±3.94[a]
Ground beans	84.2	577.21	103.16	587.65	287.02	55.06	199.17	1326.92±2.76[a]
LOX treated	85.2	538.91	92.22	548.98	286.62	54.79	209.45	1281.99±2.30[a]
Okara	74.6	315.43	71.33	351.45	224.68	51.53	162.36	896.39±2.94[c]
Soymilk[1]	90.2	1455.81	256.12	1387.95	68.26	8.06	47.19	2043.63±0.08[e]
Soymilk after TI deactivation	91.2	1371.02	243.97	1334.43	73.18	7.38	48.75	1956.43±1.94[e]
Soymilk after direct UHT	91.0	1198.88	210.33	1211.66	68.11	7.22	46.77	1745.88±1.86[d]

M % = moisture %. Din= daidzin; Gln= glycitin, Gin=genistin; Dein= daidzein; Glen= glycitein; Gein=genistein. ND = not detected.
Soymilk after TI deactivation = soymilk[2].
Soymilk after direct UHT = soymilk[3].
Total[A] = total isoflavones expressed in µg aglycone equivalents ± standard deviation.
Means with different letters in the same column are significantly different at $p<0.05$.

Table 4.3: Total isoflavone concentrations in µg aglycone equivalents per gram of the sample reported on a dry matter basis, in various samples of the DUPLICATE hot grind process trial.

Sample names (HG process)	M%	Din	Gln	Gin	Dein	Glen	Gein	Total[A] (µg/gm)
Soybeans for indirect process	10.0	749.00	147.05	864.33	ND	ND	ND	1091.52±2.02[a]
Ground beans	87.6	766.45	152.90	835.40	22.58	ND	ND	1110.40±1.12[a]
LOX treated	87.0	774.84	157.07	879.00	ND	ND	ND	1122.86±0.02[a]
Okara	74.0	426.69	100.65	524.03	18.34	15.84	1.73	688.25±1.62[b]
Soymilk[1]	90.0	941.00	194.75	1071.00	85.00	25.00	41.00	1519.38±2.10[c]
Soymilk after TI deactivation	88.6	736.99	149.35	848.77	ND	ND	ND	1075.92±0.99[a]
Soymilk after indirect UHT	90.2	927.14	195.91	1041.53	ND	21.32	0.51	1364.06±0.21[d]
Soybeans for direct process	11.0	695.73	126.51	847.41	18.87	50.00	ND	1104.17±1.01[a]
Ground beans	82.6	631.32	132.7	722.75	16.49	29.25	ND	967.72±0.42[ab]
LOX treated	82.4	686.87	135.28	810.17	19.65	ND	ND	1031.86±0.07[a]
Okara	74.0	488.34	107.15	578.23	16.80	16.23	1.96	763.01±0.28[b]
Soymilk[1]	88.0	991.58	197.66	1161.41	30.33	ND	ND	1487.97±0.92[c]
Soymilk after TI deactivation	88.5	869.56	169.3	987.39	20.43	ND	ND	1276.69±5.23[d]
Soymilk after direct UHT	89.7	923.68	188.83	1008.64	21.94	ND	0.388	1337.38±2.28[d]

M % = moisture %. Din= daidzin; Gln= glycitin, Gin=genistin; Dein= daidzein; Glen= glycitein; Gein=genistein. ND = not detected.
Soymilk after TI deactivation = soymilk[2].
Soymilk after direct UHT = soymilk[3].
Total[A] = total isoflavones expressed in µg aglycone equivalents ± standard deviation.
Means with different letters in the same column are significantly different at $p<0.05$.

Table 4.4: Total isoflavone concentrations in µg aglycone equivalents per gram of the sample reported on a dry matter basis, in various samples of the DUPLICATE cold grind process trial.

Sample names (CG process)	M%	Din	Gln	Gin	Dein	Glen	Gein	Total[A] (µg/gm)
Soybeans for indirect process	10.0	749.00	147.05	864.33	ND	ND	ND	1091.52±2.02[a]
Ground beans	84.4	239.61	46.28	345.25	291.15	50.64	184.87	918.32±0.60[b]
LOX treated	84.4	236.53	47.50	344.48	301.53	51.28	192.30	935.18±1.41[b]
Okara	73.6	213.14	47.12	290.79	166.21	30.71	105.18	644.09±1.87[b]
Soymilk[1]	89.2	846.29	173.79	973.61	71.01	22.87	32.03	1362.20±2.47[c]
Soymilk after TI deactivation	90.4	778.33	169.47	927.5	61.35	26.04	24.47	1275.06±3.26[c]
Soymilk after indirect UHT	90.2	784.38	168.57	938.06	64.38	24.89	26.83	1289.03±1.71[c]
Soybeans for direct process	11.0	695.73	126.51	847.41	18.87	50.00	ND	1104.17±1.01[a]
Ground beans	82.6	190.40	39.13	286.89	312.87	49.02	197.93	880.38±1.19[b]
LOX treated	82.2	309.21	58.59	393.25	252.58	44.10	159.43	928.14±0.38[b]
Okara	72.2	150.17	32.26	237.23	202.41	33.23	128.20	624.42±1.53[b]
Soymilk[1]	87.7	796.99	167.15	900.56	69.186	19.83	31.78	1277.09±0.70[c]
Soymilk after TI deactivation	90.5	827.47	177.15	892.21	64.94	24.00	24.94	1289.94±0.38[c]
Soymilk after direct UHT	90.0	805.00	164.50	901.20	65.30	23.15	29.70	1278.04±3.08[c]

M % = moisture %. Din= daidzin; Gln= glycitin, Gin=genistin; Dein= daidzein; Glen= glycitein; Gein=genistein. ND = not detected.
Soymilk after TI deactivation = soymilk[2].
Soymilk after direct UHT = soymilk[3].
Total[A] = total isoflavones expressed in µg aglycone equivalents ± standard deviation.
Means with different letters in the same column are significantly different at $p<0.05$.

The type of grinding process most probably caused a difference in the activity of glucan *endo*-1, 6- β–glucosidase, popularly known as the β-glucosidase. This enzyme present in soybean hydrolyzes the isoflavone glucosides, thereby producing the respective aglycones **(Xie et al., 2003).** Grinding of soybeans at a lower temperature resulted in an increase in the action of β–glucosidase, as shown by the increase in the concentration of aglycones. HPLC data showed that the samples of 'ground beans' along with the 'LOX treated beans' and the 'okara' obtained from the cold grind process had a higher concentration of aglycones namely the daidzein, genistein and glycitein (Table 4.1). The respective samples from a hot grind procedure had little amount of aglycones in them. A similar trend was observed in every trial carried out (Table 4.2, 4.3, 4.4). The cold grinding of soybeans at a temperature of 45°C favors an enhanced activity of the β–glucosidase, whereby the isoflavone aglycones, daidzein, genistein and glycitein are formed. This resulted in higher aglycone concentrations in samples of the cold grind process. Studies by **Matsuura and others (1989)** observed an activated β–glucosidase action under special conditions of processing, such as the soaking of soybeans in water. The optimum condition for β–glucosidase activity was also observed at a temperature of 45°C by these researchers **(Matsuura et al., 1995)**. It was clear from this study that the soaking of soybeans in water for a time period was not necessarily required, but the grinding of soybeans with water at 45°C was enough for an enhanced action of β–glucosidase. The length of time for which the soybeans resided within the lower temperature (45°C) was lesser than that would have happened during a soaking procedure. However, this short period of time was found enough for the β–glucosidase

enzyme to cause its action, thereby leading to the conversion of isoflavone glucosides to their respective aglycones. This may be due to size reduction during grinding, which facilitates the enzyme/substrate interaction. This also proves that the temperature (45°C) of contact of soybeans had a major influence towards the enhanced action of the enzyme causing the formation of aglycones.

Isoflavone levels in the final soymilks obtained by hot-grind and cold-grind processes are shown in Table 4.1 and 4.2 respectively. It was found that soymilk prepared by hot-grind process contained significantly higher ($p<0.05$) isoflavone concentrations than that prepared by a cold-grind process. For example, the total amount of isoflavones in soymilk prepared by a hot grind process was 1795.81µg/gm DM and that from a cold grind process was 1736.36µg/gm DM. Such a trend of increased isoflavone concentrations in hot ground samples compared with that of cold ground samples was observed for every trial independent of whether it was direct UHT treated or indirect UHT treated (Table 4.2, 4.3, 4.4). Hot grinding caused an increase in the extraction of isoflavones into the soymilk compared with cold grinding. Such an improvement in the extraction of isoflavones into the soymilk could be due to the higher solubility of isoflavones in hot water than in cold water extract **(Gugger and Grabiel, 2000)**. This highlights the need for soymilk manufacturers to give a greater preference for hot grinding over cold grinding from the point of view of an improvement in the extraction of isoflavones into the soymilk. The nutritional quality of soymilk in terms of its phytochemical concentration can therefore be improved to a certain extent by using a hot grinding procedure for the extraction of soymilk instead of a cold grinding process.

4.2 ISOFLAVONE LEVELS IN SOYMILK SUBJECTED TO DIRECT VERSUS INDIRECT UHT TREATMENT

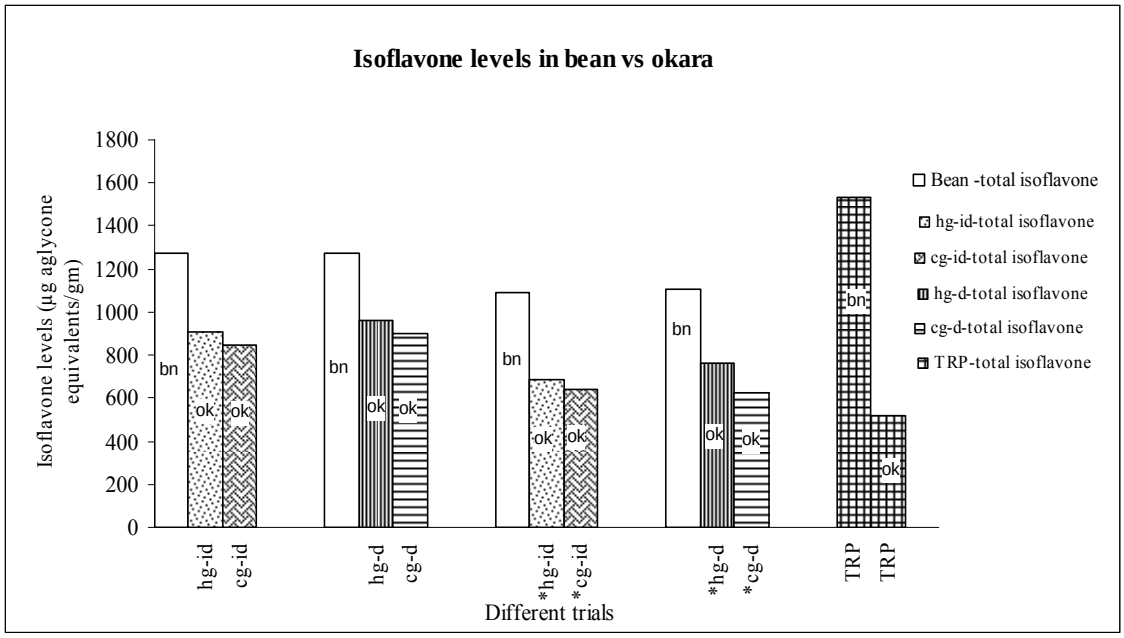
Soymilk prepared by hot grind or cold grind processes were subjected to direct or indirect UHT treatment processes so as to extend the shelf life for the product. The soymilk samples after a direct/indirect UHT treatment (soymilk^3) were also analyzed for their isoflavone concentrations. The total isoflavone amounts in soymilk obtained by the hot extraction proceeded by a direct UHT treatment was 1809.28 μg/gm, (Table 4.1) while the same by corresponding indirect UHT treatment was 1795.81μg/gm, expressed on a dry matter basis (Table 4.1). In a similar way the total isoflavone amounts in soymilk prepared by a cold extraction proceeded by a direct UHT treatment was 1745.88 μg/gm (Table 4.2), while the same by corresponding indirect UHT treatment was 1736.36 μg/gm (Table 4.2). Results of isoflavone analysis showed no significant differences in isoflavone levels ($p<0.05$) in the soymilk subjected to direct versus indirect UHT treatment. Considering the phytochemical concentration of the soymilk obtained, preference to any one of the two UHT methods is not really recommended, since both the processes results in a nearly equal impact on the isoflavone concentrations of the end product. Therefore, either a direct or an indirect UHT treatment can be chosen for the soymilk processing.

The direct and indirect UHT treatments given to the soymilk were at a temperature of 143ºC for 10 sec or 140ºC for 4 sec respectively. The differences in temperature and time period of application of heat for the direct and indirect UHT processes might have been too little to cause a huge impact on the comparative concentrations of isoflavones in the

end product. Thus the isoflavone levels in the soymilk obtained after a hot extraction followed by a direct or indirect UHT process, remained nearly the same. A similar trend was also observed for the soymilk obtained after a cold extraction followed by a direct or indirect UHT treatment. However, a UHT process either direct or indirect may have resulted in an increase in the concentrations of isoflavones in comparison to the soybeans used for extraction. A detailed mass balance study can only help with evaluating the same. Less access and confidentiality of certain processing systems utilized for this study were certain limitations in carrying out a detailed study. Therefore, only a comparative study was carried out instead of a mass balance study to evaluate the isoflavone levels in the soymilk prepared by the different UHT heat treatment processes. Results of the comparative study on the level of isoflavones in soymilk after a direct or indirect UHT treatment showed non-competitive values. Thus the soymilk manufacturers can choose either a direct or an indirect UHT treatment for the processing of soymilk. The kind of UHT treatment given to soymilk has little influence towards the level of isoflavones in soymilk and it also allows the possibility of having more choices of heat treatment options between a direct and an indirect process for the soymilk manufacturers.

4.3 OKARA AND ISOFLAVONE LOSSES

A large proportion of isoflavones were found to be lost in the soy residue (okara) after a hot grind or cold grind followed by decanting. The comparison of isoflavone levels in soybean seeds and okara after calculating them on the same basis of moisture are shown in Figure 4.1.



Total isoflavones in soybean seeds and okara are expressed in µg aglycone equivalents per gram of sample on a dry matter basis. Soybean seeds are denoted as 'bn' and okara as 'ok'. hg = Hot grind; cg = Cold grind. 'id' represent an indirect UHT process, while 'd' represent a direct UHT process. hg* and cg* represent hotgrind and coldgrind UHT duplicate trials, respectively. TRP = Traditional soak and grind process.

Figure 4.1: Comparison of isoflavone levels in soybean seed and okara during the hot and cold grinding trials and from a traditional soymilk process

Traditionally, soymilk is made by soaking soybeans in excess water followed by grinding, filtering, and cooking **(Liu, 1997)**. The preparation of soymilk was also carried out by a soak and grind process during this study. The traditional soymilk preparation was carried out in a local soymilk manufacturing factory under industrial conditions as described earlier in chapter two *(index no: 2.1.2.2).* The soymilk residue or okara is removed during the filtration process. A certain amount of fat, carbohydrate, protein and isoflavones are also removed along with the okara during such type of filtration processes. The amount of isoflavones lost into the okara during the traditional soymilk making process was therefore determined in this study. The soybeans used for the

traditional soymilk making process contained 1530.67 µg/gm of isoflavones, while the okara contained 519.27 µg/gm of isoflavones, expressed on a dry matter basis (Figure 4.1). Results of HPLC analysis showed that the amount of isoflavones fractionated into the okara during the traditional soymilk manufacturing process was comparatively lower than the amount of isoflavones being lost into the okara during a 'no soak procedure' used for soymilk making. Studies conducted by **Wang and Murphy (1996),** reported as recovering high levels of isoflavones in the soymilk by pressing the okara. The results of isoflavone analysis in okara obtained from the soak and grind process showed similar results by retaining lesser amounts of isoflavones in them. Lesser amounts of isoflavones were fractionated into the okara of a traditional soymilk making process, when compared with that of the amounts present in the soybean seeds used for its preparation.

This highlights the need to evaluate the advantages and disadvantages of automated and advanced processing systems used for soymilk preparations on an industrial scale. Retaining the isoflavones in the prepared soymilk is also an equally important parameter of nutritional significance while considering such type of processing modules. A high % of solid matter is being removed as okara and it is considered as an industrial waste with much of it being used as a landfill. Therefore, it is very essential to at least identify the processing conditions such that the losses of nutrients into these waste products can be minimized either by optimizing the desired processing steps or by choosing alternative processing methods. Okara is not utilized fully as food or feed sources, until recently; okara powder has been used to partially replace wheat flour for bread making. It is

therefore imperative to conduct further studies on the ways to minimize the loss of isoflavones into the okara and to maximize the extraction of isoflavones into the soymilk.

4.4 GENERAL DISCUSSION

Among the different processing conditions, the type of heat treatments given to soymilk during the extraction and sterilization processes are the major parameters that determine the yield and nutritive value of the final product obtained. Much of the research to date has been done on the understanding of the availability of lysine, thiamine and riboflavin of soymilk during heat treatment **(Kwok and Niranjan, 1995**; **Kwok et al., 1998)**. The available lysine content in heat-processed soymilk has a nutritional implication, where it has been correlated with the biological value (PER) and pepsin digestibility **(Van Buren et al., 1964).** High temperature heat treatments given under optimum conditions are found to improve the protein quality **(Kwok et al., 1998)**. The superiority of application of UHT heat treatment in maximizing vitamin retention was also discussed by **Kwok et al (1998).** Though isoflavones are not classified as nutrients, they reportedly affect human health as much as vitamins and minerals. But the effect of isoflavones during UHT processing has never been studied in detail.

Ideally, an optimal thermal process for soymilk is the one which results in maximum destruction of bacterial spores with minimum degradation of sensory and nutritional quality. This study reveals the fate of isoflavones during the extraction and UHT processing of soymilk. Such data are very useful for process optimization during a soymilk preparation. Nowadays, in large-scale production of soymilks, automated

extraction and UHT systems are being used. Though such systems offer time and energy savings, the question still remains whether there is a conflict between an ideal system and optimal retention of important functional and nutritional components into the soymilk during its manufacture. Since the health claim places soybean products into a selected category of foods that possess unique medicinal and nutritional values, its quality need to be maintained or not at least lowered by utilization of high technology and advanced processing systems. Under the many constraints of using such type of soymilk processing systems, some advantages and disadvantages are inevitable consequences of thermal processing. However, a comparative study on the level of isoflavones followed here, helped in identifying the process module which can be best chosen to retain the maximum amount of isoflavones in the end product. This is the first study on a continuous pilot plant unit using different extraction systems and UHT treatments to process soymilk. Moreover, the results on the fate of isoflavones are also useful while defining mathematical and kinetic models to understand the maximum retention of nutrients in the soymilk prepared by the UHT process.

CHAPTER 5

EFFECT OF DIFFERENT COAGULANTS ON THE ISOFLAVONE LEVELS AND PHYSICAL PROPERTIES OF FIRM TOFU

Tofu is a more widely accepted food among the soy food product categories. It is made by the coagulation of soymilk with salt or acid to produce a soy protein gel which traps water, soy lipids and other constituents in the matrix. Different types of coagulants are used for the coagulation of soymilk to obtain tofu. However, limited information is available about the selection of suitable coagulants for the production of tofu.

Tofus made from different coagulants cannot be easily differentiated between each other since they all appear similar but, their structural and other properties may be different. The knowledge of the effect of different coagulants in retaining isoflavones in tofu remains almost unknown. Considering the phytochemical concentrations of tofu, it was deemed essential to understand the type of coagulants that can best retain the maximum amount of isoflavones in the prepared tofu. The quantification of isoflavones in tofu samples and the separated wheys were therefore carried out using the HPLC method developed for isoflavone analysis during this study. Each coagulant may produce tofu with different textural properties and equally important are the physical properties of tofu, which characterize its acceptance. The physical properties of tofu prepared from different coagulants were also determined in this study and a comparative evaluation in physical properties between them was further carried out. Thus an understanding of the total effect of different coagulants towards the physical properties and isoflavone

contents in tofu was made possible. These studies helped in identifying the most suitable coagulant that not only resulted in a firm tofu with better physical properties but also one in which the highest amount of isoflavones were retained.

5.1 EFFECT OF DIFFERENT COAGULANTS ON THE ISOFLAVONE LEVELS IN TOFU

Identity preserved 'Harovinton' beans were used for the preparation of soymilk. The amounts of daidzin, glycitin, genistin, daidzein and genistein in soybeans used for this study were determined, and the contents were found to be 1022.19 ± 13.2, 111.52 ± 7.8, 1049.32 ± 15.4, 37.14 ± 0.77 and 16.90 ± 0.53µg/gm respectively, on a dry weight basis. Extraction followed by a mild saponification utilized in this study and as described earlier in Chapter two *(index no: 2.2.8.1)* converted all the isoflavone glucoside ester forms into their corresponding glucosides. Further to which, the total amount of isoflavones in soybeans were expressed in aglycone equivalents and this corresponded to a total of 1405.50 ± 17.3µg/gm, on a dry weight basis. Soymilk was prepared in bulk from a single batch of the above soybeans and the prepared soymilk was also analyzed for its isoflavone concentrations. The prepared soymilk had a total of 182.19 ± 0.36 µg/gm of isoflavones on a wet weight basis, which was equivalent to 1570.64 µg/gm on a dry weight basis. This particular batch of soymilk was henceforth used for the preparation of tofu.

The different coagulants used in this study for the preparation of tofu were calcium sulfate, magnesium sulfate, calcium chloride, magnesium chloride, calcium acetate,

calcium lactate and acetic acid solution. All the calcium salts, magnesium salts and the acidic coagulant (acetic acid) used in this study precipitated the soymilk proteins. Tofus were prepared using every coagulant at two different concentration levels, except for the acidic coagulant. The coagulant concentrations used for tofu preparation were 0.4 and 0.5% based on the amount of soymilk used. The lowest concentration of coagulant applied by **Lu et al (1980)** to obtain coagulation was 0.1% for calcium chloride, 0.2% for calcium lactate and 0.3% for calcium sulfate. Moreover, coagulants such as calcium sulfate, calcium lactate and calcium acetate used at 0.1 – 0.2% were found not to result in soybean curd formation by some researchers **(Lu et al., 1980; Kamel and de Man, 1982)**, while precipitating the soy proteins with 0.6% of coagulants like calcium sulfate was found to form too hard a tofu by other researchers **(Schaefer and Love, 1992; Kao et al., 2003)**. Tofu is a perishable food product and its preparation over a wider range of coagulants (0.1 - 0.6%) for many different coagulants was not possible in this study, since the physico-chemical properties along with its isoflavone evaluation need to be carried out immediately after its preparation. Hence during this study which involved many different types of coagulants, the concentration of coagulants utilized for the preparation of tofu was 0.4 - 0.5%, which is neither the least nor the highest levels of coagulant usage. Moreover, the concentration level of the coagulants so chosen was also the typical amounts used for tofu formations. Tofu was also prepared using 5% of acetic acid solution during this study.

Table 5.1: Comparison of isoflavone contents in soybean curds prepared using different coagulants

Coagulant type	Usage level (% w/w)	Tofu obtained (gm)	Isoflavones in tofu (µg/gm) on a dry wt basis
Calcium sulfate	0.5	225.36[a]	1317.88 ±6.77[e]
Calcium sulfate	0.4	232.49[a]	1424.00 ±0.67[f]
Calcium chloride	0.5	210.18[b]	1301.06 ±2.43[ei]
Calcium chloride	0.4	209.99[b]	1295.85 ±2.41[eh]
Magnesium sulfate	0.5	246.26[ac]	1333.77 ±0.19[e]
Magnesium sulfate	0.4	234.49[a]	1282.16 ±1.21[g]
Magnesium chloride	0.5	203.46[b]	1279.54 ±4.69[g]
Magnesium chloride	0.4	219.27[a]	1279.54 ±1.11[g]
Calcium acetate	0.5	195.14[d]	1280.18 ±4.24[g]
Calcium acetate	0.4	192.75[d]	1285.30 ±3.98[g]
Calcium lactate	0.5	220.55[a]	1301.81 ±6.01[ei]
Calcium lactate	0.4	232.20[a]	1292.36 ±4.20 [eh]
*Acetic acid 5%	20ml	212.50[b]	1280.18 ±7.03[g]

Isoflavones reported as µg aglycone equivalents per gram of tofu, expressed as the mean ± standard deviation. Like superscripts in the same column do not differ significantly at $p \leq 0.05$.
*Acetic acid 5% solution of 20ml was used for the preparation of a tofu.

Table 5.1 shows the effect of different soy protein coagulants on the level of isoflavones in the prepared tofu. Coagulation of soy proteins by calcium salts is caused by the cross-linking between protein molecules by the calcium ions **(Wang and Hesseltine, 1982)**. When calcium sulfate was used as a coagulating agent, it was observed that the tofu prepared from lower concentration of calcium sulfate (0.4%) gave a higher product yield than when higher concentration (0.5%) was used. The same tofu coagulated with 0.4% calcium sulfate was also found to contain a higher concentration of isoflavones than the other. The decrease in the yield of tofu with increasing calcium sulfate concentration could be due to increasing syneresis and loss of whey from the curd as more bonding occurred thus making the protein matrix denser and compact **(Sun and Breene, 1991)**. Moreover, calcium sulfate was also found to be a better coagulant than calcium chloride in terms of the yield of tofu and in the retention of isoflavones in the tofu. The differences

in the speed of the different coagulants in precipitating the soy protein might be an explanation for such an effect.

The chloride salts, calcium chloride and magnesium chloride were found to be rapid in their action of coagulating the soy proteins. After the addition of these coagulants into the soymilk, a quick formation of curd was observed whereby the wheys separated fast. In comparison and visualized by naked eye as the separation of whey occurred, the coagulating speeds of the respective sulfate salts were found to be very slow. A time period of more than 8min was required for the curd formation to occur when using the calcium and magnesium sulfate salts. However, the quick coagulating power of calcium chloride resulted in more exclusion of isoflavones into the whey during the coagulation process. The calcium chloride coagulated tofus had a lesser concentration of isoflavones compared with the calcium sulfate coagulated tofus. Using calcium chloride at 0.4% or 0.5% level did not cause a significant difference in the yield of tofu ($p<0.05$), though there was a minor difference in the concentration of isoflavones in them (Table 5.1). Thus the rapid action of certain coagulants like calcium chloride in forming the coagulum than the slow acting salts like calcium sulfate might cause a significant reduction in the amount of isoflavones in the tofu prepared from it.

Magnesium ions can also be used instead of calcium ions for soy protein coagulation, since this divalent cation can form crosslinking between the protein molecules. But the sites of crosslinking in the protein molecules may be different for both calcium and magnesium ions causing the latter to form a loose network. The exact mechanism of gel

formation together with its stabilization effect caused by magnesium ion is not clearly understood, where by its effect may be different from that caused by calcium ions. However, similar to the results of calcium salts, the magnesium sulfate coagulated tofu was also found to contain higher isoflavone concentrations than the magnesium chloride coagulated tofu.

Calcium lactate and calcium acetate are rarely used as coagulants for tofu manufacture. The possibility of using these calcium salts as suitable coagulants was explored during this study and their impact on the level of isoflavones in the prepared tofu was further evaluated. These calcium salts have a major advantage of being more soluble in water than calcium sulfate and hence its ease of handling. Results of analyses showed that calcium lactate is a better coagulant than calcium acetate, in terms of the yield and retention of isoflavones in the tofu. However, the isoflavone concentrations in calcium acetate tofu were found to be equivalent to that present in magnesium chloride and acetic acid coagulated tofu (Table 5.1), which means it could form a protein network with more isoflavone entrapment, though the yield of tofu was lower. There are no reports available on the isoflavone contents in calcium acetate or calcium lactate coagulated tofu.

A mild acetic acid solution is commonly used as a tofu coagulant, especially in Indonesia **(Shurtleff and Aoyagi, 1979b)**. The application of acetic acid is mainly to increase the shelf life of tofu and for imparting a mild acidic taste. In this study, a 5% acetic acid solution was used for the preparation of tofu. The mechanism of action of an acidic coagulant in causing the soy protein to coagulate is probably different from that of

calcium or magnesium salts, but the retention of isoflavones in acetic acid coagulated tofu was found to be similar to some calcium or magnesium salt coagulated tofu. Reports were not available on the preparation of acetic acid coagulated tofu or the analysis of isoflavone amounts in acetic acid coagulated tofu and wheys. Results of HPLC analysis showed that the concentration of isoflavones in acetic acid coagulated tofu was much similar ($p \leq 0.05$) to that of the isoflavone concentrations in magnesium chloride and calcium acetate coagulated tofu (Table 5.1).

5.2 EFFECT OF DIFFERENT COAGULANTS ON THE PHYSICAL PROPERTIES OF TOFU

Tofu being bland in taste, properties such as the texture, color and moisture are the different parameters that ultimately determine the quality of a tofu obtained. Even though the concentration of coagulants used in this study was within a narrow range of 0.4 - 0.5%, the tofu obtained differed in their physical properties, especially in their texture characteristics. The network formation of tofu generally involves the denaturing of soy protein followed by their aggregation with a coagulant and formation of a network. However, different coagulants might have resulted in the formation of tofus with different moisture, texture and yield. While the phytochemical concentrations of tofu are critical in terms of its functional food value, the physical properties of tofu are equally important attributes that affect the product acceptability.

5.2.1 Evaluation of the yield, moisture and color of tofu

Table 5.2 shows the results of the yield, moisture and color of tofu obtained after using different coagulants.

Table 5.2: Yield, moisture and color of tofus prepared using different coagulants

Coagulant type Usage level	Usage level (% w/w)	Tofu M%	Tofu obtained (gm)	Color determination		
				L	a*	b*
Calcium sulfate	0.5	79.2	225.36[a]	85.65	0.535	24.39
Calcium sulfate	0.4	79.5	232.49[a]	85.82	0.281	24.95
Calcium chloride	0.5	76.6	210.18[b]	86.66	0.421	23.01
Calcium chloride	0.4	76.1	209.99[b]	86.57	0.388	22.79
Magnesium sulfate	0.5	79.6	246.26[a,c]	86.41	0.003	23.00
Magnesium sulfate	0.4	78.7	234.49[a]	85.78	0.359	23.74
Magnesium chloride	0.5	78.3	203.46[b]	86.56	0.435	23.12
Magnesium chloride	0.4	78.7	219.27[a]	86.98	0.280	23.30
Calcium acetate	0.5	77.3	195.14[d]	85.96	0.549	23.56
Calcium acetate	0.4	77.4	192.75[d]	85.91	0.614	24.19
Calcium lactate	0.5	79.0	220.55[a]	86.53	0.320	23.30
Calcium lactate	0.4	79.7	232.20[a]	85.97	0.287	23.72
*Acetic acid 5%	20ml	76.8	212.50[b]	86.73	0.657	22.36

Tofu M% = percentage moisture in tofu.
*Acetic acid 5% solution of 20ml was used for the preparation of a tofu.
Like superscripts in the same column do not differ significantly at $p \leq 0.05$.

Comparison of the tofus coagulated with sulfate and chloride salts, the slow acting coagulants gave a better yield of tofu than the rapid acting ones. The rapid curd formation observed for coagulants like calcium chloride and magnesium chloride might have caused the formation of aggregates that are too large in size to form a good gel and a uniform distribution of coagulant might be difficult to be achieved during such a rapid reaction between the soy protein and the coagulant. The yield of tofu is very critical especially for the tofu manufacturers. To slow down the reaction with soy proteins, mixing of rapid acting coagulants with the slow acting coagulants is done by many tofu

manufacturers on an industrial scale. Used at the same concentration levels, sulfates gave a better yield of tofu than their respective chlorides. Isoflavone analysis also showed the sulfate salt coagulated tofus as retaining a higher concentration of isoflavones in them than the chloride salt coagulated tofus. Among all the different coagulants of this study, calcium sulfate at 0.4% level was found to give the highest yield of tofu. While the yield of calcium acetate tofu was the least, the yield of acetic acid coagulated tofu was found to be nearly the same ($p \leq 0.05$) as when using calcium chloride or magnesium chloride as coagulants (Table 5.2).

With regard to the color, either white or creamy white color is the desirable tofu characteristic. Every tofu produced had a creamy white color. The L values for the tofus ranged from 85.93 to 86.41 while a* and b* values ranged from 0.003 – 0.657 and 22.36 – 24.95, respectively. The different coagulating agents were found to have a little effect ($p \leq 0.05$) on the color of tofu, especially when using the same lot of bulk soymilk, as in this case. The acetic acid coagulated tofu showed the highest a* value (0.657), indicating an increase in the redness and a low b* value (22.36), indicating a decrease in the yellowness for this particular tofu.

The moisture content of tofu samples varied with the use of different coagulants and ranged from 76.1 – 79.7%. The variation in the moisture content of tofu obtained after using different coagulants is probably due to the differences in gel network influenced by different anions and its ionic strengths towards the water holding capacity of the soy protein gels. Thus the coagulant concentration and the type of anion will further affect the

hardness of an obtained tofu. The high moisture content accounted for a higher tofu yield since excess water became incorporated into the tofu **(Wang and Hesseltine, 1982; Sun and Breene, 1991)**. Such a positive correlation between tofu yield and moisture contents has also been observed by **Cai et al (1997).** Tofu with high moisture contents appeared smooth while those with low moisture contents had a coarse texture by visual examination.

5.2.2 Evaluation of the textural properties of tofu

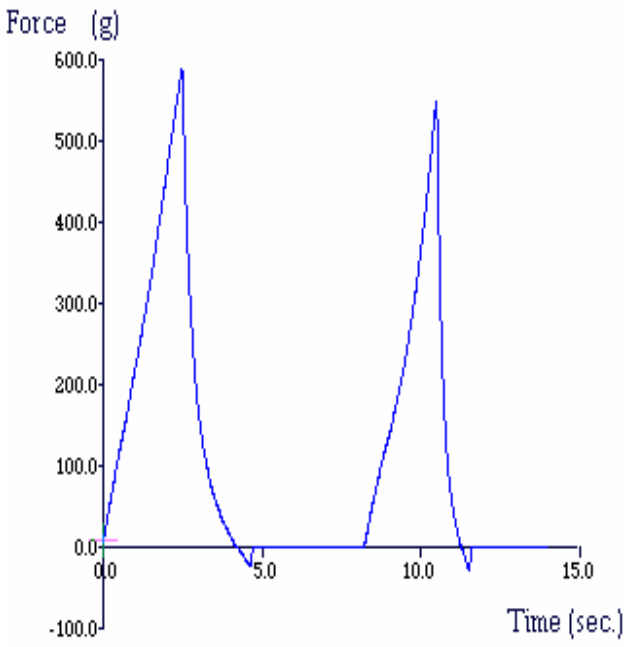


Figure 5.1: Typical texture profile analysis curve obtained for a firm tofu

The use of different coagulants appeared to primarily influence the textural parameters of a tofu obtained. The 'texture profile analysis' performed on each of the prepared tofus helped in evaluating the effects of different coagulants on the firmness of individual tofu matrix. The typical texture profile analysis (TPA) curve obtained for a tofu sample is

shown in Figure 5.1. TPA analysis helped in obtaining the hardness, cohesiveness, chewiness and gumminess of the tofus prepared. Comparing the hardness of the different tofus obtained a firm tofu with greater hardness than another would mean it is harder and firmer with greater cohesiveness. Such a tofu will possess high elasticity and greater chewiness making it more difficult to eat.

Table 5.3: Textural properties of tofu prepared using different coagulants

Coagulant type	Usage level (% w/w)	Hardness (g)	Spring* (mm)	Cohesive#	Gumminess (g)	Chewiness (kg mm)
Calcium sulfate	0.5	416.82^{a}	9.66^{f}	0.649^{g}	247.51^{h}	2.39^{m}
Calcium sulfate	0.4	458.53^{a}	9.69^{f}	0.635^{g}	262.47^{h}	2.54^{m}
Calcium chloride	0.5	515.29^{b}	9.88^{f}	0.645^{g}	301.46^{i}	2.97^{m}
Calcium chloride	0.4	484.16^{b}	9.68^{f}	0.648^{g}	286.75^{h}	2.77^{m}
Magnesium sulfate	0.5	292.49^{c}	9.86^{f}	0.644^{g}	171.89^{j}	1.69^{n}
Magnesium sulfate	0.4	293.36^{c}	9.89^{f}	0.639^{g}	168.34^{j}	1.66^{n}
Magnesium chloride	0.5	414.56^{a}	10.00^{f}	0.663^{g}	255.32^{h}	2.55^{m}
Magnesium chloride	0.4	395.49^{d}	9.88^{f}	0.661^{g}	238.94^{h}	2.36^{m}
Calcium acetate	0.5	607.77^{e}	10.00^{f}	0.657^{g}	368.84^{k}	3.68^{o}
Calcium acetate	0.4	617.68^{e}	9.81^{f}	0.652^{g}	368.69^{k}	3.61^{o}
Calcium lactate	0.5	370.54^{d}	9.82^{f}	0.660^{g}	226.41^{h}	2.22^{m}
Calcium lactate	0.4	339.42^{d}	9.84^{f}	0.657^{g}	205.39^{l}	2.02^{m}
Acetic acid 5%	20ml	436.01^{a}	9.37^{f}	0.656^{g}	263.83^{h}	2.47^{m}

Like superscripts in the same column do not differ significantly at $p \leq 0.05$.
Spring* = springiness; Cohesive# = cohesiveness.

The hardness of a tofu made on a production scale may be greater than that obtained by a lab scale process, because of the possibility of application of higher pressures for expulsion of wheys. However, an equal amount of pressure for a definite time period was applied during this study which helped in a comparative evaluation of the textural properties of the tofu prepared. The textural properties of tofu prepared using different coagulants are shown in Table 5.3.

Calcium sulfate coagulated tofu had a hardness value lesser than the calcium chloride coagulated tofu. A firm, but not too hard tofu was obtained after using calcium sulfate as a coagulant. However, when magnesium sulfate was used as a coagulant the corresponding tofu had textural properties which were much different from others. The hardness and chewiness of magnesium sulfate coagulated tofu was less than the hardness and chewiness of every other tofu prepared in this series. While the moisture content of this tofu was higher, that of its whey was the least among all the others. Probably an incomplete precipitation of soy proteins occurred and instead of having a compact protein network, it had a loose network encompassing many air gaps within it. This might be one of the reasons why magnesium sulfate is rarely used alone as a coagulant for firm tofu preparation. It is commonly used after mixing with other nigari type coagulants such as magnesium chloride and calcium chloride **(Hou et al., 1997).** However, comparatively higher levels of isoflavones were found in magnesium sulfate-coagulated tofu. Moreover the magnesium sulfate-coagulated tofu retained a higher concentration of isoflavones than its chloride salt-coagulated tofu.

Compared with the other coagulants of study, calcium acetate resulted in a tofu with greater hardness, accompanied by a maximum release of wheys and lesser amounts of tofu. TPA data revealed this tofu as the hardest tofu among all the tofus obtained during this study (Table 5.3). A tofu with lower yield contained less water and would be harder and such a condition was well observed in the case of calcium acetate coagulated tofu. The high hardness of calcium acetate tofu is probably one of the reasons that make it less acceptable for human consumption. While the lower yield of this tofu might be of

concern for the tofu manufacturing industry too. The acetic acid coagulated tofu showed comparable textural properties to the calcium sulfate coagulated tofu, though the yield of this tofu was less than the latter.

5.3 EVALUATION OF THE EXPELLED TOFU WHEYS

Tofu whey is the residual liquid separated during the production of firm tofu. Removal of whey by pressing is requisite, to obtain a compact and close network of firm tofu. The amount of whey released varied with the type of coagulant used for the preparation of the tofu. This variation in the amount of whey was due to the different water holding capacities of the tofu. Recent studies by **Jackson et al (2002)**, illustrated the probability of leaching of isoflavones into the whey, however, a clear understanding on the effect of different coagulants towards the ability to retain isoflavones in the tofu and its leaching into the wheys were described as unknown. Moreover, whey is considered as a waste stream and hence its nutritive value is always neglected. This is the first set of studies, which analyzed wheys in a very sequential manner to identify the loss of isoflavones. The pH of the expelled wheys was also determined during this study for an understanding of the coagulation effect caused by individual coagulants towards the curd formation and its influence towards the tofu properties.

5.3.1 Tofu wheys and pH effect

Table 5.4 shows the details of the amounts of wheys expelled during the different tofu preparations, together with its pH and isoflavone concentrations.

Table 5.4: Amount, pH and isoflavone contents in the wheys collected during tofu preparation

Coagulant type	Usage level (%w/w)	Whey M%	Whey obtained (gm)	Whey pH	Isoflavones in whey (μg/gm) wet wt basis
Calcium sulfate	0.5	96.2	257.08	5.76	101.30±0.47
Calcium sulfate	0.4	96.2	249.27	5.77	102.12±0.18
Calcium chloride	0.5	95.9	275.02	5.62	99.23±0.45
Calcium chloride	0.4	96.1	271.10	5.70	103.35±0.72
Magnesium sulfate	0.5	95.8	238.36	5.91	106.47±0.13
Magnesium sulfate	0.4	95.8	246.89	5.91	111.11±1.34
Magnesium chloride	0.5	95.8	279.11	5.74	103.88±0.58
Magnesium chloride	0.4	96.2	265.60	5.84	103.73±0.38
Calcium acetate	0.5	95.8	292.79	5.90	98.98±0.58
Calcium acetate	0.4	96.0	293.15	5.91	104.72±0.83
Calcium lactate	0.5	96.2	262.02	5.92	105.19±0.15
Calcium lactate	0.4	96.2	247.45	5.95	109.51±0.54
Acetic acid 5%	20ml	96.7	263.43	5.29	102.79±1.04

Whey M%= percentage moisture in whey. Isoflavone contents in tofu whey given as the mean ± standard deviation.

Comparing the sulfate and chloride salts, the pH of the chloride salt tofu wheys were found to be lower than that of the equivalent sulfate salt tofu wheys. The pH of the magnesium sulfate tofu whey was 5.91 when the concentration of coagulant used was either at 0.4 or at 0.5% level. A decrease in pH was always described as essential for the coagulation of soy proteins by many researchers **(Lu et al., 1980; Kamel and de Man, 1982)**. However, when compared to calcium sulfate and calcium chloride, the decrease in pH caused by magnesium sulfate and magnesium chloride respectively, was far less. These results suggest that calcium ions are stronger and more sensitive in combining with soy proteins than the magnesium ions.

The amount of whey released was higher for calcium acetate tofu than for calcium lactate tofu (Table 5.4). The pH of calcium acetate and calcium lactate tofu wheys were found in

a very narrow range of 5.90 – 5.95. A decrease in pH to less than 6.0 is required for soy protein coagulation to occur **(Lu et al., 1980)**, but even at the same pH, different coagulants behaved differently. For example, the hardness, gumminess and chewiness of the calcium acetate tofu were the highest while these values were the least for magnesium sulfate coagulated tofu (Table 5.3), though both the tofu wheys had a similar pH. This further proves that calcium ions derived from calcium salts like calcium acetate or calcium lactate (though not necessarily from calcium chloride or calcium sulfate), might bind more strongly to soy proteins than the magnesium ions.

The pH of the whey separated from acetic acid coagulated tofu was found to be the least (pH = 5.29) among all the others and it appeared more translucent than any of the other wheys collected. This was also confirmed by the least solids present in its wheys since its moisture content (96.7%) was the highest among all. The pH decrease caused by acidic coagulants like acetic acid might aggregate proteins by weakening the electric repulsion and liberates the hydrated water of protein, while the binding of calcium to carboxyl groups of proteins might have brought about the association of proteins by calcium salt coagulants **(Ono et al., 1976)**. But the results of this study showed the option of using a mild acetic acid solution as an alternative for calcium or magnesium salts for the preparation of tofu. However, an acetic acid coagulated tofu might not be an extra source of minerals such as calcium and magnesium in calcium or magnesium salt coagulated tofus.

5.3.2 Isoflavones in tofu wheys

Tofu wheys consist of complex sugars and are flatulence-causing factors. They are also a major disposal material of concern for tofu manufacturers **(Uzzan and Labuza, 2004)**. There are no reports about the utilization of tofu whey either as a nutritional liquid or as a modified product suitable for human consumption. Recently, it has been used as a medium for the production of lactic starters **(Thi et al., 2003)**. However, wheys are also a source of minor proteins **(Kao et al., 2003)**. Isoflavones might form complexes with protein in soymilk, which can be released together with wheys during the coagulation process. Depending on the type of coagulant and its concentration, it is also possible that more isoflavones may become excluded from the tofu and pass into the wheys during the coagulation process resulting in a lower quantity of isoflavones left in the resultant tofu. This can be partially explained as an incomplete coagulation process. Wheys were analyzed for their isoflavone concentrations in this study and the concentrations of isoflavones in released wheys are shown in Table 5.4.

A significant amount of isoflavone was found to be lost into the expelled wheys depending on the type of coagulant used for the preparation of the tofu. On an average 104.02 μg/gm of isoflavones were lost into the tofu wheys, expressed on a wet weight basis. The losses of isoflavones in whey may be due to protein-associated isoflavones being released into the whey **(Jackson et al., 2002)**. Wheys appeared very dilute and translucent with a moisture content ranging from 95.8 - 96.7%. Release of wheys might be essential during a firm tofu preparation, however, steps to minimize the drainage of proteins and associated isoflavones into the whey is crucial in order to enhance the

functional value of the tofu. The need for proper utilization of this low cost by-product as a source of isoflavones or as a functional liquid product is essential. Results of isoflavone analysis showed that, tofu wheys can serve as a potential alternative source of isoflavones.

An evaluation of the level of isoflavones in the prepared tofus and wheys helped in a total understanding of the influence of different coagulants towards the ability to retain isoflavones in the product (tofu) and the byproduct (whey) of the process. Obtaining a tofu with the desired texture, color and moisture together with its maximized functional food value might be critical, but certainly achievable. This research thus identified calcium sulfate as the best coagulant in obtaining a high-quality tofu with soft and smooth structure. The highest concentration of isoflavones was obtained in tofu with 0.4% calcium sulfate when compared to the higher percentage of the same coagulant. Yield of this tofu was also the highest among all the other different coagulants studied. Some studies have also supported the use of calcium sulfate as a highly available source of calcium for pre-menopausal women **(Martin et al., 2002).** Though calcium sulfate is the oldest and the most widely used coagulant, it shall still be considered as the most superior coagulant with its ability to form a tofu with good texture and with high nutritional properties.

A certain amount of isoflavones form complexes with proteins and are released into the wheys. But the excess outflow of isoflavones into the whey can be restricted if an appropriate coagulant is used. This can help in obtaining a tofu with higher isoflavone

content and thereby causing a minimum leaching of isoflavones into the wheys. Optionally soybean cultivars containing high isoflavone content can be utilized for the preparation of soymilk or tofu. However, limitation exists in their availability. Therefore the type of coagulant can be a viable and major option available to every tofu manufacturers in obtaining a product with improved isoflavone amounts. The study thus substantiated the relationship between the coagulant type and isoflavone amounts in the tofu prepared from it. The results of this study can further serve as a guide for process optimization of tofu making with respect to the type of coagulant, where it becomes essential to retain the maximum nutrients in the tofu prepared, especially when the choices of coagulants are plenty.

CHAPTER 6

CONCENTRATION AND PROFILE OF ISOFLAVONES IN SOY BASED SUPPLEMENTS, HEALTH PRODUCTS AND INFANT FORMULAS: EVALUATION OF ITS LEVEL OF INTAKE

Recent approaches of incorporating soy isoflavones into the diet involve the preparation of products containing different concentrated forms of soy extracts. A plethora of soy isoflavone supplements and health products are available in the market place. They are mainly targeted for the health of women, where they are commercialized as an alternative therapy for alleviating menopausal discomfort, for the prevention of osteoporosis or even for an improvement in breast health. With the increased consumption of these products, it is important to determine their isoflavone content and compositions.

Soy based infant formulas (SBIFs) are another group of foods consumed to a large extent by infants with allergies to cow's milk. Isoflavones being bioactive phytoestrogens with hormonal and non-hormonal activities, an understanding on the concentrations of isoflavones in SBIFs are also very important. Therefore, the objective of the present study was to determine the concentration and profile of isoflavones in randomly selected samples of commercially available soy isoflavone supplements, soy based health products and SBIFs obtained from South East Asia. The HPLC method developed earlier served as a convenient method for isoflavone quantification, while ESI-MS was used to establish their specific peak identities.

6.1 ISOFLAVONES IN SOY SUPPLEMENTS

The profile and concentration of isoflavones in different commercially available soy isoflavone supplements are shown in Table 6.1. The concentration of isoflavones in these supplements ranged from 405 - 57570 μg/gm of the product, expressed as aglycone equivalents.

Table 6.1: Isoflavone concentrations in commercially available soy supplements, expressed as μg aglycone equivalents per gram of the product.

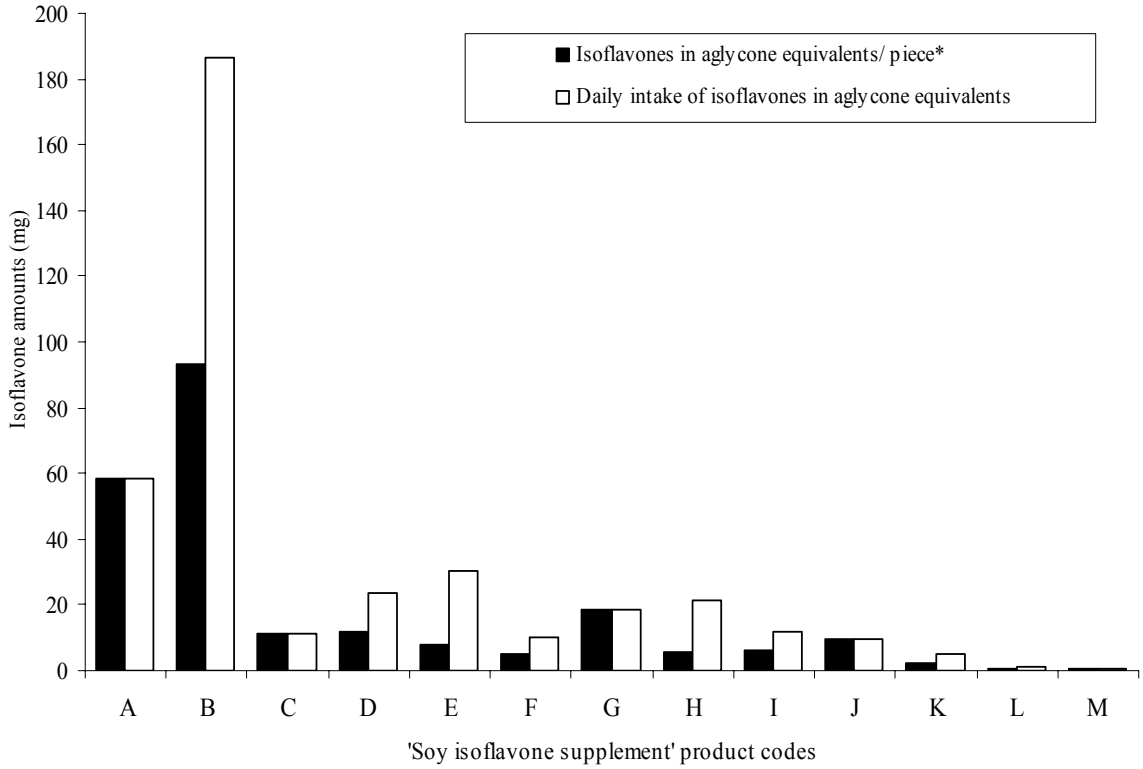
Product Code	Din	Glyin	Gin	Dein	Glyein	Gein	Total isoflavones
	$x10^3$ (μg/gm)			$x10^3$ (μg/gm)			$x10^3$ (μg/gm)
Product A	30.74	4.56	41.95	5.09	2.53	2.03	57.57±0.99[a]
Product B	21.38	3.67	62.49	1.05	1.12	0.39	57.03±1.16[a]
Product C	17.80	3.15	25.23	0.33	0.60	0.11	29.71± 0.35[b]
Product D	11.29	1.97	15.22	0.17	0.25	0.07	18.18±0.09[c]
Product E	13.34	5.03	3.66	0.14	0.28	ND	14.08±0.87[c]
Product F	5.92	0.89	8.26	0.23	0.20	0.33	10.13±0.21[cd]
Product G	6.06	0.85	8.01	0.10	0.12	ND	9.53±0.20[d]
Product H	7.36	3.94	2.08	0.31	0.15	ND	8.81±0.13[d]
Product I	3.69	0.33	9.35	0.15	0.22	0.09	8.79±0.12[d]
Product J	4.67	1.94	1.67	0.13	0.26	1.74	7.28±0.18[d]
Product K	3.75	0.99	2.76	0.18	0.11	0.07	5.02±0.05[d]
Product L	0.35	0.18	0.13	ND	ND	ND	0.466±0.02[e]
Product M	0.10	0.03	0.17	0.17	ND	ND	0.405±0.01[e]

Din =Daidzin, Glyin =Glycitin, Gin =Genistin, Dein = Daidzein, Glyein = Glycitein, Gein = Genistein.
ND = not detected. Total isoflavones reported as mean ± standard deviation.
Means with common letters are not significantly different at $p \leq 0.05$.

The highest concentration of isoflavones was found in product 'A' labeled as a 'Hi-Soy isoflavone supplement' and manufacturers of this product were the only ones who labeled the profile of individual isoflavones with concentrations and summed up the values to obtain the total isoflavones, which equaled 100mg per tablet. But, it is mainly the aglycones that are absorbed by the gut and exert their protective effects **(Izumi et al., 2000)**. Hence the total concentration of isoflavones if expressed as the arithmetic sum of 12 soy isoflavones, as done by this manufacturer, will only over-estimate the true

isoflavone concentration **(Erdman et al., 2004)**. The extraction of isoflavones followed by a saponification procedure used in this study and as described earlier in Chapter two *(index no: 2.2.8.1),* converted the different forms of isoflavone glucoside esters into their corresponding glucosides, leaving the three parent isoflavones intact. Further, the total quantified amounts of isoflavones in soy based products were expressed as µg aglycone equivalents per gram of the sample. The values provided by the manufacturers on the labels of the products mostly referred to the total of the 12 soy isoflavones. However, after expressing in aglycone equivalent concentrations, the total amount of isoflavones in product 'A' was only 58.54 mg/tablet instead of 100mg/tablet (as labeled).

The prescribed serving size for each isoflavone supplement differed from manufacturer to manufacturer, and the total amount of isoflavones, expressed as aglycone equivalents, per serving was therefore calculated from that obtained by HPLC analyses multiplied by the weight of the product contents and their serving sizes. The details of the resultant daily intake of isoflavones from the soy isoflavone supplements based on their serving sizes are shown in Figure 6.1.



Piece* refers to the product form, which is either a tablet or a capsule.
'Daily intake of isoflavones' is calculated from the quantified amounts of isoflavones of a product and based on the dosage recommendations given by the manufacturer.

Figure 6.1: Comparison of the amount of isoflavones per tablet or capsule and the possible daily intake of isoflavones, expressed in aglycone equivalents for the different soy isoflavone supplements.

Product B was found to contain 57030 µg of isoflavones per gram of capsule, and a serving of two capsules (each weighing 1.635g) per day would give a total of 186.48mg of isoflavones compared to 58.54mg of product A, served one tablet per day (Figure 6.1). This also highlights the importance of the actual weight of a tablet or capsule and the serving sizes of the individual soy isoflavone supplements. Consumers may not be aware

of this fact and may only be looking for a highly concentrated pill of isoflavones. Moreover, the manufacturers compete in showing higher figures on their product labels, especially since there is no established and standardized method of expressing the concentration of isoflavones. Much confusion also exists for the consumers, when comparing the labels of soy isoflavone supplements available over-the-counter and produced by different manufacturers. As would be obtained from product 'B', a high daily intake of isoflavones from some of these products may be of concern, since they might result in deleterious effects to humans, which have been shown in animal studies **(Leopold et al., 1976; Setchell et al., 1987).**

Product 'M' of 'multivitamins together with isoflavones' contained the least amount of isoflavones, 405µg/gm, while another product 'L' labeled as 'phytoestrogens from soy extract' had only 466µg of isoflavones per gram of the product. Every soy supplement marketed, was found to have some data on isoflavone concentrations, though most of their labeling appeared ambiguous. A generalized statement termed "soy isoflavone extract of 40%" was labeled on three of the soy isoflavone supplements (A, B & F). This labeling will be understood as the product would contain 40% of isoflavones per tablet / capsule. The amount of isoflavones per gram of supplement 'A' and 'B' was statistically not different ($p \leq 0.05$), while that in supplement 'F' of 10.13 mg/gm of sample was found to be five times lower than that of A or B (Table 6.1). This shows the huge differences in the isoflavone content of identically labeled products of soy isoflavone supplements. The wide variations in the amount of isoflavones in these products are of particular interest, since it relates to how much isoflavones are consumed with every tablet or capsule taken

in orally. Ingestion of supplements 'C' to 'M' would provide a daily intake of less than 30mg isoflavones (Figure 6.1), which is less than or equivalent to the isoflavone amounts that can be obtained from a serving of 200ml of soymilk or 100g of tofu **(Murphy et al., 1999)**. Thus according to the results of this study, the real daily doses of isoflavones in aglycone equivalents obtained by 80% of the products studied are only moderate or lower, in comparison with the amounts available from natural soy foods.

The profile of isoflavones found in different soy isoflavone supplements also varied among one another (Table 6.1). Eight out of the 13 products contained genistin as the major isoflavone while the remaining products had daidzin in higher amounts than genistin. Depending on the clinical effect being sought, the type of supplement or the profile of isoflavones may strongly influence the end point **(Setchell et al., 2001)**.

6.2 ISOFLAVONES IN SOY BASED HEALTH PRODUCTS

Several soy based health products designed for weight management are considered to be suitable for health. These products were mostly prepared from soy protein isolates and were either flavored or unflavored. Obtained in powder form (P1 to P11), they need to be reconstituted with water or even milk to obtain a diet shake. Few products are labeled as having isoflavones in them, while the concentration of isoflavones was not specified in any of these products. They being soy protein products, their primary purpose is not as isoflavone supplements, but as sources of soy protein.

HPLC analysis showed that the level of isoflavones per gram of these products was much lower than those in soy isoflavone supplements. Table 6.2 shows the composition and concentration of isoflavones per gram of the different commercially available soy based health products obtained after HPLC analysis. The level of isoflavones in these products ranged from 46.32 - 1333.80 µg per gram of the product. No two products appeared the same in their profile and content of isoflavones.

Table 6.2: Isoflavone concentrations per gram of the product and the amounts obtained from a serving of the commercially available soy based health products, expressed in aglycone equivalents

Product code	Din	Glyin	Gin	Dein	Glyen	Gein	[a]Total amount of isoflavones (µg/gm)	Isoflav (mg)/ serving (serving amount*)
P1	822.76	109.44	1148.44	nd	43.60	nd	1333.80±21.64[b]	33.34 (25g)
P2	529.57	36.17	1154.62	62.78	19.78	85.16	1235.98±0.36[c]	43.87 (35.5g)
P3	334.36	53.07	661.79	28.59	31.07	24.65	720.00±19.83[d]	25.92 (36g)
P4	181.65	215.99	455.47	7.03	23.46	4.66	568.77±5.18[e]	14.22 (25g)
P5	233.66	38.11	532.05	11.08	27.68	10.98	549.32±5.75[e]	15.38 (28g)
P6	139.28	17.06	346.28	4.02	5.52	8.65	330.59±12.10[f]	10.58 (32g)
P7	38.50	150.78	94.67	1.69	4.38	5.01	189.83±13.68[g]	14.98 (78.9g)
P8	37.89	2.54	106.65	3.71	4.85	4.95	104.95±1.13[h]	5.25 (50g)
P9	33.88	1.99	74.41	3.92	4.33	4.17	80.90±1.21[h]	2.75 (34g)
P10	6.95	47.69	22.36	1.06	4.43	nd	54.10±1.06[i]	3.02 (55.8g)
P11	5.58	41.03	26.84	nd	nd	nd	46.32±0.77[i]	2.74 (59.2g)

Din =Daidzin, Glyin =Glycitin, Gin =Genistin, Dein = Daidzein, Glyen = Glycitein, Gein = Genistein.
nd = not detected.
[a]Total amount of isoflavones is the quantified amount of isoflavones obtained by HPLC analysis and reported as mean ± standard deviation.
[b – i]Means in the same column with different superscripts are significantly different at $p \leq 0.05$.
*Isoflavone amounts per serving (mg) is calculated from the quantified amounts of isoflavones per gram of a product and based on the serving amounts stated by the manufacturer.

'Product P1' was found to contain the highest concentration of isoflavones of 1333.80 µg followed by 'Product P2' with 1235.98 µg per gram of the product. Higher amounts of genistin followed by daidzin were present in every soy based health product except for

P4, P7, P10 and P11. Product P4 showed a different profile of isoflavones with the amounts of genistin>glycitin>daidzin. Products P10 and P11 were produced by the same manufacturer, but differed in their amounts of protein, as indicated on their labels. As low as 46.32 µg of isoflavones per gram of the product was present in P11, a chocolate flavored product, while the same flavored with vanilla, P10 had 54.10µg. Product P10 had slightly higher protein content than P11 and the same trend was found for their isoflavone concentrations as well. The concentration of glycitin was found to be greater than the sum of the concentrations of daidzin and genistin for these two products. However, genistein was reported to be the most biologically active phytoestrogen **(Mazur et al., 1998)**.

From the obtained total isoflavone content of soy based health products and according to their serving amounts, the possible intake of isoflavones were calculated and shown in Table 6.2. It can be seen that the different soy based health products when prepared according to the manufacturer's directions resulted in different amounts of isoflavones in their finished products. This is the first study where several soy isoflavone supplements and soy based health products were analyzed in parallel to obtain comparative data of their isoflavone profiles and concentrations.

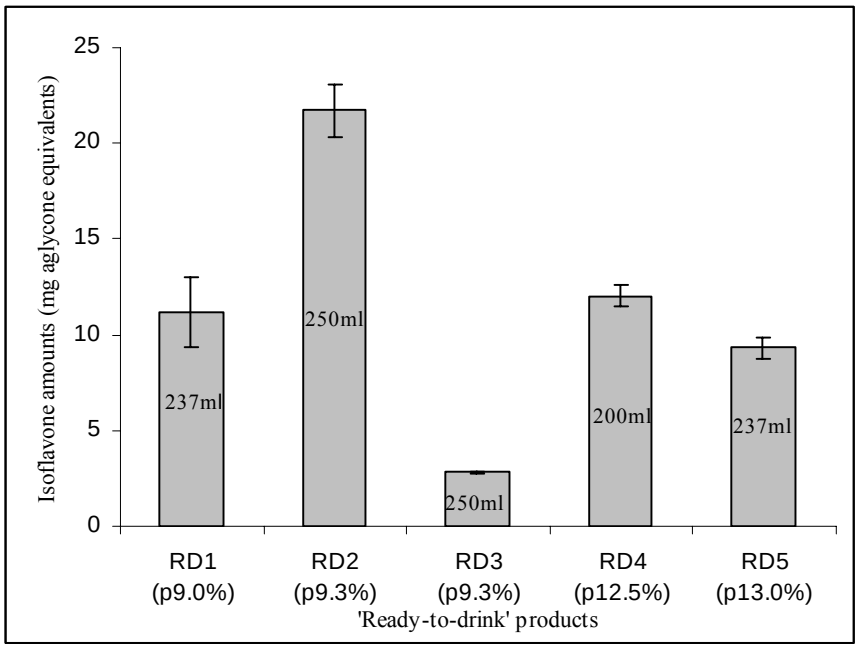
Liquid products of the same category were also obtained during this study. The concentration of isoflavones in these 'ready-to-drink' products obtained by HPLC analysis is shown in Table 6.3. These products were always found in open shelves in nutrition/ health shops and were stored at ambient temperatures.

Table 6.3: Isoflavone amounts in different ready-to-drink products, expressed as µg aglycone equivalents per gram of the product.

RD#	Din	Glyin	Gin	Dein	Glyein	Gein	Total isoflavones (µg/gm)
RD1	7.03	45.03	14.86	0.53	0.83	0.43	44.06±1.80[a]
RD2	2.29	115.43	5.87	4.22	0.45	ND	83.28±1.39[b]
RD3	1.46	9.13	5.03	0.55	ND	ND	10.40±0.01[c]
RD4	2.28	73.59	6.77	1.76	0.91	ND	55.18±0.58[ab]
RD5	7.29	31.72	15.05	0.39	0.97	0.34	35.79±0.56[a]

RD# is the ready to drink product code.
Din =Daidzin, Glyin =Glycitin, Gin =Genistin, Dein = Daidzein, Glyein = Glycitein, Gein = Genistein.
ND = not detected.
Total isoflavones reported as mean ± standard deviation.
Means with common letters are not significantly different at $p \leq 0.05$.

Product 'RD2' and 'RD3' are ready-to-drink products (RD) flavored with vanilla and chocolate respectively, and are products from the same manufacturer. A complete serving size of 250ml of RD2 and RD3 provided a total of 21.70mg and 2.80mg of isoflavones respectively (Figure 6.2). Though both RD2 and RD3 showed a protein content of 9.3g/serving, the level of isoflavones in them were drastically different from one another. Moreover, the Product RD2 appeared to be the ready-to-drink form prepared from Product P10, which is the powdered form of the product intended for the same application. But the profile of isoflavone in this product was much different, with the glycitin concentrations nearly 14 times greater than the sum of daidzin and genistin concentrations. This product might be manufactured from soy germ extract, as glycitin is the major isoflavone of the soybean hypocotyls **(Gugger, 2002).** Every RD product appeared to be made from soy germ, since they showed very high concentrations of glycitin. Little information is available about the biological activity of glycitin and glycitein, though its estrogenicity is considered lesser than that of daidzein and genistein.



The serving size (ml) for each ready-to-drink product is indicated.
Protein content in 100gm of individual product samples is represented as p%.

Figure 6.2: Total isoflavone amounts obtained from a serving of the different ready-to-drink products

The intake of isoflavones after the consumption of these RD products ranged from 2.80 - 21.70mg, per serving (Figure 6.2). Though soy protein isolate was the major protein source in these RD products, they also contained milk proteins and sodium caseinate in them. When other food components such as casein and flavouring materials are added to the soybean product, the overall isoflavone concentrations would be lowered. Moreover, in such a case, the protein content in the product cannot be directly correlated with their isoflavone concentrations.

On an average, the amount of isoflavone intake obtained from a serving size of the RD product (Figure 6.2) was lower than that obtained from a serving of the soy based health

product in the powder form (Table 6.2). However, the profile of isoflavones in these products depends on the raw materials used for their preparations, which might be soy germ or even soy protein isolate. Analyses showed that the soy based health products do not possess the same isoflavone profile or content, as that obtained from soy foods, which are produced from whole or de-hulled soybeans. These differences may however lead to markedly different plasma isoflavone profiles when ingested **(Setchell et al., 2001).**

Soymilk and tofu are the most popularly consumed soy foods in the region and are gaining popularity through out the world. Analyses of several commercially available brands of soymilk from the region (as described earlier in Chapter three) showed an average isoflavone concentrations ranging from 76 -199 μg/gm, which can provide 15.20 – 39.80 mg of isoflavones, from 200ml serving of soymilk, on a wet weight basis. Soft and firm tofu samples contained an average of 168.22 and 302.00 μg/gm of isoflavones, respectively, on a wet weight basis. Thus an intake of 100g portion of soft and firm tofu types can result in a possible ingestion of less than 16.82 and 30.20 mg of isoflavones, respectively (assuming cooking water was not thrown away). Considering the population in South East Asia as soy food eaters and good amounts of isoflavones are already available to them through these soy foods, an additional intake of soy isoflavone supplements or health products may however result in higher ingestion of these biologically active compounds.

6.3 ISOFLAVONES IN SOY BASED INFANT FORMULAS

Soy- based foods are used primarily to replace milk or milk-based formulae in the diets of children who are allergic and intolerant to milk or having galactosemia. These products are largely available in the form of powders that need to be reconstituted with water before consumption. Soy protein isolates are the most refined soy protein ingredients used primarily for the preparations of SBIFs worldwide **(Merritt and Jenks, 2004).** Only a few brands of infant formulas were obtained for this particular study and their protein content ranged from 13 - 16%. Though the major ingredient in all these infant foods was soy protein; the presence of isoflavones or their amounts was not labeled in any of them.

Table 6.4 shows the profile and concentration of isoflavones in different commercially available SBIFs. The isoflavone content in these products ranged from 59.50 - 226.84 μg per gram of the formula. These values are in general agreement with those published for different other SBIFs from USA and other parts of the world **(USDA & Iowa State University, 2002).** The observed differences in isoflavone concentrations among the different SBIFs can be due to the differences in the percentages of soy protein isolates used for their preparation. Considering the seasonal variation in the isoflavone content of soybeans themselves as a factor of two to five **(Tsukamoto et al., 1995),** its influence can be considerable for soy isolate preparations. **Murphy et al (1997)** observed a lower variation (210- 290 μg/gm of the formula) in the amount of isoflavones in SBIFs commercialized in the United States. Results of this study showed the existence of a wider variation in the concentration and profile of different soy isoflavones in SBIFs.

Table 6.4: Isoflavone concentrations in commercially available soy based infant formulae expressed in μg aglycone equivalents per gm of the product in powder form.

Product Code	Din	Glyin	Gin	Dein	Glyein	Gein	Total isoflavones (μg/gm)
IF1	94.61	14.18	217.79	9.77	4.48	9.62	226.84±1.39[a]
IF2	59.78	10.51	162.47	23.02	12.75	16.00	196.55±16.60[b]
IF3	54.60	12.86	140.20	2.82	ND	1.48	133.48±1.45[c]
IF4	25.34	4.88	75.60	26.33	5.22	29.04	126.45±6.32[c]
IF5	14.40	33.00	33.78	2.19	5.54	0.87	59.54±1.26[d]

Din =Daidzin, Glyin =Glycitin, Gin =Genistin, Dein = Daidzein, Glyein = Glycitein, Gein = Genistein.
ND = not detected.
Total isoflavones reported as mean ± standard deviation.
Means with common letters are not significantly different at $p \leq 0.05$.

The predominant isoflavone identified in majority of the SBIFs was genistin followed by daidzin and glycitin, respectively. Studies by **Setchell et al (1997)** also found isoflavone glucosides as the major isoflavones in five SBIFs collected from the U.S. However, the product IF5 of this study was found to contain an equal proportion of genistin and glycitin, while daidzin was present in very low amounts. IF5 also contained casein besides having soy protein isolate. As a result, the isoflavone concentrations cannot be directly correlated to the protein content of the product.

Product IF1, had the highest concentration (226.84μg/gm of formula) of isoflavones among the five infant formulas analyzed. The feeding instructions for infants of different age group were different for individual formulas. When prepared according to the manufacturer's directions, the calculated concentrations of isoflavones that might be ingested by the infants of different age groups are shown in Table 6.5.

Table 6.5: Daily intake of isoflavones by infants (categorized based on their age) after consuming different soy based infant formulas, expressed in mg aglycone equivalents

Infant formula (intake)	Age group 0-2 weeks	Age group 2-4 weeks	Age group 4-8 weeks	Age group 8-12 weeks	Age group >12 weeks	Age group >6months
IF1						
Serving (g/day)[a]	77.40	103.20	107.50	129.00	137.60	-
Isoflav (mg/day)[b]	17.55[c]	23.40[e]	24.38[e]	29.26[ef]	31.21[f]	
IF2						
Serving (g/day)[a]	70.40	105.60	105.60	105.60	132.00	140.80
Isoflav (mg/day)[b]	13.83[c]	20.75[e]	20.75[e]	20.75[e]	25.94[e]	27.67[e]
IF3						
Serving (g/day)[a]	-	-	-	-	-	105.90
Isoflav (mg/day)[b]						14.14[c]
IF4						
Serving (g/day)[a]	53.07 -68.23	68.23-75.82	75.82-94.77	94.77-113.73	132.68	132.68
Isoflav (mg/day)[b]	6.71 -8.62[d]	8.62 -9.58[d]	9.58-11.98[cd]	11.98 -14.38[c]	16.77[c]	16.77[c]
IF5						
Serving (g/day)[a]	-	-	-	-	-	213.00
Isoflav (mg/day)[b]						12.68[c]

Serving (g/day)[a] is the amount of infant formula consumed per day as per the manufacturer's direction.
Isoflav (mg/day)[b] is the calculated intake of isoflavones based on serving sizes and expressed in aglycone equivalents.
[c – f]Values of isoflavones bearing common letters are not significantly different at $p \leq 0.05$.

The daily intake of isoflavones for the infants consuming these SBIFs was found to range between 6.71 and 31.21mg depending on the product and age of the infant. **Setchell et al (1997)** found two SBIFs, which can result in an isoflavone intake of 47mg per day for an infant of 4-month old. However, the maximum intake of isoflavones from the SBIFs of this study for a 3 - 6 month old infant was found to be approximately 31.21 mg/ day. The product IF1 contained the highest concentration of isoflavones per gram of the product and it also would result in the ingestion of the highest levels of isoflavones through every age group. Moreover the serving sizes recommended for this formula was higher compared with those of other SBIFs. Product IF4 would provide much lower levels of intake of isoflavones for infants of every age group than those from products IF1 and IF2.

Setchell et al (1997) found in their studies that infants fed SBIFs had 13000-22000 times higher circulating concentrations of isoflavones than their plasma oestradiol concentrations, which may be sufficient to exert biological effects, whereas the contribution of isoflavones from breast-milk and cow-milk was negligible. It might also be advisable to reconsider their utilization for infants, especially in the presence of hormonal disruption **(Fort et al., 1990).**

6.4 EVALUATION OF PRODUCT LABELS, CONTENTS, INTAKE AND NEED FOR STANDARDISATION OF ISOFLAVONE LEVELS

Epidemiological studies have shown isoflavones as having various health protective effects including the lowering of cholesterol **(Anderson et al., 1999)**, decreasing the incidence of different types of cancers **(Lee et al., 1991),** menopausal symptoms **(Dixon and Ferreira, 2002)** and even osteoporosis **(Setchell and Cassidy, 1999)**. But, soy isoflavone supplements sold over-the-counter were found to be targeted mainly for women, to help postmenopausal syndromes, to support breast health, and to aid in weight control, while the other positive effects of isoflavones were never labeled. This is because women being more health conscious and especially being aware of the possible side-effects and long term health consequences from using hormone therapy for amelioration of the discomforts associated with the menopausal transition **(Kurzer, 2003)**, are more inclined towards consuming dietary phytoestrogens as an alternative. However, a wide variation was found to exist in the amount of isoflavones in soy isoflavone supplements and hence their concentrations need to be standardized. Many studies have also shown that isoflavone aglycones are absorbed better than the glucosides **(Izumi et al., 2000;**

Ruiz-Larrea et al., 1997). However, among the different soy isoflavone supplements studied, aglycones were found at much lower levels compared with their total isoflavone amounts. When expressed in aglycone equivalents, the isoflavone content in these products was much lower compared with their labeled amounts.

SBIFs have been generally shown to be nutritionally safe and adequate for normal growth of infants. However, it might also be advisable to reconsider their use for infants, especially in the presence of possible hormonal disruptions **(Fort et al., 1990)**. Humans do not normally have high concentrations of estrogens until after puberty and therefore feeding the infants with SBIFs may result in higher concentrations of isoflavones in blood and tissue **(Badger et al., 2002)**, which might be of concern. The possible chronic effects that might occur after high levels of intake of isoflavones need to be further studied to confirm their safety.

The display of isoflavone concentrations on the labels of every soy isoflavone supplement, soy based health product and SBIF, if made mandatory, could be more advantageous to consumers, researchers and health practitioners, since it can provide additional nutritional information. Expressing the isoflavone concentrations in aglycone equivalents is recommended as this will enable the consumers to make a better comparison of the active ingredients in the products available on the shelves. This study further highlights the need for standardization of these products. Urgent research is also needed to find the optimal doses of the different isoflavone forms, long term effects and its interaction with other drugs.

PART III

CONCLUSIONS AND FUTURE RESEARCH

CHAPTER 7

CONCLUSIONS AND FUTURE RESEARCH

7.1 CONCLUSIONS

The major conclusions that can be drawn from this study are the following:

A rapid, simple and reproducible method for the extraction and quantification of isoflavones in soy based products was developed using HPLC. The optimized method developed for the study separated the six soy isoflavones in a single chromatographic run and confirmation of the identified peaks was done by LC-MS. This proved the efficacy of the developed method in using it as a routine method for isoflavone analysis. The analytical method itself is also a useful tool for further study of isoflavones.

A comprehensive analysis of isoflavone and protein contents in soymilks and tofus collected from different South East Asian countries was carried out as part of this study. Higher amounts of isoflavones were found in products with high levels of protein. This is the first set of studies which give guidelines to choose soymilk and tofus based on their protein contents. However, this may not be true, if alcohol-washed soy protein was used for the preparation of soymilk. The data on isoflavone amounts in soy products, extend the results reported by other researchers like **Murphy (1981), Eldridge (1982), Wang et al (1990)** etc. The database is also useful for clinical researchers, who might wish to choose a soymilk with higher isoflavone content, so as to permit smaller volumes of soymilk to be consumed for their clinical studies and thereby facilitating compliance in dietary intervention studies.

Studying the effect of novel, automated and advanced soymilk processing systems used lately by soymilk manufacturers, it was found that hot grinding caused an improvement in the extraction of isoflavones into the soymilk compared to cold grinding, while there was no apparent differences in the loss of isoflavones due to direct UHT heating compared to indirect UHT heating.

The studies on the effect of different coagulants used for the preparation of tofu showed that the isoflavone content varied significantly with the use of different coagulants. Tofu produced using calcium sulfate as a coagulant gave the highest yield of tofu and it also retained the highest amount of isoflavones. This was the first set of studies which showed the effect of coagulants on the content of isoflavones during the preparation of tofu. Okara and wheys, the waste stream of the processes were found to carry certain amounts of isoflavones along with them. This study further highlights the need for urgent research in converting them to isoflavone ingredient products suitable for human or animal feed.

Considering the recent trend in market flooding with soy isoflavone supplements and related products, a comprehensive analysis of isoflavone concentrations in soy based supplements, health products and infant formulas from South East Asian countries was carried out. Different brands of SBIFs would result in the ingestion of different amounts of isoflavones by the infants. The amounts of isoflavones were expressed on the basis of aglycone equivalents units and a better comparison of the isoflavone amounts in the different products was made possible. Among the products analyzed, soy isoflavone

supplements had the highest isoflavone concentrations, followed by soy based health product. However, taken in moderate amounts they will most likely provide certain health benefits and it is good to educate people in this respect. This study highlights the need for standardization in expressing the concentration of isoflavones and the urgent necessity of defining specific dietary recommendation for soy isoflavones with regard to individual disease prevention.

7.2 SUGGESTIONS FOR FUTURE RESEARCH

On the basis of the results obtained from the present studies, the following studies are recommended:

- Analytical results showed the loss of isoflavones into the by-products of soymilk and tofu preparations. Considering okara and whey as rich sources of isoflavones (although diluted), these materials need to be utilized and processed further to prevent loosing these functionally significant compounds and using them as raw materials for isoflavone ingredient product development. The isoflavone ingredient of choice has to fit into the organoleptic and physical nature of the developed product (solid or liquid, colloidal or clear, powder/bar), the desired ratio of isoflavone to soy protein (especially if intending to use the FDA claim for soy proteins), stability and shelf life and finally the price.

- Information regarding the bioavailability of isoflavones from soy supplements as compared with that of soy foods are limited **(Setchell et al., 2001)**. Differences might exist in the bioavailability and metabolism of isoflavones dependent on the nature of

their chemical form and an evaluation studies will help in understanding the fate of isoflavones, its pharmacokinetic properties, bio-potencies as well as the differences based on consumption of a supplement instead of soy food.

- Toxicological studies related to isoflavones or the amount that may result in deleterious effects in human are also unknown. For the same reason, superiority of certain isoflavone components in terms of activity and bioavailability, as well as recommended dosage, cannot be clearly determined. Studies pertaining to its allowable amounts, relating them to specific disease preventions can serve as guidelines for the consumers while choosing a soy product for consumption. Determining the Effective Dose 50 (ED50), Lethal Dose 50 (LD50), or equivalent values of isoflavones with dose-effect relationships in animal and human studies are also recommended.

- With the recent advances in non-thermal preservation methods of pulsed electric field, irradiation, UV light, or chemical preservation, there is absolutely no data concerning its influence on the isoflavone profile in soy or isoflavone-enriched products. The recent advances in these technologies and the progressive increase in developed soy products imply the need for pioneering this area of research too.

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APPENDICES

Two examples of the HPLC calibration reports are shown.

Appendix A1: LC Calibration Report of genistin

Appendix A2: LC Calibration Report of glycitin

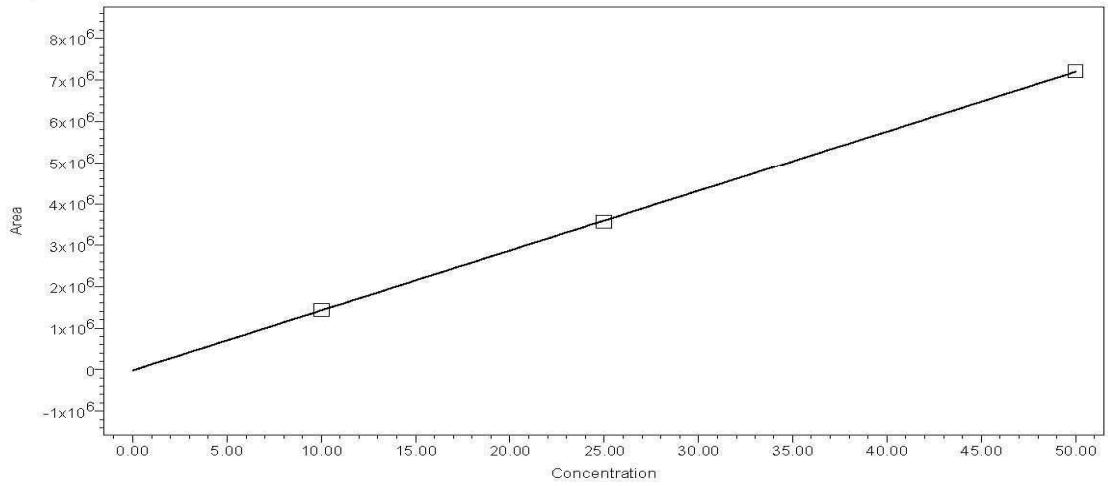
APPENDIX: A1

LC Calibration Report

Reported by User: System

Project Name: Molamma_soya

Processing Method:	genistin	Project Name:	Molamma_soya
Processing Method ID:	1241	System:	2695_2996
Calibration ID:	1246	Channel:	W2996 260.0nm-1.2
Date Calibrated:	04/06/2003 11:24:56	Proc. Chnl. Descr.:	W2996 PDA 260.0 nm at 1.2



Name: genistin; RT: 30.630; Fit Type: Linear (1st Order); Cal Curve Id: 1247; R: 0.999982; R^2: 0.999963; Weighting: None; Equation: Y = 1.44e+005 X - 2.14e+004

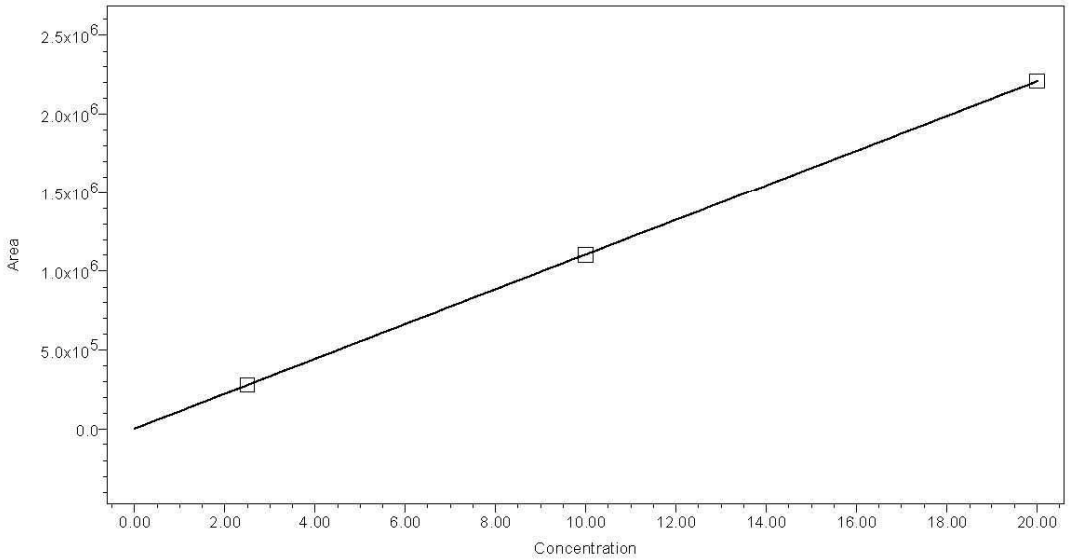
Peak: genistin

	Sample Name	Result Id	Name	Level	X Value	Response	Calc. Value	% Deviation	Manual	Ignore
1	genistin 10ppm	1245	genistin	1	10.000	1434775.987	10.088	0.88	No	No
2	genistin 25ppm	1248	genistin	2	25.000	3567123.020	24.860	-0.56	No	No
3	genistin 50ppm	1249	genistin	3	50.000	7203796.461	50.053	0.11	No	No

LC Calibration Report

Reported by User: System Project Name: Molamma_soya

Processing Method:	glycitin	Project Name:	Molamma_soya
Processing Method ID:	1299	System:	2695_2996
Calibration ID:	1301	Channel:	W2996 260.0nm-1.2
Date Calibrated:	04/06/2003 13:19:27	Proc. Chnl. Descr.:	W2996 PDA 260.0 nm at 1.2



Name: Glycitin; RT: 23.868; Fit Type: Linear (1st Order); Cal Curve Id: 1302; R: 0.999999; R^2: 0.999999; Weighting: None; Equation: Y = 1.10e+005 X + 2.28e+003

Peak: Glycitin

	Sample Name	Result Id	Name	Level	X Value	Response	Calc. Value	% Deviation	Manual	Ignore
1	glycitin 2.5ppm	1310	Glycitin		2.500	278812.497	2.506	0.26	No	No
2	glycitin 10ppm	1309	Glycitin		10.000	1104295.003	9.989	-0.11	No	No
3	glycitin 20ppm	1308	Glycitin		20.000	2209339.513	20.005	0.02	No	No

APPENDIX: A3

A representative chromatogram obtained for a sample of soymilk by LC-MS

A3