APPLICATION OF GARCINIA INDICA AS A COLORANT AND ANTIOXIDANT IN RICE EXTRUDATES

by

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ABSTRACT OF THE THESIS

Application of *Garcinia indica* as a colorant and antioxidant in rice extrudates

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Garcinia indica, commonly known as Kokum, is a tropical fruit native to India. The rinds of fruit are rich source of red anthocyanin pigments which are characterized as cyanidin-3-glucoside and cyanidin-3-sambubioside. The fruit juice is strongly acidic (pH = 1.87) due to the presence of hydroxycitric acid, an organic acid known for its weight suppressing action. Recently a new, yellow, fat soluble pigment called garcinol has been separated from the fruit rinds. Garcinol is a polyphenolic compound known to possess antioxidant activity. Presence of bioactive compounds in the fruit promotes its use as a colorant and antioxidant in a food product. The antioxidant potential of Kokum has been previously studied but the effect of processing on its antioxidant activity is still unknown. Thus effects of extrusion processing on the color and antioxidant activity of rice extrudates fortified with spray-dried encapsulated fruit powder were studied.

A lab-scale Brabender single screw extruder was used for the extrusion. The control variables were barrel temperature (120°C-180°C), feed moisture (18-24%) and Kokum powder content (3-5%). The total phenolic content (TPC) and antioxidant activity of rice extrudates were measured by Folin-Ciocalteau method and ORAC, respectively.

The color of extrudates (hue and chroma) was also measured to quantify the redness of samples. Effects of control variables on physical properties of extrudates such as bulk density, breaking strength and expansion index were also studied.

The extrudates showed excellent red color when extruded at temperatures up to 150°C beyond which anthocyanins degraded resulting in lesser red color. TPC and ORAC of samples showed maximum retention at 120°C and 180°C and minimum at 150°C. The loss of TPC and ORAC varied from 33 to 61% and 12 to 75%, respectively. Maximum breaking strength and maximum bulk density were observed at low temperature (120°C) and high moisture (21%). Expansion index was maximum at high temperature (150°C) and low moisture (18%). The effect of powder on bulk density and expansion index was not as prominent as that of temperature.

Thus, Kokum can be used as colorant and antioxidant for extruded product however; the loss of antioxidants is substantial.

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1. INTRODUCTION

1.1 Introduction to Kokum:

Kokum (*Garcinia indica)* is also known by different English names such as wild mangosteen or red mango. In India, it is known by names such as Bindin, Biran, Bhirand, Bhinda, Katambi, Panarpuli, Ratamba or Amsool etc. (Padhye et. al., 2009). *Garcinia indica* belongs to the botanical family of Clusiaceae or according to the old classification it belongs to the family of Guttiferae which has approximately 1350 species. Clusiaceae is further divided into five sub-families, one of which is Clusioideae. Sub-family Clusioideae has two tribes Clusieae and Garcicieae and Garcicieae has two genera namely Garcinia and Mammea. The genus Garcinia contains 200 species out of which over 20 are found in India (Patil, 2005).

Kokum is an evergreen tree predominantly grown in the tropical humid rain forests of Western Ghats in South India up to an elevation of around 800 meters. It is also called Kokum butter tree, brindonia tallow tree or mangosteen oil tree. The tree grows up to 10-18 meters with drooping branches (Nayak et al., 2010). It flowers from November to February with fruits ripening from April to May. After 15 years, a properly cared single plant yields about 30 to 50 kg of fruit (Patil, 2005).

The ripe Kokum fruit is red or dark purple colored containing 3-8 large seeds. The fruit is spherical, 2.5 to 3.0 cm in diameter. Seeds are usually connected to the rind by tissue and embedded in a red acidic pulp. High content of malic acid and little amounts of tartaric and citric acids give pleasant tart test to the fruit (Patil, 2005). At present, India produces 10,200 metric tons of Kokum with productivity of 8.5 tons/ha. The estimated

demand for Kokum in 2004-2005 was approximately 774.2 metric tons (Nayak et al., 2010).

Kokum fruit has longer shelf life at low temperatures. Traditionally, the fruit rinds are sun dried to reduce water activity and increase shelf-life. The fresh fruits are cut into halves and the seed is removed before sun drying for about 6-8 days. The product obtained after sun drying is commercially called Amsul. The normal shelf life of fresh fruit is about 5 days at room temperature.

Different products like dried ripe Kokum rind (Amsul), Kokum syrup are made from the fruit and rind. Kokum seed is a good source of fat called Kokum butter. It is used in chocolate and confectionary industry. Sometimes it is also used in surfactant and ointment industries. Because of the sweetish acidic test and its typical flavor, Kokum is used as an acidulant in different curries like traditional fish curries etc. The dried rind is also used to make a peculiar soup and cold drinks in summer. The dried rind is extracted with water to make syrup which is sweetened to make a cold drink. In some parts of India, rinds are spiced and sweetened with jaggery for feasts (Patil, 2005). Aqueous Kokum extract also has 4% sugar which can be fermented to make excellent quality wine. Dried kokum rind pieces are powdered, sieved and stored in airtight containers. Powder is used in coconut and fish curries as an acidulant (Nayak et al., 2010).

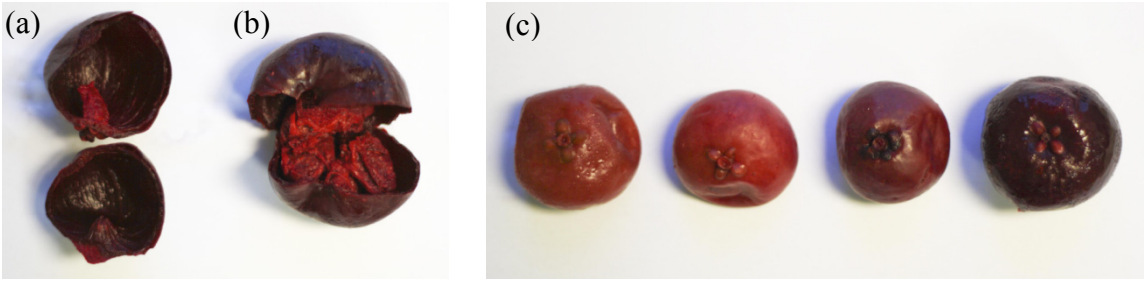


Figure 1.1: (a) Kokum Rinds (b) Rinds with seeds (c) Whole fruit with variation in color

1.2 Nutritional profile of Kokum:

Kokum fruit is naturally very acidic (pH = 1.5 to 2.0) and contains large amounts of acid. Environmental conditions cause some variation in composition of fruit. Table 1.1 gives the proximate composition of fruit on fresh weight basis.

Table 1.1 Proximate composition of Kokum:

Nutrient	Amount (g/100g fresh weight)
Moisture	80.0
Protein (N × 6.25%)	1.0
Total ash	2.6
Tannins	1.7
Pectin	0.9
Total sugars	4.1
Crude fat	1.4
Organic acid	5.9
Anthocyanins	2.4

Source: (Nayak et al., 2010)

1.2.1 Fat composition of Kokum:

Kokum seed contains about 25% edible fat commonly known as Kokum butter. It is extracted mostly by crushing seeds, boiling them in water and removing fat from top or by churning the seeds in water. Sometimes it is also separated by solvent extraction. It is used as edible fat or adulterant of ghee. Crude Kokum butter is yellowish, while when refined, it is white in color. Refined Kokum butter is comparable with high quality hydrogenated fats. Fatty acid composition of Kokum butter is given in Table 1.2. Free

fatty acids are present up to 7.2% of total Kokum butter (Patil, 2005).

Table 1.2 Fatty acid composition of Kokum butter:

Description	Value (%)
Fatty acid composition (%)	
Palmitic acid	2.5
Stearic acid	56.4
Olein	39.4
Linolein	1.7
Glycerides composition (%)	
Tristearin	1.5
Oleodistearin	68
Oleopalmitostearin	8
Palmitodiolein	20
Triolein	2

Source: (Patil, 2005)

Kokum butter has been successfully used in chocolate and cocoa industry. It helps in improving texture of chocolate without affecting the flavor (Maheshwari and Yella Reddy, 2005).

Recently a new fat soluble pigment called garcinol has been separated from fruit rind. Garcinol is a yellowish pigment. Crude fat content of fresh Kokum rind on moisture free basis is 10%. Garcinol is extracted using hexane from fruit rinds (Patil, 2005). Since then, it has been studied for its medicinal properties.

1.2.2 Organic acids in Kokum:

Major portion of organic acids in Kokum is hydroxycitric acid (HCA) (1,2 dihydroxypropane-1,2,3-tricarboxylic acid). Rinds contain about 20-30% of (-)-HCA on dry basis. Due to its presence in high amounts in Garcinia species it is also called as garcinia acid. (-)-HCA is separated from rinds of Kokum by aqueous extraction. (-)-HCA has tendency towards lactonization during purification, evaporation and concentration. So it is converted to its sodium, potassium or calcium salts, lactones or esters (Jena et. al, 2002). A liquid chromatographic method has been developed to determine the amount of HCA and HCA-lactone in Kokum leaves as well as rinds (Jayaprakasha and Sakariah, 2002).

Other minor acids found in Kokum are citric acid, malic acid and ascorbic acid. Thus acid base titration to calculate HCA content gives small error (Jayaprakasha and Sakariah, 2002).

1.2.3 Anthocyanins in Kokum:

Kokum contains 2 to 3 % of red pigment. Anthocyanins of Kokum are water soluble and possess antioxidant activity. Two major pigments characterized in Kokum are cyanidin-3-glucoside and cyanidin-3-sambubioside which are usually present in the ratio of 4:1 (Nayak et al., 2010). These two anthocyanins were first identified by thin layer chromatography using acetic acid: HCl: water in ratio of 15:3:82 (Nayak et al., 2010). The respective sugars associated with these two pigments are glucose and xylose. Thus the extract of anthocyanins contains water, pigment and sugars. Due to high water content these extracts have low shelf life and thus commercially they are concentrated. This also further reduces the transportation and storage cost. Concentration of such color

by conventional evaporation or distillation results in loss of hue and chroma. Thus membrane processes such as microfiltration, ultrafiltration or reverse osmosis are employed. But these methods have few drawbacks such as need of high pressure, membrane clogging, maximum achievable concentration and reduction in the gradient. A novel forward osmosis method is also developed for concentration of Kokum anthocyanins using semi-permeable nonporous active skin layer of cellulose triacetate embedded in a nylon mesh with NaCl solution as an osmotic agent (Nayak and Rastogi, 2010).

1.3 Nutraceutical properties of Kokum:

Kokum contains two major active compounds having nutraceutical properties namely garcinol and hydroxycitric acid. Both of these compounds are present in the rinds of Kokum. They play beneficial role in human health since they have anti-cancer and anti-obesity properties.

1.3.1 Garcinol:

Garcinol is a yellow colored, fat soluble pigment found in the rinds of Kokum at level of 2-3%. In fact all Garcinia species have some amount of garcinol (Yamaguchi et al., 2000; Bakana et al., 1987; Sahu et al., 1989). Garcinol can be separated from the fruit rinds by ethanol or hexane extraction (Yamaguchi et al., 2000; Padhye et al., 2009).

1.3.1.1 Chemistry of garcinol:

Garcinol is a polyisoprenylated benzophenone derivative and contains phenolic hydroxyl groups. This makes it active antioxidant. It is also called as camboginol, a tri-isoprenylated chalcone. It has β-diketone moiety and thus resembles a known antioxidant viz. curcumin (Pan et al., 2001). Molecular weight of Garcinol is 602 ($C_{38}H_{50}O_6$) and its

melting point is 122°C (Nayak et al., 2010). It is crystallized out from hexane extract of the fruit rind. The absorption spectral data and molecular formula indicate relation to isomeric xanthochymol and in terms of optical rotation to cambogin. The 1,3-diketone system is enolisable since presence of two isomeric trimethyl ethers. The UV spectrum of garcinol shows that 1,3-diketone system is conjugated to the 3,4-dihydroxybenzoyl moiety. The IR spectrum of trimethyl ethers shows there is presence of saturated carbonyl group and two α,β-unsaturated carbonyl groups. Some features of the garcinol molecule indicate it can be derivable from Maclurin (2,4,6,3',4'-pentahydroxybenzophenone) and five isoprenyl units (Padhye et al., 2009). The general structure of garcinol is shown in Figure 1.2.

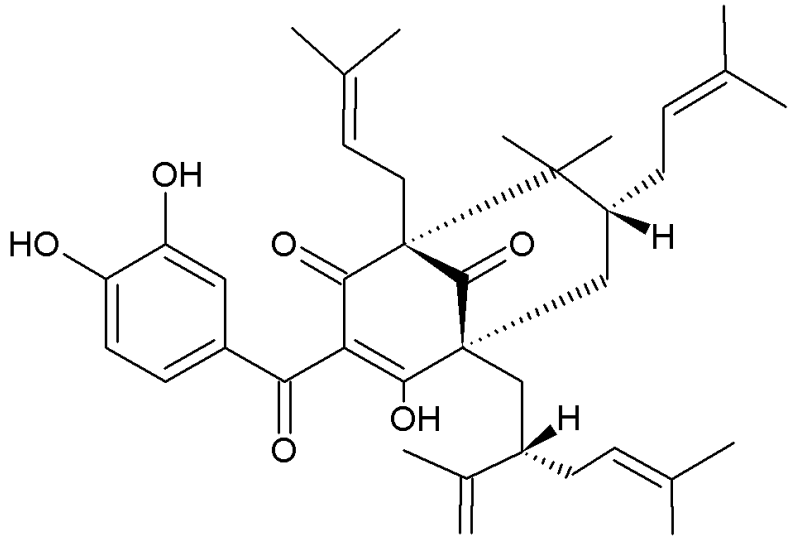


Figure 1.2: Structure of garcinol (Yamaguchi et al., 2000)

1.3.1.2 Nutraceutical properties of garcinol:

Garcinol has been studied for its anti-cancer, anti-ulcer, anti-oxidative and anti-glycation activity (Nayak et al., 2010). The antioxidant activity of Kokum syrup, aqueous and boiled extract has been measured by various techniques such as ORAC, FRAP, ABTS etc. and it is shown that these preparations have very good antioxidant potential

due to presence of garcinol and anthocyanins (Mishra et al., 2006). Garcinol can scavenge alkyl-peroxyl radicals to form hydroperoxy derivative of garcinol and cambogin or isogarcinol. Isogarcinol has similar biological activities as garcinol and is potent antioxidant as well. These compounds can induce apoptosis in human leukemia HL-60 cells; inhibit NO radical generation and LPS-induced iNOS gene expression. Thus garcinol has been shown to have better anti-tumor activity than curcumin (Sang et al., 2002; Sang et al., 2001). Garcinol has been shown to possess antioxidant activity in H_2O_2-NaOH-DMSO system and radical scavenging activity against hydroxyl radical, methyl radical and superoxide anion. The emulsified garcinol suppresses superoxide anion similar to DL-α tocopherol (by weight), while it has three times greater free radical scavenging activity against 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals than DL-α-tocopherol by weight (Yamaguchi et al., 2000). Garcinol is also shown to possess antioxidant activity against arachidonic acid metabolism and NO radical synthesis by modulation of arachidonic acid metabolism. Defective arachidonic acid metabolism and generation of NO radicals are involved in carcinogenesis and inflammation (Hong et al., 2006). Liao et al. (2005) showed garcinol prevents NO radical accumulation in LPS-induced inflammatory mediators such as iNOS and COX-2. Thus garcinol may have neuroprotective effects against brain injury. A recent study by Koeberle and co-workers (2009) showed that garcinol interferes with two enzymes, 5-lipoxygenase and microsomal prostaglandin PGE_2 synthase that play important role in inflammation and tumorigenesis. Garcinol and its oxidative products interact with colon cancer cells such as HT-29 and HCT-116 as well as normal immortalized intestinal cells such as IEC-6 and INT-407. They have potent growth-inhibitory effects on all intestinal cells but more

effective on cancer cells than normal ones. Thus at certain concentration garcinol can be used to inhibit growth of cancer cells (Hong et al., 2007). Another similar study on garcinol has shown inhibition of growth of human leukemia HL-60 cells suggesting its chemopreventive action (Matsumoto et al., 2003). Balasubramanyam et al (2004) have shown non-specific inhibition of histone acetyltransferase by garcinol suggesting anti-HIV property. Above research shows that garcinol has very promising antioxidant, anticancer, anti-inflammatory properties.

Garcinol is also reported to show some antimicrobial activity. It plays important role in treatment of gastric ulcers caused by *Helicobacter pylori* chronic infection. This bacterium along with cells from gastric mucous membrane produces hydroxyl radicals and superoxide anions. Conventional antibiotics such as Clarithromycin have side effects and thus garcinol can be a good alternative (Chatterjee et al., 2003; Chatterjee et al., 2005). Garcinol also showed antimicrobial activity against *Staphylococcus aureus* which was comparable to traditional antibiotic Vancomycin (Rukachaisirikul et al., 2005; Iinuma et al., 1996). Yoshida and co-workers (2005) reported, garcinol fortified diet decreases the incidence of tounge neoplasms and pre-neoplasms. It also induces apoptosis through the activation of caspases and thus works as anti-tumor agent (Pan et al., 2001). There are numerous reported mechanisms through which garcinol acts as antioxidant, anti-inflammatory or anti-cancer agent as explained above.

1.3.2 Anthocyanins:

The two major anthocyanin pigments found in Kokum are characterized as cyanidin-3-glucoside and cyanidin-3-sambubioside. They have been identified by thin layer chromatography as well as HPLC, mass and NMR spectroscopy (Nayak et al.,

2010; Nayak et al., 2010). Anthocyanins constitute approximately 2.4% of the total fruit biomass. These pigments can scavenge free radicals and are water soluble. They can be extracted from the fruit rind by hydraulic press using 1% acidified water as a solvent (Nayak et al., 2010). The monomeric anthocyanins in Kokum can be measured using pH differential method (Wrolstad et al., 2005).

1.3.2.1 Chemistry of anthocyanins:

Anthocyanins are group of important compounds which are part of flavonoids. They are responsible for red and purple colors in fruits. Chemically, anthocyanins are based on a C-15 skeleton with a chromane ring having a second aromatic ring B in position 2 (C6-C3-C6). Usually one or more sugar molecules are attached to different hydroxylated positions of basic structure. Substituted glycosides of salts of phenyl-2-benzopyrilium (anthocyanidins) are anthocyanins (Delgado-Vargas et al., 2000).

Basic structure of anthocyanidin is shown in Figure 1.3 where different R could be -H, -OH or $-OCH_3$. Figure 1.3 also shows the most accepted nomenclature (Delgado-Vargas et al., 2000).

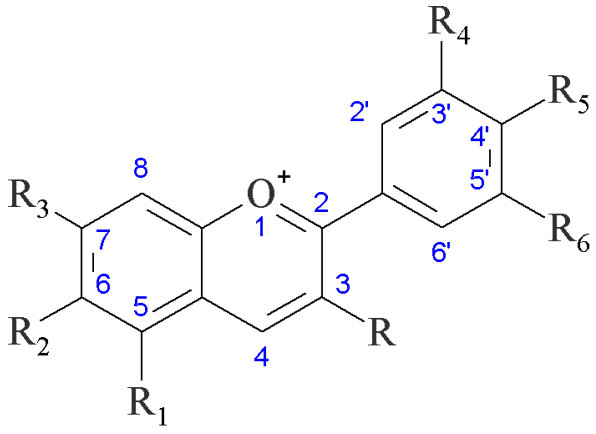


Figure 1.3: General structure of anthocyanidin pigment (Delgado-Vargas et al., 2000)

This basic structure of anthocyanidin pigment is responsible for a number of different color compounds produced by chemical combination with glycosides and acyl groups. The most common sugar groups occurring in nature are glucose, rhamnose, xylose, galactose, arabinose and fructose while common acyl groups are coumaric, caffeic, ferulic, p-hydroxy benzoic, synapic, malonic, acetic, succinic, malic, oxylic etc. Substitution of hydroxyl and methoxyl groups has influence on different shades of colors of anthocyanins. Increase in the number of hydroxyl groups gives more bluish shade while increase in methoxyl groups increases redness. Depending upon the number and position of hydroxyl and/or methoxyl groups there are total 17 anthocyanidins of which 6 are the most common ones, namely cyanidin, delphinidin, pelargonidin, malvidin, peonidin, petunidin. Cyanidins have hydroxyl groups attached at 3,5,7,3' and 4' position. It gives magenta and crimson shades (Delgado-Vargas et al., 2000). Cyanidin-3-glucoside found in Kokum has hydroxyl groups attached at the corresponding positions and glycosidic linkage at position 3. The other major pigment cyanidin-3-sambubioside is similar in structure and has disaccharide sambubiose attached instead of glucose.

1.3.2.2 Nutraceutical potential of anthocyanins:

Anthocyanins have been shown to possess strong antioxidant activity. Given their wide distribution in nature, daily intake of anthocyanins is 25 to 215 mg/person depending upon gender and age (Delgado-Vargas et al., 2000). Anthocyanins prevent ascorbic acid oxidation, scavenge free radicals, show inhibitory effects against oxidative enzymes and reduce the risk of cancer and heart diseases (Bridle and Timberlake, 1997). The 3' and 4' –OH in B-ring determine radical scavenging capacity with a saturated 2,3-double bond. Different glycosylation and hydroxylation positions determine their

potential as an antioxidant (Wang et al., 1997). With increase in hydroxyl groups in B-ring, antioxidant activity increases when present as glucosides. Corresponding aglycones have weaker activities (Tsushima et al., 1996). Azevedo et al., (2010) showed antioxidant properties of anthocyanins with DPPH, FRAP and oxygen consumption assays. They showed radical scavenging activity and reducing capacity increased with the number of hydroxyl groups present in B-ring. 3' and 4' –OH groups are important in preventing ascorbic acid oxidation by anthocyanins-metal chelation (Sarma et al., 1997). Anthocyanins also have effect on lipid peroxidation. They are better agents against lipid peroxidation than α-tocopherol. Anthocyanins also have scavenging properties against –OH and O_2^-. Bioflavonoids such as leucoanthocyanidins, catechins, flavonols etc. along with anthocyanins such as cyanidin-3-glucoside have shown activity to improve permeability and strength of capillaries, to accelerate the ethanol metabolism and to reduce inflammations and edematic reactions (Delgado-Vargas et al., 2000).

1.3.3 Hydroxycitric acid:

Hydroxycitric acid (HCA) is a major acid found in Kokum. HCA is also found in other Garcinia species such as *G. cambogia, G. atrovirdis* etc. (Lewis and Neelakantan, 1965). Kokum can contain up to 23% of HCA on dry basis. The major part is found in leaves and rinds as HCA and some quantity is present as HCA lactone. HCA which is also called as Garcinia acid can be separated from rinds by thermal as well as athermal methods. HCA has been separated as sodium salt by combination of aqueous NaOH and methanol extraction and then neutralizing with HCl. Acetone is used to obtain pure crystals of HCA. HCA has also been separated by athermal method in which HCA is extracted with deionized water and then concentrated by osmotic membrane distillation

with hydrophobic polypropylene membrane. This method being nonthermal avoids degradation of HCA and also HCA lactone formation (Nayak et al., 2010). HCA has also been produced from microbes like *Streptomyces* sp. U121 and *Bacillus megaterium* G45C (Yamada et al., 2007).

1.3.3.1 Chemistry of HCA:

HCA has hydroxyl groups at the second and third carbon atom. HCA has two asymmetric carbons and thus two pairs of diastereoisomers. Out of the four, (-)-hydroxycitric acid is found in Garcinia species. Free HCA is readily converted to HCA lactone while evaporation or concentration. Structures of HCA and its isomers are shown in Figure 1.4.

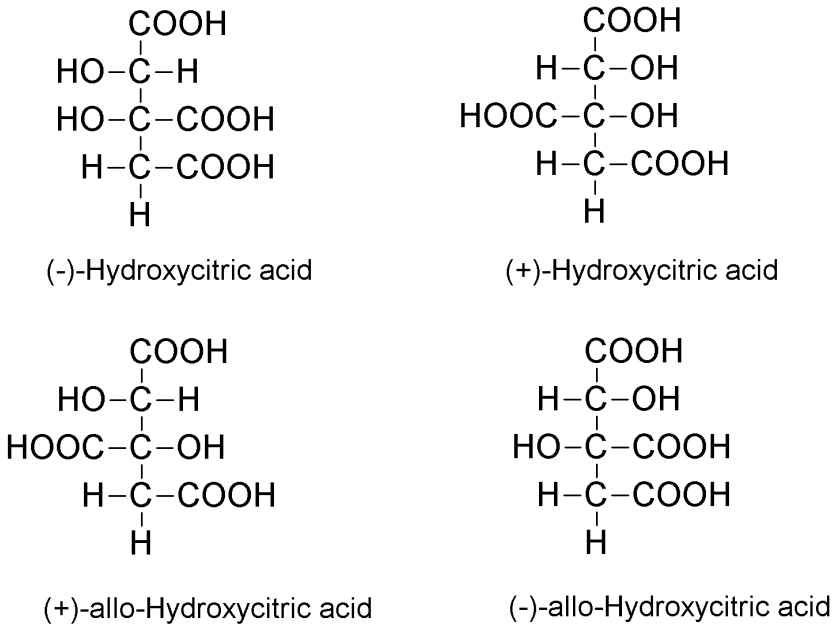


Figure 1.4: Stereoisomers of hydroxycitric acid (Jena et al., 2002)

There are two corresponding lactones for each pair namely, (-)-hydroxycitric acid lactone and (+)-allo-hydroxycitric acid lactone. Since HCA is unstable in its free form, it is

commercially available as a calcium salt. Potassium hydroxycitrate is also formed by KOH treatment of HCA (Jena et al., 2002).

1.3.3.2 Regulatory effects of HCA:

(-)-HCA has inhibitory effect on ATP:citrate lyase (ATP:citrate oxaloacetate lyase, EC 4.1.3.8). This enzyme plays influential role in fatty acid synthesis from carbohydrates. It catalyzes cleavage of citrate to acetyl-CoA and oxaloactate. The ultimate source of carbon for fatty acids is acetyl-CoA and acetyl-CoA is an important molecule in formation of fats from carbohydrates. Thus by limiting the availability of acetyl-CoA, (-)-HCA plays important role in regulating fatty acid synthesis. The knowledge of powerful inhibition of ATP:citrate lyase by HCA helps in the study of citrate cleavage reaction (Jena et al., 2002).

HCA, in some cases, has been observed to stimulate fatty acid synthesis. HCA inhibits lipogenesis only when cytoplasmic acetyl-CoA is produced by citrate cleavage enzyme otherwise if the alternate source of acetyl-CoA is available, for example, acetate, it will activate fatty acid synthesis (Jena et al., 2002). Since HCA regulates the ATP:citrate lyase enzyme and thus citrate cleavage reaction it acts as an anti-obesity agent. Due to its regulatory effect it is also known as weight controlling agent.

HCA can be used to increase activity of carnitine palmitoyl transferase (CPT 1). CPT 1 is a rate limiting factor in fat burning and thus weight loss. HCA limits production of acetyl-CoA which in turn reduces production of malonyl-CoA. Malonyl-CoA has inhibitory effect on CPT 1. Thus by reducing malonyl-CoA formation HCA works as a weight reducing agent. But since malonyl-CoA is also important in transmitting insulin signal in the cells, HCA may have adverse effect on insulin sensitivity (Jena et al., 2002).

To study effects of processing on Kokum and its active components, we chose rice flour as a base for extrusion processing. Kokum fruit powder was mixed with rice flour before extrusion and different properties of extrudates were studied. Since rice is white in color it provided good base to study the red color of extrudates.

1.4 Introduction to rice and its phenolics:

Rice is the staple food in many parts of the world. It is consumed in most of East and South Asia, the Middle East and Latin America. Rice and its different preparations are very important in terms of calories provided. Major constituent of rice is carbohydrates and due to the high content of starch, cooking results in gelatinization. This property is very useful in extrusion processing which is explained later. It is grown in areas with high rain fall or where water can be readily made available throughout its cultivation. Typical proximate composition of long grain raw rice is given in Table 1.3.

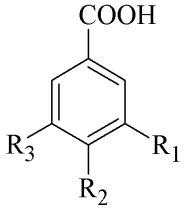
Table 1.3 Proximate composition of rice:

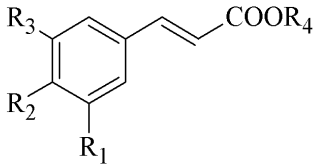
Nutrient	Percentage
Protein	7-8%
Lipid	0.5-1%
Ash	0.5-1%
Water	10-12%
Carbohydrates by difference	~80%

Source: USDA nutrient database

The analysis of white rice and brown rice has shown that it contains two major free phenolic acids as ferulic acid and p-coumaric acid. Few bound phenolics are also found along with free phenolic acids. Major portion of the phenolic acids is in rice bran

than rice germ. Thus polished rice has lesser amount of phenolics than brown rice. Harukaze and co-workers (1999) studied 21 different cultivars of rice for phenolic acids. They showed that free phenolics in all cultivars varied from 4.3 to 15.7 mg/100g on dry basis in polished rice. Tian et al. (2004) reported same numbers in white rice which were less compared to brown rice. HPLC analysis showed the other minor phenolic acids as protocatechuic acid, hydroxybenzoic acid, vanillic acid, syringic acid, chlorogenic acid, caffeic acid and sinapinic acid. These acids have similar structures and are shown in Figure 1.5.





Protocatechuic acid: R_1=H, R_2=OH, R_3=OH
Hydroxybenzoic acid: R_1=H, R_2=OH, R_3=H
Vanillic acid: R_1=H, R_2=OH, R_3=OCH_3
Syringic acid: R_1=OCH_3, R_2=OH, R_3=OCH_3

Caffeic acid: R_1=OH, R_2=OH, R_3=OH, R_4=H
p-Coumaric acid: R_1=H, R_2=OH, R_3=H, R_4=H
Ferulic acid: R_1=OCH_3, R_2=OH, R_3=H, R_4=H
Sinapinic acid: R_1=OCH_3, R_2=OH, R_3=OCH_3, R_4=H
Chlorogenic acid: R_1=OH, R_2=OH, R_3=H, R_4=quinate

Figure 1.5: Phenolic acids of rice (Tian et al., 2004)

The bound acids present in rice are phenolic-carbohydrate esters. They can be released by alkali extraction (Harukaze et al., 1999). Some of them are identified as 5,5'-diferulic acid, 5,8'-diferulic acid, 6'-O-(E)-feruloylsucrose and 6'-O-(E)-sinapoylsucorse (Harukaze et al., 1999; Tian et al., 2004).

1.5 Extrusion:

Extrusion is defined as a process where food material is forced out through a small orifice or a die of given shape under pressure. The force is applied either by using a piston or a screw. Screw extrusion is predominant in food applications. Extruders were

first used in late 19th century to manufacture sausage. In 1930s it was used to produce pasta from water and semolina mixture. It was also used to make RTE products like corn curls which use high shear. Extrusion is a combination of many operations such as mixing, shearing, cooking, grinding, homogenizing, unitizing, shaping, forming, plasticizing, melting, fluid flow and mass transfer etc. Some of these processes take place depending upon desired product. For example in pasta, shaping and forming is the main objective, in corn curls expansion and development of porous structure is important.

Extrusion is popular because of its high flexibility. Same extruder can be used to manufacture different products using either dry or moist feed. In addition, on-line adjustments can be made to get varied product characteristics. Extrusion can be performed at low moisture hence less redrying and better energy efficiency. It is low cost equipment having high productivity rate. It is a continuous process with negligible effluent. Extrusion is high temperature short time process where food is exposed to temperatures up to 200°C for 1 to 10 seconds (Riaz et al., 2000).

Extrusion is used to produce different kind of products which are broadly classified as products for human consumption, animal and pet products and non-consumables. We will discuss more about consumable products. There are different types of consumables which can be formed by extrusion processing. These include expanded, unexpanded, half-cooked, texturized, candy like or modified. Different products require different set of conditions. For example, expanded products like corn curls or rice based snacks require high shear, high temperature and low moisture content compared to unexpanded pasta type products where moisture content required is more and temperature is low. Extrusion is also used for semi-processing of foods. For example, half cooked

potato pellets, where product after extrusion is stored at appropriate conditions for further processing such as frying, cooking or baking. To make co-extruded products a different die design is needed. In this case core material and coating material are simultaneously fed. For texturized meat analogs, a long die is used to achieve fibrous texture to the extrudate. Modified starches can also be made by extrusion. Thus, as discussed, variety of products can be made in extruder.

1.5.1 Single screw extruder:

Extruders are primarily of two types namely single screw extruder and twin screw extruder. As names suggest single screw extruder uses one screw whereas twin screw extruder uses two screws. Single screw extruders have a single screw rotating within a restriction called barrel. Most of the screws have constant pitch. The barrel is usually grooved to convey and mix material uniformly. The extruder usually has three main sections known as feed section, transition section and metering section. The raw material is adjusted to desirable moisture content and then fed through the hopper. The rotating screw then conveys food material from feed section into transition section where it gets cooked. Here the screw channel becomes shallower and hence food material is compressed which results in increase in pressure. Due to dissipation of mechanical energy, temperature rises and starchy material gets gelatinized. Food is then transported through metering section under pressure and then forced out through the die. Single screw extruders are described by their characteristic properties such as length/diameter ratio or compression ratio which is the ratio of the maximum channel depth to the minimum channel depth. Extruders are also characterized by amount of shear applied.

For example cold forming extruders are low shear, whereas collet extruders are high shear extruders. Figure 1.6 shows extruder and its different components.

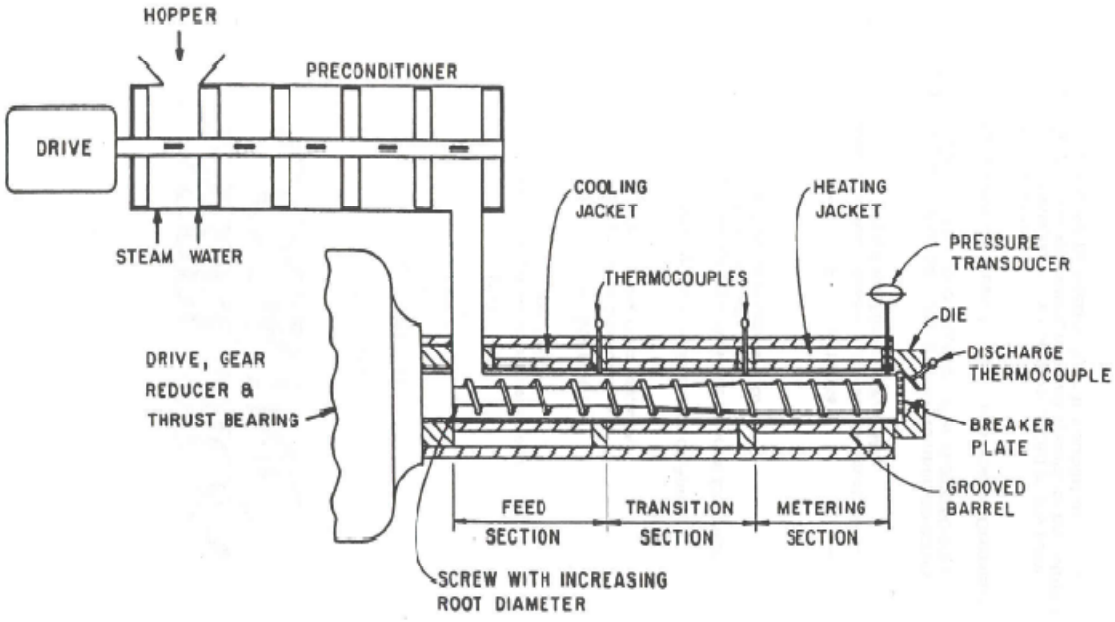


Figure 1.6: Different sections of extruder and its components (Harper, 1981)

As seen from Figure 1.6, extruder has thermocouples at each section to measure the temperature and cooling water jackets to maintain it. The pressure just before product is forced out of the die is measured by pressure transducer. The increase in root diameter of screw helps to create shear and compress the food material.

1.5.2 Effects of extrusion on nutritional quality:

Due to high temperature and shear inside the extruder, food components can undergo molecular changes including chemical reactions and physical transformations such as starch gelatinization and fragmentation, protein denaturation, flavor degradation, lipid oxidation, caramalization etc. These changes result in textural, color, flavor and nutritional developments.

1.5.2.1 Starch:

Starch undergoes various changes depending upon experimental conditions. At high moisture content gelatinization occurs. Gelatinized starch is prone to retrogradation and hence staling. Similarly at high temperature, starch fragmentation is prominent which results in sticky product. Fragmentation is due to physical damage to the structure of starch, mainly to the amylopectin. At low moisture and high temperature starch melting takes place but it has been observed that with the higher specific mechanical energy (SME) input, even at lower temperatures, melting and fragmentation of starch can occur. SME is defined as the ratio of the net power consumed by extruder and the mass flow rate in the extruder. Typical values of SME are 100 - 1000 kJ/kg. Typically, the raw material is in glassy state before extrusion. As it hydrates it becomes rubbery and then increase in temperature inside extruder makes it free flowing. At the exit through the die, moisture is flashed off and depending upon other conditions product can expand or remain the same. Thus it is either glassy or rubbery. Glassy product is crunchy whereas rubbery product is chewy. As the starch fragmentation occurs, its molecular weight is decreased and hence glass transition temperature is also decreased (Karwe, 2003).

Extrusion has different effects on amylose and amylopectin which are major constituents of rice starch as well. Since amylopectin is highly branched it is prone to shear but both amylose and amylopectin molecules may decrease in molecular weight. The more linear amylose molecule is prone to gelatinization before degradation. High molecular weight starches thus degrade at greater proportion during extrusion. Dextrinization occurs with degradation and with more and more shear; glucose can be manufactured from starch by extrusion (Riaz et al., 2000). The expansion of rice

extrudates in our experiments is due to gelatinization of amylose and then loss of moisture at high temperature.

1.5.2.2 Proteins:

Solubility of proteins in water or salt solutions is observed to decrease after extrusion. Protein denaturation also occurs due to high temperature and shear. Although denaturation and loss of solubility are attributed more to the high barrel temperature, SME also plays important role (Della Valle et al., 1994). Wheat protein solubility was decreased at temperatures less than 100°C (Ummadi et al., 1995). Disulfide linkages in protein are also affected by extrusion. They are disrupted leading to intermolecular and intramolecular reactions. Insoluble aggregates are formed due to electrostatic and hydrophobic interactions. High molecular weight proteins can break into small units. Non-enzymatic Maillard reactions with reducing sugars also take place during extrusion (Riaz et al., 2000). The possibility of protein interactions during extrusion is very less in our experiments since rice is a poor source of proteins.

1.5.2.3 Lipids:

Lipid binding and lipid peroxidation is also common during extrusion. Free lipids are bound to starch especially amylose (Riaz et al., 2000) but at higher temperatures there is decrease in binding. Lipid peroxides have been found only in free lipids so lipid binding may protect lipids from peroxidation which enhances the shelf life of extruded products (Karwe, 2003). Generally high lipid food is not extruded since it impairs extrusion performance. Large expansion during extrusion favors oxidation because of the large surface area which might be concern during storage but due to starch binding and

denaturation of hydrolytic enzymes, oxidation is limited (Riaz et al., 2000). Lipid content of powder or rice is very less hence these effects are not of concern in our experiments.

1.5.2.4 Vitamins:

Almost all vitamins are very sensitive to heat and shear during extrusion. Minimizing these factors gives maximum retention of vitamins. Lipid soluble vitamins D and K are generally more stable than Vitamin A and E. Thermal degradation is the major reason for β-carotene and at 200°C it is observed to decrease by 50% in wheat flour extrusion (Guzman-Tello and Cheftel, 1990). Amongst water soluble vitamins, Vitamin C and thiamine were observed to degrade at low moisture in wheat flour but not riboflavin and niacin (Andersson and Hedlund, 1990). Thiamine losses are highly variable though ranging from 5-100% (Killeit, 1994).

1.5.2.5 Phytochemicals:

Phenolic compounds are degraded during extrusion. Őzer and co-workers (2006) reported decrease in moisture content results in more destruction of phenolics and hence less total antioxidant activity in specially made snack food. At drier conditions shear is more which may be the reason of more destruction. Similarly high screw speed also affected phenolic content adversely. Camire et al. (2007) reported heavy losses of anthocyanins pigments from blueberry, cranberry, Concord grape, raspberry powders in extruded cornmeal at high temperatures. Destruction of phenolic compounds and lower antioxidant activity were observed. Thus extrusion has been reported to affect anthocyanins and polyphenols. We studied effect of extrusion on phenolic compounds of Kokum.

1.6 Prior work on Kokum and extrusion:

Antioxidant activity of *Garcinia indica* has been measured before by different methods such as ORAC, ABTS, FRAP etc. (Mishra et al., 2006). But nobody has incorporated Kokum and/or its constituents as an antioxidant. Similarly anthocyanin content of Kokum has been measured and *Garcinia indica* has been proved to be a rich source of color (Nayak et al., 2010), but it has not been used as a colorant in any food product. Some work has been done on antioxidant activity of aqueous Kokum extract (Mishra et al., 2006) although a lot of work has been done on importance of garcinol and hydroxycitric acid as functional ingredients. But there potency after processing has not been evaluated and needs to be studied. Camire et al. (2007) used different fruit powders in extrusion and studied their functionality. But loss in antioxidant activity and free polyphenols of Kokum during extrusion processing has not been studied yet. To summarize there is no work on the use of *Garcinia indica* as a colorant or a fortifying agent in any food process.

1.7 Hypotheses:

We had two hypotheses for our research

1. *Garcinia indica* can be used as a colorant for extruded rice product.
2. *Garcinia indica* can be used to make antioxidant rich extruded rice product.

1.8 Rationale:

Garcinia indica contains anthocyanins and garcinol which are known nutraceuticals. Boiled extracts of *Garcinia indica* have been shown to possess antioxidant activity (Mishra et al., 2006). If they are to provide health benefits, they should withstand conventional processing techniques. Since fruit powder is water soluble it can be uniformly distributed and equilibrated with flour for fortification. So antioxidant activity

and total phenolics are measured for extrudates at different temperatures, different levels of addition of powder and moisture contents. This is compared with the control to determine the loss of phenolics.

Similarly Kokum also has high concentration of anthocyanin pigments which suggests its use as a colorant. But these anthocyanins may get destroyed at high temperature hence color measurement after high temperature processing is necessary. It is also the indication of stability of anthocyanins.

1.9 Objectives:

The overall objective of this work was to investigate effect of processing on antioxidant activity and total phenolic content in extrudates made from rice flour and microencapsulated *Garcinia indica* fruit powder.

The specific objectives are as follows:

1. To find operating window for extrusion of rice flour mixed with *Garcinia indica* fruit powder.
2. To measure antioxidant activity and total phenolic content (TPC) by Oxygen radical absorbance capacity (ORAC) and Folin-ciocalteau method, respectively before and after extrusion.
3. To measure the color of extrudates at different experimental conditions.
4. To study the effect of extrusion control variables on physical properties of extrudates such as breaking strength, bulk density and expansion index.

2. MATERIAL AND METHODS

2.1 Materials:

2.1.1 Rice:

Rice flour was procured from Gulf Pacific Rice Company, Inc. (Houston, TX). Flour was made from long grain polished white rice. Composition of long grain rice was previously shown in Table 1.3.

2.1.2 Kokum powder:

The Kokum fruit powder was obtained from Frolic Foods, Mumbai, India which was used as a colorant in all experiments. Encapsulated powder was made by spray drying crushed fruit rinds with maltodextrin as a carrier. Powder contained approximately 0.6% protein, 8% maltodextrin, 1.3% fat and the rest was crushed rind.

2.1.3 Solvents:

Absolute ethanol (ACP/USP grade) was obtained from Pharmco-Aaper Products (Brookfield, CT; Shelbyville, KY). Ethanol was used as an extracting solvent for polyphenols from extrudates, rice flour and *Garcinia indica* powder.

2.1.4 Reagents for Folin-ciocalteu analysis:

Folin-ciocalteu reagent, gallic acid and anhydrous sodium carbonate powder were obtained from Sigma-Aldrich (St. Louis, MO).

2.1.5 Chemicals for ORAC assay:

AAPH (2,2'-azobis(2-amidinopropane)dihydrochloride) was bought from Sigma-Aldrich (St. Louis, MO).

Trolox and disodium fluorescein were also purchased from Sigma-Aldrich (St. Louis, MO).

Anhydrous sodium dihydrogenphosphate and disodium hydrogenphosphate were procured from Sigma-Aldrich (St. Louis, MO). These salts were used to prepare buffer solution.

2.2 Equipments and procedures:

2.2.1 Particle size distribution of rice flour and Kokum powder:

Before performing extrusion experiments, particle size distribution of both rice flour and Kokum powder was determined. Rice flour and Kokum powder were passed through different sieves and percentage distribution was calculated. Sieves used were of mesh size 70 (212 μm), 80 (180 μm), 100 (150 μm), 200 (75 μm) and 325 (45 μm). All sieves were stacked in increasing order of their mesh sizes. A certain weighed amount of rice flour or Kokum powder was placed on the uppermost sieve #70 and sieves were shaken for 30 min. After 30 min, amount of powder or rice flour on each sieve was weighed and converted into percentage of the total.

2.2.2 Extrusion of rice and powder blend:

For extrusion, a C.W. Brabender (Hackensack, NJ) (Plasticorder model) single screw extruder was used. A tapered screw with 2:1 compression ratio was used to provide enough shear and expansion. L/D ratio for screw was 20:1. Screw had root diameter of 11.35 mm, tip diameter of 18.8 mm and helix angle of 18.29°. Screw speed could be varied from 0 to 230 rpm. For our experiments screw speed was kept constant at 150 rpm. Die was a cylindrical hole 5 mm in length and 3 mm in diameter.

Figure 2.1: Single screw used for extrusion

The extruder barrel could be heated to preset temperature accurately. The barrel had two different heating zones viz. zone 1 and zone 2. The barrel was set for desired temperatures and was left to attain constant temperature at barrel and die for about 30 min. Temperature was maintained by cold compressed air and circulating water. Temperature of zone 1 was kept constant at 60°C for all experiments so that there is a gradual increase in temperature which helps in preheating the flour. Temperature of zone 2 was varied according to the design. After extruder attained constant temperature at both zones, motor was started and previously blended flour was fed automatically through hopper at constant feed rate. Flour was prepared by mixing desired amount of moisture and powder a day before the extrusion and then allowed to equilibrate in refrigerator overnight at 4°C. Hobart laboratory mixer (Troy, OH) was used to mix powder and water uniformly with the rice flour. Extruded samples were collected at constant flow rate, die temperature and barrel temperature and then were dried overnight in vacuum oven at 40°C with no vacuum. Dried samples were then placed in glass jars and flushed with nitrogen before storing in refrigerator at 4°C.

Preliminary experiments were carried out to determine the range of parameters for extrusion. To determine range of moisture content for equilibrated rice flour, different blends of rice flour were made. Starting from the blend with lowest moisture content, extrusion runs were carried out to determine lower and upper limit for further experiments. At very low moisture content there is high shear and below certain limit extrusion is not possible whereas above certain moisture content there is no expansion of the product. Based on these observations moisture range was decided to be 18 to 24% on wet basis. Similarly a temperature range was determined based on product quality at high

and low temperatures. At very low temperatures there was no cooking whereas at high temperatures it had burnt flavor. With this criteria temperature was fixed as 120°C to 180°C. Range for percentage of fruit powder was decided purely on appearance. Above 5% of addition, product was observed to be brown instead of red. Hence addition of powder was limited to 3 to 5%.

A Box-Behnken design for three parameters was used for designing experiments for extrusion. Three control variables used were moisture content, powder content and temperature of the barrel. The range for each of these parameters was decided as explained previously. This design has 12 treatment combinations at the center of edges of the cube and three repetitions at the center of the cube. Box-Behnken design is a second order rotatable or nearly rotatable design which requires three levels of each parameter. The number of experiments generated depends on number of factors in the design. $N = 2k (k-1) + C_0$ where k are number of factors, C_0 is number of central points. Advantage of BBD is, it doesn't contain the experiments for which all factors are at high level simultaneously and thus avoids experiments under extreme conditions which might yield undesirable results (Ferreira et al., 2007). The Box-Behnken design can be graphically represented as shown in Figure 2.2.

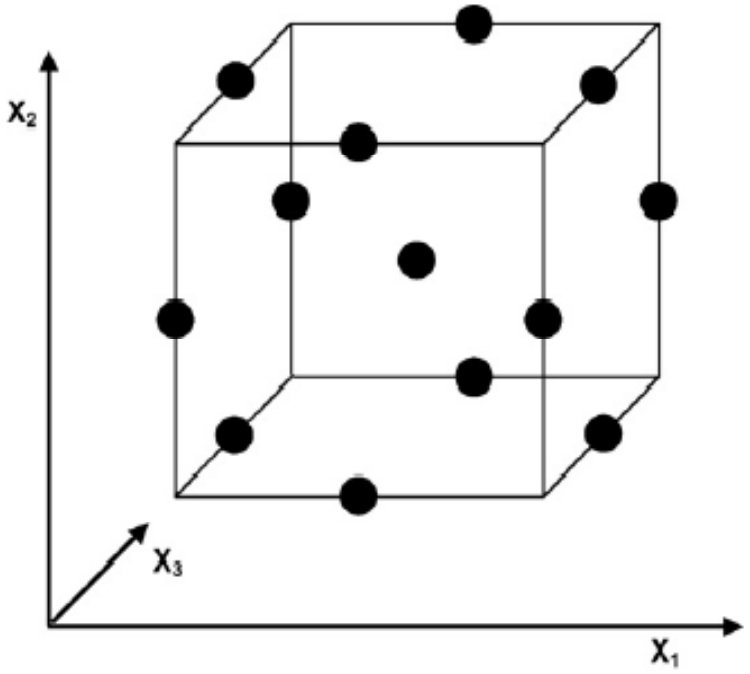


Figure 2.2: Graphical representation of three level second order factorial BBD

(Ferreira et al., 2007)

Here X_1, X_2 and X_3 represent three factors which in our case were moisture content, temperature and powder content. The black dots represent conditions of each experiment. Thus at each corner of the cube lies combination of extreme conditions of respective parameters whereas the center of each edge represents combination of midpoints.

BBD has been applied in various experiments before to optimize analytical systems. For example, it has been used in optimization of injection analysis used for determination of sulphate in ethanol automotive fuel. Other examples include optimization of chromatographic methods, electroanalytical methods, optimizing capillary electrophoresis and sorption process. It has also been used before in optimization of extrusion processing. (Doğan and Karwe, 2003). Table 2.1 shows coded and uncoded levels of Box-Behnken design.

Table 2.1 Coded and uncoded levels of BBD:

Sr. No.	Coded			Uncoded		
	X_1	X_2	X_3	Temperature (°C)	Moisture content (%)	Kokum powder content (%)
1	-1	-1	0	120	18	4
2	-1	1	0	120	24	4
3	1	-1	0	180	18	4
4	1	1	0	180	24	4
5	0	-1	-1	150	18	3
6	0	-1	1	150	18	5
7	0	1	-1	150	24	3
8	0	1	1	150	24	5
9	-1	0	-1	120	21	3
10	1	0	-1	180	21	3
11	-1	0	1	120	21	5
12	1	0	1	180	21	5
13	0	0	0	150	21	4
14	0	0	0	150	21	4
15	0	0	0	150	21	4

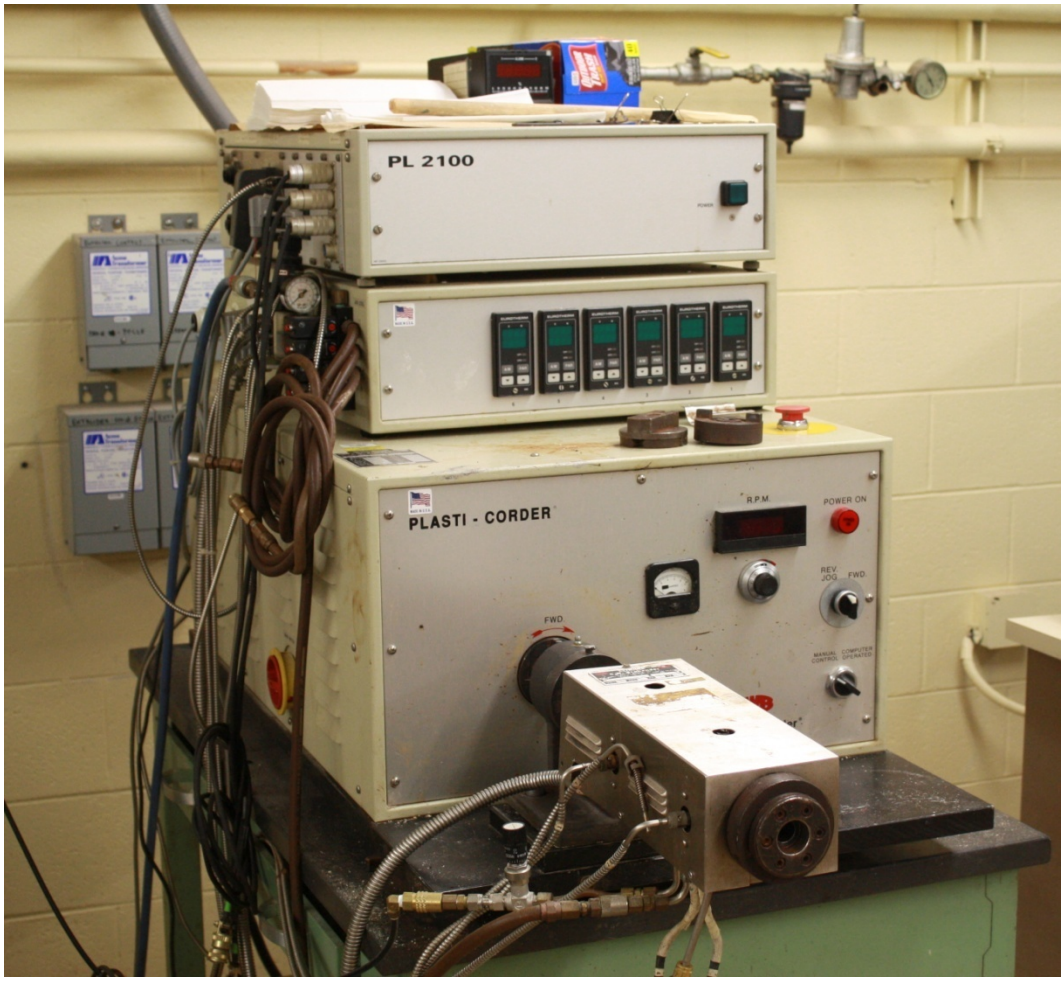


Figure 2.3: Brabender extruder in Rutgers, Food Science pilot plant

2.2.3 Grinding of samples:

A laboratory blender (New Hartford, CT) was used to make fine powder of extrudates. Extrudate powder was passed through 0.5 mm sieve (US mesh #35) procured from Gilson Company Inc, (Lewis Center, OH). Powdered form of extrudates was used for determination of color, total phenolics and ORAC.

2.2.4 Analysis of moisture:

Moisture content of extrudates or blended flour was determined by using Sartorius (MA-30) (Edgewood, NY) moisture analyzer.

2.3 Analytical techniques:

2.3.1 Color measurement:

Color of extrudates was measured using Konica-Minolta chroma meter (CR-410) (Konica-Minolta, Tokyo, Japan) as performed earlier by Berrios et al., (2004). The colorimeter was calibrated with a white D_{65} standard (Y = 94.7, x = 0.3156 and y = 0.3319) and 2º observer angle before sample measurement. All ground samples were passed through 0.5 mm sieve before analysis. Samples were placed in a small non-transparent white dish and with the help of sensing head; colorimeter was placed above the sample without any direct contact. Color was measured in triplicates for each sample and average values were reported. CIE Lab color space was used to record L^*, a^* and b^* values. Figure 2.4 shows Konica-Minolta CR-410 colorimeter.

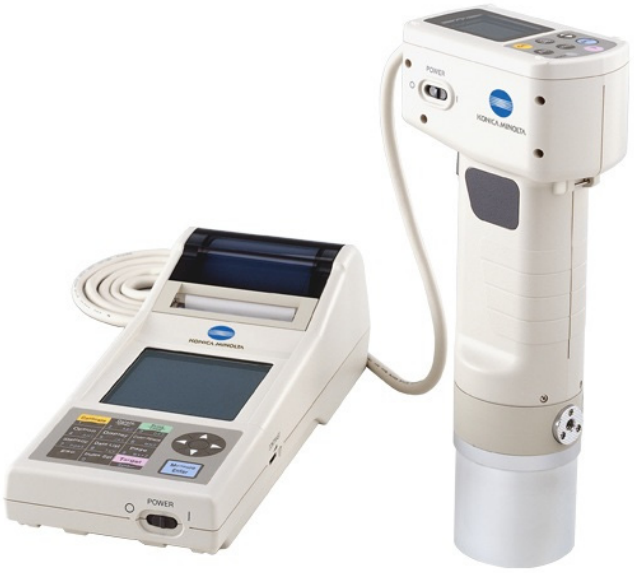


Figure 2.4: Konica-Minolta CR-410 colorimeter

L*, a* and b* values were then converted to Munsell color system. Since a^* is not the right indication of redness (Wrolstad et al., 2005), hue (description of color) and chroma

(saturation of color, dull or vivid) were calculated. Hue and chroma are calculated as,

$$h^0_{ab} = \tan^{-1}\left(\frac{b^*}{a^*}\right) \quad \text{(Eq. I)}$$

$$C^*_{ab} = \sqrt{(a^{*2} + b^{*2})} \quad \text{(Eq. II)}$$

where h^0_{ab} is hue and C^*_{ab} is chroma.

As seen from Figure 2.5, hue is the angle expressed in degrees varying from pure red at 0° to yellow at 90°, green at 180° and blue at 270°. Chroma is measured as the distance from the center of color space and thus at the perimeter lies the darkest shade of the color (Wrolstad et al., 2005). Similarly Y-axis represents the lightness and darkness. Center of the color space represents color between white and black that is grey. Munsell system is diagrammatically represented in Figure 2.5. It also shows how different shades of color lie at different points in the color space.

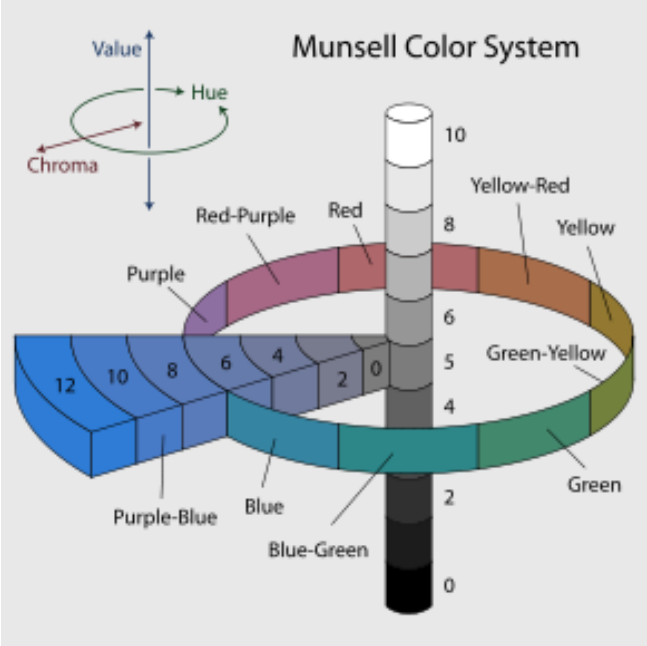


Figure 2.5: Munsell color system (http://en.wikipedia.org/wiki/Munsell_color_system)

Thus as the hue angle increases from 0°, redness decreases and color approaches towards yellow at 90°. At the same angle, distance from the center decides dullness of the

same shade of color. At the same angle, distance along Y-axis determines lightness or darkness of the color.

2.3.2 Relative humidity and color:

To determine relation between relative humidity and hue value, extrudate with 3% added powder was equilibrated with six different saturated salt solutions. Salts used were LiCl, CH_3COOK, $MgCl_2$, $Mg(NO_3)_2$, $CuCl_2$, $NaNO_3$ and K_2SO_4. Saturated solutions of these salts have fixed relative humidity values of 11.3, 22.5, 33.1, 54.5, 67.7, 75.4 and 97.6% respectively when equilibrated at 25°C. Extrudates were placed in desiccators with saturated solutions at bottom and equilibrated for two days. After two days, moisture content and color of the extrudates were measured. The graph of hue angle vs. moisture content was plotted.

2.3.3 Measurement of antioxidant activity by ORAC:

ORAC stands for Oxygen Radical Antioxidant Capacity. ORAC was first developed by Cao and Prior (1999) based largely on the preliminary work by Glazer and his co-workers (1990). The method of Glazer involved measurement of decrease in fluorescence of B- or R-phycoerythrin (PE) in the presence of reactive species (RS) like peroxyl radicals. It related rate constant of PE decay to the antioxidant activity of the added sample. The peroxyl radical generator used was 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH).

The principle behind ORAC assay is to measure the chemical damage to fluorescent compound like PE through its decrease in fluorescence. The damage is caused by different oxidative species making conformational and chemical changes to protein. Since fluorescence is sensitive towards structural integrity, changes in structure are index

of oxidative damage. Thus inhibition of reactive species by antioxidant which reflects the protection against loss of fluorescence of PE is measure of its antioxidant activity against RS (Cao and Prior, 1999). But over the period of time PE has been observed to be inconsistent and unstable resulting into variable reactivity with peroxyl radicals. Considering disadvantages of PE a new probe for ORAC was developed by Ou et al., (2001). Fluorescein was considered as a new probe which is stable. Disodium salt of fluorescein is commercially available and provides direct measure of hydrophilic chain-breaking antioxidant capacity.

In the assay, fluorescein, antioxidant and excess of AAPH are mixed in a fluoremetric cuvette and fluorescence is measured over certain period of time. Higher the antioxidant activity of sample lower is the decay of fluorescence. In the presence of antioxidant, radicals preferentially react with antioxidant than fluorescein and thus fluorescence is preserved. The relative fluorescence is plotted against time and area under the curve (AUC) is calculated from the plot. AUC is then compared with standard antioxidant like trolox to obtain relative antioxidant capacity.

2.3.3.1 Reagent preparation:

For our experiments, a methodology established by Huang et al., (2002) was used to determine ORAC.

Phosphate buffer: A 75 mM phosphate buffer (pH = 7.4) was made by dissolving 1.58 g of NaH_2PO_4 and 8.78 g of Na_2HPO_4 in distilled water and made to 1 liter.

Trolox: Trolox was used as a standard antioxidant. A 100 μM stock solution of Trolox was made in phosphate buffer and stored at -51°C. Trolox solution stored at such conditions is stable for several months. Fresh dilutions (6.25, 12.5, 25 and 50 μM) were

made from stock solution for standard curve as required.

Fluorescein: Fluorescein stock solution 1 (1 mM) was prepared in 75 mM phosphate buffer (pH = 7.4). Stock solution 2 (4.19×10^{-3} mM) was made from the first by further diluting with buffer and stored in dark at 4°C. Fluorescein stored at such conditions lasts for several months. The 8.16×10^{-5} mM fresh working solution of fluorescein was made as required by further diluting the second stock solution.

AAPH: AAPH solution (radical generator) was made by dissolving 0.414 g of AAPH in 10 ml buffer to final concentration of 153 mM and stored in dark in ice bath. AAPH solution was made fresh every time and discarded after experiments.

2.3.3.2 Sample preparation:

20 ml of 70% ethanol was added to 0.5 g of sieved extrudate powder or flour premix and kept on shaker (1 hour) for extraction of antioxidants. After the extraction, the samples were centrifuged for 5 min to separate supernatant. Supernatants were further diluted appropriately with buffer before analysis.

2.3.3.3 Experimental protocol:

The assay was performed for extruded samples and equilibrated flour before extrusion. Extracts from extrudates were diluted 1:5 whereas extracts from equilibrated flour were diluted 1:8 with buffer solution before analysis. Appropriate dilutions were necessary so that the AUC obtained later were within the range of the standard curve. 2.25 ml of fluorescein solution was taken in fluoremetric cuvette and 375 µl of trolox solution (6.25, 12.5, 25, 50, 100 µM) or diluted sample or buffer solution (for blank) was added to it. The mixture was stirred continuously with magnetic stirrer and allowed to incubate at 37°C for 10 min. This is important step since ORAC assay is sensitive to

temperature. After incubation, 375 µl of AAPH was added quickly in all four cuvettes and fluorescence was measured after every 1 min for approximately 70 min till it dropped to 5% of the starting value. During the measurement, temperature of the cuvette was maintained constant and the chemicals were stirred continuously by magnetic stirrer. Varian Cary Eclipse fluorescence spectrometer in Dr. Ludescher's laboratory was used for the kinetic study.

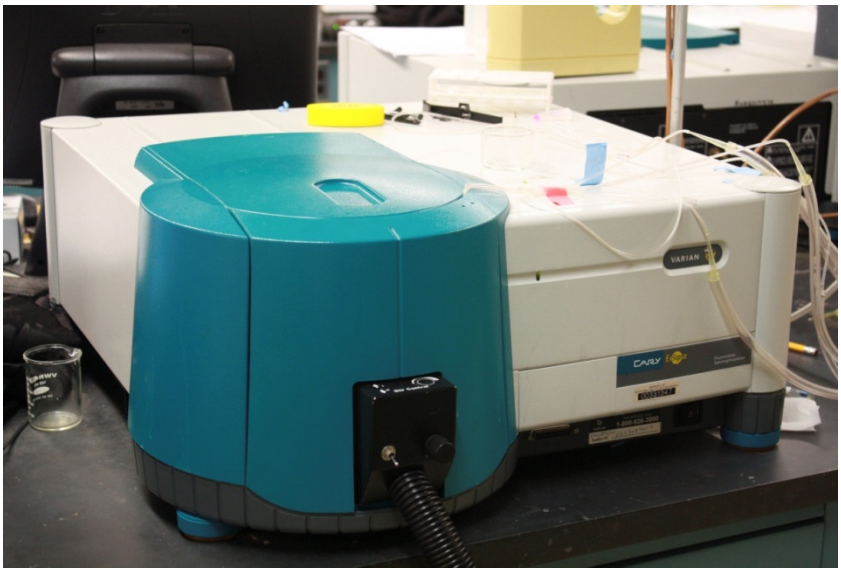


Figure 2.6: Cary Eclipse fluorescence spectrophotometer used for measuring ORAC

Table 2.2 shows the operating parameters for fluorescence spectrometer.

Table 2.2 Parameters for fluorescence spectrometer:

Parameter	Value
Excitation wavelength	485 nm
Emission wavelength	530 nm
Excitation slit width	10
Emission slit width	10
Temperature of the bolck	37°C

2.3.3.4 AUC and ORAC calculation:

Relative fluorescence was calculated as fluorescence at given time/fluorescence at t = 0. AUC was then calculated from the curve of relative fluorescence vs. time by following formula which is based on trapezoidal rule.

$$AUC = 0.5 + f_1/f_0 + \dots f_i/f_0 + \dots + f_{n-1}/f_0 + 0.5(f_n/f_0) \quad \text{(Eq. III)}$$

Where f_0 is the initial fluorescence at 0 min and f_i *is* fluorescence at time *i*. AUC thus calculated was then interpolated with the help of standard curve to calculate equivalent micromoles of trolox. For the standard curve, different concentrations of trolox were tested (6.25 µM to 100 µM) and relative fluorescence for each concentration was plotted against time. AUC of the blank was subtracted from AUC of trolox and normalized AUC was plotted against corresponding concentrations. The relative ORAC was calculated by following formula. The constant numbers appear due to dilutions of samples and expression of concentrations.

$$\text{ORAC (µMTE/100 g)} = \frac{\text{C} \times \text{Dilution factor} \times 2 \times 100}{50} \quad \text{(Eq. IV)}$$

Where:

C is concentration in µMTE, calculated by interpolation for each sample. Dilution factor was 5 for extrudates and 8 for flour premixes as stated before.

2.3.4 Analysis of total phenolic content (TPC):

Folin-Ciocalteau colorimetric method was used to determine total phenolics in extrudates and flour premix. The principle behind this method is chemical reduction of the Folin-Ciocalteau reagent which is mixture of tungsten and molybdenum oxides. The resulting reduction products are blue in color and have maximum broad light absorption at 765 nm. The oxidation reaction is slow and thus it takes some time for the

development of color. The reaction is sensitive to high temperature and color is degraded. The intensity of light absorption is directly proportional to concentration of total phenols (Singleton et al., 1999). The standard polyphenol used in the assay is gallic acid.

2.3.4.1 Preparation of reagents and sample:

Sample: 10 ml of 70% ethanol was added to 1 g of sieved extrudate powder or flour premix. The tubes were shaken on electronic shaker for 1 hour and then centrifuged for 5 min. Supernatants were then separated and volume was measured before analysis for total phenolics.

Folin-Ciocalteau reagent: Folin-Ciocalteau reagent was freshly prepared by diluting 1:1 with distilled water and stored in dark.

Sodium carbonate solution: 10 g of Na_2CO_3 was completely dissolved in distilled water and then was made to 50 ml to make 20% w/v solution. The solution was allowed to cool to room temperature.

2.3.4.2 Experimental protocol:

250 µl of sample was taken in a small test tube to which 250 µl of Folin-Ciocalteau reagent was added. To this, 500 µl of 20% Na_2CO_3 solution was added. The mixture was allowed to incubate at room temperature for 30 to 45 min in dark. After the full development of blue color, 1 ml water was added to the tube and tube was shaken well. The precipitate if any was allowed to settle and the clear solution was pipetted into 96-well plate for spectrometric reading. Absorbance was measured at 765 nm using spectrophotometer (Synergy HT) by Bio-tek instruments (Winooski, VT). For each sample, absorbance reading was corrected by subtracting the blank. Figure 2.7 shows the Bio-Tek spectrophotometer used to measure absorbance of samples.

Figure 2.7: Bio-Tek spectrophotometer with 96-well plate used for measurement of TPC

For standard curve, different concentrations of gallic acid (10, 20, 30, 40, 50 µg/ml) were analyzed for absorbance at 765 nm by same method as described above. The curve of absorbance vs. concentration was then plotted. After adjusting the dilutions and concentrations, total phenolics were reported as milligrams of gallic acid equivalents per 100 g of extrudate or premix.

2.3.5 Bulk Density:

Bulk density is defined as the total mass of the product divided by total volume occupied by the product. The volume also includes pore volume, particle volume and internal volume. Bulk density is calculated by following formula.

$$\rho = \frac{\text{Mass of the piece}}{\text{Volume of the piece}} \qquad \text{Eq. V}$$

where ρ is the bulk density.

For bulk density, extrudate was assumed to be a cylinder and thus diameter and length of each extrudate were measured. Mass of each piece was also measured. Volume of piece was calculated as $\pi r^2 l$ where l is the length and r is the radius of each piece.

2.3.6 Breaking strength:

Breaking strength is the maximum force required per unit area to break the piece of material. 10 pieces of extrudate were randomly chosen for measurement. A compression test was performed to measure breaking strength. All measurements were taken using Brookfield Texture Analyzer (CT3) (Middleboro, MA) (Figure 2.8).

Figure 2.8: Brookfield Texture Analyzer (CT3) used for measuring breaking strength

A specialized TA-VBJ (Volodkevitch bite jaw) probe was used for all measurements (Figure 2.9).

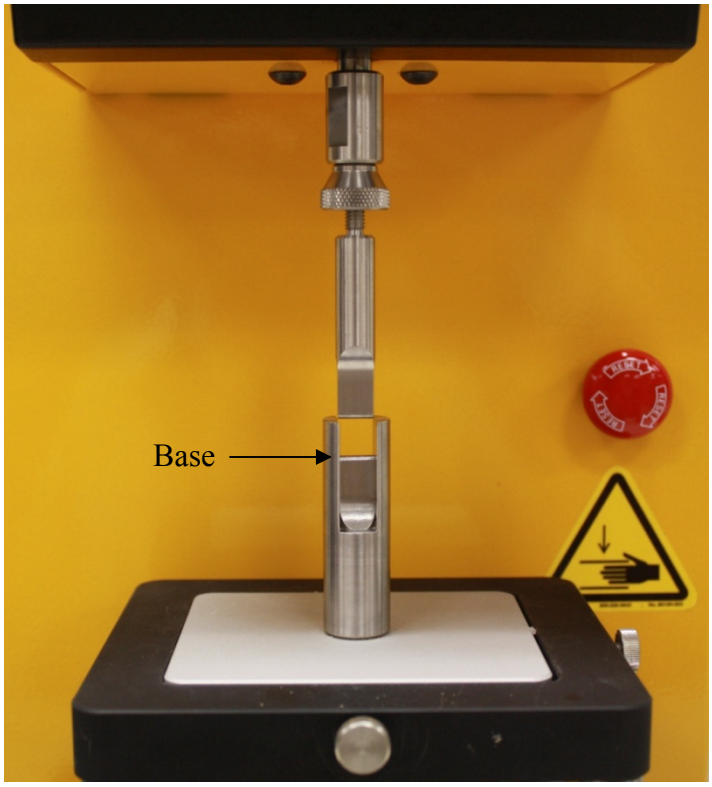


Figure 2.9: Brookfield TA-VBJ (Volodkevitch bite jaw) probe

Before any measurement, the instrument was calibrated to locate the base. After calibration, a small piece of extrudate was kept on the base and then was broken using the probe running down at constant speed. The data was recorded using TexturePro CT software provided alongwith. The parameters for texture analyzer are listed in Table 2.3.

Table 2.3 Parameters for texture analyzer:

Parameter	**Value**
Trigger Load	0.07 N
Test speed	1 mm/s
Percentage deformation target value	100%

The maximum load (g) to break the piece was noted from the software and was converted to Newtons. Cross sectional area of the extrudate was calculated as πr^2 where r is the radius of the extrudate. Breaking strength was expressed as maximum force per unit area (N/mm^2).

$$\text{Breaking strength} = \frac{\text{Maximum load to break extrudate (N)}}{\text{Area of cross section } (\pi r^2)(mm^2)} \qquad \text{(Eq. VI)}$$

2.3.7 Radial expansion index:

Radial expansion index (REI) is defined as the ratio of the diameter of the extrudate and the diameter of the die. Square of REI is called sectional expansion index (Alvarez-Martinez et al., 1988).

$$\text{REI} = \frac{D_e}{D_d} \qquad \text{(Eq. VII)}$$

where D_e is diameter of extrudate and D_d is the diameter of die.

2.3.8 Response surface methodology:

In order to study effects of control variables such as temperature, moisture content and powder content on output characteristics and minimize number of experiments, response surface methodology was used. It is a statistical and mathematical technique used to improve or optimize given process. It is used when there are several input parameters affecting some quality characteristic of the product. Usually the input variables are transformed into dimensionless coded variables commonly with mean value equal to zero. The true relation is unknown and thus it is assumed to be first order or second order polynomial. Most commonly there is interaction between two variables and thus first order model is inadequate. Second order model includes interaction effects too. Thus by adding interaction effects more accurate model can be developed. Second order

model is flexible and the coefficients of parameters can be determined easily. They also work out well to solve real response surfaces. The general equation of second order polynomial model is given by the following equation.

$$\eta = \beta_0 + \sum_{j=1}^{k} \beta_j x_j + \sum_{j=1}^{k} \beta_{jj} x_j^2 + \sum_{i<}^{} \sum_{j=2}^{k} \beta_{ij} x_i x_j \qquad \text{(Eq. VIII)}$$

where η is a specific response, β indicates different coefficients and x_1, x_2, x_3 etc. are coded variables.

Total of six responses, namely, ORAC value, total phenolics, color, breaking strength, bulk density and expansion index were studied. Three input variables were temperature (120°C-180°C), moisture content (18-24%) and powder content (3-5%). Analysis of our second order model was done using statistical analytical software (SAS) v9.2 with preset significance level of 5%.

3. RESULTS AND DISCUSSION

3.1 Proximate analysis of rice flour and Kokum powder:

Proximate analysis of rice flour was provided by Gulf Pacific Rice Company Inc. (Houstan, TX), as shown in Table 3.1.

Table 3.1 Proximate analysis of rice flour:

Component	Amount (%)
Moisture	11.62
Lipids	0.66
Protein	7.13
Carbohydrates	79.95
Ash	0.39
Vitamins and minerals	0.25

Moisture content of Kokum powder was found to be 5 to 6%. The fat content and protein content of Kokum powder (0.64% and 1.28% respectively) were obtained from Leco Corporation complying with AOAC methods (Michigan, USA).

3.2 Particle size distribution:

Table 3.2 Particle size distribution of rice flour and Kokum powder:

Mesh size	Mesh opening (mm)	Rice Flour		Kokum powder	
		Weight (g)	Percentage	Weight (g)	Percentage
70	0.212	21.76	11.12	26.08	19.38
80	0.180	17.39	8.89	92.95	69.06
100	0.150	14.92	7.62	12.33	9.16
200	0.075	134.59	68.78	3.21	2.38
325	0.045	5.19	2.65	0.01	0.01
<325	-	1.83	0.94	0.02	0.01

As seen from Table 3.2, Kokum powder was coarser than rice flour. But it was soluble in water, thus mixing of powder with rice and moisture was uniform.

3.3 Relative humidity and color:

Hue values for extrudates equilibrated at different relative humidity were plotted against their moisture content. It was found that moisture content of extrudates did not change the hue value significantly. In other words, moisture content of extrudates did not affect redness of samples.

Table 3.3 Hue values of extrudates equilibrated at different relative humidity:

Salt	Moisture (%)	Humidity (%)	Hue (Degrees)
LiCl	6.13	11.30	53.08 (0.09)
CH_3COOK	7.45	22.50	53.08 (0.18)
$MgCl_2$	8.07	33.10	52.97 (0.03)
$Mg(NO_3)_2$	9.24	54.50	54.12 (0.19)
$CuCl_2$	9.87	67.70	54.77 (0.09)
$NaNO_3$	10.57	75.40	55.84 (0.22)
K_2SO_4	13.71	97.60	58.57 (0.29)

Figure 3.1 shows the graph of hue vs. moisture content where it was seen that hue remains almost constant.

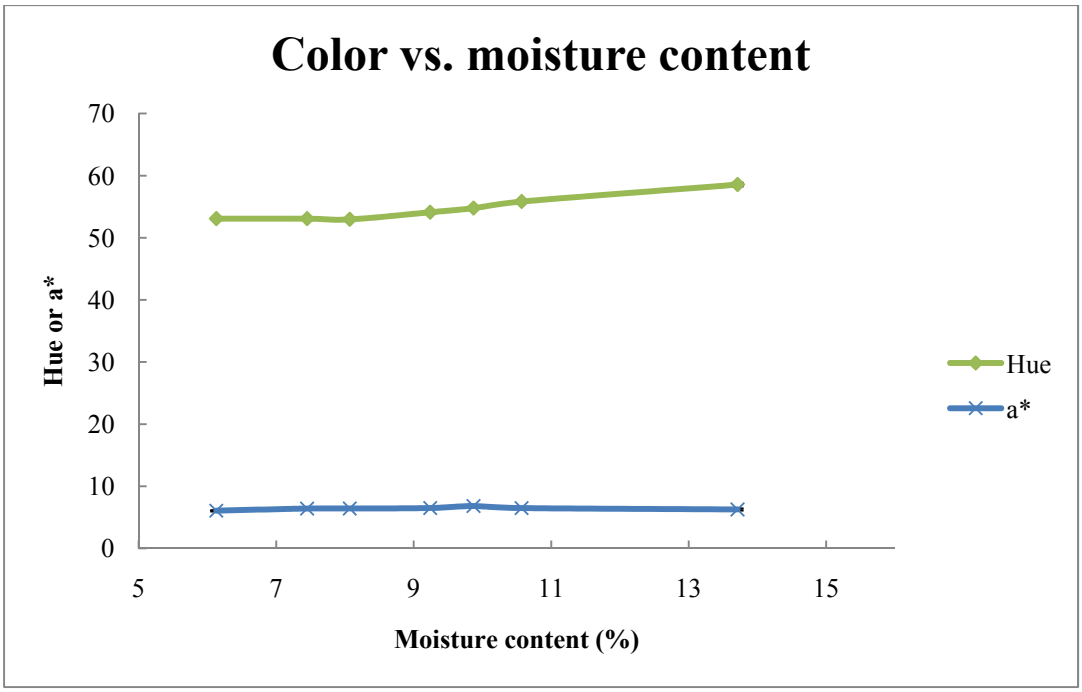


Figure 3.1: Graph of color vs. moisture content of extrudates with 3% Kokum powder

3.4 Color of extrudates:

Response surface methodology was used to determine the effect of control variables on properties of extrudates. Three dimensional response surface plots show the variation of response with any two selected factors. From preliminary study, it was observed that temperature and addition of powder were the major factors contributing to the color of extrudates. Hue and chroma values (calculated in triplicates) are shown in Table 3.4. Hue angle for Kokum powder was 13.6° (±0.1 S.D.) chroma was 46.24 (±1.04 S.D.).

Table 3.4 Hue and chroma values of extrudates:

Sr. No.	Temperature (°C)	Moisture (%)	Powder (%)	Hue	Chroma
1	150	18	5	20.97 (0.12)	13.88 (0.41)
2	150	21	4	34.08 (0.12)	12.42 (0.15)
3	150	18	3	46.76 (0.05)	10.29 (0.11)
4	150	24	3	48.71 (0.24)	10.96 (0.30)
5	150	24	5	23.69 (0.21)	14.78 (0.55)
6	120	18	4	25.50 (0.20)	12.01 (0.48)
7	120	21	3	36.08 (0.26)	10.95 (0.17)
8	180	18	4	47.33 (0.22)	11.84 (0.32)
9	180	21	3	49.53 (0.38)	11.03 (0.31)
10	150	21	4	33.34 (0.21)	12.38 (0.45)
11	180	21	5	47.95 (0.28)	12.92 (0.29)
12	120	24	4	22.49 (0.12)	12.81 (0.17)
13	180	24	4	44.14 (0.45)	12.48 (0.85)
14	120	21	5	15.80 (0.36)	14.40 (0.77)
15	150	21	4	34.11 (0.19)	12.28 (0.36)

Values indicated in brackets are standard deviations over triplicates. The data was analyzed using response surface analysis by SAS v9.2 to study the effect of input parameters on color and following equations were obtained. Equation 1 represents predictive model which includes only the major effects contributing towards the response that is hue whereas equation 2 represents master model which includes all effects. Variables are in coded levels.

$$\text{Hue} = 35.37 + 11.14*T - 9.08*P \qquad R^2 = 0.8767 \qquad \text{Eq. (1)}$$

$$\text{Hue} = 35.37 + 11.14*T - 0.19*M - 9.08*P + 1.66*T^2 - 0.05*T*M + 4.68*T*P - 0.64*M^2 + 0.19*M*P + 1.83*P^2 \qquad R^2 = 0.9361 \qquad \text{Eq. (2)}$$

Where T is temperature (°C), P is powder content (%) and M is moisture content (%).

A contour plot was generated based on the master model that is equation (2). Contour plot can be plotted by fixing one of the input variables and then varying the other

two. Variation of the response with the variation of any two input parameters is plotted. We observed that in predictive model for all responses, moisture term was generally absent or had negligible effect on response and thus for all contour plots we kept moisture content constant at 21%. Contours for hue values at fixed moisture content (21%) are shown in Figure 3.2.

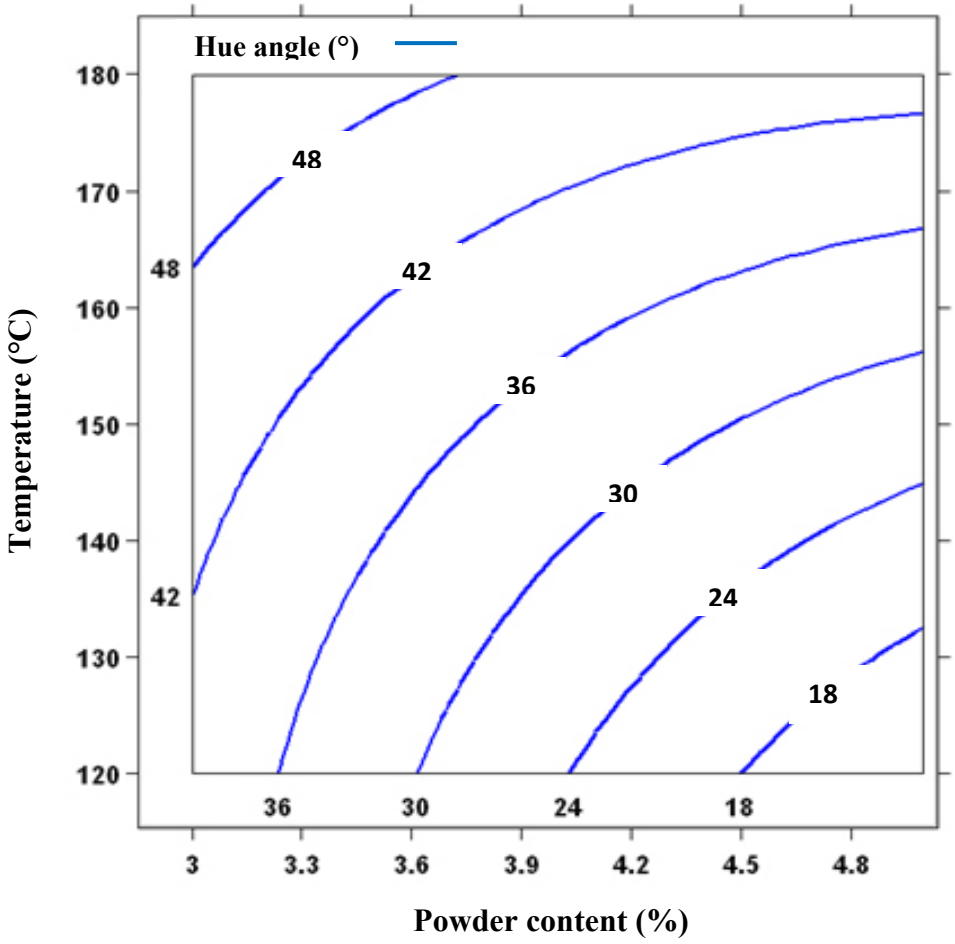


Figure 3.2: Contour plot for color of extrudates at fixed moisture content (21%)

As seen from above contours, as the powder content increased from 3 to 5% hue angle decreased and hence more redness. This result was expected since more powder was added. Also with the increase in temperature hue angle increased which means lesser

redness. This observation led to conclusion that anthocyanins might have degraded at high temperature. Figure 3.3 shows picture of different extrudates.

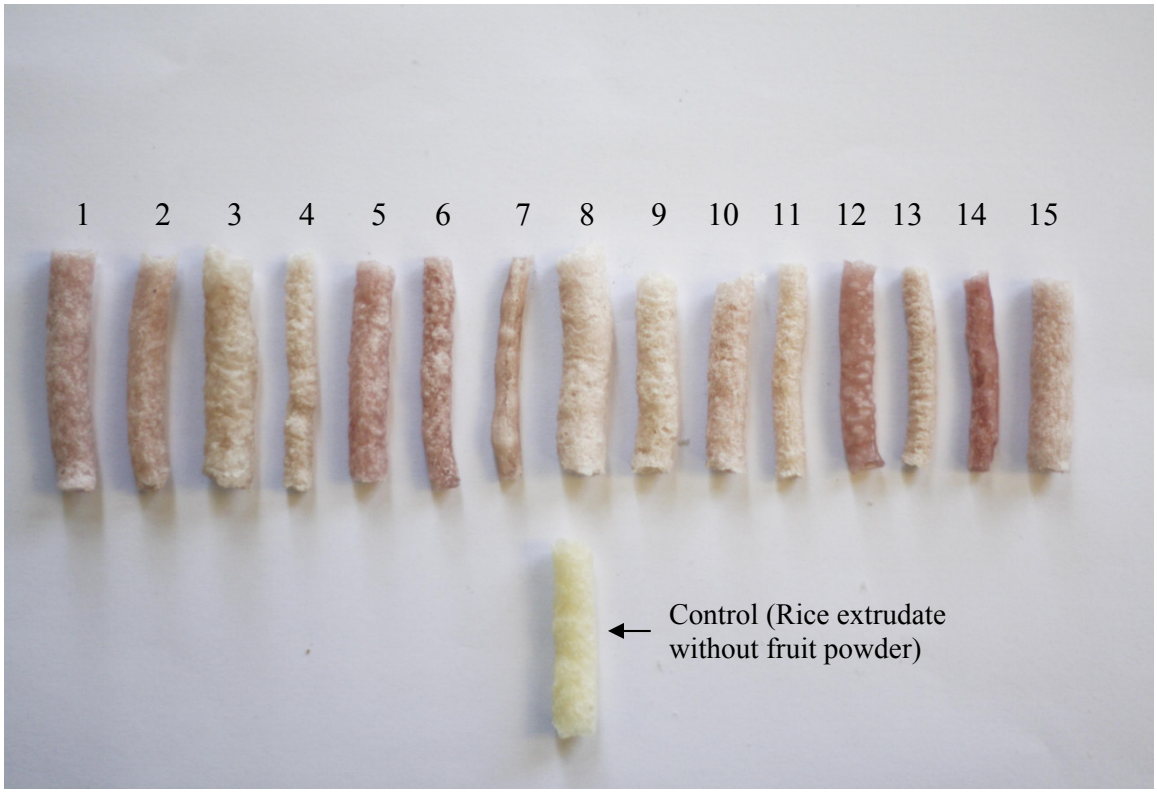


Figure 3.3: Picture of extrudates corresponding to run number

It was found that hue angle values of extrudates extruded at high temperature (180°C) with 4 or 5% powder were comparable to those at low temperature and low powder content. For example, sample extruded at 120°C, 21% moisture content and 3% powder had lesser hue angle than the one which was extruded at 180°C, 21% moisture and 5% powder. This clearly shows negative effect of temperature on anthocyanins. Lowest hue angle was found for sample extruded at low temperature and high powder content (experiment 14:- 120°C, 21% M.C. and 5% powder). At low temperature, loss of anthocyanins was less compared to that at 180°C.

3.5 ORAC of extrudates:

For ORAC values, temperature and percentage of powder were found to be the most important factors. Loss in ORAC value was different for different experiments. Figure 3.4 shows standard curves for different trolox concentrations.

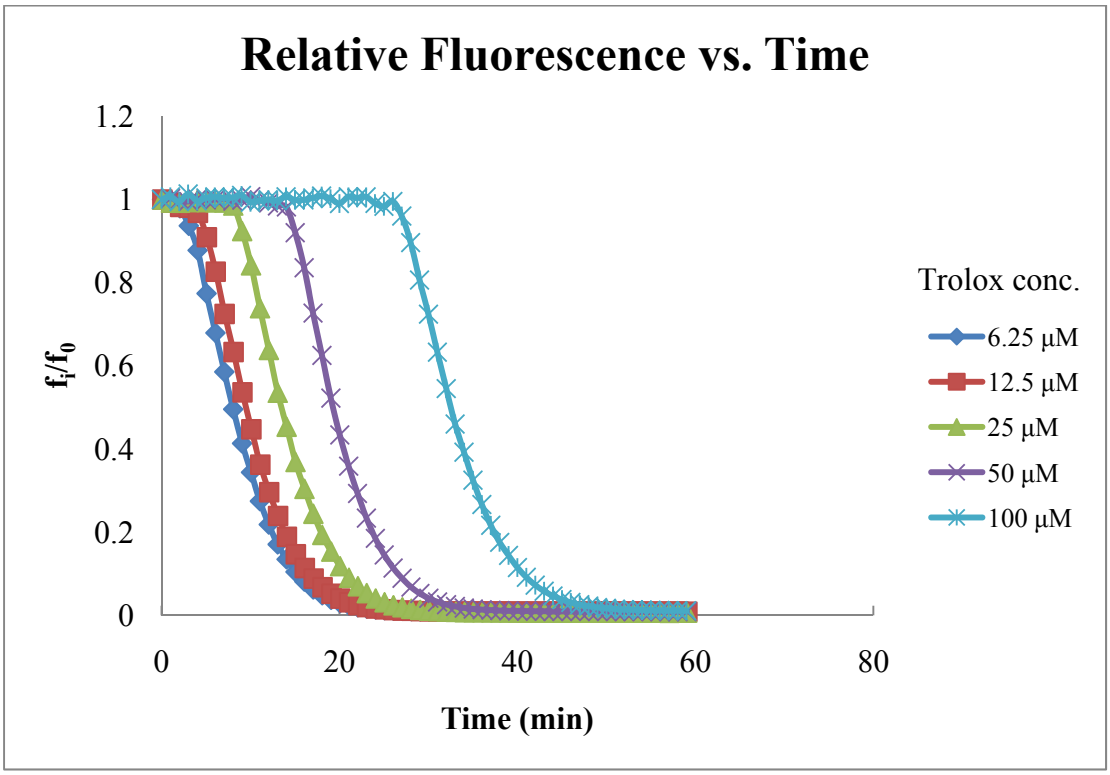


Figure 3.4 Graph showing standard curves with different trolox concentrations

This experiment was performed in triplicates so that three such graphs were obtained. (The above graph is one of the experiments). The corresponding area under the curve (AUC) for each curve was calculated and plotted against concentration. Average of three such curves was calculated and one master curve was obtained. The plot of area under the curve vs. concentration is shown in Figure 3.5.

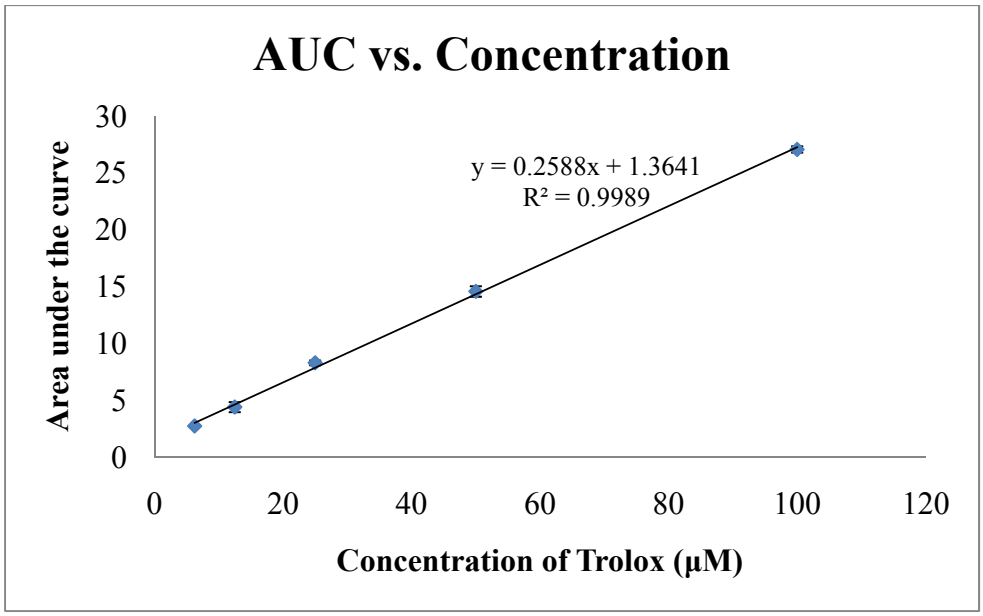


Figure 3.5 Standard curve of AUC vs. Concentration of Trolox

Standard curve shown in Figure 3.5 was used to calculate ORAC values of extrudates and flour before extrusion.

Table 3.5 ORAC values of extrudates and flour before extrusion:

Sr. No.	Temp. (°C)	Moisture Content (%)	Powder (%)	ORAC (µMTE/100g) Before	ORAC (µMTE/100g) After	Percentage Loss
1	150	18	5	1906 (284)	732 (26)	62
2	150	21	4	1637 (291)	496 (44)	70
3	150	18	3	1456 (171)	493 (58)	66
4	150	24	3	1559 (231)	521 (47)	67
5	150	24	5	2767 (119)	679 (15)	75
6	120	18	4	1211 (113)	761 (16)	37
7	120	21	3	1216 (229)	638 (17)	48
8	180	18	4	1215 (236)	674 (32)	45
9	180	21	3	718 (169)	634 (25)	12
10	150	21	4	1898 (282)	544 (32)	71
11	180	21	5	2174 (331)	840 (68)	61
12	120	24	4	1942 (133)	704 (2)	64
13	180	24	4	2049 (133)	935 (53)	54
14	120	21	5	2348 (307)	1002 (33)	57
15	150	21	4	1811 (153)	686 (56)	62

Table 3.5 shows the values of ORAC for extrudates and also for the flour before extrusion (dry basis) for comparison. Values in the bracket indicate corresponding standard deviations from triplicates. As seen from these values, ORAC was maximum at low temperature and high powder content (120°C, 21% M.C. and 5% powder). Interestingly, it was observed that loss of ORAC at 180°C was lower than that at 150°C at fixed powder content.

To study the effect of temperature and powder together as described above, a contour plot was built as shown in Figure 3.6. The corresponding equations based on predictive model (Eq. 3) and master model (Eq. 4) obtained from SAS are also given below.

$$ORAC = 593 - 2.75*T + 120.88*P + 180.5*T^2 \qquad R^2 = 0.7469 \qquad \text{(Eq. 3)}$$

$$ORAC = 593 - 2.75*T + 22.38*M + 120.88*P + 182.7*T^2 + 79.5*T*M - 39.5*T*P + 10.46*M^2 - 20.25*M*P + 20.46*P^2 \qquad R^2 = 0.8690 \qquad \text{(Eq. 4)}$$

A contour plot was built using master model equation. As seen from the equation and contours, powder content and temperature were the most important factors influencing ORAC.

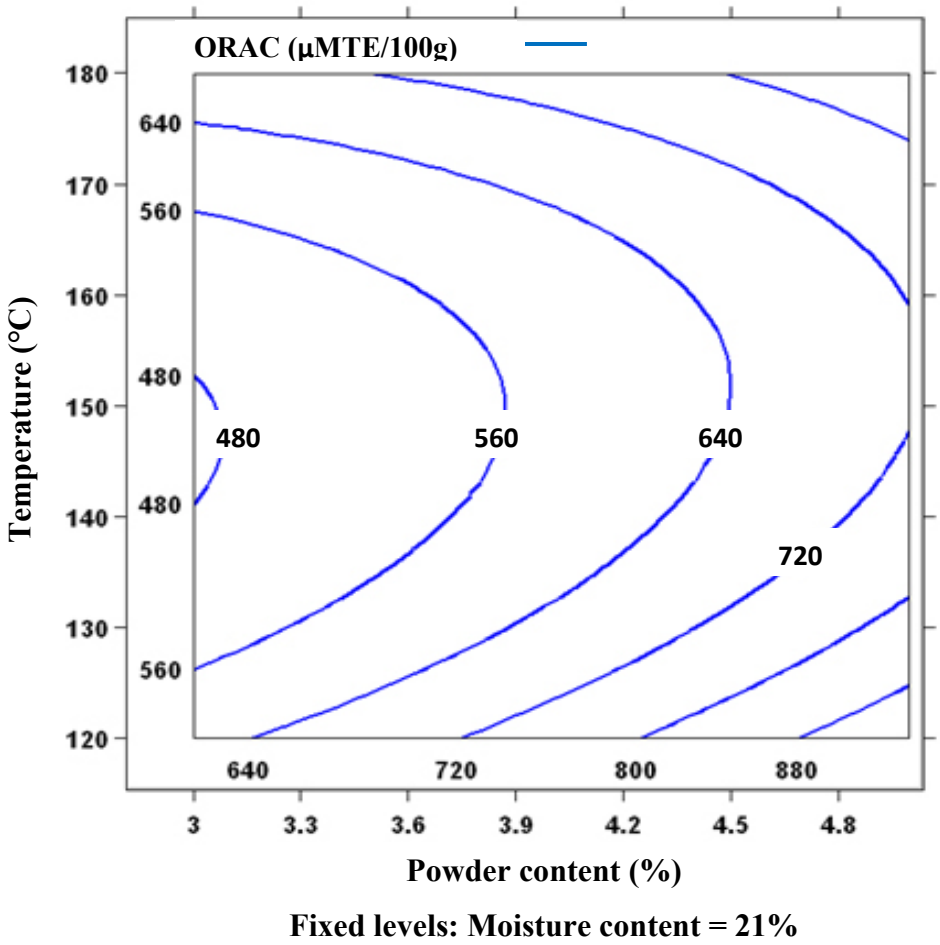


Figure 3.6: Contour plot for ORAC of extrudates at fixed feed moisture (21%)

On X-axis is the percentage of powder and on Y-axis is the temperature (°C).

3.6 Total phenolic content (TPC):

Total phenolics were determined by Folin-Ciocalteu method. It was observed that maximum amount of phenolics or the minimum loss was at low temperature and high powder content. For total phenolics, a gallic acid standard curve was built with concentration ranging from 0-50 µg/ml. Each point on the curve represents average of three. Curve shows standard concentrations of 10, 20, 30, 40 and 50 µg/ml and the corresponding absorbance. Figure 3.7 shows the standard curve built with different concentrations of gallic acid.

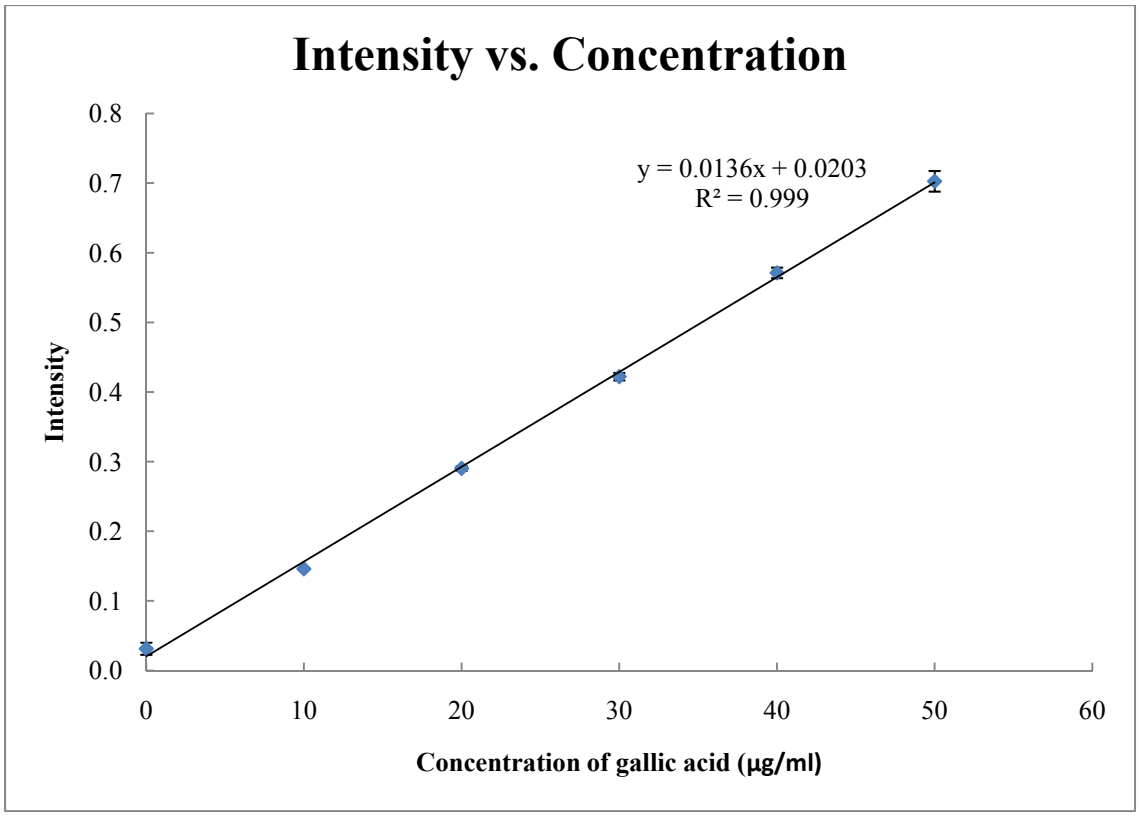


Figure 3.7: Standard gallic acid curve for determination of TPC

The curve shown in Figure 3.7 was used to measure TPC for extrudates and the flour before extrusion. The graph also shows the regression equation used for determination of TPC from samples and the corresponding R^2 value. Total phenolics for all extrudates and for flour before extrusion are given in Table 3.6.

Table 3.6 Total phenolics of extrudates and flour before extrusion:

Sr. No.	Temp. (°C)	Moisture content (%)	Powder (%)	TPC of flour (mg/100g)	TPC of extrudates (mg/100g)	Percentage loss (%)
1	150	18	5	23.17 (0.39)	11.35 (0.25)	51
2	150	21	4	22.13 (0.69)	8.6 (0.17)	61
3	150	18	3	19.14 (1.21)	8.77 (0.14)	54
4	150	24	3	20.10 (0.39)	8.80 (0.22)	56
5	150	24	5	23.66 (0.71)	10.37 (0.27)	56
6	120	18	4	20.63 (0.97)	10.39 (0.50)	50
7	120	21	3	20.16 (0.43)	10.11 (0.07)	50
8	180	18	4	22.07 (0.12)	12.61 (0.71)	43
9	180	21	3	20.39 (0.53)	10.58 (1.26)	48
10	150	21	4	21.55 (0.24)	9.96 (0.43)	54
11	180	21	5	23.63 (0.35)	15.86 (0.36)	33
12	120	24	4	22.59 (0.39)	10.01 (0.41)	56
13	180	24	4	21.70 (0.52)	14.03 (0.15)	35
14	120	21	5	24.08 (0.33)	12.05 (0.05)	50
15	150	21	4	21.84 (0.45)	8.62 (0.3)	61

TPC values indicated in Table 3.6 are on dry basis, i.e., per hundred grams of dry flour or dry extrudate powder. The numbers in brackets indicate the standard deviation over triplicates.

The effect of input variables on TPC was similar to that of ORAC and to show this effect, a contour plot at fixed feed moisture (21%) was built (Figure 3.8). The corresponding equations based on predictive model (Eq. 5) and master model (Eq. 6) are also shown below.

$$TPC = 9.50 + 1.32*T + 1.42*P + 2.46*T^2 \qquad R^2 = 0.8604 \qquad \text{(Eq. 5)}$$

$$TPC = 9.50 + 1.32*T + 0.013*M + 1.42*P + 2.52*T^2 + 0.45*T*M + 0.84*T*P + 0.19*M^2 - 0.25*M*P + 0.58*P^2 \qquad R^2 = 0.9448 \qquad \text{(Eq. 6)}$$

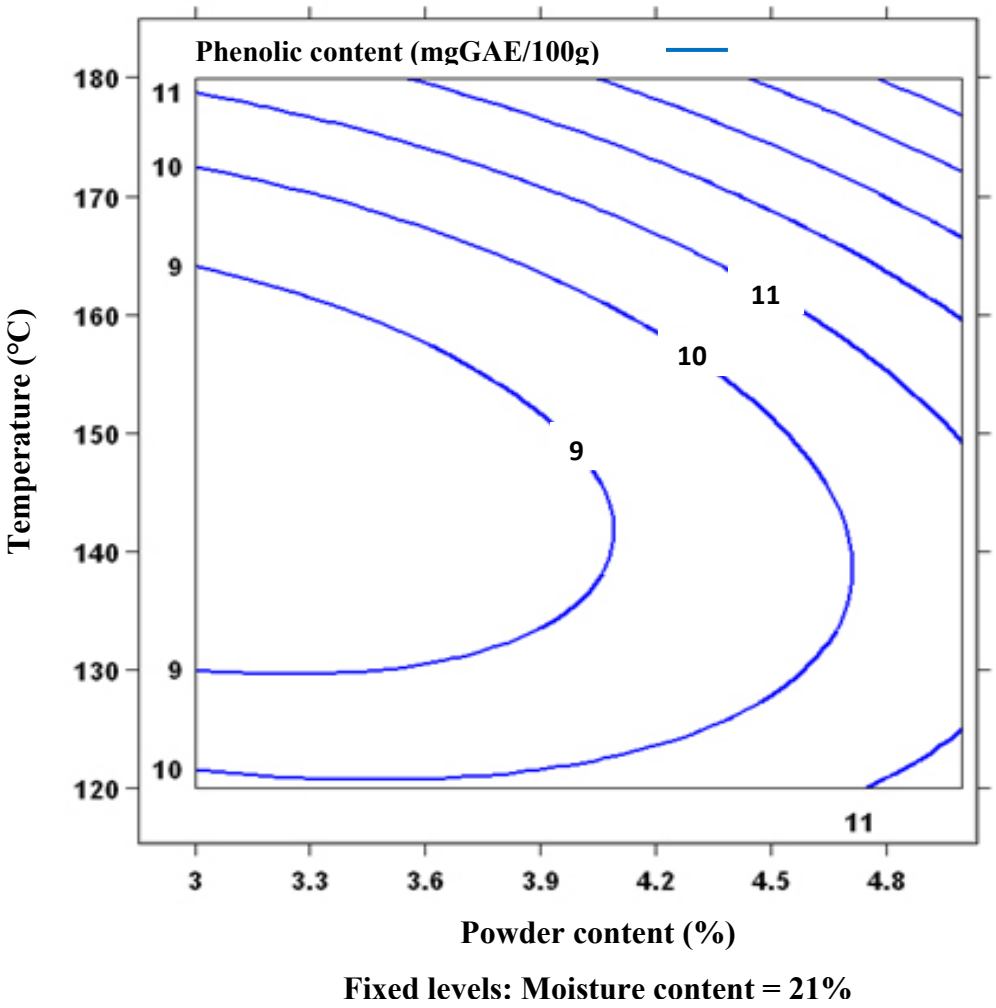


Figure 3.8: Contour plot for TPC of extrudates at fixed feed moisture (21%)

As seen from Figure 3.8, a similar trend as compared to ORAC was observed. Percentage loss of total phenolics was lesser at 180°C and 120°C. High temperature retention could be attributed to binding of phenolics through reactions making them stable where as at low temperature there is lesser degradation of phenolic compounds. As expected, when powder content increased TPC also increased. We also observed that there were significant losses of TPC from extrudates and thus addition of 3 to 5% of powder was not far enough. The TPC values ranged from 8 to 15 mg/100g which were quite low. Also since the exact mechanism behind higher retention of total phenolics at

high temperature is not known, it needs further study.

3.7 Breaking strength:

Breaking strength of extrudates over the range of parameters was studied. Breaking strength values are shown in Table 3.7.

Table 3.7 Breaking strength of extrudates:

Exp. No.	Temp. (°C)	Moisture (%)	Powder (%)	Breaking strength (N/mm^2)
1	150	18	5	0.51 (0.06)
2	150	21	4	1.41 (0.23)
3	150	18	3	0.51 (0.14)
4	150	24	3	1.52 (0.36)
5	150	24	5	0.98 (0.12)
6	120	18	4	1.34 (0.34)
7	120	21	3	5.47 (0.28)
8	180	18	4	0.39 (0.10)
9	180	21	3	1.16 (0.29)
10	150	21	4	1.11 (0.26)
11	180	21	5	0.87 (0.31)
12	120	24	4	1.44 (0.31)
13	180	24	4	1.23 (0.29)
14	120	21	5	1.70 (0.38)
15	150	21	4	1.08 (0.22)

As seen from Table 3.7, the maximum breaking strength was observed at 120°C, 21% moisture and 3% powder. To study the effect of different input variables on breaking strength of extrudates, a contour plot was developed at fixed moisture content (21%). The equations based on predictive model (eq. 7) and master model (eq. 8) are given below.

$$\text{Breaking strength} = 1.38 - 0.79*T \qquad R^2 = 0.2480 \quad \text{(Eq. 7)}$$

$$\text{Breaking strength} = 1.38 - 0.79*T + 0.30*M - 0.58*P + 0.66*T^2 + 0.19*T*M + 0.87*T*P - 0.76*M^2 - 0.14*M*P + 0.44*P^2 \qquad R^2 = 0.8184 \quad \text{(Eq. 8)}$$

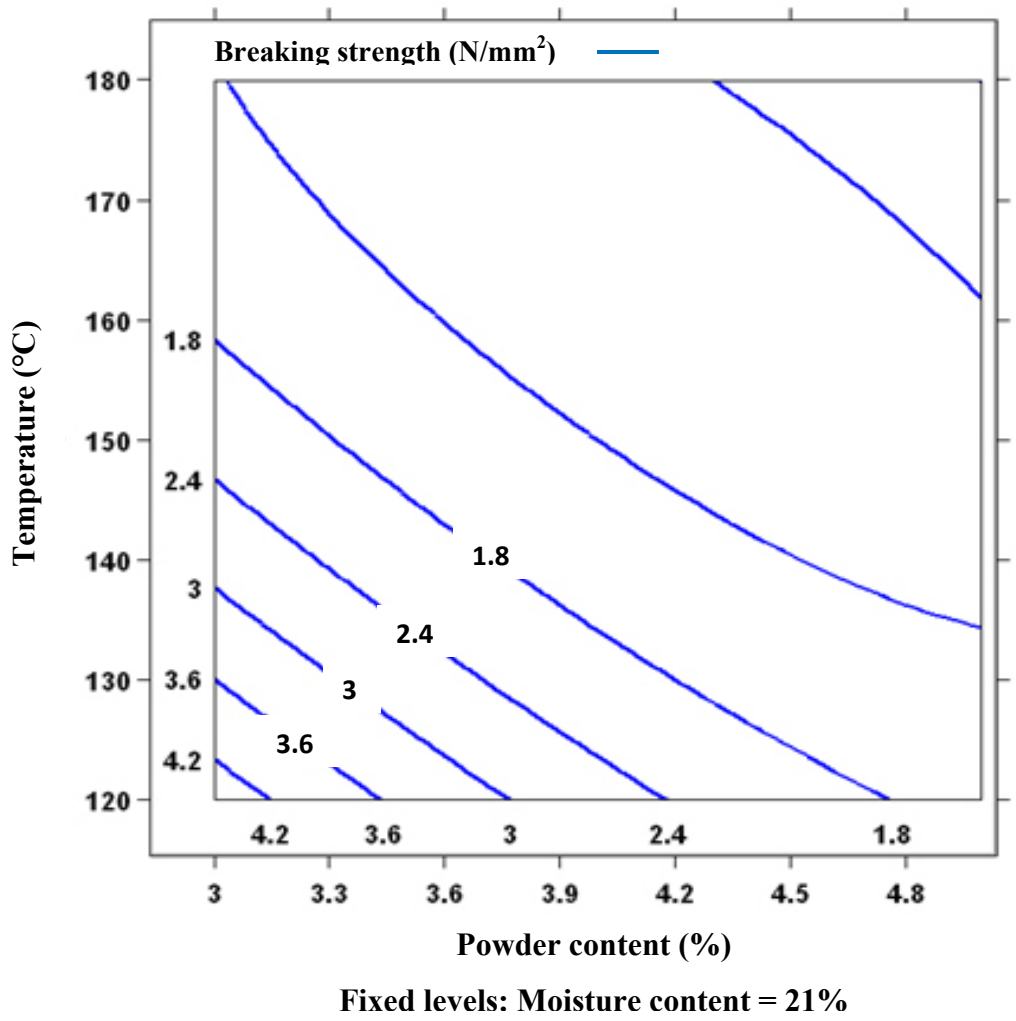


Figure 3.9: Contour plot for breaking strength of extrudates at fixed feed moisture (21%)

As seen from the contour plot (Figure 3.9), breaking strength decreases with high temperature and more powder addition. Breaking strength is higher at lower temperature because at low temperature there is less flashing of moisture and thus extrudate structure is compact. At high temperature, due to expansion and flashing of moisture, structure is porous and thus fragile. This results into lesser breaking strength. The addition of powder might have changed the microstructure of extrudates and hence change in breaking strength.

3.8 Expansion index:

Expansion index was found to vary with the temperature, moisture and powder content. Table 3.8 shows the values of expansion index.

Table 3.8 Expansion index of extrudates:

Sr. No.	Temperature (°C)	Moisture (%)	Powder (%)	Expansion index
1	150	18	5	2.59 (0.02)
2	150	21	4	2.39 (0.06)
3	150	18	3	3.38 (0.04)
4	150	24	3	2.06 (0.11)
5	150	24	5	2.32 (0.05)
6	120	18	4	2.03 (0.04)
7	120	21	3	1.59 (0.04)
8	180	18	4	2.85 (0.06)
9	180	21	3	2.44 (0.06)
10	150	21	4	2.41 (0.03)
11	180	21	5	1.85 (0.07)
12	120	24	4	1.91 (0.03)
13	180	24	4	1.79 (0.07)
14	120	21	5	1.81 (0.03)
15	150	21	4	2.5 (0.05)

The values in brackets show the standard deviation over triplicates. It was observed that expansion index increased with increase in temperature. Similarly it also increased with decrease in moisture. Expansion index is the measure of expansion of product. Figure 3.10 shows the effect of control variables on expansion index. The equations based on predictive (eq. 9) and master (eq. 10) model are given below.

$$\text{Expansion index} = 2.41 + 0.19*T - 0.35*M - 0.11*P - 0.47*T^2 - 0.24*T*M - 0.20*T*P + 0.19*M^2 + 0.26*M*P \qquad R^2 = 0.9871 \qquad \text{(Eq. 9)}$$

$$\text{Expansion index} = 2.41 + 0.19*T - 0.35*M - 0.11*P - 0.48*T^2 - 0.24*T*M - 0.20*T*P + 0.19*M^2 + 0.26*M*P - 0.03*P^2 \qquad R^2 = 0.9885 \qquad \text{(Eq. 10)}$$

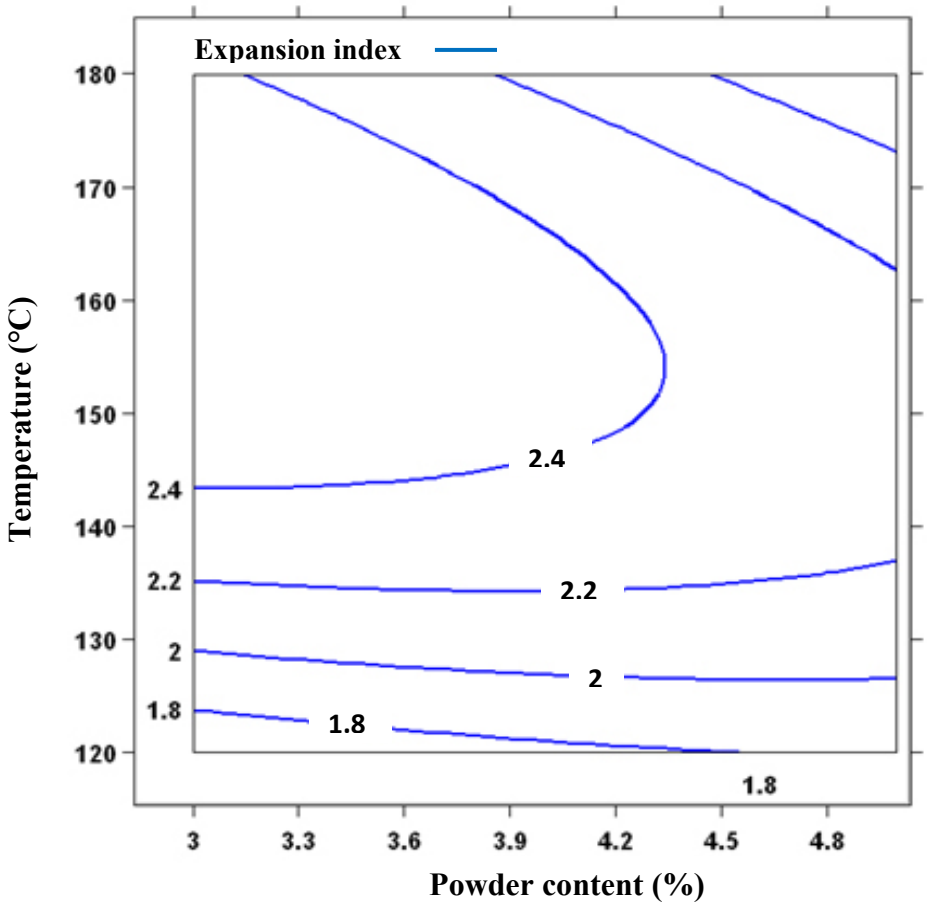


Figure 3.10: Contour plot for expansion index of extrudates at fixed feed moisture (21%)

As seen from the contour plot (Figure 3.10), expansion index increased with the increase in temperature but there was little effect of powder addition. At high temperature, moisture is flashed off under pressure as steam and hence there is increased expansion. Similarly at high moisture content there is not enough flashing and thus product is less expanded. Similarly at low temperature there is not enough heat for flashing of water and thus again lower expansion. Thus for maximum expansion of the product, temperature should be high and feed moisture should be low.

3.9 Bulk density:

Bulk density was calculated as the total mass of extrudate over its total volume. It was observed that at high temperature and low moisture, bulk density was low but with higher moisture content it was higher. Table 3.9 shows the bulk density data for extrudates.

Table 3.9 Bulk density of extrudates:

Sr. No.	Temperature (°C)	Moisture (%)	Powder (%)	Bulk Density (kg/m^3)
1	150	18	5	388 (6)
2	150	21	4	479 (12)
3	150	18	3	253 (8)
4	150	24	3	482 (17)
5	150	24	5	474 (17)
6	120	18	4	583 (21)
7	120	21	3	912 (20)
8	180	18	4	232 (13)
9	180	21	3	363 (21)
10	150	21	4	491 (15)
11	180	21	5	347 (14)
12	120	24	4	641 (14)
13	180	24	4	432 (17)
14	120	21	5	710 (13)
15	150	21	4	450 (12)

A contour plot (Figure 3.11) was built to study effects of input variables on bulk density. The equations based on predictive model (Eq. 11) and master model (Eq. 12) are as follows.

$$\text{Bulk density} = 482.47 - 184*T \qquad R^2 = 0.6267 \qquad \text{(Eq. 11)}$$

$$\text{Bulk density} = 482.47 - 184*T + 71.63*M - 11.38*P + 91.21*T^2 + 35.5*T*M + 46.5*T*P - 92.41*M^2 - 35.75*M*P + 18.46*P^2 \qquad R^2 = 0.9267 \qquad \text{(Eq. 12)}$$

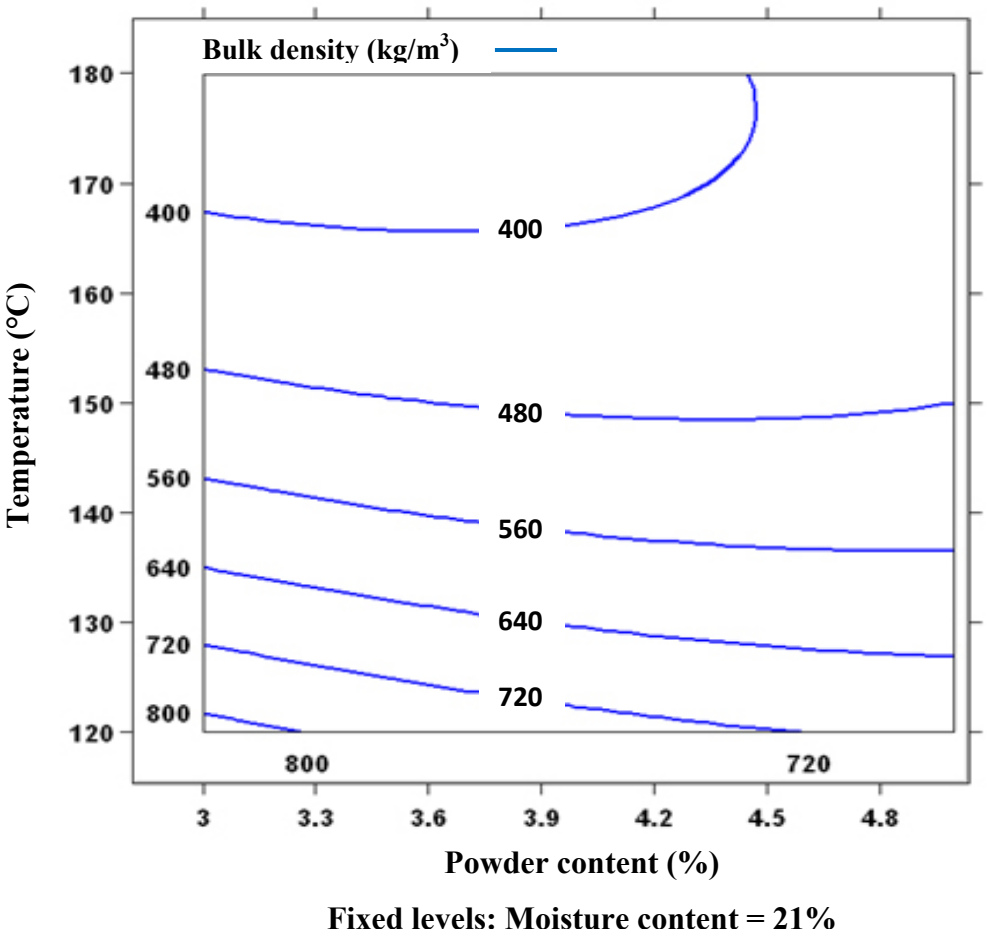


Figure 3.11: Contour plot for bulk density of extrudates at fixed feed moisture (21%)

As seen from the contour plot (Figure 3.11), bulk density decreased with the temperature. Addition of powder did not affect bulk density much. At low temperature due to less flashing of moisture, the extrudate structure is compact and dense. Thus porosity is less and bulk density is high whereas at high temperature, expansion is more due to more flashing of moisture. This results into porous structure and thus bulk density is lower. Similarly if moisture content is high, more moisture is retained compared to low feed moisture extrusion and thus compact structure. Higher bulk density also means higher breaking strength since the structure is dense and needs more force to break. At the same time, since structure is compact, expansion is the least. This can be verified

from our results since we observed least expansion index for extrudate which had maximum bulk density and maximum breaking strength (exp. 7). Minimum bulk density was 232 kg/m^3 at 180°C, 18% feed moisture and 4% powder content.

The scatter plot in Figure 3.12 shows the relation between expansion index and bulk density. The general trend is that, as the bulk density increased, expansion index decreased.

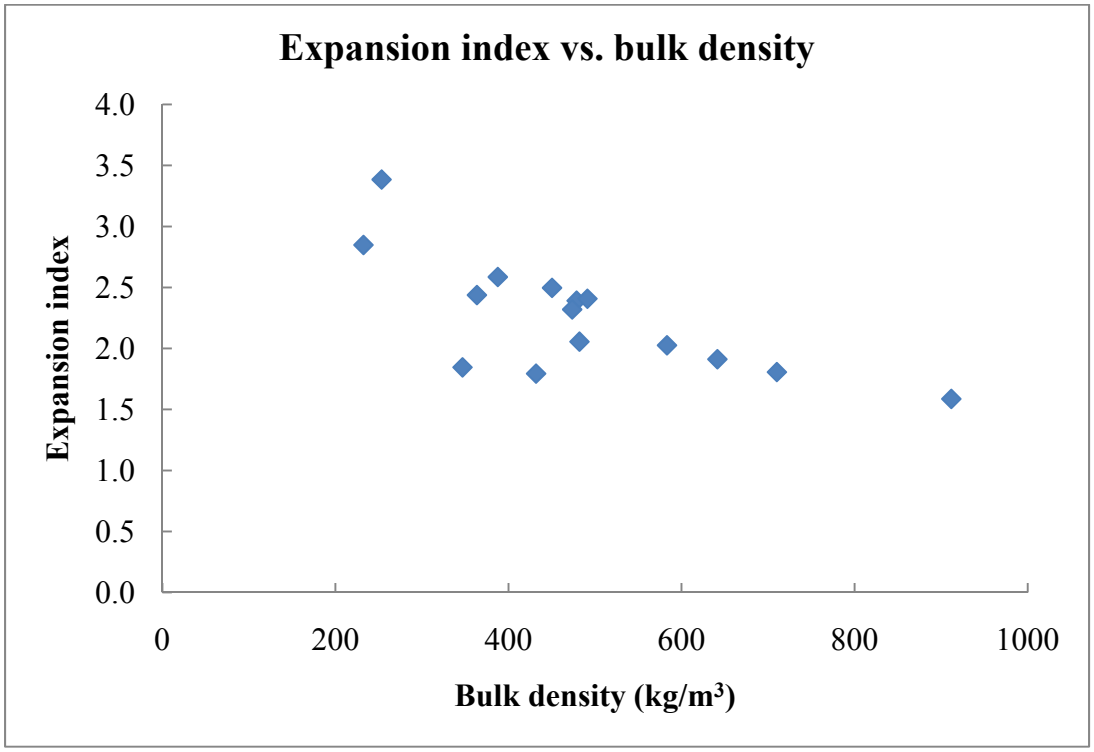


Figure 3.12: Scatter plot of expansion index vs. bulk density of extrudates

4. CONCLUSIONS

Following conclusions can be drawn from our research:

1) Single screw extrusion of rice flour with Kokum powder was possible only when moisture content was more than or equal to 18% of total feed premix. Addition of powder made extrusion slightly difficult than that of rice flour alone. When powder was added at level more than 5%, color was too dark giving unpleasant appearance. Extrusion was possible at temperatures up to 180°C beyond which product started burning and falling apart.

2) With addition of Kokum powder up to 5%, extrudates showed excellent red color. The red color of extrudates, which was due to anthocyanins, was stable at low temperatures <=150°C. At high temperature (180°C), pigments started degrading and hence product showed lesser redness. With high feed moisture, shear was less and thus some protection to anthocyanins but effect of temperature was more prominent. Thus to get good appearance and avoid loss of anthocyanins, extrusion should be carried out at low temperature (<150°C) and high feed moisture (21-24%) with appropriate addition of powder (4-5%).

3) Antioxidant activity and total phenolic content of extrudates were affected by extrusion processing. Loss of phenolic compounds was lower at 120°C and 180°C than at 150°C. One of the hypotheses for high temperature retention of phenolic compounds could be the molecular binding of phenolics with other ingredients in food matrix which make them stable from high heat and shear. Other possible reason could be release of free phenolics, e.g. hydrolysis of tannins, which is not possible at low temperature. Again, high feed moisture helped in reducing the shear and protecting phenolic compounds.

Addition of some other form of fruit may help in increasing phenolic content. The phenolic content of extrudates was very low, so more amount of powder or other form of fruit is needed to increase TPC and ORAC significantly. Extrusion processing caused heavy losses of phenolic compounds and antioxidant activity. Since losses in phenolics and antioxidant activity were minimum at 120°C and 180°C, low temperature or high temperature extrusion cooking with high feed moisture would be advisable.

4) Bulk density, expansion index and breaking strength were co-related to each other. At low temperature (120°C) and high moisture content (24%) bulk density was maximum and hence expansion index was minimum. This was observed due to the compact and dense structure and thus breaking strength was also maximum. At high temperature (150°C to 180°C) and low moisture (18%) expansion index was high and bulk density was minimum. This was observed due to high flashing of moisture under pressure resulting in expanded product. Thus breaking strength was minimum. The effect of addition of powder on bulk density and expansion index of extrudates was less prominent than temperature.

To summarize our conclusions, *Garcinia indica* can be used as a colorant and antioxidant in an extruded food product, but extrusion causes heavy losses of phenolic compounds and antioxidant activity. Also, there is no one set of optimum conditions giving the best product in terms of all physical and chemical properties. One or more properties have to be sacrificed in order to get the better product in terms of others. For example, at low temperature and high moisture we have extrudate with good color, minimal loss of phenolics and antioxidant activity but bulk density is very high giving harder product perhaps undesirable for consumption.

5. FUTURE WORK

We found 5% addition of powder was not enough to make antioxidant rich product due to losses during extrusion. Thus a different form of Kokum fruit can be used. For example, fruit pulp or fruit pomace can be tested for its extrudability with rice flour and its potential for antioxidant activity. Fruit pomace is expected to contain all bioactive compounds of the fruit.

Shelf-life study of extrudates is important which will determine stability of product and its components. Phenolics and antioxidant activity can be measured as a function of time to determine stability of phenolics in extrudates.

The reason behind higher retention of phenolics at high temperature is still not fully clear and needs further study and investigation. A further study on chemistry of phenolic compounds and their interaction with food matrix would explain this phenomenon.

The effects of twin screw extrusion on phenolic content and antioxidant capacity of extrudates can be studied.

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Appendix

Typical datasheet generated by SAS v9.2 for statistical analysis

ADX Report

Today's date: 31AUG2010
Experiment creation date: 31AUG2010

DESIGN DETAILS

Design type: Response Surface
Design description: Box-Behnken
Number of factors: 3
Number of runs: 15

Customization:

FACTORS

Factors and Levels:

Factor	Low	Center	High
T	120	150	180
M	18	21	24
P	3	4	5

RESPONSE

Response
ORAC
PHENOLICS
COLOR
BRSTRNGTH
EXINDEX
BULKDENS

DESIGN POINTS (Coded)

RUN	T	M	P	ORAC	PHENOLICS	COLOR	BRSTRNGTH	EXINDEX	BULKDENS
1	-1	-1	0	761	10.39	25.50	1.05	2.03	583
2	-1	1	0	704	10.01	22.49	1.13	1.91	641
3	1	-1	0	674	12.61	47.33	0.30	2.85	232
4	1	1	0	935	14.04	44.14	0.96	1.79	432
5	0	-1	-1	493	8.77	46.76	0.40	3.38	253
6	0	-1	1	732	11.35	20.97	0.40	2.59	388
7	0	1	-1	521	8.80	48.71	1.20	2.06	482
8	0	1	1	679	10.37	23.69	0.77	2.32	474
9	-1	0	-1	638	10.11	36.08	4.30	1.59	912
10	1	0	-1	634	10.58	49.53	0.91	2.44	363
11	-1	0	1	1002	12.05	15.80	1.33	1.81	710
12	1	0	1	840	15.86	47.95	0.69	1.85	347
13	0	0	0	686	8.60	34.08	1.11	2.39	479
14	0	0	0	544	9.96	33.34	0.87	2.41	491
15	0	0	0	496	8.62	34.11	0.85	2.50	450

DESIGN POINTS (Uncoded)

RUN	T	M	P	ORAC	PHENOLICS	COLOR	BRSTRNGTH	EXINDEX	BULKDENS
1	120	18	4	761	10.39	25.50	1.05	2.03	583
2	120	24	4	704	10.01	22.49	1.13	1.91	641
3	180	18	4	674	12.61	47.33	0.30	2.85	232
4	180	24	4	935	14.04	44.14	0.96	1.79	432
5	150	18	3	493	8.77	46.76	0.40	3.38	253
6	150	18	5	732	11.35	20.97	0.40	2.59	388
7	150	24	3	521	8.80	48.71	1.20	2.06	482
8	150	24	5	679	10.37	23.69	0.77	2.32	474
9	120	21	3	638	10.11	36.08	4.30	1.59	912
10	180	21	3	634	10.58	49.53	0.91	2.44	363
11	120	21	5	1002	12.05	15.80	1.33	1.81	710
12	180	21	5	840	15.86	47.95	0.69	1.85	347
13	150	21	4	686	8.60	34.08	1.11	2.39	479
14	150	21	4	544	9.96	33.34	0.87	2.41	491
15	150	21	4	496	8.62	34.11	0.85	2.50	450

FIT DETAILS FOR PHENOLICS:

ANOVA for PHENOLICS

	Master Model					Predictive Model				
Source	DF	SS	MS	F	Pr > F	DF	SS	MS	F	Pr > F
T	1	13.86011	13.86011	20.51268	0.0062	1	13.86011	13.86011	17.85337	0.0014
M	1	0.00125	0.00125	0.00185	0.9674					
P	1	16.15961	16.15961	23.9159	0.0045	1	16.15961	16.15961	20.81538	0.0008
T*T	1	23.35468	23.35468	34.56444	0.0020	1	22.60248	22.60248	29.11451	0.0002
T*M	1	0.819025	0.819025	1.21214	0.3211					
T*P	1	2.7889	2.7889	4.127515	0.0979					
M*M	1	0.129808	0.129808	0.192113	0.6795					
M*P	1	0.255025	0.255025	0.377432	0.5659					
P*P	1	1.220769	1.220769	1.806714	0.2367					
Model	9	57.78341	6.420379	9.50203	0.0116	3	52.62221	17.54074	22.59442	<.0001
(Linear)	3	30.02098	10.00699	14.81014	0.0064					
(Quadratic)	3	23.89949	7.966497	11.79025	0.0105					
(Cross Product)	3	3.86295	1.28765	1.905696	0.2467					
Error	5	3.378425	0.675685			11	8.539634	0.77633		
(Lack of fit)	3	2.163225	0.721075	1.186759	0.4876	5	5.749134	1.149827	2.472303	0.1506
(Pure Error)	2	1.2152	0.6076			6	2.7905	0.465083		
Total	14	61.16184				14	61.16184			

Fit Statistics for PHENOLICS

	Master Model	Predictive Model
Mean	10.808	10.808
R-square	94.48%	86.04%
Adj. R-square	84.53%	82.23%
RMSE	0.822001	0.881096
CV	7.605483	8.152259

Canonical Analysis: Stationary point for PHENOLICS

Stationary point:	Critical value is a Minimum
Predicted response at stationary point:	7.996259
Standard error of predicted value:	1.429832

Canonical Analysis: Critical value for PHENOLICS

Factor Name	Coded	Uncoded
T	0.11444	153.433
M	-1.24335	17.270
P	-1.59196	2.408

Canonical Analysis: Eigenvectors for PHENOLICS

Eigenvalues	T	M	P
2.61695	0.97749	0.08091	0.19486
0.56187	-0.14802	-0.39519	0.90660
0.09868	-0.15035	0.91503	0.37432

Ridge Analysis for PHENOLICS

Radius	Predicted Response	Standard Error	Dependent variable	Type of ridge	T	M	P
0.0	9.0600	0.47458	PHENOLICS	MINIMUM	0.00000	0.00000	0.00000
0.1	8.8841	0.47311	PHENOLICS	MINIMUM	-0.05959	-0.00036	-0.08030
0.2	8.7391	0.46887	PHENOLICS	MINIMUM	-0.10078	-0.00151	-0.17275
0.3	8.6183	0.46243	PHENOLICS	MINIMUM	-0.12459	-0.00616	-0.27283
0.4	8.5166	0.45491	PHENOLICS	MINIMUM	-0.13492	-0.01710	-0.37617
0.5	8.4301	0.44800	PHENOLICS	MINIMUM	-0.13569	-0.03668	-0.47984
0.6	8.3563	0.44389	PHENOLICS	MINIMUM	-0.12979	-0.06671	-0.58198
0.7	8.2933	0.44512	PHENOLICS	MINIMUM	-0.11917	-0.10833	-0.68122
0.8	8.2396	0.45415	PHENOLICS	MINIMUM	-0.10527	-0.16171	-0.77638
0.9	8.1937	0.47299	PHENOLICS	MINIMUM	-0.08916	-0.22600	-0.86659
1.0	8.1546	0.50287	PHENOLICS	MINIMUM	-0.07166	-0.29954	-0.95139
0.0	9.0600	0.47458	PHENOLICS	MAXIMUM	0.00000	0.00000	0.00000
0.1	9.2736	0.47311	PHENOLICS	MAXIMUM	0.07460	0.00128	0.06658
0.2	9.5300	0.46886	PHENOLICS	MAXIMUM	0.15899	0.00396	0.12126
0.3	9.8327	0.46233	PHENOLICS	MAXIMUM	0.24905	0.00789	0.16707
0.4	10.1839	0.45447	PHENOLICS	MAXIMUM	0.34227	0.01277	0.20661
0.5	10.5848	0.44673	PHENOLICS	MAXIMUM	0.43729	0.01833	0.24174
0.6	11.0361	0.44117	PHENOLICS	MAXIMUM	0.53337	0.02440	0.27372
0.7	11.5385	0.44038	PHENOLICS	MAXIMUM	0.63009	0.03083	0.30338
0.8	12.0923	0.44736	PHENOLICS	MAXIMUM	0.72720	0.03755	0.33132
0.9	12.6976	0.46507	PHENOLICS	MAXIMUM	0.82457	0.04448	0.35792
1.0	13.3547	0.49587	PHENOLICS	MAXIMUM	0.92211	0.05158	0.38348

Alias Structure for PHENOLICS

Master Model	Predictive Model
No effects aliased.	No effects aliased.

Predictive Model for PHENOLICS

Coded Levels(-1,1):

PHENOLICS = 9.495714 + 1.31625*T + 1.42125*P + 2.460536*T*T

Uncoded Levels:

PHENOLICS = 58.74286 - 0.776304*T + 1.42125*P + 0.002734*T*T

Effect Estimates for PHENOLICS

	Master Model				Predictive Model			
Term	Estimate	Std Err	t	Pr > |t|	Estimate	Std Err	t	Pr > |t|
T	1.31625	0.290621	4.529093	0.0062	1.31625	0.311515	4.225325	0.0014
M	0.0125	0.290621	0.043011	0.9674				
P	1.42125	0.290621	4.890388	0.0045	1.42125	0.311515	4.562388	0.0008
T*T	2.515	0.427783	5.879153	0.0020	2.4605357	0.456011	5.395787	0.0002
T*M	0.4525	0.411	1.100972	0.3211				
T*P	0.835	0.411	2.031629	0.0979				
M*M	0.1875	0.427783	0.438307	0.6795				
M*P	-0.2525	0.411	-0.61435	0.5659				
P*P	0.575	0.427783	1.34414	0.2367				

OPTIMIZATION

Factors:

Factor	Setting
T	150
M	21
P	4

```
Response(s):

_____________________________________
Response    Est. Value
_____________________________________
ORAC        593 [521.6855,664.3145]
PHENOLICS   9.495714 [8.762736,10.22869]
COLOR       .
BRSTRNGTH   .
EXINDEX     .
BULKDENS    .
_____________________________________

Desirability:

_____________________________________
Overall
   34.96%

ORAC
   D(ORAC) =   0 when ORAC < 300
   D(ORAC) = 0.5 when ORAC = 600
   D(ORAC) =   1 when ORAC > 900

   Function power:
   Lower half: 1
   Upper half: 1

PHENOLICS
   D(PHENOLICS) =   0 when PHENOLICS <  6
   D(PHENOLICS) = 0.5 when PHENOLICS = 11
   D(PHENOLICS) =   1 when PHENOLICS > 16

   Function power:
   Lower half: 1
   Upper half: 1


_____________________________________
```