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NEPAL FOOD SCIENTISTS AND TECHNOLOGISTS ASSOCIATION

The Association is a professional and educational organization of Food Scientists and Technologists of Nepal with its central office in Kathmandu.

There are two chapters- Purbanchal (Eastern Development Region) and Narayani (Mid Development Region) of the association within the country which are located at Dharan and Hetauda respectively. Similarly, there are three chapters of the association in abroad situated in United States of America, Austrailia and Nigeria.

Objectives

- To facilitate the development and popagation of knowledge of Food Science and Technology.
- To provide a forum for discussion and exchange of the outcomes of research work in the field of Food Science and Technology.
- To create supportive environment in order to encourage Food Science and Technological innovations.

Major Activities

- Publication of Journal of Food Science and Technology Nepal (JFSTN), Food Nepal and Newsletters.
- Arranging lectures and seminars on different aspects of Food Science and Technology for the benefit of members and the Public

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The General Secretary
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Phone: 00977-1-4262741, 00977-1-4262369; Fax: 00977-1-4-262337
Email: nefostaexcom@yahoo.com
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Altogether one hundred and one articles of five different types (Review articles 28, Research papers 48, Research notes 23, viewpoint 1 and short communication 1) are published in first five issues of JFSTN i.e 12, 27, 20, 17 and 25 articles respectively, the details is given as follows:

Summary of the papers Published in JFSTN (Vol-1, 2005 to Vol-5, 2009)

Issue	*Review Article*	*Research Paper*	*Research Note*	*View Point*	*Short Communication*	*Total*
JFSTN, 1, 2005	6	2	4	-	-	**12**
JFSTN, 2, 2006	8	16	3	-	-	**27**
JFSTN, 3, 2007	7	12	1	-	-	**20**
JFSTN, 4, 2008	3	7	7	-	-	**17**
JFSTN, 5, 2009	4	11	8	1	1	**25**
Total	**28**	**48**	**23**	**1**	**1**	**101**

On analysing country wise contribution, about half (50 out of 101) of the papers are contributed by the professionals reflecting the work done outside the country (those professionals are either international professionals or Nepalese professionals worked outside during either their study period or working period.) and rest of the papers 51 out of 101 are contributed by the professionals from home, the details is given as follows:

Country wise Contribution of papers (Published) in JFSTN (Vol-1, 2005 to Vol-5, 2009)

Country	*JFSTN, 1, 2005*	*JFSTN, 2, 2006*	*JFSTN, 3, 2007*	*JFSTN, 4, 2008*	*JFSTN, 5, 2009*	*Total*
Australia	1	1	1	1	1	5
Bangladesh	-	-	-	1	-	1
Belgium	-	-	-	-	1	1
China	3	5	4	2	2	16
India	-	5	2	1	7	15
Japan	-	1	2	1	-	4
Nepal	8	11	10	10	12	51
Nigeria	-	4	1	-	-	5
Srilanka	-	-	-	1	1	2
Thailand	-	-	-	-	1	1
Total	**12**	**27**	**20**	**17**	**25**	**101**

Submission of Manuscript to JFSTN, Volume 6, 2010

All of the professionals related to Food Science and Technology are requested to submit their valuable manuscript (both in the form of hard and soft copy) of Review Articles, Research Papers, Research Notes, Viewpoints and Short Communications to the following address. Any clarification regarding preparation of manuscript and publication can also be addressed to this address.

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JFSTN had been able to register in Nepal Journal Network (NEPJOL) at June 8, 2009 and professionals from any corner of the world can access the content of JFSTN in the website **www.nepjol.info**

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Irradiation as an Effective Way of Microbial Control in Food Preservation and Processing

RAJAPAKSHE ARACHCHILLAGE UPUL JANAPRIYA MARAPANA[1] and SOMASIRI WIJETUNGA[2]

[1]Dept of Animal Science, Faculty of Agriculture, University of Ruhuna, Mapalana, Kamburupitiya, Sri-Lanka
[2]Dept of Agricultural Engineering, Faculty of Agriculture, University of Ruhuna, Mapalana, Kamburupitiya, Sri-Lanka

Irradiation is safe method which can be used to eliminate most microbial pathogens present in the foods. The doses, at which foods are irradiated, depend on the types of food, storage duration and other environmental factors prevailing during the irradiation. Herbs, spices, and dry vegetables, which are used in very small amount for mixing with other foods, can be irradiated up to 30 kGy. Poultry and red meats can be irradiated at doses of 1.5 to 3.0 kGy and 4.5 to 7.0 kGy respectively. Use of high dose, in some cases, causes to deteriorate the food quality and irradiation in combining with other preservation methods can avoid the use of high doses. The combination methods with irradiation have shown good results for some foods. Various methods of irradiation in food preservation and processing are discussed in this paper.

Keywords: Irradiation; Preservation; Microorganism; Gamma radiation; Combination processes.

Introduction

The nutritive and fresh food is one of the basic needs for human for healthy life. People are really seeking of nutritive food now ever before. Consumers are increasingly aware of the health benefits and risks associated with consumption of food. To satisfy the expectations of consumers, the food industry is devoting considerable resources and expertise to the production of wholesome and safe products. Production of safe foods include examining materials entering the food chain, suppressing microbial growth and reducing or eliminating the microbial load by processing and preventing post contamination (Bower and Daeschel, 1999). The presence of a processing unit operation aiming at microbial destruction is of primary importance to ascertain safety and stability of food. Heat treatments are traditionally applied to pasteurize or sterilize food, generally at the expense of its sensory and nutritional qualities (Beatrice and Ahmed, 2002).

As consumers increasingly perceive fresh food as healthier than heat treated food, the industry is now seeking alternative technologies to maintain most of the fresh attributes, safety and storage stability of foods (Kevin, 2006). One of very interesting and developing alternative technology for food preservation and processing is the irradiation.

Food irradiation is the process of exposing a food item to certain types of radiation energy to bring about desirable changes of the food. Ionizing radiation is radiant energy which has the ability to break chemical bondsï (Kevin, 2006). There are major three types of radiation that can be applied for foods, namely, electron beams, x-rays and gamma rays. Gamma rays can be naturally obtained from radioactive decay of Cesium 137 or Cobalt 60 (Donald et al., 1996). Amount of radiation that has to be applied to foods depend on types of foods and time needed for preservation. The radiation is measured in Grays (Gy) and 1 Gy is equal to energy of 1 Joule absorbed by 1 kg of product (Kevin, 2006). The objective of this paper is to review the various methods of irradiation in food preservation and processing.

*Corresponding author, Email: marapana@ansci.ruh.ac.lk

Mechanism of Microbial Inactivation by Irradiation

DNA is very sensitive to irradiation. Therefore, use of irradiation makes immense damages to DNA of microorganisms in the foods. The approved level of irradiation causes to base damage, breaking of DNA strands, and cross linking of them (Beatrice and Ahmed, 2002). All these cause the microorganism to loss of their ability of reproduction. In addition to this, the free radicals produced in the irradiation also badly affect to DNA functions of microorganism.

Effect of Irradiation on Foods

Irradiation can be applied for different types of foods for preservation, while keeping the nutritive value and Organoleptic parameters are unaffected. The possibility of using irradiation for different types of foods is briefly discussed below.

Irradiation on fruits and vegetables

Fruits and vegetables can be irradiated to prevent the spoilage organisms and improve the storage ability. In addition to, insects can also be eliminated and over-ripening and sprouting can also be prevented. Minimum and maximum doses can be applied for irradiation fruits and vegetables are 0.3 kGy and 1 kGy respectively (Donald et al., 1996). They also suggested that some fruits and vegetables are sensitive to radiation and the skin of the fruits is damaged.

Combination of high pressure treatment with irradiation improves the destroying capacity of bacteria and yeast in Kefir without affecting to the structure of proteins and lipids.

Irradiation on cereals and cereal products

One of the important food stuff that lots of people consumed worldwide is the cereals. The huge amount of cereals is lost its value in quality and quantity due to poor preservation. And also, other post harvest losses causes to loss significant amount of cereal products. In addition, to preserve of different types of cereals, it is very important to find the ways of new processing technologies that provide high nutritive value and tastes with longer preservation periods and low cost.

Studies showed that irradiation can be successfully use for cereal product preservation. One of most commonly found bacteria in cereal products is *Bacillus cereus*. The studies showed that more than eight strains are sensitive to electron beam irradiation (Sarrias et al., 2003). They further reported that irradiation dose necessary to kill *Escherichia coli*, *Bacillus cereus*, sulfur-reducing Clostridia and fungi in un-husked and husked rice are 7.5 and 1.1 kGy, respectively. The 7.5 kGy dose is enough for decontamination of husked rice as well as reduced aerobic plate counts to average levels of 2.66 log cfu/g in husked rice inoculated with 10^4 spores per gram of strain EPSO-41WR. They reported that some strains have greater resistance to irradiation and the doses of irradiation needed to destroy them are different. The D_{10} value for them is in between 2.07 to 2.68 kGy.

The most popular cereal product is noodles and it is world widely accepted well popularized product. Most of the people use dried noodles since they are easily available. But consumers more prefer to use fresh noodles if they are easily available (Cai, 1998). The consumers in China are more likely to be used fresh noodles rather than dried noodles. Cai (1998) reported that gamma rays irradiation can be used to extent the preservation period of fresh noodles when they are stored in room temperature. The dose of irradiation of gamma rays required to prolong the storage period of fresh (wet) noodles at room temperature is more than 8 kGy and those irradiated noodles can stored more than 10 days without affecting to their physical properties and nutritive value.

Irradiation on meat and meat products

The worldwide consumers are used different types of meat products. Different types of processed meats products are easily available. The contaminated meat products are associated with serious health risks when they are used for consumption. The meat products provide good nutritive media for lots of microorganisms and therefore, when the meat products are once contaminated, the number of microorganisms are rapidly increased their number by proliferation (FSIS Docket 97-076P, 1999).

Relatively low dose of ionizing radiation can be used for radiation pasteurization treatments of meat to control microorganism, which are mostly pathogens and food spoilage bacteria (Donald et al., 1996). The potential for consumer infection by pathogens is decreased greatly and self life is extended by radiation pasteurization of meats. These benefits can be achieved only if the highest quality meat products are irradiated and by avoiding recontamination after packaging. Radiation pasteurized products are neither sterile nor self stable when they are handled or stored in poorly. They have to be well refrigerated after irradiation pasteurization and cooked before serving. Irradiation products also provide opportunity to avoid cross contamination also.

Irradiation on beef

Irradiation can destroy the extremely virulent bacteria, such as *Escherichia coli* O157:H7, which can not easily be destroyed using traditional methods of food preservation, in ground beef (Thayer and Boyd, 1993; Claveroet et al., 1994).

Most of the pathogens in beef can be destroyed by using irradiation dose in between 1.5 to 10 kGy depending on the types. All most all species of salmonella can be destroyed by using 3 kGy dose of irradiation while Escherichia coli O157:H7 can be destroyed by about 2 kGy of dose irradiation (Thayer and Boyd, 1993; Thayer et al., 1995).

The most important thing is that actual number and percentage of cells that will be killed by irradiation depend on various factors such as type of pathogen, growth stage, absorbed radiation dose, irradiation time temperature, oxygen presence, water content. Spore forming bacteria of the genera *Bacillus* and *Clostridium* are more sensitive to heat pasteurization doses of radiation. The few non spore forming bacteria that may survive in irradiation are injured severely and become much more sensitive to heat. They are, therefore, very unlikely to survived after cooking.

Ouattara et al (2001) reported that use of gamma irradiation on beef patties can significantly reduced the pathogens of *Enterobacteaceae,* lactic acid bacteria, *Pseudomonas*, and *Brochothrix thermosphacta*. They further reported that lactic acid bacteria and *Brochothrix thermosphacta* are more resistant to irradiation than *Enterobacteaceae* and *Pseudomonas*.

Peter and David (2002) demonstrated that possibility of use of electron bean as an irradiation technique on beef steaks. Electron irradiation doses ranging from 2 to 4 kGy can effectively reduce the population of Psychrotrophic bacteria in beef steaks.

Irradiation on poultry

Poultry can also be irradiated to destroy pathogens. Studies showed that irradiation can reduce the multiplication of Coliforms, *Escherichia coli*, Psychrotrophs, Salmonella and Campylobacter on poultry meats (Lewis et al., 2002). They further reported that 2 kGy dose of irradiation can be used but after long period of storage (more than 28 days) quality of meat is reduced significantly, texture, flavor and overall acceptability. Lipid oxidation is also increased as storage time and level of irradiation increased.

Irradiation on pork

The gamma irradiation on the physiochemical, organoleptic and microbial properties of pork was studies and reported that no any adverse effect on that properties of pork. Regardless of the type of packaging and dose rate of irradiation, all irradiated pork is prevented effectively from bacteria spoilage for at least 43 days (Lacroix et al., 2000). They further revealed that meat redness and texture of irradiated loins are relatively well preserved during longer storage periods (about 45 days), especially when they are packed under vacuum. Overall physiochemical and organoleptic changes in pork loins appeared to be relatively little affected by the 6 kGy dose. Microbial safety of pork packed in an atmosphere of 25% CO_2, 75% N_2 followed by irradiation dose of 1.7 kGy is better than that of untreated pork (Margaret, 1996). It is reported that irradiation threshold limit of pork is 1.75, above which undesirable sensory changes occur.

Irradiation on mutton

Bhide et al (2001) revealed that gamma irradiation can reduce Bacillus cereus, that grow in mutton, without adversely affects to quality of mutton with regard to the acceptability of consumers.

Irradiation on fish and fish products

Peoples in most part of the world consumed fish than meat. Small islands can easily obtain their requirement of fish from surrounding seas. But, in large countries, the consumption of fresh ocean fish is a problem in middle parts of such countries due to transportation problems. Therefore, it is essential to have a good preservation technique that can preserve fish without destroying organoleptic parameters (CAST, 1996). Most popular method is freezing but processed or unprocessed fish or fish products can be easily prevented from pathogenic growth by using irradiation treatments. Series of studies have already been done for evaluating the effects of irradiation on fish or fish products.

Savvaidis et al (2002) reported that irradiation doses of 0.5 and 2 kGy significantly affected to the population of bacteria namely, *Pseudomonas*, *Brochothrix thermosfacta*, lactic acid bacteria, sulfur dioxide producing bacteria (*Shewanella putrefacta* and *Enterobacteaceae*) at 4 and 4°C. They further stated that dose of irradiation 2 kGy at lower temperature more effective and Pseudomonas and sulfur dioxide reducing bacteria are more sensitive to gamma radiation than others. On the basis of sensory odor scores, a self life 28 days (2 kGy at 4°C) was reported for salted vacuum packaged fresh water trout, compared with a self life of 7 days for untreated sample.

Aflatoxin B1 is produced by some fungus that grows on certain foods. Direct smoked fish is a good media for the growth of fungi that produce aflatoxin B1. *Aspergillus flavus* that produce aflatoxin can be significantly reduced by gamma irradiation (Ogbadu, 1988). The production level of toxin is decreased with increasing dose of gamma irradiation. Therefore, aflatoxin B1 production from *Aspergillus flavus* can be controlled effectively by using irradiation.

Irradiation on beverages

Beverages are also very important human consumptive and they can be preserved using irradiation. Irradiation treatments are used for preservation and processing of most vegetable and fruit beverages. High intensity pulsed electric fields (HIPEF) is a non-thermal technology used in the processing of liquid foods. Inactivation of food borne microorganisms by this technology is an important alternative to traditional thermal methods with a great potential for new liquid products (Selma et al., 2002). Studies showed that growth of *Enterobactor aerogene*; which are most commonly found microorganism in horchata, kind of vegetable drink; can be retarded growth by HIPEF. And synergic effect with low temperature can delay the lag phase of microorganism. HIPEF can also be considered as new technique for food preservation, related to irradiation.

Irradiation on other foods

Various studies showed that the irradiation can be effectively used to control pathogenic organisms, in various types of foods without affecting to their acceptability as a food stuff using different doses (Table 1).

Ouattare et al (2001) demonstrated that possibility of using irradiation for controlling of *Pseudomonas putida*, and other aerobes, which are proliferation on pre-cooked shrimp.

Manninen and Sjoberg (1991) reported that possibility of using Direct Epifluorescent Filter Technique (DEFT) and aerobic plate count (APC) to detect irradiated foods.

Table1: Recommended irradiation doses for different food items

Product	*Dose (kGy)*	*Purpose*
Low dose (<l kGy)		
Wheat, wheat flour	0.2-0.5	Insect control
White potatoes	0.05-0.15	Inhibit sprouting
Pork	0.3-1.0	Control trichinella
Fruit	1 maximum	Control insect, delay ripening
Fresh Vegetables	1 maximum	Insect control
Medium dose (1-10 kGy)		
Poultry, fresh or frozen	3 maximum	Microbial control
Shell eggs	3 maximum	Salmonella control
Meat, uncooked, chilled	4.5 maximum	Microbial control
Meat, uncooked, frozen	7 maximum	Microbial control
Seeds for sprouting	8 maximum	Microbial control
Dehydrated enzymes	10 maximum	Microbial control
High dose (11-45 kGy)		
Herbs	30 maximum	Microbial control
Spices	30 maximum	Microbial control
Vegetable seasonings	30 maximum	Microbial control
Meat, frozen, packaged	44 maximum	Sterilization
Animal feed and pet food	2-25	Salmonella control

(Source: Olson, 1998.)

Anu et al (2000) showed that ice cream can be irradiated in dose of gamma radiation to prevent possible microorganism poisoning. Pathogens such as *Listeria monocytogens* 036, *Versinia enterocolitica* 5692 and *Escherichia coli* O157:H19, respectively showed the D_{10} values 0.38, 0.15 and 0.2 kGy in ice cream at -72 °C suggesting efficacy of low doses (1 kGy) in eliminating them. They further demonstrated that a radiation dose of 1 kGy is sufficient to eliminate the natural number of pathogens present in the ice cream without affecting to flavor.

Ju et al (2002) have studies the possibility of using of irradiation to improve the hygienic quality, extension of self life of "Kwamegi", which is made from semi dried Pacific Saury. They reported that irradiation dose range between 7 to 10 kGy is optimum to control the pathogenic agents such as *Salmonella* and *Shigella* species present in "Kwamegi". Even 60 days after storage at 5 °C, they found that quality of "Kwamegi" is significantly better than non irradiated ones.

Microbial Deactivation of Foods by Irradiation with Other Preservation Methods

With an increasing requirement of consumers for foods that are more convenient, easier to store, higher quality, more natural, healthier and safe, there is a trend towards the development of preservation procedures that are milder, less intensive and additive free. An important element of this trend is the use of procedure in new combinations that allow reduction in the extreme use of any single technique (Gould, 1996). There are many established and emerging preservation technologies that are increasingly and usefully employed in combinations (Gould and Jones, 1989). This numerous procedures act on foods by inhibiting microbial growth, inactivating microorganisms.

Irradiation has already been shown to be a potentially valuable component in a number of combination procedures in ways that increase antimicrobial efficacy whilst minimizing unwanted organoleptic effects (Cambell and Grandison, 1990).

Irradiation has emerged as a potential method of food preservation. It is being used to extend the self life of raw and processed foods in many countries worldwide. Like all other methods of food preservation, irradiation has a number of limitations. Irradiation, when used alone, can cause the development of undesirable sensory and chemical changes in some foods, depending on the absorbed dose and the conditions of irradiation (Thakur and Singh, 1995). The development of irradiation for food preservation is dependant on good process for choosing the doses. The reduction of doses is particularly suitable, not only for the cost, but for organoleptic qualities of food (Vincent et al., 1990).

One way of avoiding these detrimental effects of radiation is to combine irradiation with other preservation methods such as heating, cryogenic temperature and modified atmosphere or vacuum packaging. These associated processes can make the total food process more labor intensive and time consuming, resulting in the increased cost of treated products. However, the decreased cost of irradiation at low doses may

reduce the cost involved, depending upon the cost of that processes relative to the cost of irradiation (Thakur and Singh, 1995). The use of combination processes has been found to inhibit the development of undesirable sensory changes and some chemical changes in food, making food irradiation a more useful method of food preservation.

Many studies showed that the combination processes of food preservation with irradiation can be effectively used for different types of foods to evaluate the pathogenic destroy ability as well as the quality of foods that consumers are more preferred (Giroux et al., 2001; Ouattara et al., 2002a; Margaret, 1996; Gould, 1996).

Ouattara et al (2001) reported that low dose irradiation with combination of antimicrobial coating of pre cooked shrimp can prolong the self life. Antimicrobial coatings obtained by incorporating various concentration of thyme oil and trans-cinnamaldehyde in coating formulations prepared from soy or whey protein isolates have been used for the study. They further demonstrated that gamma irradiation and coating treatments have synergistic effects in reducing the aerobic plate count. *Pseudomonas putida* can be easily deactivated. No detrimental effects of gamma irradiation on organoleptic parameters have been found.

Giraoux et al (2001) have studied the combination effect of ascorbic acid and gamma irradiation on beef patties for their sensory characteristics at 4 +/- 1°C storage temperature. They further reported that although irradiation treatment, alone, had detrimental effects on redness and yellowness, the incorporation of ascorbic acid into meat before irradiation resulted in significant stabilization of color parameters. Color improvement with ascorbic acid is not related to pH reduction and also, no significant detrimental effects on taste or odor have found in irradiated meats containing ascorbic acid.

Most common pathogens present in beef products such as *Enterobacteriaceae*, presumptive *Staphylococcus aureus*, presumptive *Pseudomonas* species, B*rochothrix thermosphacta* and lactic acid bacteria can be reduced their proliferation by irradiation only and improvement of preservation can be obtained by combining irradiation with natural antimicrobial compounds such as ascorbic acid (Ouattara, 2002b).

Lactic acid bacteria and B*rochothrix thermosphacta* are more resistant to irradiation than *Enterobacteriaceae* and Pseudomonas (Ouattara et al., 2001). Irradiation dose could be reduced considerably without affecting to preservation quality of meats by introducing natural antimicrobial compounds.

Louise et al (1997) demonstrated that possibility of use of irradiation with ascorbic acid treatment to eliminate *Escherichia coli* and *Lactobacillus curvatus*. They further revealed that synergistic effects can be seen when using irradiation dose in between 0.145 and 1.1 kGy with ascorbic acid range of 0.02 and 1% for *Escherichia coli* but no any effect to be seen on *Lactobacillus curvatus* controlling up to 2% ascorbic acid with irradiation.

The acid presensitization with low dose gamma irradiation can eliminate bacillus cereus on muttons, effectively (Bhide et al., 2001). They further reported that 2% acetic acid with 3 kGy irradiation elicited most effective to lower the total viable count of *Bacillus cereus*. And combination method can increase the self life of mutton substantially without any adverse effect on acceptability of meat.

Humidity can also be used combination with irradiation to control microorganism growth on foods. Humidity of foods affects to fungus growth and some fungus produce toxins, which are very toxic when they are ingested. Himly et al (1995) reported that possibility of use combination method to control *Aspergillus flavus* on ground nutmeg and peanut. They added that growth of *Aspergillus flavus* can be controlled by reducing RH, itself, less than 85%. The irradiation dose of 3 kGy is the most effective dose that can eliminate growth and production of toxin, at any RH.

Electron beam irradiation, after preseasoning with ginseng or garlic, can enhance the quality of beef sirloin steaks with eliminating growth of several microorganisms, mostly Psychrotrophic bacteria (Peter and David, 2002).

More than two combination methods such as irradiation, heating, high pressure, can be applied to inactivate or retard the proliferation of pathogens on foods. Irradiation of Kefir at 5 kGy and high pressure treatment 400 MPa, 5 or 30 min deactivated the bacteria and yeast in Kefir and left the structure of the protein and lipid of the product unchanged (Isabelle et al., 2001).

It is reported that irradiation prior to heating is more effective to reduce pathogenic growth in beef, indicating that microbial cells are more heat sensitive after irradiation. Margaret (1996) reported that melanosis (black spots) forming in cold water prawn during storage can be avoided by dipping in a 0.005% 4hexylresorcinol for minute followed by irradiation of 1.6 kGy and can be stored at 3°C more than 15 days without spoilage. The main effect for that long storage period is the retarding the growth of microbes associated with spoilage of prawn.

Garin-Bastuji et al. (1990) showed that mild heat treatment with gamma irradiation, dose of 10 kGy can deactivate *Escherichia coli* in milk. Frozen yogurt can also be irradiated by gamma irradiation of dose 12 kGy with heat treatment and it can eliminate Bacillus species effectively (Hashisaka et al., 1990).

The combined preservation techniques can effectively used for controlling pathogenic organisms, which grow in food, without affecting to nutritive value and organoleptic parameters of food. It is revealed that low dose irradiation can play a big role in combination with other preservation methods and this provides the opportunity to the food processors to adopt this preservation process, while minimizing cost involved in preservation and improving quality of foods.

Limitations of Irradiation

Irradiation is not an only recognized method for food preservation and there some limitations. Although high doses of irradiation can eliminate most of the pathogens in the foods, this badly affects to the nutritive value and organoleptic parameters in some foods. Other important aspect of this technology is that all the foods can not be preserved using irradiation. It has found that Vitamin B1 and E in pork is destroyed in irradiation (Donald et al., 1996). And also Vitamin C is destroyed in irradiation when the fruits are irradiated. Although cost of irradiation may be low; when it is used with other preservation methods to avoids damages occurring during high dose of irradiation in foods, the cost is might be gone higher. It is very important note that the careful handling procedures have to be followed to avoid radiation exposure to users of the instruments and to surrounding environment.

Concluding Remarks

Good hygienic practices can reduce the level of contamination but the most important virulent pathogens cannot easily be eliminated from food processing. And it is not possible to destroy or inactivate them by primary processing, particularly, from foods that are sold raw. Several preservation techniques exist but the most versatile method among them is the processing with irradiation. Use of irradiation is safe, efficient, environmentally clean and energy efficient, low cost compared with other methods that give same level of decontamination as irradiation, when irradiation is properly handled. Radiation treatment at doses of 2 to 7 kGy, depending on the time of irradiation and type of food, can effectively eliminate potentially pathogenic non-spore forming bacteria including *Salmonella*, *Staphylococcus aureus*, *Compylobactor*, *Listeria monocytogenes*, *Escherichia coli* O157:H7 without affecting to organoleptic parameters, nutritional and technical qualities of the food.

High quality convenience foods can be produced and preserved, economically, easily and avoiding quality losses, by irradiation in combination with other processing and preservation methods. Dry food ingredients such as cereals, herbs can be irradiated with doses of 3 to 10 kGy and it can avoid the use of fumigants with microorganism killing gases. Irradiation at doses of 0.15 to 0.7 kGy in combination with other preservation methods can control of emerging pathogens in foods. Irradiation in combination with other preservation and processing methods has been recognized as a very effective method for controlling of microorganisms growing on most foods.

Irradiation of raw poultry, raw red meat, sea food and spices, at 2 to 10 kGy, has been approved by 41 countries in the world (Farkas, 1998). Irradiation is an emerging technology in an increasing number of countries and more and more clearances on irradiated foods are issued or expected to be issued in near future in most countries of the world for different types of foods. However, future studies should be more focused on the irradiation in combination with other methods of preservation for different types of foods.

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Ohmic Heating Applications in Food Processing: A Review

SANTOSH PATIL, ANUPAMA SINGH* and MANOJ KULSHRESTHA

Department of Post Harvest Process and Food Engineering, GBPUA & T, Pantnagar U.S Nagar India

In the era of globalization where the intensification and commercialization of food industries take place, the modern trends indicate the enhancement in fruits and vegetables processing, prevention of quality deterioration during processing and focus on the complete utilization of fruit and their by products with low energy requirement. Ohmic heating as a non thermal heating process is believed to improve the product quality with minimal structural, nutritional or organoleptic changes. Ohmic heating is a direct method and provides rapid cooking, minimum clumping, high temperature sterilization and almost 100% energy efficient. The literature relevant to ohmic heating, equipment and its application has been reviewed and presented in the present paper.

Keywords: Ohmic heating, Microbial activity, Preservation, Processing

Introduction

Recent interest in rapid methods of heating of foods has resulted in revived attention towards ohmic heating as one of the non-thermal technology. Ohmic heating is based on the passage of electrical current through a food product that serves as an electrical resistance. Ohmic heating is now regarded as highly attractive commercial technique for food processing and potential applications of ohmic heating include blanching, thawing, online detection of starch gelatinization, fermentation, peeling, dehydration.

The basic principal of ohmic heating or joule effect is the dissipation of electrical energy in the form of heat using an electrical conductor. This energy generation is proportional to the square power of the local Ohmic heating is a novel commercial process wherein electric current is passed through foods or other materials with the primary purpose of heating them due to internal energy generation within the material (De Alwis and Fryer, 1992). Heat generation in ohmic heating process is believed to be uniform. The rate of heating depends on the applied voltage, electrical conductivity and composition of the food. Fryer and Zhang (1993) reported that ohmic heating itself can be achieved in a variety of ways as electric heating, electro hydraulic shock, electro oration and electro conductive heating. Several advantages of the project have been reported such as rapid and uniform heating, continuous production, higher sterilization at lower cooking temperature, high quality products and processing of foods with high solid fractions.

In general ohmic heating can be utilized for thermal processing of almost any type of food products. However it is particularly beneficial for heat treatments of concentreated and highly viscous food products. For proteinaceous products where coagulation posses a problem during heat treatment, ohmic heating may be effective in raising the temperature above the coagulation temperature and cooling it quickly prior to coagulation (Das Gupta, 2004). Applications of ohmic heating in food processing have been reviewed in this paper.

Application of Ohmic Heating

Cooking

Uemura *et al.* (1998) used high pressure ohmic heating to cook four rice varieties (Indica, Japonica, Koshihikari and glutinous rice). Physical properties of the cooked rice were measured by compression testing, DSC, colorimetry and rapid viscometry. Indica rice became soft and sticky when heated to temperature above 120°C. Indica rice was also found to have high amylose content (28.3%) and underwent structural changes when heated above 120°C. In contrast, Japonica, Koshihikari and glutinous rice varieties had much lower amylose contents of 18.6, 18.6 and 0%, respectively, and showed little change with high temperature heating. Colour change in glutinous rice was greater than the other rice varieties. It was concluded that high temperature, high pressure ohmic heating improved the texture of high amylose Indica rice. Eliot *et al.* (1999a) investigated the effects of blanching on the texture of ohmically heated potato cubes. Three batches of potato cubes (16 × 16 mm) were blanched (90-95°C) in boiling salted water (5% NaCl) for 1, 2 or 4 min. These three batches and a non-blanched batch were then treated by ohmic heating at 135°C for 0, 2 or 4 min before texture analysis. Holding time during ohmic heating had a significant effect on texture of potato cubes. Long holding times greatly decreased firmness of the non-blanched samples. Blanching increased electrical conductivity of potato cubes and therefore, the heating rate, thus reducing quality loss during heating. Blanching also decreased texture degradation during ohmic heating. Blanched products showed better residual firmness than the non-blanched potato cubes. It was concluded that

*Corresponding author, Email: asingh3@gmail.Com

pretreatment by blanching is particularly suited to HTST processing of vegetables, as a high temperature applied for a short time, was generally insufficient to ensure complete inactivation of enzymes responsible for texture degradation. Eliot and Goullieux (2000) investigated the effect of low temperature pre-treatments of potato cubes on improving the texture quality after ohmic heating. Potato cubes were first cooked for 0 to 60 min in tap water at a temperature ranging from 55 to 70°C. Control samples were cooked at 95°C for 5 min. A firming effect (+ 25% compared to fresh samples) due to low temperature cooking (60 °C, 60 min) was clearly observed. Precooking for 1 h in brine solutions was then tried. The firming effect was emphasized at 55 and 60°C. After ohmic heating, cubes precooked at low temperature were always firmer than the control samples, but no significant differences were found due to different cooking temperatures. Low temperature pretreatments improved textural qualities of potato cubes. It was concluded that pretreatments at 70°C offered good compromise between product quality and product stability.

Piette *et al.* (2004) studied the cooking of bologna emulsion (lean and fatty pork meat, sodium chloride, sodium erythorbate and sodium nitrite) in a smokehouse (180-min cycle; to 70°C at core) and by ohmic heating (64-103 V; 3.9-10.3°C/min; to 70-80°C). The finished products were compared for colour, texture, pH, drip, and rancidity. Heating rates, final temperature and a 20 min holding time had little influence on the quality of ohmically heated sausages. Further, ohmically heated sausages were similar to smokehouse products except for texture, which was significantly softer ($P > 0.05$) in ohmically heated products. The sausages could be hardened by use of binders. Ozkan *et al.* (2004) compared the mechanical properties and composition (fat, moisture, and solids content) of hamburger patties cooked using hot grill plates (conventional cooking) with those cooked using combined ohmic and plate heating. Conventional cooking was carried out by placing frozen patties between heated plates of a grill, whereas combined cooking was carried out by applying 50 V of alternating current across two heated surfaces utilizing the principle of ohmic heating together with plate heating. Required cooking time using combined cooking was 117 s as against 163 s for conventional cooking. Flavour and texture of cooked hamburgers was assessed by an untrained panel of four. The similarities of hamburger quality were also confirmed by the panel. It was concluded that ohmic heating is comparable to conventional heating in terms of quality of cooked hamburgers. Shirsat *et al.* (2004) compared the effects of ohmic heating and conventional cooking methods on the water binding of a meat emulsion. Samples of meat emulsion batter were steam or ohmically cooked (at voltage gradient ranging from 3 to 7 V/cm) to a target end-point temperature. The samples were compared using expressible fluids (EF), dielectric properties and light microscopic techniques. EF testing revealed significantly higher levels of expressible moisture in steam-cooked samples ($P< 0.05$) and significantly greater amounts of expressible suspended solids in the highest voltage gradient (7 V/cm) ohmically cooked samples ($P < 0.05$). Dielectric constant and dielectric loss factor values at 300 and 3000 MHz were higher for steam-cooked samples, suggesting greater amounts of loosely bound water. Subsequent light microscopic evaluation of samples suggested slightly greater levels of physical disruption in ohmically cooked samples. Li-Jun *et al.* (2006) investigated the optimization of two-stage heating condition for making filled-tofu from soymilk and the effect of the two-stage heating on soft-tofu. They recommended that the combination of 70^{0}C for 10 min and 100^{0}C for 5 min was the best condition of the two-stage heating for preparing filled-tofu. Compared to the one-stage heating the two-stage heating increased the soft-tofu's apparent breaking strength by 12.2%, apparent Young's modulus by 16.2% and reduced the synergetic rate by 21.8%. Increased in the yield and the solid recovery was observed to be 4.5% and 5.4% respectively. They also mentioned that two-stage ohmic heating is a potential method for heating soymilk in tofu manufactures.

Blanching

Eliot *et al.* (1999a) investigated the effect of blanching on the texture of ohmically heated potato cubes. Three batches of blanched and a non-blanched potato cubes were treated by ohmic heating at 135ºC for 0, 2 or 4 min before texture analysis. As holding time increased, firmness of the non-blanched samples decreased. Blanching increased electrical conductivity of potato cubes which resulted in reduction in quality loss and texture degradation during ohmic heating. It was concluded that pretreatment by blanching is particularly suited to High Temperature Short Time (HTST) processing of vegetables. Sensoy and Sastry (2004) determined weight and volume losses in mushrooms during blanching under conventional and ohmic heating conditions. Ohmic blanching offered the advantage of being able to maintain high solid content (up to 50%) during blanching as compared to the use of excessive amounts of water (60 Kg/400 l) in conventional blanching. Filiz and Coskan (2005) studied the peroxidase inactivation and colour change during ohmic blanching of pea puree. The ohmic blanching was performed by application of four different voltage gradients in the range of 20 –50 V/cm. The puree samples were heated from 30^{0}C to 100^{0}C and held at 100^{0}C to achieve adequate blanching. The conventional blanching was performed at 100^{0}C water bath. They conclude that ohmic blanching can be used as an alternative method for pea puree. They also mentioned that the ohmic blanching applied by using 30 V/cm and above voltage gradient inactivated peroxidase enzyme at less time than the water blanching. The effect of voltage gradient during ohmic blanching on the colour of puree was significant ($p < 0.01$).The ohmic blanching above 20 V/cm gives better colour values than the water blanched puree.

Starch gelatinization

Wang and Sastry (1993) investigated the influence of starch gelatinization on electrical conductivity during ohmic heating. Corn and potato starch suspensions in starch: water ratio of 1:5 w/w were subjected to ohmic heating, with agitation, to 90°C by alternating current at 60 Hz with a voltage gradient of 20 V/cm. The partially and fully gelatinized starch suspensions prepared by heating were also tested. DSC at a scanning rate of 10°C/min was used to measure gelatinization energy and degree of gelatinization. DSC thermograms and electrical conductivity curves for both corn and potato starch suspensions gave endothermic gelatinization peaks with similar shapes and temperature ranges. While % gelatinization values were in agreement in low- and mid-gelatinization ranges, differences were observed at high degree of gelatinization due to high ohmic heating rates. In addition, electrical conductivity increased with temperature but decreased with degree of gelatinization, possibly because of structural changes and an increase in the level of bound water. Fa *et al.* (2004) developed a method for determination of gelatinization temperature using ohmic heating. The mechanism underlying the change in electrical conductivity during ohmic heating was investigated. Corn, mung bean and potato starch suspensions with starch: water mass ratios in the range of 1:3 to 1:10 were ohmically heated to 90°C using 50 Hz AC power and with a voltage gradient of 10 V/cm. The relationship between electrical conductivity of native starch suspensions and temperature was generally linear except in the gelatinization range. Gelatinization temperature was determined using the curve of $\frac{d\sigma}{dT}$ vs T which exhibited a shape similar to the endothermic peak on a DSC thermogram. It was suggested that the reduction in electrical conductivity within the gelatinization range of native starch suspensions may be a consequence of swelling of starch granules during gelatinization resulting in reduction in the area for the motion of the charged particles.

Sterilization and pasteurization

The passage of electric current through foods generates heat, which is used to sterilize the food. Thus it is possible to sterilize food particles as fast as liquids. De Alwis and Fryer (1992) used ohmic heating as a sterilization method. Sensoy *et al.* (1995) demonstrated the inactivation of bacteria in egg white and milk. These highly conductive fluid foods were processed using ohmic heating equipment using *Salmonella dublin* as indicator bacterium. Various square-waves, electric field pulses were applied, with different pulse durations and frequencies. The higher pulse frequency was found to have greater inactivation. Qinghua *et al.* (1995) investigated the effect of high strength (35-70 kV/cm), short duration (2-3 μs) pulsed electric fields on the viability of *Escherichia coli* suspended in simulated milk ultrafiltrate under controlled temperature of 7, 20 and 33 °C. A 10^{-9} reduction of E. coli in simulated milk ultrafiltrate was achieved by a stepwise, pulsed electric field treatment. Inactivation of *E. coli* by the pulsed electric field treatment was promoted by increasing the suspension temperature from 7 °C to 20 °C. Inactivation rate of treatment was found to be independent of initial concentration of E. coli. High-strength pulsed electric field treatment was found adequate for pasteurization of liquid foods.

Zuber (1999) has described HTST stabilization of foods by passing an electric current through the food product. The heating effect was directly dependent on the relative electrical conductivity of the individual components of the product. It was especially suited to highly viscous foods, foods containing particles, products with a tendency to browning, products susceptible to flavour loss, and au gratin products. The technique was successfully applied to HTST stabilization of 'Chilli Con Carne' and 'Pasta a la Carbonara' dishes. Eliot *et al.* (1999b) investigated the use of ohmic heating in processing of cauliflower florets. Texture and firmness of fresh cauliflower florets, and those precooked in tap water at 40 to 70°C for 0 to 60 min, were compared with a control sample cooked at 95°C for 5 min. Samples cooked at temperature greater than 60°C were less firm, but the texture of samples heated at 40-50°C was not significantly different from that of fresh florets. Effect of precooking time on floret texture and firmness was insignificant. Cauliflower florets precooked at low temperature in salt water for 30 min were subjected to ohmic heating (holding time 30 s at 135°C). After ohmic heating, florets precooked at 40 or 50°C were firmer (>300%) than florets precooked at 95°C. It is concluded that low temperature precooking of florets in salt water combined with ohmic heating is a viable alternative to HTST sterilization of cauliflower florets. Eliot *et al.* (1999b) studied ohmic heating for sterilization of cauliflower. Cauliflower florets were cut into small pieces (less than or equal to 2 cm) and mixed with a starch solution. Starch solutions were prepared from Clearam MH 10 (6% w/w) or Colflo 7 (4.8% w/w) starches, to which 0.25, 0.3 or 0.4% NaCl was added. Cauliflower was preheated at 50°C for 30 min or 60°C for 20 min in order to preserve texture during ohmic heating. Ohmic heating was performed in a 10 kW APV continuous ohmic heating pilot plant (flow rate 115-130 kg/h, approximately 130°C, minimum holding time approximately 20 s). The heated cauliflower, starch and salt mixtures prepared were tested for firmness and microbiological quality. The samples were also packaged aseptically and stored at temperature less than 25, 37 or 55°C for 7 days prior to microbial analysis using a microscope.

Other food related applications

Lima and Sastry (1999) studied the effect of ohmic heating pretreatments on hot air drying rate of yam samples and juice yield from apples, Cylinders (length, 2.35 cm; thickness 1 cm) cut from apples and yams were sandwiched between two titanium electrodes and electrically heated at two waveforms

(sine at 60 Hz and sawtooth wave at 4 Hz). Electric field strengths were varied between 20 and 40 V/cm for 4 Hz sawtooth waves and 40, 60 and 70 V/cm in case of 60 Hz sine waves. Yam and apple samples were heated to geometric centre temperature of 40 and 80°C prior to hot air drying (6.5 h) or juice extraction, respectively. The results were compared with those obtained for untreated samples. Drying rates and juice yields were higher using a 4 Hz sawtooth wave than by using a 60 Hz sine wave. For 4 Hz sawtooth wave the drying rate of yams was significantly faster at 40 V/cm than at 20 V/cm; while with 60 Hz sine wave, 40 and 60 V/cm gave faster drying rates. With apple samples, use of 4 Hz ohmic pretreatment reduced the time required to achieve enhanced juice yields compared to the 60 Hz treatment; mean times required to reach 40°C using each treatment were 33 and 109 s, respectively. Results supported the hypothesis that reduced frequency and waveform during ohmic heating increase mass transfer efficiency. These findings were claimed to be useful in designing electrical heating processes for different foods. Wang and Sastry (1995) examined the effect of thermal pretreatments on hot air drying rates of carrot, potato and yam. The vegetables were preheated by three heating methods (conventional, microwave and ohmic) to 50 or 80° and dried in a hot-air dehydrator. Results showed that drying rate increased with pretreatment temperature. Ohmic heating pretreatment increased the drying rate more than the conventional and microwave heating. For all samples, ohmic pretreatment showed stronger influences on isotherms than microwave heating, while the effect of conventional heating pretreatment was only observed in case of potato. Rao *et al.* (2004) assessed the utility of ohmic heating in improving the extraction and stability of rice bran oil in comparison to microwave heating and an unheated control. Ohmic heating increased the total percent of lipids extracted from rice bran to a maximum of 92 %, while 53% of the total lipids were extracted from control sample. Greater extraction yields were achieved upon lowering the frequency of alternating current, and it was suggested that this effect could be due to electroporation. Results also indicated that ohmic heating was effective in rice bran stabilization. Tuoxiu and Lima (2003) examined if the vacuum drying rate of sweet potato tissue was accelerated using ohmic heating. Three electrical field strengths (50, 70 and 90 V/cm) and 3 endpoint temperatures (45, 60, 80°C) were used. Immediately after ohmic heating, the samples were freeze dried. The vacuum during freeze drying was maintained at 50 x 10^{-3} bar. Moisture content data were obtained after 0, 5, 10, 15, 20, 30, 45, 60, 90 and 120 min of drying. Ohmic heating significantly increased the vacuum drying rate ($P < 0.05$). However, endpoint temperature and electrical field strength did not significantly affect the vacuum drying rate ($P > 0.05$). The required drying time was reduced by 24%. Lima *et al.* (2001) studied the diffusion of beet dye from beetroot tissue into a carrier fluid (0.1 or 0.2% NaCl) as a function of steady-state temperature (42, 58 and 72°C) during conventional and ohmic heating. Volume of beet dye diffusing into solution was enhanced over conventional heating during ohmic heating at 42 and 58°C, but not at 72°C. This could be explained by examining the differences in electrical conductivity of beet tissue during conventional and ohmic heating. At 42 and 58°C, electrical conductivities of ohmic heating beet tissues were higher than those of beet conventional heating tissue. At 72°C, however the electrical conductivities of both types and beet tissues were equal. The extent of diffusion in the case of ohmic heating was also positively correlated with voltage applied to maintain steady state temperature. The results suggest that food processes involving mass transfer can be enhanced by choosing conditions in which electrical conductivity of the food is maximized.

Ohmic Heating Systems and Equipment

The ohmic heating device employs a rectangular geometry and consists of two rectangular electrodes made of stainless steel placed on a rectangular plate of perspex sheet. Circuit diagram of electricity supply system for ohmic heating device is given in Fig 1.

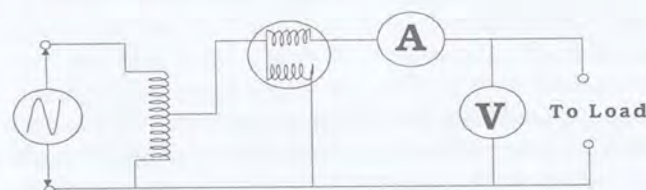

Fig 1: Circuit diagram of electric supply system for ohmic heating device

Swientek (1988) introduced an ohmic heater operating at 50 or 60 Hz. It involved no heat transfer surfaces (hence no product burn-on or fouling), which was suitable for aseptic processing of food liquids, slurries and particulates, e.g. pickles, olives, soups, milk, yoghurt, meat and fruit juices. It operated without mechanical agitation and shear-sensitive products could be processed with good particulate integrity. Qihua *et al.* (1994) designed and fabricated an ohmic heating unit for continuous thermal processing of liquid foods. The unit also had a data acquisition system for sensing the liquid temperature distribution, line voltage and current with time. A separate ohmic heating unit was also provided for batch heating tests. The data acquisition system performed well and could record temperature, voltage and current at intervals of 2 s. The performance of the ohmic heating unit was evaluated in batch and steady-state continuous-flow experiments. Tests with 0.1M aqueous sodium chloride solution showed ohmic heating to be fast and uniform. In batch heating tests, the electrical conductivity of the liquid could be determined easily as a function of temperature using instantaneous values of the voltage gradient and current density. In continuous-flow heating experiments, temperature

of liquid at the outlet was decided directly by the flow rate, electrical conductivity, other physical properties, applied voltage gradient and the dimensions of the heating unit.

Sensoy *et al.* (1995) developed a treatment chamber with increased electric resistance for reducing high current flow in ohmic processing of foods. Treatment chamber had an insulating material 2 mm thickness with small holes of 1 or 2 mm diameter. The insulating material was placed between the electrodes to reduce flow area. Highly conductive fluid foods like egg white and milk were processed using this equipment, with *Salmonella dublin* as indicator bacterium. Various levels of square wave electric field pulses were applied, by altering pulse duration and frequency. The higher pulse frequency was found to have greater inactivation. Berthou and Aussudre (2000) have described different types of equipment for ohmic heating of foods: a plate exchanger for sterilization of milk products and fruit juices; a flash heater for liquid eggs; a belt cooker for surimi; a tubular heater for fruit pieces; and a column sterilizer for prepared dishes containing discrete pieces. They have also described an equipment for sterilization of liquids by ohmic heating having one or more than one processing chamber delimited by walls. Two of the walls are electrically conductive plates, parallel to each other and spaced at a specified distance. Each chamber includes an inlet near one extremity of the conductive plates, an outlet near the other end of the conductive plates, and means for application of electrical current to the plates. The liquid to be treated circulates through the chamber.

Changes due to Ohmic Heating

Electrical conductivity

Halden *et al.* (1990) described the changes in electrical conductivity in terms of changes in structure and composition of the food material during ohmic heating of various foods including pork, pork fat, potato, mushroom (raw and blanched) and beetroot. A number of factors such as starch transition, melting of fats and cell structure changes occurring during conventional heating, affected the electrical conductivity. However, it was found that an electric field could cause changes in food properties while conventional heating did not. This was thought to be due to the electro-osmotic dehydration. Preheating of some foods (e.g. blanching of mushrooms) increased the electrical conductivity, making them suitable for ohmic processing. Palaniappan and Sastry (1991a) developed a device for determination of electrical conductivities of foods under ohmic and conventional heating conditions. Orange and tomato juices were tested in the device. Electrical conductivity of juices increased with temperature and decreased with solid content. Effect of temperature was greater for orange juice, whereas effect of solid content was greater for tomato juice. Temperature dependence of conductivity was linear, under both conventional and ohmic heating. Experiments were also conducted to determine the effect of particle size on the electrical conductivity. The electrical conductivity of the suspensions of carrot juice solids, and polystyrene spheres in sodium phosphate solution showed an increase with decreasing particle size.

Palaniappan and Sastry (1991b) used a static ohmic device for determining electrical conductivities of three vegetable samples (potato, carrot, yam) and two meat samples (chicken, lean beef) using constant voltage power supply. Conductivities of vegetable samples were increased by soaking them in salt solutions, while soaking in water resulted in reduced conductivity due to leaching of electrolytes. Under ohmic heating at a voltage gradient of 60 V/cm electrical conductivities increased linearly with temperature. As field strength was decreased the electrical conductivity-temperature (ó-T) curve gradually became nonlinear. Under conventional heating solutions, a sharp transition was observed at about 70°C, coinciding with significant softening of sample tissue. Wang and Sastry (1993) investigated salt diffusion as a pretreatment for ohmic heating. The potato tissue was soaked in aqueous NaCl solutions of various concentrations. The result indicated that the electrical conductivity was sensitive to salt concentrations at levels greater than 0.01 g/cm-3 below 0.01 g/cm3, electrical conductivity was nearly constant. Vacuum infusion was only effective in increasing salt concentration in outer layers, indicating that its usefulness was primarily limited to small particles. The ó-T relations were linear for low concentrations of NaCl. At higher salt concentrations, the ó-T curve became nonlinear, possibly as a result of salt equilibration during the heating process. Wang and Sastry (1995) studied electrical conductivity changes of raw vegetable samples (potato, carrot and yam) in cyclic ohmic heating or precooked by either conventional heating prior to ohmic heating. Samples precooked by either conventional or ohmic methods had higher heating rates than raw materials.

Castro *et al.* (2003) investigated effects of field strength, soluble solid content and particle size on electrical conductivity of strawberries and strawberry products (strawberry pulps and jelly). Electrical conductivity increased with temperature for all the products and conditions tested. Electrical conductivity varied greatly between strawberry based products. Castro *et al.* (2004) studied effect of multiple thermal process (conventional + ohmic heating or ohmic + ohmic heating) on electrical conductivity of strawberry pulp. Electrical conductivity decreased after the 1st heat treatment when pulps were subjected to multiple thermal treatments. Filiz and Coskan (2005) studied the temperature dependent electrical conductivities of fruit purees during ohmic heating. In this study, the apricot and peach purees were heated on a laboratory scale static ohmic heater by applying voltage gradients the range of 20–70 V/cm. The linear temperature dependent electrical conductivity relations were obtained. They found that bubbling was observed above 60^{0}C especially at high voltage gradients. They also mentioned that the ohmic heating system performance coefficients were in the range of 0.49–1.00.

Microbial activity

Sensoy *et al.* (1995) demonstrated the inactivation of bacteria in egg white and milk. These highly conductive fluid foods were processed using ohmic heating equipment using *Salmonella Dublin* as indicator bacterium. Various square waves, electric field pulses were applied, with different pulse durations and frequencies. The higher pulse frequency was found to have greater inactivation. Qinghua *et al.* (1995) investigated the effect of high strength (35-70 KV/cm), short duration (2-3 μs) pulsed electric fields on the viability of *Escherichia coli* suspended in simulated milk ultrafiltrate under controlled temperature of 7, 20 and 33°C. A 10-9 reduction of *E. coli* in simulated milk ultrafiltrate was achieved by a stepwise, pulsed electric field treatment. Inactivation of *E. coli* by the pulsed electric field treatment was promoted by increasing the suspension temperature from 7°C to 20°C. Inactivation rate of treatment was found to be independent of initial concentration of *E. coli*. High strength pulsed electric field treatment was found adequate for pasteurization of liquid foods. Zuber (1999) has described High Temperature Short Time (HTST) stabilization of foods by passing an electric current through the food product. The heating effect was directly dependent on the relative electrical conductivity of the individual components of the product. It was especially suited to high viscous foods, foods containing particles, products with a tendency to browning, products susceptible to flavour loss.

Ascorbic acid degradation

Castro *et al.* (2004) studied the ascorbic acid degradation kinetics and ohmic heating of strawberry products (i.e. strawberry pulps, topping, filling and strawberry-apple sauce). They mentioned that electrical conductivity increased with temperature for all the products and conditions tested following linear relationship. The ascorbic acid degradation kinetics in strawberry pulps for the temperature range of 60 to 97°C were unaffected by lower values of the electric field strength (<20 $V.cm^{-1}$). They also mentioned that ascorbic acid degradation followed first order kinetics for both conventional and ohmic heating treatments and the kinetic constants were in the range of the values reported in the literature for other food systems. Alhussein *et al.* (2005) studied the degradation kinetics of ascorbic acid. It was determined in pH 5.7 buffer solution using an isothermal batch ohmic heater with stainless steel electrodes. Variables included in this study were temperature (40, 60 and 80 -C); power (0, 100,150 and 300 W); and electrical conductivity (varied using 0.25%, 0.5% and 1.0% NaCl). Ascorbic acid concentration was detected by using a HPLC technique. The results indicated that ascorbic acid degradation can be described successfully by a first order model during both conventional and ohmic heating. They also found that at the same power level, degradation rate constants tended to increase with increasing field strength (applied voltage). Buffer capacity loss at pH 5.7 was significant but not as pronounced as at pH 3.5.The degradation rate decreased with increasing electrical conductivity (which is linearly related to temperature and salt content). On the basis of above review, it can be concluded that ohmic heating can be utilized for thermal processing of almost any type of food products. In general, ohmic heating is an important development of aseptic processing. Most important factor that controls ohmic heating is the electrical conductivity of the medium. Also during conventional heating of aonla, considerable amount of quality losses occur which may be prevented by application of non thermal technology. Based on the above review, study has been planned to investigate approach of ohmic heating towards maintaining quality parameter of aonla during preparation of aonla pulp.

Concluding Remarks

Ohmic heating leads to effective blanching due to rapid heating rates and enhancement of mass transfer, even at relatively low temperature. Also electrical conductivity of product increases due to percentage of salt level and blanching which reduces quality loss during heating. It has also been observed through reviews that there is considerably loss of vitamins during the blanching or any other heat treatment. It must be necessary to develop a non-thermal heat treatment so as to produce a nutritive product. Ohmic heating could be the suitable approach for maintaining the quality of food as it offers several other advantages over the conventional heating methods as it is a direct method and provides rapid cooking, minimum clumping, high-temperature sterilization and almost 100% energy efficient.

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Biobased Packaging Materials for the Food Industry

RACHANA SHALINI[1,*] and ANUPAMA SINGH[2]

[1]Agri Business Division, National Productivity Council, SCO 40 Ist floor, Sec 7C, Chandigarh 160019, India
[2]Department of Post Harvest Process and Food Engineering, G.B. Pant University of Agriculture and Technology, Pantnagar 263145, India

Biobased food packaging materials are derived from renewable sources and are potentially biodegradable that is composting (which is a technique for waste management). Biobased packaging materials include both edible coatings and edible films along with primary and secondary packaging materials. At the turn of the last century most non-fuel industrial products; dyes, inks, paint, medicines, chemicals, clothing, synthetic fibers and plastics were made from biobased resources. During the last years, the leading world research teams have been working on developing new biodegradable and edible packaging based on renewable biological sources, the so called "regulated life cycle materials". By the 1970s petroleum derived materials had, to a large extent, replaced those materials derived from natural resources. Recent developments are raising the prospects that naturally derived resources again will be a major contributor to the production of industrial products. Biobased /green polymers in food packaging are the wave of the future. The Scientific challenge is to find such applications and thus to create the demand for large scale production of biopolymers/ biomaterials that would help in attaining the sustainable development of green materials in contrast to petroleum.

Keywords: Biobased polymers, Biomass, Food packaging

Introduction

At the turn of the 20th century, most non-fuel industrial products like inks, dyes, paints, medicines, chemicals, clothing, synthetic fibres and also plastics were made from biologically derived resources. However, 70 years later petroleum derived chemicals replaced these to a major extent. But now in the 21st century, the recent developments are raising the prospects that naturally derived resources again will be a major contributor to the production of industrial products. Currently, scientists and engineers successfully perform developments and technologies that will bring down costs and optimize the performance of Biobased products. At the same time, environmental concerns are intensifying the interest in agricultural and forestry resources as alternative feedstocks (Weber *et al.* 2002).

Food is dynamic system with limited shelf –life and specific packaging requirements. While the issues of food quality and safety are first and foremost in the mind of food producers and retailers, a range of other issues surrounding the development of any food package must be addressed before a particular packaging system becomes a reality (Otles and Otles, 2004). The necessity of export orientation of agriculture and increasing the competitiveness of food industry requires utilization of contemporary packing. The problems of packing are connected with the irrational utilization of material resources and energy environmental contamination and low competitiveness of product because of their inefficient packing (Aladjadjiyan *et al.* 2002).

* Corresponding author, Email:rachanã.shalini@npcindia.org

Recently, the leading world scientific and research teams work on the creation of new biodegradable and /or edible packing materials, on the basis of reproducing renewable biologicl resources i.e. "materials having a regulating life cycle" (Bastioli, 2001; Peterson *et al.*, 1999; Haugaard *et al.*, 2001). Designing and manufacturing of packaging materials is a multi–step process and involves careful and numerous considerations to successfully engineer the final package with all the required properties. The properties to be considered in relation to food distribution are manifold and may include gas and water vapour permeability, mechanical properties, sealing capability, thermoforming properties, resistance (towards water, grease, acid, UV light, etc.), machinability (on the packaging line), transparency, anti fogging capacity, printability, availability and, of course, costs. Moreover, a consideration of the "cradle to grave" cycle of the packaging material is also required; hence, the process of disposal of the package at the end of its useful life must also be taken into consideration (Coombs and Hall, 2000). This paper wishes to review the application of biobased packaging materials for the food industry.

Definition of Food Biobased Materials

Definition of biobased food packaging materials based on their origin and use, leading to the following definition. "Biobased food packaging materials are materials derived from renewable sources. These materials can be used for food applications". In addition, packaging materials recognized as biodegradable according to the standards outlined by the EU Standardization Committee. This amendment was included

not to exclude materials which currently, of practical and economical reasons, are based on non-renewable resources, but at a later stage these materials may be produced based on renewable resources (Chandra and Rustgi, 1998; Weber *et al.*, 2002).

Origin and Description of Biobased Polymers

Biobased polymers may be divided into three main categories based on their origin and production (Petersen *et al.* 1999)

Category 1: Polymers directly extracted/removed from biomass. Examples are polysaccharides such as starch and cellulose and proteins like casein and gluten.

Category 2: Polymers produced by classical chemical synthesis using renewable biobased monomers. A good example is polylactic acid, a biopolyester polymerized from lactic acid monomers. The monomers themselves may be produced via fermentation of carbohydrate feedstock.

Category 3: Polymers produced by microorganisms or genetically modified bacteria. To date, this group of biobased polymers consists mainly of the polyhydroxyalkonoates, but developments with bacterial cellulose are in progress. The three categories are presented in schematic form in Fig 1.

In general, compared to conventional plastics derived from mineral oil, biobased polymers have more diverse chemistry and architecture of the side chains giving the material scientist unique possibilities to tailor the properties of the final package.

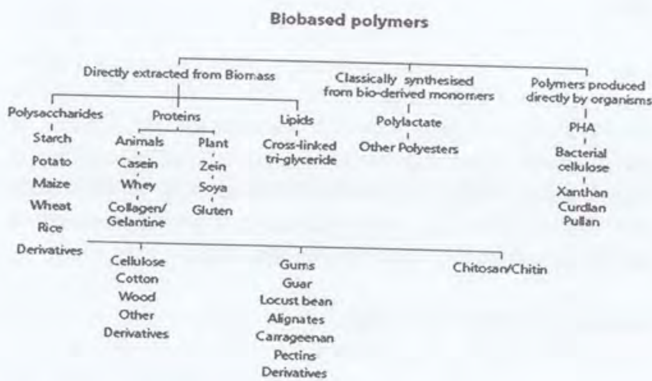

Fig1. Schematic presentation of biobased polymers based on their origin and method of production (Chandra and Rustgi, 1998)

Category 1: Polymeric materials

The natural Category 1 polymers, "Polymers directly extracted from biomass", most commonly available, are extracted from marine and agricultural products. Examples are polysaccharides such as cellulose, starch, and chitin and proteins such as casein, whey, collagen and soy. All these polymers are, by nature, hydrophilic and somewhat crystalline, factors causing processing and performance problems, especially in relation to packaging of moist products. On the other hand, these polymers make materials with excellent gas barriers (Marron *et al.* 2000). The principal polysaccharides of interest for material production have been cellulose, starch, gums, and chitosan. Likely, the more complex polysaccharides produced by fungi and bacteria (Category 3 biobased polymers) such as xanthan, curdlan, pullan and hyaluronic acid, will receive more interest in the future (Otles and Otles, 2004).

Starch, the storage polysaccharide of cereals, legumes and tubers, is a renewable and widely available raw material suitable for a variety of industrial uses. As a packaging material, starch alone does not form films with adequate mechanical properties (high percentage elongation, tensile and flexural strength) unless it is first treated by either plastization, blending with other materials, genetic or chemical modification or combinations of the above approaches (Otles and Otles, 2004). Corn is the primary source of starch, although considerable amounts of starch are produced from potato, wheat and rice starch in Europe, the Orient and the United States. Starch is economically competitive with petroleum and has been used in several methods for preparing compostable plastics. However, a challenge to the development of starch materials is the brittle nature of blends with high concentrations of starch. Overcoming the brittleness of starch while achieving full biodegradability in blends can be accomplished by the addition of biodegradable plasticizers. Common plasticizers for hydrophilic polymers, such as starch, are glycerol and other low molecular weight – polyhydroxy – compounds, polyethers and urea. Plasticizers lower the water activity, thereby limiting microbial growth. When starch is treated in an extruder by application of both thermal and mechanical energy, it is converted to a thermoplastic material. Cellulose is the most abundantly occurring natural polymer on earth and is an almost linear polymer of anhydroglucose. Because of its regular structure and array of hydroxyl groups, it tends to form strongly hydrogen bonded crystalline microfibrils and fibres and is most familiar in the form of paper or cardboard in the packaging context. Waxed or polyethylene coated paper is used in some areas of primary food packaging; however the bulk of paper is used for secondary packaging (Marron *et al.* 2000).

Cellulose is a cheap raw material, but difficult to use because of its hydrophilic nature, insolubility and crystalline structure. To make cellulose or cellophane film, cellulose is dissolved in an aggressive, toxic mixture of sodium hydroxide and carbon disulphide ("Xanthation") and then recast into sulphuric acid. The cellophane produced is very hydrophilic and, therefore, moisture sensitive, but it has good mechanical properties. It

is, however, not thermoplastic owing to the fact that the theoretical melt temperature is above the degradation temperature, and therefore cannot be heat-sealed (Petersen *et al.* 1999). Chitin is a naturally occurring macromolecule present in the exoskeleton of invertebrates and represents the second most abundant polysaccharide resource after cellulose. In general, chitosan has numerous uses; flocculant, clarifier, thickener, gasselective membrane, plant disease resistance promoter, wound healing promoting agent and antimicrobial agent. Chitosan also readily forms films and, in general, produces materials with very high gas barrier, and it has been widely used for the production of edible coating. Furthermore, chitosan may very likely be used as coatings for other biobased polymers lacking gas barrier properties. However, as with other polysaccharide based polymers, care must be taken for moist conditions. (Kittur *et al.*, 1998).

Proteins can be divided into proteins from plant origin (e.g. gluten, soy, pea and potato) and proteins from animal origin (e.g. casein, whey, collagen, keratin). A protein is considered to be a random copolymer of amino acids and the side chains are highly suitable for chemical modification which is helpful to the material engineer when tailoring the required properties of the packaging material. Due to their excellent gas barrier properties, materials based on proteins are highly suitable for packaging purposes. However, like starch plastics mechanical and gas properties are influenced by the relative humidity due to their hydrophilic nature. One of the ways to modify protein properties is by chemical modification and, as seen in Fig 2, proteins contain a wide variety of chemical moieties which may help tailoring protein properties towards specific applications (Graaf and Kolster,1998).

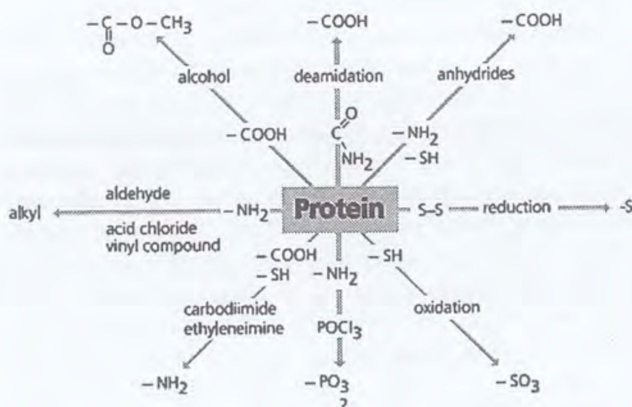

Fig 2: The numerous and diverse side chains of proteins (Graaf and Kolster, 1998)

Casein is a milk-derived protein. It is easily processable due to its random coil structure. Upon processing with suitable plasticizers at temperatures of 80–100 °C, materials can be made with mechanical performance varying from stiff and brittle to flexible and tough performance. Casein melts are highly stretchable making them suitable for film blowing. In general, casein films have an opaque appearance. The main drawback of casein is its relatively high price. Casein was used as a thermoset plastic for buttons in the 1940's and 50's. It is still used today for bottle labelling because of its excellent adhesive properties (Otles and Otles, 2004).

Gluten is the main storage protein in wheat and corn. Wheat is an important cereal crop because of its ability to form viscoelastic dough. Mechanical treatment of gluten leads to disulfide bridge formation formed by the amino acid cysteine which is relative abundant in gluten. Processing temperatures are, depending on the plasticizer contents, in the range of 70–100 °C. Mechanical properties may vary in the same range as those for caseins. Gluten plastics exhibit high gloss (polypropylene like) and show good resistance to water under certain conditions. They do not dissolve in water, but they do absorb water during immersion. Due to its abundance and low price, research on the use of gluten in edible films, adhesives, or for thermoplastic applications is currently being carried out (Otles and Otles, 2004).

Soy proteins are commercially available as soy flour, soy concentrate and soy isolate, all differing in protein content. Soy protein consists of two major protein fractions referred to as the 7S (conglycinin, 35%) and 11S (glycinin, 52%) fraction. Both 7S and 11S contain cysteine residues leading to disulphide bridge formation and processing is, therefore, similar to gluten with similar mechanical properties. The most successful applications of soy proteins were the use in adhesives, inks and paper coatings (Fossen, and Mulder 1998).

Keratin is by far the cheapest protein. It can be extracted from waste streams such as hair, nails and feathers. Due to its structure and a high content of cysteine groups, keratin is also the most difficult protein to process. After processing, a fully biodegradable, water-insoluble-plastic is obtained. However, mechanical properties are still poor compared to the proteins mentioned above (Shukla1992).

Category 2: Polymeric materials

For category 2 polymers, "*produced from classical chemical synthesis from biobased monomers*" using classical chemical synthesis for the production of polymers gives a wide spectrum of possible "bio-polyesters". To date, polylactic acid is the Category 2 polymer with the highest potential for a commercial major scale production of renewable packaging materials (Weber *et al.* 2002). The PLA materials have a good water vapour barrier and have also relatively low gas transmittance. The feedstock can be agricultural resources, example corn or wheat, or alternatively agricultural waste products, such as whey or green juice, may be used (Garde *et*

al. 2000, Sodergaard, 2000). However, a wide range of other biopolyesters can be made. In theory, all the conventional packaging materials derived from mineral oil today can in the future be produced from renewable monomers gained by e.g. fermentation. Today, this approach is not economically feasible due to the cost of the production of the monomers (Garde *et al.*2000).

Lactic acid, the monomer of polylactic acid (PLA), may easily be produced by fermentation of carbohydrate feedstock. The carbohydrate feedstock may be agricultural products such as maize, wheat or alternatively may consist of waste products from agriculture or the food industry, such as molasses, whey, green juice, etc. Recent results point out that a cost-effective production of PLA can be based on the use of green juice, a waste product from the production of animal feeds. PLA is a polyester with a high potential for packaging applications. The properties of the PLA material are highly related to the ratio between the two mesoforms (L or D) of the lactic acid monomer. Using 100% L-PLA results in a material with a very high melting point and high crystallinity. If a mixture of D- and LPLA is used instead of just the L-isomer, an amorphous polymer is obtained with a Tg of 60°C, which will be too low for some packaging purposes (Sinclair, 1996).

Category 3: Polymeric materials

In this category the polymer *"are produced directly by natural or genetically modified organisms"* (Otles and Otles, 2004). Poly (hydroxyalkanoates) (PHAs), of which poly (hydroxybutyrate) (PHB) is the most common, are accumulated by a large number of bacteria as energy and carbon reserves. Due to their biodegradability and biocompatibility these biopolyesters may easily find industrial applications. A general overview of the physical and material properties of PHAs, along with accomplished applications and new developments in this field, can be found in a recent review. The properties of PHAs are dependent on their monomer composition, and it is, therefore, of great interest that recent research has revealed that, in addition to PHB, a large variety of PHAs can be synthesized by microbial fermentation. The monomer composition of PHAs depends on the nature of the carbon source and microorganisms used. PHB is a typical highly crystalline thermoplastic whereas the medium chain lengths PHAs are elastomers with low melting points and a relatively lower degree of crystallinity. A very interesting property of PHAs with respect to food packaging applications is their low water vapour permeability which is close to that of LDPE. Recent application developments based on medium chain length PHAs range from high solid alkyd-like paints to pressure sensitive adhesives, biodegradable cheese coatings and biodegradable rubbers. Technically, the prospects for PHAs are very promising. When the price of these materials can be further reduced, application of biopolyesters will also become economically attractive (Westhuis *et al.* 2000, Walle *et al.*, 2001).

To date, bacterial cellulose is rather unexploited, but it represents a polymeric material with major potential. Bacterial strains of *Acetobacter xylinum* and *A. pasteurianus* are able to produce an almost pure form of cellulose (homo-beta-1,4-glucan) (Iguchi *et al.* 2000). Its chemical and physical structure is identical to the cellulose formed in plants. Plant cellulose, however, has to undergo a harsh chemical treatment to remove lignin, hemicellulose and pectins. This treatment severely impairs the material characteristics of plant cellulose: the degree of polymerization decreases almost ten-fold and the form of crystallization changes. Bacterial cellulose is processed under ambient conditions and the degree of polymerization is 15000, 15 times longer than cellulose from wood pulp. Bacterial cellulose is highly crystalline. In bacterial cellulose, 70% is in the form of cellulose I and the rest is amorphous. This composition results in outstanding material properties: a modulus as high as 15–30 GPa was determined across the plane of the film. Production costs of bacterial cellulose are high due to the low efficiency of the bacterial process; approximately 10% of the glucose used in the process are incorporated in the cellulose. The high price of bacterial cellulose of approximately 20 Euro/kg hampers its applicability in low-added-value bulk products. Several high-added-value specialty applications have been developed. The material has been used as an artificial skin, as a food grade non-digestible fiber, as an acoustic membrane, and as a separation membrane (Brown, 1996).

Concluding Remarks

Cellulose- based materials are being widely used for food-packaging, but apart from these materials, very few other biologically based packaging materials have been commercially introduced. However, developments are taking place at a rapid and increasing speed and commercial trials with starch based packagings for pasta (Italy) and PLA-based pots for yoghurts (Germany) have already been performed. So, biobased polymers have increasing importance. The main reason is they are produced from renewable resources and also they can be recycled. Biobased polymers have different categories according to different production methods and different applications in food industry. The researches about applications of biobased polymers show that not only they have suitable properties for applications in food industry but also they have a low cost. If we compare them with petroleum products; having recycle option, products of renewable resources, having low cost and having suitable properties for packaging applications are going to make them the most preferable material in the near future.

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Pesticide Residue Monitoring and Quarantine System of Some Selected Asian Countries with Reference to Nepal

PRAMOD KOIRALA* and ANANDA SHOVA TAMRAKAR

Tribhuwan University, Kathmandu Nepal

Pesticides have contributed to dramatic increase in crop yields and in the quantity and variety of diet. Pesticides owing to their pest-destroying properties are required in global food production but they remain present as residues in food from both plant and animal origin. Pesticide despite their known toxicity, are widely used in developing countries. Pesticide use in the production and distribution of food produce has become an important public policy issue. Nepal's average consumption of pesticides is far lower than many other developed countries, but the problem of pesticide remains high in Nepal. Pesticide residues in several crops have also affected the export of agriculture commodities in the last few years. In this context, pesticide safety, regulation on pesticide use, judicious application is some key strategies for minimizing pesticide problems. This paper reviews the lessons what Nepal can learn from some of the selected Asian countries like China, Malaysia, Cambodia, the Philippines and Indonesia in the control of pesticides in food products.

Key words: Pesticide, Public policy, Judicious application, Pesticide residue monitoring, Nepal

Introduction

Pesticides have profoundly improved the human livelihood. Their dramatic effort in preventing, crop loss and controlling vectors of diseases have led to their acceptance and expanded use throughout the world. However, the powerful chemicals for killing pests have raised concern that they are agents of human diseases and environmental pollution. It has been observed that their long term, low dose exposure is increasingly linked to human health effects such as immune-suppression, hormone disruption, diminished intelligence, reproductive abnormalities and cancer (Gupta, 2004; Wiles et al., 1998).

Pesticides classified as being extremely or highly hazardous by FAO and WHO, including banned by other countries, continued to be used in developing countries. According to WHO, developing countries use about 30 percent of the pesticides in the worlds, and this use is increasing, this intrinsically dangerous technology is being promoted in a setting without technical and human resources to control it properly (Keshavchandran et al., 2009).

Agriculture work is one of the most prevalent types of employment in the world. Nearly 50 percent of the world labor is employed in agriculture. Agriculture includes farming, ranching, fishing and forestry together they carry significant risk for development of pesticide risk (Das et al., 2001). Over the last 50 years, agriculture deeply changed with a massive utilization of pesticides to enhance crop protection. For many reasons, the severity of pesticides hazards is much pronounced in third world countries (Maroni et al.,2006).

Use of pesticides has become inevitable to sustain and improve current level of crop production by protecting crops from pests. Subtropical countries observe varying temperature and humidity profile throughout the year, which brings a vast array of pests to be tackled. Some pests are found to attack multiple targets (various crops) and have acquired resistance from prolong use of common pesticides. In these countries, literacy is low, particularly rural mass that triggers the improper and non-judicious use of pesticides by farmers. Additionally, the use of incorrect (high) dose of pesticides by farmers leads to the contamination of their products, which in turn causes a greater risk for consumers or traders (Bhanti et al., 2004). The objective of this paper is to review the monitoring and quarantine system of some selected Asian countries like China, Malaysia, Cambodia, the Philippines and Indonesia with reference to Nepal for the control of pesticide residues level in various foods and food products.

Use of Pesticides in Agriculture

The economy of Nepal is largely based on agriculture. It contributes about 38% to the national economy, provides employment for over 65% of the labor force and is main source of income in rural areas, which accounts for 85% of total population (APBSD 2008). The well being of the economy depends largely on the production, processing and distribution of major products such as rice, wheat, maize, vegetables, milk and meat. In the last decade, agriculture grew at an annual average rate of 3.0 per cent and exhibited fluctuating trend mainly on account of weather conditions, pest attacks on crops, shortage of inputs and little attention given to its sub-sectors other than crop farming.

*Corresponding author; Email: pramodkoirala2002@yahoo.com

Agriculture development continues to remain the most important objectives of Nepal planning and policy. In the process of development of agriculture, pesticides have become important tools as plant protection or boosting food production. Although Nepal average consumption of pesticides is far lower than many other developed economies, the problem of pesticide remains high in Nepal. Pesticide residues in several crops have also affected the export of agriculture commodities in the last few years. Until the 1950s, the people of Nepal remained unaware of modern chemical pesticides and were dependent upon traditional organic techniques for killing pests. Chemical pesticides were first introduced to Nepal in 1955 when Paris Green, gamaxane, and nicotine sulphates were imported from USA for malaria control. DDT made its first impact in 1956. This was soon followed by a variety of other organochlorines (in 1950s), organophosphates (in 1960s), carbamates (in 1970s), and synthetic pyrethroids (in 1980s) (Koirala and Tamrakar, 2008).

In Nepal, national pesticides surveillance data (1995-2005) revealed that 12.1% of the food samples were detected with the residues of pesticides which included malathion (3.9%), BHC (3.1%), Methyl parathion (2.8%), DDT (1.8%) and parathion (0.3%). Commodity-wise detection of pesticide residues showed the highest level of residues in root vegetables (11.9%) followed by leaf vegetables (10.9%) (Koirala et al., 2009a)

In the past, Nepal government lack regulations governing import, distribution disposal & use of chemical pesticides. But, the pesticide Act 1991 and the pesticide Regulations, 1993 came into effect as of 16 July 1994 (Pesticide act, 1991 and pesticide regulation, 1993). However, the effectiveness of these regulations is poor. Still persistent organochlorines are imported, and used within the country. Organochlorines insecticide such as BHC, aldrin, dieldrin, endrin, chlordane, lindane and heptachlor are already banned for normal use in Nepal's market. But a persistent Organochlorine BHC, is very popular and widely used by farmers. (Koirala and Tamrakar, 2008). Food Act 1967 and Food Regulation 1970 has established MRLs for pesticides in food products, but limited to cereals, pulses and their products, processed water and infant food (Government of Nepal 2000). Department of Food Technology & Quality Control constantly monitors pesticide residues level in food products where MRLs has been laid down. (Koirala et al., 2008a; Koirala et al., 2008b)

According to general consensus, the global climate is changing, which may also affect agricultural and livestock production. Global warming will increase pest population including weeds, invasive species, insects and insect vectors and plant diseases, which lead to increase pesticides on crop production (Palikhe, 2007). This will create a promising threat in food safety and human health. The potential impact of climate change on food safety is a widely debated and investigated issue. (Bailey, 2008; Brussel, 2006; Camell and Knight, 1991).

Nepal has export potential for agriculture and processed products in the international market. Nepal's specific geographic, agro-climatic, environment friendly agricultural system, less use of fertilizer and pesticides are main reasons for the export possibilities. This is not possible unless and until the country doesn't have safe food production. As Nepal has already become a member of WTO, this opportunity can be utilized (Koirala et al., 2007).

The presence of pesticide residues in food commodities has always a matter of serious concern. The level of pesticide residues in foodstuffs are generally legislated so as to minimize the exposure of the consumer to harmful or unnecessary intake of pesticides (Zorka and Maja, 2009). Pesticides safety in food products is one of the prime concerns of every country. In recent years relevant progress has been made in food policy and regulation for pesticide control. Food ingredients and contaminants (such as pesticides) are extensively scrutinized before their approval. Health protection of consumers is the absolute priority of all countries (Abhinash and Singh, 2009; Mansour 2004). Some of the selected informations on pesticide residue monitoring and quarantine system of some of the selected Asian countries with reference to Nepal are given in Table 1.

Legislation regarding monitoring and quarantine system of Pesticides

Some countries like China, Indonesia and the Philippines introduced new food safety law in 2009. The penalty provision has been increased and the new law is more strict for export and import of food products. In these countries, different law and orders are in place to control different food products. In Philippines, like Nepal, different administrative orders for the control of pre-packed foods, bottled drinking water, breast milk substitutes, salt iodination are implemented. So, in order to control safety of food products, different orders are implemented. In china, the legal measures are very strict. Company without domestic market experiences are not allowed to export food products to other countries.

Indonesia depend on Codex standard and don't carry out research for fixation of Maximum Residue Level (MRLs), however, some countries have their MRLs for example China and Malaysia. However, these countries depend on codex standard on pesticide for international food trade.

Pesticide residue monitoring scheme is necessary for exporting country for the export of their agriculture produce. In some countries, the country doesn't require risk analysis certificate during entry and exit of food products of smaller quantities. Developed countries have more focus on pesticides residues in food. European community is upcoming with more strict measures on pesticides than US and other developed countries.

Table 1: Selected information* on pesticide residue monitoring and quarantine system of some selected Asian Countries with Reference to Nepal

Selected parameters	*China*	*Malaysia*	*Cambodia*	*Philippines*	*Indonesia*	*Nepal*
Major food export	Garlic, apples and other fruits and vegetables	Vegetables	Rice, soybean, maize, cassava	Rice, coconut, corn, tropical fruits	Mango, mango stone, banana, papaya	Tea, coffee, cardamom, vegetables
Major food import	Grains	Rice, pulses, fruits and vegetables	Rice, tapioca	Fresh fruits, milk, luncheon meat	Apples, pears, grape, orange	Fruits and vegetables
Law and regulation	Food safety Law 2009 & Ministerial regulation	Food act 1983, Food regulation 1985	Food safety law 1984	Food and drug act 2009	Food safety law 2009	Food law 1967
Standard	National standard, Professional standard & Local standard	National and Codex standard	Codex	National and Codex standard	National and Codex standard	National and Codex standard
Monitoring residues in primary produces	MOA	MOA	MOA	MOA	MOA	None
Monitoring residues processed products	MOH	MOH	MOI	PM's Office BFAD	FDA	DFTQC
Import and Export	AQSIQ	MOH	MOC and MOI	MOC	MOC	MOA
Pesticide residue monitoring program	Developed	Developed	Developed	Developed	Developed	Under process
Laboratory analysis of pesticide residues	MOA	MOA	MOA	MOA	MOA	MOA
Monitoring for entry and exit for pesticides	AQSIQ	MOA and MOH	MOA	PM's Office BFAD	FDA	MOA
Past experience of trade dispute due to pesticides residues in export	Malaysia	Singapore	No	No	No	No
Past experience of trade dispute due to pesticides residues in import	No	Chilies from India and Thailand		No	No	No
Use of test kit to monitor pesticide residues in food	No	No	Yes	No	No	No

*Personal communication with Food Quarantine and Food Safety Officials of China, Malaysia, Cambodia, Philippines and Indonesia at 3-20 September, 2009 in jiangsu, China; MOA : Ministry of Agriculture, MOH : Ministry of Health, MOI : Ministry of Industry, MOC : Ministry of Commerce, PM: Prime Minister, BFAD : Bureau of Food & Drug, FDA : Food & Drug Administration, DFTQC : Department of Food Technology & Quality Control, AQSIQ : General Administration of Quality Supervision, Inspection and Quarantine.

Technical Capacities and Test Procedure

Different government agencies are working with the objective to control raw, processed and import and exit of food products. Therefore, clear cut roles of different regulatory agencies have been identified at different stages of food chain. In order to control pesticides, regulatory agencies are generally working at three different levels. Usually, the ministry of agriculture is responsible for monitoring pesticide residues for fresh produces. Either ministry of health or ministry of industry monitoring is responsible for monitoring of pesticides in processed food. And in monitoring of pesticides in export and import of food products, ministry of finance or health is responsible. For example, in Malaysia, monitoring of pesticides in fresh agriculture produce-fruits and vegetables, ministry of agriculture is involved whereas in the case of processed food ministry of industry is involved and with monitoring of export and import of food products ministry of health is responsible.

In some countries, regulatory institutes have very good strength of human resources with many technical staff working with this sector. In China, there are 10, 000 staff working for 110 food laboratories designed with the facility of pesticides residue analysis. Some countries have introduced qualitative test kit to control import of fresh fruits and vegetables. Cambodia uses test kit to test pesticides residues at entry point.

Pesticide Residues and Food Trade

The countries reported presence of pesticides residues in domestic food as well as imported food products. Malaysia reported that chilies imported from India has the presence of ethion above MRLs fixed by Malaysia. Similarly, Malaysia is facing problem with the presence of pesticides in food imported from Thailand and China. Therefore, in Malaysia, food products liable for pesticide use imported from Thailand needs 10 percent mandatory sampling and testing for quarantine. So is the case with Cambodia. There is a problem of pesticides in imported food products from Thailand. Different agricultural produce imported from Thailand have the pesticides above MRL fixed by Codex.

Lessons Learnt by Nepal

Legislation

Pesticide residues in food are global problems. However, the common problem for these Asian countries is cross boarder import of even banned pesticides from their neighboring countries. This has affected food trade as well as the health of the consumers. Government has policy aimed at limiting pesticide exposure and usage, but doing so without damaging the yields of food production. The primary need, currently, like other countries is creation and implementation of sound national polices to effectively articulate appropriate guidelines for managing farm pest control activities. In these countries, law and regulations are in place and updated periodically.

Government of Nepal may update pesticide act and regulation and extend it to all types of pesticides including biopesticides, veterinary pesticides and pesticides used in household purposes. The labeling of pesticide containers may be in local language with full information. Manufacturer may also need to register their products in the government. The Government may deregister class 1b pesticide. The law may cover the advertise provision for pesticides. Similarly, storage of pesticides may meet FAO standard. Pesticide registration list may be published through mass media and publicly available. Government of Nepal may stop import of unregistered pesticides. Similarly, the database of import may be established. In order to do it, the capacity of customs should be strengthened. There may be bilateral cooperation to return illegal pesticides between countries. The government may also review taxation policy to sustain control of pesticides and encourage alternatives such as biopesticides

Effective quarantine system

Nepal has already the system of quarantine for plants, animal and food. Different regulatory agencies under the ministry of agriculture are working with different custom points mandated to check pesticide safety of the food products. However, in practice no check of pesticides is made so far at custom points due to technical problems associated with inadequate laboratory facilities. Food quarantine is necessary for domestic as well as entry and exit of food products.

Organizations

In these Asian countries, for control pesticides, regulatory agencies are working at three different levels. Usually the ministry of agriculture is responsible for monitoring fresh agriculture produce. Either ministry of health or ministry of industry for processed food products and for monitoring of pesticides in export and import ministry of finance or commerce is involved. Government of Nepal may strengthen pesticide inspectors as a separate unit form plant protection and the enforcement mechanism to address the substandard pesticides, labels, advertising, controlling improper storage, checking retailers, collecting samples for testing and import inspection.

Laboratory analysis

Laboratory strength is enough to monitor domestic market and import and export of food products. However, in order to minimize cost and effort most of the countries have introduced

test kit method at entry point as screening test for pesticides. Systematic monitoring is necessary to monitor quality of pesticides. Pesticide quality control laboratory may be established and strengthened. Similarly, the government may develop the capacity to build the pesticide residue monitoring. The sustainability of these laboratories may be financed through levy on pesticides.

Standards

Some of the Asian countries developed their own MRLs for pesticides, however, some depend on Codex standard due to their limited capability on pesticide research. Nepal can follow CODEX standard for MRL in order to minimize cost and effort.

Awareness

Government may raise awareness on different aspects of pesticides with the partnership with different development partners such as NGOs/INGOs. The awareness message may highlight on management of empty containers at household level, the malpractices related to pesticide use. Similarly, pesticide residues may also be highlighted in the awareness campaign. The alternatives to chemical pesticides may also be promoted and the use of Personal protective equipments (PPE) at users level may be promoted through mass education.

In Nepal, the pesticide retailers are the major source of information for pesticide users. Therefore, regularly updating them with training is necessary. License should be made necessary for all sellers. The Government may inspect regularly the license with strict measures. The government may construct and manage the storage for safe disposal of pesticides according to FAO guideline. The government may raise awareness with users "Triple rinsing". The government may establish a container management scheme. The sustainability may be financed through levy on pesticides.

Concluding Remarks

An important component of any food safety program is the control and monitoring of pesticide residues. Pesticide residues in food have become increasingly important world-wide in recent years, not only in terms of protecting the health of consumers, but also to meet requirements for international trade. This is especially important for many developing countries that export food to the major trading blocks of the developed world, or that have the potential to do so. To facilitate such trade, it is necessary to have safe and quality assured pesticide free foods.

Effective management of pesticides with clear cut roles and responsibilities of public agencies and established quarantine system with mandatory sampling in food trade for pesticides prone food products is necessary. Codex standard for pesticide residues is useful. Similarly, test kits are useful for screening pesticides to avoid costly and troublesome procedure for pesticide analysis. Further, RMP needs to be developed for exportable food items. These were the some best practices adopted in the studied countries.

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Investigation on the Thermal Stability of Folic Acid and 5-Methyltetrahydrofolic Acid in Model Liquid Food Systems

ASHOK KUMAR SHRESTHA[1,*], PETER TORLEY[2] and PIERRE-EDOUARD FORISSIER[1]

[1]Centre for Nutrition and Food Sciences, University of Queensland, St Lucia, Queensland 2072, Australia
[2]School of Agriculture and Wine Sciences, Charles Sturt University, Wagga Wagga, NSW, Australia

Folic acid and 5-Methyltetrahydrofolic acid are two major folate vitamers. Although folic acid is commonly used for food fortification to prevent the incidence neural birth defects, 5-methyltetrahydrofolic acid (5-MTHF) is considered to be more bioavailable than folic acid. Therefore, the fortification of foods with 5-MTHF is under consideration. A comparative study on the thermal stability of folic acid and 5-MTHF in various model liquid food matrices such as milk, soymilk, starch-water and water during boiling and autoclaving at various time intervals was performed. The thermal degradation of 5-MTHF was much severe than folic acid in all food matrices e.g., almost 70% loss in 5-MTHF and 17% loss in folic acid. The average retention of folic acid and 5-MTHF (at 5 heating times) was lowest for starch-water and highest for soymilk. The kinetic data showed that there is no linear decrease in concentration of these vitamins with heating temperatures or heating times indicating other factors besides heat such as dissolved oxygen, non-buffered system, binding of folates by proteins, entrapment of vitamins and poor recovery during purification might affect the folate loss.

Keywords: Fortification; Folic acid; 5-Methyltetrahydrofolic acid; Food matrix; Stability

Introduction

Folate is the term most commonly used to refer a family of vitamers with related biological activity. The term folic acid and folate or folacin is often used interchangeably. While folate refers to all forms of vitamin including the naturally occurring forms of the vitamins (polyglutamates), folic acid refers to the most oxidized, stable, and easily absorbable synthetic form (monoglutamate) (Hoffpauer and Bonnette, 1998). Several other designations assigned for folic acid in the past were "*Lactobacillus casei* factor", "Vitamin B_c", "Vitamin U", "norite eluate factor", "Vitamin B_{10}", and "Vitamin B_{11}" (as reviewed by Hawkes and Villota, 1989 and Selhub and Rosenberg, 1996). Among about 100 of similar folate compounds, folic acid and 5-methyl-5, 6, 7, 8-tetrahydrofolic acid, are the most commonly reported forms (Gregory, 1989). In nature, most of the folate compounds are covalently bonded by one to seven glutamic acid residues by peptide linkage to the of L-glutamic acid end of folate structure. Folate deficiency in the diets has been linked to malformation of the embryonic brain/spinal chord development, a condition referred as *neural tube defects or NTDs* (manifested by still-births, mental retardation, swollen head, and poor bladder control) and also to megaloblastic anemia, atherosclerosis, stroke, cancer, Alzheimer's disease (as reviewed by Arcot & Shrestha, 2005). Folate exists in a significant amount in green leafy vegetables, fruits, legumes, fermented vegetables, egg yolk but most of the cereals and their flours are poor source of folate (Shrestha *et al.*, 2003). Considering the significant number of NTD cases, fortification of cereal based foods with folic acid (synthetic form) has been made mandatory in 57 countries including USA and Australia.

The use of folic acid as a fortificant, however, has been controversial as it can mask the haematological abnormalities of vitamin B12 deficiency while the neurological complications remain in progress (NHMRC, 1995). There is now consideration for the use of 5-MTHF (Fig 1) which cannot possibly mask the Vitamin B12 deficiency as an alternative to folic acid (Wright *et al.*., 2001).

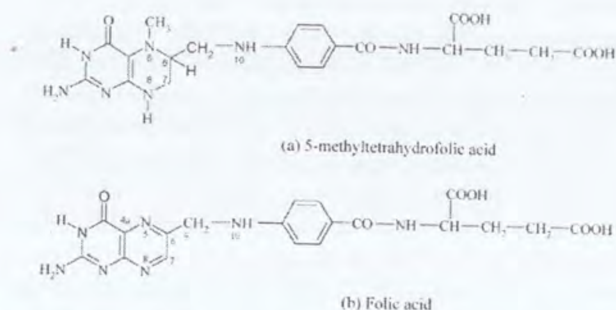

Fig 1: Chemical structures of 5-methyltetrahydrofolic acid and folic acid

Historically, 5-MTHF has not been considered as it has been difficult and expensive to synthesize and less stable. Folic acid and pure natural isomer 6S-5-MTHF found to have similar bioavailability (Pentieva *et al.*, 2004). All folate compounds are highly sensitive and easily destroyed under high

*Corresponding author: Email: amarceley@gmail.com

temperature, air, light, low pH, and reducing agents (as reviewed by Arcot and Shrestha, 2005). Various studies have shown that 5-MTHF is more sensitive to thermal degradation compared to folic acid (Nguyen *et al..*, 2003; Indrawati *et al.*, 2004a & b, 2005; Chen and Cooper, 1979) and easily destroyed during food processing. There have been a number of studies where thermal degradation kinetics of folic acid and 5-MTHF has been determined under various conditions *e.g.*, presence at various levels or absence of oxygen (O'Broin *et al.*, 1975; Chen and Cooper, 1979; Paine-Wilson and Chen, 1979; Ruddick *et al.*, 1980; Mnkeni and Beveridge, 1983; Barret and Lund, 1983); high hydrostatic pressure (Nguyen *et al.*, 2003; Indrawati *et al.*, 2004a); and pH, buffer type and presence of antioxidants (Paine-Wilson and Chen, 1979; Indrawati *et al.*, 2004b; Mnkeni and Beveridge, 1982). The thermal degradation kinetics of 5-MTHF and other folate derivatives followed first-order reaction kinetics (Nguyen *et al.*, 2003; Indrawati *et al.*, 2004b; Virberg *et al.*, 1997)

There have been several studies which reported significant loss of folic acid in fortified foods during processing (Osseyi *et al.*, 1998; Arcot *et al.*, 2001; Shrestha *et al.*, 2003). So far, very few studies have been done to evaluate the effect of processing on 5-MTHF fortified foods (Nguyen *et al.*, 2003; Indrawati *et al.*, 2005; Virberg et al., 1997). Cereal based foods are the major target of folate fortification, but liquid drinks such as flavoured starch based drinks, dairy and soy milks, fruit juices, carbonated drinks etc., have also been used as a vehicle for folic acid fortification. Liquid food is one of the alternatives to improve the bioavailability of folate compounds through diets, instead of grains and flours. Liquid foods have easy passage to the digestive system since it does not depend on rigorous mechanical digestion in gut and easily absorbed through blood. Besides, the liquids are easy to formulate for fortification works as the fortificants are more homogenously distributed in solution as compared to the solid foods.

Processing these fortified drinks involve heating at elevated temperatures as well as exposure to air and light. Considering the sensitivity of 5-MTHF to harsh physical and chemical environments, a significant loss during processing is expected. The knowledge of retention/loss of folate in fortified processed foods is important to ensure adequate 5-MTHF present in target foods (for manufacturers as well as consumers) It is also important so as to predict the final concentration of 5-MTHF in processed liquid foods. The effort has also been made to describe the reaction kinetics of these two vitamers. Therefore, the major aim of the present study is to evaluate the stability 5-MTHF in different simulated liquid foods systems processed under different heating conditions. As folic acid is the only fortificant used for folate fortification in foods, this study used folic acid stability as a reference. The current research is expected to be beneficial to food manufacturers involved in folate fortification works as well as to food and nutrition professionals involved in dietary planning.

Materials and Methods

Materials **:** Soymilk (Sanitarium) and low fat milk (Coles) were purchased from local supermarket. Normal maize starch (amylose content approximately 27%) was purchased from Penfords Australia, Lane Cove, Sydney, Australia. Milli-Q Grade, distilled deionized was used throughout the analysis.

Folic acid (PteGlu-Na_2) and 5-methyltetrahydrofolate ((6S)-5-CH_3-H_4PteGlu-Na_2) were purchased from Merk Eprova AG, Switzerland. Standard solutions were prepared under subdued conditions. The purity of each folate standard was checked using a spectrophotometer at appropriate wavelengths (Blakley, 1969) with a pH 7 phosphate buffer as the blank. All other reagents were of analytical grades, purchased from local suppliers.

Stock solution of folic acid and 5-MTHF (1mg/mL in 0.01M NaOH in 20% ethanol) was flushed with nitrogen. The solution was distributed in a number of small brown bottles, about 10 mL capacity, and flushed with nitrogen and stored at -18°C until further use. For the HPLC calibration curve, standard solutions of both folate vitamers (200 ng/mL in 0.1 M acetate buffer or elution buffer) were prepared on the day of HPLC analysis from stock solution. The solution was further diluted into a series of standards with the same buffer, within the range of 5 to 200 ng/mL in elution buffer. All the folate containing glass wares were wrapped in aluminium foil to prevent photo-oxidation of folate.

Thermal treatments **:** Four different matrices were chosen for stability studies. Water, soymilk and skim milk were accurately weighed (about 50 g) in 250 mL Schott bottles in duplicates. Starch suspension was prepared by dissolving 2 g of starch in 50 g of hot (boiling) water in Schott bottle and cooled. The bottles were wrapped with aluminium foil. One mL each of folic acid and 5-MTHF stock solution together (~1 mg/mL) was added to 4 different matrixes and mixed well. These samples were heated for 10, 30, 60, 90 or 120 min at 100°C in a boiling water bath. Another set of similar samples were autoclaved (121°C and 14.7 *psi*) at similar time intervals. At the end of heating cycle, all samples were cooled in an ice-water bath to stop the degradation of the folate and then transferred in brown bottles, flushed with nitrogen and stored in the freezer (-18°C). All the sample preparation, extraction, purification and HPLC analysis were performed under protection from direct daylight.

Folate Extraction and Purification **:** Considering folic acid and 5-MTHF were externally added, it was expected that these vitamins could easily be extracted from the food matrices (Arcot *et al.*, 2002). Therefore, no further treatment such as enzyme hydrolysis etc. was done on sample to extract the folate from the matrix, as reported previously (Shrestha *et al.*, 2000; Rader *et al.*, 1998). For extraction, the folate extracts were thawed and 0.2 mL sample was mixed in 9.8 mL of

extraction buffer [phosphate buffer (0.1M, pH 6) containing 2% ascorbic acid and 0.1% 2-mercaptoethanol]. The solution was heated for 10 min in a boiling water bath, cooled in an ice bath and centrifuged at 10,000 rpm for 10 min to remove any suspended solids. An aliquot (~1 mL) of supernatant was micro-filtered (0.45 µM pore size, 25 mm id, no 4614, Pall Corporation) using a micro-syringe in a 1.5 mL in Eppendorf tubes. The filtrate was flushed with nitrogen and stored at -20°C until purification (within few days).

The sample purification was done according to method described by Jastrebova *et al.* (2003) with slight modification. The samples extract were purified using a solid phase extraction (SPE) with a strong anion exchange (SAX) cartridge (3mL/500 mg of quaternary amine N^+, counter-ion Cl^-, no 57017, Supelco, Inc.) under vacuum. The cartridges were first pre-conditioned twice with 2.5 mL methanol and twice with 2.5 mL water at a flow-rate of 1–2 drops/s. Aliquots (2.5 ml) of the sample extracts were applied to the cartridges and passed slowly with flow-rate not exceeding 1 drop/s. The cartridges were washed twice with 2.5 mL water to remove matrix interfering components (flow-rate 1–2 drops/s). The elution of retained folate was performed slowly (flow-rate not exceeding 1 drop/s) with 0.1 M sodium acetate containing 10% (w/v) sodium chloride, 1% (w/v) ascorbic acid and 0.1% (v/v) 2-mercaptoethanol. The first portion (0.7 ml) of eluate was discarded and the second portion (3.8 ml) was collected and weighed. The extract was diluted with elution buffer before injected into HPLC.

HPLC Analysis : An Agilent 1100 Series Standard Higher Performance Liquid Chromatograph (Agilent Australia) equipped with a model 717 plus autosampler, a model 600 controller pump (gradient pump), and a UV-photodiode array (PDA) detector (model 996) was used for folate analysis. Recording of the chromatograms and evaluation of the peak areas were performed using the Millennium 32 data acquisition system (Waters). The folate compounds were separated with a Phenomenex C18 (2) column (5 mm X 150 mm L X 4.6 mm i.d.; Luna), which was protected with a guard column (Phenomenex C18 ODS, octadecyl, 4 mm L and 3.0 mm i.d.; Luna). UV absorbance at a wavelength of 290 nm was used to detect the elution of folate compounds. Gradient elution with acetonitrile and 30 mM phosphate buffer, pH 2.2, was performed to separate folate compounds at a flow rate of 0.8 mL/min. The gradient was started at 5% acetonitrile, which was maintained isocratically for the first 8 min, and then the acetonitrile concentration was raised linearly to 24% within 23 min and decreased back to 5% after 5 min. The injection volume was 100 µL. The running time was 36 min, and the time between injections was 46 min. A calibration curve for each folate standard was obtained by the external standard method in which peak height was plotted against six concentrations of folate standards injected (5, 25, 50, 100, 150 and 200 ng/mL). These calibration plots were prepared on the day of use. Quantification of folate contents in the samples was performed using linear regression procedures. Peak identification was based on the retention time and spiking (addition of standard compounds into the purified sample extract) to confirm peaks for any samples in which identification using the retention time was considered to be inadequate.

Degradation kinetics : From the HPLC results, the final folic acid and 5-MTHF concentrations of each liquid food matrices under both heating conditions (C) was measured against the original concentration of added vitamers (C_0). There have been several studies that showed the degradation of both folic acid and 5-methyl tetrahydrofolic acid follow a first order reaction kinetic given by following equations 1, 2, and 3. For each food matrix, a curve indicating the logarithmic value of C/C_0 against the heating time [$\ln(C/C_0)$ vs t] was drawn to describe the kinetic order of the reaction.

$$\frac{dC}{dt} = k \, x \, C \qquad (1)$$

$$\ln \frac{C}{C_0} = -k \, x \, t \qquad (2)$$

$$k = \frac{Ea}{R * T} \qquad (3)$$

Where,

C is the residual or final folate concentration at treatment time (µg/mL), C_0 is the initial folate concentration (µg/mL); t is heating time, min, Ea is activation energy ($kJ.mol^{-1}$), T is absolute temperature (K) and R = Gas constant, 8.31 $J.K^{-1}.mol^{-1}$.

Quality control : Recovery study was performed to check the reliability and reproducibility of the method and validity of data. Each assay was accompanied by a set of recovery tubes. The assays with recovery levels above or below the range of 95-105% were not considered for the study and were discarded.

For the recovery study, 30 ng both vitamers were added into final diluted samples tubes just before analysis. The % recovery was calculated as follows:

$$\%\,Recovery = \frac{ng\ folic\ acid\ in\ spiked\ sample\ tube - ng\ folic\ acid\ in\ unspiked\ sample\ tube}{ng\ folic\ acid\ added\ to\ spiked\ sample} x100$$

Besides, a known concentration of folic acid and 5-MTHF (from final standard) were injected in between the sample injections so as to ensure the repeatability of the assay.

RESULTS AND DISCUSSION

Sample preparation, extraction and purification

The stock solution containing folic acid and 5-MTHF was used for fortification of food matrices for stability studies. Some of the stock solutions were analysed for folic acid and 5-MTHF contents during HPLC analysis of treated samples which gave close to 100% original values. The processed samples were extracted in the extraction buffer containing combined antioxidants of 2-mercaptoethanol and ascorbate which is expected to be were effective in preventing folate losses during the whole analysis (Ginting *et al.*, 2002).

Considering folate vitamers were externally added on each food matrices in very high quantity (1 mg folic acid or 5-MTHF/50 mL liquid food or 20,000 ng/mL), preliminary HPLC analysis was performed on centrifuged, micro-filtered samples. These clear filtrates (from all 4 matrices) were diluted with elution buffer before being injected to HPLC. However, these samples did not show any trace of folic acid and 5-MTHF in HPLC chromatogram, indicating the need for sample purification. The solid phase extraction (SPE) technique using strong anion exchange (SAX), however, ensured separation and detection of folate vitamers by HPLC. The SPE-SAX procedure for purification of folate compounds was also shown a good recovery of folate standards.

HPLC performance

HPLC analysis of standards showed folic acid and 5-MTHF are well separated by the current protocol of gradient elution and the Phenomenox C18 column. The retention time for folic acid and 5-MTHF were 20.41 and 16.76 min, respectively (Fig 2). It was also noted that the retention time for both vitamers change slightly between injections. However, it did not affect the absolute value of the folate vitamers in the samples. These slight variations could be due to difference in concentration of mobile phase, elution buffer, column temperature and other factors that possibly influence the mobility of the analyte in the column. Some minor peaks also appeared in the vicinity of the folic acid and 5-MTHF peaks indicating possible breakdown of these compounds.

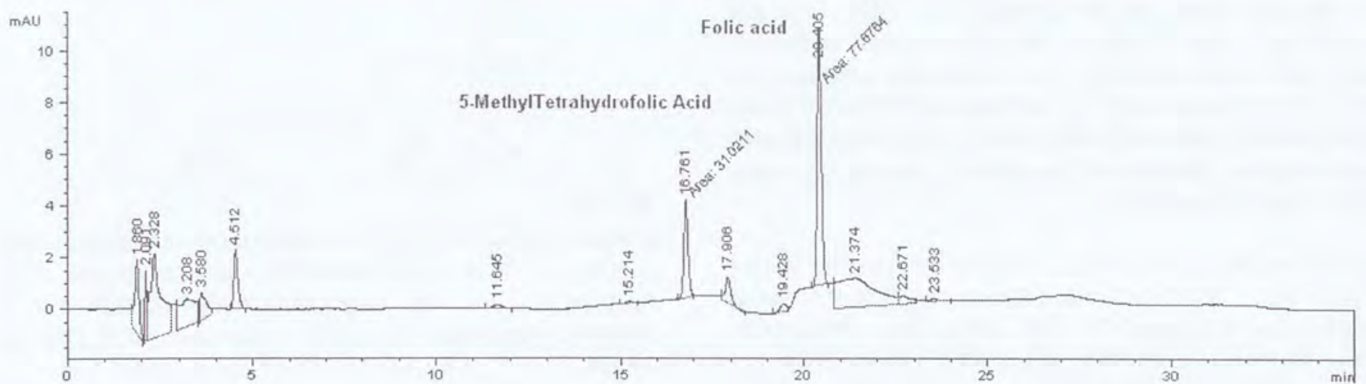

Fig 2: Chromatogram of standard mixtures of folic acid and 5-methyl tetrahydrofolic acid at 200 ng/mL.

The detection limits for the HPLC system used in this study for folic acid and 5-MTHF were 0.3 and 0.5 ng (in 100 ìL injection). Calibration curves showed a linear response (Regression coefficient, $R2 > 0.99$) for both vitamers (Fig 3). The detector response was equally good for peak area and peak height. However, peak height of the analyte was chosen for the calculation of folate content of sample as it was less subjective (sometimes peak broadening render it difficult to measure area) and slight more accurate.

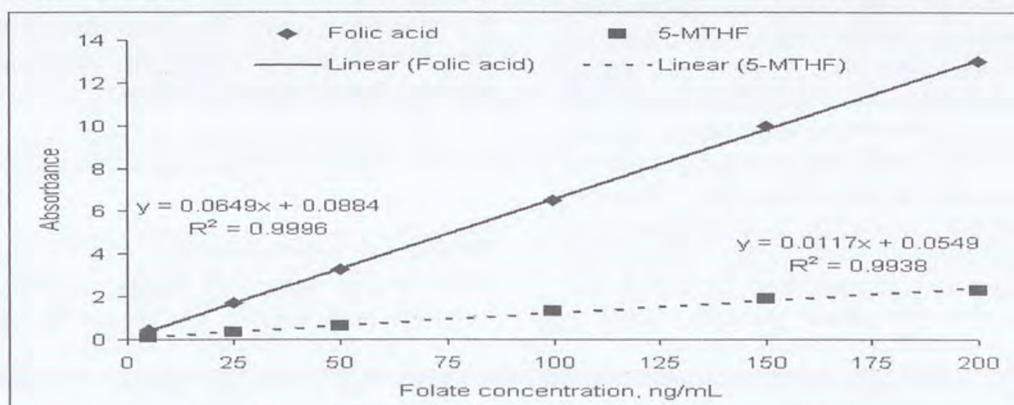

Fig 3: Calibration curve of folic acid and 5-MTHF using peak height.
(Regression equation of each vitamer is given beside the predicted lines.)

The recovery study of both vitamins showed less than expected %recovery for both vitamins, ranging from 70 to 100%. The recovery found to vary between the matrices with higher recovery in water and lower in milk. Considering the lower stability of these vitamins and possible binding with folate binding proteins (in milks), the current level of recovery is considered to be acceptable.

Thermal treatment on folic acid in model foods

Soybean (and hence soymilk) and milk are known to contain significant amount of indigenous folate (Ginting and Arcot, 2004; Arcot *et al.*, 2002; NHMRC, 1995). However, our analysis showed very low amount of folic acid and 5-MTHF. This is probably that milk and soymilk were diluted significantly during extraction and HPLC analysis, the concentration of these vitamins were at the low level of detection by HPLC. Ginting and Arcot (2004) have reported that soybean seed contains very low amount of 5-MTHF and zero amount of folic acid. They reported the total folate content of soybean is 192 ìg/100g (dry basis) where 5 and 10-formyltetrahydrofolates form 90% of the total folate and rest is 5-MTHF. Milk is reported to contain about 10ìg 5-MTHF/100g (NHMRC, 1995) which is very low.

Table 1 shows the folic acid content of soymilk, skim milk, starch-water and water matrices, boiled and autoclaved at 10, 30, 60, 90 and 120 minutes. The retention of folate vitamers varied from 19% to 99.4% which indicates variation in their susceptibility to heat.

Table 1: Folic acid content of various matrices boiled and autoclaved at various time intervals

Heating time, min	*Soymilk*		*Milk*		*Starch-water*		*Water*	
	Boiling	*Autoclave*	*Boiling*	*Autoclave*	*Boiling*	*Autoclave*	*Boiling*	*Autoclave*
10	88.7±2.9	88.7±2.9	90.7±4.6	91.2±0.4	86.2±4.9	88.4±5.4	93.0±4.3	91.7±3.5
30	94.2±1.1	94.2±1.1	88.1±1.0	94.9±3.9	73.2±11.7	87.6±7.6	92.3±1.3	96.4±11.3
60	86.9±2.7	86.9±2.7	92.6±7.8	99.4±0.8	89.7±4.0	84.8±8.4	95.6±2.4	88.5±6.4
90	91.5±3.2	91.5±3.2	91.3± 2.0	78.6±11.5	83.4±4.5	76.0±0.1	94.0±2.3	96.1±4.5
120	90.1±0.8	90.1±0.8	84.5±2.5	84.7±2.6	NA	84.7±9.5	92.2±2.8	90.1±6.2
Average	90.3	90.3	89.4	89.8	83.1	84.3	93.4	92.6

This study also showed folic acid is less affected by thermal processing as compared to 5-MTHF. Folate vitamers have been reported to susceptible to oxidation *e.g.*, from dissolved oxygen (Day and Gregory, 1983; Virberg *et al.*, 1997). In this study, no effort has been made to de-aerate the liquid matrix, therefore, the loss of folic acid and 5-MHTF may have been partly contributed by dissolve air too. The effect is likely to be more significant in current study as boiling condition (and high pressure) release of air bubble from the liquid, potentially oxidizing the folate compounds.

Table 1 shows the percentage retention of folic acid in all food matrices were high, irrespective of matrices, heating condition and time. The average folic acid retention in soymilk, milk, starch-water and water after boiling from 10 to 120 min were 90.3, 89.4, 83.1 and 93.4%, respectively, and retention after autoclaving at the same time intervals were 90.3, 89.8, 84.3, and 92.6% respectively. It showed that boiled water has the highest retention (93.4%) and the autoclaved starch had the lowest retention (84.3%). Besides, the comparison of percentage retention of folic acid between boiled and autoclaved samples, for each heating time, showed almost identical values. This indicates folic acid is equally stable in pressure cooking. A number of liquid food products such as liquid breakfast cereals, fruit juices and several types of milk are heated for processing as well as pasteurization/sterilization purposes. One of the most commonly used thermal processes for liquid food nowadays is UHT (ultra high temperature) that involves heating foods in the range of 135-150°C for few seconds for sterilization of milk. Considering higher stability of folic acid at autoclaved condition at significantly longer time, its retention under UHT is also expected to be high.

Starch and soluble complex polysaccharides such as various gums, pectin, arabinoxylan, alginates etc. form important ingredients of many liquid based drinks. It is not known how these ingredients affect the retention of folate compounds during boiling or high pressure treatment. The possibility of folate vitamers embedding into these carbohydrate matrices and the subsequent effect of heat on their stability is not known. Various studies have shown that foods have to be treated with amylolytic enzymes to release folate from the starch matrices during extraction and analysis (Shrestha *et al.*, 2000; Tamura, 1998; Rader et al, 1998). In current study, starch matrix showed the lowest retention of folic acid in both boiled and autoclaved samples. It might be true that equal to

folic acid loss due to heat, entrapment of folic acid in starch matrix (and not releasing during centrifugation) likely to be the cause of low folic acid content in this matrix.

In unprocessed milk, the native folate is mostly bound to the folate binding proteins, FPB (Wagner, 1985). FBP may also serve to facilitate folate uptake by interacting with mucosal receptors, as shown *in vitro* in intestinal cells (Colman *et al.* 1981). However, it has also been shown that heat treatment; condensation and evaporation release protein-bound folate compounds. Losses of folate in milk during UHT processing are generally less than 20%, but losses of up to 43% have been observed (Andersson, 1993). This study showed about 90% retention of added folic acid in boiled and autoclaved milk. The value is consistent with the percentage folic acid loss comparable to other matrices indicating FPB do not play any role in protection of folates during thermal processing. Therefore, it is highly unlikely that FPB helps to protect thermal degradation of folate vitamers in milk. There is no data available on the complex formation between soy protein and folate. Similar to milk, soy protein does not seem to affect the retention of folic acid in soymilk. Our previous study showed that UHT (140°C for 10s) of soymilk lower folate content from 320 to 276 μg/100, moisture free basis (a loss of 86%) (Arcot *et al.*, 2002). The most probable cause of low folate content in UHT milk would be degradation of other heat-sensitive folates such as 5-MTHF, 10-formyl folic acid, 5-formyl-5,6,7,8-tetrahydrofolic acid, 5,6,7,8-tetrahydrofolic acid, 7,8-dihydrofolic acid (DHF) and so on.

There have been several studies that showed folate vitamers are less susceptible to thermal degradation if heated in presence of reducing agents such as ascorbic acid and 2-mercaptoethanol (Arcot *et al.*, 2002; Chen and Cooper, 1979; O'Brien *et al.*, 1975). It is likely that the protective effect offered by ascorbic acid during extraction varies with the food matrix. For example, Day and Gregory (1983) investigated the effects of ascorbic acid on stability of folic acid (fortified) in a model liquid food system when heated from 100 to 140°C for various times. The authors found almost the same degree of folic acid retention for the food system with or without ascorbic acid *e.g.*, about 70% retention if heated for 25 min at 120°C.

We previously studied the effect of extraction conditions (100°C *vs* 121°C) on stability of folic acid in nine fortified breakfast cereals. It showed that although the extraction method using boiling temperature was expected to retain more folic acid than autoclaving, only four samples showed significantly ($p<0:05$) higher values and no significant difference in values were observed for the rest of the samples. Day and Gregory (1983) reported 0% loss of folic acid when the model liquid food system (fortified) was heated at 100°C, 30% at 120°C and 60% at 140°C, when heated for 25 min. Paine-Wilson and Chen (1979) reported that pure folic acid solution was stable for up to 10 h heating at 100°C (pH 4–12). Therefore, the current research further established that the folic acid in liquid foods is highly stable, irrespective of heating conditions.

Thermal treatment on 5-MTHF in model foods

The percentage retention of 5-MTHF in all food matrices were much lower than folic acid, irrespective of food matrices, heating condition and time. The retention of 5-MTHF was lowest (19.3%) in 60 min autoclaved water and highest (67.4%) in 90 min autoclaved soymilk (Table 2). The average 5-MTHF retention in soymilk, milk, starch-water and water after boiling from 10 to 120 min were 48.6, 29.4, 29.6 and 33.7%, respectively and retention after autoclaving at the same time intervals were 56.5, 34.9, 35.4, and 32.2%, respectively. It showed that autoclaved soymilk has the highest average retention (56.5%) and the boiled milk had the lowest average retention (29.4%) (Average values). The higher 5-MTHF content in all soymilk was most probably contributed by higher amount of indigenous folates present in soymilk. The HPLC analysis of raw soymilk showed a 30 μg 5-MTHF/100 mL (wet basis). The comparison of percentage retention of 5-MTHF between boiled and autoclaved samples for each heating time showed a noticeable difference. It was noticed that 5-MTHF values for autoclaved soymilk, milk and starch-water were higher than their boiled counterparts and no difference observed for water.

Table 2: 5-Methyltetradhydrofolic acid content of various matrices boiled and autoclaved at various time intervals

Heating time, min	*Soymilk*		*Milk*		*Starch-water*		*Water*	
	Boiling	*Autoclave*	*Boiling*	*Autoclave*	*Boiling*	*Autoclave*	*Boiling*	*Autoclave*
10	NA	41.9±0.1	43.0± 9.7	43.9±9.1	31.3±1.5	27.1±5.8	23.2±0.6	28.5±2.0
30	48.5±10.6	55.1± 5.0	19.7±0.5	30.7±5.0	22.6±2.9	23.7±3.8	27.0±1.1	29.6±0.6
60	45.4±3.5	65.0±2.5	27.9±10.3	36.3±0.1	39.1±5.2	42.7±0.4	41.1±2.1	19.3±1.3
90	44.1±8.0	67.4±3.6	23.6±2.9	24.3±3.9	20.6±7.9	30.5±5.5	31.0±4.6	33.2±3.4
120	56.1±4.2	53.0±8.4	32.7±6.4	39.1±2.8	34.2±8.9	52.8±4.9	46.0±7.1	50.3±8.4
Average	44.0	56.5	29.4	34.9	29.6	35.4	33.7	32.2

There was no distinct trend of increasing loss of this vitamer with increased heating time, for both boiled and autoclaved samples. It was expected that the loss of vitamin would be higher when autoclaved, and or when processed for longer times which is not observed in current study. In this context, it is preferable to know about the effect of other factors besides thermal treatment that might have contributed towards the losses of the nutrient of interest i.e., 5-MTHF. A number of factors might have led to these unexpected results as described below.

Dissolved Oxygen

Besides high temperature, oxygen is known to be a factor in degradation of 5-MTHF (Viberg *et al.*, 1997; Mnkeni and Beveridge, 1983; Barret and Lund, 1983). Virberg *et al.* (1997) heated L-5-MTHF solution (phosphate buffer, pH 7) at normal and low oxygen concentration (0.3 and 60 ppm) at 110 to 150°C. The investigation showed that a low oxygen concentration during processing at these temperatures decrease the folate degradation but would not completely prevent it. The ability of gas to dissolve in liquid is affected by several factors, such pressure, temperature and solid content. If pressure increases the solubility then the higher temperature expand the gas and decrease the solubility of gas in the solvent. On the other hand, gases are released from the solvent when salts or solid is introduced. Therefore, the level of oxygen in the current liquid matrices are expected to be vary due to varying composition, ranging from water to milk and to quite viscous starch-water mixture. The exposure of 5–MTHF to oxygen in the wide ranging liquid food matrices at higher temperature have *domino effect* on their degradation rate (possibly randomly). Therefore, most of the sample preparation and purification methods use a suitable reducing agents *e.g.* ascorbic acid, 2-mercaptoethanol etc., in a buffered system to scavenge the dissolve oxygen from the solution. In one of such studies, Chen and Cooper (1979) reported that an aqueous solution of 5-methyltetrahydrofolate containing 0.1% ascorbic acid lost 20% of its activity when heated for 3 h at 100°C. However, under the same conditions without ascorbate, it lost 90% of its activity in 65 min of heating.

Buffering System

In most of the stability studies pertaining folates a buffered system is used as they are stable only at the limited range of pH (Nguyen *et al.*, 2003; Indrawati *et al.*, 2004b; Virberg *et al.*, 1997). However, in the current study the samples were not buffered as these model foods were studied in their original condition (approximately at neutral pH). Previous study have shown that even the choice of buffer solution could affect the stability of 5-MTHF (Paine-Wilson and Chen, 1979). They reported that when 5-MTHF is heated at 100°C the degradation rate increased by a five fold between the buffer that gave the lowest degradation rate and the buffer that gave the highest degradation rate, and that the choice of buffer would probably have a greater influence on the degradation rate at higher temperatures than at lower temperatures. Other investigators also reported various pH maxima *e.g.*, pH 7, 9 and 3-6 (and various buffering systems) for the stability of 5-MTHF (Paine-Wilson and Chen, 1979; O'Broin *et al.*, 1975; Mnkeni and Beveridge, 1983). These investigators also suggested that in aqueous solutions, 5-MTHF may have a lower stability than folic acid. It is likely that in absence of buffer, a significant a swing in the pH of the solutions (or matrices) might have occurred further contributing towards the degradation of 5-MTHF.

Folate binding proteins

It is likely that (enzymatic) binding of folate compounds by milk protein and their incomplete release during extraction and purification affected the folate measurement. Wigertz et al., (1997) found that the recoveries after spiking most of the dairy samples with 5-MTHF prior to heat extraction were considerably lower (53%). Further heat treatment followed by enzymatic deconjugation needed for further recovery of the vitamin. Very few data are available on the folate binding properties of plant proteins. Ginting *et al.* (2002) reported a recovery of 80% 5MTHF spiked to *Tempeh*, a mold fermented soybean food. Therefore, it is likely that certain protein in milk and milk products, soybean etc. bind folates rendering the under-estimation of 5-MTHF in foods.

Extraction and purification

The test samples (after heat treatment) were stored (at -20°C) for before extraction. These defrosted samples were further boiled for 10 min to ensure complete folate extraction, cooled and centrifuged. In starch-water matrix, a significant amount of starch aggregates left behind in centrifuge tube. It is likely that a significant amount of folate might have been physically entrapped by the starch aggregates. This could be one of the causes of lower 5-MTHF in starch-water matrix, besides thermal degradation. The protocol for sample extraction and purification using SPE-SAX (Vahteristo *et al.*, 1996) is long and tedious which is also likely to under-estimate the folate level in analyte. Particular concern is a number of washing and elution steps with varying flow rates that affect the final concentration of the eluted analyte. Vahteristo *et al.* (1996) in her recovery study reported that up to as much as 25% folates could be lost during SPE-SAX purification.

Chromatography

It was noted that compared to chromatogram of the pure folate standards, the samples, including water matrix, had a significant number of peaks around the peak of interest, particularly 5-MTHF (Fig 4).

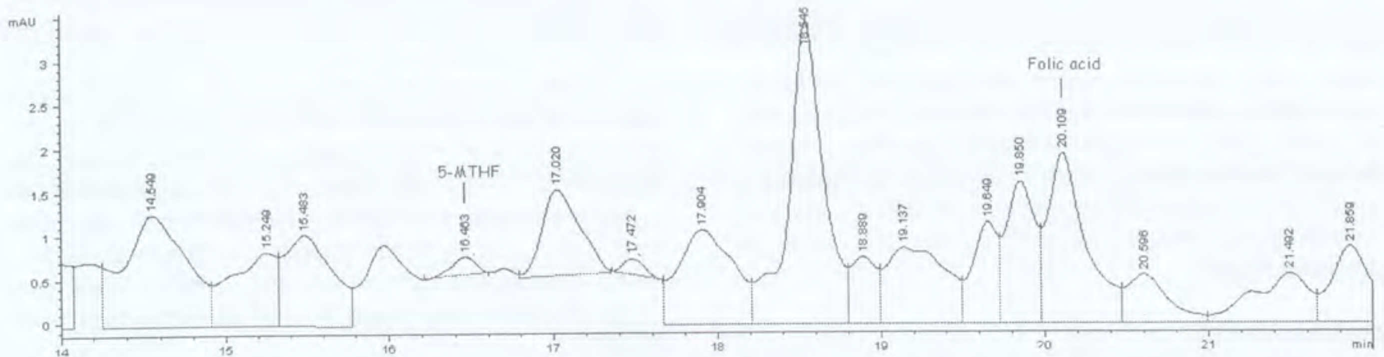

Fig 4: Chromatogram showing 5-MTHF and folic acid in processed fortified soymilk

In some instances, these peaks affected to the judgment for the picking of right peak for the calculation. It is also likely that high temperature processing may have degraded 5-MTHF (and folic acid) into several other folate vitamers with similar structures, generating several peaks close to the 5-MTHF. It has been reported that there are about 100 folic acid derivatives exist naturally due to their chemical diversity (Gregory, 1989), therefore, overlapping of peaks cannot be discounted. Wallin (2005), in her Chemical and Technical Assessment report for FAQ/WHO, has reported at least 4 breakdown and oxidation products of calcium salt of L-5-MTHF. So, there is every possibility of under-estimation of 5-MTHF in the processed food matrices due to the thermal breakdown of vitamin.

Autoclaving operation

Autoclaving has been used to study the effect of sterilization condition (121°C and 14.7 *psi* pressure) on folate vitamers in liquid foods. Although 10 to 120 min heating time was used for autoclaving, the actual heating time was quite longer. It was estimated that at least extra 10-15 min heating 'come up' time (up to 121°C) and about 10 min cooling time (~100°C) were needed besides actual autoclaving time. Therefore, the vitamins have been exposed much longer heating time than actually reported. The particular concern is for shorter heating periods, such as 10 and 30 mins where the effect is expected to be more significant.

Kinetics of folic acid and 5-MTHF degradation

Current study showed the significant degradation of folic acid and 5-MTHF when heated at higher temperatures. One of the objectives of the study were to determine the order of a reaction to construction a kinetic model for the given food matrix. Paine-Wilson and Chen (1979) initially showed that the degradation kinetics of folate vitamers at 100°C followed the first order kinetics in a wide pH range. The effect of boiling and autoclave temperatures on the folic acid degradation kinetics at various heating times is studied. The natural logarithmic value of folate concentrations in final and original food matrix [$\ln(C/C_0)$] as a function of constant temperatures 100°C and 121°C at various times is shown in the Fig 5. As previously mentioned, the thermal degradation of both folate vitamers with heating time was not linear. Therefore, the curves in degradation kinetic of most of the matrices appeared to be more or less random. The trend lines for the retention of folic acid in all the food matrices were almost horizontal with very low regression coefficient ($R^2 \sim 0.1$) indicating almost no change in folic acid concentration with heating time. It showed the concentration of folic acid in autoclaved starch-water mixture and autoclaved milk slightly increased retention with time ($R^2 \sim 0.37$). However, the kinetic study of 5-MTHF in food matrices, in general, showed that increasing heating time causes logarithmic degradation of this vitamer, although at much lower scale (R^2 up to 0.69). Milk and starch-water (boiled), however, did not show any change in the concentration of 5-MTHF with heating time. In most of the kinetic study on thermal degradation of 5-MTHF, increasing incubation times led to gradual destruction folates: the $\log(C/C_0)$ plot *versus* time going downward from upper left corner to lower right corner with the regression coefficient close to unity (Nguyen *et al.*, 2003; Indrawati *et al.*, 2004a and b). Unfortunately, the kinetic data on thermal degradation of folic acid and 5-MTHF on the given food matrices did not conform the previous findings. Therefore, the much expected first-order reaction kinetics of folate degradation could not be established. No effort has been made to calculate the degradation rate coefficients (k) and activation energy (Ea) as the order of the reaction could not be established.

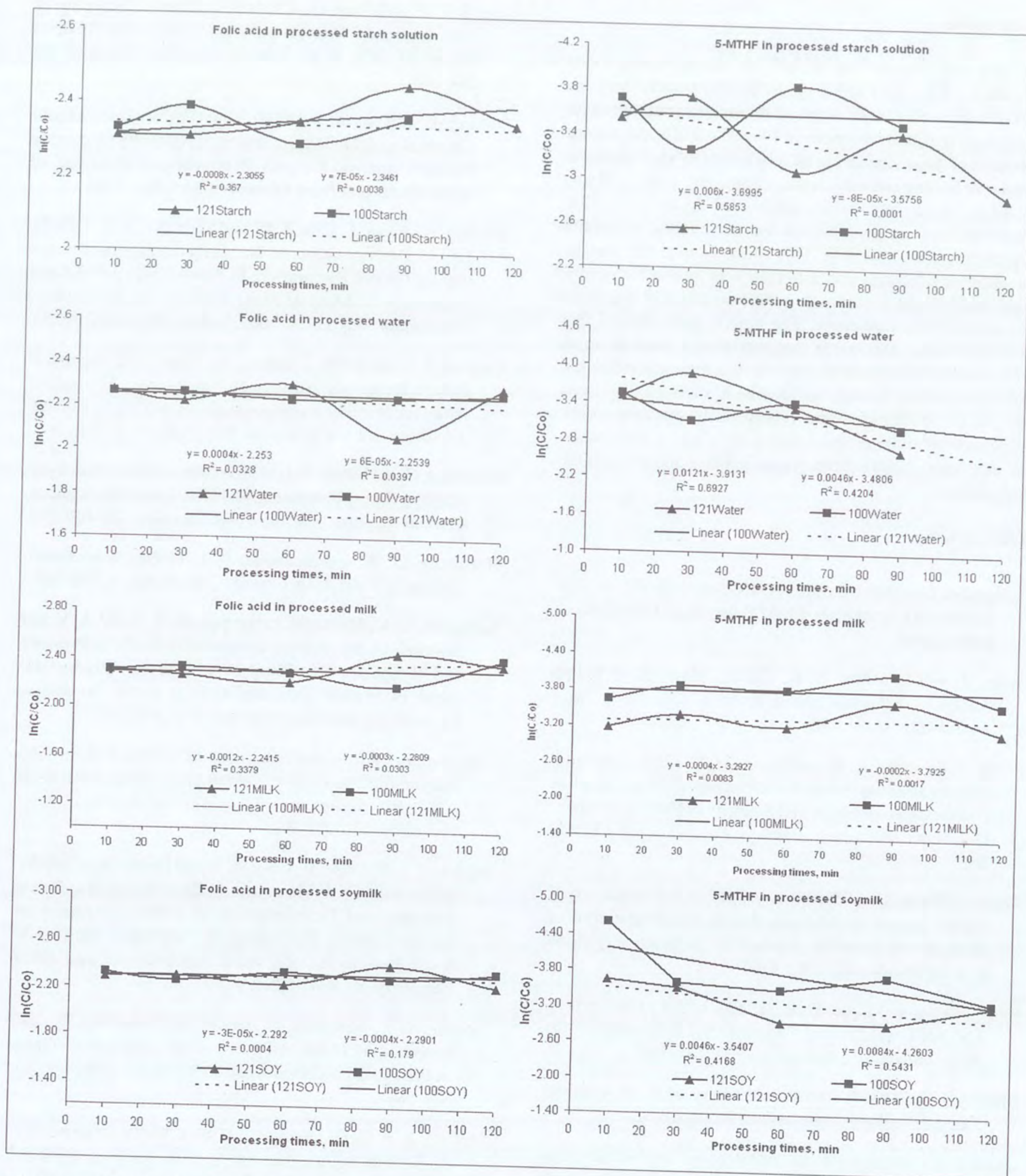

Fig 5: Reaction kinetics of folic acid and 5-methyltetrahydrofolic acid retention in various model food systems: starch-water, water, milk and soymilk, at 100 and 121°C.

Conclusions

Boiling and autoclaving of various model liquid foods containing folic acid and 5-methyltetrahydrofolic acid (5-MTHF) showed a large range of thermal degradation with percentage retention ranging from 19 to 99.4%. The percentage retention of these vitamers in all four model foods was almost similar in boiling and autoclaving conditions. Folic acid was relatively stable even when autoclaved up to 2 h (~90% retention). In contrast, 5-MTHF was more labile to thermal degradation than folic acid. This study showed the average retention of folic acid and 5-MTHF was lowest for starch-water and highest for soymilk indicating effect of matrix on retention of these vitamers. The kinetic data showed that there is no linear decrease in concentration of these vitamins with mode of heating and also heating temperatures and times, indicating other factors besides heat such as dissolved oxygen, non-buffered system, binding of folates by proteins, entrapment of vitamins and poor recovery during purification etc. may have played important role in folic acid and 5-MTHF degradation.

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Low Salt Effects on Gelation and Textural Properties of Pork Batter Gel Enhanced by Microbial Transglutaminase and Cooking Method

UPUL MARAPANA* and BO JIANG

Key Laboratory of Food Science and Safety, Southern Yangtze University, 170 Huihe Road, Wuxi, Jiangsu Province, P. R. China 214036

The influence of three cooking methods (water bath, retort and microwave) on the mechanical properties of low salt pork gel with (2.5 or 5 g kg^{-1}) or without (control) microbial transglutaminase (MTGase) was investigated using dynamic rheological measurements. The MTGase treatment significantly increased ($P<0.0001$) breaking force, gel strength and texture properties (hardness, fracturability, and chewinesss) as measured by punch and compression tests. Microwave cooked low salt gels had the lowest hardness and fracturability, however highest chewiness than retort and water bath cooked samples. The mechanical properties were related to the dynamic rheological properties of the low salt raw batter which exhibited an increased storage modulus upon the MTGase treatment during heating. The results suggested that MTGase treatment influenced the rheological and mechanical properties of low salt pork gels; however the efficiency of the enzyme treatment depended on the cooking methods.

Keywords: MTGase, Cooking method, Mechanical properties, Gelation, Salt

Introduction

Sodium chloride is one of the most widely used ingredient in processed and comminuted products to enhance the functional properties of meat proteins including thermal properties (Barbut and Findlay, 1991), heat induced gelation (Careche et al, 1991; Nuckles et al, 1991), swelling of muscle fibres and increase the viscosity of the continuous protein matrix (Wilding et al, 1986), water holding capacity (Richardson and Jones, 1987; Bernthal et al, 1991; Acton et al, 1983), emulsifying properties (Gaska and Regenstein, 1982; Gillett et al, 1977), preservative effect due to lower water activity (Marsh, 1983; Sofos, 1984), texture or binding together of the cooked product (Huffman et al, 1981; Coon et al, 1983; Pepper and Schmidt, 1975; Kenney et al, 1992; Terrell, 1983) and flavour (Gillete, 1985; Ruusunen et al, 2001). Meat becomes a sticky paste, when ground with certain amounts of NaCl. Usually, the concentration of NaCl to produce such sticky meat paste is 2-3% (w/w). However, excessive intake of sodium has been linked to hypertension (Dahl, 1972) and increased risk of stroke and premature death from cardiovascular diseases (Tuomilehto et al, 2001). Therefore, in many countries, the demand for a variety of low salt meat products has increased. Application of heat for cooking of further-processed meats and addition of salt are two major factors involved in denaturation and gelation of muscle proteins. Gelation of meat paste during incubation was due to the formation of network structures or polymerization of myosin heavy chain (MHC), a main component of muscle protein. Tsukamasa et al., (1993) suggested that such polymerization of MHC and gelation of salted meat paste were partly induced by transglutaminase. It is a protein cross-linking enzyme, which catalyses the acyl-transfer reaction between the ã-carboxyamide group of peptide bound glutamine residues and various primary amines. In addition, cross-linking of food proteins can influence many properties of food including texture viscosity, solubility, emulsification and gelling properties (Kuraishi, et al, 1997). However, most gelation and textural studies have been conducted with model system with dilute protein solution and or constant heating rate. Hence, it is doubtful whether the observations obtained from the model system would exactly represent the real food processing techniques such as retort and microwave cooking. Therefore, the objective of this study was to examine the dynamic rheological and textural properties of low salt pork batter gels treated with microbial transglutaminase as a function of cooking methods.

Materials and Methods

Post rigor pork leg meat (semimembranosus muscles) was purchased from a local market 24-36 h post mortem (pH 5.9). The pork was trimmed of visible fat and connective tissue, and then ground in a laboratory grinder (JCW 6 Shanghai Instrument Company LTD) through a plate with 3 mm diameter orifices. The ground meat was portioned, vacuum packaged and frozen at -20°C until product formulation. The ingredients used in the homogenate formulations included sodium chloride, sodium nitrite, sodium ascorbate, sodium tripolyphosphate. All the ingredients were of analytical grade (Sinopharm Chemical Reagent Co Ltd, Shanghai, P. R. China). Microbial transglutaminase (MTGase) (Yiming Fine Chemical

* Corresponding author; Present Address: Dept of Animal Science, Faculty of Agriculture, University of Ruhuna, Mapalana, Kamburupitiya, Sri-lanka.
Emai: umarapana@yahoo.com

Plant, Gensi, Jiangsu, P.R.China) was a mixture containing 900, 90, and 10 g kg $^{-1}$ maltodextrin, sodium caseinate and microbial transglutaminase, respectively (activity of 65 u g^{-1}). Enzymatic activity was measured by the hydroxamate procedure (Folk and Cole, 1966) with CBZ (Carbobenzoxy)-L-glutaminylglycine (Sigma-Aldrich Chemie, GmbH Germany). The enzyme concentration is reported in the present study as the commercial concentration.

Protein analysis **:** The protein concentration of the ground pork was determined by the AOAC (1997) procedures (928.08). Nitrogen values were converted to protein using a conversion factor of 6.25. The protein content of the pork meat was 217 g kg^{-1}.

Preparation of pork batter gel **:** Pork batter gel was prepared by the method of Pietrasik and Li-Chan (2002). Before processing meat was tempered at 4°C and meat protein content was adjusted to a constant level of 60 and 100 g kg^{-1} with added ice and water (50:50) in all formulations. Composition of low salt pork batter formulations are shown in Table 1. Treatments (300g each) were prepared by mixing ground meat and other ingredients for 15 seconds on high speed food blender (model DS-1 Shanghai Instrument Company LTD, P.R.China). The levels of sodium chloride, tripoly phosphate, nitrite and ascorbate were 15, 2, 0.1 and 0.5 g kg^{-1}, respectively. Concentrations of MTGase in separate formulations were MTGase free as control, 2.5 and 5 g kg^{-1}. Immediately after homogenate preparation, the batter was stuffed in to poly-vinyl casings (Yurun Meat Company, Nanjing, P. R. China) (30mm×120mm). The casings were tightly closed and allowed to stand overnight in a cold room at 4°C.

Table 1: Composition (G Kg^{-1} by Weight) of the Low Salt Pork Batter Formulations

Protein level g kg^{-1}	*Meat*	*MTGase*	*Ice & Water*	*Others**
	276.5	Control	705.9	17.6
60	276.5	2.5	703.4	17.6
	276.5	5	700.9	17.6
	460.8	Control	521.6	17.6
100	460.8	2.5	519.1	17.6
	460.8	5	516.6	17.6

Note: * 0.1, 0.5, 2 & 15 g kg^{-1} sodium nitrite, sodium ascorbate, sodium tripolyphosphate and sodium chloride respectively

The homogenate samples were then cooked in a three different heating method, separately, isothermally at 90°C in a water bath for 15 min or autoclaved at 121°C for 15 min (pressure 1.05MPa) or cooked in house hold Microwave oven (model Galanz WD 800T with an adjusted electric output power at medium and operating frequency 2450 MHz (85°C)) for 15 min, and thereafter cooled down in ice water until a core temperature of 20°C was reached. The gel samples stores at 4°C until analyzed. Three replicate pork batter gels were prepared on different days and three measurements were taken from each replicate.

Gelation and measurement of dynamic rheological parameter : Low salt (10 g kg^{-1}) meat paste samples (60 and 100 g kg^{-1} protein) with (2.5 and 5 g kg^{-1}) or without MTGase (control) were incubated overnight at 4°C and treated samples were subjected to dynamic rheological testing using a model AR 1000 rheometer (England) equipped with parallel plates. Specifically gels were formed by heating the protein mixtures from 20 to 80°C at a rate of 1°C/min. The gelling samples were continually sheared in an oscillatory mode at a fixed frequency of 0.1Hz with a maximum strain of 0.02, changes in the storage modulus (G', namely rigidity due to elastic response of the material, phase angle ä, phase difference between the stress and strain under imposed sinusoidal conditions) were monitored throughout the gelling process.

Mechanical properties **:** Gel samples (25 mm diameter and 30 mm height) were equilibrated to room temperature for 30 min in a plastic bag to avoid dehydration before the mechanical properties were measured. The mechanical properties were determined using a TA-XT2i Stable Micro Systems Texturometer (England). The texture expert version 1.2 software was used to collect and process the data. The puncture test was performed, compressing samples to 75% of the initial height using a compression speed of 60 mm min^{-1} and a cylinder probe (P/20) with 1.2 cm diameter. The breaking force (g), deformation (cm) and gel strength (g × cm) for each treatment were measured. Nine samples were analyzed for each treatment.

Texture profile analysis (TPA) (Bourne, 1978) was performed using aluminium cylindrical probe (P/50) with 50 mm diameter. Gel samples were compressed twice to 25% of their original height at a constant cross head speed of 60 mm min^{-1}. The TPA parameters, namely hardness [peak force on first compression (g)], springiness [distance the sample recovered after the first compression (mm)], fracturability [first bite, the

force required to produce the first fracture (g)], cohesiveness [ratio of the active work done under the second force-displacement curve to that done under the first compression curve (dimensionless)], gumminess (hardness × cohesiveness) and chewiness [hardness × cohesiveness × springiness (g×mm)] were computed.

Microstructure **:** The microstructure of raw batters (100 g kg^{-1} protein) with (5 g kg4^{-1}) or without MTGase were analyzed by scanning electron microscopy (SEM). Samples were fixed with a mixture (1:1 vol/vol) of 1% glutaraldehyde and 4% paraformaldehyde in 0.1% phosphate buffer pH 7.2, post-fixed with OsO_4, washed, de-hydrated in acetone, critical-point dried, sputter-coated with gold-palladium and scanned by SEM at 10 kV (Quanta 200, AFEI Co. Holland).

Sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE) **:** SDS-PAGE was performed to determine protein changes induced by MTGase and cooking method, and the procedure described by Laemmli (1970). Using a Mini-PROTEAN[R] 3 cell slab gel electrophoresis unit (Bio-Rad USA), 10% acrylamide separating gel width×height×thickness (80mm×73mm×0.75mm) with a 4% acrylamide stacking gel on the top was made. Myofibrillar protein was prepared according to Eisele and Brekke (1981); Xiong and Brekke (1989), and all preparation steps were carried out at 2-4°C. Myofibrills were suspended in the phosphate buffer (0.6M NaCl in 50 mM Na_2HPO_4, pH 6.0) for final protein concentration of 20 mg mL^{-1}. Protein concentration was determined by the Biuret method using bovine serum albumin as standard (Gornall et al, 1949). Three samples were prepared for each cooking method and each sample treated with (2.5 or 5 g kg^{-1}) or without (control) MTGase. After over night incubation at 4°C, each set of samples were heated in a different cooking method separately as described in previously. Two grams of resultant gel were blended with 18 mL dissolving solution (5% Sodium dodecyl sulphate, 0.1% â-mercaptoethanol) and incubated for 1 hr at 80°C, in order to allow maximal solubilization of aggregated protein and subsequently centrifuged at 3000rpm for 15 min. The supernatants, after measuring protein concentration by Biuret method, were diluted to a 2 mg mL^{-1} protein concentration with water and then mixed (1:1 ratio) with SDS-PAGE sample buffer and dissolved by heating in boiling water for 3 min. Aliquots of 15 ìg of protein per lane were loaded on to the gel. Electrophoresis was done at a constant voltage of 20 mA per gel. Gels were stained with Coomassie brilliant blue R-250 to visualize the protein bands.

Statistical analysis **:** The experimental design was a complete randomized 2×3×3 factorial with two muscle protein levels (60 and 100 g kg^{-1}), three MTGase levels (MTGase free as control, 2.5 and 5.0g kg^{-1}) and three cooking methods (Water bath, Retort and Microwave). Reported data are means of nine measurements (except gelation), three replicates with triplicate measurements. Data were analyzed by analysis of variance (ANOVA) using the generalized linear model procedure of the SAS system for windows version 8.1. When ANOVA showed significant treatment effects ($P<0.05$), mean separation was done by using the Duncan test.

Results and Discussion

Gelation and measurement of dynamic rheological parameter

Fig 1 (a) and (b) show the variation in the storage modulus and phase angle of the control and MTGase treated low salt batter samples tested in the course of the heating process. Each point on the curve is the average of at least two determinations obtained from separate experiments. Dynamic rheological measurement is a useful tool for studying the gelation mechanism as it allows direct comparison of the gelation with the aggregation process (Xiong, 1994). Storage modulus (G'), an elastic component of protein gels which can be measured with small-strain dynamic testing, is also a useful rheological parameter for delineating gel network properties.

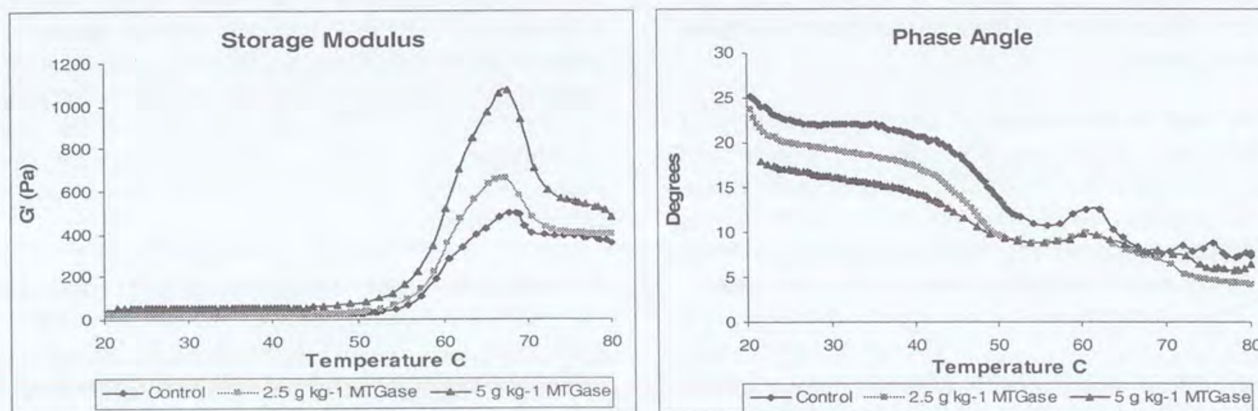


Fig 1 (a)

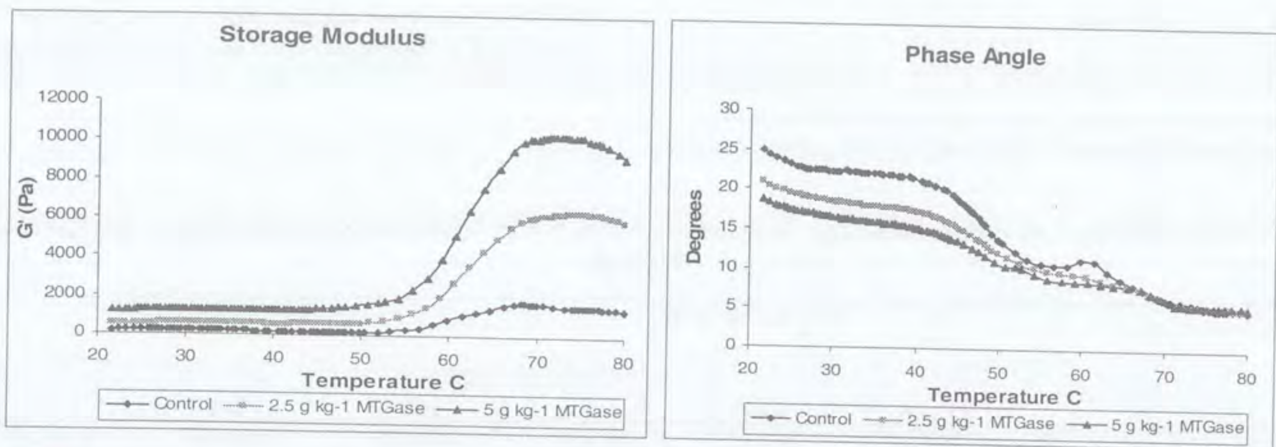


Fig 1 (b)

Fig 1: Representative rheograms of heat-induced low sodium (10 g kg^{-1}) batter gel [(a) 60 g kg^{-1} muscle protein (b) 100 g kg^{-1} muscle protein] after being treated with (2.5 or 5 g kg^{-1}) or without (control) MTGase.

Storage modulus of the control and MTGase treated low salt batters began to increase at around 53°C. The increase in G' in the 50-60°C region resulted from the aggregation of myosin oligomers, apparently involving tail-tail cross-linking, and form particles that probably make up the strands of the gel networks (Sharp and Offer, 1992) or result of intramolecular cross-links during incubation (gln-lys cross-linking) (Ramirez-Suarez and Xiong, 2003) to produce a more permanent network among MTGase treated low salt batter samples. MTGase treated low salt batter samples develop more elastic gel structure (greater storage modulus) with a lower onset gelling temperature than MTGase free batter sample on both protein level. The earlier onset of gelation for MTGase treated batter, when compared to control, suggests that a cross-linked protein have a lower temperature requirement for producing an elastic structure. Comparison between G' values for control and MTGase treated batters showed that addition of 2.5 or 5 g kg^{-1} MTGase resulted in up to 11 and 34% increase in G' values on heating at 80°C for lower protein batters while higher protein (100 g kg^{-1}) batters showed that 3 and 5 fold increase in shear values on same MTGase and temperature levels. The decline in G' at 68°C may attribute from decrease in gel elasticity resulting from unfolding of myosin domains and rearrangements of intermolecular linkages, followed by formation of a more permanent network structure.

However, storage modulus of high salt (25 g kg^{-1}) batters (control and MTGase treated) began to increase at 43°C (data not shown) whereas in low salt batters shown slight increase of G' at same temperature as a result of less extraction of salt soluble proteins. The increase in G' in the 40-50°C region resulted from the interfilamental association between heavy meromyosin (Egelandsdal, et al, 1986). The myosin head portion was denatured within the temperature range of 45-55°C and will result in temporary association of myosin filaments, thereby increasing the gel elastic response. As prolong heating, G' decreased, probably caused by denaturation of the light meromyosin which resulted in the redistribution of inter- and intramolecular forces (Xiong and Blanchard, 1994). During thermal gelation, myosin and salt-soluble myofibrillar protein exhibit complex changes in rheological characteristics depending on specific temperatures, pH and ionic strength (Egelandsdal et al., 1986; Xiong, 1993; Xiong and Blanchard, 1994). Gordon and Barbut (1992) have also indicated that different NaCl level (1.5 to 2.5%) resulted in different protein extraction levels in chicken meat batters. Further, they suggested that different ratios of soluble meat proteins were involved in the network formation process at the different salt levels.

Gelation of muscle protein results from transformation of an amorphous viscous solution to a three dimensional, relatively elastic network structure. Hence, dynamic gelation process could be monitored by measuring changes in stress-strain phase angle during oscillatory testing. The dynamic changes in the phase angle (ä) of the low salt batter samples exhibited two major peaks during the sol to gel transition temperature at 43 and 63°C respectively. However, high salt batter in similar condition reached one peak during the sol to gel transition at 43°C (data not shown). The occurrence of the peak would involve at least unfolding of salt soluble protein, which resulted in increased viscosity, followed by the intermolecular association of the salt soluble protein components to produce a more elastic gel network (Xiong and Blanchard, 1994). Phase angle values of low salt batter were recorded within the range 4.2-7.6 degrees, whereas high salt batter shown 2.4-4.4 degrees (data not shown) on final heating to 80°C. However, control and MTGase treated low salt batter samples were shown to be elastic. For an ideal viscous solution, ä equals 90°, while for an ideal elastic component, ä equals 0° (Ferry, 1980).

Mechanical properties

Puncture test parameters of heat induced low sodium batter gels are shown in Table 2, as a function of protein, MTGase level and cooking method. There were significant differences ($P<0.0001$) between muscle protein level and MTGase treatment on breaking force and gel strength of pork batter gel.

Table 2: Puncture Test Properties of Low Sodium Pork Batter Gel as Influenced by Protein, Mtgase and Cooking Method

		60 g kg^{-1} protein			*100 g kg^{-1} protein*		
		MTGase (g kg^{-1})					
		*Control**	*2.5*	*5*	*Control**	*2.5*	*5*
Breaking force (g)	W	$233.77^{a/x}$	$380.15^{b/x}$	$493.31^{c/x}$	$897.80^{d/x}$	$1668.90^{e/x}$	$1748.59^{f/x}$
		(20.45)	(9.70)	(39.87)	(74.02)	(107.88)	(85.81)
	R	$323.33^{a/y}$	$481.18^{b/y}$	$778.88^{b/y}$	$808.31^{b/y}$	$1740.04^{c/y}$	$1941.42^{d/y}$
		(9.71)	(39.33)	(35.49)	(61.87)	(162.34)	(49.34)
	M	$210.80^{a/x}$	$363.93^{b/x}$	$375.13^{b/z}$	$601.05^{c/z}$	$1151.41^{d/z}$	$1365.64^{e/z}$
		(20.90)	(26.05)	(16.70)	(54.42)	(74.30)	(51.42)
Deformation (cm)	W	$1.30^{a/x}$	$1.46^{b/x}$	$1.52^{b/x}$	$1.63^{c/x}$	$1.25^{a/x}$	$1.05^{d/x}$
		(0.05)	(0.04)	(0.02)	(0.01)	(0.08)	(0.06)
	R	$1.49^{a/y}$	$1.55^{a/x}$	$1.23^{b/y}$	$1.47^{a/y}$	$1.24^{b/x}$	$0.97^{c/x}$
		(0.11)	(0.03)	(0.12)	(0.10)	(0.20)	(0.09)
	M	$1.27^{ac/x}$	$1.35^{ab/y}$	$1.39^{b/z}$	$1.25^{c/z}$	$1.11^{d/y}$	$1.42^{b/y}$
		(0.05)	(0.06)	(0.06)	(0.01)	(0.07)	(0.20)
Gel strength (g × cm)	W	$302.90^{a/x}$	$555.72^{b/x}$	$748.85^{c/x}$	$1467.40^{d/x}$	$2092.77^{e/x}$	$1841.26^{f/x}$
		(29.58)	(11.95)	(72.56)	(131.97)	(189.98)	(174.23)
	R	$481.54^{a/y}$	$719.96^{b/y}$	$961.49^{c/y}$	$1195.57^{b/y}$	$2165.01^{d/x}$	$1871.70^{e/x}$
		(46.93)	(44.61)	(95.21)	(114.65)	(212.45)	(164.56)
	M	$269.04^{a/x}$	$491.78^{b/x}$	$520.51^{b/z}$	$748.93^{c/z}$	$1283.79^{d/y}$	$1936.36^{e/x}$
		(25.64)	(57.07)	(5.48)	(64.12)	(125.67)	(197.58)

Note: * no MTGase. W = water bath; R = retort; M = microwave ^{abcdef} Different letters in the same row indicate significant differences ($P<0.05$) at each treatment level; ^{xyz} Different letters in the same column indicate significant differences ($P<0.05$) at each treatment level.

Puncture test parameters were significantly increased ($P<0.0001$) in both types of protein formulations by addition of MTGase. The effect of low salt level was compensated by the addition of MTGase. Reducing salt limits protein extractability and alters thermal protein denaturation and/or aggregation patterns of the muscle proteins (Trouf and Schmidt, 1986), which affects the binding properties of meat products. Because salts play a key role in the solubilization of myofibrillar proteins for subsequent denaturation/ aggregation to give good water-fat-retention and acceptable rigidity/elasticity of the meat gels (Gordon and Barbut, 1992). In addition, this exudate protein serves as a binding agent and good substrate for cross-linking reactions by MTGase (Kuraishi et al, 1997; Ghavimi et al, 1987). However, Olson (1982) reported that a 25% reduction in NaCl is probably can be achieved without affecting product flavor and texture. Ruusunen and Puolanne (2005) also reported that 1.4% NaCl in cooked sausages and 1.75% in lean meat products are enough to produce a heat stable gel with acceptable firmness. Our results also support the findings of above authors. The low salt (1.5%) pork batter gel shows the acceptable gel strength and addition of MTGase enhanced the batter stability.

Table 3 shows the results of texture profile analysis of low salt batter gel samples in different MTGase and cooking methods. The hardness, fracturability, and chewiness values decreased when the protein level and MTGase was decreased from 100 to 60 g kg^{-1} and 5 to 0 g kg^{-1}, respectively. The use of MTGase improved the textural properties of the low salt batter gels as a function of the protein level and cooking method used. The highest values corresponded to 100 g kg^{-1} protein and 5 g kg^{-1} MTGase batter samples cooked in water bath. The increase in mechanical properties with MTGase levels was related to the decrease in intensity of the myosin heavy chain band in SDS-PAGE gels.

Table 3: Textural Properties of Low Sodium Pork Batter Gel as Influenced by Protein, Mtgase and Cooking Method

MTGase (g kg^{-1})		60 g kg^{-1} protein			100 g kg^{-1} protein		
		*Control**	*2.5*	*5*	*Control**	*2.5*	5
Hardness (g)	W	180.80$^{a/x}$ (17.32)	308.49$^{b/x}$ (24.36)	431.63$^{c/x}$ (40.23)	735.48$^{d/x}$ (46.10)	1323.26$^{e/x}$ (104.57)	1534.39$^{f/x}$ (69.27)
	R	293.49$^{a/y}$ (20.21)	510.94$^{b/y}$ (60.48)	561.14$^{c/y}$ (51.23)	844.89$^{d/y}$ (74.65)	1416.31$^{e/y}$ (131.82)	1434.80$^{e/y}$ (121.29)
	M	204.24$^{a/x}$ (19.65)	212.72$^{a/z}$ (25.31)	374.10$^{b/z}$ (28.69)	671.75$^{c/z}$ (52.98)	1268.57$^{d/z}$ (83.81)	1089.01$^{e/z}$ (107.25)
Fracturability (g)	W	147.32$^{a/x}$ (30.16)	257.91$^{b/x}$ (36.16)	372.64$^{c/x}$ (54.52)	697.80$^{d/x}$ (74.31)	1229.48$^{e/x}$ (142.69)	1444.65$^{f/x}$ (158.38)
	R	276.09$^{a/y}$ (47.36)	357.40$^{b/y}$ (40.71)	530.03$^{c/y}$ (57.41)	820.36$^{d/y}$ (81.47)	1299.48$^{e/y}$ (121.65)	1412.46$^{f/x}$ (153.25)
	M	160.12$^{a/x}$ (11.82)	167.15$^{a/z}$ (33.89)	325.86$^{b/x}$ (31.25)	666.40$^{c/x}$ (80.18)	1342.38$^{d/y}$ (104.17)	1226.87$^{e/y}$ (123.65)
Springiness (mm)	W	0.96$^{a/x}$ (0.02)	0.90$^{a/x}$ (0.14)	0.47$^{b/x}$ (0.10)	0.53$^{c/x}$ (0.06)	0.46$^{b/x}$ (0.05)	0.50$^{b/x}$ (0.06)
	R	0.90$^{a/x}$ (0.11)	0.80$^{b/y}$ (0.23)	0.44$^{c/x}$ (0.09)	0.53$^{d/x}$ (0.04)	0.41$^{c/x}$ (0.06)	0.39$^{c/y}$ (0.06)
	M	0.94$^{a/x}$ (0.04)	0.94$^{a/x}$ (0.04)	0.80$^{b/y}$ (0.09)	0.87$^{c/y}$ (0.14)	0.74$^{b/y}$ (0.2)	0.77$^{b/z}$ (0.14)
Cohesiveness	W	0.31$^{a/x}$ (0.01)	0.32$^{a/x}$ (0.05)	0.23$^{b/x}$ (0.04)	0.26$^{c/x}$ (0.03)	0.22$^{b/x}$ (0.02)	0.29$^{d/x}$ (0.01)
	R	0.32$^{a/xz}$ (0.02)	0.30$^{a/x}$ (0.03)	0.22$^{b/x}$ (0.04)	0.27$^{c/x}$ (0.04)	0.22$^{b/x}$ (0.01)	0.24$^{b/y}$ (0.01)
	M	0.34$^{a/z}$ (0.06)	0.31$^{b/x}$ (0.03)	0.30$^{b/y}$ (0.05)	0.35$^{a/y}$ (0.03)	0.21$^{c/x}$ (0.04)	0.20$^{c/z}$ (0.03)
Chewiness (g × mm)	W	53.04$^{a/x}$ (11.99)	78.30$^{b/x}$ (18.44)	61.77$^{a/x}$ (22.05)	94.27$^{c/x}$ (20.49)	155.89$^{d/x}$ (18.67)	190.57$^{e/x}$ (16.25)
	R	82.66$^{a/y}$ (13.60)	119.92$^{b/y}$ (24.64)	68.35$^{c/x}$ (12.03)	113.27$^{b/y}$ (13.64)	117.03$^{b/y}$ (15.24)	118.58$^{b/y}$ (18.23)
	M	67.41$^{a/x}$ (22.03)	70.84$^{a/x}$ (18.60)	110.03$^{b/y}$ (6.81)	206.06$^{c/z}$ (19.54)	232.61$^{d/z}$ (20.78)	243.05$^{d/z}$ (18.45)

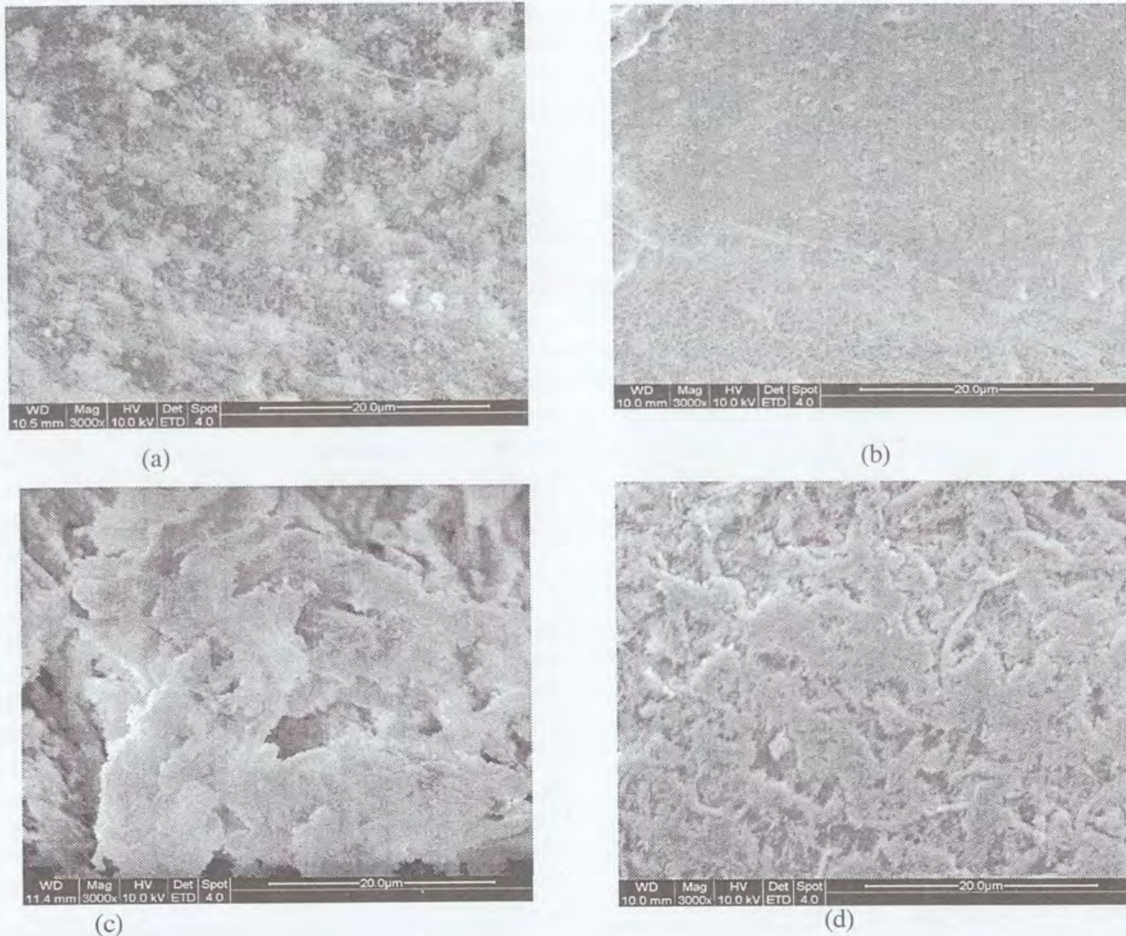
* no MTGase. W = water bath; R = retort; M = microwave. ^{abcdef}Different letters in the same row indicate significant differences (P<0.05) at each treatment level; ^{xyz} Different letters in the same column indicate significant differences (P<0.05) at each treatment level at each parameter.

The results agreed with the findings of Téllez-Luis et al (2002). Significant effects were observed for protein, MTGase level and cooking method on hardness, fracturability, springiness, chewiness and cohesiveness (P<0.0001 and P<0.001, respectively). Except springiness and cohesiveness, other parameters generally, increased with increases in protein and MTGase levels. Regardless of the protein content the gels processed with increasing MTGase levels exhibited higher hardness, fracturability, and chewiness in relation to ones manufactured without or with lower levels of added MTGase. The cohesiveness and springiness values decreased when the protein and MTGase levels were increased from 60 to 100 g kg^{-1} and 0 to 5 g kg^{-1}, respectively. The influence of protein and MTGase levels on textural parameters were additionally affected by interaction with cooking method. A number of authors have found higher salt level in comminuted meat products to be harder than low-salt ones. Kuraishi et al (1997) found that adding salt (1–3%), with MTGase caused an increase in binding strength when compared to MTGase alone.

Jiménez-Colmenero et al (1998) reported that textural properties (hardness and chewiness, and lesser extent springiness and cohesiveness) of low fat meat batters were influenced by internal batter temperature as well as the salt level. High internal temperatures produced harder and chewier meat batters. Similarly, 2.5% salt were produced harder, chewier and springier batters than those produced with 1.5% salt. Similar results have been reported (Foegeding and Ramsey, 1987; Barbut and Mittal, 1990; Carballo et al, 1996; Camou et al, 1989; Cofrades et al, 1997). Uresti et al (2004) reported that hardness and springiness values increased with increasing salt level from 0 to 2% in fish products. However, in our study, there were noticeable variations with respect to cooking methods (Table 3). At present study, using heat-induced aggregation, we found that 15 g kg^{-1} NaCl allows MTGase to promote cross-linking reactions, which improved the mechanical properties of the meat batters.

Microstructure

The effect of salt and MTGase on microstructure of the raw batter gels may be explained on the basis of molecular interactions. The structure of a batter gel in the presence of 25 g kg^{-1} salts shows (Fig 2 a & b) that the protein has solubilized to form a uniform network while low salt (15 g kg^{-1}) batter (Fig 2 c & d) illustrate that sponge like network structure.



(a) (b) (c) (d)

Fig 2: Scanning electron micrographs (magnification: 3000×) showing the microstructure of 100 g kg^{-1} pork muscle protein batter gel formulated with 25 g kg^{-1} salt (Fig 2. a & b) or 15 g kg^{-1} salt (Fig 2. c & d) in the presence (Fig 2. b & d) or absence (Fig 2. a & c) of 5 g kg^{-1} microbial transglutaminase.(Bar = 20 ì m)

The sponge like structure is less suited to harbor water or attain appreciable gel strength (Siegel and Schmidt, 1979). Comminution of muscle at high ionic strength causes swelling of muscle fibers (Wilding et al., 1986), solubilization of myosin, and extraction of myofibrils from the muscle fibers. Low-temperature gelation of the solubilized myofibrillar proteins may increase the viscosity of the protein matrix (Nakayama et al., 1983). Salt increase the ability of salt soluble protein to bind meat pieces primarily by solubilizing the protein. This facilitates the molecular interactions that are necessary to produce uniform network structure of protein which gives the greater strength. However, addition of MTGase shows (Fig 2 b & d) that the protein has aggregated to form a compact network while absence of MTGase (Fig 2 a & c) demonstrate that looser network. MTGase treatment led to aggregate gel networks through the formation of intermolecular å-(ã-glutamyl)-lysyl cross-links. This compact network of protein batter contributes to the strength of the gel due to the occurrence of a greater number of molecular interactions.

SDS-PAGE

The electrophoretic profiles of myofibrillar proteins obtained from the different cooking methods are shown in Fig 3. The high intensity of myosin band in control samples indicates no interactions or even low non-disulfide covalent protein interactions.

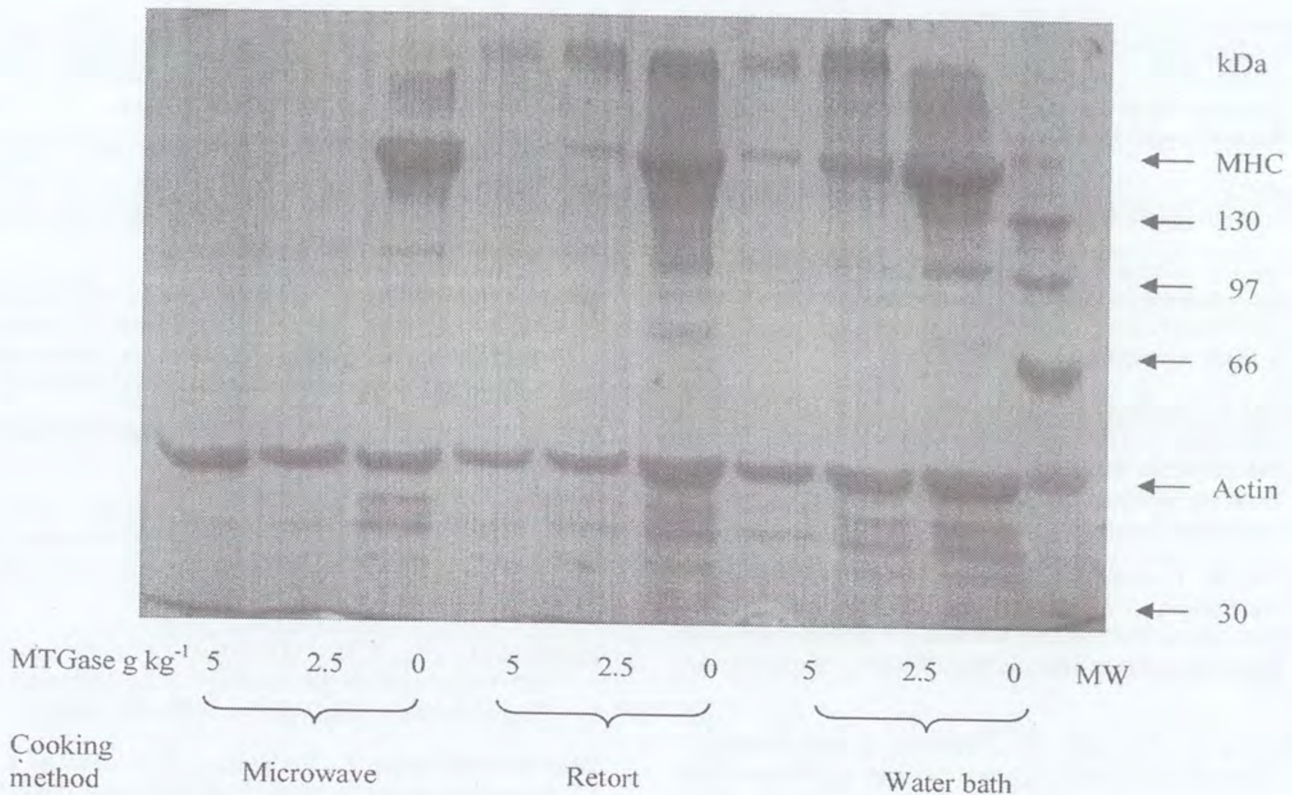


Fig 3 Effect of three cooking method on electrophoretic profile of pork leg myofibrillar protein with (2.5 or 5 g kg-1) or without (control) MTGase treatment. (MHC = Myosin heavy chain, MW = molecular weight standard)

Regardless the cooking method, the intensity of myosin band was reduced gradually using 5 and 2.5 g kg^{-1} MTGase. The decrease in intensity indicates increasing myosin aggregation during the over night incubation. MTGase induce covalent protein-protein interactions could be reason for the above effect. This type of covalent interactions cannot be disrupted by sample buffer containing sodium dodecyl sulfate and â-mercaptoethanol during sample preparation. The increase in breaking force, hardness and fracturability with MTGase levels was related to the decrease in intensity of the myosin heavy chain band in SDS-PAGE gels.

Conclusions

The gelation process was different in low salt batters. However, addition of MTGase increased the gel viscosity. At the same cooking method MTGase treated batters formed different gel matrix with stronger binding properties than control samples. In addition, three cooking methods also had dissimilar effect on punch test and textural properties due to temperature variations and heating rate encounter in three methods. Whatever meat and salt level used, addition of MTGase improved the mechanical properties. Regardless the salt concentration gels processed with MTGase treatment influenced the rheological and textural properties; however the efficiency of enzyme treatment depended on the cooking methods.

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Shelf Stability and Sensory Quality of a Fried Chick-Pea Snack Incorporated With Soy Protein Concentrate

REENA KUMARI R.K. and JAMUNA PRAKASH*

Department of Studies in Food Science and Nutrition, University on Mysore, Manasagangotri, Mysore, 570 006, India.

The objective of study was to incorporate soy protein concentrate (SPC) to a chick pea based deep fried traditional product namely 'Seviya' and study its oil absorption, keeping quality and sensory quality. SPC replaced the base flour at 10, 15 and 20% levels in products, which were analyzed for oil uptake, free fatty acid contents (FFA) and sensory attributes by standard techniques. Products without SPS served as controls. Results revealed that incorporation of SPS increased the fat absorption in products in proportion to the amount of SPS added ranging from 30.3 to 40.9%. The FFA throughout the storage period was very low indicating the shelf stability of product. Products were stored in PET and steel containers at low and room temperature and subjected to sensory analysis. Soy incorporated products scored consistently higher for all sensory attributes indicating SPS can be used to improve the sensory quality of fried snacks.

Key words: Oil uptake, expansion ratio, free fatty acids, sensory attributes, packaging materials.

Introduction

Deep frying of foods in oil is one of the traditional technologies used very commonly for production of shelf stable snack foods in India. Deep fried snacks are sold as ready-to-eat foods and are specially prized for their sensory appeal despite their high fat and high calorie content. In general, people from different cultures prefer deep fried snacks for their crunchy texture and delicious flavour (Boskou *et al*., 2006). During deep frying, the oil acts as a medium to transfer heat to the food and removes moisture in the process, thus altering the textural quality of foods (Choe and Min 2007). This also increases the oil content of food which could be anywhere between 20-40%. Therefore, from the health point of view, the high fat content may be undesirable due to various adverse effects associated with high fat intakes and resultant obesity. Another potential harm could come through the use of oil for frying at high temperatures either for a long time or for refrying which could probably increase the polar components and secondary oxidation products in fried foods (Mellema 2003; Saguy and Dana 2003).

The process of frying comparatively needs shorter cooking time and is easier as high oil temperatures cook the food quickly. The oil absorption depends on many factors such as the composition of food being fried, the composition of oil, the frying temperatures, the moisture content, and use of shortening and leavening agents in the product. The moisture in the product is replaced with oil, however the water soluble components do not leach in oil (Saguy and Dana 2003). Continuous heating of oil during frying gives rise to deteriorative reactions such as hydrolysis, oxidation and polymerization. These result in formation of several volatile and non-volatile decomposition products, hence it is necessary to monitor the quality of oil being used for frying and of the product being fried. The shelf stability of the product being fried also depends on the composition of oil and of the ingredient (Choe and Min 2006; Paul and Mittal 1997). The formation of decomposition products is mainly due to thermal oxidation and polymerization of unsaturated fatty acids present in the oil. These products remain in the frying oil to promote further degradation and absorbed by the fried food and could be eaten by the consumer (Saguy and Dana 2003).

Despite an increase in the consumption of deep-fried products due to their taste quality and shelf stability, consumers demand low fat products for health reasons (Lloyd et al., 2004). Hence, exploration of technologies which can reduce fat absorption in foods is beneficial to the food processor. For formulating low fat baked products different types of fat substitutes have been used. However, for fried products, a different approach is required to reduce fat absorption. Research work has been done on the use of proteins and hydrocolloids as ingredients to reduce oil uptake in deep fried foods (Mallikarjunan et al., 1997). Huse et al., (1998) evaluated the effectiveness of edible coatings formulated from hydroxypropyl methylcellulose, methylcellulose, corn zein and amylose in restricting oil absorption from a deep- fried traditional West African snack and reported that coated sample absorbed significantly lesser oil. Many studies report the use of edible ingredients for batter and beading mix to improve the coating performance and reduce fat absorption (Duxbury 1989). Different ingredients used for such purposes are powdered cellulose (Pinthus et al., 1993); methylcellulose (Williams and Mittal, 1999) and other additives and gums (Bajaj and Singhal 2007; Gowri et al., 2008; Puyed and Prakash, 2009). The use of proteins, such as soy protein isolate (Martin and Davis, 1986;

*Corresponding Author; Emil: jampr55@hotmail.com

Rayner *et al*,. 2000), milk powder and egg albumin as ingredients in fried products is also known (Pradeep et al., 1999).

The base ingredients used for the preparation of fried snacks are many. Some are just vegetable based as potato and banana chips or legumes fried in whole or decorticated form while some use cereal or legume flours as batters or dough for preparing products. In cereal and legume based products, the protein and starch components help in providing structure and texture to fried products. The most popular legume used for deep frying is chick pea (*Cicer arietinum*) which in the flour form serves as a base for many products '*Pakora*', '*Boondi*', '*Mathi*', '*Seviya*' etc (Singh and Tyagi 2001). *Seviya* is a deep, salted, and spiced snack item prepared all over India in different forms. Traditionally, it is prepared from chick pea (also known as *Bengal gram*) flour with additives such as spices, rice flour and sodium bicarbonate to impart crisp and crunchy texture to the fried product. Incorporation of full fat soy, defatted soy and soy protein concentrate as well as other ingredients to fried products including '*seviya*' has been reported by many workers. These have been attempted with a view to improve product quality, increase shelf life and reduce cost of the products (Ahluwalia et al., 1995; Anantha and Daya 2006; Pallavi et al., 1993; Rakshpal and Gurumukh, 1989; Simmi and Gurumukh 1991).

A reduction in oil uptake on incorporation of soy flour to a cereal based deep fried product was observed in our earlier studies (Puyed and Prakash 2007, 2009). Soy flour was also effective in reducing oil uptake in dehydrated preserved products which are consumed after deep frying (Puyed and Prakash 2008). Hence it was of interest to see whether soy used in the form of its concentrate also has the same effect. Hence, we chose the product commonly called as '*sev*'or '*seviya*' to study the effect of incorporation of soy protein concentrate on the oil uptake, shelf stability and sensory quality of product.

Materials and Methods

Materials **:** The raw ingredients used for the study namely chick-pea flour (*Cicer Arietinum*), omum, (*Trachyspermum ammi*) salt and rice (*Oryza Sativa)* bran oil were purchased from the local market in packed form. Chick-pea flour is milled from whole chick-pea after removal of outer brown colour husk and is yellow in colour. Soy protein concentrate (SPC) was procured from Dupont-Solay Company. Rice bran oil was used as the frying medium. The chemicals used for the analysis were from Sd Fine Chemicals Ltd., Mumbai. Glass double distilled water was used for all analysis. All analysis was carried out in duplicate.

Methods **:** The study was designed using the traditional recipe to prepare *seviya*. SPC was incorporated to base flour at three different levels replacing the base flour. The products were analysed for fat absorption and sensory quality initially. The shelf stability was also studied by storing the product in different packaging containers and analyzing free fatty acid content and sensory attributes at periodic intervals.

Preparation of products **:** For the preparation of control product (P-1) the procedure was as follows, Chick-pea flour was sieved through 60 mesh sieve. It was mixed well with shortening (10% heated and cooled rice bran oil), salt (2%) and omum (0.5%). It was allowed to rest for 10 min and then a very soft dough was made using water. The dough was filled into a mould which had small round multiple holes of 1.0 mm diameter. The dough was extruded through the mould into hot rice bran oil (175°C) and fried for 5-6 min. The product was drained of excess oil and removed from oil after development of golden brown colour (cessation of bubbling signified the end of frying). The control product (**Product-1**) was prepared only with chick-pea flour. For experimental products, chick-pea flour was replaced with 10% (**Product-2**), 15% (**Product-3**) and 20% (**Product-4**) of SPC. In a typical batch, 500g of flour and 500 ml of oil were used for preparation of product. Each product was fried separately and fried oil was not reused for any of the product. The composition of products prepared is given in Table 1.

Table 1: Composition of formulated snack products

Products	*Bengal gram flour (g)*	*Soy protein concentrate (g)*	*Salt (g)*	*Omum (g)*	*Oil shortening (ml)*	*Water (ml)*
Product-1 Control	500	-	10	2.5	50	750
Product-2 (SPC -10%)	450	50	10	2.5	50	430
Product-3 (SPC -15%)	425	75	10	2.5	50	410
Product-3(SPC-20%)	400	100	10	2.5	50	400

SPC: Soy protein concentrate.

Analysis: The prepared dough was analyzed for its moisture content and the products were analyzed for their frying time, expansion ratio, fat uptake and free fatty acid contents (FFA). The FFA contents of fresh and fried oils were also analyzed. For analysis of moisture, the samples were placed in a dry air oven at 60°C for 6-8 hours and weighed repeatedly after cooling in a desiccator till a constant weight was recorded. Moisture was determined as loss in the weight of sample (AOAC, 1990). The expansion ratio of product was determined as follows - a piece of fried sample was taken and the diameter measured using screw gauge in mm. The expansion ratio was calculated as percent increase over the diameter of the orifice of the extrusion mould. The fat absorption of the fried product was determined using Soxhlet apparatus by repeated extraction of oil with petroleum ether (Boiling Point, 60-80°C), removing the residual solvent over a water bath, drying the sample in oven for 1-2 hours and weighing the oil extracted (AOAC 1990). Fresh and fried oils were also measured for free fatty acid content. Acid value of a fat/oil is the number of milligrams of potassium hydroxide required to neutralize the free acids in known amount of sample under prescribed conditions and is expressed as free fatty acids (FFA) present in the sample (AOCS 2000). For determination of FFA as indicative of keeping quality, the oil from the fried products was extracted in petroleum ether (boiling point, 60-80°C) and subjected to analysis.

Storage study: Since fried products are generally stored and used over a period, two different packaging material i.e, PET (Polyethylene terephthalate) and stainless steel container were selected for storage study. The prepared products were stored at room temperature and in refrigerator (4°C) using two materials. All the products were analyzed for free fatty acid contents on 0th, 7th, 14th and 21st days of storage.

Sensory analysis : The sensory quality of products was determined by subjecting the prepared products to sensory analysis on the day of preparation and on storage at the end of I and II week. The attributes tested were appearance, color, texture, aroma and taste with the help of a score card by 25 panel members. The evaluation was done using a 10-point hedonic scale for sensory parameters. Coded samples were presented to panel members with a score card with a rating scale representing quality grade description as 1-2, poor; 3-4, fair; 5-6, good; 7-8, very good and 9-10, excellent (ISI 1972). The panel members were students of the institution, who were familiar with the sensory analysis techniques. They were selected based on a screening test conducted using standard threshold tests for salt solution.

Statistical analysis : The analytical and sensory analysis data were subjected to statistical analysis making use of Minitab statistical software using suitable tests. Mean and standard deviation were calculated individually for all parameters of analysis and sensory attributes for each product. Analysis of variance was done to find out significant difference between the free fatty acids in stored products and quality attributes of prepared snacks.

Results and Discussion

The results of the study are presented in Tables 2- 5 and Fig.1. The dough parameters and certain observations on yield of product are presented in Table 2. The estimated moisture content of dough ranged between 43.6 to 53.7%. The moisture content of control product was highest due to higher content of water required for dough formation. The weight of the dough was also highest for control product at 1175 and decreased on incorporation of SPC.

Table 2: Dough parameters and yield of prepared products

Variation	*Weight of dough (g)*	*Weight of product (g)*	*Expansion ratio (%)*	*Frying time (min)*	*Moisture (%)*
Product-1 (Control)	1175	684	10	30	53.7
Product-2 (SPC-10%)	947	694	30	30	45.6
Product-3 (SPC-15%)	926	764	30	30	44.2
Product-4 (SPC-20%)	916	768	30	30	43.6

SPC: Soy protein concentrate.

This shows that chick-pea flour had a higher water absorption capacity in comparison to SPC, this can be attributed to the presence of starch in chick-pea flour which reduces on addition of soy protein concentrate. This is in contrast to what has been observed for incorporation of defatted soy flour in the products. Many workers report an increase in water absorption capacity of soy incorporated dough (Singh and Singh, 1989; Ahluwalia et al., 1995; Simmi and Gurumukh 1991). The weight of product however, was slightly lesser in control and increased with 15 and 20% SPC incorporation. The expansion ratio of product showed 10% increase (1.1mm) for control and showed an increase of 30% (1.33mm) for SPC incorporated products indicating an increase in expansion ratio of soy incorporated products. The cooking time was more or less similar for all variations. Singh and Singh (1989) studied incorporation of defatted soy flour in preparation of *seviya* and its effect on quality characteristics of product. Bengal gram flour was blended with defatted soy flour at 10 to 30% level. During product preparation, the blends absorbed greater volumes of water than control for dough making. The results revealed that the hardness of the product as well as diameter and expansion ratio increased up to 15% level of defatted soy flour and thereafter decreased.

The oil absorption of product presented in Table 3 shows that it was least in control product at 30.3%. On addition of SPC, the products absorbed a remarkably high content of oil, this is in contrast to many observations regarding soy flour which is known to reduce fat absorption including in our laboratory (Puyed and Prakash 2007 & 2008).

Table 3: Initial chemical and sensory analysis of products

Parameter	*Products*				
	Product-1	*Product-2*	*Product-3*	*Product-4*	*P value*
Chemical analysis					
Fat absorption (%)	30.3± 0.199	37.1± 0.020	36.7± 0.143	40.9± 0.403	-
FFA in fried oil[a] (%)	0.024	0.027	0.048	0.049	-
FFA in product (%)	0.344	0.434	0.411	0.408	-
Sensory analysis					
Appearance	5.52±1.33	7.36±0.99	7.56±1.23	7.52±1.12	0.000***
Color	5.36±1.35	7.32±1.07	7.28±1.34	7.16±1.25	0.000***
Texture	6.48±1.69	7.36±0.95	7.08±1.32	6.88±1.39	0.145ns
Aroma	6.48±1.66	7.16±1.11	7.16±1.18	6.84±1.65	0.028*
Taste	6.44±1.61	7.64±1.11	7.28±1.28	7.16±1.43	0.022*

[a: FFA in fresh oil - 0.017 %]
P-1: Control product, **P-2:** 10% soy protein, **P-3:** 15% soy protein and **P-4:** 20% soy protein.
Values represent mean± standard deviation of sensory responses of 25 panelists.
F- Ratio: ns – not significant, * - $P<0.05$, ** - $P<0.01$.

However, it may be noted that in this study, SPC was used which could influence the fat absorption and was distinctly different from defatted soy flour. Since soy proteins have a large number of hydrophobic groups, it is possible that a higher number of these in protein concentrate increased fat absorption. The product with 20% SPC absorbed 35% higher fat in comparison to control. According to Narayana and Narasinga Rao, (1982) processing of SPC increases the fat absorption capacity due to dissociation of proteins that occurs on heating and also due to denaturation which may occur on most the non polar residue of protein molecules. Singh and Seetha (1993) examined the oil absorption properties of *seviya* prepared from different grain legumes and reported fat uptake ranging from 31.5 to 35.6%, which is very similar to what was seen in our study.

The FFA content of fresh rice bran oil was found to be 0.017% and in fried oil a slight increase was observed. The values ranged from 0.024 to 0.049%. However, the increase was negligible from health point of view. On the preparation day, the products had very low FFA content which was 0.344 for control, in SPC incorporated product the values were slightly higher ranging from 0.408 to 0.434%. This shows that rice bran oil can be used as a frying medium and is quite stable to frying temperatures.

The prepared product was stored in PET and steel containers both at room temperature and under refrigeration. These were analyzed for FFA on 0th, 7th, 14th and 21st days of storage. The results are presented in Fig. 1. On storing the product there was an increase in FFA content, the increase varied depending upon the storage and temperature of storage. The control product stored at room temperature had values of FFA which were 0.491 and 0.550% for PET and stainless steel containers respectively at the end of storage period. Similar product stored under low temperature showed 0.449 and 0.485% FFA. On incorporation of SPC there was a slightly higher FFA formation in all products. In the products stored at room temperature in PET bottles the values were between 0.501 and 0.560% and in the product stored in stainless steel containers the values were 0.544 and 0.626%. It may be noted that these FFA content are presented for extracted oil and the amount in actual product will be much lesser. In the product stored under refrigeration, the FFA values ranged between 0.489 to 0.563 for all containers, differences were not noted between the type of containers as low temperature inhibited FFA formation. In comparison to control, SPC incorporated product had higher FFA formation.

On analysis of variance it was found that there were no significant differences in the free fatty acid content of any stored products. Hence it can be said that as per the chemical analysis the products were stable for the duration of storage study and the storage conditions did not influence the free fatty acid contents of products.

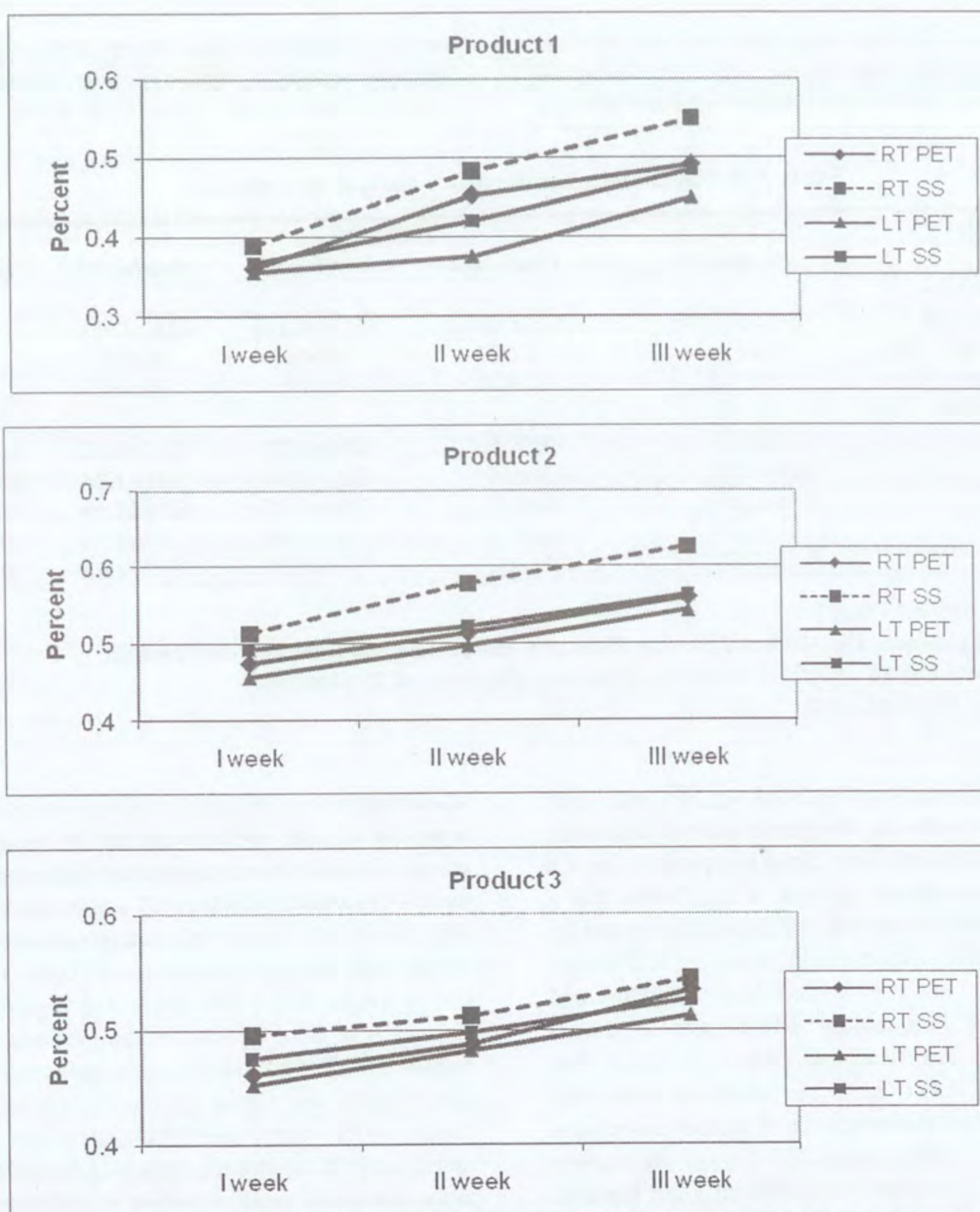

Fig.1. Effect of storage on free fatty acid contents of products stored in PET and steel containers [RT- Room temperature, LT- Low temperature, SS – Stainless steel]

Sensory analysis :

The mean sensory scores of products and statistical analysis for the scores are presented in Table 3-5. The initial mean sensory scores of the product presented in Table 3 show that the control product had scores ranging from 5.36 to 6.48 for various quality attribute. On incorporation of SPC, a remarkable increase in sensory parameters was seen wherein the values ranged from 7.16 to 7.64 for the product with 10% SPC, 7.08 to 7.56 for product with 15% SPC and 6.84 to 7.52 for product with 20% SPC. The addition of SPC seemed to improve all the sensory quality attributes of chick-pea based snack product. This was found to be very surprising as the control product itself is known for high acceptability. On incorporation of SPC the acceptability scores increased further. The differences were found to be highly significant for the quality of appearance and color, marginally significant for aroma and taste and non significant for texture. A significant increase in the acceptability of soy incorporated products has also been reported by Singh and Singh (1989). An improvement in the textural quality of a similar fried product on incorporation of defatted soy flour was observed by Ahluwalia et al. (1995).

The mean scores of product stored in steel container are presented in Table 4. In this range of products the values obtained by control samples were lesser than what was given for SPC incorporated product. For the quality of appearance, control had a score of 5.94 whereas soy SPC incorporated product were given scores between 6.76 to7.32, for the quality

of color, the control obtained low value of 5.0 with the experimental products obtaining scores between 6.9 to 7.92. According to the grading scale described the differences were in a scale of fair to good. Statistically these differences were highly significant (P≤ 0.001). For texture, the control sample was given a score of 6.04 and SPC incorporated sample had scoring of 6.96 to 7.36, although all scores were within the quality grade description of 'good', the differences were found to be marginally significant (P≤0.05). For the quality of aroma the scores given were lesser than what was seen on the initial day. The control value was given a scoring of 5.32, whereas the SPC incorporated products had higher value of 6.52 to 7.0. Similar scoring was also given for the quality of taste. The differences were highly significant for aroma and significant for taste.

Table 4: Sensory scores of products stored in steel container

Sensory attributes	*Product-1*	*Product-2*	*Products* *Product-3*	*Product-4*	*'P' value*
Low Temperature					
I week					
Appearance	5.94±1.34	6.76±1.27	7.32±1.14	7.12±1.17	0.00***
Color	5.04±1.40	7.00±1.50	7.20±0.82	6.92±1.50	0.00***
Texture	6.04±2.01	6.96±1.31	7.12±1.30	7.36±1.29	0.015*
Aroma	5.32±1.60	6.52±1.69	6.88±1.05	7.00±1.55	0.0004***
Taste	5.76±1.69	6.76±1.92	7.20±1.35	7.00±1.41	0.0107*
II week					
Appearance	4.76±1.20	7.60±1.53	7.48±1.53	8.12±1.20	0.000***
Color	4.92±1.08	7.68±1.55	7.40±1.53	8.52±1.22	0.000***
Texture	6.48±2.06	7.20±1.44	7.68±1.44	7.88±1.33	0.000***
Aroma	5.72±1.79	7.36±1.66	7.72±1.28	8.04±1.39	0.000***
Taste	5.68±1.77	7.36±1.73	8.00±1.15	8.00±1.29	0.000***
Room Temperature					
I week					
Appearance	5.08±1.43	6.56±1.46	7.12±1.64	7.16±1.42	0.000***
Color	5.00±1.31	7.32±1.20	7.36±1.75	7.16±1.25	0.000***
Texture	5.76±1.61	6.68±1.65	7.28±1.52	6.76±1.67	0.019*
Aroma	5.76±1.29	6.40±1.49	6.80±1.74	6.80±1.63	0.097ns
Taste	5.92±1.42	7.12±1.72	7.52±1.70	7.00±1.60	0.004**
II week					
Appearance	4.64±1.26	7.36±1.41	7.00±1.39	7.56±1.75	0.000***
Color	4.56±0.90	7.36±1.55	7.48±1.53	7.76±1.50	0.000***
Texture	5.60±1.72	7.24±1.21	7.68±1.37	7.24±1.56	0.000***
Aroma	5.64±1.65	7.16±1.38	7.60±1.57	7.12±1.88	0.000***
Taste	5.52±1.77	7.80±1.24	7.84±1.22	7.44±1.47	0.000***

P-1: Control product. **P-2:** 10% soy protein. **P-3:** 15% soy protein and **P-4:** 20% soy protein.
Values represent mean± standard deviation of sensory responses of 25 panelists.
F- Ratio: ns – not significant, * - P≤0.05, ** - P≤0.01, *** - P≤0.001.

Sensory analysis for the product was continued for another week and samples were analyzed on 14th day. The sensory scores for products stored in steel container under low temperature indicate that the trend of scoring was similar in comparison with 7th day results. The control products were given very low scores of 4.76 and 4.92 for appearance and color and slightly higher score of 6.48 for texture. For aroma and taste the scores were 5.72 and 5.68 respectively. The product with 10% soy obtained scores in the range of 7.2 to 7.68. The product with 15% soy had still higher values ranging from 7.4 to 8.0 and the product with 20% soy was given highest scores ranging between 7.88 to 8.52. For some of the quality attributes, the scores obtained were higher than what was seen on 7th day. The differences were highly significant for appearance, color, aroma and taste and marginally significant for texture.

The mean sensory scores of the product stored in steel containers at room temperature are also presented in Table 4. The scores were almost similar to product stored under low temperature. The lowest scores were given for appearance and color of control product. The scores improved significantly for SPC incorporated product. The quality of texture was marginally different among products with highest score for product with 15% soy followed by product with 20% soy, 10% soy and control. The quality of aroma ranged between 5.76 to 6.8 and there were no significant differences among products for their attributes, the taste differed significantly with SPC incorporated product obtaining higher values. The product stored for 14th days also showed a similar profile with control obtaining lesser score and experimental obtained higher scores. However, the scores were slightly

lesser than obtained by products stored under refrigeration, the range being 7 to 7.84.

The mean scores and ANOVA of product stored in PET containers are presented in Table 5. The control product stored for 7 days under low temperature obtained low scores for appearance and color. The scores given to SPC products were higher in both the categories. For the quality of texture, significant differences were seen between control and experimental product, the grading being fair and good respectively. The aroma of control product were found to be similar to texture for all product, however the differences were marginally significant. The quality of taste was also better for soy product with higher score (7.48 to 7.64) and the control having a lower value of 6.24. The differences were significant at P≤0.001. The products which were stored in PET containers for 14 days showed similar sensory responses as seen in steel container stored at low temperatures. Here also the values ranged between 4.96 to 6.24 for the control product and 6.96 to 8.24 for SPC incorporated products, the differences were statistically significant for all attributes.

Table 5: Sensory scores of products stored in PET container

Sensory attributes	*Products*				
	Product-1	*Product-2*	*Product-3*	*Product-4*	*'P' value*
Low Temperature					
I week					
Appearance	4.84±1.60	6.64±1.85	6.96±1.46	7.08±1.29	0.000***
Color	4.76±1.39	6.72±1.95	7.04±1.72	7.36±1.47	0.000***
Texture	5.72±1.65	6.92±1.63	7.12±1.42	7.40±1.22	0.001***
Aroma	5.72±1.54	6.76±1.81	6.56±1.26	7.00±1.22	0.017*
Taste	6.24±1.64	7.64±1.50	7.48±1.58	7.60±1.32	0.003**
II week					
Appearance	4.96±1.31	7.56±1.13	8.12±0.91	7.36±1.26	0.000***
Color	5.08±1.35	7.92±1.02	8.04±0.87	6.96±1.59	0.000***
Texture	6.24±2.01	7.80±0.89	7.96± 1.15	7.56±1.20	0.000***
Aroma	5.84±1.71	7.84±1.19	7.80±1.41	7.60±1.44	0.000***
Taste	6.20±1.67	8.02±1.13	8.24±1.10	8.04±1.46	0.000***
Room Temperature					
I week					
Appearance	4.92±1.41	6.96±0.98	6.92±1.32	7.12±1.56	0.000***
Color	4.48±1.19	6.80±1.26	7.40±1.91	7.28±1.17	0.000***
Texture	6.00±2.04	6.44±1.45	7.16±1.21	7.36±1.19	0.006**
Aroma	5.72±1.57	6.64±1.35	7.28±1.49	7.20±1.44	0.000***
Taste	6.04±1.90	6.96±1.23	7.24±1.30	7.24±1.45	0.016*
II week					
Appearance	5.04±1.18	7.20±1.26	7.76±1.42	7.96±1.64	0.000***
Color	4.56±1.44	7.08±1.26	7.88±1.48	7.68±1.67	0.000***
Texture	6.04±2.10	7.08±1.19	7.60±1.17	8.28±1.04	0.000***
Aroma	5.60±2.00	7.12±1.39	7.64±1.47	7.40±1.50	0.000***
Taste	5.64±1.72	6.96±1.28	7.60±1.65	7.68±1.54	0.000***

P-1: Control product, **P-2:** 10% soy protein, **P-3:** 15% soy protein and **P-4:** 20% soy protein.
Values represent mean± standard deviation of sensory responses of 25 panelists.
F- Ratio: ns – not significant, * - P≤0.05, ** - P≤0.01, *** - P≤0.001.

The mean sensory scores of the product stored in PET containers for 7days at room temperature indicated that products obtained a lower scores for appearance and color for control product and higher scores for other products. The quality of texture differed significantly with control and 10% soy product scoring lower values, and the other two products scoring higher values. Aroma differed significantly with lowest value for the control followed by product with 10% soy, 20% soy and 15% soy. However, for the quality of taste the differences were marginally significant, the trend of scoring being similar to other attributes. The product stored in PET containers at room temperature for 14 days also showed a similar response. However, the scores obtained were better than what was given to product stored at room temperature for 14th days. The values were highly significant and different from each other.

Simmi and Gurumukh (1991) prepared *seviya* from blends of Bengal gram, defatted soy and rice flour and studied their organoleptic characteristics. They reported that products made from blends containing 10-15% soy and rice flour were similar to control with respect to overall acceptability. A similar study done in our laboratory using Sorghum flour instead of chick pea flour for soy protein incorporation revealed an increased fat uptake in soy incorporated products. Fat content of control product was 27.5% whereas that of soy incorporated products ranged from 30.8 to 40.9%. The free fatty acid levels, though showed a gradual increase with storage time, were

not affected by soy incorporation. The initial free fatty acid levels were in the range of 0.243 – 0.465% and at the end of 21 days, they increased to 0.961-0.987%. The products stored in PET containers at low temperature were better. The sensory quality of soy incorporated products was better in terms of higher sensory scores and storage of products did not lower the sensory quality of products (Kumari and Prakash, 2009).

Conclusion

In conclusion, it can be said that incorporation of SPC to chick pea flour at different levels for preparing a fried snack resulted in increased fat absorption in product. The extent of increase was proportional to the level of soy proteins added. The products were stable for 3 weeks as judged by monitoring the free fatty acid levels during storage. Sensory analysis of products indicated that incorporation of SPC to chick-pea flour improved all the sensory attributes of the products, the product stored at low temperature was better than product stored at room temperature. Among steel and PET containers, PET containers were better for storage. Soy protein incorporation to fried products can be recommended for improving sensory quality.

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Genetic Variability of Three Xylanase-Inhibiting Proteins Present in Wheat Grain

PRAKASH OLI* and EVI CORES

Laboratory of Food Chemistry, Katholiek University of Leuven, Belgium

Study on three xylanase inhibitors namely TAXI, XIP and TLXI found in the wheat grains were studied in proteomics approach by using high resolution 2-DE gel electrophoresis combined with Mass Spectrophotometry. Three European wheat varieties were taken for studying the qualitative variability of abovementioned xylanase inhibitors. The 2-DE separation combined with MS identification revealed many isoforms for all of the xylanase inhibitors. Five genetic variants were identified for TAXI, three for XIP, and one for TLXI-type proteins. The genetic variability among these three cultivars did not differ qualitatively thereby suggesting the presence of the same pool of isoforms of xylanase inhibitors in wheat irrespective of the genetic differences in the wheat cultivars.

Keywords: Polymorphism, Wheat, Xylanase inhibitors, 2 DE gel electrophoresis

Introduction

Non Starch Polysaccharides (NSPs) in wheat comprise mainly cellulose, â-glucans, arabinoxylans, arabinogalactan-peptides and glucomannans. They have predominantly a structural role in the cell walls of the wheat grain. From the nutritional point of view, these are believed to be important dietary fibre components (Hoseney, 1994 and Fincher and Stone, 1986).

Arabinoxylans (AX) are cell wall NSPs present in several cereals such as wheat, barley, rye, oat, rice and sorghum (Izydorczyk and Biliaderis, 1995). These are a heterogeneous group of polysaccharides and hence only a general structure exists for them. Basically these have the backbone of â-(1,4)-linked-D-xylopyranosyl residues (xylose), substituted mainly with á-L-arabinofuranosyl residues (arabinose) at the C(O)-3 and/or the C(O)-2 position. Phenolic acids such as ferulic and p-coumeric acid can be coupled to the C(O)-5 position of arabinose units through an ester linkage (Perlin, 1951). Other minor, but common substituents bound to the xylan backbone are 4-O-methyl-glucuronic acid and glucuronic acid. These uronic acids are bound to the C(O)-2 position of the xylose residues (Fincher, 1975).

Endo-(1,4)-â-D-xylanases (EC 3.2.1.8, further referred to as endoxylanases) are key enzymes in the degradation of xylan and arabinoxylan. As they generate (arabino)xylo-oligosaccharides by degrading the chain internally, these have a strong impact on AX structure and functionality (Dekker and Richards, 1976; Reilly, 1981).

It has been reported that endogenous endoxylanases are present in a number of cereals including wheat (Preece and McDougall, 1958; Cleemput *et al.*, 1995). During germination, they degrade the aleurone and starchy endosperm cell walls making starch and storage proteins accessible for amylases and proteases, respectively (Mares and Stone, 1973). Endoxylanases are also found in bacteria, fungi, insects, snails, crustaceans and marine algae (Dekker and Richards, 1976). Reduction of endoxylanase activity is reported for a number of substances like metal ions, organic materials (glycerol, ethanediol, several sulfahydryl reagents) and sugars (xylose, arabinose, xylotriose) (Dekker and Richards, 1976). In addition to these factors, endogenous proteinaceous inhibitors of endoxylanases were found in wheat while studying the solubilization of AX during the production of Belgian white beer (Debyser et al., 1997). Three distinct types of xylanase inhibiting proteins have been isolated from wheat so far; these are TAXI (*Triticum aestivum* L. xylanase inhibitor), XIP (xylanase inhibiting proteins) (Debyser and Delcour, 1998) and TLXI (Thaumatin-like xylanase inhibitor) (Fierens et al., 2004).

*Corresponding author; Present Address: Agro Enterprises Center, Federation of Nepalese Chamber of Commerce and Industry, Kathmandu, Nepal; Email: oliprakash@hotmail.com

TAXI-type xylanase inhibitors have been purified from wheat, rye, durum wheat and barley. The highest amount of this inhibitor has been isolated from wheat (ca. 38 ppm) and rye (ca. 22 ppm), while lower levels were purified from durum wheat (ca. 11 ppm) and barley (ca. 4-5 ppm). The inhibitors isolated from these cereals were heterogeneous in nature comprising a mixture of multiple isoforms. TAXI-type xylanase inhibitors have not been detected in maize, rice, oats and buckwheat (Goesaert *et al.*, 2003). XIP-type xylanase inhibitors were purified from both wheat flour and bran as well as from other cereals such as barley and rye. Beaugrand et al., (2006) reported that XIP-type xylanase inhibitors are present in maize and oats. Durand et al., (2005) have also reported a XIP-type inhibitor protein from rice which they called RIXI (Rice Xylanase Inhibitor).

TLXI-type xylanase inhibitor was first isolated from wheat meal using a combination of cation exchange chromatography and affinity chromatography. The yield was approximately 2.5 ppm in whole meal and thus much less than for TAXI- and XIP-type xylanase inhibitors (Fierens *et al.*, 2005).

In the breadmaking process, it has been shown that AX increase dough consistency and farinogaph water absorption

(Jelaca and Hlynka, 1971). These have a strong impact on the viscosity of the dough aqueous phase, thereby stabilizing the gas cells during fermentation and baking (Gan *et al.*, 1995). This in turn enhances bread loaf volume and results in a finer crumb structure. In contrary, the other types of AX (generally referred to as water unextractable arabinoxylans: WU-AX) are believed to destabilize dough structure since they can form physical barriers for the gluten network and are able to perforate gas cells resulting in coalescence and decreased gas retention for the dough (Courtin and Delcour, 2002).

In this respect, endoxylanases that preferably solubilize WU-AX can have a positive effect on loaf volume. However, endoxylanase overdosage, which causes hydrolysis of high molecular AX that are particularly water extractable can have a negative effect on loaf volume by changing dough viscosity. Hence, optimization of AX functionality in breadmaking can be achieved by selecting the appropriate dosage and the right type of microbial endoxylanase (Courtin and Delcour, 2002). The presence of endogenous xylanase inhibitors in wheat significantly affects the performance of these enzymes. Debyser et al., (1997) showed that TAXI retarded the increase in loaf volume despite the addition of nd:*A. niger* endoxylanase whereas Sibbesen and Sørensen (2001) showed that the combined addition of TAXI and an endoxylanase, resulted in better dough handling properties, a decreased stickiness, an increased dough viscosity and a decreased solubilisation and degradation of AX. A study also showed that the functionalities of two *A. niger* endoxylanases in wheat breadmaking were strongly dictated by their sensitivities towards TAXI- and XIP-type xylanase inhibitors. Whether inhibitor-sensitive endoxylanases are able to perform their activity on flour AX during breadmaking highly depends on the timeframe for complexation with the xylanase-inhibitors. Possibly, the more restricted mobility of these bio-active molecules in dough than *in vitro* might allow some endoxylanases to hydrolyse the AX population to some extent during breadmaking before it becomes inactivated (Gebruers *et al.*, 2005).

During storage of refrigerated dough, enzymatic hydrolysis of AX by endogenous xylanases give rise to syruping and drip loss from the dough (Atwell, 1998). Release of water from the dough is mainly caused by a reduction in water holding capacity following solubilization of AX to yield low MM components. When an excess of TAXI-type xylanase inhibitors was added to the dough recipe, up to 65% of the flour endoxylanase activity was inhibited and, after 10 days of storage at 10 ^{0}C, syruping was reduced up to 50%. Hence, xylanase inhibitors with broad endoxylanase specificities can have a positive impact against this phenomenon (Gys, 2005). In animal feed, it is generally admitted that AX impart the anti-nutritional effects due to their property to increase viscosity in the intestine and marked decrease in nutrients uptake. The use of endoxylanases that solubilise the AX population into low molecular units in animal feed has been found to decrease the anti-nutritive effect imposed by AX thereby increasing the efficiency of cereal based diets (Fengler and Marquardt, 1988). Despite extensive characterization of TAXI-, XIP-, and TLXI-type xylanase inhibitors, there is a lack of knowledge on their polymorphism in wheat grain. The aim of this study was to elucidate this unknown heterogeneity using high-resolution 2-DE and subsequent MS analysis. The study also aimed in knowing the variability of these three types of xylanase inhibitors due to genetic variation.

Materials and Methods

Three different European wheat cultivars Claire (harvest 2005), Koch and Zhora (harvest 2003) were obtained from AVEVE (Landen, Belgium) and ground by using a Cyclotec 1093 sample mill (Tecator, Hogänäs, Sweden). All reagents used were from Sigma-Aldrich (Bornem, Belgium) and were of analytical grade unless specified otherwise. *Bacillus subtilis* xylanase (GH family 11) was supplied by Danisco (Braband, Denmark) and *Aspergillus niger* xylanase (GH family 11) was from Megazyme (Bray, Ireland).

Extraction and purification of Xylanase Inhibitor Proteins : Extraction of wheat whole meal (100 g) and subsequent depectinization of the extract from wheat whole meal was done as suggested by Gebruers et al., (2002). From the whole meal extract xylanase inhibitors were purified by performing cation exchange chromatography (CEC) followed by affinity chromatography (AC) with immobilised xylanases according to the protocol described by Gebruers et al., (2002) with a few modifications. In the first AC column, which has the immobilized GH family 11 *B. subtilis* xylanase, the TAXI-type proteins were bound whereas the XIP- and TLXI-type proteins were bound in second affinity-based step in which the GH family 11 *A. niger* xylanase was present as biospecific ligand. The bound proteins were eluted with sodium acetate buffer. Thus eluted proteins were further desalted and concentrated to ca. 2.0 mg/ml by ultrafiltration using Vivaspin 15R concentrators with a molecular mass cut- off of 5 kDa.

2-DE and staining : For 2-DE separation, 40 microgram of purified xylanase inhibitor proteins were taken which were denatured and applied to rehydrated Immobiline Drystrips of pH 6-11 (18×0.3×0.5 cm). Samples were cup-loaded near the anode and focused at 20°C using the Ettan IPGphor II IEF unit (GE Healthcare). The running parameters for IEF were followed as described by Croes and co-workers (2008). The IPG strips were then transferred to 15% homogenous polyacrylamide (PAA) gels (25×20×0.1 cm) and SDS-PAGE was performed at 20°C using the Ettan Daltsix vertical electrophoresis system in conjunction with the Tris-glycine buffer system. The system was run as optimized by by Croes et al., (2008). The gels were stained with the sensitive CBB G-250 method as described by Candiano et al., (2004) and scanned by the ImageScanner II system with accompanying Labscan 5.00 software (GE Healthcare).

Protein identification by tandem mass spectrometric analysis : Protein spots were picked manually from CBB stained gels, and trypsin-digested according to the method of Shevchenko et al., (2006). Tryptic digests were analyzed by LC-ESI-MS/MS on a LCQ Classic (Thermo Electron, San Jose, CA, USA) ion trap MS equipped with a nano-LC column switching system as described by Dumont et al., (2004).

Results and Discussion

Two DE of TAXI-, XIP-, AND TLXI

A number of isoforms were seen for all the three types of xylanase inhibitors. According to the basis of purification of TAXI-type proteins by xylanase affinity chromatography, Gebruers et al., (2002) reported six isoforms of TAXI-I and one isoform of TAXI-II. And on the basis of genetic sequences, it has been reported that TAXI-I consist of three isoforms (Ia, Ib and III) and TAXI-II also consists of same numbers of isoforms (IIa, IIb and IV) (Fierens et al., 2003; Fierens et al., 2004; Igawa et al., 2004). Similarly the XIP has been reported to exist in two genetic variants namely XIP-I and XIP-III. Multiple isoforms have been reported for XIP- and TLXI-type proteins (Elliott et al., 2003; Fierens et al., 2004; Igawa et al., 2005).

From this experiment, the resulting 2-DE gels revealed large families of isoforms for each of these xylanase inhibitors which are expressed within the wheat grain at maturity. The pattern of those isoforms revealed in 2-DE gels are shown in Fig 1 (TAXI type) and Fig 2 (XIP and TLXi type). This high variation in spots was never seen before for these types of proteins.

The uncleaved form of TAXI-type xylanase inhibitors (40 kDa) appear in the 2-DE gels as several spots which were located in MM range of 42.0 to 46.0 kDa and pI range of 7.0 to 9.5 whereas the cleaved form were present in the MM range of 27.0 to 33.0 kDa and pI range of 8.9 to 10.0 (Fig 1). The C-terminal parts of the cleaved form, which has a MM of around 10.0 kDa was not seen in the 2-DE gels because they have lower pI-values (5.0-5.3). The values of MM thus detected in 2-DE were slightly higher than calculated from their amino acid sequences.

XIP-type xylanase inhibitors were seen in several spots differing in MM of 30.0 to 35.0 kDa and pI of about 7.5 to 9.5. Various isoforms were found as vertical rows of spots with the same pI but different MM, which may be due to different degrees of glycosylation. TLXI-type proteins were seen as four different spots, all at the same pI of ~9.8 but differing in MM of 18.0 to 21.0 kDa.

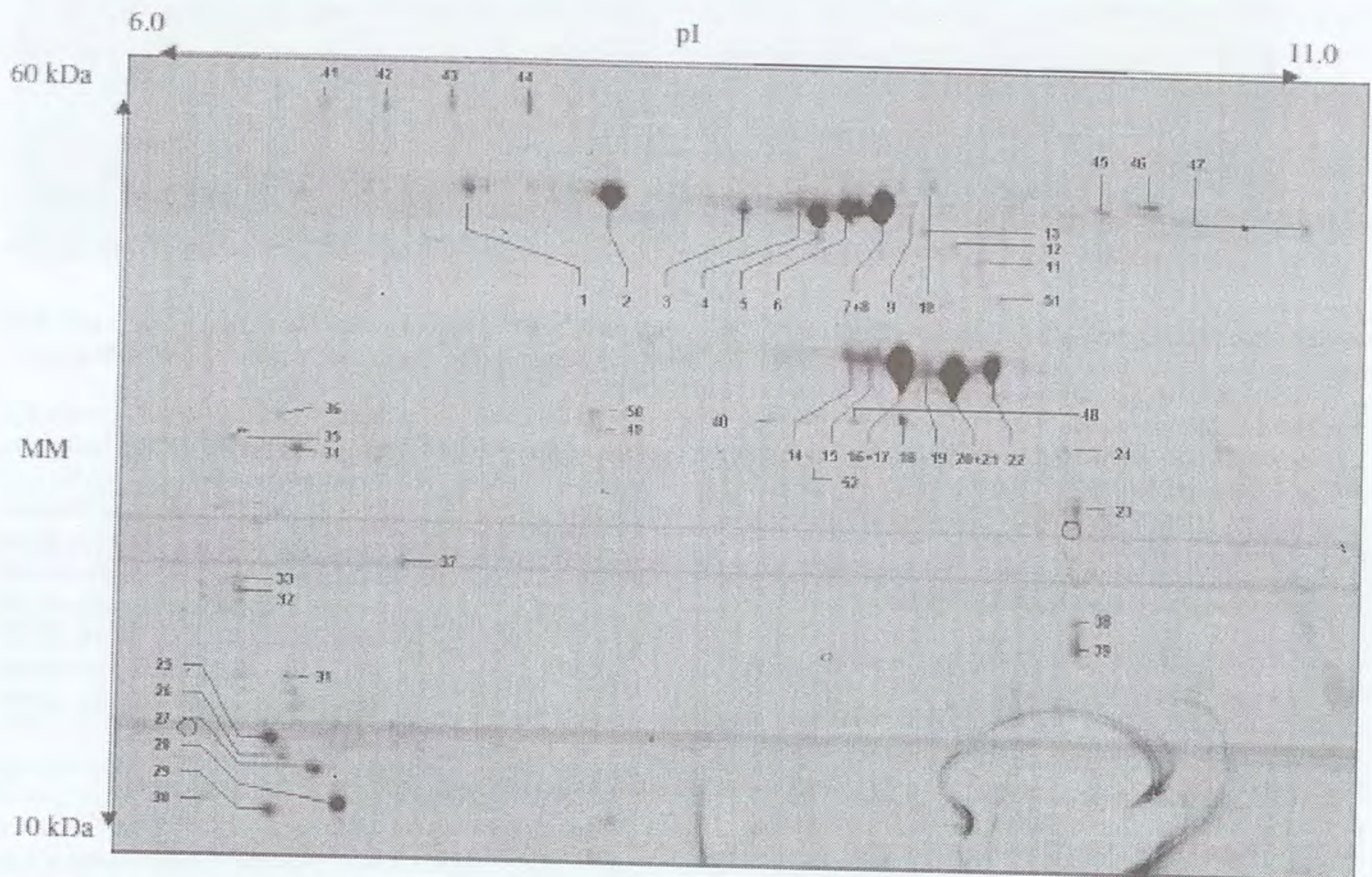

Fig 1: Colloidal CBB stained 2-DE patterns (pH 6-11, 15% PAA-gels) of TAXI-type proteins (40 μg) present in wheat WM from variety Claire. The numbered spots were cut, tryptic digested and analyzed with mass spectrometry (LC-ESI-MS/MS).

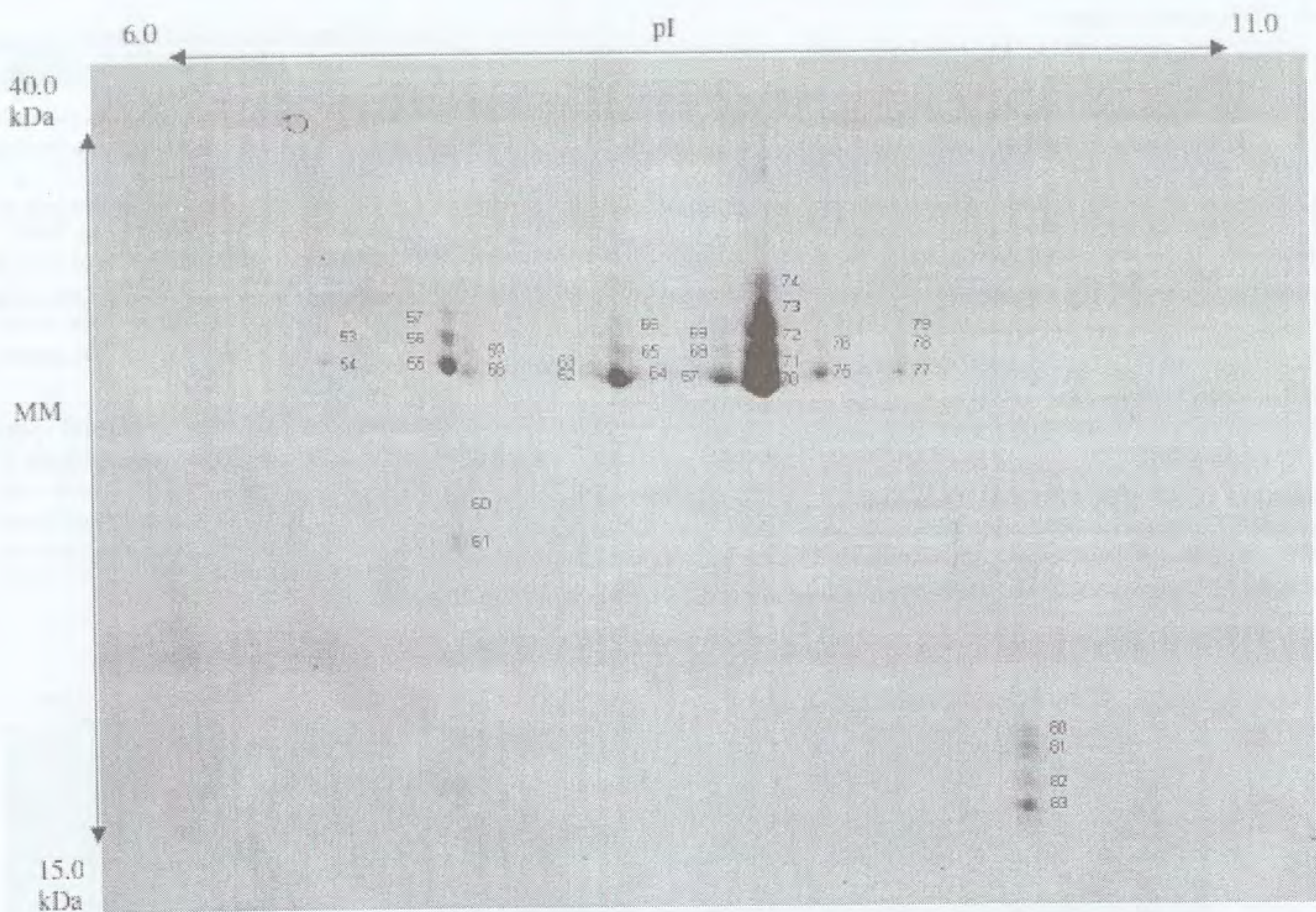

Fig 2: Colloidal CBB stained 2-DE pattern (pH 6-11, 15% PAA-gels) of XIP/TLXI-type proteins (40 μg) present in wheat WM from variety Claire. The numbered spots were cut, tryptic digested and analyzed with mass spectrometry (LC-ESI-MS/MS).

For XIP, three different genetic variants (XIP-I, XIP-III and XIP-CD 919957) could be identified whereas only one genetic variant was detected for TLXI. The different spots identified from the 2-DE gel from XIP/TLXI-type proteins are shown in Fig 2.

Identification of Xylanase inhibitors by MS

The MS result indicated different genetic variants exist for all three types of xylanase inhibitors. The results after identification of these isoforms are shown in the Table 1 and Table 2 for TAXI and XIP & TLXI type inhibitors respectively. For TAXI-type xylanase inhibitors, the uncleaved form exists in various spots which correspond to five different genetic variants, whereas for the N-terminal polypeptides of the cleaved-TAXI form only four genetic variants remain.

Among the different genetic variants described in literature, four of them namely, TAXI-Ia, TAXI-IIa, TAXI-IIb/TAXI-IV were identified to be expressed in the mature wheat kernel. In addition to these four genetic variants, one "putative" sequence which was very similar to TAXI-type proteins but not recorded before in wheat was found to be expressed in mature wheat kernel. TAXI-IIa was solely observed for the uncleaved form of TAXI-type proteins. Whereas, TAXI-Ib as reported by Raedschalders et al., (2005) in the gene sequence of wheat and expressed in *P. pastoris* has not been found expressed in mature wheat kernel. Another genetic variant, namely TAXI-III also could not be observed expressed in the mature wheat kernel. As reported by Igwa et al., (2004), TAXI-III, which is expressed during pathogen attack and wounding, was also not detected to be expressed in mature wheat kernel.

Besides the different isoforms of TAXI-type xylanase inhibitors, some other spots were observed in 2-D gels which were identified as â-glucosidase (spots 41, 42, 43 and 44 in Fig 1), low molecular weight glutenin subunits (spots 45, 46 and 47 in Fig 1), class II chitinase (spot 48 in Fig 1), Thaumatin-like protein (spots 52 in Fig 1) and á-amylase inhibitors (spots 25, 26, 27, 28, 29 and 30 in Fig 1).

For XIP-type xylanase inhibitors also, many spots were detected and identified as three different genetic variants, namely XIP-I, XIP-III and XIP-CD919957. These isoforms were

Table 1: Identification of spots detected in 2-DE gel (pH 6-11, 15% PAA-gel) of proteins purified by an affinity chromatography on a *B. subtilis* xylanase column from wheat whole meal of variety Claire.

Spot number[1]	*Name of protein*	*Theoretical MM -pI*	*Observed MM- pI*	*Accession number in genebank (NCBI/SWISSPROT)*
TAXI form A (40 kDa)				
1	TAXI ACCN1	39.1-7.6	45.0 - 7.3	"Putative"[2]
2	TAXI ACCN1	39.1-7.6	45.0 - 7.9	"Putative"[2]
3	TAXI Ia+ACCN1 (100:1)[3]	38.8-8.2	45.0 - 8.4	AJ 438880.1+"Putative"[2]
4	TAXI IIb/IV[4]+Ia (2:1)[3]	40.0-8.5	45.0 - 8.7	AJ 697850.1/AB114628.1+ AJ 438880.1
5	TAXI Ia+IIb/IV[4] (10:1)[3]	38.8-8.2	45.0 - 8.8	AJ 438880.1+ AJ 697850.1/AB114628.1
6	TAXI IIb/IV[4]	40.0-8.5	45.0 - 8.9	AJ 697850.1/AB114628.1
7	TAXI IIb/IV[4]	40.0-8.5	45.5 - 9.0	AJ 697850.1/AB114628.1
8	TAXI IIb/IV[4]	40.0-8.5	45.0 - 9.0	AJ 697850.1/AB114628.1
9	TAXI IIa	40.0-8.2	45.0 - 9.2	AJ 697849.1
10	TAXI IIa	40.0-8.2	46.0 - 9.3	AJ 697849.1
11	TAXI IIb/IV[4]	40.0-8.5	42.0 - 9.5	AJ 697850.1/AB114628.1
12	TAXI IIb/IV[4]	40.0-8.5	43.0 - 9.4	AJ 697850.1/AB114628.1
13	TAXI IIb/IV[4]	40.0-8.5	44.0 - 9.3	AJ 697850.1/AB114628.1
TAXI form B (N-terminal fragment) 30 kDa				
14	TAXI ACCN1+Ia (100:1)[3]+15 kDa globulin	27.0-8.6	33.0 - 8.9	"Putative"[2]+ AJ 438880.1
15	TAXI ACCN1+Ia (10:1)[3]	27.0-8.6	33.0 - 9.0	"Putative"[2]+ AJ 438880.1
16	TAXI ACCN1	27.0-8.6	33.0 - 9.1	"Putative"[2]
17	TAXI ACCN1+Ia (100:1)[3]	27.0-8.6	30.0 - 9.1	"Putative"[2]+ AJ 438880.1
18	TAXI ACCN1	27.0-8.6	28.0 - 9.1	"Putative"[2]
19	TAXI ACCN1+Ia+IIb/IV[4] (5:1:1)[3]	27.0-8.6	32.0 - 9.2	"Putative"[2]+ AJ 438880.1+ AJ 697850.1/AB114628.1
20	TAXI Ia+IIb/IV[4] (3:1)[3]	26.8-8.7	32.0- 9.3	AJ 438880.1+ AJ 697850.1/AB114628.1
21	TAXI Ia+IIb/IV[4] (10:1)[3]	26.8-8.7	31.0 - 9.3	AJ 438880.1+ AJ 697850.1/AB114628.1
22	TAXI IIb/IV[4] + Ia (100:1)[3]	27.2-8.8	33.0 - 9.5	AJ 697850.1/AB114628.1+ AJ 438880.1
23	TAXI ACCN1	27.8-8.6	25.0 - 9.9	"Putative"[2]
24	TAXI ACCN1	27.8-8.6	27.0 - 9.8	"Putative"[2]
Wheat α-amylase inhibitor				
25	IAAC3	18.2-7.4	15.0 - 6.5	P17314
26	IAAC3	18.2-7.4	14.0- 6.6	P17314
27	IAA1	13.3-6.7	13.0- 6.7	P01085
28	IAAC1+IAAC2	15.5-7.5	12.0- 6.9	P16850+P16851
29	0.19	13.2-6.5	12.0- 6.6	AY729677.1
30	IAAC2	15.5-6.9	12.0 - 6.3	P16851

[1]Spot numbers as indicated in Fig 1.
[2]Raedschelders, et al (2005). Molecular, genetic and functional analysis of TAXI-type xylanase inhibitors in cereals. Doctoral thesis, KULeuven.
[3]Quantitative ratio, roughly estimated with MS based on intensities of specific peptide ions.
[4]TAXI IIB/IV: not distinguishable with identified peptides

varying both in MM and pI. For XIP-III, five different isoforms were detected, which ranged in MM from 30.0 to 32.0 kDa and pI of 7.6 to 8.0 whereas XIP-I has at least 22 different isoforms which varied in MM and pI from 25.0 to 36.0 kDa and 8.1 to 9.4 respectively. Three spots could also be matched with EST-sequence XIP-CD919957 which were observed at the same pI of ~9.0 but had different MM ranging from 30.0 to 33.0 kDa. XIP-type xylanase inhibitors were reported in literature as glycosylated proteins which could be responsible for this broad vertical range of MM.

TLXI, which has been reported in literature as containing multiple isoforms, also exists as four different isoforms which have MM from 18.0 to 21.0 kDa and a pI of ~9.8. The TLXI isoforms only differed in their MM, suggesting that they are glycosylated to different extent.

Table 2: Identification of spots detected in 2-DE gel (pH 6-11, 15% PAA-gel) of proteins purified by affinity chromatography on *A. niger* xylanase column from wheat whole meal of variety Claire.

Spot number [1]	*Name of protein*	*Theoretical MM -pI*	*Observed MM- pI*	*Accession number in genebank (NCBI/SWISSPROT)*
XIP-type xylanase inhibitors				
53	XIP-III	30.4-6.9	32.0 - 7.6	AB 204556.1
54	XIP-III	30.4-6.9	33.0 - 7.6	AB 204556.1
55	XIP-III	30.4-6.9	32.0 - 8.0	AB 204556.1
56	XIP-III	30.4-6.9	33.0 - 8.0	AB 204556.1
57	XIP-III	30.4-6.9	34.0 - 8.0	AB 204556.1
58	XIP-I	30.3-8.3	32.0 - 8.1	Q8L5C6
59	XIP-I	30.3-8.3	33.0 - 8.1	Q8L5C6
60	XIP-I	30.3-8.3	27.0 - 8.0	Q8L5C6
61	XIP-I	30.3-8.3	25.0 - 8.0	Q8L5C6
62	XIP-I	30.3-8.3	32.0 - 8.5	Q8L5C6
63	XIP-I	30.3-8.3	33.0 - 8.5	Q8L5C6
64	XIP-I	30.3-8.3	32.0 - 8.6	Q8L5C6
65	XIP-I	30.3-8.3	33.0 - 8.6	Q8L5C6
66	XIP-I	30.3-8.3	34.0 - 8.6	Q8L5C6
67	XIP-I	30.3-8.3	32.0 - 8.8	Q8L5C6
68	XIP-I	30.3-8.3	33.0 - 8.8	Q8L5C6
69	XIP-I	30.3-8.3	34.0 - ~8.8	Q8L5C6
70	XIP-I+XIP-CD 919957	30.3-8.3	30.0 - 9.0	Q8L5C6+EST-sequence
71	XIP-I+XIP-CD 919957	30.3-8.3	31.0 - 9.0	Q8L5C6+EST-sequence
72	XIP-I+XIP-CD 919957	30.3-8.3	33.0 - 9.0	Q8L5C6+EST-sequence
73	XIP-I	30.3-8.3	34.0 - 9.0	Q8L5C6
74	XIP-I	30.3-8.3	36.0 - 9.0	Q8L5C6
75	XIP-I	30.3-8.3	30.0 - 9.2	Q8L5C6
76	XIP-I	30.3-8.3	32.0 - 9.2	Q8L5C6
77	XIP-I	30.3-8.3	30.0 - 9.4	Q8L5C6
78	XIP-I	30.3-8.3	32.0 - 9.4	Q8L5C6
79	XIP-I	30.3-8.3	33.0 - 9.4	Q8L5C6
TLXI-type xylanase inhibitors				
80	TLXI	15.6-8.4	21.0 - 9.8	AJ 786602[2]
81	TLXI	15.6-8.4	20.0 - 9.8	AJ 786602[2]
82	TLXI	15.6-8.4	19.0 - 9.8	AJ 786602[2]
83	TLXI	15.6-8.4	18.0 - 9.8	AJ 786602[2]

[1] Spot numbers as indicated in Fig 2
[2] Not available in NCBI-database (Fierens et al., 2005)

The differences in pI and MM of spots from same variants suggest for the possibilities of some modification in the parent xylanase inhibitor proteins. *In vivo* post-translational modifications such as glycosylation, phosphorylation, acetylation or others might be the reasons for this variation. Likewise, the action of endogenous wheat protease on those xylanase inhibitors might also be the cause to produce different isoforms within the same genetic pools.

Genetic variability of xylanase inhibitors in three wheat varieties

The 2-DE patterns of the xylanase inhibitor proteins obtained from three European varieties, namely Koch, Zhora and Claire were compared and no differences was detected in the number of isoforms and in their corresponding MM or pI. The resulting pictures of the gels from theses three varieties are shown in Fig 3 and Fig 4.

This indicates that after extraction and purification of the three types of xylanase inhibitors, the same pool of isoforms could be found for three varieties. However, this does not imply that there were no any quantitative differences between these three types of xylanase inhibitors and their isoforms present in the three wheat cultivars.

Conclusions

The study, with the help of 2-DE gel electrophoresis and MS, could reveal the multiple isoforms present in xylanase inhibitors present in the wheat grain which were observed as several spots in the 2-DE gels. The qualitative indifferences of those variants in the 2-DE from three different wheat

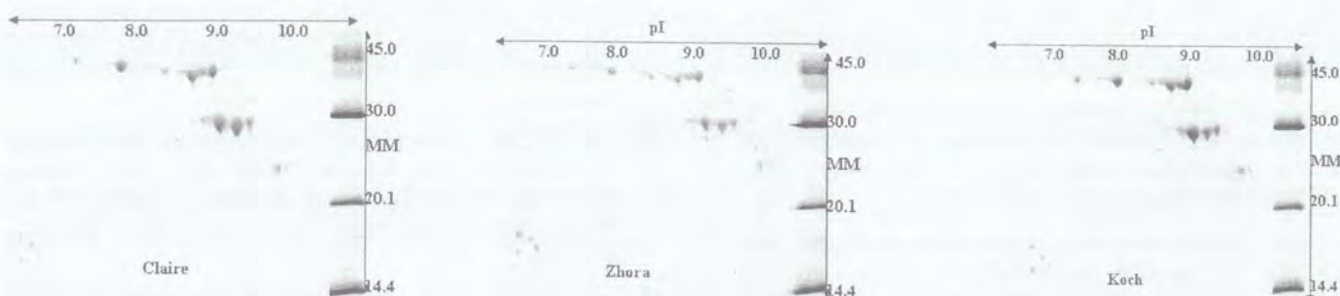

Fig 3: Colloidal CBB stained 2-DE (pH 6-11, 15% PAA) patterns of TAXI-type inhibitors (40 μg) from three wheat varieties Claire, Koch and Zhora. The estimated pI-values and MM (kDa) markers are indicated on top and at the right side respectively for each gel.

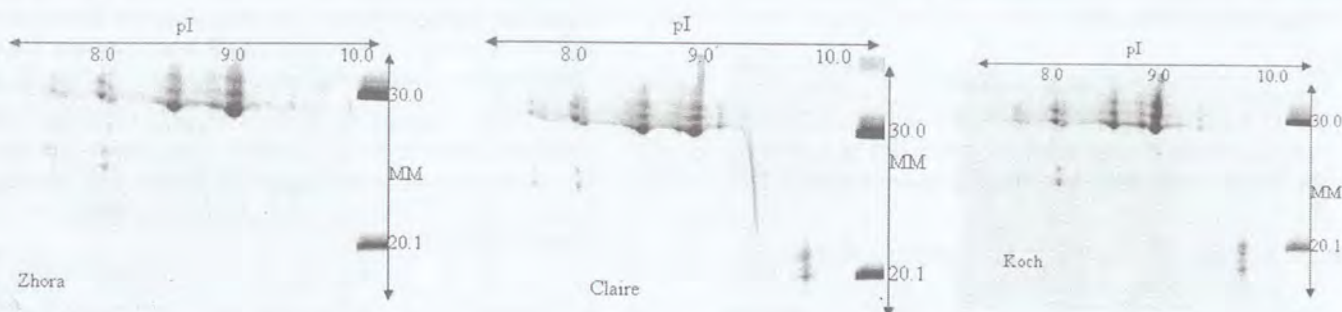

Fig 4: Colloidal CBB stained 2-DE (pH 6-11, 15% PAA) patterns of XIP-/TLXI-type inhibitors (40 μg) from three wheat varieties Claire, Koch and Zhora. The estimated pI-values and MM markers (kDa) are indicated on top and at the right side respectively for each gel.

varieties might be the indication that all wheat cultivars contain the same variants of xylanase inhibitors and one the 2-DE gels can be used as the fingerprint for further study in this area. Using this method, temporal and spatial distribution of xylanase inhibitors in wheat kernel can be studied during different physiological and pathological conditions. This will enable to know how these three types of xylanase inhibitors evolve during the course of development and pathological attacks and what are their physiological and plant defense role.

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Study on the Fractionation of Protein from *Masyaura* and their Nutritional Evaluation[#]

NAWA RAJ DAHAL* and LI QI

The Key Laboratory of Industrial Biotechnology of Education Ministry,
Southern Yangtze University, Wuxi 214036, PR China

Masyaura, a legume based traditional fermented food of Nepal, prepared from blackgram (Phaseolus mungo) dhal and colocosia (Colacosia esculanta) tuber as per the traditional sun-drying as well as alternative controlled fermentation and mechanical drying method were subjected to the fractionation of proteins. Nutritional evaluation of major protein fractions from Masyaura as well as blackgram dhal were also carried out. Results indicated that albumin and globulin were the major protein fractions in Masyaura and albumin fractions were nutritionally superior to the globulin fractions. SDS-Page of those fractions indicated that the major protein band at about 50KDa incase of blackgram protein. Sun dried and controlled fermented Masyaura showed bands at less than 50KDa indicating that the major protein in blackgram dhal degraded into small molecular fractions during the course of fermentation which leads to the improvement of digestibility of protein.

Keywords: *Masyaura*, Fermentation, Protein, Albumin, Globulin, Nutritional evaluation

Introduction

Masyaura is an important legume based traditional fermented food of Nepal, is being used by all classes of people (Karki, 1986). It is usually prepared in cottage or home scale and used as an adjunct in curry. *Masyaura* is considered as a substitute of meat especially for poor people (Karki, 1986). *Masyaura* is a good source of protein as comparable to animal protein (Dahal et al, 2003a). The raw materials used in the preparation of *Masyaura* are legumes such as blackgram or greengram and vegetables like colocasia tuber, ashgourd or raddish based on the availability in the local market. Preparation of *Masyaura* is a traditional method of preserving perishable vegetables (Gajurel and Baidya, 1979, Dahal et al, 2003a).

Masyaura is similar to Indian *Wari* are friable and spongy dried balls about 2-5 cm diameter in size. Once it is prepared, the dried balls are stored for future use. At the time of cooking, it is mixed with curry to make soup and served with rice as side dish (Gajurel and Baidya, 1979). At present, informations on biochemical, nutritional, antinutritional, sensory and digestibility characteristics of protein and starch of *Masyaura*; bioavailability characteristics of minerals such as calcium and zinc in *Masyaura*; organic acids and volatile components in *Masyaura* as well as polyphenol and antioxidant characteristics of *Masyaura* are available in the literature (Karki, 1986; Dahal et al, 2003a; Dahal et al, 2003b; Dahal et al, 2005; Dahal and Qi, 2005; Dahal and Swamylingappa, 2006; Dahal and Qi, 2006; Dahal and Qi, 2007, Dahal and Qi, 2008). In this paper, an attempt was made to explore the changes in protein during processing and to evaluate the nutritional quality proteins in *Masyaura* since such information is lacking in the literature.

*Corresponding author; Present address: Department of Food Technology and Quality Control, Babarmahal, Kathmandu, Email: nawarajd@yahoo.com

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Materials and Methods

Blackgram (*Phaseolus mungo*) dhal was purchased from local market of Kathmandu, Nepal and Colocosia (*Colacosia esculanta*) tuber was purchased from the local market of Wuxi, China.

Preparation of Masyaura: Traditional method: Cleaned split blackgram dhal was washed thoroughly with water to remove husk and foreign matter and soaked for 16h in water. The soaked dhal was hand washed in tape water to remove husk and ground into a thick paste (Moisture around 70%) using a wet grinder. The colacosia tuber was washed peeled and wet grinded to get thick paste (Moisture of colocasia paste was maintained about 70% with colocasia powder. Colocasia tuber was sliced, blanched at 70°C for 5 min dried at 50°C and was grinded to get powder). The blackgram dhal paste and colocasia paste were then mixed in a ratio of 1:1. The dough was made into small lumps weighing 20-30g, were distributed 1-2 inches apart on steel trays between lumps, left to ferment overnight at ambient temperature. The spongy textured balls are then sun dried for 5 days (Room Temp.20°C, Humidity 70%).

Controlled fermentation and mechanical drying technique: Dough was prepared and distributed on steel trays similar to traditional method. Trays were then subjected for fermentation at 30° C at 24 h (Relative humidity 80-90%) and dried at 50°C for 12-16 h. The time and temperature of fermentation was

selected based on the sensory characteristic of from based on the previous study (Dahal and Qi, 2005). The dried product was packed and stored in sealed polythene bags at ambient temperature. The dried *Masyaura* was powdered in a grinder to pass through 60 mesh sieve and used for chemical analysis. *Fractionation of Black-gram and Masyaura Protein:* Basic study on proteins from blackgram dhal and *Masyaura* were carried out. Water soluble albumin, salt soluble globulin, alcohol soluble prolamine and acid soluble glutenin were fractionated. Protocol given in Fig 1 was used for the fractionation of proteins (Reddy et al., 1982). *Moisture* and *Protein* were determined by the standard AOAC (1990) method. The Protein content in the fraction was determined according Bradford Method given by (Sawhney and Singh 2000). *Amino acid analysis* was done according to HPLC method of Godel et. al., 1995. Tryptophan was estimated by the ninhydrin method of Swakais and Pest (1990) after extracting protein according to the method of Concon (1975). *Chemical score* was calculated according to the method of Block and Mitchel (1946) *Essential Amino acid Index* was calculated according to the procedure of Oser (1951) taking into account the ratio of EAA in the test protein relative to their respective amounts in whole egg protein. *Biological value (BV)* was calculated according to the method of Oser (1959). *Nutritional Index (NI)* was calculated according to Crisan and Sands (1978). *SDS-PAGE* Sodium dodecyl sulfate Polyacrylamide Gel Electrophoresis) was done according to the method given by Sawhney and Singh, (2000).

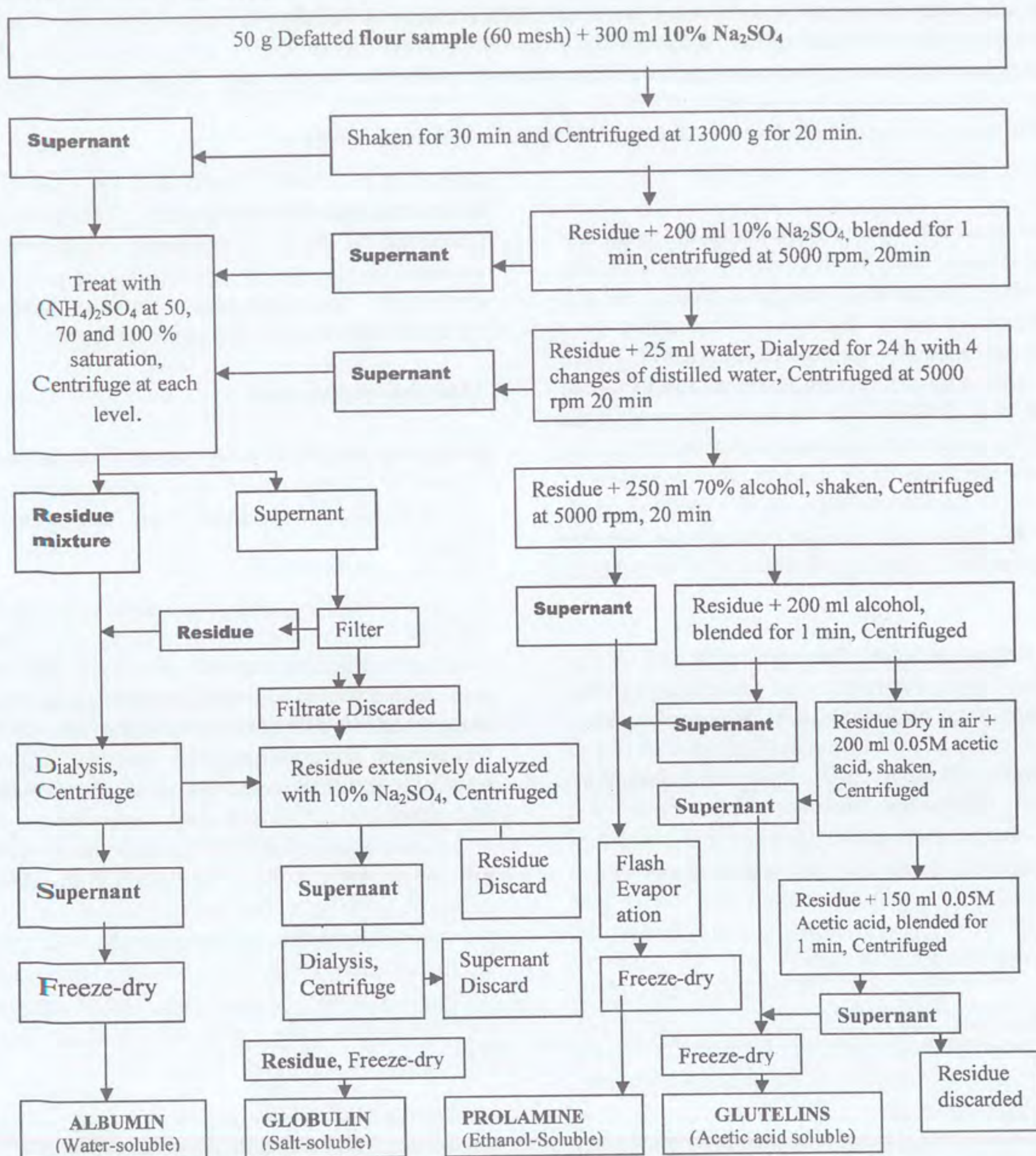


Fig 1: Protocol used for the fractionation of protein from Blackgram dhal and *Masyaura* protein

Results and Discussion

Quantitative analysis of Protein fraction

Albumin, globulin, prolamine and glutelin fractions from blackgram dhal, unfermented *Masyaura*, sun-dried *Masyaura* and controlled fermented *Masyaura* were obtained. The data obtained from the quantitative analysis of protein is given in Table 1.

Table 1: Quantitative analysis of blackgram and *Masyaura* protein fraction

Protein Fraction	*Volume of Extract, ml*	*Freeze-dried fraction, g*	*Protein* in dried fraction, %*	*Extracted protein, mg (%)*
		Blackgram Dhal		
Albumin	130	1.04	44.2 ± 1.2	461 (32.5)
Globulin	90	1.28	69.8 ± 1.5	894 (63.0)
Prolamine	80	0.06	81.7 ± 0.8	48 (3.3)
Glutelin	24	0.04	40.3 ± 0.6	17 (1.2)
		Unfermented *Masyaura*		
Albumin	150	1.38	55.1 ± 0.8	766 (42.8)
Globulin	120	1.11	86.0 ± 2.6	955 (53.9)
Prolamine	94	0.04	71.5 ± 3.1	29 (1.6)
Glutelin	44	0.07	40.1 ± 1.0	29 (1.7)
		Sun-dried *Masyaura*		
Albumin	184	1.98	43.0 ± 1.3	854 (41.4)
Globulin	130	1.15	95.6 ± 2.7	1105 (53.5)
Prolamine	107	0.09	79.3 ± 1.8	76 (3.7)
Glutelin	40	0.06	44.0 ± 0.5	29 (1.4)
		Controlled Fermented *Masyaura*		
Albumin	164	1.82	45.5 ± 0.9	832 (43.3)
Globulin	124	1.02	95.1 ± 1.1	973 (50.6)
Prolamine	156	0.11	80.2 ± 1.3	89 (4.6)
Glutelin	40	0.06	43.5 ± 0.6	27 (1.5)

[Note: * Protein determined by Bradford Method]

Blackgram dhal showed the major protein fraction of globulin accounting 63% of total protein followed by albumin 32.5%. Prolamine and glutelin fractions were lower at 3.3 and 1.2% respectively. Unfermented *Masyaura* (blackgram paste mixed with colocosia paste before drying at 50°C for 16h) showed the value of 53.9, 42.8, 1.6 and 1.7 % of globulin, albumin, prolamine and glutelin respectively. Sun dried *Masyaura* sample showed % of 53.5, 41.4, 3.7 and 1.4% respectively. Similar results were also observed in case of controlled fermented *Masyaura* sample indicated the value of globulin, albumin, prolamine and glutelin as 50.6, 43.3, 4.6 and 1.5% respectively. The result clearly indicated that albumin and globulin are the major protein fractions in *Masyaura* samples and in blackgram dhal. Reddy et al, 1982 was also reported the major protein fraction of blackgram dhal as globulin 81% and albumin 13% with minor amount of prolamine and glutelin.

Nutritional Evaluation of Protein fractions

Nutritional evaluation was carried out for the protein fractions from blackgram dhal and *Masyaura* samples. Total amino acid composition, essential amino acid composition, chemical score and nutritional parameters for the major protein fractions albumin and globulin was studied.

Amino Acid Composition

Amino acid composition for major protein fractions i.e. albumin and globulin from blackgram dhal & *Masyaura* protein were analyzed. Amino acid composition of albumin fraction is given in Table 2 and the composition of globulin fraction is given in Table 3 respectively.

Total essential amino acid of the albumin fraction from blackgram dhal; unfermented *Masyaura*, sun-dried *Masyaura* and controlled fermented *Masyaura were* found to be 43.9, 42.8, 41.6 and 41.0 g/100g protein respectively. The composition for albumin fraction was found to be slightly higher as compared to the total protein from those albumin fraction samples analyzed (Table 2). Similarly, the total essential amino acid for globulin fraction from black gram dhal, unfermented *Masyaura*, sun-dried *Masyaura* and controlled fermented *Masyaura* were found to be 43.5, 39.9, 42.4 and 38.4 g/100g protein respectively (Table 3). The composition for globulin fraction was found to be slightly

Table 2: Amino acid Composition* of albumin fraction from blackgram and *Masyaura Protein*

Amino Acid	*BGD*	*UFM^*	*SDM*	*CFM*
Aspartic acid	9.4 (13.0)	12.2 (14.9)	11.6 (14.4)	11.2 (11.7)
Glutamic acid	14.2 (16.8)	15.3 (12.9)	14.2 (15.8)	11.0 (12.7)
Serine	3.5 (5.4)	5.4 (5.5)	5.1 (5.7)	2.4 (4.4)
Histidine	3.0 (2.6)	2.5 (2.1)	2.3 (2.4)	2.2 (2.0)
Glycine	5.4 (3.4)	4.5 (3.5)	4.3 (3.8)	4.2 (3.1)
Threonine	5.1 (3.5)	3.8 (3.6)	3.6 (3.8)	3.5 (3.2)
Alanine	1.8 (4.0)	3.3 (3.8)	3.2 (4.3)	3.0 (3.5)
Arginine	11.7 (5.6)	8.7 (6.4)	8.7 (6.4)	8.4 (5.5)
Tyrosine	3.3 (2.5)	4.0 (3.1)	4.0 (2.9)	3.9 (2.3)
Valine	5.6 (4.2)	5.5 (4.0)	5.3 (4.1)	5.1 (3.9)
Methionine	1.2 (1.1)	0.4 (0.7)	1.0 (0.9)	1.1 (0.6)
Cysteine	1.7 (0.5)	1.6 (1.0)	1.5 (0.8)	1.7 (0.6)
Phenylalanine	5.0 (6.0)	6.7 (5.3)	7.0 (6.0)	7.5 (4.9)
Isoleucine	4.5 (3.6)	4.7 (3.0)	4.3 (3.3)	4.1 (3.1)
Leucine	7.1 (8.0)	7.8 (7.0)	7.2 (8.2)	7.9 (7.2)
Lysine	8.8 (6.5)	5.9 (5.1)	5.1 (5.6)	4.2 (4.5)
Proline	5.2 (3.9)	4.9 (3.8)	4.5 (4.2)	3.8 (3.6)
*Tryptophan***	1.6 ± 0.25 (0.7 ± 0.04)	2.4 ± 0.16 (1.0)	2.6 ± 0.17 (1.0 ± 0.04)	2.0 ± 0.06 (0.9±0.12)
% Total EAA	43.9 (36.6)	42.8 (33.8)	41.6 (36.6)	41.0 (31.2)

- * (g/100g protein)
- BGD: Blackgram Dhal; UFM: Unfermented *Masyaura*; SDM: Sun dried *Masyaura*; CFM: Controlled Fermented *Masyaura*.
- ** Tryptophan determined by ninhydrin method and result is expressed as mean of the duplicates.
- Amino acids represented by italics indicate essential amino acids.
- Values in the parenthesis indicate the amino acid composition of total protein from respective samples.
- ^ Amino acid composition of total protein for UFM is computed from raw materials formulation.

Table 3: Amino acid Composition* of globulin fraction from blackgram and *Masyaura Protein*

Amino Acid	*BGD*	*UFM^*	*SDM*	*CFM*
Aspartic acid	12.5 (13.0)	11.9 (14.9)	12.9 (14.4)	12.2 (11.7)
Glutamic acid	17.0 (16.8)	17.8 (12.9)	17.2 (15.8)	19.2 (12.7)
Serine	5.0 (5.4)	5.5 (5.5)	4.9 (5.7)	4.9 (4.4)
Histidine	2.9 (2.6)	2.7 (2.1)	2.8 (2.4)	2.7 (2.0)
Glycine	2.9 (3.4)	3.3 (3.5)	3.3 (3.8)	3.2 (3.1)
Threonine	2.2 (3.5)	2.7 (3.6)	2.4 (3.8)	2.3 (3.2)
Alanine	3.4 (4.0)	3.2 (3.8)	3.5 (4.3)	3.5 (3.5)
Arginine	6.7 (5.6)	6.8 (6.4)	6.9 (6.4)	6.5 (5.5)
Tyrosine	3.1 (2.5)	3.3 (3.1)	3.1 (2.9)	3.0 (2.3)
Valine	6.1 (4.2)	5.0 (4.0)	6.1 (4.1)	5.4 (3.9)
Methionine	0.5 (1.1)	0.4 (0.7)	ND (0.9)	0.2 (0.6)
Cystine	0.2 (0.5)	0.9 (1.0)	0.6 (0.8)	0.9 (0.6)
Phenylalanine	7.1 (6.0)	6.3 (5.3)	6.8 (6.0)	6.2 (4.9)
Isoleucine	4.9 (3.6)	3.9 (3.0)	4.9 (3.3)	4.2 (3.1)
Leucine	9.2 (8.0)	8.3 (7.0)	9.1 (8.2)	8.5 (7.2)
Lysine	7.1 (6.5)	6.2 (5.1)	6.6 (5.6)	5.8 (4.5)
Proline	4.7 (3.9)	4.4 (3.8)	4.5 (4.2)	4.5 (3.6)
*Tryptophan***	3.1 ± 0.05 (0.7 ± 0.04)	2.9 ± 0.04 (1.0)	2.8 ± 0.01 (1.0 ± 0.04)	1.9 ± 0.04 (0.9±0.12)
% Total EAA	43.5 (36.6)	39.9 (33.8)	42.4 (36.6)	38.4 (31.2)

- * (g/100g protein)
- BGD: Blackgram Dhal; UFM: Unfermented *Masyaura*; SDM: Sun dried *Masyaura*; CFM: Controlled Fermented *Masyaura*.
- ** Tryptophan determined by ninhydrin method and result is expressed as mean of the duplicates.
- Amino acids represented by italics indicate essential amino acids.
- Values in the parenthesis indicate the amino acid composition of total protein from respective samples.
- ^ Amino acid composition of total protein for UFM is computed from raw materials formulation.

higher as compared to the total protein from those globulin samples (Table 3). The slightly increase in the total essential amino acids in both the albumin and globulin fractions compared to total protein may be due to the purity of those fractions,. Reddy et al, (1982) have reported that similar amino acid composition for the black gram albumin and globulin fractions as well as total protein of black gram. Minor variation may be due to the maturity and varietal difference. The black gram seed proteins and their fractions, with the exception of glutelins, have also been reported to be higher E/T ratio than the ideal protein of 36% proposed by the WHO of United Nations (FAO, 1968) albumin ranking the highest with an E/T value of 48.7%, whereas, glutelins with the lowest value of 34.2%.

Protein Score

Protein scores for major protein fractions albumin and globulin from black gram dhal, unfermented *Masyaura*, sun-dried *Masyaura* and controlled fermented *Masyaura* were calculated and presented in Table 4.

The Protein score suggested that the first limiting amino acid was sulphur amino acids in albumin, globulin and total protein from all samples of blackgram dhal and *Masyaura* prepared by different methods. Protein score for globulin fractions was observed lower (12.5-29.7) as compared to albumin fractions (42.0-58.7) as given in Table 4. Similar observations were also made in case of blackgram seed protein in previous study carried by Reddy et al, 1982.

Table 4: Protein Score* of major protein fraction of Blackgram and *Masyaura* Protein

Score	*Albumin*	*Globulin*
	Blackgram Dhal	
Protein Score	57.8 (38.2)	16.3 (38.2)
1st Limiting	Sulphur (Sulphur)	Sulphur (Sulphur)
2nd Limiting	Isoleucine (Isoleucine)	Threonine (Isoleucine)
3rd Limiting	Valine (Tryptophan)	Isoleucine (Tryptophan)
	Unfermented *Masyaura*	
Protein Score	42.0 (43.8)	29.7 (43.8)
1st Limiting	Sulphur (Sulphur)	Sulphur (Sulphur)
2nd Limiting	Isoleucine (Isoleucine)	Isoleucine (Isoleucine)
3rd Limiting	Valine (Valine)	Threonine (Valine)
	Sun-dried *Masyaura*	
Protein Score	51.6 (40.8)	12.5 (40.8)
1st Limiting	Sulphur (Sulphur)	Sulphur (Sulphur)
2nd Limiting	Isoleucine (Isoleucine)	Threonine (Isoleucine)
3rd Limiting	Lysine (Valine)	Isoleucine (Valine)
	Controlled-Fermented *Masyaura*	
Protein Score	58.7 (35.8)	26.7 (35.8)
1st Limiting	Sulphur (Sulphur)	Sulphur (Sulphur)
2nd Limiting	Isoleucine (Isoleucine)	Threonine (Isoleucine)
3rd Limiting	Lysine (Valine)	Isoleucine (Valine)

(Note: Values and Amino acid in parenthesis indicates the protein score and limiting amino acids for total protein from the respective samples)

Nutritional parameters

The calculated nutritional indices such as essential amino acid index, biological value and nutritional index of blackgram, unfermented *Masyaura*, sun-dried *Masyaura* and controlled-fermented *Masyaura* are shown in Table 5. EAA index and biological value for albumin fraction from black gram dhal and *Masyaura* samples was slightly higher than globulin fraction and total protein. *Masyaura* samples were comparable the nutritional parameters of albumin, globulin and total protein fractions. However the results indicated that albumin fraction is nutritionally superior to globulin fraction in *Masyaura* protein (Table 5). Biologically assayed PER values of 1 to 1.9 for seeds of black gram proteins have been reported by Reddy et al, (1982). Comparison between three nutritional

Table 5: Nutritional parameters of major protein fraction of Blackgram and *Masyaura*

Parameters/Sample	*Albumin*	*Globulin*
	Blackgram	
EAA Index (%)	87.6 (66.1)	70.1 (66.1)
Predicted BV (%)	83.8 (60.4)	64.7 (60.4)
Nutritional Index	38.7 (15.9)	48.9 (15.9)
	Unfermented *Masyaura*	
EAA Index (%)	77.4 (64.8)	70.1 (64.8)
Predicted BV (%)	72.7 (59.0)	64.7 (59.0)
Nutritional Index	42.6 (13.2)	60.3 (13.2)
	Sun-dried *Masyaura*	
EAA Index (%)	76.0 (67.7)	68.0 (67.7)
Predicted BV (%)	71.1 (62.1)	62.5 (62.1)
Nutritional Index	32.7 (13.6)	65.1 (13.6)
	Controlled-fermented *Masyaura*	
EAA Index (%)	75.5 (59.8)	68.7 (59.8)
Predicted BV (%)	70.6 (53.5)	63.1 (53.5)
Nutritional Index	34.3 (12.1)	65.3 (12.1)

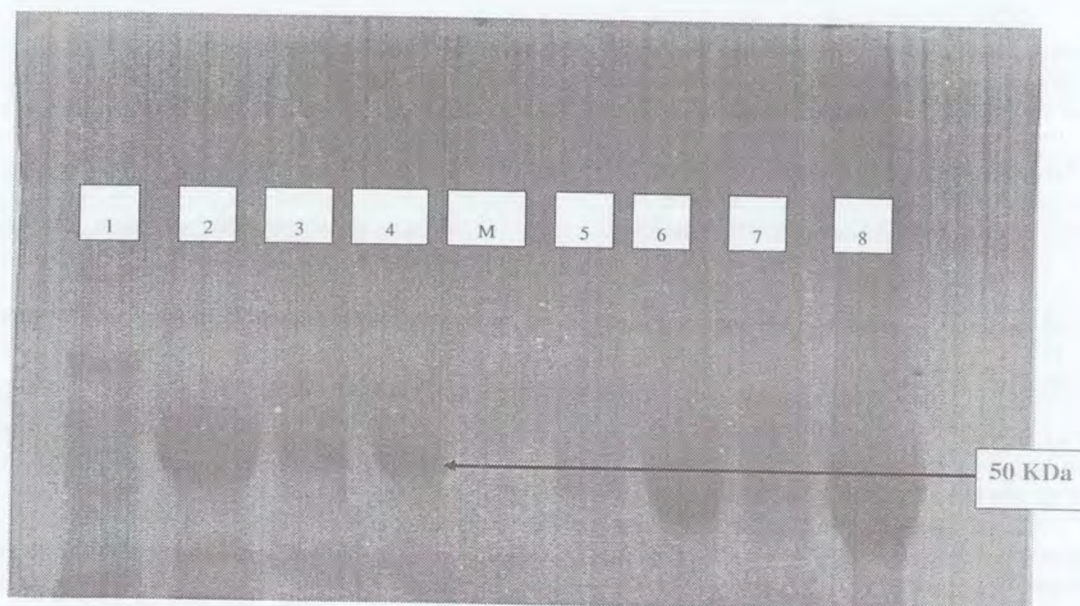
(Note: Values in the parenthesis indicate the value for the nutritional parameters for total protein from the respective samples)

parameters (i.e. E/T %, chemical score, and predicted biological value) was found to be projected some interesting conclusions previously. The ranking of protein fractions for the nutritional quality based on E/T% had found not to be correlated significantly with those based on chemical score or predicted biological value. Unlike E/T%, both chemical score and predicted biological value depends on the proportion of essential amino acids in a sample to that of its proportions in reference protein. Both of these parameters assumed existence of an optimal ratio of amino acids. Unlike chemical score, predicted biological value is a cumulative term wherein the deviations from the optimal contents for most of the essential amino acids were accounted (Reddy et al, 1982). However nutritional index for albumin fraction was found to be slightly lower than that of globulin fraction for all the samples (Table 5). This may be due to the low protein content in albumin fraction compared to globulin fraction of those samples (Table 1).

SDS-PAGE of major protein fractions

The gel electrophoresis (SDS-PAGE) pattern of major protein fractions albumin and globulin and of total proteins from black gram and from *Masyaura* fermented by different methods is given in Fig 2. The gel electrophoresis of albumin from black gram dhal showed 4 major bands and 4 minor bands. Blackgram globulin showed one major band at the molecular weight range with a molecular weight of about 50 KDa and with 5 minor bands.

Albumin and globulin fractions from unfermented *Masyaura* showed similar results to the black gram protein fractions. Protein fractions from sun-dried *Masyaura* sample showed two major bands for albumin fractions and with other 6 minor bands at the lower molecular weight range similar to albumin from black gram. Globulin fraction in sun-dried *Masyaura* showed one major band at the molecular weight range of less than 50KDa and other 8 minor bands. The results of albumin and globulin fractions from controlled-fermented *Masyaura* were similar to sun-dried *Masyaura*. Result indicated that the major protein fraction in black gram degraded to lower molecular weight protein fractions during fermentation, which reflected in increased solubility and in-vitro digestibility of protein. Sathe, (1996) has reported that black gram albumin had four major polypeptides in with molecular weight of 14,000, 84,000, 56,000 and 139,000 (indicate units). Similarly Reddy et al, (1982) have reported that albumin fraction of 8 subunits and globulin fraction of 6 subunits in black gram protein. The electrophoresis results suggest that the black gram proteins are degraded into the lower molecular weight fractions during the course of fermentation of the products. The hydrolysis of high molecular weight globulin to lower weight protein during *Oncom* fermentation has been reported (Sathe 1996).



[1. Blackgram Albumin, 2. Blackgram Globulin, 3. Unfermented *Masyaura* Albumin, 4. Unfermented *Masyaura* Globulin, M. Marker Protein, 5. Sun-dried *Masyaura* Albumin, 6.Sun-dried *Masyaura* Globulin, 7. Controlled-Fermented *Masyaura* Albumin, 8. Controlled Fermented *Masyaura* Globulin.]

Fig 2: SDS-PAGE Pattern of major protein fractions of Blackgram and *Masyaura*

Conclusions

In conclusion, albumin and globulin proteins are the major protein fractions in *Masyaura*. The Nutritional parameters such as essential amino acid index and predicted biological value were superior in albumin compared to globulin fraction. The major protein fractions were degraded to lower molecular weight fractions that reflected in the improvement of protein digestibility of *Masyaura* compared to the protein from raw ingredients. However, further studies on the albumin and globulin fractions of *Masyaura* proteins are essential to know the biochemical characteristics at molecular level.

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Antioxidant and Antibacterial Properties of Jatropha (*Jatropha curcas*) Meal Extracts

RAKSHIT DEVAPPA.K [1], SANJAY K. RAJESH [2] and BHAGYA SWAMYLINGAPPA [1,*]

[1] Department of Protein Chemistry & Technology, Central Food Technological Research Institute, Mysore, 570020, India.

[2] Department of Biotechnology, Sri Jayachamarajendra College of Engineering, Mysore, 570 006, India.

Jatropha meal was extracted with polar and non-polar solvents. The organic solvents extracted higher phenolics (35-38%) compared to aqueous extraction (24%). The antioxidant and antibacterial activities of the extracts were evaluated. Organic solvent extracts showed highest activity in all the tested models. The activity in the order of ethanol > Acetone > Isopropanol > Methanol > Water. In addition to the antioxidant activity, jatropha meal has been shown to have antimicrobial activity.

Keywords: *Jatropha; extract; phenolics; antioxidant; antibacterial activity*

Introduction

In the recent years research on antioxidants has been intensified with the aim of finding new effective natural compounds having higher antioxidant activity. In the present scenario, revenues of antioxidant industry are predicted to grow from $55m in 2004 to 58m $70m in 2008 (Aehle et al., 2004). Scientific research suggests that antioxidant serves in health protection and also used as stabilizers in food, cosmetic, pharma, plastic, chemical and lubricant industries. The main feature of antioxidants is its ability to trap free radicals during lipid peroxidation.

In living organisms, excess of oxygen-derived free radicals such as superoxide radical ($O_2^{\cdot -}$), hydroxyl radical ($^{\cdot}OH$), hydroperoxyl radical ($HO_2^{\cdot}$) and nitric oxide radical ($^{\cdot}NO$) can initiate lipid oxidation through 'ene' reaction where olefenic groups of fatty acids are converted to their corresponding $^{\cdot}OH$ generation and allyl hydroperoxides (Amarowicz et al., 2004; AOAC, 2000). These Reactive oxygen species (ROS) can form endogenously by normal aerobic respiration or by exogenous sources such as organic solvents, pesticides, tobacco smoke and other pollutants (Aregheore et al., 2003; Baruda and Oleszek, 2001). Uncontrolled syntheses of ROS are clinically associated with aging, inflammation, heart disease, atherosclerosis, diabetes, immunosuppression, neurodegenerative diseases etc. (Cheeseman and Slater, 1993; Cao et al., 1997,). The antioxidant consumption has been proven effective in preventing these diseases (Cao et al., 1997; Cohly, 1998, Vinson et al., 1995).

Majority of the commonly used antioxidants are of synthetic origin such as BHA, BHY, TBHQ, Propyl galate (Winata and Lorenz, 1996). Although these molecules are effective, they are suspected to be carcinogens (Madsen and Bertelsen, 1995). This situation created a necessity and importance for industries, which are directly related to food additive production, cosmetics and pharmaceuticals to increase their efforts in preparing bioactive compounds from natural products by extraction and purification, which may be used in place of synthetic antioxidants. Currently, natural antioxidant industries growth is pushed by easier consumer acceptance and legal requirements for market access. Among the natural antioxidants, phenolic antioxidants are in the forefront, as they are widely distributed in the plant kingdom. Plants produce phenolic compounds to deal with ROS and substrate derived-free radicals produced during the process of photosynthesis (Lu and Foo, 1995). Plant polyphenols have multifunctional properties and can act as reducing agents, singlet oxygen quenchers and hydrogen donating antioxidants (Pratt, 1992). They are also reported to have anti-microbial, colorants, antifungal, antipathogenic, anti herbivore and allelopathic properties (Shahidi and Naczk, 1995; Shahidi et al., 1997). Different types of natural antioxidants have already been isolated from different plant materials such as oilseeds, cereal crops, vegetables, fruits, leaves, roots, spices, and herbs (Ramarathnam et al., 1995) Phenolic compounds occur in oilseeds as the hydroxylated derivatives of benzoic and cinnamic acids, coumarins, flavonoid compounds, and lignins (Oomah et al., 1995)

The extracts of various oilseed meals possess antioxidant properties which in some cases showed better antioxidant properties than those observed for synthetic antioxidants at the same concentration levels (Mahinda and Shahidi, 1999). Meals obtained from oilseeds, such as canola, mustard, flax, sesame, peanut, borage, and evening primrose, after oil extraction, contained a variety of antioxidative factors

*Corresponding author; Email: bhagguru@yahoo.com

(Shahidi et al., 1997). The antioxidative effect of source materials, their extracts or their fractions will depend on the seed type, the content and chemical nature of their active components (Kozlowska et al., 1983; Fukuda et al., 1985; Amaronicz et al., 2001; Pratt and Hudson, 1990).

Jatropha curcas (physic nut) is a genus of Euphorbiaceae, native of Mexico and South America. The kernel contains 56.7% oil and the cake obtained after the oil extraction is rich in protein (Rakshit and Swamy Lingappa, 2008). Presently, Jatropha is cultivated widely for biodiesel production and after extraction of oil huge amount of biomass or cake are obtained. There has been several attempts to utilize the biomass as by product (as a feed ingredient after detoxification) from biodiesel industry (Rakshit and Bhagya, 2007). The phenolic compounds in Jatropha defatted meal is considered to be undesirable because of their antinutritional and sensory properties (e.g. bitter taste, dark color, astringency). Removal of phenolic compounds could improve the meal quality as well as it can be a new source of natural antioxidants. The total phenolics in toxic variety of Jatropha whole seed were ranged from 2.0-2.3 % (Makkar et al., 1998). Studies have reported that Jatropha plant exhibits bioactive activity for fever, mouth infection, Jaundice, and joint rheumatism, (oliver-Bever, 1986). Fagbenro-Beyioku et al., (1998) has reported antiparasitic activity of the sap and crushed leaves of *J. curcas*. In the present study, it was of interest to study the utilization of Jatropha meal antioxidants as a value added product for biodiesel industry. These antioxidants could be concentrated either as crude extracts or individual phenolic compounds. Therefore, the objective of this study was to optimize the extraction conditions to extract antioxidant and antibacterial properties from Jatropha meal and to utilize them in cosmetic/pharmaceutical or for industrial applications.

Materials and Methods

Commercial varieties of *J. curcas* seeds were obtained from local vendor, Karnataka, India. Reagents namely â-Carotene, Linoleic acid, Tween-20 (Polyoxyethylene (20) sorbitan monolaurate) emulsifier, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), Pyrogallol (1, 2, 3, benzenetriol), Sodium carbonate, DPPH (2, 2, diphenyl-1-picryl hydryzyl), Gallic Acid, were obtained from Sigma Aldrich. All other chemicals and solvents used were of analytical grade.

Preparation of Defatted Ghani Pressed Meal (DGPM): The pre-cleaned seeds were mechanically pressed using power Ghani (a large motorized pestle and mortar) to extract the oil. The resulting cake was solvent extracted with hexane in a ratio of 1:5 (w/v) using stainless steel columns. The extraction was repeated 4 to 5 times, with a soaking time of 12 h each (Rakshit and Swamylingappa, 2008). The fat content of the cake was less than 1g/100g (AOAC, 2000). The cake was air dried, powdered and passed through 22-mesh sieve. The resulting defatted ghani pressed meal was stored at room temperature for analysis.

Preparation of extract: The DGPM was extracted with different solvent systems in a ratio of 1:5 (w/v). The solvents used for the extractions were (A) Acetone/Water, (B) Methanol/Water, (C) Water, (D) Ethanol/Water and (E) Isopropanol /Water at 80°C for 30min under reflux conditions. All solvents were mixed in the ratio 70:30 (v/v) except water (C). The slurry was filtered through Whatman No. 4 filter paper. This procedure was repeated two times, and the filtrate was pooled. The residual solvent was removed under vacuum at 40°C. The resulting concentrated solution was freeze dried and stored at - 20°C for further analysis.

Total Phenolic content: The concentration of the total phenolic compounds was determined by the method of Ranganna (1995). The crude extracts were dissolved in water to obtain the concentration of 0.5 mg/ml in water. Sample 0.5 ml extracts was mixed with 0.5ml (1:1(v/v)) of diluted of Folin-ciocalteus phenol reagent and 1 ml of saturated sodium carbonate solution. Tubes were allowed to stand for 30 min at room temperature and the absorbance was measured at 660 nm. The results are expressed as Tannic acid equivalents.

Radical scavenging activity using DPPH method: The free radical scavenging activity was measured according to the method of Hatano et al. (1998). The extracts (5 ìg to 50 ìg) were added to 0.5 ml methanolic solution of DPPH radical (final concentration of DPPH was 0.2 M). The mixture was shaken vigorously and allowed to stand for 30 min at room temperature. The change in absorbance was measured spectrophotometrically at 517 nm. The control was prepared without extract and methanol was used for baseline correction. Radical scavenging activity was expressed as % inhibition and calculated using the following equation:

% Radical scavenging activity = (Control OD – Sample OD/ Control OD) x 100

Evaluation of Antioxidant activity in â-Carotene-linoleate model system: The Antioxidant activity was determined according to the method reported by Miller (1971). 2 mg of â-carotene is dissolved in 10 ml of chloroform. A 2 ml aliquot of â-Carotene was pipetted into a 250ml round bottomed flask. The chloroform was removed under vaccum at 40 ºC. To this 40 mg of purified linoleic acid and 400 mg of Tween 20 (Polyoxy ethylene sorbitan mono palmitate) are added and mixed. To this emulsion 100 ml of aerated distilled water were added and shaken vigorously. Aliquots (4 - 8 ml) of this emulsion were transferred into a series of tubes containing 100 or 200 μl of extracts, BHT (in methanol), so that the final concentration of extracts and BHT (20 – 80 ppm in the medium). BHT was used for comparative purposes. The tubes were placed at 50ºC in a water bath and the absorbance was measured at 470 nm at zero time using

spectrophotometer (UV- 1601, Shimadzu). Measurement of absorbance was continued at an interval of 15 min till the end of 2 h. A mixture prepared as above without â carotene served as blank.

The antioxidant activity of extracts was calculated in terms of bleaching of the â carotene using the equation.

$$\% \text{ANT} = 100 \times (R_{\text{control}} - R_{\text{sample}}) / R_{\text{control}}$$

Where, $R_{control}$ and R_{sample} are the average bleaching rates of â-Carotene in the emulsion without antioxidant and with extract, respectively.

Determination of reducing power: The determination of reducing power was performed as described by the method of Oyaizu (1986). Various concentration of Jatropha extracts (0-30 ìg) were taken in 1 ml of distilled water, mixed with 2.5ml phosphate buffer (0.2 M, pH 6.6) and 2.5ml of 1% potassium ferricyanide. The mixture was incubated at 50 °C for 20 min. Aliquots of 2.5 ml of 10% trichloroacetic acid were added and the mixture was centrifuged at 3000 rpm for 10 min. The upper layer of solution (2.5 ml) was mixed with distilled water (2.5 ml) and 0.5ml of $FeCl_3$ (0.1%) and the absorbance was measured at 700 nm. Increase in the absorbance of reaction mixture indicates increased reducing power.

Antibacterial Bioassay by disc diffusion method: Antibacterial bioassay was carried out on ten strains of food borne pathogens by means of disc diffusion method. The assay was carried out according to the method of Murray et al. (1998) with some modification. The bacterial cultures used are *Bacillus cereus, Pseudomonas aeruginosa, Yersinia enterocolitica, Proteus vulgaris, Listeria monocytogen, Staphylococcus aureus, Streptococcus pyogenes, Escherichia coli, Klebsiella pneumoniae and Shigella sonnei.* A loopful of organisms was pre-cultured in 10 ml nutrient broth for 6 hours. The turbidity of the culture was adjusted to 0.5 McFarland optical density. From the seed culture 0.1ml of bacterial suspension was inoculated on Mueller Hinton agar plates by spread-plated method. Sterilized Whatman No.42 filter paper discs (6mm diameter) impregnated with 10 µl of the methanol extract of *Jatropha curcas* seed meal extracts (A, B, C, D and E) at the concentration 10, 20, 30 µg/disc. The disks were placed on the seeded agar plates and diameters of inhibitory zones were measured after the plates were incubated at 37 °C for 24 h. Oxacillin (1 µg/disc), and norfloxacin (10 µg/disc) were used as positive controls for gram positive and gram-negative bacteria respectively. The tests were carried out in triplicate. Methanol was included in all experiments as negative controls.

Minimum inhibitory concentration determination: Minimum inhibition concentration of the methanol extract of *J. curcas* meal extracts (A, B, C, D and E) against food borne pathogens was determined according to the method of Vairappan (2003). The extracts were diluted in 200 µl methanol to different concentration levels (25 to 500 µg/ml). Diluted seed extract was mixed with the desired bacterial strains cultured in 9.8 ml of nutrient broths (10^4 colony forming unit). 200 µl of Methanol and sterile physiological saline without seed meal extracts were used as solvent and culture control respectively. Bacterial growth was monitored at 4, 8, 12, 16, 20 and 24 h and quantified as colony forming unit (CFU) by serial dilution method using plate count agar.

A second test was performed by transferring 1ml of each bioassay culture (bacterial culture with pigment) to a new test tube containing nutrient broth (9 ml). Observation was made for 24 h, to determine possible bacterial growth on secondary culture broths. Plate count method was used to determine the bactericidal or bacteriostatic mode of action of the pigment. Bacterial growth on plate count agar will suggest the tested compound as bacteriostatic, while lack of growth will indicate that the compound as bactericidal.

Statistical Analysis: All measurements were performed in triplicate, and analysis of variance were conducted by the General Linear Model procedure using SAS software Student–Newman–Keul's multiple-range tests were used to test for significant differences between the mean values for the treatments ($P < 0.05$).

Results and Discussion

The complete screening or assessment of antioxidant should include its interaction against a wide range of species, which are more directly responsible for oxidative damage like, scavenging superoxide, hydroxyl radical and ferryl species. Antioxidants may be water soluble, lipid soluble, insoluble or bound to cell walls. Hence, extraction efficiency is an important factor in quantification of antioxidant activity. In the study, the organic solvent systems (A, B, D, and E) were chosen because they were less polar and they can extract more hydrophobic components. The presence of water in the solvent system increases permeability of seed tissue and thus enabling a better mass transport by molecular diffusion. Water (C) was chosen as it might extract highly polar constituents and it would certainly be the most practical solvent in future in industrial applications.

The amount of extractable components (extract yield) obtained with the organic solvent extraction system was 5.90%, 7.55%, 6.30%, 5.95% respectively for acetone: water, Methanol: water, ethanol: water and Isopropanol: water respectively (Table1). However, the yield for water extraction was higher (9.8%) than those for organic solvent extraction systems. The increased extraction may be due to soluble sugars and proteins, which were extracted in aqueous system.

The phenolic and polyphenolics compounds constitute the main class of natural antioxidants present in plants and are

Table 1: Yield and total phenolic content of Jatropha defatted Ghani Pressed meal

Solvent systems	Yield (w/w)	Total Phenolics (%)
(A) Acetone: Water	5.90±0.19	38.58±1.2
(B) Methanol: Water	7.55±0.20	35.06±1.0
(C) Water	9.80±0.26	24.04±1.0
(D) Ethanol: Water	6.30±0.30	36.23±1.6
(E) Isopropanol: Water	5.95±0.21	36.21±1.4

Values are the mean±standard deviation (SD) of three determinations

usually quantified using Folin's reagent. The total phenolic content of extracts (A, B, D, and E) was in the range of 35.06 – 38.58% respectively (Table 1). However, the low phenolic content of extract C (24.04%) may be attributed to co extraction of other water-soluble compounds like proteins and carbohydrates. The percent extraction of total phenolics in the extracts was 82, 79.04, 86.39, 83.82, and 79.4 % for ABCD and E respectively. In the oil seeds, phenolic compounds are responsible for dark color, bitter taste and flavor of the cake meal after oil extraction.

The ability of compounds to scavenge hydrophilic free radicals is reflected by DPPH assay (Aehle et al., 2004). The results were evaluated and relative activity was compared (Fig 1).

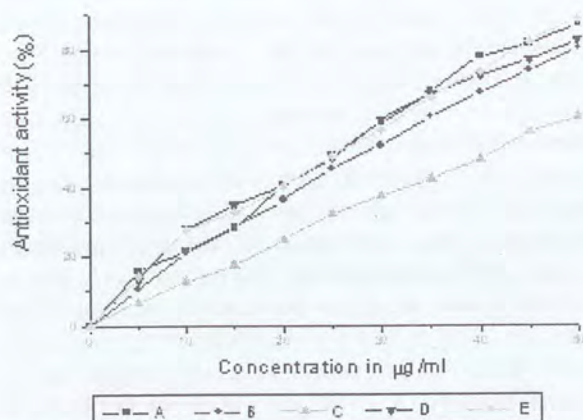

Fig 1: DPPH scavenging activity of Jatropha extracts in various solvents

The results showed that the antioxidants derived from plants may be polar or non-polar not necessarily available to react with the DPPH. Hence, they react at different reaction kinetics and the reaction will often not go to completion in a assay time. Therefore, the sample concentration that can lower the initial absorbance of DPPH solution by 50% is chosen as endpoint for measuring the antioxidant activity. The IC_{50} (µg/ml) of extracts ABDE was 25.42, 28.87, 25.21, and 26.44 respectively. Whereas extract C, showed higher IC_{50} of 41.49 µg/ml (Table 2). In DPPH assay, lower the IC_{50} the better is its capacity to scavenge the radicals like peroxy radicals (Frankel, 1991).

Table 2: Free radical scavenging activity (IC_{50} value) of Jatropha extracts in different solvents

Sample Extract	IC_{50} mg/ml
(A) Acetone: Water extract	25.54 ± 0.12
(B) Methanol: Water extract	28.94 ± 0.07
(C) Water extract	41.56 ± 0.06
(D) Ethanol: Water extract	25.82 ± 0.61
(E) Isopropanol: Water extract	26.49 ± 0.04

Values are the mean±standard deviation (SD) of three determinations

In the present study organic solvent extracts showed higher scavenging capacity than water extract and the order of activity was B>E>A>D>C. The activity of extracts is attributed to their hydrogen donating ability and these are believed to counter free radical chain of oxidation and to donate hydrogen from the phenolic hydroxyl groups, thereby forming stable end product, which does not initiate or propagate further oxidation (Sherwin, 1978). The data reveals that extracts are free radical inhibitory and primary antioxidants that react with free radicals. The scavenging potential of antioxidant extract is indicated by degree of discoloration of DPPH.

The antioxidant activity of the Jatropha extracts in the β-carotene-linoleate model system at 20 – 80 ppm was measured by the bleaching of β-carotene, is presented in Fig 2. ß-carotene bleaching occurs in multiple pathways so interpretation of results may be complicated (Prior et al., 2005) and also there are no standard formats for expressing results. Hence, studies may utilize different methods for calculating inhibition kinetics (Baruda and Oleszek, 2001; Amarowicz et al., 2004; Prior et al., 2005).

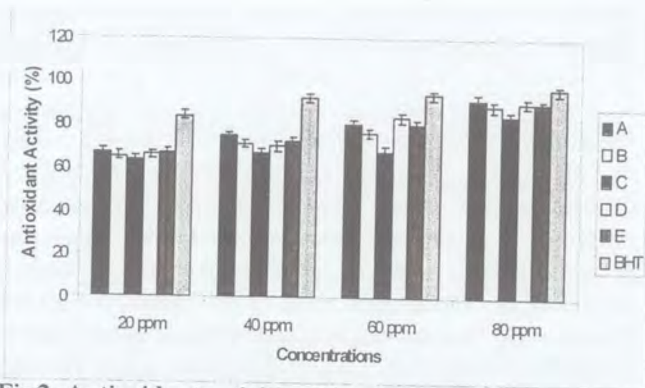

Fig 2: Antioxidant activities (%ANT) of extracts by beta-carotene bleaching method

The average rate (15-20 min) antioxidant activity is presented as % ANT. As the oxidation progressed the absorbance of ß carotene at 470 nm decreased and its yellow color faded. The effectiveness of extracts preventing bleaching of ß-carotene increased at higher concentrations. The extracts A, B, C, D and E showed 84-92 % (ANT) activity at 80-ppm concentration (Table 3).

Table 3: Antioxidant activities (%ANT) of extracts assessed by beta- carotene bleaching method

Extract	20 ppm	40 ppm	60 ppm	80 ppm
A	66.80	74.64	80.43	92.04
B	65.34	71.30	76.26	88.87
C	63.58	66.61	67.83	84.39
D	66.00	69.86	83.43	90.41
E	66.75	72.15	80.36	90.20
BHT	84.08	92.49	94.03	96.65

The higher activity may be attributed to the phenolics. However, the order of extracts activity was found to be A>D>E>B>C. BHT was comparatively more effective antioxidant than all the other extracts examined. The oxidation is induced by light, heat or by peroxyl radicals (Ursini et al., 1998). In this system, the linoleic acid free radical formed from abstraction of hydrogen atom from one of its diallylic methylene groups attacks the highly unsaturated ß-carotene molecules. As ß-carotene molecules lose their double bonds by oxidation, the compound looses its chromophore and reduction in orange color is monitored spectrophotometrically. The presence of extracts can hinder the linoleate free radical and other free radical formed in the system.

The reducing capacity of a compound is the measure of extracts ability to donate electron to Fe (III) and the capacity of a reduction of a compound serves as a significant indicator of its antioxidant potential (Mier et al., 1995). Thus, reducing power evaluation was taken as an important parameter for the assessment of antioxidant activity. Reducing power of the Jatropha extracts as a function of their concentration is shown in Fig 3. The reducing ability of the Jatropha extracts increased with increasing concentration. All the solvent extracted samples showed very high reducing activity of absorbance or EC_{50} 17.25, 16.0, 15.5, and 17.10 for A, B, D and E. whereas, the reducing power of water extract was lower when compared with the organic solvent extracts. This data reveals that the reducing ability of Jatropha extracts is in part a contributor to antioxidant activity and the reduction power decreases inversely to the polarity of extraction solvent. The order of reduction power of the extracts was D>B>E>A>C. The reducing power might be due to hydrogen donating ability (Shimada et al., 1992) and is generally associated with the presence of reductones (Pin-Der Duh, 1998). However, the reducing power of plant extract is lower than that of BHT. Furthermore, the antioxidant activities of putative antioxidants have been attributed to various mechanisms such as the prevention of chain initiation, the binding of transition metal ion catalysts, the decomposition of peroxides, the prevention of continued hydrogen abstraction and radical scavenging (Diplock, 1997). Hence, there may not always be linear correlation between total antioxidant activity and reducing power activity.

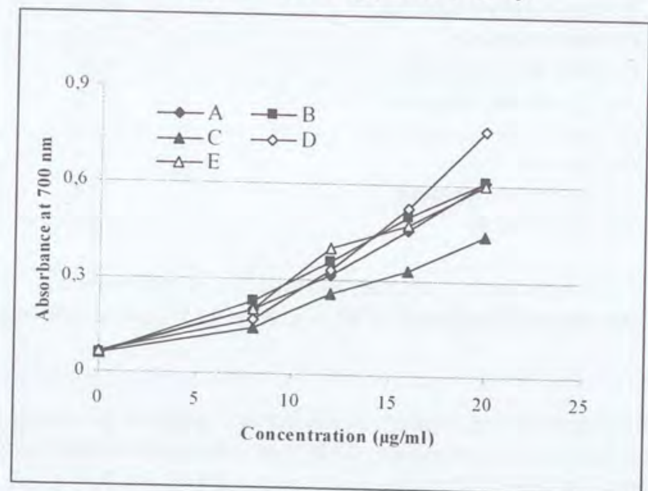

Fig 3: Antioxidant Activity of Jatropha extracts by reducing power method

Antibacterial activity of *J. curcas* DM extracts were tested against 10 species of food borne pathogens (*Bacillus cereus, Pseudomonas aeruginosa, Yersinia enterocolitica, Proteus vulgaris, Listeria monocytogen, Staphylococcus aureus, Streptococcus pyogenes, Escherichia coli, Klebsiella pneumoniae, Shigella sonnei*) using disc diffusion assay. Preliminary screening results showed that the methanol extract of *J. curcas* was effective against all the microbes tested except *Bacillus cereus* (3mm). A clear zone of inhibition by the pigment against *Proteus vulgaris* is shown in **Fig 4**.

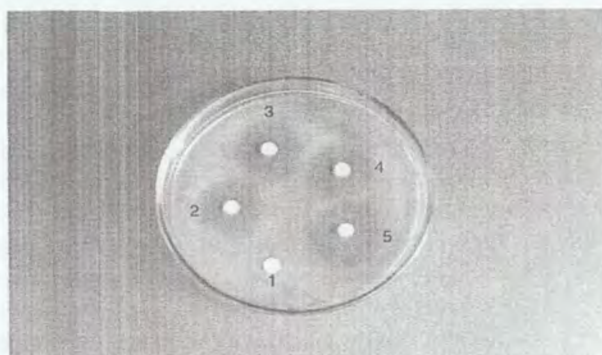

Fig 4: Inhibition zone formed by Jatropha meal extracts against *Proteus valgaris*; (1. Control , 2. Ethanol extract, 3. Methanol extract, 4. Water extract and 5. Acetone extract)

Results of disc diffusion assay of pigment with Oxacillin and Norfloxacine as positive controls are shown in Table 4.

Methanol extract of seeds showed antibacterial activity against *Bacillus cereus, Pseudomonas aeruginosa, Yersinia enterocolitica, Proteus vulgaris, Listeria monocytogen, Staphylococcus aureus, Streptococcus pyogenes, Escherichia coli, Klebsiella pneumoniae, Shigella sonnei,* with the significant inhibition against *Yersinia enterocolitica, Proteus vulgaris, Pseudomonas aeruginosa, Streptococcus pyogenes,* and showed intermediate inhibition against *Staphylococcus aureus, Escherichia col,i Listeria monocytogen.* The greatest zone of inhibition (14mm) was displayed by *Proteus vulgaris* and *Yersinia enterocolitica.* The most susceptible bacterial species were *Yersinia enterocolitica, Proteus vulgaris, Pseudomonas aeruginosa, Streptococcus pyogenes, Staphylococcus aureus, Escherichia col,i* and *Listeria monocytogen.* The solvent used for dissolving (Methanol) the extract and the standard antibiotics gave negative results, showing that they did not influence in the antimicrobial activities observed for the extract (Table 4).

Table 4: Inhibition zone (mm) formed by the Jatropha meal methanol extract against different pathogenic strains

Microorganism	*Methanol extract*	*Oxacillin*	*Norfloxacine*	*DMSO*
Bacillus cereus	3.0	12.0	-	R^b
Pseudomonas aeruginosa	12.0	-	26.0	R^b
Yersinia enterocolitica	14.0.	-	18.4	R^b
Proteus vulgaris	14.0	-	22.0	R^b
Listeria monocytogen	6.0	18.2	-	R^b
Sta Phylococcus aureus	10.0	14.0	-	R^b
Streptococcus pyogenes	13.0	21.0	-	R^b
Escherichia coli	8.0	-	20.0	R^b
Klebsiella pneumonia	10.0	-	22.0	R^b
Shigella sonnei.	5.0	-	18.5	R^b

Disc size: 6mm, concentration of the methanol extract: 30 µg /disc, Oxcillin 1 µg /disc, Norfloxacine 10 µg /disc, R^b Absence of inhibition. Values are mean ± standard deviation of three replicate analyses

Investigation was further carried out to determine minimum inhibitory concentration (MIC) of methanol extract of Jatropha seeds. Viability test was conducted on five food borne pathogens: *Yersinia enterocolitica, Proteus vulgaris, Pseudomonas aeruginosa, Streptococcus pyogenes,* and *Staphylococcus aureus.* Methanol extract showed MIC in the following tendency; *Yersinia enterocolitica, Proteus vulgaris* and *Pseudomonas aeruginosa* (50 µg/ml) < *Streptococcus pyogenes* (200 µg/ml) < *Staphylococcus aureus* (400 µg/ml). Fig 5 and Fig 6 show the results of time course experiments carried out to determine MIC value for methanol extract against *Yersinia enterocolitica* and *Staphylococcus aureus.*

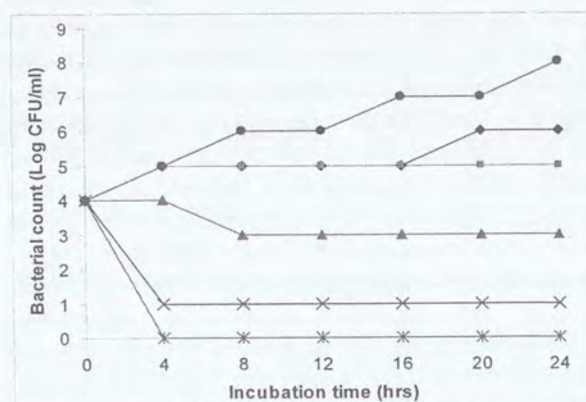

Fig 5: Dynamics of *Staphylococcus aureus* growth as a function of time in different concentration of pigment (Ï%) Control, (◆) 25 µg/ml, (%) 50 µg/ml, (²%)100 µg/ml, (X) 200 µg/ml, (–) 400 µg/ml

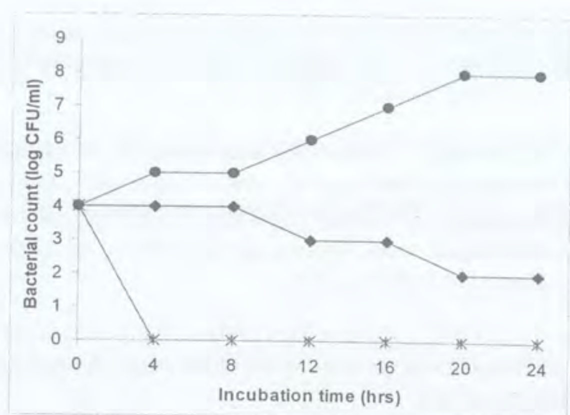

Fig 6: Dynamics of *Yersinia enterocolitica* growth as a function of time in various concentration of pigment (Ï%) Control, (◆) 25 μg/ml, (%) 50 μg/ml, (²⁰%) 100 μg/ml, (X) 200 μg/ml, (–) 400 μg/ml

The results showed that as the concentration of the pigment (both pure and crude) increases the viability of the organisms decreases gradually. Organisms such as *Yersinia enterocolitica, Proteus vulgaris, Pseudomonas aeruginosa* shown drastic decrease in viability at lesser concentration of pigment (50.0 μg/ml) where as in *Streptococcus pyogenes* and *Staphylococcus aureus,* decrease in viability was found at higher concentration of pigment (200 μg/ml). The minimum Inhibitory concentration for the pigment lies in between 50 to 400 g/ml (Table 5).

Second set of test to determine bactericidal or bacteriostatic activities was performed by culturing 1ml culture broth of bioassay test tubes that show no growth samples. Test tube that show no growth samples contain bacteria that are dead or just inhibited and sub culturing them with out chemotherapeutic agent will enable us to differentiate methanol extract with bactericidal or bacteriostatic activity. All sub cultured test tubes showed no bacterial growth. Therefore it could be assumed that the Jatropha seed extract is having bactericidal activity.

Table 5: Minimum inhibitory concentration (μg/ml) of Jatropha meal extract on different food borne pathogens

Bacterial strains	*Acetone extract*	*Ethanol extract*	*Methanol extract*
Staphylococcus aureus	300	400	400
Yersinia enterocolitica	25	50	50
Proteus vulgaris	25	50	50
Pseudomonas aeruginosa	25	50	50
Streptococcus pyogenes	150	200	200

Conclusions

The broad range of antioxidant activity of the extracts indicates the potential application of the meal extracts as a source of natural antioxidants or neutraceuticals. The present investigation clearly showed that Jatropha extracts as effective antioxidants and antimicrobial preservatives, which could be used in cosmetic and pharmaceutical applications or as an effective stabilizer for increasing the shelf life of oils and oil based applications.

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Effectiveness of *Bojo* (*Acorus calmus*) as Natural Insecticide in Wheat Grain[#]

EKARAJ PAUDEL* and PASHUPATI MISHRA

Central Campus of Technology, Dharan, Nepal

Effectiveness of Bojo (Acorous calamus) was studied as natural wheat grain protectant against infestation by rice weevil (Sitophilus oryzae). Bojo was prepared in three different forms as Dried Powder (DP), Chloroform Extract (CE) and Petroleum Ether Extract (PEE). LT_{90} (time required for 90% mortality of the weevils) value was taken as an indicator of effectiveness. Similarly effects of Bojo treatment on progeny development of rice weevil was also studied by observing growth of the weevil in the second generations. Result indicated that DP and PEE were effective as natural wheat grain ptotectant.

Key Words: Bojo (*Acorous Calamus*), Rice weevil (*Sitophilus oryzae*), Lethality, LT_{90}, Dose level.

Introduction

In Nepal, agricultural sector is suffering from its low contribution in Gross Domestic Productivity, GDP (39%) in spite of the involvement of a very large part of the population i.e. 66 % (CBS, 2007). Agricultural sector in Nepal, though considered as the leading sector, is not keeping pace with the rapidly growing population of the country. This is not only due to low productivity of the sector but also due to a high post harvest loss of the harvested produce which is affecting the produce both in terms of quality and quantity. Various studies have indicated that post harvest loss in cereal grains are up to 33% (NARC,1997).

The use of insecticide, in this context, seems to be inevitable. Use of insecticide and pesticide in storage bin and bags are quite common. The effectiveness of the pesticides and insecticides in storage bin is quite good to control infestation but at a heavy cost. The cost of these synthetic chemical pesticides can not only be limited to the heavy price the farmers are paying for, but should also be linked with hygienic, environmental and ecological aspects. Pests may develop resistance after repeated use. Resistance of the pests towards the chemical due to the repeated application reduces the effectiveness of the chemicals year after year. Toxic and non degradable residues of these chemical even after drying of the grain and environmental pollution caused by these chemicals has always threatened the healthy living of the people (Borsdorf *et al,* 1992).

Some botanical plants which can be obtained very easily at the local level can be a very good option to the synthetic chemical pesticides. The use of botanical pesticides in Nepal, irrespective of confirmed doses and application methods, is from centuries. Many plants in local vegetation of Nepal have shown promising insecticidal activity. Some common ones are *Banmara* (*Lantana camera*), *Bojo* (*Acorous calamus*), Neem (*Azadirachta indica*), *Tulsi* (*Osmium basilicum*), *Titepati* (*Artemesia vulgaris*), *Sarifa* (*Anona squamosa*) Rayo (*Brassica nigra*) *Lasun* (*Allium sativum*) Tobacco(*Nicotana tubacum* L) *Pire ghans* (*Cambopogan citrates*) *Asuro* (*Adhatoda vasica*) *Simali* (*Vitex meundo* L) *etc* (Paneru, 1996).

In spite of superiority of botanical pesticides to the chemical pesticides, the bulkiness of the botanical pesticides makes them practically impossible to be applied in large grain storage. Moreover, they cannot be applied in the disinfection of spaces, corners and crevices till they are extracted in a suitable solvent so that they can be used in form of emulsions as sprays or fumigants as fumes (Hill, 1990). The presesnt study wishes to assess the effictiveness of *Bojo* (*Acorus calmus*) as natural insecticides in wheat grain.

Materials and Methods

Preparation of Dried Powder (DP): Cleaned *Bojo* rhizomes were cut in transverse section to obtain circular rings of thickness 2 mm. *Bojo* pieces so obtained were oven dried at temperature of 55 ± 5^0C in a hot air oven to obtain final moisture content of 10%. It was grinded and then screened in screens of mesh no. 15 and 18 to obtain particles of 0.3607 mm average size. Dried powder such prepared were used in the amount of 0.5, 1.0, 1.5, 2.0 and 2.5g (Total 7.5 g) for each 100g of wheat, and labeled as Dose Level 1, 2, 3, 4 and 5 respectively. For the control, Dried Powder was not added.

Preparation of Chloroform Extract (CE) : Dried *Bojo* powder prepared was used. 7.5 grams of dried powder was extracted in 100 ml of chloroform at 60°C for 1 h. It was then, cotton filtered and the residue left was washed with 6ml chloroform for three times. 5 grams of rice husk, which was oven dried at 80°C for 1 h to kill hidden infestation, if present any, was mixed with the extract and stirred properly. Chloroform in the control and sample was removed by evaporation in hot water

* Corresponding author, Email: pyogen2002@gmail.com

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bath maintained at 60±5°C for 1 hour. Combined weight of rice husk and chloroform extract was taken. $\frac{1}{15}, \frac{2}{15}, \frac{3}{15}, \frac{4}{15}$ and $\frac{5}{15}$ th part of the weight were used in the experiment as chloroform extract and labeled as dose label 1, 2, 3, 4 and 5 respectively.

Preparation of Petroleum Ether Extract : Same as Chloroform Extract; petroleum ether was taken in place of chloroform.

Cultivation of Test Insect : Rice weevils (*Sitophilus oryzae*) of the age not older than 15 days were used to assess effectiveness of *Bojo* as natural insecticide. Wheat grain heavily infested with the organism was used. All the adults were removed. Calculated amount of water was added to the wheat grain and it was conditioned at room temperature for 24h to get final moisture content of 14%. Conditioned wheat grain sample was incubated at temperature of 28±3°C for 15 days. Weevils present in the incubated sample were used.

Preparation of Wheat : Wheat sample free from any visible infestation was used. The sample was disinfested as suggested by Hond (2003). Sample was cleaned properly and dried in a hot air oven at temperature of 80°C for 1h to destroy the hidden infestation, if present any. Wheat sample was cooled; calculated amount of water was added, and finally conditioned at room temperature to get final moisture content of 14%.

Experimental Procedure : One hundred grams of wheat sample was kept in a plastic jar with 7.5 cm diameter and 8cm height. Various doses of Dried Powder, Chloroform Extract and Petroleum Ether Extracts were kept in the separate jars and mixed thoroughly with 100g of wheat sample. Twenty rice weevils cultivated at laboratory were added. Mouths of plastic jars were covered tightly with double layers of muslin cloth with help of rubber. Plastic jars were incubated at 28±3°C.

Assessment of effectiveness

Mortality of weevils: Number of live insects in each of the plastic jars was counted in each 24 h interval for 10 days. A graph of Number of the live insects Vs days was plotted. Curve fitting was also carried out by regression analysis best fitted curve was obtained (Gupta and Kapoor, 2004). From the curve, time in hours, required for 90% mortality, i.e. Lethal time (LT_{90} i.e., for the number of live insects to become 2), was also calculated. LT_{90} value was used as an index of effectiveness.

Progeny development: After 10 days, all survivors in each plastic jars were removed and jars were left for another 35 days with their mouth covered. After 35 days jars were again observed to check presence of weevils from second generations. For each treatment, Maximum Dose Level at which growth was observed was noted to find minimum Dose Level which is sufficient to check the progeny development.

Germination of wheat: Wheat sample which was free from any visible infestation was used. Grains were cleaned properly and 2.5% dried powder was mixed. Germination of grains was tested in each 5 days interval for 45 days. Wheat grains were soaked in water for 24 h to remove dormancy. Fifty soaked wheat grains were kept in a Petri dish containing wet cotton. Four such replicates were taken. Dishes were incubated at 30°C. Water was added periodically with help of a dropper to keep the cotton wet. After 2 days, number of grains capable of producing sprouts was counted and germination energy was expressed as %.

Statistical tests: Statistical data analysis was carried out as described by Gupta and Kapoor (2004).

Result and Discussion

Mortality of weevils

Plot of number of survivor of the treatment with *Bojo* sample is presented in Fig1, Fig 2 and Fig 3 for DP, CE and PEE respectively. All the Dose Levels used in the experiment for various treatments showed similarities that in a particular day, number of live insects were least for dried powder.

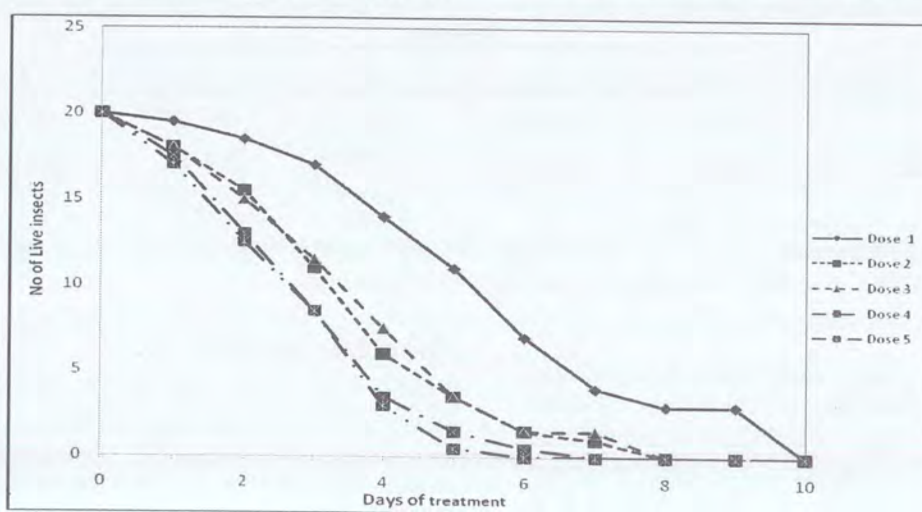

Fig 1: Changes in number of live weevils with time (in days) for dried powder

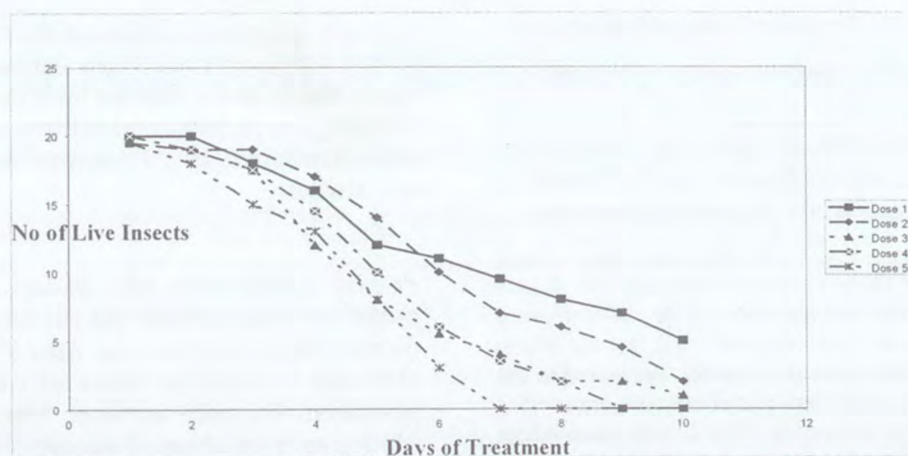

Fig 2: Changes in number of live weevils with time (in days) for chloroform extract.

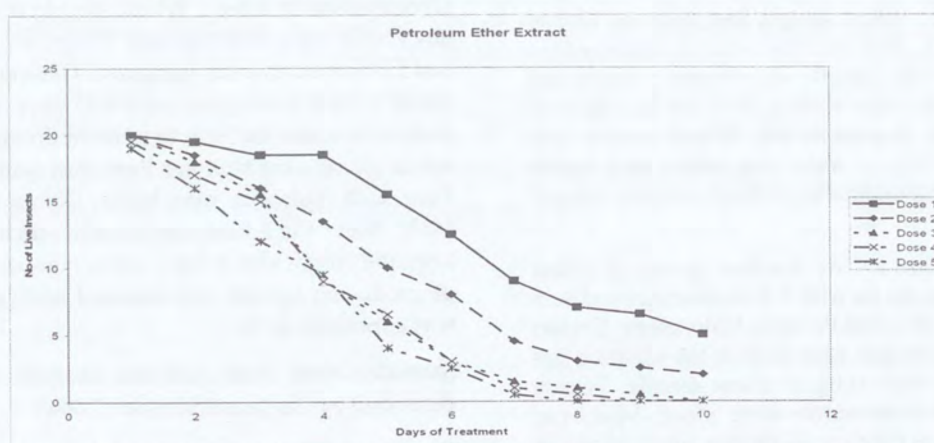

Fig 3: Changes in number of live weevils with time (in days) for Petroleum Ether extract.

Effect of various treatments on LT_{90} values of different dose level is presented in Table 1. In case of dried powder minimum dose level not significantly different ($p<0.05$) with the highest dose level was determined to be dose level 2 which equals 1% dried *Bojo* by weight.

Table 1: Effect of treatments on LT_{90} values of different dose level

Treatments	*Dose level*				
	1	*2*	*3*	*4*	*5*
Dried Powder	279.8^{a1}	158.1^{ab2}	152.5^{ab1}	126.32^{ab1}	115.99^{b1}
Chloroform Extract	489.69^{a2}	359.9^{ab1}	226.99^{bc1}	228.31^{bc1}	199.36^{c1}
PE Extract	518.06^{a2}	259.28^{b12}	185.27^{b1}	177.29^{b1}	163.01^{b1}

Note: Different alphabetic superscript (a, b, c) along the row and numeric superscript (1, 2) along the column indicate the samples are significantly different ($p < 0.05$) in their LT_{90} values

The value obtained is in good agreement with the experiment carried out by Paneru (1996) who concluded that 1% dried powder of *Bojo* by weight was sufficient to control the infestation of rice weevil. Similarly, for CE and PEE minimum dose level not significantly different with the highest dose level were found to be dose level 3 and 2 respectively.

Progeny development of weevil

Result of the observation for progeny development in the second generation is presented in Table 2. For DP none of the Dose Level used in the experiment showed growth of weevils in second generations except for the control. For PEE, and CE all the Dose Levels at and below 1, and 2 respectively and their respective controls growth was observed.

Table 2: Maximum Dose Level for different treatments allowing weevils to growth in second generations.

	DP	*PEE*	*CE*
Dose Level	0	1	2

(DP: Dried Poweder, PEE: Petroleum Ether Extract, CE: Chloroform Extract)

Chander et al., (1990) has noted that *A. calamus* powder at the rate of 1% did considerable reduction in progeny emergence of *S. oryzae* whereas quite lower dose than the mentioned level was found to affect adversely on the attempt to control the infestation resulting not to check the infestation but to enhance the progeny development of the rice weevil.

Risha et al., (1990) has indicated that lower doses of calamus powder do not affect immature stage, rather seemed to encourage the progeny emergence of *S. oryzae*. No such evidence was noticed in any of the lower doses used in the experiment in this study. But according to Nahal *et al* (1989), variation in insecticidal property of *Bojo* not only depends on the part from which powder is made but also depends on maturity and climatic condition under which the plant was grown. Similarly Paneru et al (1997) has indicated that effectiveness of *Bojo* as insecticide depends highly on the altitude at which it is grown. Therefore, all the deviations from aforementioned results obtained in this experiment were regarded as variations created due to different insecticidal characteristic of *Bojo* which was created due to variation in the altitude, climatic condition during growth and the part of the plant used.

Germination of wheat

From the mortality data, the most effective treatment at its highest Dose Level was selected to test whether treatments with *Bojo* changes the germination power of wheat grains or not. Wheat grains were admixed with *Bojo* dust at the rate of 2.5% concentration. Germination test for wheat grains was carried out for 50 days in an interval of each 5 days. A graph of % germination and days is presented in Fig. 4

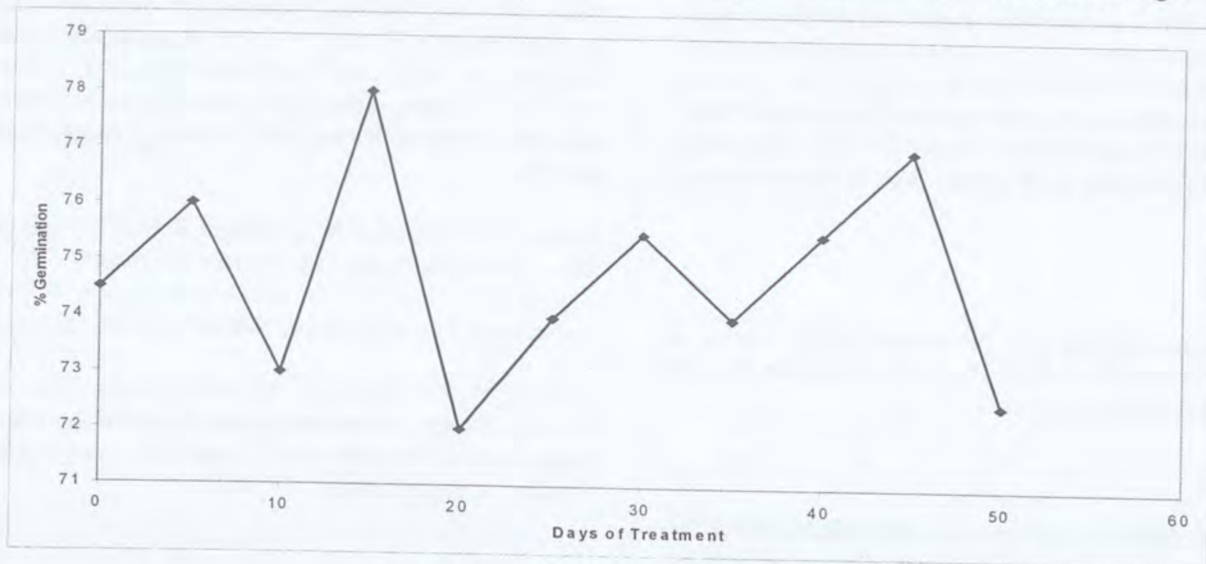

Fig 4: Changes in % germination with time.

Average germination of wheat sample used was found to be 74.5 % at the beginning (0th day) of the experiment. Values of % germination of wheat sample was found maximum at 15th day and minimum at 20th day with the respective values of 78 and 72%. The mean value of % germination of wheat sample during the experimental period was calculated to be 74.8%.

A regression analysis was carried out by using Karl Pearson's coefficient. The value of Karl Pearson's coefficient was found to be -0.01535, which indicates that there is a negligibly small negative correlation between No. of days for which wheat is stored treated with *Bojo* sample and % germination of the sample. Further analysis for probable error (P.E.) in the experiment shows its value 0.203322. Since Karl Pearson's coefficient is lower than probable error it can be concluded that there is no significant relationship between no of days and % germination of the grain.

From this study, it is found that dried Bojo powder as such is very effective to control infestation and can be separated with the wheat grain before milling, if cleaned properly. But it has its own drawbacks. It is not handy to use, it can't be applied uniformly on the large scale, and similarly it can not be used in space treatment in corners and crevices. The number of consumers of organic foods, who are very conscious towards handy but hazardous chemical pesticides, is continuously increasing. There is an immediate need to develop an handy form of organic insecticide, for example in

the form of emulsions as sprays or fumigant as fumes, which can be applied in the large scale uniformly throughout the stock and for space treatment purpose.

Highest mortality rate is not only a single factor to determine whether or not a treatment method is effective to be used as infestation control means. Equally or more important factor than high mortality is that the treatment used must be capable to check the progeny development. The effectiveness of the treatment with botanical insecticide goes on fading with passage of the time due to the loss of volatile oil. Therefore, newly formed progeny from subsequent development of egg to larva, pupa and adult insect can survive easily and damage grain. Nahal *et al* (1989) has reported that the percentage mortalities, whilst apparently not dose related, increased significantly with the exposure period as well as with the post treatment period. This indicates that the duration of exposure is more important than the Dose Level. Taking in consideration this fact, relatively lower doses of PEE and CE can be used except at the condition, where due to a very heavy infestation an immediate control is necessary. Though from the analysis of mortality data for chloroform extract and petroleum ether extract minimum Dose Level not significantly different from the highest mortality were 1.5 and 1 % respectively, from the observation for the progeny development it can be concluded that the Dose Level greater than 1.0 and 0.5 % for chloroform extract and petroleum ether extract is sufficient to control infestation.

Conclusion

It can be concluded that *Bojo* can successfully be used as a natural wheat grain protectant to check the infestation without any hindrance in germination of the wheat.

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Effect of Detoxified Expeller Pressed and Dehulled Jatropha (*Jatropha curcas*) Meals in Poultry Feed Formulations

MELVIN PRASAD[1], RAKSHIT KODEKALRA DEVAPPA[1], SERVA PEDDHA MUTHUKUMAR[2] and BHAGYA SWAMYLINGAPPA[1,*]

[1]Department of Protein Chemistry and Technology, Central Food Technological Research Institute, Mysore, 570020, India.
[2]Department of Biochemistry and Nutrition, Central Food Technological Research Institute, Mysore, 570020, India.

Jatropha has been identified as a suitable tree borne oil seed for the production of biodiesel in India and other countries. The seed contains 30-32% protein and 60-66% oil. The cake obtained after oil extraction is rich in protein, is not being used as food or feed due to the presence of highly toxic phorbol esters and antinutritional constituents. In view of the high toxic nature of phorbol esters, expeller pressed as well as dehulled seed meal was subjected to alkali and heat treatments to reduce the phorbol esters and anti nutrients. The detoxified meals were used in poultry feed formulations at 10% level. The diets containing untreated meal and meals treated with calcium hydroxide and sodium hydroxide were fed to 4 weeks old chicks as starter diet followed by finisher diet. Dehulled meal treated similarly was used in finisher diets in the second set of experiment. The performances of birds fed on diet containing NaOH treated meals were comparable to the groups fed on control diet. The microscopic examination of intestine, liver, heart and kidney of the birds fed on diet containing NaOH treated meal did not differ significantly ($P<0.05$) compared to control diet fed group. However, the histological examination revealed that the birds fed on diet containing dehulled meal treated with $Ca(OH)_2$ and NaOH showed normal architecture than similarly treated expeller pressed meal diets . Jatropha meals treated with NaOH improved the poultry performance. However, dehulling and treating the meal has positive benefits in poultry feed formulations compared to un-dehulled expeller pressed meal. Hence, dehulling of jatropha seeds before oil extraction results in producing better quality meal.

Keywords: Jatropha meal; Detoxification; Poultry feed; Growth performance; Histopathology

Introduction

Jatropha curcas (family *Euphorbiaceae*) popularly called Ratanjyot is a small tree bearing oil seed grown on wastelands and hedges in India and tropical America (Makkar et al., 1997 and 1998). The seeds contain 30-32% protein and 60-66% oil. Jatropha is being cultivated in central and western parts of India, mainly for biodiesel production, as their indigenous production do not meet the currant demand. Jatropha has been identified as a suitable tree born oil seed for the production of biodiesel (Aderibigbe et al., 1997). The cake obtained after oil extraction is presently used as fertilizer. The presence of high levels of toxic phorbol esters and antinutrients restricts the use in animal feed. However, the protein rich cake could be used as a potential source of protein for livestock after detoxification (Aregheore et al., 2003; Chivandi et al., 2004; Rakshit et al., 2006; Rakshit and Bhagya, 2006).

Jatropha curcas seed fed to calves at 2.5, 1 and 0.25 g/kg body weight showed rapid onset of toxic manifestations and death within 19 hours of administration (Ahmed and Adam, 1979). The seeds of *Jatropha curcas* are known to be toxic in mice and rats (Adam, 1974; Stripe et al., 1976). Adam and Magzoub (1975) have reported that the Nubian goats fed *Jatropha curcas* seeds at doses ranging from 0.25 – 10 g/kg/day, were found to be toxic with mortality between 2-21 days. El Badwi et al., (1995) have also reported high degree of mortality in Brown Hisex chicks, which were fed diet containing 0.5% jatropha seed. The phorbol esters have been identified as main toxicants in this meal and heating at temperature of 160°C for 30 minutes did not destroy the phorbol esters (Makkar and Becker, 1999; Makkar et al., 1997). Becker and Makkar (1998) have reported that threshold level at which phorbol esters appeared to cause adverse effects in carp was 15 ¼g /g feed. Carp (*Cyprinus carpio*) fed diets containing the non-toxic, fat free Jatropha kernel meal showed lower weight gain than the heat-treated Jatropha meal (Makkar and Becker, 1999). Various chemical and physical methods have been reported to reduce the toxic phorbol ester up to 90% and the antinutritional constituents in the jatropha meal. Protein isolate prepared by steam injection heating has been shown reduce the antinutritional components more than 90% and eliminates the toxic phorbol esters to a negligible (trace) level (Rakshit and Bhagya, 2007, 2008a). The toxicity and growth studies carried out on rats indicated that feeding the rats with NaOH and $Ca(OH)_2$ treated meals at 10% protein level showed better performance than the untreated meal diet (Rakshit and Bhagya, 2008b).However, there are no reports available in literature that the detoxified jatropha meal has been used in poultry feed formulations. Therefore, the objective of this study was to evaluate the replacement of

*Corresponding author; *E-mail*: bhagguru@yahoo.com

detoxified expeller pressed and dehulled defatted jatropha meals in poultry feed formulations.

Materials and methods

Jatropha curcas seeds of the commercial variety were procured from a local vendor, Karnataka, (India). Expeller pressed cake was obtained from DI oils India Pvt. Ltd (Coimbatore, India). Vaccines were obtained from ventri biologicals, vaccine division (Pune, India), Diagnostic kits were purchased from Agappe diagnostics Ltd (Kerala, India),Tannic acid was obtained from Loba Chemicals Pvt. Ltd (Bombay, India). Phytic acid, trypsin, benzoyl-DL-arginine-*p*-nitroanilide hydrochloride (BAPA), 2- amino-2-(hydroxymethyl)-1, 3-propanediol (Trizma base), phorbol ester –12 —myristate 13- acetate were purchased from Sigma Chemical Co. (St Louis, USA). Mineral and vitamin premix were purchased from Virbac animal health India Pvt Ltd (Mumbai, India) and Aamoda pharmaceuticals (Bangalore, India), respectively .All chemicals used were of analytical grade.

Preparation of defatted meals: The seeds were crushed using hammer and the kernels and hulls were separated. The kernels were powdered and defatted in a stainless steel column, with hexane in a ratio 1:5 (w/v). The extraction was repeated 4-5 times with a soaking time of 12 h each till the fat content was less than 1 g/100g (AOAC, 2000). The expeller cake was made in to small grits and extracted with hexane by following the same procedure. The meal was air dried and powdered to pass through a 40-mesh sieve and stored at room temperature for further use.

Detoxification procedure: Dehulled defatted meal and expeller pressed cake meal was treated with aqueous solution of 2% calcium hydroxide and 2% NaOH in a ratio 1:1 (w/v) and mixed till it becomes a thick paste covered with a lid and kept for 30min. at room temperature. Autoclaved at 121°C for 15min.The material was dispersed in water in a ratio of 1:5 (w/v) and kept for 1h filtered through a muslin cloth. The process was repeated once and dried at 90°C. The meal was powdered to pass through a 60-mesh sieve for analysis. (Rakshit and Bhagya, 2007).

Chemical composition : The detoxified samples were analysed for moisture, protein (N x 6.25), fat, ash and crude fiber (AOAC, 2000).

Toxic and antinutritional constituents : Phytic acid: The procedure of Thompson and Erdman (1982) was used for phytic acid estimation by converting it to ferric phytate and the phosphorus content was analyzed by the method of Tausky and Shorr (1953). The phytate phosphorus derived was converted to phytic acid by multiplying the values with a factor of 3.55. *Tannins:* Tannin content was estimated colorimetrically according to the procedure of Ranganna (1995) and expressed as percentage of tannic acid equivalents. *Trypsin inhibitor activity:* Trypsin inhibitor activity was determined according to the procedure of Kakade et al. (1974) using benzoyl-DL-arginine p-nitroanilide hydrochloride as substrate and 2x crystallized bovine trypsin. Results are expressed as trypsin inhibitor units (TIU) in milligram of sample. *Estimation of phorbol esters:* The phorbol ester concentration was estimated in the untreated and treated meal as described by Makkar et al. (1998). The treated and untreated meals were extracted with methanol quantitatively. HPLC was carried out using Waters Symmetry 300™, C_{18} 5μm, 4.6 x150 mm i.d., column was controlled at 25°C, flow rate 1ml/min, with Waters 1525 HPLC binary pump, Waters 2996 photodiode assay detector at 280nm, and millennium software. The solvent used were: (A) 1.75 mL of o-phosphoric acid (85%) in 1 litre of distilled water; (B) acetonitrile. All solvents were degassed by ultrasonification and application of vacuum. The gradient used was as follows: 60% A and 40% B at start, 40% -50% B in 10 min, 50% - 75% B in 30 min, 75% to 100% B in the next 15 min. (Makkar et al., 1997). The results were expressed as equivalents to phorbol ester –12 –myristate 13- acetate. Saponins: The saponin content was determined according to the modified method of Gestetner et al. (1966).

Birds and experimental design: Fifty-six day old chicks (BV-300) procured from a commercial hatchery (M/S Venkateshwara Hatcheries Pvt. Ltd.), Hosur (India), were wing banded, weighed and housed in brooders provided with water, feeder and maintained under standard management procedures. The birds were fed on standard starter diet for four weeks. Then, they were randomly distributed into 4 groups of 8 chicks each for experiment 1. The groups were designated as control (C1), untreated (T1), $Ca(OH)_2$ treated (T2) and NaOH treated (T3) jatropha meal. Similarly, for experiment 2, the chicks were grouped into 4 groups of 6 birds each; control (C2), dehulled untreated (T4), jatropha meal treated with $Ca(OH)_2$ and NaOH as T5 and T6, respectively. All chicks were vaccinated against New Castle disease at 7th, 14th and 28th days of age and on the 21st days of age with Gumboro disease. The animal experiment was carried out following guidelines laid down by the Committee for the Purpose of Control and Supervision of Experiments on Animals by the Government of India, Ministry of Social Justice and Empowerment.

Experiment 1: Diets, feeding and managements :
The chemical composition of untreated expeller pressed cake and treated meals for the experiment 1 is given in Table 1.

The expeller pressed cake meal was commercially available and it contained lowest concentration of phorbol ester (Rakshit and Bhagya, 2008a). The meals were used along with other ingredients to formulate diets as given in Table 2.The untreated and treated meals were used at 10% levels as source of protein and computed to meet the Bureau of Indian Standards (BIS) requirement in the diet. The standard diet

Table 1: Chemical composition and antinutritional constituents of treated and untreated jatropha meal

Constituents (%)	Expeller pressed meal			Dehulled defatted meal		
	Untreated	*Ca (OH)₂ treated*	*NaOH treated*	*Untreated*	*Ca (OH)₂ treated*	*NaOH treated*
Moisture	7.3±0.1	8.0±0.09	8.2±0.2	7.0±0.1	7.9±0.1	8.26±0.2
Protein (N x 6.25)	24.20±0.3	16.58±0.3	20.66±0.5	56.92±0.4	55.32±0.2	56.06±1.0
Fat	0.4±0.01	0.4±0.03	0.3±0.03	0.5±0.01	0.4±0.0	0.5±0.01
Ash	5.63±0.16	5.88±0.09	5.88±0.09	8.43±0.02	11.85±0.03	8.86±0.14
Crude fiber	8.0±0.1	12.5±0.5	13.0±0.1	4.0±0.01	8.0±0.1	7.5±0.5
Carbohydrate (By difference)	54.47±0.5	56.64±0.5	51.96±0.5	23.15±0.5	16.53±0.5	18.82±0.5
Trypsin inhibitors (TIU/mg Sample)	15.0±0.5	2.9±0.1	2.3±0.1	72.5±1.50	11.25±0.5	9.5±0.5
Tannins	0.947±0.02	0.448±0.015	0.453±0.02	0.85±0.04	0.458±0.01	0.528±0.03
Phytic acid	3.20±0.05	3.12±0.03	2.74±0.03	3.93±0.07	3.85±0.03	3.32±0.08
Saponin	0.198±0.002	0.185±0.002	0.136±0.002	2.79±0.012	2.335±0.025	1.926±0.021
Phorbol esters	0.072±0.003	0.0076±0.0002	0.0102±0.001	0.135±0.0003	0.0145±0.0002	0.0187±0.001

Mean ± standard deviation (SD) of (n=3) determinations, TIU, trypsin inhibitor units

Table 2: Composition of experimental diets prepared from untreated and treated expeller pressed jatropha meal

Ingredient composition (g/100g)	Starter diet (0-4 weeks) (C1)	Starter (5-7 weeks) diet				Finisher (8-9 weeks) diet			
		Control (C1)	Untreated (T1)	Ca (OH)₂ (T2)	NaOH (T3)	Control (C1)	Untreated (T1)	Ca(OH)₂ (T2)	NaOH (T3)
Yellow Maize	32.53	32.53	19.08	23.2	25.72	44.63	31.28	35.31	38.04
Ground nut cake (De-oiled)	20.3	20.3	16.6	17.04	17.19	17.09	13.17	13.71	13.74
Soy bean meal (De-oiled)	18.37	18.37	16.26	16.11	15.77	13.08	11.07	10.72	10.49
Ragi	9.67	9.67	16.1	13.15	11.61	6.38	12.81	10.17	8.43
Broken Rice	13.72	13.72	16.55	15.09	14.3	13.41	16.26	14.68	13.89
Test Sample (Jatropha meal)	0.0	0.0	10.0	10.0	10.0	0.0	10.0	10.0	10.0
Salt	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Vitamin mixture	1.66	1.66	1.66	1.66	1.66	1.66	1.66	1.66	1.66
Mineral mixture	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5
Total	100	100	100	100	100	100	100	100	100

without jatropha meal served as control (diet C1). The untreated jatropha cake meal (diet T1), meal treated with $Ca(OH)_2$ and NaOH constitute diet T2 and diet T3, respectively .The ingredients were milled, mixed in a food processor and stored in a air tight container at room temperature. The birds were fed on standard starter diet for 4 weeks. The birds were fed on experimental starter diet from 5th -7th week followed by finisher diets from 8th to 9th week.

Experiment 2: Diets, feeding and managements

In experiment 2, the dehulled and defatted jatropha meal was treated similar to the experiment 1.The chemical compositions of meals are given in Table 1. The diet (Table 3) was not only low in concentration of phorbol esters but also devoid of hulls, which contributes high fiber and other antinutritional constituents such as tannins.

Table: 3 Composition of experimental diets from untreated and treated dehulled defatted jatropha meal

Ingredient composition (g/100g)	*Starter diet (0-4 weeks) (C2)*	*Finisher (5-7 weeks)*			
		Control (C2)	*Untreated (T4)*	*$Ca(OH)_2$ (T5)*	*NaOH (T6)*
Yellow Maize	32.53	44.63	22.19	22.59	12.17
Ground nut cake (De-oiled)	20.3	17.09	8.64	8.85	12.31
Soy bean meal (De-oiled)	18.37	13.08	7.43	7.61	12.77
Ragi	9.67	6.38	22.19	22.71	24.81
Broken Rice	13.72	13.41	24.14	22.83	22.53
Test Sample	0.0	0.0	10.0	10.0	10.0
Salt	0.25	0.25	0.25	0.25	0.25
Vitamin mixture	1.66	1.66	1.66	1.66	1.66
Mineral mixture	3.5	3.5	3.5	3.5	3.5
Total	100	100	100	100	100

The diet formulated without jatropha meal served as control (diet C2). The diet prepared with untreated dehulled and defatted jatropha meal (diet T4), meal treated with $Ca(OH)_2$ and NaOH are diet T5 and T6, respectively. The birds were fed on standard diet for 4 weeks followed by finisher diet from 5th to 7th week.

Data collection : Feed consumption and BW of chicks were recorded weekly for 9 and 7 weeks of age for experiment 1 and 2, respectively. Feed conversion efficiency was calculated on the basis of unit feed consumed to unit body weight gain. The mortality of the birds was recorded. The postmortem examinations were carried out on dead birds to record the symptoms.

Histopathology studies: At the end of experiment, the chicks were starved for 18 h to empty their crops, slaughtered by cervical dislocation, exsanguinated, de-fathered and eviscerated. The tissues of the target organs were collected weighed and stored in 10% formalin. The tissues were processed to obtain 5 μm sections and stored with hematoxylin and eosin and a detailed microscopic examination was carried out.

Statistical analysis : The experimental data were statistically analyzed by Duncan's (1955), new multiple range test to determine the significant ($P< 0.05$) differences among treatments (Steel and Torrie, 1980).

Results and Discussion

The chemical composition of the defatted expeller pressed meal and dehulled, defatted meal is given in Table1. The protein content of the untreated expeller pressed and dehulled defatted meals were 24.2 and 56.5%, respectively. The fat content was 1% in all cases. The phytic acid content of the untreated expeller pressed and dehulled defatted meal was 3.2 and 3.9% respectively, which reduced by 2.25 and 14.95% after treating the meal with $Ca(OH)_2$ and NaOH respectively. The saponin content was reduced after treatment by 6.6 to 31.3% respectively. While the tannin content was reduced by 53 and 52% after treating the meal with $Ca(OH)_2$ and NaOH, respectively. The phorbol esters content of defatted expeller pressed meal was 0.072% (72 mg%) treating the meal with Ca(OH)2 and NaOH reduced the phorbol esters to 7.6 and 10.2 mg% respectively. The phorbol ester content of dehulled defatted meal was 135 mg% which reduced to 14.5 and 18.7 mg% after treating the meal with $Ca(OH)_2$ and NaOH respectively (Table1).

Table 4 presents the data on feed intake; growth rate and feed conversion efficiency of chicks fed with control, untreated and treated diets in experiment 1. The feed intake in control standard diet (C1) fed group chicks was 19.6 g/bird/day. In the case of diet T2 and T3 the feed intake was 15.14 g/bird / day and 17.89 g/bird/day, respectively. On the other hand, the feed intake of diet containing untreated (T1) and $Ca(OH)_2$ treated meal (T2) marginally low. The feed conversion efficiency did not show any difference between the diets T1, T2 and T3.

The body weight gain and feed intake of the birds fed with T3 were better and closer to the standard control diet. The performance of the birds fed with diet T3 was comparable to the feed intake (Fig 1 and 2).

Table 4: Feed intake and growth rate of chicks fed on diets containing untreated and treated jatropha meal

Parameters	*Expeller pressed meal*				*Dehulled defatted meal*			
	Control (C1)	*Untreated (T1)*	*$Ca(OH)_2$ (T2)*	*NaOH (T3)*	*Control (C2)*	*Untreated (T4)*	*$Ca(OH)_2$ (T5)*	*NaOH (T6)*
Initial mean body weight (g)	45.25	45.08	45.36	44.67	45.08	45.71	45.35	44.93
Final mean body weight (g)	597.5	441.92	479.48	549.42	424.16	327.33	366.2	398.33
Body weight gain (g/bird/day)	9.48	7.01	7.61	8.72	8.65	6.68	7.47	8.12
Feed intake (g/bird/day)	19.58	13.60	15.14	17.87	17.89	11.69	15.01	16.65
Feed conversion efficiency	2.06	1.94	1.99	2.04	2.06	1.75	2.01	2.05
Mortality	0	3	3	1	0	3	1	0
Concentration of phorbol ester consumed (mg)	0	31.32 ± 0.3	4.04 ± 0.1	7.18 ± 0.2	0	20.38 ±0.1	4.55 ±0.0	7.36±0.1

Average values of each group

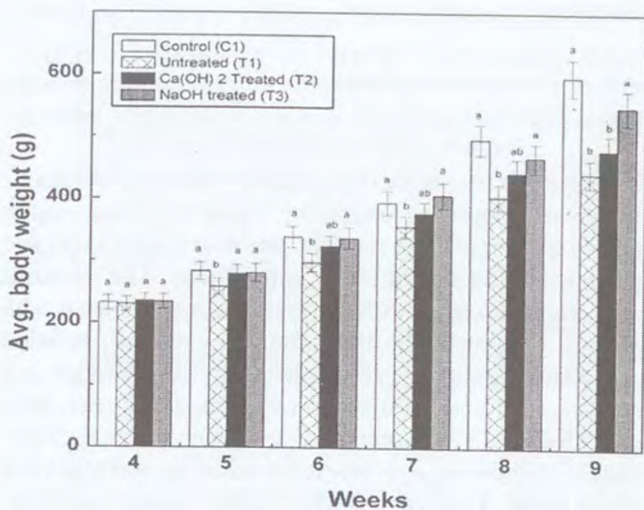

Fig. 1: Growth performance of birds fed with diets containing expeller pressed jatropha meal. Means with no common superscript letters (a, b and ab) differ significantly ($P<0.05$).

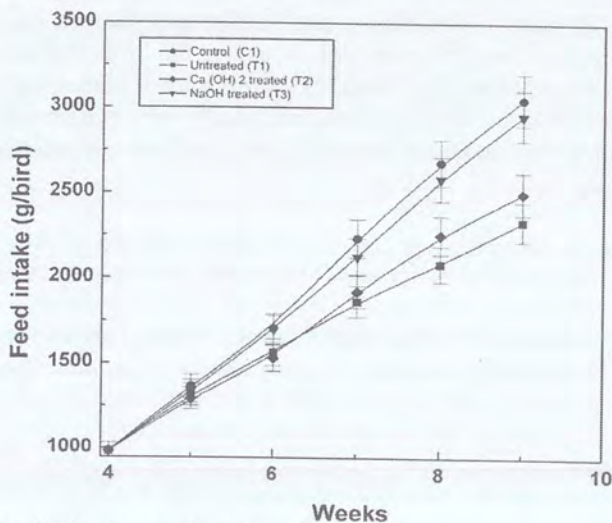

Fig. 2: Weekly feed intake of birds fed on diets containing expeller pressed jatropha meal

In experiment 2, the chicks fed with the diet containing untreated dehulled defatted jatropha meal and meals treated with $Ca(OH)_2$ and NaOH was compared with standard control diet (Table 4). The feed intake groups fed with $Ca(OH)_2$ and NaOH treated meal diets T5 and T6 were slightly lower than the control standard diet (C2). The feed conversion efficiency of diets T5 and T6 were similar to control diet but diet T4 showed lower feed conversion efficiency of 1.75. The body weight gain of T6 group was comparable to control group and it was low for groups T4 and T5 (Table 4). These birds had poor feed intake compared to birds fed with control standard diet and T6. The poor performance of T4 and T5 was comparable to the results obtained for diets T1 and T2 in experiments 1(Fig.3 and 4).

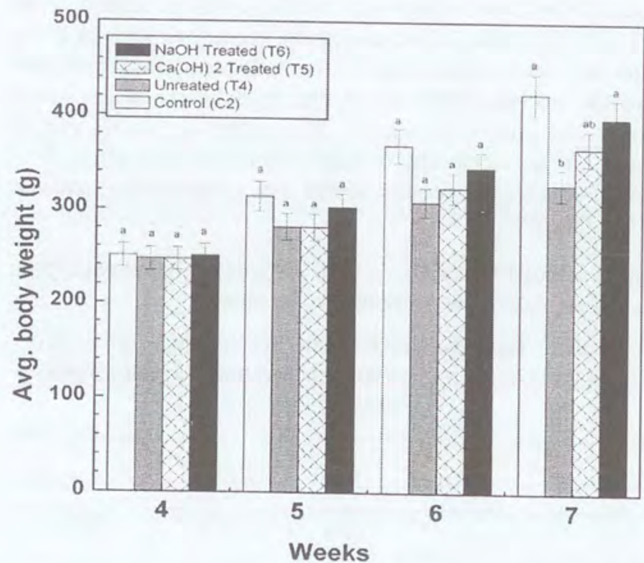

Fig. 3: Growth Performance of birds fed with diets containing dehulled jatropha meal

Means with no common superscript letters (a, b and ab) differ significantly ($P<0.05$)

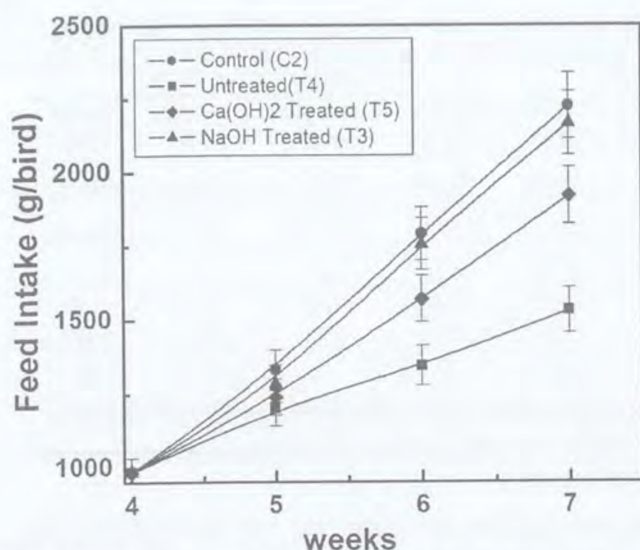

Fig. 4: Weekly feed intake of birds fed on diets containing dehulled jatropha meal.

In experiment 1, the physical appearance of the birds fed with T1 diet containing untreated expeller pressed meal showed severely stunted growth, ruffled feathers, acute enteritis with purging watery diarrhoea, soiled abdomen, dyspnoea, lordosis and recumbency before death. While the birds fed with diet T2 containing $Ca(OH)_2$ treated expeller pressed meal showed moderate growth, ragged feathers, watery diarrhoea, soiled back and mild dyspnoea. However, birds fed with diet T3 containing NaOH treated meal showed semisolid droppings otherwise looked healthy and active like control birds (C1).

In experiment 2, the birds fed with diet T4 containing dehulled meal showed medium growth, ruffled feathers, enteritis with watery diarrhoea, soiled abdomen, dyspnoea and lordosis. The diet T5 containing $Ca(OH)_2$ treated meal showed moderate growth, smooth feather cover, and mild diarrhoea and soiled back. The birds fed with diet T6 containing NaOH treated meal did not show any changes compared to control (C2) group which were healthy, active with groomed feathers and solid droppings.

Table 5 shows the relative organ weights of liver, heart, brain and pancreas to the body weight of chicks.

Table 5: Relative weight of target organs of birds fed on diets containing expeller pressed Jatropha meal g/100g body weight

Groups	*Liver*	*Heart*	*Brain*	*Pancreas*
Control (C1)	2.25^{a}	0.64^{a}	0.42^{a}	0.42^{a}
Untreated (T1)	2.41^{a}	0.96^{b}	0.51^{a}	0.49^{a}
$Ca(OH)_2$ (T2)	2.03^{a}	0.72^{ab}	0.59^{a}	0.48^{a}
NaOH (T3)	2.65^{a}	0.67^{ab}	0.51^{a}	0.46^{a}

Means within a column with no common superscript letters (a, b and ab) differ significantly (P<0.05).

The weights of liver, brain and pancreas of chicks fed with $Ca(OH)_2$ (diet T2) and NaOH treated (diet T3) expeller pressed meal did not differ significantly (P<0.05) compared to control. However, the weight of heart significantly increased (P<0.05). Incase of chicks fed diets containing dehulled untreated and treated meals did not show any significant (P<0.05) difference in the weights of liver, heart, brain and pancreas (Table 6).

Table 6: Relative weight of target organs of birds fed on diets containing dehulled jatropha meal g/100g body weight.

	Liver	*Heart*	*Brain*	*Pancreas*
Control (C2)	2.92^{a}	0.78^{a}	0.58^{a}	0.50^{a}
Untreated (T4)	2.17^{a}	0.79^{a}	0.45^{a}	0.39^{a}
$Ca(OH)_2$ Treated (T5)	2.53^{a}	0.83^{a}	0.60^{a}	0.38^{a}
NaOH Treated (T6)	2.25^{a}	0.72^{a}	0.55^{a}	0.37^{a}

Means within a column with no common superscript letters (a, b and ab) differ significantly (P<0.05).

On necropsy, the relevant vital organs revealed the difference among the groups. In the groups (T1 and T4), the intestines showed severe catarrhal enteritis, petechial hemorrhages and severe extravasations of blood in the lumen. The intestinal walls were severely swollen, hyperemic and inflamed with necrotic erosions. The liver showed wrinkled, mottled appearance with severe congestion, fatty degeneration and necrosis. The heart showed severe congestion, petechial hemorrhages and focal necrosis. The kidneys were congested, mottled, and friable with severe petechial hemorrhages and inflammation. The birds fed with $Ca(OH)_2$ treated meal diets (T2 and T5) showed enteritis, hemorrhages with hyperemic with inflamed intestinal walls. The liver was mottled with moderate congestion, fatty degeneration and necrosis. Mild necrosis of cardiac fibres and congestion were also observed in the heart. The kidneys are friable and showed mild congestion and inflammation. Whereas, the birds fed with NaOH treated meals (T3 and T6) showed smooth, homogenous intestinal walls. The liver, heart and kidneys were also smooth, firm, normal in colour and appearance similar to the control birds.

The microscopic examination of organs from the birds fed untreated meal diets (T1 and T4) showed complete denudation of intestinal villi, severe necrosis, inflammation and degeneration of surface epithelium and mucosal layers. The liver evinced extensive hepatic necrosis, degenerated hepatocytes, and fatty vacuoles in parenchymal cells. The heart showed severe infarction and nuclear degeneration of cardiac muscle fibres. In kidney, severe necrosis and degeneration of glomerular endothelial cells and convoluted tubules were noticed (Fig.5 and 6) In the birds fed with $Ca(OH)_2$ treated meal groups (T2 and T5) mild denudation of intestinal villi and necrosis of epithelial cells were noticed.

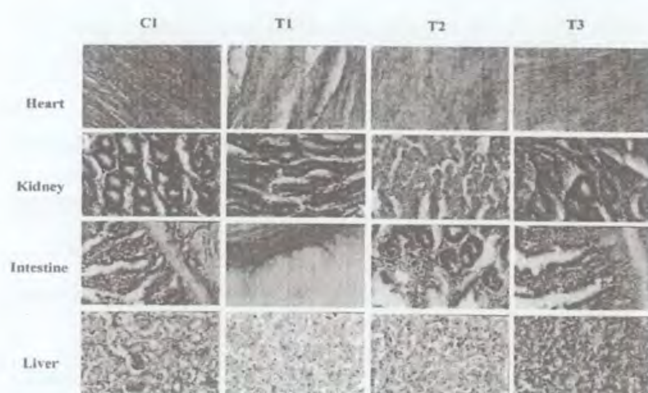

Fig. 5: First row to fourth row, the microscopic findings of heart, kidney, intestine and liver of birds fed on diets containing untreated and treated expeller pressed jatropha meals compared to the control diet fed birds.

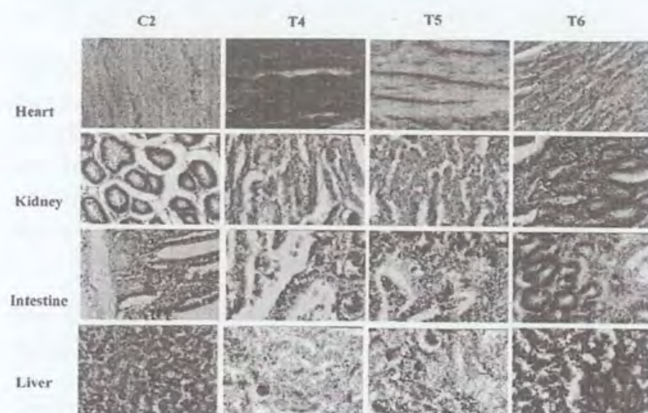

Fig. 6: First row to fourth row, the microscopic findings of heart, kidney, intestine and liver of birds fed on diets containing untreated and treated dehulled jatropha meals compared to the control diet fed birds

The liver showed mild necrosis and degeneration of hepatic cells. The cardiac muscle fibres were seen congested and the kidney revealed mild necrosis. In the case of the birds fed with NaOH treated meal groups (T3 and T6), integrated villi, clear mucosal cells, surface epithelium and mucosal layers could be seen. In liver, hepatocytes with clear nuclei and sinusoids were observed. The heart also showed clear muscle fibres with nuclei. The kidneys revealed structured glomeruli with convoluted tubular structure (Fig 5 and 6). The histological architecture of (T3 and T6) groups could be comparable to the birds fed with control standard diets (C1 and C2). The histological examination revealed that the birds fed with diet T5 and T6 were better architecture than the similarly treated expeller pressed meal diets (T2 and T3).

There are very limited reports in the literature with respect to detoxification procedures and very few *in vivo* studies of the same. The short-term toxicity studies with kernel have been attributed to phorbol esters content (Makkar et. al., 1997). The oral toxicity causing manifold clinical symptoms has been attributed to phorbol esters (Gandhi et. al., 1995). Heat treatment alone has not been able to decrease the concentration but also chemical treatment was necessary to bring down the concentration of phorbol esters. The presence of phorbol esters up to 16 mg% in the diet had significant adverse effect on feed intake and growth in rats (Rakshit and Bhagya 2008a).

In this study, the feed intake and feed conversion of birds fed treated meals T2, T3, T5 and T6 were higher compared to the groups fed untreated meals (T1 andT4). The concentration of phorbol esters consumed by group T2 and T3 was 4.04 and 7.18 mg/bird. Out of 8 birds, only one bird died in group T3 where as 3 birds were dead in group T1 and T2 indicating that it may be due the presence of antinutritional constituents in the meal. The birds fed with diet T5 and T6 showed lower 16.6 and 0% mortality rate respectively compared to 50% in untreated meal fed groupT4. The results indicate that not only the concentration of phorbol esters in the diet but also the other constituents present in the hull contribute to the negative effect on birds. In the analogues experiment Rakshit and Bhagya (2008b) have reported that the rats fed with dehulled meal with higher concentration of phorbol esters (9.02 mg) lived longer than the ghani pressed meal with lower concentration of phorbol esters (1.73 mg). However, the phorbol esters are highly toxic in nature even at low concentration levels. Abdel Gadir et al. (2003) reported that feeding the goats with ground jatropha seed orally at a concentration of 1 g/Kg or 0.25 g/Kg showed reduced body weight at the end of 18 and 21 days. Pigs fed with diet containing different levels of detoxified jatropha meal (1.3-5%) with phorbol esters content of 10.4 to 40 µg/Kg for 4 weeks resulted in 20% mortality (Chivandi et al., 2006).

The relative organs weight of liver, brain and pancreas of control (C1) and untreated groups (T1, T2 and T3) did not show any significant differences ($P<0.05$). However, the weight of heart was significantly different from control group indicating the enlargement of heart and accumulation of fluid. In the case of the groups (T4, T5 and T6) fed with diet containing untreated and treated dehulled jatropha meals did not show any significant difference in the weight of organs compared to control group indicating that the hulls present in the expeller pressed meal has some negative effect on the vital organs like heart.

Microscopic examination of some of the vital organs indicated severe necrosis, inflammation and degeneration of surface epithelium and mucosal layer. The liver showed hepatocytes and fatty cytoplasmic vacuoles. The heart showed congestion, petechial hemorrhage. Abdel Gadir et al. (2003) reported that goats fed with jatropha kernel showed similar hemorrhage or congestion in heart and intestine and necrosis of liver has been attributed to *jatropha curcas* toxicity. Similar

histopathological symptoms have been reported when pigs were fed with detoxified jatropha meal (Chivandi et al., 2006). Adam (1974) reported high mortality in mice fed with high concentration of jatropha meal. However, feeding the jatropha meal to mice at a concentration of 1% caused no death. In our study, the percent mortality was very low and the performance of birds fed diet with treated meal was better than untreated one. The performance of birds was much better in the case of diet containing dehulled untreated meal and treated meals than the diet containing untreated expeller pressed meal and treated meals. The study clearly showed that NaOH treatment was better in detoxifying expeller pressed or dehulled jatropha meals. The results obtained are comparable to those reported by Rakshit and Bhagya (2007, 2008b).

Conclusions

Both $Ca(OH)_2$ and NaOH treatments are effective methods in reducing the phorbol esters in expeller pressed meal and dehulled jatropha meals. Dehulling the jatropha seeds and treating meal with sodium hydroxide showed better growth performance on the birds. However, the study clearly showed that the phorbol esters may not be the major factor responsible for poor growth and mortality but also other antinutritional factors present in the meals. Further investigations are currently under way for total removal of phorbol esters and other antinutritional factors from *Jatropha curcas,* which seems to be a promising source of protein in poultry feed formulation on an industrial scale.

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Effect of Pre Treatment on the Bacteriological and Physical Qualities of Spices and Herbs used in Thai Green Curry and Antibacterial Property of Curry Extract

BEATRICE OLAWUMI TEMILADE IFESAN[1,*], SUNISA SIRIPONGVUTIKORN[1], PAIBOON THUMMARATWASIK[2] and DUANGPORN KANTHACHOTE[3]

[1]Department of Food Science and Technology, Prince of Songkla University, Songkhla, Thailand.
[2]Agro-Industrial Technology Department, Prince of Songkla University, Songkhla, Thailand.
[3]Department of Microbiology, Prince of Songkla University, Songkhla, Thailand.

Ingredients used in Thai Green Curry were investigated for their bacteriological, physical and antibacterial qualities. Pre treatment methods of washing with water and blanching were investigated as techniques for decreasing the bacterial loads and improving the green colour of the ingredients. Blanching of the ingredients yielded at least one log reduction in the total viable counts and the same effect was noticed on coliform counts except for big finger chili, sweet vegetable and root of corriander. Garlic was the ingredient with the least total viable count of 10^2 cfu/g. The total viable count of the curry paste decreased along with increase in storage days. The study of the antibacterial activity of the curry for 18 storage days revealed that the curry aqueous extract produced inhibitory effect on foodborne pathogens with largest zone of inhibition on Staphylococcus aureus followed by Pseudomonas fluorescens DMST20076 and Escherichia coli 0157: H7 with no inhibition on Listeria monocytogenes DMST17303.

Keywords: Antibacterial, Blanching, Foodborne pathogen, Pretreatment, Thai green curry extracts

Introduction

Spices and herbs have been used for thousands of centuries by many cultures to enhance the flavor and aroma of foods and are becoming more popular ingredients with a tendency of replacing synthetic antimicrobial agents. Many spices which are herbal products and their essential oils extracts have been reported. The essential oils and terpenoid alcohols of spices contribute to their smell, taste and tactile sensation while the antibacterial properties and potential application of essential oils in foods is documented (Burt, 2004). Extracts of garlic, cinnamon, curry, mustard, basil, lime, ginger and other spices and herbs has been demonstrated to possess antimicrobial properties (Arora and Kaur, 1999; Marino *et al.*, 1999; Onyeagba *et al.*, 2004; Siripongvutikorn *et al.*, 2005).

However, spices and herbs may be contaminated with many microorganisms because of conditions under which they were grown and harvested (Pafunmi, 1986; Giese, 1994; Kneifel and Berger, 1994). The microbial analysis of cumin seeds and chili pepper were carried out where aerobic plate counts ranging from 2×10^6 to 2×10^8 cfu/g were detected on chili powder and 1×10^4 to 1×10^8 cfu/g from cumin seeds (Bhat *et al.*, 1987). Samples of curry, paprika, white and black peppers, ginger, basil, mixed spices, and curcuma in Netherlands were reported to contain total viable counts of $\geq 10^7$ cfu/g (De Boer and Boot, 1983). Use of spices without appropriate intervention steps to eliminate or reduce the microflora could result in contamination and spoilage of the end product or survival of pathogens with the potential for the product to cause food borne illness. To ensure a safe product, it is important to consider the microbiology of fresh produce during harvesting and marketing, and the possible way of altering the microbial load through processing methods.

Minimal processing methods which include washing and decontamination to reduce microbial population on fresh produce have been reported (Nicholl *et al.*, 2004; Zhang and Farber, 1996). The use of high frequency and microwave treatments for pasteurization of spices was carried out and resulted in decrease of microbial load from log 7 to log 3 cfu/g (Dehne and Bogl, 1993). Other processing methods reported include, rinsing with various mixtures of vinegar (Karapinar and Gonul, 1993; Adebolu and Ifesan, 2001), X-ray sterilization (Robinson *et al.*, 1954), and the use of an irradiation dose of 7.5kGy (Sharma *et al.*, 1984). Blanching is a heat process used to destroy enzymatic activity in vegetables prior to further processing. As such it is not intended as a sole method of preservation but as a pre treatment which is normally carried out between the preparation of raw material and later operations. Also it reduces the number of contaminating microorganism on the surface of foods and hence assists in subsequent preservation operations (Fellows, 1997).

Thai Green Curry is one of the popular curries not only in Thailand but Worldwide and some researchers have discovered that some of the ingredients used in preparing the curry have medicinal value. Garlic one of the ingredients is said to possess allicin a volatile compound which is unstable in the presence of heat but highly reactive (Ankri and

*Corresponding author, E-mail: bodewunmi2001@yahoo.co.uk

Mirelman, 1999), and has been reported to have antimicrobial and antioxidant compounds with health benefits (Nishimura *et al.*, 2000). Galangal root, lemon grass, and kaffir fruit peels are other ingredients used in the curry and have been reported to be effective in inhibiting tumors in the digestive tracts (Murakami *et al.*, 1994; Murakami *et al.*, 1995). However no scientific report has been given on the effect of pre treatment on the ingredients used in Thai green curry and the antibacterial effect of the curry aqueous extract. The objectives of this study were to assess the efficiency of the pre treatment methods to reduce contamination on the ingredients and to determine the antibacterial activity of the curry extract during storage.

Materials and Methods

Sample Preparation: Fresh spices used for the curry include the following; Big finger chili (*Capsium annuuum*), Lemon grass (*Cymbopogon citratus*), Galangal (*Alpinia galangal*.L.), Phak wan ban (*Sauropus androgyus*(L), Shallot (*Allium ascalonicum* L), Garlic (*Allium sativum*), Root of corriander (*Corriandrum sativum)* , Kaffir fruit peel (*Citrus hystrix)* and Hot chili (*Capsium frutescens*) were purchased from the fresh market in Hat-Yai, Songkhla Province of South Thailand. The ingredients were processed as follows. *(A) Initial load:* Samples were sorted and trimmed before taken for analysis. *(B) Unblanched:* Samples were washed under running tap water for 2 min and rinsed with sterile distilled water before they were taken for analysis. *(C) Blanched*: Samples except garlic and shallot were subjected to two separate blanching processes. Blanched for 1 min in boiling water and blanched for 1 min with boiling solution of 0.5% potassium metabisulphite (KMS) (Negi and Roy, 2000). They were then allowed to drain for 1-2 m and the antimicrobial effectiveness of the blanching process was carried out by determining the viable counts. *(D) Blended:* Samples were blended separately after blanching and were examined for total viable count. All these samples were sealed in sterilized plastic bags until analyzed.

Bacteriological and Physical Quality Analysis: Total Viable Counts: Twenty five g of each of the treatments above (A, B, C, D) were added to 225 ml of sterile peptone water. Serial dilutions were done and 1 ml of each dilution were pour plated using Plate Count Agar (PCA; Merck), allowed to gel and incubated at 37°C for 24 h (APHA, 1984). ***Coliform bacterial count:*** This was determined for all the treatments using Lauryl Sulphate Tryptose broth (LST; Merck) inoculated with dilutions 10^1 to 10^3 of samples and Durham tubes were inserted to detect gas production. Positive tubes were inoculated into Brilliant Green Lactose Bile broth (BGLBB; Merck) and incubated at 35°C for 24 to 48 h to confirm presence of coliform. Positive tubes were then inoculated into EC broth (EC; Merck) and incubated at 44.5°C for 24 to 48 h to detect fecal coliform. Positive tubes were streaked on EMB agar (EMB; Merck) and IMViC test was carried out to confirm *E. coli* (Feng *et al.*, 2002). ***Determination of color:*** The colour of samples from all treatments unblanched, blanched, and blanched with (KMS) solution were investigated by using colour meter. Color analysis was based on the International Commission on Illumination (CIE) L (lightness), a (redness), and b (yellowness) values, which were measured with a colorimeter (Hunter Lab Universal Software, Hunter Associates Laboratory Inc., Reston, VA). ***pH determination:*** The pH of the samples was determined with a pH meter (Beckman Instruments, Fullerton, CA) following the modified method of Osundahunsi *et al.* (2007). It was carried out on the initial load quality sample (A). Samples were blended and the extract used for determination of pH was obtained by pressing the paste from the blend aseptically with sterile white cloth.

Preparation of curry paste: Fresh spices including; garlic, shallot, kaffir fruit peel, big finger chili (green), chili (green), galangal, lemon grass, root of corriander, and galangal were treated as mentioned above; unblanched , blanched with water only, and blanched with KMS. Samples of shallot and garlic were not blanched before they were added to the curry. Black pepper, corriander seed and cumin powder were bought as prepared spices (packaged from the grocery). The ingredients were blended in a blender and were sealed in sterile plastic bags and stored at 4°C for 18 days.

Curry extracts preparation: To obtain the extract from the curry, the blended curry paste was brought out from the refrigerator and after opening the sealed polythene bag, the curry was aseptically placed in a sterile piece of white cloth used as sieve. The curry paste was pressed by hand covered with gloves and sterile polythene bag to remove the water from the curry. The aqueous extract from the curry was collected in a sterile beaker and was used for antibacterial activity.

***Antibacterial activity*:** Evaluation of the antibacterial activity of chemotherapeutic agents using agar diffusion method was used (Barry and Thornsberry, 1991). Stock cultures of all test organisms *Escherichia coli* 0157: H7, *Pseudomonas fluorescens* DMST 20076, *Listeria monocytogenes* DMST 17303 were obtained from National Institute of Health, Department of Medical Science, Ministry of Public Health. Bangkok. Thailand. *Staphylococcus aureus* was obtained from Teaching Hospital of Prince of Songkla University. Two loopful of each test organisms were aseptically transferred into 5 ml Brain Heart Infusion broth (BHI; Merck KgaA Darmstadt, Germany) and incubated at 37 °C for 18 h. These overnight cultures were adjusted to final concentrations of 10^6–10^7 cfu/ml using McFarland standard. One ml of this final concentration was taken into 7 ml BHI soft agar and then poured on 15 ml already gel Mueller Hinton agar (MHA; Merck) and allowed to solidify. Wells were made using 6 mm cork borer and 90 µl of each aqueous extract was filled into

the wells. The plates were then incubated at 35°C for 24 h after which they were observed for zones of inhibitions and these were measured using vernier caliper. Antibacterial activity of curry was investigated every 3 days interval of storage (18 days).

***Statistical Analysis*:** Data were subjected to analysis of variance (ANOVA). Comparison of means was carried out by Duncan's multiple- range test (Steel and Torrie, 1980). Statistical analysis was performed using the Statistical package for Social Science (SPSS 11.0 FOR Windows, SPSS Inc., Chicago, IL). All experiments were determined in triplicate.

Results and Discussion

Total viable counts and pH of spices

Bacterial load and pH value of the individual ingredients are presented in Table 1. The result indicated that big finger chili, lemon grass, phak wan ban, root of coriander, and hot chili were heavily contaminated with microorganisms with total viable counts of ≥ 10^6 cfu/g while peeled galangal, shallot, garlic, and kaffir fruit peel had lower loads. Spices and herbs may be contaminated with microorganisms because of the conditions under which they were grown, harvested, and transported. However, the number and type of microorganism may vary with materials, origin, climatic conditions, harvesting, storage methods, packaging and general environmental handling circumstances can also affect the quality (Almelo *et al.*, 2002).

Table 1: Total viable count (cfu/g) and pH of the spices

Spices	*Viable Count (Cfu/g)*				*pH*[a]
	Initial	*Unblanched*	*Blanched*	*Blended*	
Big finger chili	$\geq 10^6$	$\geq 10^4$	10^3	10^4	5.29 ± 0.01
Lemon grass	10^5	10^4	10^3	10^3	5.29 ± 0.01
Galangal	10^3	10^3	$\leq 10^2$	$\leq 10^2$	4.86 ± 0.03
Phak wan ban	$\geq 10^6$	$\geq 10^5$	10^3	10^4	6.53 ± 0.04
[b]Shallot	10^5	10^4	ND	ND	5.56 ± 0.01
[b]Garlic	$\leq 10^2$	NG	ND	ND	6.08 ± 0.02
Kaffir lime peel	$\geq 10^4$	10^4	NG	10^2	4.58 ± 0.02
Root of coriander	$\geq 10^6$	$\geq 10^5$	10^2	10^4	5.77 ± 0.02
Hot chili	$\geq 10^6$	$\geq 10^4$	10^2	10^3	6.02 ± 0.01
[c]Black pepper	$\leq 10^5$	ND	ND	ND	ND
[c]Corriander seed	NG	ND	ND	ND	ND
[c]Cumin powder	NG	ND	ND	ND	ND

- [a]mean pH values ± standard error of triplicate results. [b]Samples were not blanched. [c]processed spices bought fror grocery and not blanched. ND, Not determined. NG, No growth.

The washing of the ingredients under running tap water was able to reduce the microbial load by at least 1 log for all the spices and about 2 log in big finger chili. Blanching either with water or KMS was also able to reduce the microbial population on the spices with about 3 log reduction from root of coriander (Table 1). From the result, recontamination might have occurred after blanching or during blending where a log increase in the viable count was observed in big finger chili, phak wan ban, and hot chili. Also, after blanching kaffir lime peel there was no microbial growth but growth was observed after blending. The source of recontamination may be from the equipments, air, or from the handler.

All the ingredients bought as prepared spices from the grocery were examined for total viable count at the initial load stage, and only black pepper was found to contain microorganisms. These spices were not further investigated for total viable count because they were not subjected to blanching. It was observed that the low pH of both galangal and kaffir fruit peel could have contributed to their low microbial counts (Table 1). Though the pH of garlic is about 6 it is still able to maintain a low bacterial count at the initial load and no growth at all when the samples were washed and crushed. This may be due to the presence of allicin, the active ingredients as reported by many researchers (Ankri and Mirelman, 1999; Onyeagba *et al.*, 2004; Siripongvutikorn *et al.*, 2005).

Coliform count on each of spices

All the ingredients except kaffir fruit peel, lemon grass, and garlic were heavily contaminated with coliforms (Table 2). This may be as a result of the water used for irrigation and various processes involved in harvesting and handling. However, it was found that there was no coliform growth in kaffir fruit peel after washing under running tap water. Coliform populations on lettuce were reduced by 1.4 log cfu/g with soaking in tap water (Nicholl *et al.*, 2004).

Table 2: Coliform count on each of the spice (MPN/g)

Samples	*Initial load*	*Unblanched*	*Blanched*	*Blanched with KMS*
Big finger chili	≥ 1,100	≥1,100	≥ 1,100	≥1,100
Lemon grass	290	290	210	210
Galangal	≥1,100	≥1,100	150	No growth
Phak wan ban	≥1,100	≥1,100	≥1,100	≥1,100
Shallot	≥1,100	≥1,100	ND	ND
Kaffir fruit peel	23	NG	ND	ND
Root of corriander	210	210	NG	NG
Black pepper	≥1,100	≥ 1,100	≥1,100	≥1,100
Hot chili	≥1,100	≥1,100	43	23

- ND: Not determined. NG: No growth.

Blanching was able to reduce the coliform count on lemon grass, root of coriander, hot chili and galangal (Table 2). *Escherichia coli* were isolated from the initial loads of some of the ingredients which include finger chili, lemon grass, washed samples of sweet vegetables and root of coriander (data not shown). However, after blanching there was no isolation of *E. coli* from any sample. Kaferstein (1976) reported that washing of fresh parsley in cold water was not able to reduce *E. coli* load but blanching markedly reduced the bacterial load.

Effect of treatments on colour of samples

There are significant differences between the blanched and unblanched samples as shown on the Table 3. Blanching was able to preserve the green colour (a value) of the ingredients compared to the unblanched samples. Blanching with KMS produced results significantly different from blanching with water alone in some spices such as, lemon grass, galangal, kaffir lime peel, and root of coriander.

Table 3: Color (L, a and b values) of various Treatments (Unblanched, Blanched and Blanched with potassium metabisulp (KMS)

Samples	*Unblanched*			*Blanched*			*Blanched with KMS*		
	L	*a*	*b*	*L*	*a*	*b*	*L*	*a*	*b*
Big finger chili	31.34 ± 0.04^{a}	-1.73 ± 0.07^{c}	12.75 ± 0.04^{a}	39.28 ± 0.15^{c}	-7.48 ± 0.02^{a}	19.67 ± 0.04^{c}	37.95 ± 0.97^{b}	-6.68 ± 0.18^{b}	18.09 ± 0.63^{b}
Lemon grass	54.06 ± 0.15^{a}	8.24 ± 0.16^{c}	26.92 ± 0.11^{b}	65.83 ± 0.21^{c}	2.10 ± 0.02^{b}	23.62 ± 0.67^{a}	64.68 ± 0.73^{b}	0.69 ± 0.13^{a}	24.56 ± 0.49^{a}
Galangal	57.89 ± 0.20^{a}	10.12 ± 0.19^{c}	18.62 ± 0.24^{c}	60.30 ± 0.50^{b}	6.64 ± 0.38^{b}	15.19 ± 0.53^{b}	67.94 ± 0.85^{c}	3.05 ± 0.37^{a}	15.12 ± 0.19^{a}
Phak wan ban	7.99 ± 0.21^{a}	-5.59 ± 0.09^{a}	5.21 ± 0.14^{a}	8.70 ± 0.31^{a}	-5.30 ± 0.24^{a}	5.67 ± 0.20^{a}	9.89 ± 0.87^{b}	-5.08 ± 0.32^{a}	6.46 ± 0.58^{a}
Shallot	39.51 ± 0.10	3.48 ± 0.03	3.50 ± 0.06	ND	ND	ND	ND	ND	ND
*Garlic	57.29 ± 0.28	1.23 ± 0.10	3.50 ± 0.06	ND	ND	ND	ND	ND	ND
Kaffir fruit peel	41.70 ± 0.24^{a}	-3.07 ± 0.04^{b}	20.53 ± 0.17^{a}	46.24 ± 0.46^{b}	-2.33 ± 0.04^{c}	23.01 ± 0.23^{b}	46.05 ± 0.66^{b}	-3.22 ± 0.02^{a}	22.77 ± 0.42^{b}
Root of coriander	39.14 ± 0.02^{a}	3.68 ± 0.01^{c}	14.47 ± 0.03^{a}	44.84 ± 0.28^{b}	1.02 ± 0.01^{b}	16.29 ± 0.11^{b}	46.31 ± 0.08^{c}	0.44 ± 0.03^{a}	16.40 ± 0.06^{b}
Hot chili	34.89 ± 0.36^{c}	-3.73 ± 0.10^{a}	15.36 ± 0.37^{b}	32.14 ± 1.49^{b}	-3.21 ± 0.12^{b}	15.30 ± 0.23^{b}	31.10 ± 0.30^{a}	-0.82 ± 2.28^{c}	14.38 ± 0.43^{a}

- Each value represents the mean from triplicates ± standard deviation. $^{a-c}$means within a row with a different letter are significantly different for each value, i.e. L, a, b ($P<0.05$). L, means degree of lightness of the samples. a, means redness /greeness (+) = red, (-) = green. b, means yellowness/ blueness (+) = yellow; (-) = blue. *samples were not blanched. ND: Not determined.

Blanching with KMS reduced losses of ß-carotene, ascorbic acid, and chlorophyll during blanching of vegetables (Negi and Roy, 2000). Samples of galangal, lemon grass and root of corriander turned brown when the tissues were exposed to the air during process of trimming and cutting due to the action of the polyphenoloxidase enzyme on the phenolic compounds. It could be explained that the enzyme was destroyed during blanching resulting to samples retaining their green colour (Tables 3). Samples of garlic and shallot were not blanched due to the possibility of the loss of the active ingredients as they are not heat stable (Ankri and Mirelman, 1999; Siripongvutikorn *et al*., 2005).

Total viable count of curry paste

The total viable count (TVC) of the bacterial load on the curry paste while it was stored at Temp 4°C for 18 days is shown in Fig 1. At day 0, the TVC of unblanched curry paste was higher than that of curry blanched with water and curry blanched with KMS with 0.14 log and 0.17 log cfu/g, respectively.

Curry pastes from the three treatments appeared to be stable with little or no differences in the total viable counts throughout the period of storage. This may be as a result of the storage at low temperature and interaction of the active compounds present in the spice.

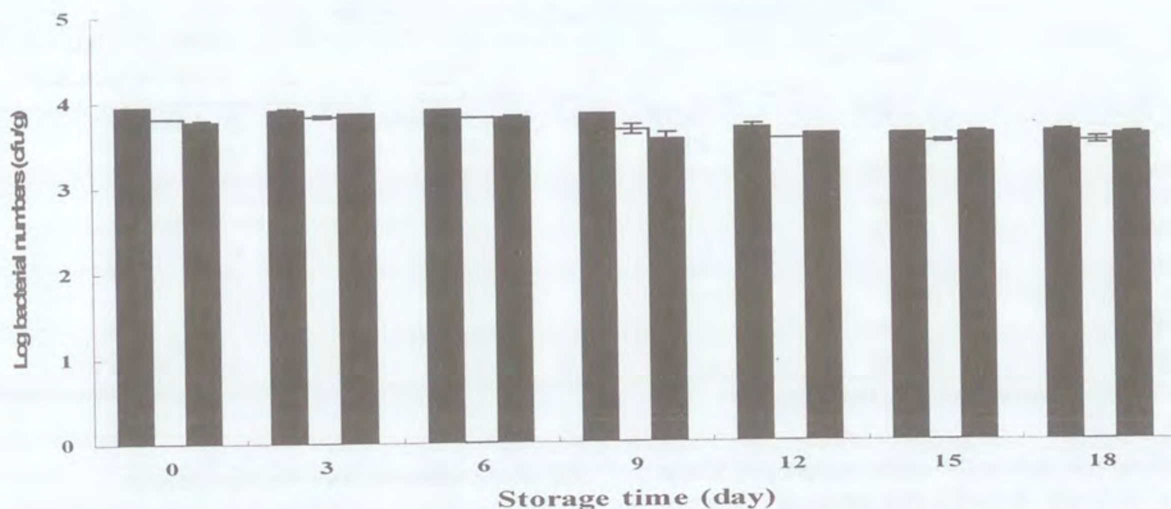
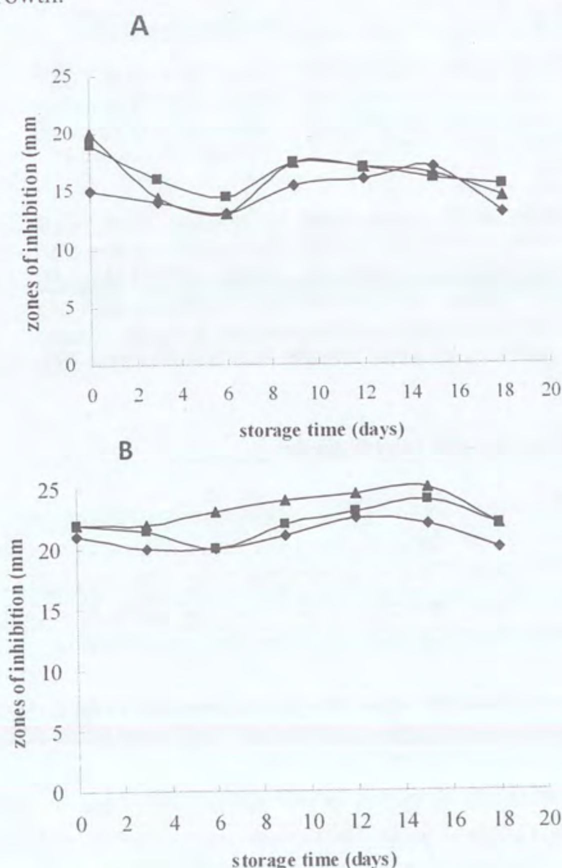


Fig 1: Total viable count (cfu/g) on the curry paste during 18 days storage at 4°C. The unblanched extract (black bars with dots), the blanched extract (white bars), and extract blanched with potassium metabisulphite (KMS) (black bars).

Antibacterial activity of curry extracts

The antibacterial activities of the extracts are shown on Fig 2. The ability of the extracts to inhibit bacterial growth is due to the bioactive ingredients present in the spices. This activity could be as a result of quite acidic pH of samples of galangal and kaffir fruit peel and also garlic which has been reported to possess an active compound which is able to inhibit microbial growth.



C

zones of inhibition (mm

storage time (days)

Fig 2: Antibacterial activity of the various curry extract against (A) *Pseudomonas fluorescens*, (B) *Staphylococcus aureus*, and (C) *Escherichia coli* during 18 d storage at 4°C. The blanched extract (²%), the extract blanched with potassium metabisulphite (KMS) (%) and the unblanched extract (f&).

Galangal extracts has been reported to possess antibacterial activity against foodborne pathogens (Mayachiew and Devahastin, 2008; Oometta-aree *et al*., 2006). The curry exhibited antibacterial activity against *S. aureus,* followed by *P. fluorescens and* then *E. coli* but could not inhibit *L. monocytogenes.* The blanched extracts were found to exhibit better antibacterial activities than the unblanched extracts and this may be due to the reduction of the bacterial load on the blanched samples by the blanching process. The data from this research revealed that the aqueous extract from Thai green curry possess antibacterial activity against foodborne pathogens.

Conclusion

Ingredients used in Thai green curry like any other spices and herbs are contaminated with microorganisms but the various processing methods of washing with potable water and blanching could reduce the microbial load and preserve the green colour. However, recontamination of ingredients is possible after blanching either from the equipments, air, or the hands of the processor. Therefore, exposure of ingredients and handling after blanching should be minimized. Aqueous extract from the curry paste was found to inhibit the growth of some pathogenic bacteria and this could mean that the curry has some medicinal value which would contribute to the health of the consumer. The extract may be considered as a food additive or the curry paste as a marinade. However the effectiveness of the antibacterial activity could be improved upon by varying the concentration of some of the ingredients especially kaffir lime peel and garlic but consumer taste and acceptability should be put into consideration.

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An Alternative Simplified Attribute Sampling Plan for Food Product Control

RAM PRASAD KHATIWADA*

Central Campus of Technology, T.U., Nepal

Acceptance sampling is concerned with the acceptance or rejection of the lot of goods. It is essential for the assurance of the quality of the food products. In this study, an alternative single acceptance sampling plan for the attributes is proposed based on the sample information. The attribute sampling plan designed for the predetermined producer's risk and consumer's risk. AQL, LQL are estimated using sample information. Probability of acceptance computed using binomial probability, the proportion non-conforming, the size of sample n, and the acceptance number c. The performance of the sampling plan measured using OC curves. For an increase in sample size, with the increase in acceptance number, the shape of OC curve changes and hence the consumer's point (LQL) drops down, for some predetermined value of a, b and producer's point. If quality management feels troubled to use the standard table of acceptance sampling, it is expected to select, to implement or to optimize the sampling plan, setting some parameters fix and using an OC curve for the performance measure of the plan, as proposed.

Key words: acceptance quality level, limiting quality level, producers' risk, consumers' risk, OC curve.

Introduction

An acceptance sampling plan is a scheme for the acceptance or rejection of the lot when cent percent inspection is impossible. The purpose of the plan is to develop decision rules to accept or reject lots based on sample data. It is capable of employing for the variables as well as attributes. In acceptance sapling plan, an item is classified as conforming or nonconforming. The conformity of the characteristics of the article is measured in some physical scale. The physical scale used may be a notional (supposed) or numeric. In variable sampling plan, the characteristics are measured on a numeric scale (i.e., in an interval or in a ratio scale). In an attribute sampling plan quality characteristics are quantified in notional scale (i.e., in a nominal or an ordinal scale). The acceptance sampling plan for the attribute results in generally binary outcomes such as pass/fail of the lot to demonstrate the conformance to specifications (Taylor,1996).

Acceptance sampling plan in a food industry can be employed in the arrival of new material, at the time of ingredients used in processing, at the end-product inspection or in the vending system. Commonly, the plan is used in manufacturing to decide whether to accept (release) or to reject (hold) lots of product. It can also be used during validation to accept (pass) or to reject (fail) the process. Moreover, sampling plan is suitable for the case of destructive inspection. It facilitates inspecting in short duration with low cost. In acceptance sampling plan, the lots of the materials are accepted for the processing or returned to the vendor during the control of raw material. When the plan is employed for the product control, the lots of the product is accepted for the supply or retuned to the producer for the necessary correction (Taylor, 1997).

In acceptance sampling, a small sample is inspected rather than inspecting total population, and so, there will be a diminution of inspection error and hence enhances the product or the material quality. The acceptance sampling plan has limitations, if it is not handled properly, of having risk of accepting a lot with poor quality (consumers' risk) or rejecting a lot of acceptable quality (producers' risk). According to ANSI/ASQC (1987), the maximum percentage (or proportion) of nonconforming items or number of non conformities in a lot that can be considered satisfactory as a process average is called acceptance quality level (AQL). It is the numerical definition that a good lot associated with producers' risk. The percentage or the proportion of nonconforming items or nonconformities in a lot for which the consumer wishes the probability of acceptance to be specified low value is called limiting quality level (LQL). It is the numerical definition of a poor lot associated with consumers' risk.

The acceptance sampling plan is classified as single sampling plan, double sampling plan and multiple sampling plan (Montgomery, 2005). A single sampling plan is the method of lot judgment, inspecting a single sample of n_1 units from a lot. A double sampling plan is the method of taking decision, inspecting the second sample of size n_2 if it is difficult to decide whether the lot is accepted or rejected based on the result of the first sample inspection. The multiple sampling plan is the extension of the double sampling plan. If, it is insufficient to make a decision in double sampling plan a multiple sampling plan is employed. A sequential sampling plan is similar to multiple sampling plan, a sequence of samples are taken from the lot and allow the number of samples to be determined entirely by the results of the sampling process. It is usually an item by item inspection process, based on the cumulative inspection results. The decision is made to either accept the lot or reject the lot or continue sampling.

Acceptance sampling is a widely used method of quality control and quality management. It is extensively used in manufacturing of electronic equipments, food and drug production, military research, and other technology of production. Acceptance sampling is used for acceptance decisions such as to decide whether the process is out of control or not. It admires both the capability and product

*Corresponding author; Email: ramktd@gmail.com

specification. If it is impossible to make a complete inspection, or it is very expensive to inspect, or inspection is destructive, then the acceptance sampling procedure is used to assure not to flow non-acceptable (bad) quality to the customer.

The efforts on development of acceptance sampling scheme found in several literatures in classical approach. Some of them are Dodge (1943), Grubbs (1946), Wald (1947), Dodge (1955), Freund (1957), Hald (1964), Wetherill & Chiu (1974), Grant and Leavenworth (1980), Taguchi and Wu (1980), Duncan (1986), Montgomery (2005). Some contributions on acceptance sampling for food and drug production system can be found in Taylor (1996, 1997, 2005), Taylor and Hansen (1997). There are different tables for sampling plans such as MIL-STD (1989), ANSI/ASQ Z1.4 (2003) and ISO-2859 (1999) in practice for the purpose of single, double or multiple sampling.

The objective of this study is to find out an easy way to make an acceptance sampling plan based on sample data. The main reason of this study is to propose a method of planning single acceptance sampling for the attributes step by step. The quality managers, who want to implement and optimize acceptance sampling procedures, but feel uneasy to understand or follow standard sampling plans already in print may set up plan using this method.

Materials and Methods

Basics of acceptance sampling : The ground rules of an acceptance sampling plan are the method of sampling, the sample sizes and the appropriate decision rules for attainment of some decision. Acceptance sampling plans are designed to ensure that the probability of accepting lots with high nonconforming proportion is low, and rejecting lots with low nonconforming proportion is low as well. In conventional method, the following assumptions are made for the development of acceptance sampling.

1. The samples are independently distributed [the performance of current item is independent of the performance of previous item, Vance and McDonald (1979)].
2. The proportion of nonconforming items remains constant lot to lot.

In a single sampling plan, the information obtained from one sample of specified size is used to make a decision to accept or reject the lot. Two parameters: n_1 (the sample size) and c_1 (the acceptance number) are specified in such plan. A sample of size n_1 is selected from a batch and the nonconformities or the number of nonconforming items d_1 is counted up. The lot is accepted if $d_1 \leq c_1$, and rejected otherwise (Montgomery, 2005). In a double sampling plan, the information obtained from the first sample is used to make the decision to accept the lot, reject the lot, or take a second sample. If the conclusion from the first sample is that the lot quality is good, the lot is accepted. If the conclusion is poor lot quality, the lot is rejected. If the first sample gives no clear idea of the quality, a good or a poor, a second sample is drawn. Based on the combined number of nonconforming items on the both samples, a decision is made to accept or reject the lot. The parameters of the double sampling plan are: n_1 (size of the first sample), c_1 (acceptance number of first sample), r_1 (rejection number of the first sample), n_2 (size of the second sample), c_2 (acceptance number of second sample), and r_2 (rejection number of the second sample). A first sample of size n_1 is selected from a lot of size N, and if, the number of non conforming items found to be d_1, the lot is accepted if $d_1 \leq c_1$, rejected if $d_1 \geq r_1$ and a second sample is selected if $c_1 < d_1 < r_1$. A second sample of size n_2 is selected, and if, the no of nonconforming items be d_2, the lot is accepted if $(d_1+d_2) \leq c_2$, and rejected if $(d_1+d_2) \geq r_2$, usually $r_2 = c_2+1$ (Montgomery, 2005).

Sampling Plan Parameters for the proportion nonconforming: Sampling plans for the proportion nonconforming are commonly designed using four parameters p_0, p_1, $\propto$ and β. The sample proportion nonconforming, p, is the ratio of the number of nonconforming to the sample size $\left(p = \frac{x}{n}\right)$. The specified proportion nonconforming levels, p_0 and p_1, are such that $p_0 \leq \frac{X}{N} \leq p_1$, where X is the actual nonconforming in the lot of size N. The proportion nonconforming level p_0 is such that lots having actual nonconforming proportion, p, will be accepted with probability $1\text{-}\propto$, if $p \leq p_0$. Another specified proportion nonconforming level p_1 is such that lots having sample nonconforming proportion p will be accepted with probability β, if $p \geq p_1$. It is summarized in Table 1.

Table 1. Producers' and consumers' risk in acceptance sampling

Lots having nonconforming proportion	*Probability of*	
	Accepting the lot	***Rejecting the lot***
$p \leq p_0$	$1\text{-}\alpha$	α
$p \geq p_1$	β	$1\text{-}\beta$

Where, p_0 is defined as a point at acceptable quality level (AQL) and p_1 the point at the limiting quality level (LQL) or lot tolerance proportion defective (LTPD). AQL is set up recognizing the current process performance or process capability. LQL is set up as enforcement of the consumer demand or market requirement. Customarily, in single sampling plan the two levels are selected for a common point i.e., $p_0 = p_1$. The probabilities $\propto$ and β are known to be as producers' risk and consumers' risk respectively. The risk of rejecting an AQL lot is the producer's risk ($\propto$ β) and the risk of accepting a lot, if it is an LQL lot, is the consumer's risk (β).

The probability that the lot will be accepted for the actual proportion nonconforming (p) falling between p_0 and p_1 lies between $1\text{-}\propto$ and β. Using operating characteristics (OC) curve, the variation in the probability of acceptance can be shown.

In practice, the values of α and β are frequently taken as 0.05 and 0.1 respectively (Hald, 1981).

OC curve: OC curve is a typical measure of the performance of the acceptance sampling plan. The probability of accepting the lot is plotted against the proportion nonconformities. In classical method, an OC curve is developed using the binomial probability. For developing an OC curve, the binomial probability of acceptance plotted against the proportion nonconformities, if the lot size is large enough, at least 10 times of the size of the sample (Mitra, 1998). Conventionally, the OC curve is used to measure the probability of lot acceptance or rejection. This can be used to measure the power function, the producers' risk and the consumers' risk as well.

If the predetermined acceptance number is *c* for a sample size *n*, the probability of acceptance *P(a)* for *x* number of nonconforming item is

$$P(a) = B(c,n,p) = \sum_{x=0}^{c} b(x,n,p) = P(x \le c) = \sum_{x=0}^{c} P(x) \quad \text{(1) (Duncan 1986)}$$

where,

$$P(x) = b(x,n,p) = {}^{n}C_{x} \; p^{x} \; (1-p)^{n-x} \quad \text{(2) (Duncan 1986)}$$

b(x,n,p) represents probability mass function of a binomial variable *x*.

To determine the maximum number of nonconformities (*c*) that can occur in *n* trials for the LQL, we can use the relation

$$\beta \ge \sum_{x=0}^{c} \Pr(X = x) \quad \text{(3) (Duncan 1986)}$$

To determine the maximum number of nonconformities (*c*) that can occur in *n* trials for the AQL, we can use the relation

$$1-\alpha \le \sum_{x=0}^{c} \Pr(X = x) \quad \text{(4) (Duncan 1986)}$$

Sample Data: Sample data were collected from an instant noodle factory named Himalayan Snax and Noodle Industries Pvt. Ltd. situated in Kavre District of Nepal. Data collection point used was the Packaging section in the processing line of the plant. Defects occurred during packaging such as crack, breakage or rupture of packaging materials were noted as the non conformance of the sampling which were screened out before packaging. To observe the product control process and sample data a scrutiny was made in online product flow of packaged instant noodle. 50 samples of different sample sizes were taken from online product flow of different lots (of same size) as convenience. The sample proportion items nonconforming was calculated and is tabulated in the Table 2 and plot of the distribution of nonconforming proportion is shown in Fig 1.

Table 2. Sample proportion of nonconforming items

Sample size	*16*	*20*	*20*	*24*	*24*	*28*	*28*	*28*	*32*	*32*	*32*	*36*	*36*	*36*	*40*	*40*	*40*	*40*	*total*
Number of item nonconforming	1	1	2	1	2	1	2	3	1	2	3	1	2	3	2	3	4	5	
frequency	3	8	2	6	1	3	4	2	2	1	2	1	6	1	3	3	1	1	50
proportion	.0625	.0500	.1000	.0417	.0833	.0357	.0714	.1071	.0313	.0625	.0938	.0278	.0556	.0833	.0500	.0750	.1000	.1250	

Results and Discussion

Sample mean nonconforming proportion is found to be 0.0617 with a standard deviation of 0.023. The minimum nonconforming proportion is 0.0278 and maximum is 0.125. The 95% confidence interval for mean nonconforming proportion is (0.00 - 0.0957). The producer's point controls the acceptance of lots that are at an AQL. We expect that lots at the producer's quality level will be accepted most of the time and prevent acceptable (good) lots from being rejected. So, AQL =0.031 (the half of the sample average point) is taken observing sample information and historical quality levels.

The consumer's point controls the rejection of lots that are at an LQL. We expect that lots at the consumer's quality level will be rejected most of the time and prevent non-acceptable (bad) lots from being accepted. We fixed LQL= 0.062 (the sample average point). Several values of *c* and *n* are calculated using binomial distribution (Equation 1, 2, 3 and 4) for a=0.05 and b =0.1 and tabulated in Table 3.

Probability of acceptance, P(a) = Pr (x $\le$c), calculated for different plan (c, n) for different nonconforming proportion based on AQL and presented in Table 4 and that for different

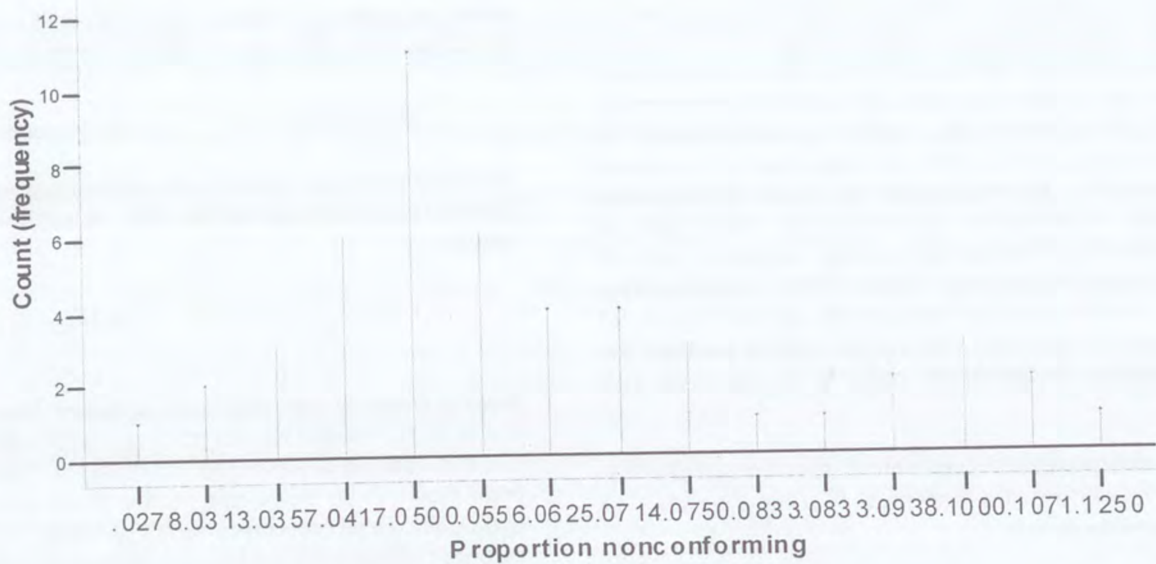

Fig. 1. Distribution of sample nonconforming proportion

[Note: Fig 1 depicts the distribution of the sample nonconforming proportion summarized from Table 2]

Table 3: Proposed sampling plan for α=0.05 and β =0.1

	c	*0*	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*
p_0 = 0.031 [1- α = 0.95]	*n*	1	11	26	44	64	85	107	129	153	176	200
p_1 = 0.062 [β = 0.1]	*n*	36	62	85	106	127	148	168	188	208	227	246

Table 4. Probability of acceptance for the plan (*c, n*) = (0, 1), (1, 11), ..., (10, 200)

	P(a)= Pr (x ≤c) calculated for (c, n) =										
p	*(0,1)*	*(1,11)*	*(2,26)*	*(3,44)*	*(4,64)*	*(5,85)*	*(6, 107)*	*(7, 129)*	*(8,153)*	*(9,176)*	*(10,200)*
0.01	0.9900	0.9948	0.9978	0.9990	0.9995	0.9998	0.9999	0.9999	1.0000	1.0000	1.0000
0.02	0.9800	0.9805	0.9852	0.9885	0.9908	0.9927	0.9941	0.9954	0.9961	0.9969	0.9975
0.03	0.9700	0.9587	0.9580	0.9575	0.9569	0.9572	0.9574	0.9588	0.9580	0.9594	0.9599
0.04	0.9600	0.9308	0.9160	0.9016	0.8871	0.8747	0.8624	0.8536	0.8388	0.8306	0.8200
0.05	0.9500	0.8981	0.8614	0.8235	0.7844	0.7482	0.7121	0.6822	0.6426	0.6145	0.5831
0.06	0.9400	0.8618	0.7973	0.7298	0.6611	0.5979	0.5371	0.4865	0.4275	0.3844	0.3407
0.07	0.9300	0.8228	0.7272	0.6287	0.5326	0.4481	0.3721	0.3118	0.2493	0.2059	0.1661
0.08	0.9200	0.7819	0.6543	0.5277	0.4117	0.3168	0.2387	0.1816	0.1294	0.0961	0.0691
0.09	0.9100	0.7399	0.5813	0.4324	0.3064	0.2125	0.1430	0.0971	0.0606	0.0398	0.0251
0.1	0.9000	0.6974	0.5105	0.3466	0.2205	0.1360	0.0806	0.0482	0.0260	0.0149	0.0081
0.12	0.8800	0.6127	0.3814	0.2099	0.1043	0.0492	0.0218	0.0098	0.0038	0.0016	0.0006
0.14	0.8600	0.5311	0.2743	0.1186	0.0444	0.0154	0.0049	0.0016	0.0004	0.0001	0.0000
0.16	0.8400	0.4547	0.1907	0.0630	0.0173	0.0043	0.0010	0.0002	0.0000	0.0000	0.0000
0.18	0.8200	0.3849	0.1285	0.0317	0.0062	0.0011	0.0002	0.0000	0.0000	0.0000	0.0000
0.2	0.8000	0.3221	0.0841	0.0151	0.0021	0.0002	0.0000	0.0000	0.0000	0.0000	0.0000

Table 5. Probability of acceptance for the plan (*c, n*) = (0, 36), (1, 62), ..., (10, 246)

	P(a)= Pr (x ≤c) calculated for (c, n) =										
p	*(0,36)*	*(1,62)*	*(2,85)*	*(3,106)*	*(4,127)*	*(5,148)*	*(6, 186)*	*(7, 188)*	*(8, 208)*	*(9, 227)*	*(10,246)*
0.01	0.6964	0.8721	0.9460	0.9778	0.9907	0.9961	0.9971	0.9993	0.9997	0.9999	1.0000
0.02	0.4832	0.6474	0.7580	0.8366	0.8878	0.9221	0.9185	0.9635	0.9748	0.9830	0.9885
0.03	0.3340	0.4414	0.5289	0.6064	0.6665	0.7146	0.6745	0.7946	0.8245	0.8524	0.8756
0.04	0.2300	0.2852	0.3342	0.3834	0.4228	0.4556	0.3823	0.5202	0.5471	0.5767	0.6035
0.05	0.1578	0.1773	0.1963	0.2181	0.2338	0.2456	0.1739	0.2726	0.2833	0.2976	0.3107
0.06	0.1078	0.1070	0.1090	0.1143	0.1160	0.1156	0.0663	0.1184	0.1184	0.1210	0.1230
0.07	0.0733	0.0630	0.0579	0.0560	0.0527	0.0487	0.0220	0.0441	0.0415	0.0403	0.0390
0.08	0.0497	0.0363	0.0296	0.0260	0.0222	0.0187	0.0065	0.0144	0.0126	0.0114	0.0103
0.09	0.0335	0.0206	0.0146	0.0115	0.0088	0.0067	0.0017	0.0042	0.0034	0.0028	0.0023
0.1	0.0225	0.0115	0.0070	0.0049	0.0033	0.0022	0.0004	0.0011	0.0008	0.0006	0.0005
0.12	0.0100	0.0034	0.0015	0.0008	0.0004	0.0002	0.0000	0.0001	0.0000	0.0000	0.0000
0.14	0.0044	0.0010	0.0003	0.0001	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
0.16	0.0019	0.0003	0.0001	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
0.18	0.0008	0.0001	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
0.2	0.0003	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000

[Note: In Table 4 and 5, *p* is the proportion nonconforming. For proposed different sampling plan (c, n), probabilities of acceptance, P(a) are computed for the corresponding non conforming proportion. The sampling plans are taken from Table 3]

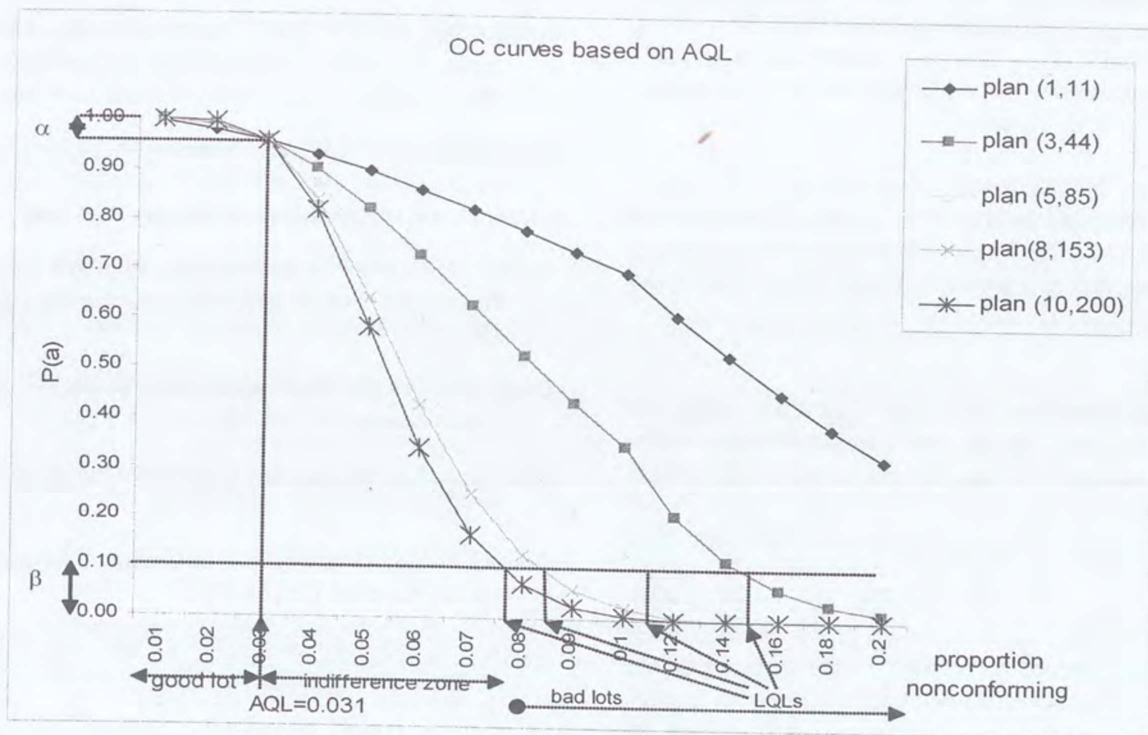

Fig. 2. OC curves based on AQL for different plan

[Note: Figure 2 shows the OC curves for proposed sampling plan (c, n) based on fixed AQL = 0.031. For ∝ = 0.05 the curve for the plan (10, 200) seems to be more striper than other plans. It cans protect consumers risk β= 0.01 up to 0.078 proportion nonconforming.]

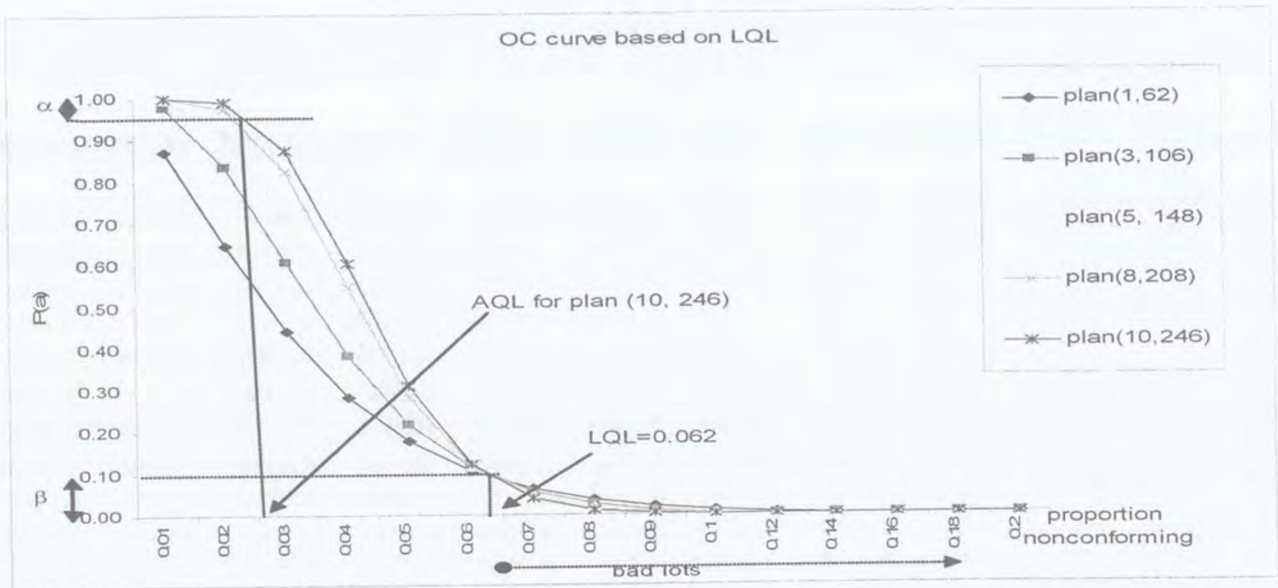

Fig. 3. OC curves based on LQL for different plan

[Note: Figure 3 shows the OC curves for proposed sampling plan (c, n) based on fixed LQL = 0.062. For β= 0.1 the curve for the plan (10, 246) seems to be more striper than other plans. It can protect producers risk ∝ = 0.0.05 up to 0.025 proportion nonconforming.]

nonconforming proportion based on LQL is given in Table 5. OC curves of the plan, based on AQL, are given in the Figure 2 and that of the plan based on LQL is presented in Figure 3. For predetermined AQL= 0.031 at ∝ = 0.05, to maintain consumer's risk β = 0.1 it is investigated that LQL= 0.076, 0.097, 0.107 and 0.145 for the plans (10, 200), (8,153), (5, 85) and (3, 44) respectively. This indicates that, if n increases the consumer's point drops down.

For fixed LQL= 0.062 at β= 0.1 to maintain producer's risk ∝ = 0.05 it is investigated that AQL= 0.025, 0.022, 0.018, 0.013 for the plans (10, 246), (8,208), (5, 148) and (3, 106) respectively. This indicates that, if n decreases the producer's point also decreases.

Conclusion

The conclusion made from the study is that, if the sample size n is increased with c, the OC curve would change in order that LQL decreases, while AQL is fixed at a point. If the sample size n is decreased with c, the OC curve would change so that AQL decreases, as LQL is fixed at a point. For different AQL and LQL the sampling plans can have different OC curves and it is difficult to deal with sampling plan adjusting both quality levels and risks in a single plan. In such cases alternatives to it should be investigated. If AQL and LQL are same, we can find a nearly equivalent OC curves. In such situation the proportion of acceptance of good lot will be equal to bad lot rejected.

Acceptance sampling does not have to be complicated. The quality manager, who wants to implement and optimize acceptance sampling procedures should not follow hard and fast rule. Who do not follow or do not want to use already printed standards, can set up a plan, as proposed, using LQL, AQL and ∝ and β.

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Mathematical Modelling of Drying Kinetics of Apple Pomace

RACHANA SHALINI[1,*], DEVENDRA KUMAR GUPTA[2] and ANUPAMA SINGH[2]

[1]Agri business Group, National Productivity Council, SCO 40, I Floor, Sector 7C, Chandigarh 160 019 India
[2]Department of Post Harvest Process and Food Engineering, G.B. Pant University of Agriculture and Technology, Pantnagar 263 145 India

Drying kinetics of apple pomace was experimentally determined in a laboratory scale dryer. The experimental study was conducted at three drying air temperatures (50, 60 and 70 °C) and three layer thickness (2, 4 and 6 mm) at a drying air velocity of 1.5 m/s on the hot air convective thin layer drying characteristics of apple pomace. Five commonly used mathematical models were evaluated with the experimental data to describe the phenomena of drying process using linearized regression technique. The results indicated that the Page's model can present better predictions with high coefficient of determination for the moisture transfer than the others. The analysis of variance of n of Page's model indicated that the effect of temperature and thickness on n was insignificant; therefore average value of n was taken (1.1163). Values of k were recalculated which ranged from 0.0043 to 0.0485. The values of activation energy were 58.406 kJ/mole, 41.983 kJ/mole and 49.339 kJ/mole for 2, 4 and 6mm layer thickness respectively of the apple pomace.

Keywords: Apple pomace, Hot air convective drying, Thin layer drying, Modelling, Activation energy

Introduction

Apple pomace is a by-product of apple processing industries, which represents a significant source of carbohydrates, acids, Vitamin C, minerals and dietary fiber (Sun *et al.,* 2007). It accounts for about 25% of the original fruit mass at 85% wet basis moisture content (Walter and Sherman, 1976). In India, the apple processing industries produce about one million tons per annum apple pomace which is the main by-product of such industries and only approximately 10,000 tons of apple pomace is utilized (Manimehalai ,2007). However, the pomace is highly is biodegradable in nature because of high bio-chemical oxygen demand (BOD) and abundant saccharide (Kaushal *et al.,* 2002).

Seasonal production and large quantity of apple pomace makes it difficult for the industries to dispose it timely and effectively. Therefore, disposing of such a large amount of apple pomace has become a challenge for environment protection. The commercial utilization of pomace shall ultimately be determined by economics of products and the cost of waste disposal coupled with pressure from environment protection agencies in implementing the laws (Kaushal *et al.,* 2002).

Drying of the apple pomace seems to be a promising utilization way for animal feed or for further processing such as nutrient recovery. Drying of moist materials is a complicated process involving heat and mass transfer simultaneously. Thin layer drying is the process of drying in one layer of sample particles or slices (Sun *et al.,* 2007). Drying techniques have been used for centuries, undergoing important evolutions (Moreira *et al.,* 2005). Studies on the drying processes are numerous because it is one of the most common industrial operations and involves high energy consumption, 10-25% of the total energy used in manufacturing processes worldwide (Majumdar and Passos, 2000). Particularly, in industrial food processing, drying operations have additional importance because, in many cases, the quality of dried food material is strongly dependent on the operational conditions used. Considering this, in order to optimize the drying process, it is necessary to reduce the reactions dependent on temperature and to increase the efficiency during dehydration. Both criteria are subordinate to producing the necessary water removal to a level at which it is possible to guarantee material preservation during storage. This allows disposing and consuming the dry product outside the traditional season. Also, a reduction in the mass and volume of the product makes transportation cheaper (Jayaraman and Das Gupta, 1992).

Several mathematical modelling and experimental studies on the drying characteristics of apple products have been conducted such as apple slices (Sacilik and Elicin, 2006; Nowak and Lewicki, 2004; Ramaswamy and van Nieuwenhuijzen, 2002; Karathanos *et al.,* 1995; Uretir, 1995; Akpinar *et al.,* 2003), apple cubes (Lewicki and Korezak, 1996; Uretir *et al.,* 1996), apple puree (Bains *et al.,* 1989), cylindrical shaped apple lump (Andrés *et al.,* 2004), and rectangular shaped apple lump (Velic *et al.,* 2004).

However, apple pomace was only used as a test material to compare two moisture determination methods, i.e., infrared drying technique and conventional oven technique (Fenton and Kennedy, 1998). So far, there is little information available about modelling, effective diffusivity and activation energy of drying process of apple pomace. Therefore, study on drying

*Corresponding author; Email: rshalini2003@rediffmail.com

of apple pomace is of great significance for environmental protection and resource utilization.

Mathematical modelling of thin layer drying of apple pomace assumes absence of moisture and temperature gradients. Although this assumption is rarely met in practical drying, the modelling of thin layer drying has been a subject of considerable research activity over the past two decades or so. This is primarily due to the fact that modelling of real life drying operations requires a reliable thin layer-drying model. The aim of this paper is to evaluate five different commonly used mathematical models with the drying data for predicting wet apple pomace drying rates.

Materials and Methods

Effective modelling the drying behavior is important for investigation of drying characteristics of apple pomace (Sun *et al.,* 2007). As there was absence of constant rate period in experimental drying of apple pomace, so only the models which describe the phenomenon of drying in falling rate period were attempted. Various researchers have suggested different grain drying models, which were further used by other research workers in drying of high moisture foods. These models could also describe the drying of apple pomace also. The following commonly used models were tested for validity of apple pomace drying

$MR = Ae^{-kt}$ (Generalized exponential model) (1)
(Henderson and Pabis 1961, Bruce 1985)

$MR=e^{-kt}$ (Exponential model) (2)
(Lewis 1921, Brooker *et al.* 1974)

$MR=e^{-kt^n}$ (Page's model) (3)
(Page 1949, Brooker *et al.* 1974)

$MR=At^B$ (Power law model) (4)
(Bruce, 1985)

$t= A(\ln MR) + B(\ln MR)^2$ (Thompson's model)(5)
(Thompson *et al.* 1968)

These models were fitted to the experimental data in their linearized forms using regression technique.

Equilibrium Moisture Content

Experimental data was also used to calculate the equilibrium moisture content of wet apple pomace by Issacs and Goudy (1968) method. It was determined as follows:

When a hydrophilic (which means material which has an affinity towards water) material is exposed to a given set of conditions, it tries to reach an equilibrium with the conditions of the surrounding medium. From earlier evidence, Hall (1971); Udani, *et al.,* (1968) and Blakesley (1974); it was reported that the instantaneous rate of drying at any time is proportional to the difference ($M-M_e$) at that instant.

$$\frac{dM}{dt} = -k(M-M_e) \qquad (6)$$

Integration of equation (10) gives the solution:

$$\frac{M - M_e}{M_o - M_e} = e^{-kt} \qquad (7)$$

For the computation of equilibrium moisture content, Singh *et al.,* (1981); Singh *et al.,* (1986) and Dwivedi (1984) used the following approach:

For any two successive observations, M_n and M_{n+1}, corresponding to drying times t_n and t_{n+1}, equation (7) leads to

$$\frac{M_e - M_n}{M_e - M_o} = \exp(-kt_n) \qquad (8)$$

$$\frac{M_e - M_{n+1}}{M_e - M_o} = \exp(-kt_{n+1}) \qquad (9)$$

By combining these expressions

$$\frac{M_e - M_n}{M_e - M_{n+1}} = \exp k(t_{n+1} - t_n) \qquad (10)$$

Since, weight values were taken after equal time intervals, therefore the right hand side of equation (10) is a constant, thus,

$$M_{n+1} = ZM_n + M_e(1-Z) \qquad (11)$$

If the data follows equation (7); then the plot of M_{n+1} against M_n should be straight line which was plotted. Equation (11) being linear in the unknowns, Me and k could also be used for determination of the corresponding values. However, if k is small then the errors in determination of Z will be considerably magnified when antilog of Z is taken. This will also cause large errors in determination of M_e value because of factor (1-Z). Therefore, equation (7) was not used for determination of k and M_e. A method developed by Issaacs and Goudy (1968) was used to calculate the constant m_e. For the present case, Issaacs and Goudy equation comes to

$$W_e = \frac{W_n . W_{n+2i} - (W_{n+i})^2}{W_n + W_{n+2i} - 2W_{n+i}} \qquad (12)$$

with W_e values, M_e values were calculated using the relationship:

$$M_e = \frac{W_e}{W_o}(100 + M_o') - 100 \qquad (13)$$

$$M_o' = \frac{W_o}{W_s}(100 + M_o) - 100 \qquad (14)$$

Once the equilibrium moisture content values were known, the rate constant k for wet apple pomace could be easily calculated through the "least square" method of analysis using drying models (Dwivedi, 1984, Singh *et al.,* 1981).

Moisture Ratio : Moisture Ratio is defined as follows

$$MR = \frac{M - M_e}{M_o - M_e} \quad (15)$$

The moisture ratios at different time intervals were calculated by equation (15) to study the drying characteristics of apple pomace.

The moisture ratio values were used to predict the drying model for apple pomace. Generalized Exponential model (Henderson and Pabis, 1961; Bruce, 1985), Exponential model (Lewis ,1921; Brooker *et al.*, 1974), Page's model (Page, 1949; Brooker *et al.*, 1974), Power law model (Bruce, 1985) and Thompson's Quadratic model (Thompson *et al.*, 1968) for thin layer grain drying were fitted to the drying data using linear regression analysis. The dependence of model constants on temperature and layer thickness was analyzed. These models were analyzed in terms of standard error (SEE) and coefficient of determination (R^2) and the best fit model was the one having lowest SEE and highest R^2. The regression was carried out using software Curve Expert 3.0 and MS Excel on personal computer. The models were plotted using Sigma Plot 10.0.

Calculation of Activation Energy : It is an index to demonstrate how much energy we need for removing one mole of water from product; it represents a combination power of the water in the product. Temperature dependence of the drying rate constant, k was calculated in accordance to Arrehenius law type relationship as follows.

$$k = k_0 \exp(-\frac{E}{RT}) \quad (16)$$

$$\ln k = \ln k_0 + (-\frac{E}{R})\frac{1}{T} \quad (17)$$

Error analysis: Error analysis for the best fit model was carried out on the basis of average mean error and E_{90} corresponding to maximum value at 90% of the data points. The average mean error was calculated using the following formula:

$$E_m = \left(\frac{\sum_{i=1}^{n} \frac{y_i - y'}{y_i}}{N} \right) X\ 100 \quad (18)$$

The maximum error at 90% of the data point was calculated as follows:

E_{90} = value of error at 90% of N (19)

Juice Expression and Apple Pomace Recovery : Culled apples of Red Delicious variety were procured from local market of Pantnagar and thoroughly cleaned with potassium permanganate solution to remove any kind of infections and then washed with tap water to remove soil and dust particles (Agrahari and Khurdiya ,2003; Shah and Bhatia, 1983). Washed apples were sliced into four equal parts and coring was done (Shah and Bhatia, 1983; Langthasa and Khurdiya, 2001). After slicing the apple slices were blanched with potassium meta bisulphite solution for fifteen minutes at the rate of 100 mg per liter of solution in one kilogram of apples, fixed from preliminary experiments. After blanching, the sliced apples were crushed in a fruit crusher and apple juice was removed through muslin cloth manually first before further processing.

The juice expression of crushed apple was done in a cylinder with the help of a laboratory hydraulic press. The carver press (Model M-25, Fred S. Carver Inc. USA) mainly consists of a moving cross-head on column, automatic load indicating gauge, a motorized package containing electro-hydraulic semi-automatic power control unit and a hand operated press loading pump system. The press had a maximum loading capacity of 25 tones and a range of pressure was available (e.g. 0-2.5 tones, 0-10 tones and 0-25 tones). The designed gauge could be attached to the press to give the required range and accuracy. The speed of pumping and the desired load set point could be adjusted using the controls provided for the same. The Cylinder and piston assembly used was designed by (Mohan, 2004). The cylinder had the height 26 cm, the inner diameter of 20.32 cm, thickness of 6.5 mm and weight of 10.77 kg of cylinder.

The juice expression from crushed sample was done by filling the cylinder with crushed apples, jute bag cutting and muslin cloth (2 sheets) between the crushed sample and the bottom end cap to reduce the solid loss. It was observed that some juice loss from top took place between the cylinder wall and the piston. This could not be completely reduced. The pressing of crushed apple was carried out at 59.7 kg/cm^2 pressing load for one hour and 45 minutes which was standardized from preliminary experiments. The juice coming out from the top was wiped off with a piece of cotton. Apple pomace was recovered in the form of cake from the hydraulic cylinder

Experimental Procedure : The SATAKE dryer was used for drying studies on apple pomace which consisted of air delivery system with a centrifugal air blower, Three phase, 1.0 HP motor and a control valve to regulate air flow rate @ 90 m/min, air heating system with a capacity of 12 x 1 kW with a 250 volt three phase electric heater and a three phase auto transformer of 1000 Volt to control and obtain the desired temperature and drying system with 20 trays of 20x10x15 cm mild steel.

Drying of Apple Pomace Samples : The SATAKE dryer was warmed up for about 0.5 h to achieve a steady state in terms of the pre-set experimental drying conditions before each drying. The weight of three levels of layer thickness 2, 4 and

6 mm was fixed to 50, 90 and 130 g respectively to maintain uniform bulk density of pomace in all the experiments. The apple pomace samples of sizes 50, 90, 130 g ($\pm$ 0.01g) of 2, 4, 6 mm thickness respectively were spread uniformly for thin layer drying in the dryer. These levels were selected to cover the expected range of variables in commercial applications as well as on the basis of the limitations of the equipment and levels used by earlier investigators for apple pomace. Trays with perforations to allow air to pass through with size 18.5×7.5×1 cm were used. Relative humidity was measured using sling psychrometer (range 0-50 °C, make: International Refrigeration Corporation, New Delhi, India).

In the first phase of experiments, the air velocity varied from 1.5 to 2.5 m/s with an increment of 0.5 m/s and the temperature and bed thickness is kept constant. In the second phase of experiments, the drying air temperature ranged from 50 to 70°C with an increment of 10°C and the bed thickness ranged from 2, 4 and 6 mm and the air velocity kept as constant 1.5 m/s. Weight of the sample was recorded at an interval of 3 minutes for first half hour of drying and at subsequent hours of drying, the interval of observation was increased from 3 minutes to 5, 10, 20 minutes. The drying was carried out till there was insignificant relative error (< 0.1%). The dried samples were then packed in polythene bag to avoid moisture migration from atmosphere (Goyal, 2002). The experiments were performed in triplicates.

Mass Measuring Method : For measuring the weight of the sample during experimentation, each tray with sample was taken out of the drying chamber, weighed on the digital top pan balance with a precision of $\pm$ 0.01 g and placed back into the chamber. The digital top pan balance was very close to the drying unit. Each process of weight measurement lasted about 10 s.

Results and Discussion

The drying behavior of the apple pomace was mathematically analyzed by testing the validity of selected models. Mean prediction error and maximum error corresponding to 90% of the data points were calculated for the best fit model.

Equilibrium moisture content computation

For the computation of equilibrium moisture content, a method developed by Issaacs and Goudy (1968) was used to calculate equilibrium moisture content. First the approach of the equation (14) was followed. The plot of M_{n+1} against M_n gave a straight line (Fig. 1-3). But in case of apple pomace, k was small (less than 1), so the error in determination of Z was considerably magnified when antilog of Z was taken, which caused large errors in determination of M_e values because of factor (1-Z). Therefore, equation (14) was not used for determination of k and M_e.

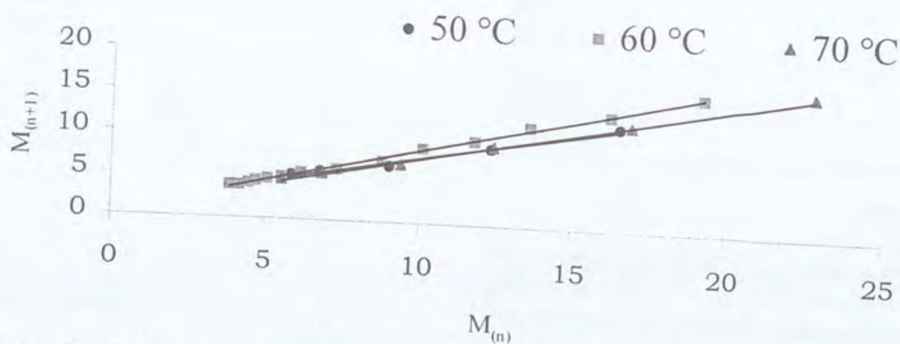

Fig.1: Plot of Data in terms of Equation (14) For Apple Pomace at 2 mm Thickness

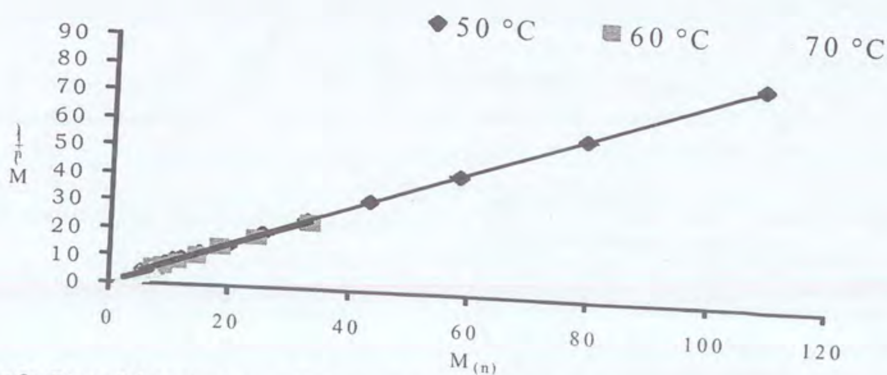

Fig 2: Plot of Data in terms of Equation (14) for Apple Pomace at 4 mm Thickness

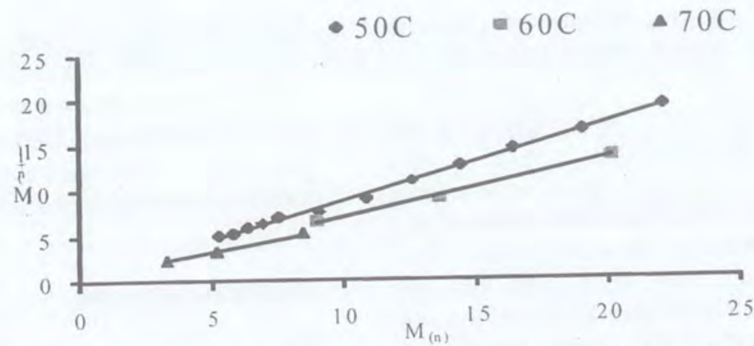

Fig 3: Plot of Data in Terms of Equation (14) for Apple Pomace at 6 mm Thickness

A method developed by Isaacs and Goudy (1968) was used to calculate equilibrium moisture content. The values of m_e were in the range of 5.09% d.b. to 5.69% d.b. for 50 °C; 4.50 to 4.86% d.b. for 60°C and 2..38 to 2.75 % d.b. for 70°C for the experimental layer thickness. The relative humidity of heated air varied from 6.6% to 20%. The values of M_e and relative humidity of heated air are given in Table (1).

Since, no published literature on equilibrium moisture content of apple pomace was found; the validity of this method for apple pomace could not be verified. However, as the temperature increased, equilibrium moisture content decreased (Table 1) which is true in case of equilibrium moisture content.

Table 1: Values of Equilibrium Moisture Content (M_e) and Relative Humidity of Heated Air at Different Levels of Temperature and Layer Thickness

Temperature (°C)	*Layer thickness (mm)*	*Equilibrium moisture content m_e (% d.b.)*	*Relative Humidity (%)*
50	2	5.4559	20.00
	4	5.3108	15.00
	6	5.1176	18.33
60	2	4.1672	11.25
	4	4.4958	12.33
	6	4.8625	14.17
70	2	2.4136	7.80
	4	2.2633	6.60
	6	2.3824	7.80

Fitting of drying models

The moisture content data observed at the drying experiments of apple pomace were converted into the moisture ratio (MR) and fitted to the five models namely, Exponential model, Page's model, Generalized exponential model, Thompson's model and Power law model. The change of moisture ratio (MR) with time of low temperature drying for various experiments of drying kinetics of apple pomace is shown in Fig. (4-6). These figures show a rapid decrease in moisture ratio in initial 50 to 70 min in all cases but in later stage of drying the decrease in moisture ratio is at a slower rate. These figures show the exponential drying curve.

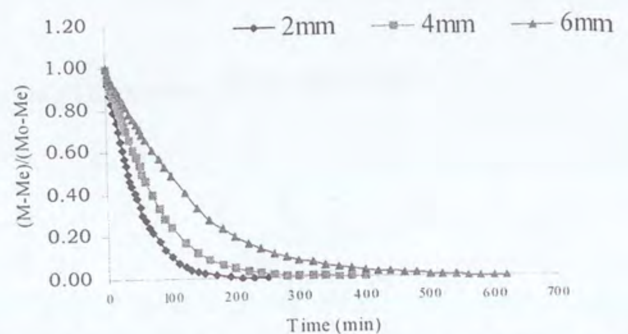

Fig 4: Interrelation between Moisture Ratio and Time at 50 °C

The statistical regression results of the different models, including the model coefficients and the comparison criteria used to evaluate goodness of fit, namely, Standard error of estimation (SEE) and coefficient of multiple determination (R^2) were obtained. The detailed statistical analysis including coefficient of determination and standard error of estimation along with model constants are shown in Table 2-3.

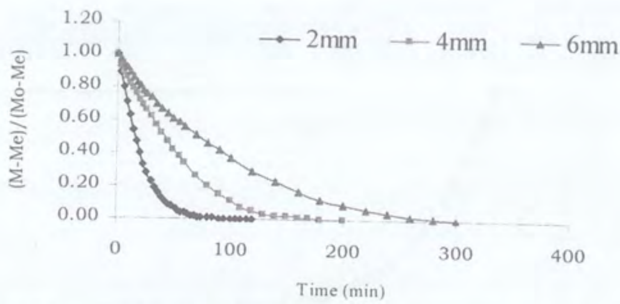

Fig 5: Interrelation between Moisture Ratio and Time at 60 °C

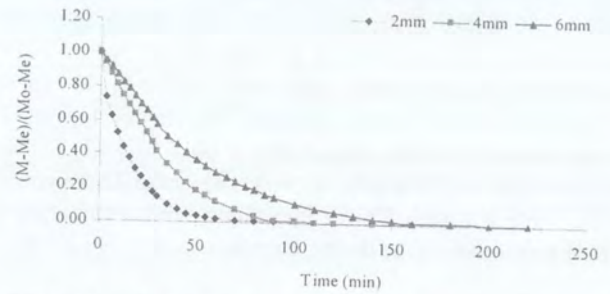

Fig 6: Interrelation between Moisture Ratio and Time at 70 °C

Table 2: Comparison of Statistical Parameters of Various Drying Models Used For Predicting Drying Behavior of Apple Pomace

Temp.(°C)	Thickness (mm)	General exponential model		Exponential model		Page's model		Power law model		Thompson's quadratic model	
		R^2	SEE	R^2	SEE	R^2	SEE	R^2	SEE	R^2	SEE
50	2	0.9989	0.0106	0.9987	0.0112	0.9995	0.0068	0.9990	0.1386	0.9993	1.8564
	4	0.9977	0.0173	0.9954	0.0193	0.9994	0.0084	0.7645	0.1697	0.9972	6.7517
	6	0.9985	0.0144	0.9978	0.0173	0.9997	0.0062	0.7479	0.1850	0.9989	6.6811
60	2	0.9945	0.0215	0.9922	0.0253	0.9994	0.0065	0.8133	0.1122	0.9955	2.3493
	4	0.9894	0.0358	0.9873	0.0385	0.9965	0.0198	0.7379	0.1715	0.9909	5.8560
	6	0.9953	0.0232	0.9949	0.0239	0.9969	0.0184	0.7428	0.1684	0.9988	3.2056
70	2	0.9952	0.0200	0.9921	0.0250	0.9944	0.0174	0.8610	0.0868	0.9935	2.1661
	4	0.9919	0.0309	0.9879	0.0369	0.9991	0.0095	0.7885	0.1485	0.9816	6.2432
	6	0.9969	0.0184	0.9943	0.0247	0.9996	0.0061	0.7795	0.1491	0.9975	3.0925

Table 3: Comparison of Regression Coefficients of Various Drying Models Used For Predicting Drying Behavior of Apple Pomace

Temperature (°C)	Thickness (mm)	General exponential model $MR=A.exp(-kt)$		Exponential model $MR=exp(-k.t)$	Page's model $MR=exp(-k.t^n)$		Power law model $MR=a.t^b$		Thompson's quadratic model $t=a(\ln MR)+b(\ln MR)^2$	
		A	k	k	k	n	a	b	a	b
50	2	1.0121	0.0216	0.0213	0.0171	1.0583	2.0899	-0.4792	-50.2375	-2.4663
	4	1.0230	0.0136	0.0132	0.0082	1.1144	2.1307	-0.4233	-78.0751	-3.3520
	6	1.0230	0.0078	0.0076	0.0047	1.0988	2.2213	-0.3868	-142.3264	-7.9324
60	2	1.0525	0.0493	0.0469	0.0375	1.1977	2.4887	-0.7287	-21.0411	-0.8144
	4	1.0402	0.0193	0.0184	0.0097	1.2114	2.0836	-0.4532	-52.5910	-3.6056
	6	1.0151	0.0106	0.0104	0.0070	1.0885	1.9321	-0.3526	-105.7906	-9.5827
70	2	0.9483	0.0638	0.0676	0.0747	0.8789	1.8471	-0.6991	-13.7252	0.4650
	4	1.0599	0.0324	0.0303	0.0128	1.2480	2.1913	-0.5493	-28.1590	-1.2775
	6	1.0468	0.0210	0.0199	0.0110	1.1510	2.1761	-0.4807	-51.0686	-2.9757

Table 2 shows the statistical parameters for different models and it was observed that coefficient of determination values ranged from 0.8133 to 0.9995 for 2 mm layer thickness, 0.7379 to 0.9994 for 4 mm layer thickness and 0.7428 to 0.9997 for 6 mm layer thickness of apple pomace. The standard error of estimation for all models ranged from 0.0062 to 6.7517. The plot for models viz, Generalized exponential model, Exponential model, Page's model, Power law model and Thompson's quadratic model with the drying data is shown in Fig. (7-15)

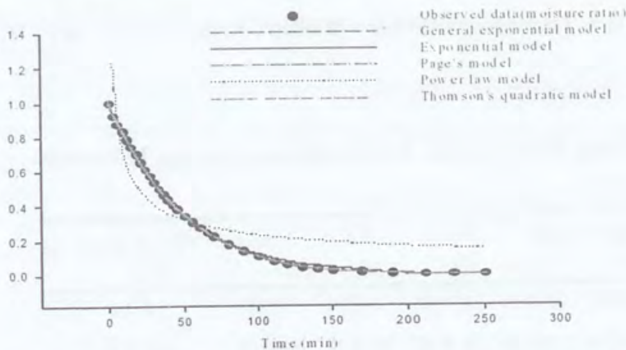

Fig 7: Comparison of various drying models for predicting the drying behavior of Apple Pomace at 50°C at layer thickness of 2 mm and air velocity of 1.5m/s

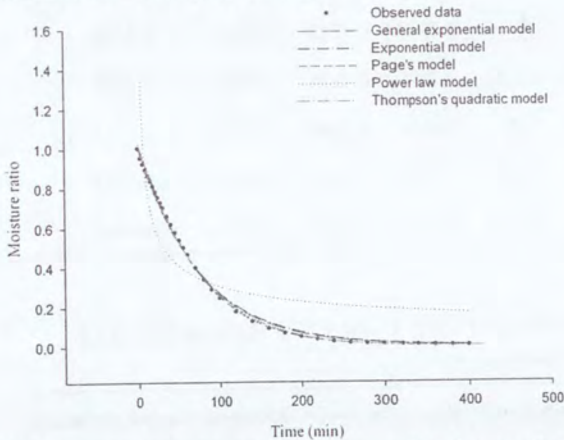

Fig 8: Comparison of various drying models for predicting the drying behavior of Apple Pomace at 50°C at layer thickness of 4 mm and air velocity of 1.5m/s

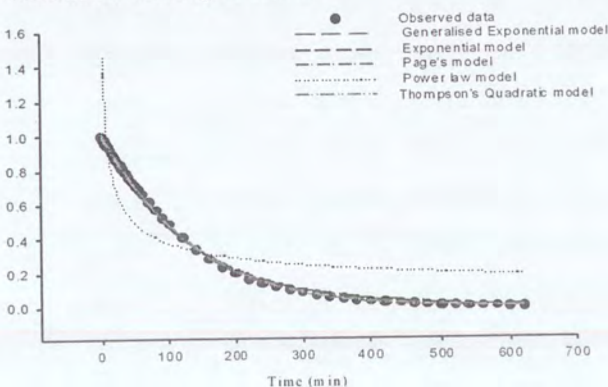

Fig 9: Comparison of various drying models for predicting the drying behavior of Apple Pomace at 50°C at layer thickness of 6 mm and air velocity of 1.5m/s

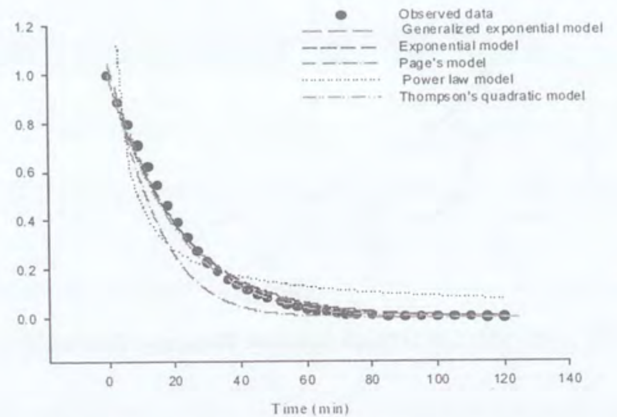

Fig 10: Comparison of various drying models for predicting the drying behavior of Apple Pomace at 60°C at layer thickness of 2 mm and air velocity of 1.5m/s

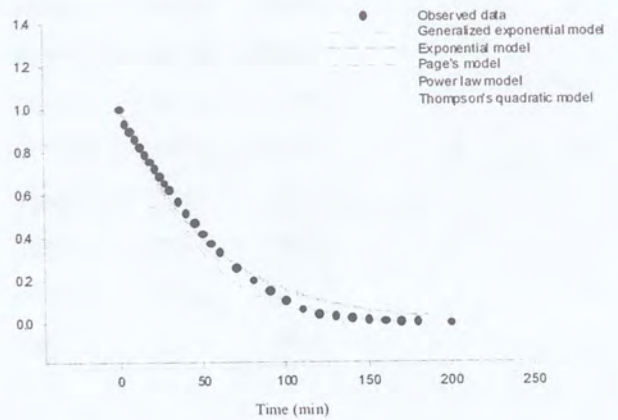

Fig 11: Comparison of various drying models for predicting the drying behavior of Apple Pomace at 60°C at layer thickness of 4 mm and air velocity of 1.5m/s

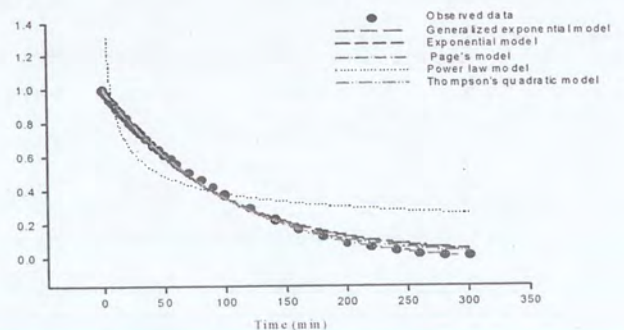

Fig 12: Comparison of various drying models for predicting the drying behavior of Apple Pomace at 60°C at layer thickness of 6 mm and air velocity of 1.5m/s

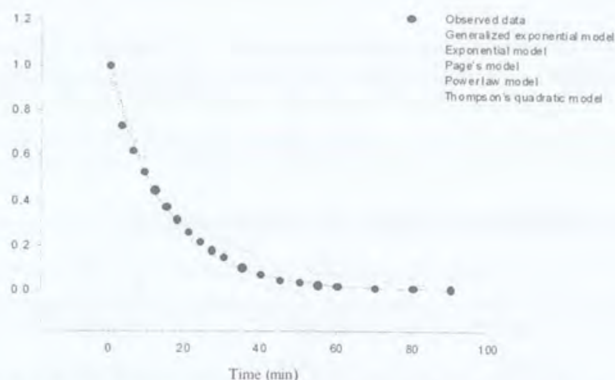

Fig 13: Comparison of various drying models for predicting the drying behavior of Apple Pomace at 70°C at layer thickness of 2 mm and air velocity of 1.5m/s

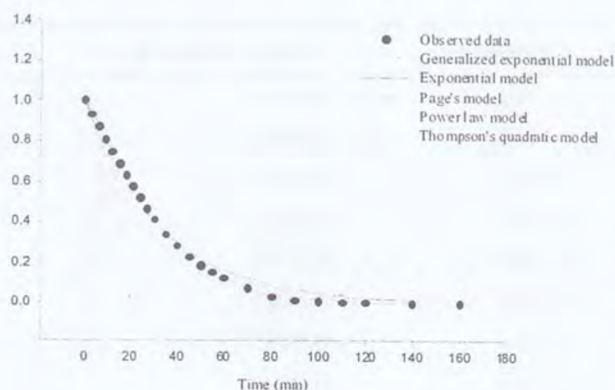

Fig 14: Comparison of various drying models for predicting the drying behavior of Apple Pomace at 70°C at layer thickness of 4 mm and air velocity of 1.5m/s

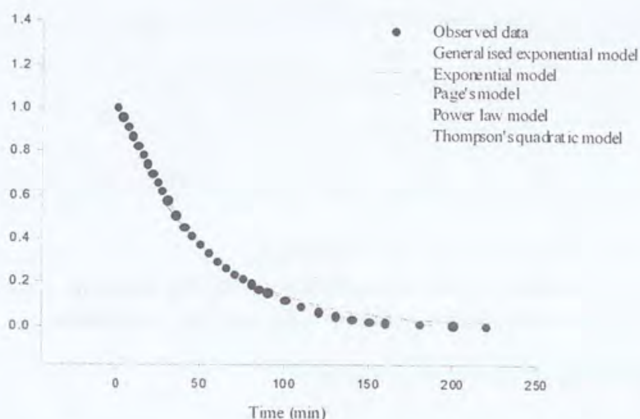

Fig 15: Comparison of various drying models for predicting the drying behavior of Apple Pomace at 70°C at layer thickness of 6 mm and air velocity of 1.5m/s

The coefficient of determination values for Generalized exponential model varied in the range of 0.9945 to 0.9989, 0.9894 to 0.9977 and 0.9953 to 0.9985 and SEE values ranged from 0.0106 to 0.0215, 0.0173 to 0.0358 and 0.144 to 0.0232 for 2 mm, 4 mm and 6 mm layer thickness of apple pomace for the temperature range of 50 to 70°C respectively while for Exponential model, the coefficient of determination values ranged from 0.9921 to 0.9987, 0.9873 to 0.9954 and 0.9943 to 0.9978 and the SEE values ranged from 0.0112 to 0.0253, 0.0193 to 0.0385 and 0.0173 to 0.0247 for 2 mm, 4 mm and 6 mm layer thickness respectively of apple pomace for the temperature range of 50 to 70°C (Table 2).

From Table 2, it can be noted that for Page's model, coefficient of determination values varied in the range of 0.9944 to 0.9995, 0.9965 to 0.9994 and 0.9969 to 0.9997 and the SEE values ranged from 0.0065 to 0.0174, 0.0084 to 0.0198 and 0.0061 to 0.0184 for 2 mm, 4 mm and 6 mm layer thickness respectively of apple pomace for the temperature range of 50 to 70°C.

The coefficient of determination values for Power law model ranged from 0.8133 to 0.9990, 0.7379 to 0.7885 and 0.7428 to 0.7795 and the SEE values ranged from 0.0868 to 0.1386, 0.1485 to 0.1715 and 0.1491 to 0.1850 for 2 mm, 4 mm and 6 mm layer thickness respectively of apple pomace for the temperature range of 50 to 70°C while the coefficient of determination values for Thompson's quadratic model ranged from 0.9935 to 0.9993, 0.9816 to 0.9972 and 0.9975 to 0.9989 and the SEE values ranged from 1.8564 to 2.3493, 5.8560 to 6.7517 and 3.2056 to 6.6811 for 2 mm, 4 mm and 6 mm layer thickness respectively of apple pomace for the temperature range of 50 to 70°C (Table 2).

From the values, the standard error of estimation was the lowest for Page's model as compared to other models for all conditions of experiments as well as the coefficient of determination was high but closely near to Generalized exponential model. Therefore, on the basis of coefficient of determination (R^2) and standard error of estimation (SEE) values, the Page's model was found to be most satisfactory than other models for entire range of experimental data. This observation is similar to the results by Doymaz (2004), Syarief *et al.* (1984), Diamante and Munro (1991), Pandey (2000), Arora *et al.* (2003) and Faisal (2003) for high moisture products; they have concluded that Page's model could adequately describe the thin layer drying behavior of high moisture prodeucts similar to apple pomace.

The regression coefficients for Page's model are tabulated in Table 3. The value of n was close to 1, which is in agreement for high moisture products indicated in literature i.e. 0.65-0.92 for dehydrated peas (Burande, 1992); 1.01 for button mushroom (Singh, 1997); 0.98-1.13 for vegetable kofta (Khiste, 1997); 0.7-0.95 for carrot halwa (Basantpure, 1998) and 1.032 for cauliflower (Pandey, 2000).

Determination of activation energy

The analysis of variance of n indicated that the effect of n is insignificant with reference to temperature and thickness (Table 4) and therefore, an attempt was made to take the average values of n and recalculate the values of drying constant k. This average value of n and recalculated values of k is given in Table 5 for Page's model. In further analysis, the average value of n and recalculated values of k were used.

Table 4: ANOVA for Effect of Temperature and Thickness on Page's Model Constant n

Source of Variation	*SS*	*df*	*MS*	F_{cal}	*P-value*	F_{crit}
Temperature	0.03216	2	0.0161	1.2413*	0.3807	6.9443
Thickness	0.0111	2	0.0055	0.4267*	0.6792	6.9443
Error	0.0518	4	0.0130			
Total	0.0950	8				

*5% level of significance

Table 5: Recalculated Values of Page's Model Constant K with Average Value of n

Temperature (°C)	*Layer Thickness (mm)*	*Average n*	*k with average n*
	2	1.1163	0.0137
50	4	1.1163	0.0081
	6	1.1163	0.0043
	2	1.1163	0.0321
60	4	1.1163	0.0116
	6	1.1163	0.0062
	2	1.1163	0.0485
70	4	1.1163	0.0202
	6	1.1163	0.0126

Fig 16 shows the relation between the natural logarithm of k and the reciprocal of absolute temperature for the drying of apple pomace and the following equations were obtained:

For 2 mm layer thickness of apple pomace:

$$\ln k = -7025\frac{1}{T} + 17.524 \qquad (R^2 = 0.9678)$$

For 4 mm layer thickness of apple pomace:

$$\ln k = -5049.7\frac{1}{T} + 10.782 \qquad (R^2 = 0.9805)$$

For 6 mm layer thickness of apple pomace:

$$\ln k = -5934.5\frac{1}{T} + 12.863 \qquad (R^2 = 0.9607)$$

The results showed a linear relationship derived from the Arrehenius –type equation. According to the slopes of the straight lines, the values of activation energy were 58.406 kJ/mole, 41.983 kJ/mole and 49.339 kJ/mole for 2, 4 and 6mm layer thickness respectively of the apple pomace. They should be nearly equal and their difference comes from different initial moisture content and the experimental error.

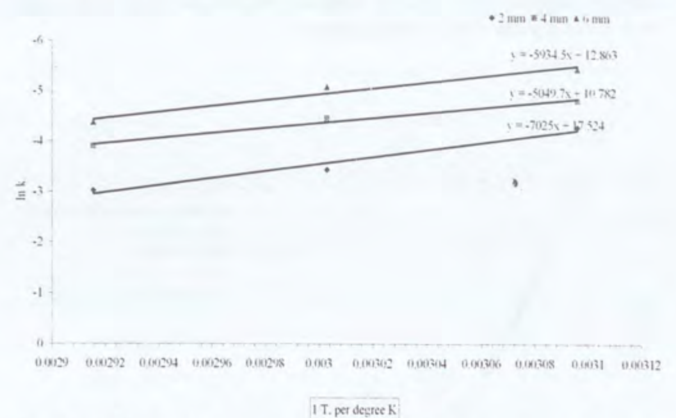

Fig 16: Effect of air temperature on drying constant k for average n for Page's model for different layer thickness

Error analysis for Page's model

The goodness of fit of Page's model was further confirmed by error analysis which indicated that associated errors were small as the average error ranged from 2.823 to 6.096% and maximum error corresponding to 90% of data point ranged from 6.335 to 11.717% for all the experiments (Table 6).

The maximum value of E_{90} was 11.72% for 60 °C and 4 mm layer thickness of apple pomace. This suggests that the drying rates in the falling rate period could be best described by Page's model.

Table 6: Error Analysis of Page's Model

Drying conditions			
Temperature (°C)	*Thickness (mm)*	E_m %	E_{90} %
50	2	4.209	9.932
	4	3.441	6.794
	6	3.935	7.197
60	2	5.508	9.887
	4	5.581	11.717
	6	3.577	7.391
70	2	6.096	6.335
	4	5.378	9.775
	6	2.823	8.668

Conclusions

The main conclusions from the mathematical modelling of hot air convective drying kinetics of apple pomace are:

- Drying of apple pomace takes place in falling rate period.
- As the temperature increased, equilibrium moisture content of the apple pomace decreased.
- Page's model as compared to other models (Generalized exponential model, Exponential model, Power law model and Thompson's quadratic model) most closely predicted the drying behavior of apple pomace with maximum error at 90% data points (E_{90}) less than 12% and average mean error in the range of 0.084 to 0.305 % for the different individual set of conditions.
- The values of activation energy were 58.406 kJ/mole, 41.983 kJ/mole and 49.339 kJ/mole for 2, 4 and 6mm layer thickness respectively of the apple pomace.

These above findings are applicable to design and operation of hot air convective thin layer drying of apple pomace.

Nomenclature

$\frac{dM}{dt}$	=	Drying rate
Σ	=	Summation
$\nabla^2(\frac{\partial^2 M}{\partial X^2})$	=	Second order differential
%	=	percent
β	=	Dt/S^2, Dimensionless
α_1	=	$(\pi/2)^2$
@	=	At the rate of
°C	=	Degree Celsius
°K	=	degree Kelvin
A	=	Constant
B	=	Constant
cm	=	centimeter
D	=	Diffusion coefficient, cm^2/h
d.b.	=	dry basis
div	=	Second partial differential
E_{90}	=	Maximum error at 90% of data points
E_m	=	Average mean error
et al	=	And others
Fig.	=	Figure
g	=	gram (s)
h	=	hour (s)
HP	=	Horse power
k	=	Drying rate constant (min^{-1})
kg	=	kilogram
kg/cm^2	=	kilogram per square centimeter
kW	=	kilowatt
M	=	Average moisture content at time t, % (d.b.)
m/min	=	meter per minute
m/s	=	meter per second
M_e	=	Equilibrium moisture content, % (d.b.)
mm	=	millimeter
M_n	=	Moisture content at time t_n (% d.b.)
M_{n+1}	=	Moisture content at time t_{n+1} (% d.b.)
M_o	=	Initial moisture content, % (d.b.)
MR	=	Moisture Ratio
n	=	Model constant
N	=	Total readings
R^2	=	Coefficient of multiple determination
S	=	One-half of slab thickness, cm
s	=	second (s)
SEE	=	Standard error of estimation
T	=	Temperature (°C)
t	=	Time, h
W_e	=	Equilibrium weights of sample (g)
W_i	=	Initial weight (g)
W_n, W_{n+1}, W_{n+2i}	=	Sample weights taken at time interval i (g)
W_s	=	Initial solid weight of sample
W_t	=	Final weight (g)
y	=	predicted value
y_i	=	observed value
Z	=	Constant =exp (-kt) and t=20 min

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Is Nepalese Honey Free from Pesticides?

PRAMOD KOIRALA[1,*], NAWA RAJ DAHAL[1], MEGH RAJ BHANDARI[1], GANESH DAWADI[1], JIWAN PRAVA LAMA[1] and UTTAM KUMAR BHATTARAI[2]

[1]Department of Food Technology and Quality Control, Kathmandu, Nepal

[2]Ministry of Agriculture and Cooperatives, Government of Nepal

Nepal produces a wide range of specialized honey noted for their purity and high medicinal value and is regarded as one of the most potential country to produce high quality honey due to its diverse climatic conditions and rich honeybee flora. Therefore, honey production is venerable tradition and potential source of rural income and employment. The demand of Nepalese honey is increasing in international market but international trade requires certain obligations. Presence of pesticide residues in honey affected the global trade of honey in the last few years. A field survey study on use of pesticides in beekeeping was carried out and honey samples were collected for laboratory analysis. The field survey result revealed that there is a threat for twenty three types of pesticides in honeybees. Laboratory analysis of seventeen types of pesticides results revealed that pesticide residues in Nepalese honey were below MRLs established by EU.

Keywords: Honeybee, Beekeeping, Honey export, Pests and pesticide

Introduction

Honey is the natural sweet substance produced by honeybees from the nectar of plants or from secretions of living parts of plants or excretions of plant sucking insects on the living parts of plants, which the bees collect, transform by combining with specific substances of their own, deposit, dehydrate, store and leave in the honey comb to ripen and mature (CAC, 2001; CAC, 2006). According to Food Act of Nepal (1967) , honey is the sweet and thick liquid food product produced by honey bees by collecting nectar of blossoms or from secretions of any part of plants and specially transforming into honey in honeycomb (Nepal gazette, 2000). According to PFA, honey means the natural sweet substance produced by honey bees from the nectar of blossoms or from secretions of plants which honey bees collect, transform store in honey combs for ripening. (PFA, 2006). According to EU, honey is the natural sweet substance produced by *Apis mellifera2* L. bees from the nectar of plants or from secretions of living parts of plants or excretions of plant sucking insects on the living parts of plants, which the bees collect, transform by combining with specific substances of their own, deposit, dehydrate, store and leave in honeycombs to ripen and mature (European union, 2006). EU definition mainly focuses honey produced by *A. mellifera*, whereas, the Codex definition need to cover honey produced by other honey bees (progressing) ,however, *A. cerana* Fab. is the prominent indigenous honeybees of Nepal.

Almost every society on earth has known and used honey. Cave paintings near Valencia in Spain from 15 000 years ago depict men gathering honey (Crane, 1999). In language and literature, religion and folk beliefs, honey symbolizes sweetness of every kind. The Bible, the Veda and Koran all extol the virtue of honey as a valuable and nourishing food. In Hinduism, honey is considered to be one of the five heavenly food" *Panchamrit*". The Koran has devoted a special chapter on honey and its uses (Sura XVI-NAHL; Ali 1989). Honey is also mentioned in Bible. Solomon in his proverbs (24:13) advises, 'my son, eat thou honey for it is good' (Kesang and Shrestha 2009).

*Corresponding author; Email: pramodkoirala2002@yahoo.com

Honey can be consumed directly or used in cakes, pastries, candies, chewing gum and toffees etc. It also has laxative properties, increases appetite helps to control gastritis and relief to allergy sinus arthritis and asthma, and one of the most effective against treating burns and cuts. It is, therefore, considered as one of the most effective and inexpensive home remedies. In many areas of Nepal, where sugar is not easily available and honey is used as a substitute of sugar. It is also used as an energy food in preparing special dishes such as pancake, *laddu* and *sel roti*. These dishes are prepared as festival dishes and in some special ceremonies. (Joshi, 1999)

In Nepal, honey is mostly sold as a general, homogenous commodity regardless of its botanical or geographical origin. Honey processors and traders especially bee companies mix all kinds of honey and sell it under uniform label and don't mention the species of honey flora origin. This type of honey is categorized mostly as blended honey or commercial honey. (Halwai and Poudel, 2007)

Nepal is only country in the world where honey is produced between the ranges of 70 -4200 meter above the sea level. Owing to the wide variation in altitude and climate condition Nepal has very broad ranges of plant diversity. Nepal has four species of Indigenous honeybees-*Apis laboriosa* S. (the Himalayan cliff bee) *Apis dorsata* (the giant bee of subtrophic) , *Apis florae* (dwarf honey bee) and *Apis cerena* (the Asian hive bee) and one exotic bee spices –the European honeybee *Apis mellifera*. The indigenous honeybee species (especially

the wild honey bee species) are already endangered because of the import of the exotic *Apis mellifera* and their imported diseases and the unsustainable management of the bee species. (Pokhrel, 2009; Joshi, 1999)

China is the world's largest producer of honey, followed by USA, Argentina, Turkey and Mexico. In world honey production, Nepal's share is about 0.05% In Nepal over 50,000 households are engaged in bee keeping and about 130,000 bee hives are in the country with three major species of bee (90,000 *cer*, 10,000 *mel* and 30,000 wild honeybees) colonies. The current production of honey in Nepal is 1000 tons per year (ABPSD 2008). Most of the honey produced in Nepal is sold in the domestic market and only a very small quantity is exported. One conservative estimate made by a study has indicated that Nepal has a potentiality to produce more than 10,000 tons of honey per year (FNCCI/AEC, 2006).

Pesticides have contributed to dramatic increase in crop yields and in the quantity and variety of diet (Williams 2000). Pesticides have profoundly improved the human livelihood (Abhinash and Singh, 2009). Their dramatic effort in preventing, crop loss and controlling vectors of diseases have led to their acceptance and expanded use throughout the world (Sharp and Peter, 2005). Pesticides owing to their pest-destroying properties are required in global food production but they remain inevitably present as residues in food from both vegetal and animal origin. (Mansour, 2004; Maroni et al., 2006; Maroni 1990). Pesticide despite their known toxicity, are widely used in developing countries. However, the powerful chemicals for killing pests have raised concern that they are agents of human diseases and environmental pollution. It has been observed that their long term, low dose exposure is increasingly linked to human health effects such as immune-suppression, hormone disruption, diminished intelligence, reproductive abnormalities and cancer (Gupta, 2004; Wiles et al., 1998). Pesticides classified as being extremely or highly hazardous by FAO and WHO, including banned by other countries, continued to be used in developing countries (WHO and UNEP, 1990; WHO, 2003). According to WHO, developing countries use about 20 percent of the pesticides in the worlds, and this use is increasing. Although, Nepal average consumption of pesticides is far lower than many other developed economies, the problem of pesticide remains high in Nepal.

Until the 1950s, the people of Nepal remained unaware of modern chemical pesticides and were dependent upon traditional organic techniques for killing pests. Chemical pesticides were first introduced to Nepal in 1955 when Paris Green, gamaxane, and nicotine sulphates were imported from USA for malaria control. DDT made its first impact in 1956. This was soon followed by a variety of other organochlorines (in 1950s), organophosphates (in 1960s), carbamates (in 1970s), and synthetic pyrethroids (in 1980s) (Koirala and Tamrakar, 2008)

In Nepal, use of pesticides has become inevitable to sustain and improve current level of crop production by protecting crops from pests. Being subtropical, Nepal observe varying temperature and humidity profile throughout the year, which brings a vast array of pests to be tackled. Some pests are found to attack multiple targets (various crops) and have acquired resistance from prolong use of common pesticides. In Nepal, literacy is low, particularly rural mass that triggers the improper and non-judicious use of pesticides by farmers. Additionally, the use of incorrect (high) dose of pesticides by farmers leads to the contamination of their products, which in turn causes a greater risk for consumers or traders (Karlman, 1987).

In the past, Nepal government lack regulations governing import, distribution disposal & use of chemical pesticides. But, the pesticide Act 1991 and the pesticide Regulations, 1993 came into effect as of 16 July 1994 (Government of Nepal 1993). Food Act 1966 and Food Regulation 1970 has established MRLs for pesticides in food products, but limited to cereals, pulses and their products, processed water and infant food. MRLs has not established for honey yet. Department of Food Technology & Quality Control constantly monitors pesticide residues level food products where MRLs has been laid down (Food Act, 1966; Food Regulation, 1970; DFTQC, 2008). However, the effectiveness of these regulations is poor. Still persistent organochlorines are imported, and used within the country. Organochlorines insecticide such as BHC, aldrin, dieldrin, endrin, chlordane, lindane and heptachlor are already banned for normal use in Nepal's market. But a persistent Organochlorine BHC, is very popular and widely used by farmers (Koirala, 2008a).

Increasing use of agro-chemicals especially pesticide use in crop production is an emerging threat (Caldas and Souza, 2004; Zorka and Maja, 2009). Pesticide like organochlorine, organophosphate and carbamates are observed poisonous to honey bees as well as pesticide residue in honey. The objective of this study was to assess the common use of pesticides during honey production and pesticide residues level in Nepalese honey.

Materials and Methods

Two major honey producing districts in Nepal are *Chitwan* and *Nawalparasi,* two VDCs from each districts were selected for study purpose. Field survey with structured questionnaire was administered. Two honey samples from each VDCs (altogether four samples) were collected and sent for laboratory analysis in Central Food Technological Research Institute (CFTRI), Mysore, India. AOAC17th Edn 2000, 970.52 test method were followed to analyze organochlorine and organophosphorous, whereas AOAC17th Edn 2000, 970.52 and PAM Vol-1 Sec 401-1 test method were used for analysis for synthetic pyrethroids and carbamates.

Results and Discussion

The result of field survey results revealed that eighteen types of pesticides were applied in different agro-products locally. Honeybees visit different cultivated and wild plants to collect nectar and pollen – according to the studies, the major portion of pesticide are used specially in rice, vegetables, vegetable seed and other commercial fruits – most of theses crops are visited by honeybee to collect nectar and pollen – apart from these crops, Nepal's beekeeping industry is mainly based on brassicae species which includes mustard cauliflower and cabbage seed production – pesticide are frequently used in these crops to save from variety of pest. These pesticide may have the chance of resides in honey. The lists of pesticides are presented in Table 1.

Table 1: Pesticides used in Honey Production in Nepal

Pesticide group	*Type of pesticide used*
Organochlorines	Chlorobenzilate, Hexachlorobezene, pp – DDT, op-DDT, pp –DDE, pp-DDD, alpha-HCH, beta-HCH, Lindane, Vinclozolin
Organophosphorous	Coumaphos, Malathion, Phosalone
Synthetic pyrethroids	Cyfluthrin, Cypermethrin, Deltamethrin, Permethrin, Fenvalerate, Fluvalinate, Cyhalothrin
Carbamates	Carbofuran, Propoxeur, Carbaryl

Samples collected during the field survey were analyzed for pesticide residues and the result of analysis is presented in Table 2. Pesticide residues for organochlorine / organophosphorous i.e. Cypermethrin, DDT, DDE, DDD, Malathion, Phosalone, Diacofol and Lindane; Pyrethroids pesticide residues i.e. Permethrin, Cypermethrin, Fenvelerate and Deltamethrin as well as Carbamates pesticide residues i.e. Oxamyl, Methomyl, Aldicarb, Carbofuran, Carbary and Methiocard were found below detection level for honey

Table 2: Analytical Result ^of Pesticide Residues Level in Honey

Pesticide	*S1*	*S2*	*S3*	*S4*
Organochlorine/Organophosphorous Pesticide Residues* ^*, #^				
DDT	BDL	BDL	BDL	BDL
DDE	BDL	BDL	BDL	BDL
DDD	BDL	BDL	BDL	BDL
Malathion	BDL	BDL	BDL	BDL
Phosalone	BDL	BDL	BDL	BDL
Diacofol	BDL	BDL	BDL	BDL
Lindane	BDL	BDL	BDL	BDL
Synthetic Pyrethroids Pesticide Residues **, ##				
Permethrin	BDL	BDL	BDL	BDL
Cypermethrin	BDL	BDL	BDL	BDL
Fenvelerate	BDL	BDL	BDL	BDL
Deltamethrin	BDL	BDL	BDL	BDL
Carbamates Pesticide Residues ***, ###				
Oxamyl	BDL	BDL	BDL	BDL
Methomyl	BDL	BDL	BDL	BDL
Aldicarb	BDL	BDL	BDL	BDL
Carbofuran	BDL	BDL	BDL	BDL
Carbaryl	BDL	BDL	BDL	BDL
Methiocard	BDL	BDL	BDL	BDL

^ Samples analyzed at Central Food Technological Research Institute (CFTRI), Mysore, India, S1and S2- Honey sample from *Chitwan*, S3 and S4- Honey sample from *Nawalparasi* * BDL - Below Detection Level of 0.005 ppm ** BDL - Below Detection Level of 0.01 ppm., *** BDL- Below Detection Level of 0.05 ppm., # Test Method - AOAC 17th Edn. 2000, 970.52, ## Test Method-AOAC 17th Edn. 2000, 998.01, ### Test Method-PAM Vol-1 Sec 401-1

The results of analysis were compared with the MRL's established by EU (Table 3).

Table 3: EU MRLs for Pesticides in Honey

Pesticide group	*Pesticide type*	*MRL in ppm*	*Pesticide group*	*Pesticide type*	*MRL in ppm*
Organochlorines	Chlorobenzilate	0.02	Synthetic pyrethroids	Cyfluthrin	0.007
	Hexachlorobezene	0.005		Cypermethrin	0.017
	pp – DDT, op-DDT, pp –DDE, pp-DDD	0.025		Deltamethrin	0.017
	alpha-HCH, beta-HCH, Lindane	0.0005		Permethrin	0.017
	Vinclozolin	0.01		Fenvalerate	0.017
Organophosphorous	Coumaphos	0.05		Fluvalinate	0.007
	Malathion	0.02		Cyhalothrin	0.007
	Phosalone	0.02	Carbamates	Carbofuran	0.001
Missllaneous	Cymiazol	0.5		Propoxeur	0.0001
	Amitraz	0.1		Carbaryl	0.003
	Brompropylat	0.05			
	Chinomethionat	0.02			

(Source : DFTQC 2008)

samples. The result of laboratory analysis result showed that the pesticide residues for organochlorine and organophosphorous is was below 0.005 ppm, synthetic pyrethroids less than 0.01 and carbamates less than 0.05 ppm (Table 2).

Because of the limited laboratory facility, seventeen types of pesticides were analyzed. Among the analyzed seventeen types of pesticides, thirteen types of pesticides in EU MRL list, the level of which is compared. DDT, its isomers (DDD, DDE) and lindane fall under POPs so it is banned for normal agriculture use. The laboratory result for DDT, DDE and DDD was below 0.005 ppm, which was below EU MRL of 0.025 ppm. Similarly, Malathion is a quite popular pesticide among Nepalese farmers for general agriculture use, however, the level of it was detected below EU MRL of 0.02 ppm. So is the case with phosalone.

Similarly, among the group of synthetic pyrethroids, cypermethrin and permethrin residue level in honey level for EU MRL is 0.017 ppm and laboratory analysis result revealed that these there pesticides were below 0.01 ppm. Among carbamates, two pesticides namely carbofuran and carbyrl was analyzed. The levels of these pesticides detected were below 0.05 which were below EU MRLs of 0.1 ppm and 3.0 ppm respectively.

The economy of Nepal is largely based on agriculture. It contributes about 32% to the national economy, provides employment for over 65% of the labor force and is main source of income in rural areas, which accounts for 85% of total population (APBSD, 2008). Agriculture development continues to remain the most important objectives of Nepal planning and policy. In the process of development of agriculture, pesticides have become important tools as plant protection or boosting food production. Although Nepal average consumption of pesticides is far lower than many other developed economies, the problem of pesticide remains very high in Nepal (Koirala et al., 2008b; Koirala et al., 2009a; Koirala et al., 2009b). Pesticide residues in several crops including honey have also affected the export of agriculture commodities in the last few years. Pesticide residues in food are global problems (Arthur 2004). Preventing pesticides in honey is a major concern of food regulators in Nepal because it is necessary to provide safe honey to the consumers. Therefore, it is essential that adequate monitoring should be in place to eliminate the possibility of the presence of the residues in honey.

Globalization has brought unique opportunities for developing countries in terms of access to markets for their products (Koirala et al., 2007). Nepal is exporting honey to different overseas markets for the last few years, but the volume and the value of export is not optimal. Until 2001/2002, Norway used to be the largest buyer of Nepalese honey. The core requirement for importing honey into EU market is for the country in question to have a Residue monitoring plan, approved by EU. To gain access to EU markets for animal products, exporting countries must have in place a residue control programme. Approved list of countries for residue monitoring plans should be submitted by exporting countries in accordance with Council Directive 96/23/EC. According to EU criteria, Nepal has to submit an implementation plan for the control of residues in food products. The work for residue monitoring plan has already initiated and draft report already submitted to EU commission (Lund et al., 2004). At present, the major countries importing honey from Nepal are Japan and South Korea. However, for the last few years, countries like UAE, Thailand and Bangladesh are also emerging as the new markets (Bhandari, 2005)

Conclusion

The level of pesticides residues in Nepalese honey for certain organochlorine, organophosphorous, synthetic pyrethorids and carbamates pesticides was found to be within the MRL limit of EU standards. In this study, the laboratory detection was in the level of 0.005 to 0.05 ppm for different pesticides group. Some pesticides for example Propoxeur and Cyfluthrin EU MRL level were below the detection level and therefore, was not discussed here. Further investigation on the level of pesticide residues are encouraged including those pesticides which were not covered in this study.

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Fast Food (Noodle) and Insulin Resistance in Mice

SUNIL SITOLE, SANTOSH GAHERWAL* andD MAN MOHAN PRAKASH

Post Graduate Department of Biotechnology, Govt. Holkar Science College, Indore-452 017, India

The present paper deals with the impact of sixty days feeding of fast food – Noodle (prepared) on the blood sugar level of mice. The result obtained showed increase in blood sugar. The increase in pre meal blood sugar after 7, 15, 30 & 60 days feeding of only noodle were 2.34, 36.71, 40.62 & 51.18 percent and in post meal blood the level of blood sugar were 48.46, 101.55, 112.5 & 125.75 percent respectively. This clearly indicates that the fast food - Noodle increase the risk factor of diabetes.

Key words: Fast food, Noodle, Blood sugar, Pre meal and Post meal

Introduction

Fast food consumption has increased greatly in the developing and developed countries during the past three decades due to its good test and availability. Generally Fast foods are nutritionally unbalanced and therefore, consider unhealthy in the long run if consume on a regular basis. Fast foods are rich in calories (Produced from refined sugar and fats), sodium (coming from common salts) and other additives, but are deficient in dietary fiber and essential micro-nutrients (like vitamins and minerals). Consumption of fast food on a regular basis leads to many health hazards like obesity, hypertension, hiper cholesterol, cardiac diseases and diabetes (Pereira *et. al.*, 2005; Shrivastava *et. al.*, 2009).

People whose diet consists primarily of fatty food such as hamburgers, pizza and French fries - weigh more and have an increased risk of insulin resistance compared to people who limit their consumption of foods high in fat. Insulin resistance is a condition in which peoples' insulin does not process food effectively and abnormal amounts of sugar circulate in the blood stream. The result is high blood sugar levels that can make such individuals more prone to high blood pressure, heart disease and diabetes. Looking to the importance of fast food in causing diabetes authors worked to observe the effect of fast food specially noodle on blood sugar.

Material and Method

Experimental Anima: Mice *MUS-MUSCULUS ALBINUS* were collected from the college of Veterinary Science and Animal Husbandary, Mhow and acclimatized to the laboratory condition for fifteen days during which they were regularly fad with normal food diet (supplied by Veterinary College). ***Test food:*** In test food only prepared noodle was used which was purchased from Indian Coffee House, Bhanvar kuan Square, Indore (M.P.). ***Experimental Design:*** Total 60 mice were used in the present investigation. They were divided in to the following two groups. *(A)* ***Control group***: In this group 30 mice were kept under normal food diet and aqua guard water. ***(B) Experimental group***: In this group 30 mice were kept on only prepared noodle (fast food) diet and aqua gaurd water. ***Autopsy:*** Mice (five) of experimental and control group were sacrificed after 0, 7, 15, 30 and 60 days for blood collection by cardiac puncture-technique. ***Blood sugar estimation:*** Quantitative blood sugar estimation was done by Nelson-Somogyi method.

*Corresponding author; Email: psgaherwal@yahoo.com

Results and Discussion

Results obtained in the present investigation are summarized in Table-1. In pre meal mice (i.e. 15 hrs. fasting) control value of blood sugar were noted in between 126 to 128 mg/dl, while in pre meal experimental group blood sugar value were found in between 126 to 192 mg/dl within 0 to 60 days of experimental duration. In pre meal blood, the sugar level after 7, 15, 30 & 60 days feeding of only noodle were observed 131, 175, 180 and 192 mg/dl. The increases in term percent were 2.34, 36.71, 40.62 & 51.18 respectively. This showed that constant use of noodle for 60 days (as food) increased the blood sugar level upto 51.18 percent in pre meal experimental animal.

In post meal mice control value of blood sugar were noted in between 128 to 132 mg/dl, while in experimental group blood sugar value were found in between 189 to 298 mg/dl within 60 days of experimental duration. In post meal blood sugar level after 7, 15, 30 & 60 days feeding of only noodle was observed 193, 260, 272 and 298 mg/dl respectively. The increases in blood sugar level were found 48.46, 101.55, 112.5 & 125.75 percent respectively. This showed that constant use of noodle for 60 days (as fast food) increased the post meal blood sugar level upto 125.75 percent.

Worldwide consumption of fast food is steadily increasing. In the United States alone, sales of fast food reached a value of $161 billion in 2004 (Pereira *et. al.*, 2003). Today in the United States, Up to 37% of adults and up to 42% of children regularly consume fast food. Fast food has been shown to promote weight gain and insulin resistance (Pereira *et. al.*, 2003; Anderson and Whitaker, 2006; Edelstein *et. al.*, 1997)

Table 1- Blood sugar level in mice (fad with only noodle)

Duration	*Control Value*		*Experimental Value*		*Difference*		*% Alter*	
	Pre Meal	*Post Meal*	*Pre Meal*	*Post Meal*	*Pre Meal*	*Post Meal*	*Pre Meal*	*Post Meal*
0 Day's	126	128	126	189	0	61	0	47.65
7 Day's	128	130	131	193	3	63	2.34	48.46
15 Day's	128	129	175	260	47	131	36.71	101.55
30 Day's	127	128	180	272	52	144	40.62	112.5
60 Day's	127	132	192	298	65	166	51.18	125.75

Blood sugar value in mg/dl

Post meal reading was taken after two hours of routine and experimental feeding.

WHO reports shows that by year 2015 obesity and diabetes disease will create a problem to human being worldwide.

Fast foods contain large amounts of partially hydrogenated oils, and this class of fatty acids may cause insulin resistance and increase risk of type 2 diabetes. Fast food also contains large amounts of highly refined starchy food and added sugar, carbohydrates that have been characterized as high in glycemic index. Consumption of a high glycemic index or high glycemic load diet has been linked to risk for diabetes (Pereira *et. al.*, 2005).

In the present investigation, mice fad on only fast food noodle (a kind of fast food) diet for sixty days showed increase in the blood sugar level. The value of blood sugar in pre meal and post meal control group were 126 to 128 mg/dl and 128 to 132 mg/dl respectively. In pre meal mice group the quantity of blood sugar was found increased from 126 to 192 mg/dl and in post meal mice group from 189 to 298 mg/dl as compare to control group. The increase in blood sugar was gradual and duration dependent.

The blood sugar level in the present investigation increased up to 51.18% in pre meal mice group, while in post meal mice group it was increased up to 125.75%. The increase in blood sugar level clearly indicate that constant use of noodle (fast food) arrested the functioning of insulin in experimental mice and increase the risk of diabetes. This finding corroborates with the finding of listed references that use of fast food develops insulin resistance and increase the risk factor of diabetes.

Conclusion

Thus on the basis of present study authors can conclude that the constant use of noodle in diet may increases the high risk factor of insulin resistance in consumer, which may cause diabetes. Thus it is advisable to user of such diet that they should restrict or avoid regular use of such diet for better health condition.

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Microbial Quality Evaluation of Probiotic Yoghurt with Reference to Market Yoghurt Collected from Kathmandu Valley

CHOODAMANI BHANDARI*

Himalayan College of Agricultural Sciences (HICAST), Gatthaghar, Bhaktapur

A study was carried out to evaluate the microbiological quality of laboratory prepared probiotic yoghurt to compare its quality with market yoghurt and juju dhau dahi collected from Kathmandu valley to monitor the presence of health hazard organism (coliform, yeast and moulds) as well as probiotic organisms (Lactobacillus acidophilus and Lactobacillus casei). The average coliform content in market yoghurt, juju dhau dahi and laboratory prepared probiotic yoghurt were found to be 3.24, 2.30 and 0.55 log cfu/g respectively, which varied significantly ($p<0.05$) to each other. Similarly, the average yeasts and moulds content in the market yoghurt, juju dhau and laboratory prepared probiotic yoghurt were found to be 1.80, 4.46 and 0 log cfu/g respectively, which also varied significantly ($p<0.05$) to each other. Probiotic count of the above samples at first day of production and after storing it at 4°C for 7 days were found to be 10.3 and 9.8 log cfu/g, which did not vary significantly ($p<0.05$) to each other. It was interesting to note that juju dhau dahi was found to contain substantial amount of probiotic organisms.

Keywords: probiotic, *juju dhau*, *dahi*, *Lactobacillus acidophilus* and *Lactobacillus casei*

Introduction

Since time immemorial, milk and milk products have been produced inseparable part of the sociocultural life (Bandyopadhyay and Khamrui, 2007). Milk, *dahi* (set yoghurt), butter, *mahi* (buttermilk), and ghee were reported in several epics of Hinduism and Buddhism. The tradition of making *dahi, mahi*, butter and ghee has been handed down from generation to generation in the farming community. They all were made in small quantity in the household scale ever since (Adhikari, 2007). It has been reported that the total milk supply in developing countries has been expanded by 122% from 1980 to 2002 (FAO/IDF, 2005).

About 0.1369 million MT of milk per year is produced, of which12% of the total milk production is processed in the formal sector (AIPC, 2007). The contribution of agriculture sector in national GDP in 40%, which provides employment to about 65% of the total population (AIPC, 2007). The livestock sector alone contributes about one third of agricultural GDP, which contribute to 4% in the national export. Dairy sector alone contributes about two third to livestock sector GDP (NDDB, 2001b and AIPC, 2007).

Milk product diversification has been observed in limited scale in Nepal (Sharma, 2007). More than 90% of the processed milk in the country is sold as pasteuriesed milk with small share (10%) of which is converted into other milk products (Bhagat, 2007). The increase in milk production and the availability of surplus milk to be used for the fulfillment of growing demand of expanding urban market development by diversifying milk products which promotes dairy enterprises, social milk market problem and milk holiday (Achhami, 2007). The consumption of yoghurt has shown trend is increasing every year as it has numerous benefits for human health. The quality attributes of yoghurt in terms of high digestibility, better taste, flavor and religious, medicinal and cosmetic values have enhanced the demand of yoghurt (Lamsal, 2007). The industrially produced yoghurt is sold in urban areas to fulfill the demand of urban people. The large scale producers are government owned Dairy Development Corporation (DDC) and private dairies (NDDB, 2001b).

For thousands of years, it has been known that when milk is allowed to stand, it will ferment spontaneously and become sour, and more resistant to putrefaction. No knowledge of microbiology existed but because of the same utensils and vessels, a typical micro flora began to involve. Starter cultures were unknown until 1878, when Lister isolated pure cultures of the lactic acid bacteria (LAB) responsible for milk acidification. The yoghurt starter bacteria and some other lactic acid bacteria (LAB) present in milk and fermented milk products have potential of being probiotic (Salminen *et al.*, 1998). The general functions of starter culture are to produce lactic acid, acetic acid, aromatic flavor components and carbon dioxide. Additionally possibilities relative to enzymatic activities in the product include lipolytic and proteolytic changes, which are desirable and undesirable depending on the products (Adhikary, 1999).

Probiotic are organisms and substances, which contributed to intestinal microbial balance (Parker, 1974). Fuller (1992) defined probiotics as "foods containing live microorganisms;

*Corresponding author ; Present Address : Food and Agriculture Organisation, UN Building, Pulchowk, Lalitpur, Nepal ; Email: chooda2002@yahoo.com

which actively enhance health of consumers by improving the balance of micro flora in the gut, when ingested live in sufficient numbers". Salminen and Isolauri in 1996 revised the definition of probiotics as "a live microbial culture or cultured dairy product, which beneficially influences the health and nutrition of the host". Over the years, many species of microorganisms have been used as probiotics. They mainly consist of lactic acid producing bacteria (*Lactobacilli, Streptococci, Enterococci,* and *Lactococci*), Bifidobacteria, some Bacillus species, some fungi like *saccharomyces* spp. and *Aspergillums* spp. In human, lactobacilli either single or in mixed culture with other bacteria like Bifidobacteria and or Streptococci are known as common probiotics. Probiotics are generally taken either in a form of food or a non-food format. Usually, they subsist in the intestinal tract and stomach, which can potentially boost the immune system and help in treating conditions like lactose intolerance, diarrhea, colitis, hypertension, cancer, constipation, food allergies, inflammatory bowel diseases etc. The probiotic bacteria not only needed to remain viable during processing, handling and storage of the food it contains, also until it reaches the intestinal tract and colonizes the surface of human intestine (Cruce and Goulet, 2001). It is thought that the mechanisms by which LAB exert their effect on cholesterol is through bile acids. The liver uses cholesterol to produce bile acids, which are secreted into the small intestine, and then absorbed again and sent back to the liver. Whilst these bile acids are in the intestine, however they can be broken down by certain kinds of bacteria that inhabit the gut (Sindhu and Kheltarpaul, 2003). Antibodies such as Immunoglobulin A (IgA) are produced by plasma cells of the immune system and are involved in protecting the body from potentially harmful microbes. Secretory immunoglobulin A (sIgA) is specifically found at the surface of the intestinal mucosa (the outer membrane layer coated in mucous that is exposed to the guts contents) and works by preventing pathogens from binding with and penetrating the gut wall. *Lactobacillus casei, Lactobacillus acidophilus* and yogurt have been shown to enhance the number of IgA-producing plasma cells in a dose-dependent manner as well as increasing sIgA levels in mice and humans. It has been found that IgA response against *Salmonella typhi* strain ingested to mimic enteropathogenic infections in human volunteers (Link *et al.*, 1994).

Microbial strains for probiotic use must be representative of microorganisms that are generally recognized as safe (GRAS). It should survive under environmental conditions where it must be active and proliferate and colonize where it is active. Probiotic strains, their fermentation products should not exhibit any pathogenic, toxic, allergic, mutagenic or carcinogenic reaction. It should be genetically stable and must not have plasmid transfer. It should be viable during processing and storage. It has been reported that the most satisfactory results are obtained from the ingestion of probiotic products containing 1×10^8 to 1×10^9 cfu/mL viable cells daily.

During fermentation, lactic acid bacteria produce a range of secondary metabolites, some of which have been associated with health promoting properties. The most notable of these are the B vitamins and the bioactive peptides. It is now well established that physiologically active peptides are produced from several food proteins during gastrointestinal digestion and fermentation of food by LAB (Gobetti *et al.*, 2002 and 2004, Shah 2000 and 2006).

Materials and Methods

Sample collection*:** A total of 15 samples of *juju dhau* were collected from the *Bhaktapur* area and a total of 15 samples of market yoghurt were collected from *Baneshwore, Hariharbhawan*, and *Koteshwore* for the study. ***Preparation of probiotic yoghurt*:** The standardized milk (3% fat and 8% SNF) was mixed with SMP and sugar at 45°C. It was then filtered in a muslin cloth. The milk was agitated minimum to avoid the production air bubbles. Six different types of probiotic yoghurt were prepared. P1, P2, P3 and P4 product were added with 2% sugar and 2% SMP (P1 and P2), 3% sugar and 3% SMP (P3) and 4% sugar and 4% SMP (P4). Similarly P5 and P6 probiotic yoghurt were added with 2% sugar and 2% SMP respectively. The milk was heated at 95°C for 5 minutes. It was then cooled for 68°C to homogenize at 2000 pressure psi. It was then cooled to 45°C to add the direct vat set (DVS) probiotic starter culture. The culture was mixed for 15 minutes and incubated at the same temperature till the yoghurt reached the pH of 4.7. After the yoghurt milk attainted the required pH, it was immediately transferred to the cold store. The laboratory prepared yoghurt was analyzed at HICAST laboratory (Nutrish, 2006). ***Coliform counts: The coliform count by method as prescribed by International Dairy Federation (IDF):1998. The number of colonies in one plate between 30 and 300 and less than 30 were also reported. The colonies exceeding 300 were reported as too numerous to count and with no colonies were reported as no growth (NDDB, 2001a). ***Enumeration of L. acidophilus and L. casei:*** The probiotic cell counts for probiotic yoghurt were done by using the selective of MRS for counting probiotic - *L. acidophilus* and *L. casei* in fermented milk products (ISO 20128 and IDF 192). ***Enumeration of yeasts and molds:*** A standard methods of international dairy Federation was followed to enumerate the yeast and molds in the samples (IDF, 1990). ***Statistical analysis:*** The data obtained were statistically analyzed by using SPSS (version 12).

Results and Discussion

Coliform count

The coliform count of market yoghurt, *juju dhau* and probiotic yoghurt is shown in Table 1. The average coliform count of market yoghurt, *juju dhau* and probiotic yoghurt were found to be 3.24, 2.30 and 0.55 cfu/mL, with standard deviation 1.737, 2.002 and 0.03 respectively. In 4 out of 6 samples of probiotic yoghurt, the presence of coliform was nil, which may be due

to the strict hygienic measures being practiced during the process of manufacturing. But in the market yoghurt and *juju dhau*, the higher count of coliform bacteria might be probably due to contamination at packaging, storage and displays/ sales outlets respectively. The presence of Coliform in market yoghurt and *juju dhau* may be due to poor hygienic conditions and improper sanitation during manufacturing process. It can be seen from Table 1 that the various samples except probiotic samples, coliform bacteria was detected in majority of the samples. According to the Turkish Standards Institute (TS 1330) (24) a maximum count of 10 cfu/g of coliform is allowed in yogurt. However, yogurt sold commercially in Nepal had much higher coliform count than that of standard value. The result agrees with result of NDDB's benchmark survey of quality of milk and milk products (2001).

Table 1: Coliform present in different yoghurt samples

Product	*Coliform**	
	Number of sample	*Count (log_{10} cfu/mL)*
Market yoghurt	15	3.24±1.737
Juju dhau (market)	15	2.30±2.002
Probiotic yoghurt (Lab prepared)	60^	0.55±0.030

*Values are mean of samples with standard deviation
^ Product P1 to P6 of 10 Batches each

The coliform counts in market yoghurt, *juju dhau* and probiotic yoghurt showed significant difference ($p<0.05$). The variation in coliform counts between different samples may be due to different in production condition of *dahi* and yoghurt. The probiotic yoghurt was produced in strict hygienic condition while the production of *juju dhau* is not very hygienic as it is produced traditionally. The packaging material, personnel attitude also matters in quality of yoghurt and *dahi*.

Yeast and molds

Yoghurt and *juju dhau dahi* samples were analyzed for the determination of presence of yeast and molds. The number of yeast and molds were extremely high in *juju dhau*, lesser in market yoghurt and nil in probiotic yoghurt samples (Table 2). Perhaps this reflects the ability of more yeast to grow when the *juju dhau* were improperly incubated and storage (distribution) condition was also extremely poor, while the probiotic yoghurt was prepared in extreme hygienic condition. The high yeast and mold count in the *juju dhau* sample could be attributed to contamination from the air, the old *dahi* culture used for *dahi* manufacturing and inadequate cleanliness during manufacturing process. The result agrees with result of NDDB (2001b), which conducted benchmark survey of quality of milk and milk products to assess the overall quality of milk and milk products from production to consumer level. The survey showed that high number of yeasts and molds present in the dahi and yoghurt samples. The presence of yeast and molds in yoghurts and *dahi* in many samples indicates that the processors need to follow up the overall high quality of the hygiene condition in preparing the *dahi* and yoghurt. The higher the number of yeasts and molds present in the given samples, the higher degree of cleanliness and sanitation in the yoghurt production plant is required and vice versa.

Table 2: Yeast and molds present in different yoghurt samples

Product	*Yeast and molds**	
	Number of sample	*Count (log_{10} cfu/mL)*
Market yoghurt	15	1.80±1.583
Juju dhau (Market)	15	4.46±0.401
Probiotic yoghurt (Lab prepared)	60^	0.00±0.000

*Values are mean of samples with standard deviation
^ Product P1 to P6 of 10 Batches each

The yeast and molds counts in market yoghurt, *juju dhau* and probiotic yoghurt showed significant variation ($p<0.05$). The variation yeast and molds counts between different samples may be due to different in production condition of *dahi* and yoghurt. The probiotic yoghurt was produced in strict hygienic condition while the production of *juju dhau* is not very hygienic as it is produced traditionally. The packaging material, personnel attitude also matters in quality of yoghurt and *dahi*.

Total viable count of probiotic organisms

Table 3 shows the total viable counts of six different varieties of lab prepared probiotic yoghurt (P1, P2, P3, P4, P5 and P6). The weighted mean values of probiotic organisms present in the probiotic sample in the first day production was 10.30 log cfu/ml and the products stored for 7 days at 4°C showed weighted mean value of 9.80 log cfu/ml for probiotic count which were not significantly different ($P=0.05$) to each other (Table 3).

Table 3: Probiotic organism present in probiotic yoghurt

Probiotic yoghurt (Lab prepared)	*Probiotic (L. acidophilus and L. casei)**			
	At production day		*After 7 days of storage at 4°C*	
	Number of sample	*Count (log_{10} cfu/mL)*	*Number of sample*	*Count (log_{10} cfu/mL)*
P1	10	10.34±0.984	10	9.67±0.269
P2	10	10.33±1.143	10	10.16±0.266
P3	10	10.03±0.945	10	9.69±0.503
P4	10	10.31±1.122	10	9.71±0.369
P5	10	10.57±0.333	10	10.01±0.575
P6	10	10.23±0.714	10	9.57±0.815
Mean	60	10.30±1.162	60	9.80±0.965

*Values are mean of samples in duplicate with standard deviation

In order to have a probiotic effect, the probiotic strains must be present in the products in sufficient numbers. It is generally recognized that the daily dosage of probiotic strains must be between $1x10^8$ to $1x10^9$ cells with a serving size of 100 gram yoghurt which means that the probiotic product must contain between $1x10^6$ to $1x10^7$ cfu/mL of product (Nutrish, 2006) (Table 3). After storing the laboratory prepared yoghurt for 7 day at 4°C, the total viable counts of six different varieties of lab prepared probiotic yoghurt showed that there is not significant variation in probiotic organism present in the sample ($p<0.05$). This result agrees with the results of CHR-HANSEN (2007) company which concluded that there was required number of viable probiotic organism present in the yoghurt sample even after 35 days of storage at 4°C with the inoculation rate of 0.02% probiotic DVS culture (NDDB, 2001b).

Presence of Probiotic organisms in juju dhau

It is interesting to note that *juju dhau* were studied for the presence of *Lactobacillus acidophilus* and *L. casei* (Table 4).

Table 4: Isolation of *L. acidophilus* and *L. casei* in *juju dhau*

Product	*L. acidophilus* and *L. casei**	
	Number of sample	*Count (log_{10} cfu/mL)*
Juju dhau	10	0.821±0.874

*Values are mean of samples with standard deviation

A total of 10 samples were studied and the mean values were 0.821 with standard deviation 0.874 and 5 samples did not show the presence of probiotic organisms. This result does not agree with findings of CHR-HANSEN (2007) (NDDB, 2001b). It is because the number of probiotic organism present in the *juju dhau* samples was far less to provide probiotic effect.

Conclusions

Market yoghurt and *juju dhau dahi* samples collected from Kathmandu valley showed higher number of coliform as well as yeast and mold count as compared to the laboratory prepared probiotic yoghurt. Probiotic count of laboratory prepared yoghurt at first day of production and after storing it at 4°C for 7 days was not significantly different to each other. The presence of probiotic organism in *juju dhau dahi* has opened tremendous opportunities for further research. The process optimization with special focus to incubation and packaging needs to be taken care of. Similarly, the market pouch yoghurt is disappointing as it has numerous spoilage organism i.e. presence of coliform bacteria, yeast and moulds have drawn the attention of the researcher, dairy industries, and dairy experts as it has direct connection with public health hazard. The production potential of probiotic yoghurt due to its numerous health benefits to the consumer is equally important to the dairy industrialists and researchers of country like Nepal as it will be the first effort to bring such products in the market by the Nepali dairy industry.

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Bacteriological Analysis of Drinking Water of Kathmandu Metropolitan City

INDRA BAHADUR PACHHAI*, OM PRAKASH PANTA, VIJAY SINGH CHHETRI and PRAMOD POUDEL

Department of Microbiology, National Institute of Science and Technology (NIST), Kathmandu, Nepal.

In the present study, altogether 130 drinking water samples collected randomly from different places of Kathmandu Metropolitan

count and isolation of bacteria to assess the drinking water quality. Among 130 total water samples, 97(74.61%) were found to be contaminated with coliform bacteria. Out of 97 coliform positive water samples, (53.07%) water samples were found to be faecally contaminated. Out of all coliform positive water Samples, 163 enteric bacteria were isolated. Among these bacteria, *E.coli* was found to be identified as highest (37.42%) followed by *Enterobacter spp* (17.17%), *Citrobacter spp* (14.11%), *Pseudomonas spp* (12.26%), *Klebsiella spp* (7.97%), *Shigella spp* (6.13%) and *Salmonella spp* (4.90%) respectively.

Key Words: Bacteria, Coliform, Water quality, Water borne disease.

Introduction

Water is very important for all living beings and is consumed by all and as such it is essential that all should get pure and clean drinking water. Simple access to safe water and adequate sanitation is an essential first step to protect human health, and a basic human right. Lack of access to safe drinking water and poor sanitation still threatens the health of millions of people.

Historically, water has played a significant role in the transmission of human disease. Typhoid fever, cholera, infectious hepatitis and many varieties of gastrointestinal disease can all be transmitted by water. The WHO (1997) has suggested that up to 80% of all human illness in developing world is caused by inadequate sanitation or polluted water. The problem of drinking water is responsible for more than 15 million children below five die each year duet to water borne disease like typhoid, dysentery, hepatic as well as many protozoan and helminthes infestation. Diseaes related to water, sanitation and hygiene risk factor kill more people that AIDS, Malaria or Tuberculosis (NHRC/WHO,2000).

Of all contaminants in drinking water, human and/or animal faeces, which may enter water routes through different means present the greatest danger to public health. Bacteriological testing provides a sensitive means for the detection and control of such pollution. Although modern microbiological techniques have made the detection of pathogenic bacteria, viruses and protozoa in sewage and sewage effluents possible, it is not practical to attempt to routinely isolate them from drinking water. Even during waterborne outbreaks, pathogens are greatly outnumbered by normal intestinal bacteria, which are easier to isolate and identify. The presence of non-pathogenic faecal indicator bacteria, such as *Escherichia coli*, indicates that pathogenic enteric bacteria could be present; if faecal indicator bacteria are absent, pathogenic enteric bacteria are probably also absent. The overall concepts adopted for microbiological quality is that no water intended for human consumption shall contain *E coli* in 100 ml sample. Treated water entering the distribution system should be 0 faecal coliforms and 0 coliform organisms per 100 ml of water. This study was conducted to assess the bacteriological quality of drinking water samples collected from kathmandu valley.

*Corresponding author; Email: ip1479@gmail.com

Materials and Methods

This study was conducted in Kathmandu Metropolitan city area from February 2008 to May 2008. One hundred and thirty water samples were randomly collected from four different sources viz. tap water, ground water, household filter water, bottled water, and stone spout. The samples were aseptically collected in 300ml sterile BOD bottle and transported by standard method as mentioned in APHA, 1998. The bacteriological analysis of samples was done MPN (3 sets 3 tubes method) on the same day immediately after its delivery and always within 6 hours of collection. When immediate analyses were not possible, the samples were preserved at 4^0C.

Detection of *Salmonella* and *Shigella* species were done by the enrichment of water samples on Selenite F broth, followed by isolation of the typical organism on selective medium, Xylose Lysine Deoxycholate Agar (XLD). (Collee *et al.*, 1996) All colonies with different characteristics on M-Endo agar, Xylose Lysine Deoxycholate Agar (XLD) agar were sub cultured onto Nutrient agar (NA) for purification. Enteric bacteria isolated on respective selective or differential media were identified on the basis of their colonial, morphological and Biochemical properties following Bergey's Manual of Determinative Bacteriology, 1994 (Holt et al, 1994).

Result and Discussion:

A total of 130 drinking water samples were collected randomly from different places of Kathmandu valley (tap water 40, ground water 40, household filter water 20, bottled water 20, and Stone spout 10) were examined.

The bacteriological analysis of drinking water samples revealed that 30 out of 40 (75 %) tap water , 34 out of 40 (85 %) ground water, 10 out of 2O (50%) household filter water samples, 3 out of 20 (15%) bottled water and 10 of 10 (100%) stone spout were contaminated with coliforms. Out of 97 coliform positive water samples, (53%) water samples were faecally contaminated (Table 1). The rate contamination of drinking water samples collected from different sites of Kathmandu Metropolitan city (74.61%) is in agreement with those reported earlier in Nepal; 88% in Kathmandu Valley (Adhikari *et al*, 1986) and 92.4% (Prasai *et al*. 2007).The present finding clearly indicates that alarming situation, when WHO guidelines for drinking water is taken into consideration.

Fecal pollution of drinking water introduces a variety of intestinal pathogens (WHO, 1997 and Cheesbrough, 1993). The presence of indicator organisms calls a need for further survey, investigation and examination of drinking water sources.

Table 1: Total and faecal coliforms in different water samples

Sample	*Total (n)*	*Coliform +ve (%)*	*Faecal coliform +ve (%)*
Tap water	40	30 (75)	27(67.5)
Ground water	40	34(85)	29(72.5)
Household filter water	20	10(50)	5(25)
Bottled water	20	3(15)	0(0.0)
Sprout water	10	10(100)	8(80)
Total	130	97(74.61)	69(53.07)

Out of 97 coliform positive water samples, 163 enteric bacteria were isolated and identified. The identified organism include *E.coli, Enterobacter spp, Citrobacter spp, Pseudomonas spp, Klebsiella spp, Shigella spp* and *Salmonella spp*. Among these bacteria , *E.coli* being the highest (37.42%) followed by *Enterobacter spp* (17.17%), *Citrobacter spp* (14.11%), *Pseudomonas spp* (12.26%), *Klebsiella spp* (7.97%), *Shigella spp* (6.13%) and *Salmonella spp* (4.90%)(Table 2). The results also showed that 30.06%, 38.03%, 7.36%, 1.84 % and 7.36% of isolates were obtained from water samples of Tap water, Ground water, Household filter water, Bottled water and stone spout sources (TABLE 2).

Table 2: Source wise distribution of bacterial isolates of water samples

Sample	*E.coli*	*Enterobacter spp*	*Citrobacter spp*	*Pseudomonas spp*	*Klebsiella spp*	*Shigella spp*	*Salmonella spp*	Total
Tap water	18	9	7	6	4	3	2	49(30.06%)
Ground water	22	12	9	7	5	4	3	62(38.03%)
Household filter water	8	0	2	2	0	0	0	12(7.36%)
Bottled water	2	0	0	1	0	0	0	3(1.84%)
Stone Spout	11	7	5	4	4	3	3	12(7.36%)
Total	61 (37.42%)	28 (17.17%)	23 (14.11%)	20 (12.26%)	13 (7.97%)	10 (6.13%)	8 (4.90%)	163 (100%)

The detection of pathogenic enteric bacteria in different sources of drinking water in Kathmandu Metropolitan city also reveals the alarming situation for water borne epidemics in the valley. So this finding revealed that it was unsafe for drinking mainly due to fecal contamination and hence the residents are exposed to a wide range of diseases (WHO, 1996). This is therefore can be correlated with diarrhoeal disease which occupied the second place among the top ten diseases in Nepal.(MOH, 2005) and periodic outbreak of gastroenterits including cholera particularly in rainy season (Pokharel and Thapa, 2004 and Ise *et al* ,1996) which has long been a major public health problem in Nepal. The recent outbreak of diarrheal disease in Jajarkot and Rukum strongly supported these findings.

The quality of water supply is not safe mainly because of the bacteriological i.e. faecal coliform contamination. The situation has still not improved since several years back (CBS/HMG, 2001, DWSS/UNICEF, 2000 and ENPHO, 2002). Although there were numerous activities related to safe drinking water awareness program on the part of government, these attempts have remained to be ineffective. This could be due to rapid population growth, unplanned urbanization, poverty and poor sanitation of its infrastructure. There are several reason associated with the poor quality of water delivered to the households. Not all water distributions have appropriate treatment facilities. Either, water is improperly disinfected or not disinfected at all. Because of intermittent supply and leakages, negative pressure often draws contaminated

material from the surface. Even good quality of water delivered from the source gets polluted due to infiltration of contaminated water through leakage points. The problem is worsened by the old distribution network (DWSS/UNICEF, 2000, NHRC/WHO, 2000 and Pradhan, 2000). All the natural water sources, such as wells, stone spouts and tube wells are neither treated nor protected properly. Surprisingly, even household filtered water was also unsafe for drinking .The finding indicated that level of awareness about handling of filter and collection water pot about sanitation issue among house wives needs to be improved. There contamination of drinking water has long been a major public health problem in Nepal, and it is therefore can be correlated with Diarrhoeal disease which occupied the second place among the top ten diseases in Nepal. (MOH, 2005)

The high level of *E.coli* can also be explained by the fact that poor sanitation habit and hygiene education influences the use of protected water supplies. Study conducted in Bangladesh revealed that 95% of the urban population had access to safe drinking water and 35% of the population had access to sanitation. But data on the level of hygiene education was much lower (IWSC, 1989). As a result health impact reduction in diarrhea was marginal. In addition, analysis of 144 water and sanitation projects worldwide concludes that sanitation has great impact than water supply (IWSC, 1989) Environmental sanitation is prerequisite for good health. Improved hygiene and sanitation have more impact than drinking water quality on health outcomes, specifically reductions in diarrhoea, parasitic infections, morbidity and mortality, and increases in child growth (Esrey *et al*, 1991, and Hutley *et al,* 1997).

All water sources are grossly polluted. Poor quality of drinking water increases the health risk of water use. Because of poor quality delivered to the household, drinking water quality is a major public health issue for Kathmandu valley. Diseases caused by contaminated water are the most common diseases in Nepal (HMG/DOHS, 1998). So a regular monitoring the water quality for improvement not only prevents disease and hazards but also checks the water resources from going further polluted (Trivedy and Goel, 1986).

Conclusion:

Out of 130 drinking water samples collected from Kathmandu Metropolitan City, 97 water samples were found to be bacterial contaminated. The bacteriological analysis of water samples revealed the presence of total coliform in 74.61% of samples (tap75 % water, ground water 85 %, household filter water samples 50%, bottled water15% and stone spout 100%). The detection of pathogenic enteric bacteria in different sources of drinking water clearly showed that it is major and serious threat to human health and environment.

Regular quality control mechanisms such as Conservation of water sources, proper sanitary survey, design and implementation of water and/or sanitation projects; regular disinfections, maintenances and supervisions of water sources; overhauling of water distribution system by replacing the old pipes and regular bacteriological assessment of all water sources for drinking purpose should be well planned and implemented.

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Selection of Appropriate Wine Yeast Strain for Plum Wine Fermentation

EILEEN SHRESTHA, SRISHTI RANA, KRIPA DAHAL and TIKA BAHADUR KARKI*

Department of Biotechnology, Kathmandu University (KU), Dhulikhel, Kavre, Nepal

This study was carried out to determine suitable wine yeast strains out of 42 isolated yeasts from grape, murcha and plum samples which were collected from local market of Kathmandu valley and Bardia district of Nepal. Using wine yeast differentiating media (WYDM), 21 wine yeast strains were selected among the isolated 42 yeast strains. Out of these 21 strains, 3 strains were selected as appropriate wine yeast on the basis of brix, % acidity (malic acid), pH and flavor. Plum wine prepared by inoculating those strains was found to be sensorily comparable to market wine.

Key words: Murcha, Wine yeast, Brix, pH, Malic acid, Plum wine

Introduction

Wine is simply an alcoholic beverage made from the fermented grape juice. Alcoholic beverages with characteristics flavor of the particular fruit like apple, pear, plum, apricot, cherry and mango are broadly termed as fruit wines (Joshi and Attri, 2005). Fruit wines are beverages obtained by alcoholic fermentation of fruits or of juices thereof, with an alcohol content amounting to between approximately 8-9 and 18% (v/v), and sometimes even more (Jarczyk and Wzorek, 1977). Fruit wines are made mainly out of pome, berry and stone fruits, and less frequently out of citrus and other fruits. Production of fruit wines differs essentially from that of grape wines, particularly with regard to the highly differentiated raw material, the need for sweetening and dilution of fruit musts, and the resulting differences as regards the process of fermentation and mellowing. Auxiliary agents used in the production of fruit wines include: pure yeast culture, carbon dioxide, sulphur dioxide, sulphites, filter aids, clarifying agents like bentonites, ascorbic acid, nitrogen, pectolytic preparations and other substances . Compared to grapes, the sugar content originally present in the fruits is not attractive, but the color and flavor make plums acceptable.

During wine fermentations, yeasts are responsible for the conversion of fermentable sugar into ethanol and other valuable by-products such as higher alcohols, organic acids and esters, while lactic acid bacteria convert malic acid into lactic acid and carbon dioxide by malolactic fermentation, resulting in the reduction of acidity of wine (Reed and Nagodawithana, 1991; Ayogu, 1999; Zarzoso et al., 2000).The objective of the study was to isolate appropriate wine yeasts from grape, plum and murcha and then utilize the selected strains for plum wine fermentation.

Materials and methods

Sample collection: Three samples of grapes and two samples of plums were collected from local vegetable markets within Kathmandu valley namely *Kalimati tarkari bazzar, New Baneshwore, Banepa, New Road* and *Khusibu*. Two samples of murcha were collected from *Bardia* (*Tharu* community) and *Lubhu* which were of manapu category. ***Preparation and preservation of must:*** For the fermentation purpose fresh and ripe undamaged plum were used. Plums were first washed with clean water and boiled for 15-30 minutes to make peeling easier. After that the pulp of the plums were cut out and the seeds were discarded. Then the separated pulps were placed on juicer and the juice was collected in sterile container. The extracted juice was heated at 70^0 C for 15-20 seconds and then cooled to 40^0 Celsius. For the extraction of plum juice in higher quantity pectinase enzyme (0.2%) was added and let to stand at 40^{0}C for 2 hours, and then the juice was strained with the help of muslin cloth. The clear juice was separated out and stored in refrigerated condition for 24 hours, finally to use for the fermentation purpose. ***Isolation of yeast:*** The murcha sample was well powdered by clean mortar and pestle and the fruit juice was obtained by properly blending fruits in mixer. 1gm of murcha and 1ml of fruit juice was taken as sample and YM agar was used for isolating yeasts (Refai, 1992). ***Screening of isolated yeast:*** The colonies isolated from pour plate technique in YM agar were selected and purified by streaking on YMA media incubated at 25^{0}C for 48 hours. The so obtained non-contaminated strains were checked by gram staining method for its conformation to be yeast. The preliminary screening of fermentative yeast was carried out in YM broth. Durham's tube in each of the test tubes were filled with broth and kept in inverted position and then sterilized. Yeast strains were then inoculated and the test tubes and incubated at 28^{0}C for 3 days. Then the yeasts with higher production of carbon dioxide were categorized as fermentative yeasts and those that did not produce carbon dioxide were categorized as non-fermentative yeasts. Further, only the fermentative yeasts were let to grow in wine yeast differentiating media (Lower Layer: glucose 2%, Peptone 0.2%, Yeast extract 0.1%, Potassium dihydrogen phosphate 0.1%, Magnesium sulphate heptahydrate 0.4%, Agar 3%, pH 5.5-5.7; Upper Layer Medium : glucose 0.5%, 2,3,5-tris phenyl tetrazolium chloride 0.005% and Agar 1-1.5%) .This way the yeasts could be found out

*Corresponding author, Email: tbk@wlink.com.np

whether they are wine yeasts or wild yeasts. ***Inoculation and fermentation:*** Final fermentations were carried out taking 1000ml plum juice in each of the three 2000ml volumetric flasks at 20^{0}brix and at 25^{0}C. Alcohol content was estimated using the colorimetric method (Jones and Markow, 2004) followed by the sensory evaluation. For the clarification of wines, 0.1% bentonite was used.

Results and Discussion

Isolation and Screening of wine yeast

Around 60 yeast-like colonies were isolated from grape, plum and indigenous murcha samples. Morphological characteristics of those yeasts like isolates were found to be whitish or creamy, smooth, circular or irregular, entire or lobate margin, convex or flat elevation. Out of these 60 yeast-like isolates, 42 of them were found to be gram positive with generally large oval, round and elliptical shapes. Among 42 gram positive yeast isolates, 26 had good fermentative capacity as they produced high carbon dioxide, good flavor and turbidity. These 26 fermentative yeasts were grown in WYDM using the pour plate technique and incubated at 28^{0}C for 48-72 hours. In WYDM, 7 fermentative yeasts gave pure red colonies, 13 gave mixed colonies of pink and red colonies, and 6 did not show any color. Pure red colonies refer to pure wine yeasts whereas pink indicates wild colonies. These 7 isolated wine yeasts were individually inoculated in 20^0 brix plum juices and let to stand for up to 8 days as a part of first round fermentation carried out in mini scale. Among these 7 strains, one strain each from murcha, plum and grape coded as MY 3, PY 6 and GY2 were selected after preliminary investigation based on sensory and chemical parameters. Strains coded MY3, PY6 and GY2 were recoded as WY1, WY2 and WY3 respectively. Those three strains were used for the preparation of wine.

Fermentation

The selected three wine strains were inoculated separately in 20^0 brix plum juices and the fermentation was performed at room temperature (25^{0}C) for a period of 15 days. During the course of fermentation, 3 main physico-chemical characteristics i.e. changes in pH, decrement in T.S.S (total soluble solid) or ^{0} brix and changes in % acidity (malic acid) were studied. The results for brix, pH and acidity are given in Tables 1, 2 and 3 respectively.

The brix of WY1 and WY3 reduced from 20^{0}brix to 8^0 brix, whereas in case of WY2, it reduced to 10^{0}brix only at the end of fermentation period. The standard yeast (YGRC, BY2001) reduced 20^{0}brix to 8^0 brix in 9 days of fermentation and remained constant for the remaining period of 15 days. The pH did not show any substantial change during the course of wine fermentation. The initial acidity of plum juice was 0.70 (as malic acid) ,finally reached to 0.73%, 0.64%, and 0.68% each in case of wines that had been inoculated with WY1, WY2 and WY3 wine yeast strains respectively. The brix and titrable acidity (% malic acid) obtained at final fermentation from these three wine strains are comparable with the brix of 8.0-12.0 and titrable acidity (% malic acid) of 0.62-0.88 of plum wine (Vyas and Joshi, 1982 and Joshi and Bhutani, 1990).

Table 1: Change in ^{0}Brix of plum wine fermented with different yeast strains at room temperature (25^0 C)

Isolate Code	*Fermentation time (days)*									
	0	*2*	*4*	*5*	*6*	*7*	*9*	*12*	*13*	*15*
WY1	20±0.0	18.6±0.2	18.4±0.4	18.0±0.2	17.9±0.1	17.4±0.2	14.0±0	11.0±0	9.0±0.21	8.0±0
WY2	20±0.0	19.0±0.1	18.2±0.1	18.0±0	17.2 ±0	15.0±0.3	13.0±0.3	12.0±0	11.0±0.2	10.0±0
WY3	20±0.0	18.4±0	18.0±0.2	17.0±0.2	16.0±0	15.4±0.1	12.0±0	10.0±0	9.0±0.1	8.0±0
YGRC(BY2001)	20±0.0	18.4±0	17.6±0	16.2±0.1	14.0±0	12.0±0	8.0±0.0	-	-	-

- YGRC (BY2001): *Saccharomyces cerevesiae* (standard)
- Values are the means obtained from the triplicate data

Table 2: Change in pH of plum wine fermented with different yeast strains at room temperature (25^0 C)

Isolate Code	*Fermentation time (days)*									
	0	*2*	*4*	*5*	*6*	*7*	*9*	*12*	*13*	*15*
WY1	3.3±0.0	3.3±0.2	3.2± 0.02	3.2±0	3.2±0.01	3.2±0.01	3.1± 0.2	3.3±0	3.3±0.02	3.4±0
WY2	3.3±0.0	3.2±0	3.2±0	3.2±0.02	3.2±0.2	3.1±0.01	3.3±0.02	3.3±0.02	3.3±0.03	3.3±0.1
WY3	3.3±0.0	3.2±0.03	3.2±0.1	3.2±0	3.2±0.01	3.2±0.0	3.3±0.01	3.3±0.01	3.4±0.01	3.4±0.2
YGRC(BY2001)	3.3±0.0	3.3±0.01	3.3±0.02	3.2±0.01	3.3±0	3.3±0.01	3.2±0	-	-	-

- Values are the means obtained from the triplicate data

Table 3: Change in acidity of plum wine fermented with different yeast strains at room temperature (25^0 C)

Isolate Code	*Fermentation time (days)*									
	0	*2*	*4*	*5*	*6*	*7*	*9*	*12*	*13*	*15*
WY1	0.70±0.0	0.92±0.01	1.07±0.01	0.60±0.001	0.89±0.0	0.90±0.01	0.80±0.01	0.70±0.0	0.72±0.01	0.73±0.01
WY2	0.70±0.0	0.80±0.02	0.97±0.03	0.72±0.02	0.96±0.02	0.93±0.01	0.80±0.0	0.70±0.01	0.72±0.01	0.64±0.01
WY3	0.70±0.0	1.00±0.01	1.12±0.02	0.73±0.01	0.97±0.02	1.01±0.02	1.00±0.01	0.76±0.01	0.67±0.02	0.68±0.13
YGRC(BY2001)	0.70±0.0	0.80±0.01	0.86±0.02	0.58±0.007	0.82±0.0	0.87±0.01	0.80±0.01	-	-	-

- Values are the means obtained from the triplicate data

In the initial phase of fermentation acidity was found to increase with progression of fermentation, while acidity decreased slowly that eventually increased during fermentation till it reached the stable phase. The decrease in pH was suspected to be due to the activation of bacteria that are responsible for the malolactic fermentation. The presence of malolactic bacteria was observed in plum juice with some few colonies in the selective MRS agar media which were separated with transparent circular boundaries. It did not occur in case of the plum juice that had gone under few days of fermentation while it did re-occur towards the final days of fermentation.

Alcohol estimation

The amount of alcohol content present in the so produced wines was estimated by colorimetric method. The alcohol contents (% v/v) produced by selected wine yeast strains WY1, WY2, WY3 were found to be 6.5, 4.5 and 7.0 respectively with the value of 6.0 for the standard yeast (YGRC). (Table 4). The ethanol % (v/v) in plum wine is 8.5-11.0 as reported by Joshi and Attri (2005). The plum wines produced in this work were found to be significantly low. The alcohol content in plum wine is affected by the pasteurization. In this work plum wines were pasteurized at 70°C for 15 seconds. Pasteurizing the wines at 70 or 80°C may reduce alcohol content (Gill et al, 2009).

Table 4: Alcohol Content in Plum wine fermented by different yeast strains

Strains	*Alcohol % (v/v)*
WY1	6.5±0.1
WY2	4.5±0.2
WY3	7.0±0.6
YGRC (BY2001)	6±0.5

Note: Values are the mean of three determinations

Sensory evaluation of wine

Sensory evaluation of wine prepared was done based on 3 categories: a) color b) aroma and c) flavor by the panelists of 10 judges. The mean scores for color, aroma and flavor of wine fermented by WY1, WY2 and WY3 were found to be 3.2, 3.2 & 2.9; 2.9, 3.2 & 2.8 and 3.3, 2.8 & 2.8 respectively (Table 5). The scores for commercial wine were found to be 3.57, 3.57 and 3.28 respectively.

Table 5: sensory score of Plum wine prepared by inoculating different yeast strains

Parameters	*Wine yeast strains*			
	WY1	*WY2*	*WY3*	*Market wine*
Color	3.2^a±0.78	2.9^a ±1.03	3.3^a ±0.94	3.57^a ±1.13
Aroma	3.2^a ±1.39	3.2^a ±1.03	2.8^a ±0.91	3.57^a ±1.39
Flavor	2.9^a ±1.19	2.8^a ±1.31	2.8^a ±1.03	3.28^a ±1.1

Note: 'a' indicates that mean values for the color, aroma and flavor are not significantly (P=0.05) different

It was found that wine prepared by inoculating different strains of yeast (WY1, WY2 and WY3) were not significantly different (P=0.05) to each other and were comparable to market wine for all of the sensory parameters that is color, flavor and aroma. It shows that the quality of wine prepared in this study was comparable to the quality of market wine.

Conclusions

Plum wine prepared by inoculating three yeast strains isolated from murcha, grape and plum was found to be sensorily comparable to market wine. The present study may help to select wine yeast strains for the development and promotion of winery from various fruit substrate. These yeast isolates may need further study at genetic level for proper taxonomic identification. Additional studies are also needed on flocculation test, hydrogen sulphide production and yeasts that are tolerant to high ethanol concentration for enhancing alcohol content of wine.

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Clostridium perfringens in Meat Products Collected from Kathmandu Valley

VIJAY SINGH CHHETRI*, INDRA BAHADUR PACHHAI and TIKA BAHADUR KARKI

Department of Microbiology, National Institute of Science and Technology (NIST), Tribhuvan University, Kathmandu, Nepal

A study was carried out to examine Clostridium perfringens in meat products. The total C. perfringens was determined by plating serially diluted sample on the Tryptose Sulfite Cycloserine Agar (TSC Agar) followed by anaerobic incubation at 37° C for 48 hrs. A total of 43 meat products (17 sausages, 13 buff chhoyela and 13 Kachila) were examined for C. perfringens contamination. The present study showed that 69.23% chhoyela with highest count 1.17 x 10^4 CFU/g, 88.23% sausages with highest count 2.4x 10^6 CFU/g and 100% Kachila with highest count 3.4 x 10^6 CFU/g were found to be contaminated with C. perfringens. Among contaminated samples, 5% were found to be containing C. perfringens load that meets infectious dose. High incidence of contaminated samples with high count of C. perfringens indicates the poor sanitary practices in meat industries; so, the food borne diseases by C. perfringens may be highly prevalent in the Kathmandu valley.

Key words: *Clostridium perfringens, Sausages, Kachila, Chhoyela*, Kathmandu valley

Introduction

Clostridium perfringens is a contaminant in meat and poultry products and continues to be a major concern to the food Industries (Smith and Schaffner, 2004). *C. perfringens* type A food poisoning ranks as the third most commonly identified food borne illness in United States (Wen and McClane, 2004). It was estimated that in England and Wales in 2000, *C. perfringens* was second to campylobacter in terms of number of cases of bacterial food borne illness and secondly only to salmonellas in number of deaths associated with food borne bacteria (Adak *et al.*, 2002). The typical pathogenesis of *C. perfringens* involves survival of spores in the food due to inadequate cooking or incomplete warming of the food. They then rapidly multiply in the food and are consumed with the contaminated food. When the food vehicle is sufficiently contaminated, these vegetative cells survive the stomach acidity and enter the small intestine where they multiply and sporulate releasing CPE, which is the cause of most of the symptoms of food poisoning (McClane, 2001). *Kachila* is spicy marinated and raw minced meat and *Chhoyela* is spicy marinated and roasted meat item popular in the Kathmandu valley. The present study was carried out to assess the occurrence of *C. perfringens* in Sausage, *Kachila, Chhoyela.*

Materials and method

Collection of Samples: The study was conducted in Kathmandu valley, Nepal, from 2007 September to 2008 January. A total of 43 meat samples (17 sausages, 13 *buff chhoyela* and 13 *Kachila*) were collected aseptically from the retail shops and Restaurants. The samples were then transported to the laboratory in ice box for processing within 2-3 hours.

Samples processing and preparation: Twenty five gram of each sample was homogenized with 225 ml 0.1% buffered peptone water (BPW) using sterile scissors and blender. The homogenized samples were serially diluted in BPW. One milliliter from representative dilution was placed on Petriplates containing a thin layer of Tryptose Sulfite Cycloserine Agar without egg yolk (TSC, Hi- Media, India) and subsequently mixed with 15 ml of TSC agar. After solidification an additional 5 ml of TSC agar was overlaid (Velugoti et al., 2007). The plates were incubated at 37°C for 24- 48 hrs in an anaerobic condition (Anaerobic jar, Hi-m Media, India). Ten presumptive *C. perfringens* isolates from each sample were confirmed as Gram-positive, non-motile rods, positive for nitrate reduction test, lactose fermentation and gelatin liquefaction in motility-nitrate and lactose gelatin media (Labbe and Harmon, 1992).

Result and Discussion

Among three meat products, *Kachila* was found to be highly contaminated (100%) with *C. perfringens* with highest count 3.4x 10^6 CFU/g that meets infectious dose as specified by USDA/FSIS (Chhetri, 2008). Similarly 88.23% sausage and 69.23% *chhoyela* were found to be contaminated with *C. perfringens* (Table 1). A study (Shrestha and Rao, 2007) of same types of products did not identify the presence of *C. perfringens* isolates. The present study was revisited this issue using revised methodology.

***Corresponding author;** Email:chhetrivijay@gmail.com

Table: 1: C. *perfringens* in meat product

Meat Product	*No. of samples*	*Positive samples*	*Positive percentage (%)*	*High count (CFU/g)*
Sausage	17	15	88.23	2.4×10^{6}
Chhoyela	13	9	69.23	1.17×10^{4}
Kachila	13	13	100	3.4×10^{6}

The contamination that meets the infectious dose, >10^{6} CFU/g (Chhetri, 2008) was found in 5% meat products. Similarly 81% meat products were found to have count less than infectious dose and 14% revealed negative result (Fig. 1). Average of 43.1% samples among total raw, unprocessed beef, veal, lamb, pork, or chicken and processed meats and meat dishes showed positive for *C. perfringens* with highest occurrence (82%) in veal cuts (Hall and Angelotti, 1965). Singh *et al.*, 2005 reported occurrence of *C. perfringens* in poultry (70.40%) and buffalo meat (65.70%) conducted in Bareilly city, India.

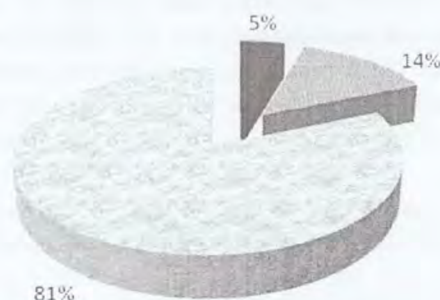

Fig 1: *Clostridium perfringens* load in meat products

Kachila is spicy marinated and raw minced meat and *chhoyela* is spicy marinated and roasted meat item popular in the kathmandu valley. The high level of contamination (up to 100%) with *C. perfringens* meeting infectious dose in more than 4% samples exhibits terrifying condition in meat sector. Besides the unhygienic processing steps, incorporation of other ingredients like spices may also cause contamination. Spices may have big role to contaminate with *C. perfringens*. Sarkar and Banerjee, 2003 reported 59% contamination with *C. perfringens* out of 27 of meat spices. Similarly Aguilera *et al.*, 2005 isolated and characterized a total of 12.17% C. *perfringens* type A strains from spices samples and among which 28.60% turned out to be enterotoxigenic.

All the isolates that are cpe positive carry one single copy of the cpe gene either on the chromosome or on a plasmid (Cornillot *et al.*, 1995). *C. perfringens* carrying enterotoxin gene (cpe) is necessary for food poisoning. (Wen and McClane, 2004). It was found that 1.4% surveyed non outbreak American retail foods were positive for *C. perfringens* type A carrying cpe gene (Wen and McClane., 2004). Approximately 70% of the Japanese retail raw meat samples were contaminated with *C. perfringens* bacteria and 4% were contaminated with cpe-positive *C. perfringens* (Miki *et al.*, 2008).

The typical pathogenesis of *C. perfringens* involves survival of spores in the food due to inadequate cooking or incomplete warming of the food. They then rapidly multiply in the food and are consumed with the contaminated food. When the food vehicle is sufficiently contaminated, these vegetative cells survive the stomach acidity and enter the small intestine where they multiply and sporulate releasing CPE, which is the cause of most of the symptoms of food poisoning (McClane, 2001).

The result suggests that sanitary conditions in different production stages of meat items must be improved to reduce or control health hazards. These results exhibit an urgent need to make proper legislation and its strict implementation to avoid problem by consumption of such types of products. Another study is very essential to confirm enterotoxigenic types of the isolates and current prevalence of food poisoning by this organism.

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Preparation of *Sinki* as an Intermediate Moisture Food and Its Quality Evaluation

PRAVIN OJHA* and SURENDRA BAHADUR KATAWAL

Central Department of Food Technology (CDFT), Central Campus of Technology (CCT), Hattisar, Dharan, Nepal

The present work was focused on developing intermediate moisture Sinki. Sinki prepared by fermenting radish at temperature of 26.7°C and relative humidity 70.5% for 8 days was found to develop acidity of about 12% (db). Steaming for 20 minutes was found to reduce acidity and volatile acidity in Sinki. Steamed Sinki was dried to 20%, 30%, 40%and 50% moisture content, product having 50% moisture content was found to be sensorily superior ($p<0.05$). Sinki was then dried to 50% moisture content at 40°C, 50°C and 60°C, product dried at 60°C was found to be sensorily superior ($p<0.05$). Water activity of Intermediate moisture Sinki thus produced with 50% moisture content was found to be 0.8.

Keywords: *Sinki,* Intermediate moisture food, Steaming, Drying, Water activity

Introduction

Sinki is one of the most prized indigenous fermented foods of Nepal. *Sinki* is a traditional, Nepalese non-salted vegetable food product prepared by spontaneous lactic fermentation of radish (tap roots). *Sinki* is believed to have existed in Nepalese culture since time immemorial. It is valued for its uniquely appetizing flavor (Steinkraus, 1996). Quality of *Sinki* was judged on basis of acidic taste and typical *Sinki* flavor. These two characteristics have been embodied as key indicator of quality (Shrestha, 2005). However, the final acidity ultimately depends on the indigenous flora present in vegetables (Shrestha, 2002). The water activity of food influences the multiplication and metabolic activity of microorganisms, also their survival and resistance (William, 1976). According to Davies, 1976, water activity must be approximately from 0.6 to 0.84 for intermediate moisture food. An inhibition of microorganisms in IMF cannot solely depend on water activity, but also on the pH, Eh, temperature, preservatives etc. If the palatability of product permits, the water activity of IMF should be below 0.85 or the pH below 5.0, since either of these *hurdles* protects the product against staphylococcal enterotoxin production (Leistner and Rodel, 1976).

Sinki is prepared from surplus radish or radish, which had lost its freshness in village and some part of urban region. Despite of its good nutritional source, urban class suspects its hygienic quality because of methodology adapted and due to its appearance. The art of traditional processes need to be refined to incorporate objectives methods of process control and to standardize quality of the final product without losing their desirable attributes such as improved keeping quality, taste and nutritional quality. According to Anon, 1996, the food and agricultural organization of United Nations has stated that value added through marketing and processing raw products can be much greater than the value of primary production. Generally, villagers produce *Sinki* from surplus radish. If this surplus radish could be utilized to produce pickle from *Sinki* by using available technology in hygienic way than low value radish could be converted to high value product. This research may also lead a way in formulating other product from *Sinki*. The present study focused on effort to utilize surplus radish to modify traditional technology and to develop product to increase value of raw materials.

Materials and methods

Preparation of Sinki: Freshly harvested radishes were bought from local market of Dharan. Radishes were cleaned and were cut into size of length 3 inch to 4 inch and breadth of 0.5 inch. Fragmented radishes were then dried in cabinet dryer for 6 hours at temperature 33°C (±2°C) and relative humidity was 43.8% (±2.8%). Around, 400-450 gm of wilted radish thus obtained were tamped gently and uniformly (placing little amount at a time) in plastic container. The headspace allowed was around 5 cm and pounding from top was achieved by pressing with salted water over plastic and capped to maintain anaerobic condition. *Sinki* was fermented at constant temperature 26.7°C (±1°C) and relative humidity 70.5% (±0.6%) for 8 days as described by Shrestha, 2005. The acidity developed was found to be 12% (db). *Steaming of Sinki:* The prepared *Sinki* was steamed at *momo* cooking utensils for 20 min. Moisture content, acidity and lactic acid bacteria count before and after steaming was carried out to access effect of steaming. *Optimization of moisture content of Sinki:* Steamed *Sinki* was dried at 40°C to 50%, 40%, 30% and 20% moisture content. *Optimization of drying temperature of Sinki: Sinki* was then dried to optimized moisture content at 60°C, 50°C and 40°C.

*Corresponding author: Present Address: National College of Food Technology, Khusibu, Kathmandu, Nepal.
Email: projha84@yahoo.com

Analytical method: Moisture, protein, fat, ash, crude fiber, acidity, fixed acidity and volatile acidity were analyzed as per the method of Ranganna, 2002. Iron and calcium were analyzed as per the method of KC and Rai, 2007. Water activity was measured by using Powkit water activity meter (Decagon). pH was measured by using glass electrode pH meter. LAB count was carried out according to Harrigan and Margret, 1979. Sensory evaluation was carried out as per the method described by Linda, 1984. Data analysis was carried out according to Gomez and Gomez, 1983 and Gupta, 2005.

Results and discussion

Effect of steaming on Sinki

When *Sinki* was steamed for 20 min, moisture content was found to be significantly increased whereas LAB counts, acidity, volatile acidity and fixed acidity was found to be significantly decreased ($p<0.05$) (Table 1).

Table 1: Effect of steaming on *Sinki*

Parameter	*Fresh Sinki*	*Steamed Sinki*
Moisture (%)	85.4±0.8^{a}	88.0±1.1^{b}
LAB count (log CFU/g)	5.1±3.2^{a}	3.2±0.8^{b}
pH	3.5±0^{a}	3.5±0^{a}
Acidity (% db) as lactic	12.0±0.1^{a}	10.1±0.1^{b}
Fixed Acidity (% db) as lactic	4.6±0.1^{a}	4.3±0.1^{b}
Volatile Acidity (% db) as lactic	7.4±0.1^{a}	5.7±0.1^{b}

- Values are the mean of three determinations ± standard deviation and different letters in superscripts (a, b) in the same row indicates significantly ($p<0.05$) different.

The moisture uptake might be due to continuous exposure of *Sinki* to steam and absorption of moisture by *Sinki*. Volatile acidity was significantly ($p<0.05$) decreased during steaming. The possible reasons might be due to loss of volatiles compound with steam. Similarly, fixed acidity was also significantly ($p<0.05$) reduced which might be due to loss of soluble compound with condensed water through *Sinki* into boiling water. There was also significant ($p<0.05$) reduction of total acidity but no difference in pH value during Steaming.

Effect of moisture on sensory score of Sinki

Sinki was dried at 40°C to various moisture content i.e. (50%, 40%, 30% and 20%), and effect of variation on moisture on sensory attributes of *Sinki* was studied. It was found that there was no significant difference in color ($p<0.05$) between 50% and 40% moisture content and between 30% and 20% moisture content. But other sensory attributes were significantly ($p<0.05$) different between each other (Table 2). The average value of sensory score for flavor was found to be greater for 50% moisture content and least for 20% moisture content as shown in Table 2. According to Desroisier and Desroisier, 1978, longer the drying period, the more the pigments will be altered. So to dry to lower moisture content, it takes more time resulting in more alteration on pigment. Thus, higher the moisture content better may be retention of color. The variation on sensory score of sensory attributes may be due to variation in moisture content. The extent of volatiles loss depends on temperature, moisture content and degree of exposure (Fellows, 2000).

Table 2: Effect of moisture on sensory attributes of *Sinki*

Parameter	*50% Moisture content Sinki*	*40% Moisture content Sinki*	*30% Moisture content Sinki*	*20% Moisture content Sinki*
Color	7.6±0.5^{a}	7.0±0.3^{a}	5.3±0.3^{b}	4.4±0.3^{b}
Flavor	8.0±0.7^{a}	7.3±0.4^{b}	6.7±0.4^{c}	6.3±0.3^{c}
Texture	7.7±0.6^{a}	6.9±0.3^{a}	5.0±0.5^{b}	3.9±0.3^{c}
Taste	7.3±0.5^{a}	6.7±0.4^{b}	6.5±0.4^{bc}	6.1±0.6^{c}
Overall Acceptance	8.0±0.6^{a}	7.1±0.3^{b}	4.9±0.3^{c}	4.5±0.4^{c}

- Values are the mean of three determinations ± standard deviation and different letters in superscripts (a, b, c) in the same row indicates significantly ($p<0.05$) different.

The average value of sensory score for texture was also found to be higher for 50% moisture content sample and least for 20% moisture content sample. Texture of fruits and vegetable becomes soggy as the cell wall breaks down and the cell loose water (Potter and Hotchkiss, 1996). The texture of food is a natural attribute, and does not remain constant. Water changes play a major role. Texture becomes dry, tough and chewy when cell lose water (Potter and Hotchkiss, 1996). According to Herpich a*et al*., 2004, product water status, cell wall physical properties and cell wall interdependently determine tensile strength, firmness and elasticity, which all together characterize texture and there is also significant correlation with both water status factors for radish tubers.

Flavor factors include both taste and odor; sweet, salty, sour, acidic etc (Potter and Hotchkiss, 1996). So taste factor might be influenced by flavor attribute. The average value of sensory score for overall acceptance was found to be significantly ($p<0.05$) superior for 50% moisture content *Sinki*.

Effect of temperature on sensory score of *Sinki*

After the optimization of moisture content, the sample was then subjected to optimize temperature at various levels of 60°C, 50°C and 40°C. The variation on sensory score due to variation in drying temperature is shown in Table 3.

Table 3: Effect of drying temperature on sensory attributes of *Sinki*

Parameter	*Sample dried at 60°C*	*Sample dried at 50°C*	*Sample dried at 40°C*
Color	7.5±0.5^{a}	7.1±0.3^{a}	7.1±0.3^{a}
Flavor	7.1±0.21^{a}	6.8±0.3^{b}	6.7±0.2^{b}
Texture	7.7±0.6^{a}	7.5±0.5^{a}	7.5±0.6^{a}
Taste	6.8±0.3^{a}	6.7±0.4^{a}	6.7±0.4^{a}
Overall Acceptance	7.7±0.6^{a}	7.1±0.4^{b}	7.1±0.3^{b}

- Values are the mean of three determinations ± standard deviation and different letters in superscripts (a, b) in the same row indicates significantly ($p<0.05$) different.

The average sensory score was found to be greater for sample dried at 60°C and least for sample dried at 40°C (Table 3). According to Desroisier and Desroisier, 1978, longer the drying period, the more the pigments will be altered. So to dry to 50% moisture content, it takes more time at lower temperature resulting in more altering in pigment. It was found that there was no effect on texture due to variation in drying temperature (Table 3). According to Herpich *et al.*, 2004, there was no significant difference in texture of radish on drying at temperature up to 70°C whereas; there was significant effect on texture of carrot on drying to 70°C than at lower temperature. It was found that there was no effect on taste due to variation in drying temperature (Table 3). As taste was related to flavor, the difference in flavor might have affected by odor. The average sensory score was found to be greater for sample dried at 60°C (Table 3). The variation in result might be due to the cumulative effect of other attributes.

Chemical analysis of Intermediate Moisture *Sinki*

Sinki dried at 60°C to 50% moisture content so called intermediate moisture *Sinki* was subjected for chemical analysis and the result is presented in Table 4.

Table 4: Chemical Analysis of Intermediate Moisture *Sinki*

Parameter	*Value*
Moisture content (%)	50.0±1.1
Water activity	0.8±0
Ash % (db)	13.6±0.5
Acidity % (db as lactic)	13.0±0.6
Crude fiber % (db)	52.8±0.9
Protein % (db)	1.8±0.1
Fat % (db)	1.8±0.1
Calcium (mg/100g *Sinki*)	785±12
Iron (mg/100g *Sinki*)	96±4

- Values are the mean of three determinations ± standard deviation

Water activity of *Sinki* at 50% moisture level was found to be 0.8 which showed the property of intermediate moisture food according to Davies et al., 1976 who stated that water activity must be approximately from 0.6 to 0.84 for intermediate moisture food. Acidity of intermediate moisture *Sinki* (13.0% db as lactic) was found to be greater than that of fresh *Sinki* (12.0% db as lactic). The increase in acidity might be due to incomplete inhibition of lactic acid bacteria during steaming. Other values of chemical parameters such as ash, crude fiber, protein, fat, calcium and iron were found to be similar to that of fresh *Sinki* prepared by Shrestha, 2005.

Conclusion

The study revealed that fresh Sinki with about 85 % moisture content when dried at 60°C to 50% moisture after steaming for 20 minutes showed the property of intermediate moisture food having water activity of 0.8. Intermediate moisture *Sinki* thus produced was found to retain better texture and appearance and can be used for formulation of ready-to-eat pickle.

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Microbial Quality of Jar Water Sold in Kathmandu Valley

PASHUPATI BHANDARI[1,*], SANTOSH KHANAL[1], MINU MITTLE[2], MALVIKA GUPTA[2], REENA MAHARJAN[2], RAHISHA MAHARJAN[2], RUBINA SHAKYA[2] and DEV RAJ JOSHI[1]

[1] Central Department of Microbiology, Tribhuvan University, Kathmandu, Nepal
[2] Department of Microbiology, Saint Xavier Campus, Kathmandu, Nepal

To assess the possible risks from the consumption of jar water, a survey study of the microbiological quality of bottled jar water was conducted. Randomly 30 different samples of jar water with 15 different brands were collected and examined for total bacterial count, total coliform count, Salmonella spp and Vibrio cholerae. All samples were found to contain total bacterial and total coliforms count exceeding WHO guidelines. The identified isolates included E. coli (36.5%), followed by Citrobacter spp (13.5%), Salmonella spp (12.1%) Klebsiella spp (10.8%), Enterobacter spp (8.1%), Proteus spp (8.1%), Shigella spp (6.7%), Vibrio cholerae (2.7%) and Erwinia spp (1.3%). The finding of this study indicated that jar water sold in Kathmandu is contaminated with high load of enteric bacteria and Vibrio cholerae in water is alarming and concern from public health point of view.

Key Words: Water, Microbial quality, Coliforms, Enteric pathogens

Introduction

Safe drinking water is essential to health and is equal right of all. The WHO estimates that over 1.1 billion population in developing world lack access to improved water supplies (WHO, 2006). The lack of proper purification and sanitation of drinking water in the developing countries leads to the scarcity of safe drinking water among one-third populations along with the increased prevalence of water borne disease, diarrhea being the major cause for death mostly among the children under the age of five years (WHO, 2006)

Water, though is an absolute necessity for life, can also be a carrier of many water borne disease such typhoid, cholera, hepatitis, dysentery and other diarrhea related diseases. The disease caused by contaminated water is among top ten most prevalent diseases in Nepal (DoHS, 2005). Diarrheal illness causes 44,000 childhood deaths/year in Nepal making water borne disease the leading cause of childhood death. The reported cases of water borne communicable disease in Nepal were typhoid (2,15,191), diarrheal disease (9,21,901), intestinal worms (6,11,072) and jaundice and infectious hepatitis (25,686) (DoHS 2006). It is more difficult to manage packaged drinking water because it is stored in plastic container for longer periods that may be reused without adequate cleaning or sterilization (ICPS, 2000)

The microbial quality of bottled water is of great interest as many consumers use it as an alternative to municipal water and consider it to be better and safer (Mavridou, 1992). Most of the people have attraction towards jar water as a drinking water source, may be, due to its taste, convenience or fashion, influence from foreigners and international media, awareness to safety and potential health benefits (Gleick, 2004; WHO, 2006), and scarcity of adequate public water supplies as well as chemically unfit underground water to drink in some regions. Fourty eight packaged water industries are registered in and licensed by Department of Food Technology and Quality Control (DFTQC) producing sixty five brands of packaged or bottled water that are available in local market of Kathmandu valley (DFTQC, 2005/2006). Ideally, drinking water should not contain any microorganisms known to be pathogenic or any bacteria indicative of faecal pollution. Detection of faecal indicator bacteria in drinking water provides a very sensitive method of quality assessment and it is not possible to examine water for every possible pathogen that might be present (WHO, 1993).This study has been conducted to assess the microbial quality of commercially available drinking jar water available in Kathmandu.

Materials and Methods

Thirty samples from 15 different brands were randomly collected from the six different sites (*Chabahil, Dhumbarahi, Kalanki, Lazimpat, Nardevi*, and *New Baneshwor*) of Kahmandu from August 2008 to September 2008. Samples were stored at 4°C in case of any delay in analyzing and were analyzed in microbiology laboratory of National College, Lainchour, Kathmandu, Nepal. Labels on jars were noted for its brand names and expiry date. Any leaked and or expired samples were rejected.

The total plate count was done by pour plate technique on plate count agar (PCA) and counting the colonies developed

* Corresponding author, Email: bhandari97@yahoo.com

after the incubation at 37°C for 24 hours (APHA, 1998). The total coliforms were enumerated by the membrane filtration (MF) technique as described by APHA, 1998. Detection of *Salmonella* and *Shigella* species were done by the enrichment of water samples on Selenite F broth, followed by isolation of the typical organism on selective medium, Xylose Lysine Deoxycholate Agar (XLD) (Collee *et al.*, 1996). Detection of *Vibrio cholerae* was done by enriching the samples in 1% alkaline peptone water for 6 to 8 hours followed by isolation on Thiosulphate Citrate Bile salt sucrose (TCBS) agar medium (Collee *et al.*, 1996). All colonies with different characteristics on M-Endo agar, Xylose Lysine Deoxycholate Agar (XLD) agar and Thiosulphate Citrate Bile salt sucrose Agar (TCBS) were sub-cultured onto Nutrient agar (NA) for purification. Entericbacteria isolated on respective selective or differential media were identified on the basis of their colonial, morphological and Biochemical properties following Bergey's Manual of Determinative Bacteriology, 1994. (Holt et al, 1994).

Results and Discussion

Among the 30 samples tested, all of them were contaminated with bacteria as heterotrophic count was detected >10 in each sample. Similarly, coliform bacteria were also detected in all the 30 samples and have crossed the WHO guideline value (0 cfu/100 ml). All the five samples form *New Baneshwor* and *Nardevi* had bacterial count >300 cfu/ml but samples from *Chabahil* and *Dhumbarahi* have exceeded same value in only three samples. Bottled water is generally of good quality for drinking, but if not properly protected during bottling and transit, could be a subject of contamination (Mavridou, 1992). High bacterial contamination in water indicates poor sanitary condition of water sources and failure of the disinfection of raw water in the treatment plant (Gautam *et al.,* 2007). Although HPC bacteria have been considered harmless, epidemiological studies conducted in countries such as Canada and the USA suggested the potential health risk associated with HPC bacteria present in drinking water, which comply to water quality standards (Payment et al., 1994;).

Table 1: Bacterial count of different microbial parameters in different sampling sites

Sites	*No of samples*	*Parameter*	*ND*	*< 10*	*10-300*	*> 300*	*Substandard samples*
Chabahil (1)	5	TPC	0	0	2	3	5
		CC	0	0	3	2	5
		FC	2	0	3	0	3
Dhumbarahi (2)	5	TPC	0	0	2	3	5
		CC	0	0	2	3	5
		FC	1	0	2	2	4
Kalanki (3)	5	TPC	0	0	1	4	5
		CC	0	0	2	3	5
		FC	0	0	3	2	5
Lazimpat (4)	5	TPC	0	0	1	4	5
		CC	0	0	1	4	5
		FC	1	0	1	3	4
Nardevi (5)	5	TPC	0	0	0	5	5
		CC	0	0	1	4	5
		FC	1	0	1	3	4
New Baneshwor(6)	5	TPC	0	0	0	5	5
		CC	0	0	1	4	5
		FC	2	0	1	2	3
Overall	30	TPC	0	0	6	24	30
		CC	0	0	10	20	30
		FC	7	0	11	12	23

ND: Not detected
Values in the parentheses indicates number assigned for sampling site

Table 2: Bacteria isolated from different sampling locations

	Site 1	*Site 2*	*Site 3*	*Site 4*	*Site 5*	*Site 6*	*Total*
E. coli	3	2	8	2	6	7	28
Citrobacter spp	1	0	4	2	3	0	10
Salmonella spp	2	2	0	1	3	2	9
Klebsiella spp	0	0	1	4	0	3	8
Enterobacter spp	2	0	0	1	3	0	6
Proteus spp	1	1	2	0	1	1	6
Vibrio spp	2	0	0	0	0	0	5
Shigella spp	0	1	1	0	0	0	2

Total colifrorm load in *Lazimpat, Nardevi* and *New Baneshwor* jar water samples was found relatively in higher number. Fecal coliform count was above the WHO guideline value in the samples of all sampling sites and was detected in about 77% of total samples. Detection of feacal coliform is more important than total coliform because their sources are feaces of the humans and animals. Collee *et al.*, 1996 suggested that presence of high proportion of coliform in water samples when coupled with detection of *E. coli* is a matter of concern, since *E. coli* is an enteric pathogen responsible for gastroenteritis in human. Though, *E. coli* is normally a harmless commensal, it may cause gastro-intestinal disease ranging in severity from mild, self-limiting diarrhoea to haemorrhagic colitis.

In the study, 82 bacteria were isolated, and on the basis of morphological, cultural and biochemical characteristics only 74 were identified. Among the identified isolates, *E. coli* (37.8%) was found to be predominant followed by *Citrobacter* spp (13.5%), *Salmonella* spp (12.1%) *Klebsiella* spp (10.8%), *Enterobacter* spp (8.1%), *Proteus* spp (8.1%) *Shigella* spp (6.7%), *Vibrio cholerae* (2.7%). E. coli was isolated in higher number from jar water samples of *Kalanki, Nardevi* and *New Baneshwor* than other sites, whereas *Shigella spp* was isolated only from Dhumbarahi and Kalanki water samples and in least number.

In *Chabahil*, jar water samples six different types of bacteria were isolated. More importantly, some of them were *Salmonella* spp, *Vibrio* spp and *E. coli. Salmonella spp* was isolated from all sites water sample except from *Kalanki.* Drinking of Salmonellae contaminated water can cause enteric fever, gastroenteritis food poisoning, bacteraemia etc (Greenwood *et al*, 2008). *Vibrio cholerae* can cause abdominal cramps, profuse and watery colorless diarrhea with excessive loss of electrolytes from the body (Greenwood *et al.*, 2008). When *Vibrio cholerae* is present in water bodies it is potentially able to cause the epidemic in the community using the same water (Madigan *et al.,* 1997). Isolation of pathogens like Salmonellae and *Vibrio spp* in water is the matter of concern for the authorities and industries to maintain the recommended quality and for public to be aware of such quality of drinking water.

Conclusion

Microbiological quality of jar water sold in Kathmandu valley was found to be highly contaminated with enteric bacteria and *Vibrio cholerae* which shows the alarming situation and serious concern from public health point of view. Proper treatment of water, management of packaging system, use of clean plastic container, preservatives is necessary for quality assurance. Similarly, public awareness, media communication and proper inspection of the water quality by the authorities are helpful in maintaining the water quality.

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Relation Between Health and Food: View Point of Commonsense

UMESH SHRESTHA*

Nature Lovers' Group, Nirogadham, Satdobato, Lalitpur, Nepal

If health is our prime concern we should know about various food items wholly. To know wholly there is no other way than to use commonsense. The commonsense, based on natural laws and natural instincts, analyzes and realizes reality of own self wholly. Tools of the commonsense are our five sense organs and the special, sixth sense, the mind. By using our commonsense it becomes clear to us that 1) the assumption of daily requirement of certain amount of various nutrients is wrong, 2) the result of taking food is not dependent on calorie value of the food taken but on the status of the body of the person who takes the food and 3) heavy food items generate dullness and heaviness; light food items generate feeling of energetic-ness and lightness and, in case of sickness, not to take any food items generates comfort.

Keywords: commonsense, microanalysis, clearing value of food, law of 'Three Equal', experiments by self

Introduction

Health and Food is directly interrelated. But nowadays, it seems that, direct and actual relation between Health and Food is ignored. Even when relation between Health and Food is accepted, that is only in terms of nutritional components derived from microanalysis of foods. It is a simple truth that Health is not only related to nutritional components of a food, but the Whole Food. If we stress only nutritional components (percentage of carbohydrate, protein, fat, minerals and vitamins) of a food, it will be fragmented point of view. And, it is a blunder mistake to adopt fragmented point of view.

Food Science has analyzed chemical composition and nutritional value of almost all of food items long ago (Shrestha, 2008a). Nowadays, it seems that practice of Food Science is concentrated only on taste, flavor and nutritional value. This is the so-called modern trend. And, this trend is far away from people's Health concerns. And it is obviously a deviation from the purpose of a Food. Correction of this deviational trend in Food Science has been urgency. Until and unless this deviation in Food Science is corrected no one can prevent health hazards of modern society.

Certainly, microanalysis of food items is noteworthy work, but if we have to realize real connection between Food and Health, we must use our commonsense. The view point based only on microanalysis of food items is always misleading (Shrestha, 2008a) May be, it is because of fragmented view point of microanalysis or incompleteness of microanalysis. Even though micro-analysis of almost all of food items has been done, this is a fact that, it is still incomplete and perhaps, it may never be completed. So importance of the commonsense remains forever.

*Corresponding author; Email: ushrestha@gmail.com

What is Commonsense?

Human being has five sense organs, namely eye, nose, ear, tongue and skin. Apart from these five sense organs, there is one special sense organ, which is mind. Mind is invisible but the work of mind is visible. Mind analyzes information from five sense organs and senses or information from within own body and realize the reality. The decision-making power that comes from analysis and realization of reality is the commonsense. In another word the commonsense is the combined senses of five sense organs and Sixth Sense organ that is mind. Commonsense and science are not contradictory things; rather these are the same things. The base of commonsense is science and science is the knowledge of natural laws; in short, commonsense is based on natural laws and natural instincts. Purpose of human commonsense is to safeguard own self, that is own body. Commonsense analyzes and realizes reality of own self wholly.

How to use Commonsense?

In relation to Health and Food, use of commonsense is, perhaps, the simplest work in the world. Except human being, all living beings of this world are doing this work easily (Shrestha, 2008a) These living beings ever did perform microanalysis of food items but they do eat only those which fit for their body and health and, they remain always healthy. This is the fact of the Nature. Human being should take lesson from this fact. It is good to do microanalysis of food items but to loose the commonsense in the name of microanalysis is wrong. The result of microanalysis should be used with support of commonsense and should be defined with holistic viewpoint to know the actual relation between Food and Health. Indeed, to use holistic point of view is to use the commonsense.

Body of human is as complex as that of any living beings. Till now we don't know thoroughly about the life force that regulates body of all beings. Life force or body itself regulates

billion and billion of cells in their work continuously and simultaneously. Until now there is no any equipment to observe so many kinds of functions of body and their effects at the same time (Shrestha, 2008b) and it seems that mankind never sees that kind of equipment. All sophisticated observation and diagnostic equipments are very limited in their capacity. Thus, if we rely only on these equipments, our observation will be limited and fragmented, and our conclusion based on limited and fragmented observation will be wrong. This is the practice what is happening nowadays in the name of science and technology in relation to human body and health. Contemporary food science and technology is no exception. To know actual relation between Food and Health, this problem should be solved. And, being the circumstances as it is, it seems that only our commonsense can solve this problem.

Only our commonsense can analyze outer and inner circumstances of our body simultaneously and observe the body as a whole unit. Without observing the body as a whole unit we can't know actual relation between any food and the body, that is, relation between Food and Health. If we use view point of taste bud (tongue), ignoring commonsense, and take food accordingly, that may hamper our stomach and whole body. That is, we should use not only the sense of taste bud but also sense of all sense organs, including the Sixth Sense, simultaneously. This is the method how the commonsense is used.

Food and Health, Relation — 1

What is the purpose of a food? We have been told that the purpose of a food is to nourish our body. According to this view point we have been told to take certain amount of carbohydrate, protein, fat, minerals and vitamins daily. According to this view point, based on microanalysis of food items and human body, 'balanced' diet charts are used all over the world. This fashion is rampant in the field of modern medical treatment and science of nutrition. But what is the result?

The result is open in front of us– this fashion is not working to make people healthy, instead, diseases like high blood pressure, heart disease, diabetes, cancer, arthritis, respiratory problems and various kinds of bowel problems are becoming also a fashion among the present day people. This is the reality of present day world. This reality openly and loudly reveals that assumption of so-called 'balanced' diet is wrong. Certainly, body needs these nutrients in different amounts but it dose not mean that we should take fixed amounts of these nutrients (so-called balanced diet) daily. Body needs food but assumption of daily requirement of fixed amount of various nutrients is wrong. This is the Relation 1 - Food and Health. We can understand this relation by using our commonsense. For example, animals in the nature do not take various kinds of food items combined. Human beings are also creation of the nature, so to make theory and chart of 'combined balanced' diet is to neglect our commonsense.

Food and Health, Relation — 2

'Balanced' diet charts used nowadays are also based on microanalysis of food items according to calorie value. It is assumed that body needs certain amount of calories daily and certain nutrient generates a fixed amount of calories. For example, it is assumed that 1 gram of carbohydrate generates 4 calories, 1 gram of protein generates 4 calories and 1 gram of fat generates 9 calories. But this assumption is always misleading in relation to human body. When people take food according to calorie value it is noticed that result is always different person to person. It means that the result is not dependent on the assumed calorie value of the food taken but on the status of the human body who takes the food (Shrestha, 2008a) This is the relation 2 - Food and Health. Thus the theory of 'balanced' diet based on calorie value is misleading.

Food and Health, Relation — 3

On the basis of the theory of 'daily requirement' of nutrients, it is a common practice in the field of medical treatment to recommend certain amount of various nutrients daily. But, it is noticed that when the patient dose not take any food or take certain type of fruits or light food items defying the theory of 'daily requirement', s/he feels more energetic or comfortable. This is a fact. Let's see another example— when someone goes for hiking or trekking or picnic and takes heavy breakfast or lunch according to theory of 'daily requirement' of nutrients, then s/he feels dullness and heaviness. But if someone takes fruits or raw food or light food during that period, irrelevant of 'daily requirement' theory, s/he feels more energetic and lightness. This is also a fact. What indicate these facts? These facts indicate that heavy food items generate dullness and heaviness; light food items generate feeling of energetic ness and lightness. And, in case of sickness, not to take any food items generates comfort. This is the relation 3 - Food and Health. Thus theory of 'daily requirement' of nutrients is misleading.

Three Laws and Some Health Problems

Let's see mechanism of some health problems in light of aforementioned 3 Laws (relations) of Food and Health. These Laws of Food and Health are not manmade laws but derived from the nature observing natural process, that is, interaction between food and body through the commonsense. Let's observe the natural process during **common cold/flu**—

During **common cold** nose becomes runny. If we take food and medicine, homemade or prescribed by authorized doctor,

nose becomes stuffy within 12 to 24 hours. But if we do not take any food or medicine, and take a glassful of hot or warm water every one or two hour, runny nose become normal within 12 to 24 or 48 hour. That is, **common cold** is cured without any food and medicine. Same is the result if we take 1 to 2 kgs of orange or *mausami* in a day, 250 gram to 500 gram at a time, after interval of 3 to 4 hours and take one glass of water in-between. Or we can take a glassful of hot or warm water with a teaspoon of honey and juice of one eight part of a medium sized lemon. The result will be the same. What indicate these processes? These processes indicate that the theory of daily requirement of various kinds of nutrients, the theory of daily requirement of certain amount of calories and the theory of daily requirement of nutrients are misleading. If those 'theories' were correct the result of taking food items and medicine during **common cold** wouldn't be stuffy nose. Basing ourselves on the result of the process (that is law of the cause and effect) our commonsense and law of logic proves that those 'theories' are manmade, not the natural, and wrong in relation to human body.

Let's observe example of natural process during **loose motion/ diarrhea/dysentery**. During loose motion, if we ingest foodstuff problem becomes aggravated. But if we don't ingest foodstuff and take only pure water or water with honey and fresh lemon juice or water with salt and sugar hourly or according to frequency of loose motion, problem begins to normalize and within 12 to 24 hour loose motion stops and the person becomes quite normal. This natural process also indicates that those 'theories' are wrong, not natural but hypothetical and not based on the facts and commonsense.

Let's observe example of natural process during **vomiting**. During vomiting it is purely nonsense to ingest any kind of food items. If we ingest any, the problem becomes more aggravated. During that period if we take hot or warm water slowly, that is 10 to 20 ml in every 5 minute, problem begins to normalize and within 12 to 24 hours the person becomes quite normal. This natural process also clearly indicates that 'daily requirement' theory is not only irrelevant but also dangerous to health.

Let's observe another example of the natural process during **fever**. During fever, if we ingest foodstuff the degree of fever rises. But if we fast, taking only water, degree of fever does not rise highly and begins to subside within 12 to 24 hours and the person becomes normal within 72 hours. During fever, if we take *mausami* (a kind of citrus fruit) fruit 1to 2 kegs within 24 hours, result of the process would be the same. This process also indicates that the theory of daily requirement of certain amount of nutrients is not correct.

After observing the natural process during these four types of preliminary health problems (**common cold, diarrhea, vomiting and fever**) we obviously notice that the body can function more effectively without any kind of foodstuff during abnormal condition and at the same time body's condition become aggravated if we ingest foodstuff. Another point is that without any foodstuff or with nominal food items (juicy fruit or fruit juice) irrelevant of 'calorie theory' during abnormal condition body heads toward normalization. This is the crux of the matter we should understand in relation to Body and Food or Health and Food.

Let's see this process from another angle— during abnormal condition of the body, if ingestion of foodstuff aggravates the condition, it must be true that ingestion of various kinds of nutrients according to theory of daily requirement make the body abnormal. Isn't it? Yes, it is true. If it is not true it must be impossible to normalize abnormal condition of the body without any foodstuff or with nominal food items. This is a fact. On the basis of this fact, it seems that, we must search purpose of food.

Purpose of Food

If we search on the basis of aforementioned fact we certainly notice that the purpose of food is to maintain body's activities at normal level or pave the way for circulation of life force within body uninterruptedly. Circulation of life force within body may sound mystic but actually it is not mystic. Circulation of life force means circulation of blood and respiration. Normal condition of the body means normal condition of circulation of blood and respiration. Thus the purpose of food is to pave the way for normal condition of blood circulation and respiration. It is noticed that if circulation of blood and respiration remain normal, all functions within body remain normal and no symptoms and syndromes of diseases arise and seen. This is also a fact. Standing on this fact, it seems that to take food items according to nutritious value is wrong. Let's clarify this point—

According to value of nutrition, meat, egg, diary products, protein based and high fat items are very nutritious and daily intake of these items is recommended. According to this kind of recommendation, food items are being taken in present day developed countries and developed areas of underdeveloped countries. Result of this practice is blockage of life force circulation in different manifestations— obesity, diabetes, heart disease, high blood pressure, arthritis, respiratory problems, bowel problems, cancer etc. Thus, it is obvious that this kind of practice of food intake dose not meet purpose of the food. So to meet the purpose of the food, it seems that, we should take food not on the basis of nutrition value but on the base of clearing value.

What is clearing Value of Food?

To be clear about the clearing value of the food, we should firstly know what is the mechanism of the blockage? Let's

observe this mechanism— when we ingest mainly food items like meat, egg, dairy products and products of white flour or white rice, we can't defecate faeces easily and wholly. That is, constipation becomes daily practice. Stool becomes sticky and foul smelling. This is the symptom of initial blockage. Then, on the base of constipation, bowel problems develops, diarrhea occurs. If we use medicine to stop diarrhea, result will be constipation. Constipation and diarrhea occurs one after another. If we frequently use medicine to stop diarrhea then obesity develops. And, if we use laxatives medicine time and again to clear constipation, then various kinds of bowel problems develop. In both cases the circulation of life force to clear the digestive system from unwanted matter is hindered. Then the unwanted matter accumulated in digestive system goes to other parts of the body, to be fatty liver, fatty heart, fatty pancreas, fatty spleen, fatty arteries, gall stone, kidney stone, polyps, cyst, mucus, deposit of uric acid, tumor etc. Thus, this is the mechanism of blockage development or disease development. And it is clearly noticed that this is the inevitable outcome of practice of taking mainly those food items assumed as highly nutritious (Shrestha, 2008a)

Now we arrived at the point from where we clearly see what the meaning of clearing value of food. Constipation, that is, accumulation of unwanted matter is the blockage whereas easy, whole, daily and regular defecation of faeces and urination is clearance of unwanted matter. So the food items that help to clear the unwanted matter from the digestive and excretory system is clearing food and the capacity to clear the unwanted matter is clearing value. We have been obviously known that meat, egg, dairy products, white flour and white rice retain unwanted matter in the body; hence from the view point of clearing value those food items are not good for health.

Clearing Food Items

Instead of blocking food items, if we use mainly whole flour, whole grain, brown rice, green and leafy vegetables and fruits regularly, we will not face any problem, loose motion or constipation, to defecate. It is a fact that easy, daily, regular and normal defecation is basic requirement for good health. For this purpose, clearing food items are the best. To take clearing food items it is not necessary to calculate according to nutritious or calorie value. Anyone can take clearing food items without calculating calorie and nutritious value. Yes, one yardstick or standard must be followed. That yardstick is yardstick of commonsense or yardstick of the body or yardstick of own belly, not the yardstick of gram or milligram. Our belly tells that how much clearing food we can take (Shrestha, 2008a)

If we take food items according to calorie and nutritious value only; it will be blunder mistake in relation to water and roughage. Water and roughage are must for digestion, metabolism and excretion but it has been told to us that water and roughage have no calorie and nutritious value. Being without calorie and nutritious value there is danger of misinterpretation that taking water and roughage is matter of one's like or dislike. It has been already become clear to us that the purpose of food is to pave the way for circulation of life force within the body. From this fact it must be clear to us that body does not care our like or dislike. Instead, we must care what the body wants? If we do not care body's like or dislikes, body reacts through various symptoms and syndromes. And with those symptoms and syndromes, we will be in uncomfortable state of various forms. So if we want to remain out of uncomfortable state, we must heed body's dictate in relation to food. There is no other way.

If we take whole grain, whole flour, brown rice, leafy and green vegetables and fruits, we get roughage and pure water from those food items according to body's need. From this fact we must know that roughage and water are necessary components of the food. Processed and dried food items lack roughage or water or both. So if we take processed and dried food only or mainly; we lack clearing roughage and life giving, nourishing and clearing natural water. Yes, we can add roughage in processed food and take plenty of extra water but that does not work as products of whole grain and fresh vegetables and fruits. This is a fact. This fact can be observed through our stool and body's hints (Shrestha, 2008a) without fresh vegetables and fruits, our stool never become normal and body never feels light after taking meal. The feeling of dullness after taking meal is direct proof of the fact that processed food and extra water do not work as natural food and natural water in vegetables and fruits.

The Law of 'Three Equal'

After becoming clear about clearing food, it also must be clear how to take those food items. We already mentioned above that it is not necessary to weigh clearing food items to know how much we should take. In this case our belly becomes our guide. If our belly becomes uncomfortable after taking clearing meal that indicates that we took more than necessary (Shrestha, 2008a) How to combine various clearing food items is another subject matter that we must be clear. It is noticed that if we take only cooked food items like whole bread or brown rice and cooked vegetables and do not take any raw vegetables (salad) or fruits then the result is also not so good. Only taking cooked food items always leads to dullness or heaviness. But when we take cooked whole grain food items and cooked vegetables with raw vegetables and fruits, result become complete different— no dullness and heaviness. Combination of those three items (whole grain items, cooked vegetables and raw vegetables or fruits) should be almost equal in quantity. And total quantity should be determined according to hints of the belly. That is, after taking meal there should not be any kind of discomfort in the belly.

We must realize that any kind of discomfort is sign of over eating. Over eating in all form is disadvantageous to health.

Meat and Dairy Product and the Law of 'Three Equal'

In the law of 'Three Equal', is there any room for meat and diary product or only food grain, green vegetables and fruits? It has become clear to us that meat and dairy products are blocking food items but it does not mean that those items are uneatable. Actually, meat and dairy products are neither necessary nor uneatable items. In normal state of our body, we can take those items according to the law of the 'Three Equal'. That is, we can take meat and dairy product as component of first part of the 'Three Equal'. That is, meat, dairy product and whole grain product should not cross the line of 33% of the total meal; another 67% should be cooked vegetables, raw vegetables and fruits. It should be clear again that we can take meal according to this equation only in the normal state of the body. If the state of the body is abnormal, we should avoid meat and dairy product because it is noticed during experiment that meat and dairy products always aggravate the abnormality of the body. If we search causes of chronic diseases, we will find that almost all of chronic diseases are linked with daily habit of taking meat and dairy product. So meat and dairy product is strictly avoidable during almost all of health problems (Shrestha, 2008a)

Peas and Beans and the Law of 'Three Equal'

In the law of 'Three Equal' we should understand that peas and beans are under the category of food grain. But the quality of peas and beans (double partitioned grain, legumes) are different from wheat and rice (single partitioned grain, cereals). So, even we can take legumes with cereals but the quantity of legumes should be restricted to about 10% of cereals. It is noticed that if we take more than that, discomfort follows after taking meal. Occasionally, we can take food items from legumes only but daily habit of this kind hampers digestive system, disturbs metabolism and excretion of metabolites. So, daily intake of peas and beans is no necessary and taking should be stopped when body face problem of metabolites excretion and hyper-blood acidity. We can take items of peas and beans daily only if body permits us. Main part of the first 33% of 'Three Equal' should be from items of single partitioned food grain like wheat, rice, maize, millet, barley etc. (Shrestha, 2008a)

Dry Nuts and Fruits and the Law of 'Three Equal'

Dry nuts like cashew nut, pistachio nut, walnut, coconut, almond and ground nut are high fat and high protein items; so these items can be taken limitedly. That is, if the state of the body is normal, according to the law of 'Three Equal' these items can be taken as a small portion (5 to 10%) of the first part (33%) of the total meal. If the state of the body is abnormal, dry nuts are avoidable or can be taken selectively. Over dose of these items always hampers the normal functioning of the body. If we use our commonsense to know about the result of overdose of these items; we can know easily.

Dry fruits like date, fig and raisins are high glucose, low fat and low protein items; these items can be taken more than dry nuts. Occasionally, we can take (except in case of diabetes mellitus) dates and raisins only for some days or weeks or months. These items can work as complete food for body. Or these items can be taken as breakfast. Or these items and dry nuts can be taken combined. When we take these items combined; part of dry nuts should not exceed 10%. People can remain healthy in this way for long time.

It is also ideal to take dry nuts, dry fruits and fresh fruits combined. In this way people can remain healthy forever. If this can be realized in practice, there is no need to take food grain because this pattern can fulfill body's needs completely— that is, about 90% glucose,4% amino acids, 3% minerals, 1+% fat and under 1% vitamins (Harvey and Marilyn, 1987) This division of percentage is an ideal composition of a complete food. To know about an ideal composition of a complete food we should not depend on microanalysis but on our own commonsense first. Later we can make microanalysis. If we take dry nuts, dry fruits and fresh fruits, the law of 'Three Equal' becomes irrelevant. That is, to take nuts and fruits only, there is no need to follow the law of 'Three Equal'. The law of 'Three Equal' is only for balancing present day prevailing food items. That is, for the items of food grain, meat and dairy product.

Use of Extra Salt

Habit to take extra salt is rampant nowadays. Salt is inherent component of any food item according to microanalysis. And any food item can be taken without extra salt. To take extra salt is only a habit. If people take food items according to the law of 'Three Equal' or take only dry nuts, dry fruits and fresh fruits only; there is no need to take extra salt. Extra salt is necessary only for processed, unnecessarily cooked and packed food items and not for the body but for habit. When we take extra salt our body becomes dull and heavy. That is, extra salt blocks the path for circulation of life force; it is clear to us that symptoms of blockage are dullness and heaviness. When we do not take extra salt for few days we feel very light and when we take food according to the law of 'Three Equal' and do not take extra salt for long period of time (for few months) any kind of hamper to body is not seen. And when we take 2—3 grams of extra salt in cooked vegetables for one day and do not take extra salt in items of food grain and salad our body does not become so dull and heavy. So, it can be said that to take 2—3 grams of extra salt in the 'Three Equal' meal per day is not dangerous but to take extra salt is not necessary.

Timing of Taking Various Items

Taking food items according to the law of 'Three Equal' does not mean that we should take various food items simultaneously. It is noticed that during taking meal, to take salad (raw vegetables and fruits) at the first and then items of whole grain and cooked vegetables at the last is not so good. It is also not so good to take salad, cooked whole grain items and cooked vegetables simultaneously. In practical experiment, it is noticed that to take cooked items of grain and vegetables simultaneously at the first and then salad at the last is proper manner to take various food items in a single meal. If we manage timing of taking various food items in this way we will not face any discomfort. Proper and improper are not dependent on our like or dislike. But it is dependent on the law of the body, law of the digestive system. If our manner of taking food items is in line with that law, we will not face any discomfort and if our manner is in defiance of the law of digestive system we will face various kind of discomfort. This is the crux of the matter in relation to timing of taking various food items. According to this law, anyone can experiment what is suitable for oneself. The sole condition is that no discomfort should prevail on our body (Shrestha, 2008a)

Timing of Water

It has become clear to us that water is a necessary component of our food. Without water, proper digestion, metabolism and excretion are impossible. But to take advantage from water, we should take it timely and properly. Water taken improperly creates discomfort for body. It is seen that when we take water and solid food simultaneously, discomfort begins in the stomach. Degree of discomfort is dependent on the quantity of water taken with solid food— more water more discomfort. Same is the case when we take water before 30 to 60 minutes to meal and 30 to 60 minutes after meal. In this case water means we should understand normal drinking water. In between two meals if we take plenty of water time and again at the interval of 10 to 20 minutes same is the result, that is, discomfort like flatulence prevails. But if we take a glass (250-300 ml) of water after 1 hour of taking meal; almost all we don't feel any discomfort. And if we take more than one or more than two glass of water after one hour of taking meal at one time; we feel discomfort. But if we take one glass of water, after one hour of taking meal, hourly we do not feel any discomfort. From these facts it becomes clear to us that to take water during and within one hour before and after of a meal is wrong (Shrestha, 2008a)

Then how to manage time table for water? It is seen that to take 3 to 4 glass of water early in the morning before to toilet, one glass hourly after one hour of taking meal up to 5 to 6 o'clock in the evening or before two hour to evening meal and taking last glass of water right before to bed is proper time to take water. Taking 3 to 4 glass of water early in the morning before toilet is advantageous for easy and whole defecation. It is also seen that to take more than one or two glass of water at one time is only suitable early in the morning before to toilet. After meal to take more than one or two glass of water at one time is not advantageous. But to take one glass of water at one time hourly or one and half hourly after meal is proper. And it is avoidable before two hour of evening meal. This is a general timetable for water taking but this timetable may not work occasionally. At that point we should use our commonsense. Demand of water is dependent on the quality of food we take. So, if we take food items occasionally of low water component then the timing of taking water may be different slightly. In that case water taking gap before and after a meal may be narrowed. But, it is always disadvantageous to take water during a meal. And to take more than one or two glass of water before and after a meal is also always disadvantageous.

Method of Deriving Conclusion

When we use our commonsense to determine about our food and health, it is out of question to fragment food items in micro parts like carbohydrate, protein, fat, minerals and vitamins because to fragment in that way is the job of microanalysis. Using commonsense, food items are divided wholly like as food grain (single partitioned or double partitioned), vegetables (leafy or other), fruits (citrus or sweet or nuts or dates), meat, (animal or bird or fish), dairy product (cow's or buffalo's or other) and water (spring or tap or other) etc. That is, we use our five sense organs directly and derive conclusion or find out relation between food and body using our sixth sense organ, the mind. All of abovementioned relations, conclusions and determinations are derived in this way. This way is simple, direct and reliable because to experiment there is no need of any equipment, and the result of any experiment can be experienced instantly or within few days. And there is no need of any guinea pig because in this method any experiment can be done on human body directly. This method's another plus point is that to experiment there is no necessary to be a specialist. Anyone can experiment on own self. And last but not the least, there is no any danger to experiment using the food items that are used by all from the time immemorial or for the long time.

To use this method is to be free from all types of assumptions and superstitions. That is, to be scientific. To be scientific is to follow the law of cause and effect, nature's main law, in all aspects of life. Or, say, using this method, anyone can develop scientific view point in all aspects of life and lead healthy and happy life. This is the miracle of use of the commonsense. To be more clear about this let's use this method on some prevailing health problems of present day world.

High Blood Pressure

It is said in modern medical science that the special etiology of essential hypertension is not known. Yes, it is unknown

subject for fragmented viewpoint but if we use viewpoint of our commonsense, it is not unknown subject. If we see from the holistic viewpoint apart from thinking pattern and activities of the person with hypertension, the food taken by her/him is directly related to hypertension. It is seen that when the person with hypertension starts to take food items according to the law of 'Three Equal', avoiding meat, egg, dairy product, extra fat and extra salt, the degree of blood pressure slowly goes down within 24 to 72 hour. And it is possible to be completely free from essential hypertension following the diet pattern of 'Three Equal' consistently and regularly and taking water properly. This is a noticed fact and we have live examples of this fact. Anyone with essential hypertension can experiment this on own self without any side effect or danger. Yes, s/he should check level of blood pressure regularly during period of experiment or say treatment by self (Shrestha, 2008c)

Diabetes Mellitus

As is the case of essential hypertension, diabetes mellitus is also directly related to the diet pattern the person takes. It is also seen that when the person with diabetes mellitus starts to take food items according to the law of 'Three Equal', avoiding products of sugar, white flour and sweet items and taking water properly, degree of blood sugar level slowly goes down within 72 hour. In this way, regularly following the proper diet pattern, a person can become free from diabetes mellitus and remain healthy. But it is impossible to be free of diabetes and remain healthy without following proper diet pattern, that is, according to the law of 'Three Equal'. This is a noticed fact of experiment on persons with diabetes mellitus. (Shrestha, 2008d)As in the case of essential hypertension, anyone can experiment on diabetes mellitus also, using viewpoint of commonsense and taking 'One's Health at Own Hand'. During period of experiment and after few months of that period, blood sugar level should be checked regularly. After a long period of experiment or practice of proper diet pattern, use of commonsense becomes day to day matter or common practice. Then microanalysis of blood sugar level will not be necessary.

Arthritis

Arthritis, in various manifestations, is also a major health problem of present day world. This problem is also directly linked with food. Over intake of highly nutritious food items like meat, dairy products, legumes, dry nuts and acidic vegetables is the main cause of arthritis. So, when a person with arthritis problem starts to take food according to the law of No:'Three Equal', avoiding those 'nutritious and acidic' items, arthritic pain subsides slowly. (Shrestha, 2008e) To know the fact about this, anyone with arthritis problem can experiment on own self. Along with proper bodily activities, proper diet pattern can make an arthritic person free from arthritis problem. For this result s/he should use proper diet pattern, that is, the diet according to the law of 'Three Equal' regularly.

Asthma

Asthma and all other kind of respiratory problems are also directly related to food. As in the case of arthritis, improper intake of those highly 'nutritious and acidic' items is the main cause of respiratory problems. So, it is seen that anyone with respiratory problems can remain comfortably only when s/he avoids those items. After a long period of cleansing process s/he can take those items on trial basis. In case of asthma, the law of 'Three Equal' also should be carefully followed, specially, when taking raw vegetables and fruits. It is seen that some kind of raw vegetables and fruits exacerbates respiratory problems so use of such items should be avoided during first stage of cleansing process. And at the latter stage, those items can be used on trial basis. It is clearly seen that asthma is curable problem through using proper diet pattern (Shrestha, 2008f) This experiment is also can be done on anyone with respiratory problem by self without any side effect.

Coronary Heart Disease (CHD)

Coronary heart disease is also prevailing disease of modern day world. And this is directly related to the diet pattern prevailing in the modern day world. It is seen that high consumption of meat, dairy product and products fried in oil is the main cause of this problem (Dean, 1991) It is also seen that persons who do not take those items and take proper diet are out of danger of this disease. On the base of these seen facts, anyone with problem of coronary heart disease can experiment proper diet pattern on own self. It is also seen that use of proper diet can clear the blockage in the arteries. Always there is no any danger of taking food properly. Proper food combination according to the law of 'Three Equal', avoiding meat, dairy products, products of white flour and oily items, is suitable for this problem.

Conclusion

Relation between food and health can be known easily by everyone using commonsense than sophisticated equipments. And, it is enough to use commonsense to maintain health. But, it should not be understood that to use sophisticated equipments and microanalysis, to know the relation between food and health, is wrong. And, it is

necessary for health to see whole aspect, not only fragment of the result noticed through sophisticated equipments and microanalysis. If we see the reality about various food items, in relation to health, using this method, it is almost certain that there is no chance to be diseased because of intake of food items.

According to present day fashion of modern medical practitioners, treatment by self is something that should not be practiced by general public. So, it should not be assumed that the writer of this article is provoking general public for self treatment because to take 'One's Health at Own Hand' is not only natural right but also accepted legal right of a person in present day world. And it should be clear that the writer is not presenting prescription to general public to practice self treatment but facts in relation to health and food. Anyone is free to take advantage from the presented facts in this article.

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Mushroom Poisoning Problem in Nepal

UPENDRA RAY

Regional Food Technology and Quality Control Office, Biratnagar, Nepal

Nepal is rich in mushroom diversity. Local people have been using wild mushrooms in their diet as well as a source of income, but they do not have proper knowledge about identification of edible and poisonous mushrooms. This practice has caused severe poisonings and even death. The reports of death from the various parts of Nepal come at an alarming rate, but still a large proportion of such incidents go unreported by the local news. Mushroom poisoning refers to the severe and often deadly effects of various toxins that are found in certain types of mushrooms. One type known as Amanita phalloides, appropriately called "death cap," accounts for the majority of cases. The toxins initially cause severe abdominal cramping, vomiting, and watery diarrhea, and then lead to liver and kidney failure.

Key Words: *Mushroom poisoning, abdominal cramping, vomiting, watery diarrhea, liver and kidney failure*

Introduction

Nepal is rich in biodiversity due to the variation topography, climate, and latitudinal changes that are found within a short distance. The prevalence of interesting mycodiversity in this Himalayan region has been attracting many enthusiastic investigators over the years. The collection and survey on mycoflora from the Nepalese Himalayan belt was first done by J. D. Hooker from eastern Nepal in 1850 (Adhikari, 1990a; Adhikari, 1990b). Nepalese mycoflora includes 585 genera and 1822 species. These wild species include the fungi of various economic importances (edible110 sp, poisonous 48 sp, medicinal 17 sp, mycorrizal 50 sp, decoration 12 sp, and parasitic 970 sp. (Adhikari, 1990a; Adhikari, 1991; Adhikari and Manandhar, 1992).Nepal is regarded as a country of ecological mosaics. The different ethnic groups in Nepal possess rich knowledge of local Non-Timber Forest Products (NNTP's) as a cultural heritage; these are listed as food, medicine and on various socio-religious purposes. In Nepal various mycophagous groups such as Serpa, Tamang, Gurung, Magar, Tharu, Danuwar, Newar, Kami, Damai, and Sarkiare directly concerned with the collection and consumption of mushrooms historically due to mushrooms delicious taste. Besides, these wild mushrooms are also locally traded as minor forest Product at local market. Out of 110 species of edible mushrooms, 40 species are sold in local markets every season. Picture of non-poisonous and poisonous mushrooms is given in Fig 1.

About 90% of the deaths due to mushroom poisoning in the United States and Western Europe result from eating Amanita phalloides. This mushroom is recognized by its metallic green cap (the color may vary from light yellow to greenish brown), white gills (located under the cap), white stem, and bulb-shaped structure at the base of the stem. A pure white variety of this species also occurs. Poisoning results from ingestion of as few as one to three mushrooms. Higher death (mortality) rates of more than 50% occur in children less than 10 years of age (Adhikari and Manandhar, 1992).

(a) (b)

Fig 1: Non-poisonous (a) and Poisonous (b) mushrooms

Causes and symptoms

Poisonous mushrooms contain at least two different types of toxins, each of which can cause death if taken in large enough quantities. Some of the toxins found in poisonous mushrooms are among the most potent ever discovered. One group of poisons, known as am toxins, blocks the production of DNA, the basis of cell reproduction. This leads to the death of many cells, especially those that reproduce frequently such as in the liver, intestines, and kidney. Other mushroom poisons affect the proteins needed for muscle contraction, and therefore reduce the ability of certain muscle groups to perform (Weekly Report, 1997; O,Brein and Linh, 1996) Symptoms of ***Amanita poisoning*** occur in different stages or phases. These include:

First phase: Abdominal cramping, nausea, vomiting, and severe watery diarrhea occur anywhere from 6-24 hours after eating the mushroom and last for about 24 hours. These intestinal symptoms can lead to dehydration and low blood pressure ***(hypotension).***

Second phase: A period of remission of symptoms that lasts 1-2 days. During this time, the patient feels better, but blood tests begin to show evidence of liver and kidney damage.

*Corresponding author; Email: rflbrt@cworld.com.np

Third phase: Liver and kidney failure develop at this point and either led to death within about a week or recovery within two to three weeks. Other symptoms are due to either a decrease in blood clotting factors that leads to internal bleeding or reduced muscle function, with the development of weakness and paralysis.

Diagnosis: In most cases, the fact that the patient has recently eaten wild mushrooms is the clue to the cause of symptoms. Moreover, the identification of any remaining mushrooms by a qualified mushroom specialist (mycologist) can be a key to diagnosis. When in doubt, the toxin known as ***alpha-amantin*** can be found in the blood, urine, or stomach contents of an individual who has ingested poisonous ***Amanita mushrooms***.

Treatment

It is important to remember that there is no specific antidote for mushroom poisoning. However, several advances in therapy have decreased the death rate over the last several years. Early replacement of lost body fluids has been a major factor in improving survival rates (Adhikari and Manandhar, 1992).

Therapy is aimed at decreasing the amount of toxin in the body. Initially, attempts are made to remove toxins from the upper gastrointestinal tract by inducing vomiting or by gastric lavage (stomach pumping). After that continuous aspiration of the upper portion of the small intestine through a nasogastric tube is done and oral charcoal (every four hours for 48 hours) is given to prevent absorption of toxin. These measures work best if started within six hours of ingestion. In the United States, early removal of mushroom poison by way of an artificial kidney machine (dialysis) has become part of the treatment program. This is combined with the correction of any imbalances of salts (electrolytes) dissolved in the blood, such as sodium or potassium. An enzyme called ***thioctic acid*** and ***corticosteroids*** also appear to be beneficial, as well as high doses of ***penicillin***. In Europe, a chemical taken from the milk thistle plant, ***Silybum marianum***, is also part of treatment. When liver failure develops, liver transplantation may be the only treatment option (Weekly Report, 1997).

Prognosis:- The mortality rate has decreased with improved and rapid treatment. However, according to some medical reports death still occurs in 20-30% of cases, with a higher mortality rate of 50% in children less than 10 years old.

Prevention:-The most important factor in preventing mushroom poisoning is to avoid eating wild or noncultivated mushrooms. For anyone not expert in mushroom identification, there are generally no easily recognizable differences between nonpoisonous and poisonous mushrooms. It is also important to remember that most mushroom poisons are not destroyed or deactivated by cooking, canning, freezing, drying, or other means of food preparation.

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Conclusions

Concise conclusion is to be provided.

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Acknowledgements can also be provided if any.

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