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Identification of Superior Parents and Hybrids from Diallel Crosses of Bread Wheat (*Triticum aestivum* L.)

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Abstract. Five parents of bread wheat (*Triticum aestivum* L.) viz. TD-1, SKD-1, Marvi, Moomal and Mehran were crossed in a half diallel design; hence 10 F_1 hybrids were developed. Parents alongwith hybrids were evaluated for combining ability and heterosis for tillers/plant, spike length, spike density, grains/spike, grain yield/plant and seed index. The experiment was conducted in a randomized complete block design with four replications at Botanical Garden, Department of Plant Breeding & Genetics, Sindh Agriculture University, Tandojam, during 2010. The analysis of variance due to genotypes, parents, hybrids and parents vs. hybrids was significant for all the characters which revealed presence of significant amount of genetic variability in the material. The results also indicated significant differences among the parents for their general combining ability (GCA) and hybrids for specific combining ability (SCA) suggesting the importance of both additive and non-additive genes in the expression of traits studied. The greater magnitude of SCA variances over GCA were recorded for tillers/plant, grains/spike and grain yield/plant which indicated the importance of additive gene action while the involvement of non-additive genes was evident in the inheritance of spike length, spike density and seed index. Among the parents, generally TD-1, Mehran, Moomal and Marvi were the best general combiners for tillers/plant, spike length, spike density, grains/spike, grain yield/plant and seed index. Whereas, the hybrids like SKD-1 × Mehran, Marvi × Mehran, Marvix Moomal and TD-1 × SKD-1 were the best specific combiners for majority of yield traits. Positive heterosis was expressed by the hybrid SKD-1 × Moomal for tillers per plant; TD-1 × Moomal for spike length; TD-1 × SKD-1 for grains per spike; Marvi × Mehran for spike density and Marvi × Moomal for seed index. The best parents and hybrids could be effectively utilized in hybridization and selection programmes and also for hybrid crop development, respectively.

Keywords: diallel analysis, combining ability, heterosis, wheat genotypes

Introduction

Contributions of both i.e. nature and selective process of man have made tremendous genetic improvements in wheat plant. Increasing crop yield has still remained main concern to wheat breeders. The knowledge regarding the genetic basis of yield and its components helped wheat breeders to sort out promising parents to be used in hybridization and selection programmes. A diallel technique is one of the most powerful quantitative genetic analysis by which plant breeders estimate combining ability and heterotic potential of fixed lines or varieties. Among various diallel forms, the half-diallel provides maximum information about genetic architecture of a trait, potentiality of parents in crosses and type of gene action controlling traits (Griffing, 1956).

The combining ability was further partitioned into general combining ability (GCA) and specific combining ability (SCA) effects; hence components of gene action were obtained. The average performance of a genotype in a series of hybrid combinations is called GCA and is used to measure additive variances and additive genes. Whereas, the performance of genotypes in crosses is determined by SCA and measures non-additive gene action (Sprague and Tatum, 1942). Importance of general combining ability for grain yield per plant was observed by Akram *et al*. (2011) and Khan *et al*. (2007) and suggested additive type of gene action for this trait. While, Shabbir *et al*. (2011) and Akbar *et al*. (2009) believed that non-additive genetic effects were high for grain yield, revealing the prevalence of SCA effects. Additive type gene action with high values of general combining ability for tillers per plant (Mahpara *et al*., 2008; Rahim *et al*., 2006), spike length (Yucel *et al*., 2009), spike density (Mahpara *et al*., 2008), grains per spike (Shabbir *et al*., 2011; Yucel *et al*., 2009) and 1000-grains weight (Dhadhal *et al*., 2008 and Mahpara *et al*., 2008) were recorded. On the contrary, substantial specific

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combining ability effects were recorded for spike length, grains/spike, 1000 grain weight (Akram *et al*., 2011; Shabbir *et al*., 2011; Cifci and Yagdi, 2010) and spike density (Iqbal and Khan, 2006).

Proper choice of parents for hybrid wheat development is an important factor to enhance grain yield potential in existing germplasm. Nagarajan (2001) suggested the use of wheat hybrid varieties at commercial scale because of its higher production. Utilization of heterotic effects for more yield were largely attributed to cross-pollinated crops, yet evidences are now available to prove the presence of such effects in self-pollinated crops like wheat. Several plant breeders worked on heterosis in wheat and reported that grain yield can be maximized from 6% (Borghi *et al*., 1986) to as high as 41% (Zehr *et al*., 1997). Maximum and positive heterosis was found in spike length (Jaiswal *et al*., 2010); grains per spike (Khattab *et al*., 2010); 1000-grain weight and grain yield per plant (Abdel-Moneam, 2009). The objective of present investigation was to explore general combiners and mark crosses with better specific combining ability for wheat yield and its related traits by employing diallel genetic analysis.

Materials and Methods

Five bread wheat cultivars of diverse origin *viz*. TD-1, SKD-1, Marvi, Moomal and Mehran were crossed in half-diallel design. The seeds of parent and their 10 F_1 hybrids were sown in randomized complete block design with four replications in the experimental field, Department of Plant Breeding and Genetics. The sowing was done during mid December, 2010 with drill method and the distance between plant to plant and row to row was kept at 6 and 9 cm, respectively. The inorganic fertilizer like one bag (50 kg) of DAP per hectare was applied at the time of sowing whereas two bags of urea, one with third irrigation and second dose was applied at the time of grain formation. The crop was harvested in April, 2010. To reduce the intensity of harmful weeds, weedicide like Buctril Super at the rate of 1000cc was applied after first irrigation. The analysis of variance was carried out according to statistical methods developed by Gomez and Gomez (1984). Diallel analysis was carried out according to Griffing's Method-11, Model-1 (Griffing, 1956) by numerical approach as adopted by Singh and Choudhary (1979). Heterotic effects in F_1 hybrids were determined as the percent increase (+) or decrease (-) of F_1 hybrids over mid or better parents according to Fehr (1987) as under:-

$$\text{Mid parent heterosis \%} = \frac{F_1 - MP}{MP} \times 100$$

$$\text{Better parent heterosis \%} = \frac{F_1 - BP}{BP} \times 100$$

Where, F_1 = hybrid performance, M.P. = mid parent value (the mean of both parents) B.P. = Better parent value (the mean of better parent). Ten plants per replication from each F_1 hybrid and parent were randomly selected and treated as index plants to record the data. The traits studied were tillers/plant, spike length (cm), spike density, grains/ spike, grain yield/plant and seed index (1000-grain weight in g).

Results and Discussion

The present experiment was conducted so as to estimate the combining ability and heterotic effects in F_1 hybrids developed from five parent half diallel of bread wheat. The characters studied were tillers/plant, spike length, spike density, grains/spike, grain yield/plant and seed index. The mean squares due to genotypes, parents, hybrids and parents vs. hybrids were significant for all the characters indicating the presence of considerable amount of variability in the breeding material (Table 1). These results further suggested that the parental lines and their hybrids were quite variable in their performance and presence of overall heterosis for most of the characters. The significant mean squares due to general combining ability (GCA) and specific combining ability (SCA) suggested that additive as well as non-additive genes were advocating the traits under study (Baloch *et al*., 2008). The magnitude of GCA variances was higher than the SCA variances indicating preponderance of additive gene effects for spike length, spike density and seed index. While high SCA variances over GCA for tillers/plant, grains/spike and grain yield/plant confirmed the prevalence of non-additive genetic effects for these traits. Importance of general combining ability variance for grain yield per plant was also observed by Akram *et al*. (2011) and Baloch *et al*.(2011) who suggested that additive type of gene action was advocating grain yield per plant, while Shabbir *et al*. (2011) believed that non-additive genetic effects were high for grain yield, revealing the prevalence of SCA effects. For other traits, additive type gene action with high values of general combining ability for tillers per plant was reported by Mahpara *et al*. (2008); spike length by

Yucel *et al*. (2009); spike density by Mahpara *et al*. (2008); grains per spike by Shabbir *et al*. (2011) and 1000-grains weight by Dhadhal *et al*. (2008) and Mahpara *et al*. (2008). On the contrary, substantial specific combining ability effects rather than general combining ability were recorded for spike length and seed index (Akram *et al*., 2011; Shabbir *et al*., 2011; Cifci and Yagdi, 2010); spike density (Iqbal and Khan, 2006) and grains per spike (Baloch *et al*., 2011).

With respect to average performance, the parents TD-1 developed maximum tillers/plant, gave maximum grain yield/plant and recorded maximum seed index. While Mehran set maximum grains/spike and Marvi measured longer spikes and also recorded maximum spike density (Table 2). It is quite interesting to note that parents like TD-1 and Marvi which performed well as *per se* also performed well for GCA effects. These results therefore suggested that both the parents could reliably be used in hybridization and selection programmes to improve various yield traits. Regarding F_1 hybrids' performance, cross TD-1 × SKD-1 set highest grains per spike and at the same time produced maximum grain yield/plant. Hybrid TD-1 × Mehran developed maximum tillers per plant and Marvi × Moomal measured longest spikes; Marvi × Mehran recorded

Table 1. Mean squares from diallel analysis for various yield traits in bread wheat (*Triticum aestivum* L.)

Source of variation	D.F.	Mean squares					
		Tillers/plant	Spike length	Spike density	Grains/spike	Grain yield/plant	Seed index
Genotype	14	20.05**	9.052**	334.39**	129.69**	18.03**	33.57**
Parents	4	11.20**	10.92**	185.44**	113.30**	12.12**	52.05**
Hybrids	9	4.82**	8.74**	430.21**	28.36**	10.62**	16.40**
Parents vs. hybrids	1	192.53**	4.38**	67.72**	1106.56**	108.3**	114.15**
GCA	4	14.40**	22.89**	764.14**	34.18**	17.40**	36.95**
SCA	10	22.31**	3.52**	162.49**	167.83**	18.28**	32.21**
Error	42	0.477	0.524	34.901	0.780	0.676	0.763

** = significant at 1% probability level

Table 2. Mean performance of parents and F_1 hybrids for various yield traits in bread wheat (*Triticum aestivum* L.)

Parents	Mean performance					
	Tillers/plant (cm)	Spike length (%)	Spike density	Grains/spike (g)	Grain yield/plant	Seed index (g)
TD-1	9.50	11.38	75.85	52.75	19.25	45.50
SKD-1	6.00	12.50	70.21	57.25	15.50	38.75
Marvi	5.50	15.75	87.05	60.75	14.75	35.50
Moomal	7.50	14.00	81.42	64.75	16.00	40.00
Mehran	8.50	13.00	72.71	65.50	17.00	39.87
F_1 Hybrids						
TD-1 × SKD-1	12.50	11.90	61.51	73.60	21.50	42.00
TD-1 × Marvi	11.50	13.50	85.79	70.50	20.75	41.25
TD-1 × Moomal	11.00	14.75	90.99	66.75	19.00	44.00
TD-1 × Mehran	12.75	13.50	75.69	71.50	19.00	40.50
SKD-1 × Marvi	9.50	14.50	85.80	68.00	17.00	42.50
SKD-1 × Moomal	11.25	12.00	72.36	69.00	17.50	44.00
SKD-1 × Mehran	12.00	12.75	67.41	71.00	21.50	42.00
Marvi × Moomal	10.75	16.50	86.63	66.75	18.75	45.25
Marvi × Mehran	11.25	15.50	92.55	71.00	20.50	40.50
Moomal × Mehran	9.50	14.00	81.12	65.00	18.00	46.50
L.S.D (5%)	0.986	0.974	8.42	1.26	1.17	1.247

maximum spike density; Moomal × Mehran gave higher seed index. It is generally assumed that F_1 hybrid performance is reflected in specific combining ability, but such assumption did not hold true (Baloch and Bhutto, 2003). These results suggested that F_1 performance may not be taken as granted for specific combining ability of such hybrids. Concerning GCA effects, TD-1 expressed maximum positive GCA effects for tillers/plant and grain yield/plant. Cultivar Marvi manifested maximum positive GCA effects for spike length and spike density; Mehran for grains/spike (1.694); Moomal for seed index. These results therefore suggested that parents TD-1, Marvi, Mehran and Moomal may be preferred for hybridization and selection programmes so as to improve majority of the characters studied. The results for specific combining ability (SCA) effects revealed that maximum positive SCA effect was displayed by hybrid SKD-1 × Mehran for tillers/plant and grain yield/plant; TD-1 × Moomal for spike length and spike density; TD-1 × SKD-1 for grains/spike and Moomal × Mehran for seed index (Table 2). These results suggested that various hybrids may be considered for hybrid crop development to improve above traits.

The GCA effects presented in Table 3 revealed that for tillers/plant, TD-1 demonstrated highest positive GCA effects of 1.021 while, Mehran ranked next (0.414), yet Marvi manifested maximum negative GCA effects of -0.800. As far as SCA effect is concerned, hybrids, SKD-1 × Mehran (2.09), SKD-1 × Moomal (2.05) and Marvi × Moomal (2.01), respectively were among the top three rankers for tillers per plant (Table 4). With respect to spike length, Marvi and Moomal established maximum positive GCA effects of 1.299 and 0.406, respectively while SKD-1 exhibited highly negative GCA effect of -0.894. Eight hybrids manifested positive SCA effects while other two crosses gave negative SCA effects for spike length (Table 4). Nonetheless, top three rankers in SCA effects were; TD-1 × Moomal (1.58), Marvi × Moomal (1.19) and TD-1 × Mehran (0.83).

Regarding GCA effects of spike density, Marvi and Moomal expressed maximum positive effects of 7.158 and 2.714, respectively. Whereas SKD-1 (-6.751) and Mehran (-1.795) demonstrated negative GCA effects (Table 3). Concerning SCA effects, six hybrids manifested positive SCA effects and four crosses gave negative SCA effects (Table 4). Nonetheless, three top rankers in SCA effects were TD-1 × Moomal (11.25), Marvi × Mehran (8.92) and SKD-1 × Marvi (7.10). GCA effects of grains/spike (Table 3) revealed that Mehran and Marvi genotypes expressed positive GCA effects of 1.694 and 0.016, respectively, while TD-1 (-1.399), SKD-1 (-0.220) and Moomal (-0.091) displayed negative GCA effects. With respect to SCA effects, the top three scoring hybrids were TD-1 × SKD-1 (9.62),

Table 3. The GCA effects of various yield traits in bread wheat (*Triticum aestivum* L.)

Parents	Tillers/ plant	Spike length	Spike density	Grains/ spike	Grain yield/ plant	Seed index
TD-1	1.021	-0.787	-1.326	-1.399	1.193	1.072
SKD-1	-0.336	-0.894	-6.751	-0.220	-0.271	-0.464
Marvi	-0.800	1.299	7.158	0.016	-0.557	-1.535
Moomal	-0.300	0.406	2.714	-0.091	-0.736	1.215
Mehran	0.414	-0.023	-1.795	1.694	0.371	-0.287
S.E. (gi.)	0.014	0.015	0.99	0.02	0.02	0.02

Table 4. The SCA effects of F_1 hybrids for various yield traits in bread wheat (*Triticum aestivum* L.).

F_1 hybrids	Tillers/plant	Spike length	Spike density	Grains/spike	Grain yield/plant	Seed index
TD-1 × SKD-1	1.99	0.03	-8.79	9.62	2.37	-0.05
TD-1 × Marvi	1.45	-0.55	1.66	6.28	1.91	0.27
TD-1 × Moomal	0.45	1.58	11.25	2.64	0.33	0.28
TD-1 × Mehran	1.49	0.83	0.46	5.61	-0.77	-1.72
SKD-1 × Marvi	0.80	0.48	7.10	2.61	0.39	3.04
SKD-1 × Moomal	2.05	-1.13	-1.95	3.72	0.29	1.81
SKD-1 × Mehran	2.09	0.11	-2.39	3.94	3.19	1.30
Marvi × Moomal	2.01	1.19	-1.52	1.23	1.82	4.12
Marvi × Mehran	1.80	0.69	8.92	3.71	2.47	0.87
Moomal × Mehran	-0.44	0.07	1.88	-2.18	0.15	4.13
S.E. (si.)	0.06	0.06	4.15	0.09	0.08	0.09

Table 5. Heterotic effects of F_1 hybrids over their mid and better parents for various traits in bread wheat (*Triticum aestivum* L.)

F_1 hybrids	Tillers per plant		Spike length		Spike density		Grains per spike		Grain yield per plant		Seed index	
	R.H. (%)	B.P. (%)	R.H (%)	B.P. (%)	R.H. (%)	B.P. (%)	R.H. (%)	B.P. (%)	R.H. (%)	B.P. (%)	R.H. (%)	B.P. (%)
TD-1 × SKD-1	61.29	31.58	-0.31	-4.80	-15.77	-18.90	33.82	28.56	23.74	11.69	-0.30	-7.69
TD-1 × Marvi	53.33	21.05	-0.46	-14.29	5.33	-1.44	24.23	16.05	22.06	7.79	1.85	-9.34
TD-1 × Moomal	29.41	15.79	16.26	5.36	15.72	11.76	13.62	3.09	7.80	-1.30	2.92	-3.30
TD-1 × Mehran	41.67	34.21	10.77	3.85	1.92	-0.19	20.93	9.16	4.83	-1.30	-5.12	-10.99
SKD-1 × Marvi	65.22	58.33	2.65	-7.94	9.13	-1.43	15.25	11.93	12.40	9.68	14.48	9.68
SKD-1 × Moomal	66.67	50.00	-9.43	-14.29	-4.55	-11.13	13.11	6.56	11.11	9.38	11.75	10.00
SKD-1 × Mehran	65.52	41.18	0.00	-1.92	-5.66	-7.28	15.68	8.40	32.31	26.47	6.84	5.34
Marvi × Moomal	65.38	43.33	10.92	4.76	2.84	-0.48	6.37	3.09	21.95	17.19	19.87	13.13
Marvi × Mehran	60.71	32.35	7.83	-1.59	15.87	6.32	12.48	8.40	29.13	20.59	7.47	1.58
Moomal × Mehran	18.75	11.76	3.70	0.00	5.27	-0.37	-0.19	-0.76	9.09	5.88	16.44	16.25

R.H. = relative heterosis; B.P. = better parent heterosis.

TD-1 × Marvi (6.28) and TD-1 × Mehran (5.61). For grain yield/plant**,** TD-1 and Mehran expressed maximum positive GCA effects of 1.193 and 0.371, respectively whereas Moomal (-0.736), Marvi (-0.557) and SKD-1 (-0.271) demonstrated negative GCA effects (Table 3). Concerning the SCA effects for grain yield/plant, eight crosses manifested positive SCA effects while remaining two hybrids showed negative SCA effects (Table 4). Seed index (1000-grain weight in g) results revealed that Moomal and TD-1 expressed maximum positive GCA effects of 1.215 and 1.072, respectively whereas other parents such as Marvi (-1.535), SKD-1 (-0.464) and Mehran (-0.287) displayed negative GCA effects (Table 3). From 10 crosses, eight hybrids manifested positive SCA effects yet, top three hybrids were Moomal × Mehran (4.13), Marvi × Moomal (4.12) and SKD-1 × Marvi (3.04) (Table 4).

Heterotic effects revealed that hybrids SKD-1 × Moomal exhibited maximum positive heterobeltiosis for tillers per plant; TD-1 × Moomal for spike length; Marvi × Mehran for spike density; TD-1 × SKD-1 for grains/spike; SKD-1 × Mehran for grain yield/plant; Marvi × Moomal for seed index (Table 5). Regarding heterobeltiosis, maximum positive heterobeltiosis was displayed by hybrids SKD-1 × Marvi for tillers per plant; TD-1 × Moomal for spike length and spike density; TD-1 × SKD-1 for grains/spike; SKD-1 × Mehran for grain yield/plant and Moomal × Mehran for seed index. These crosses revealed good scope for their commercial exploitation of heterosis as well as isolation of promising progenies in later segregating generations. Analogous to our heterotic effects, Jaiswal *et al*. (2010) obtained mid parent and better parent heterosis for tillers per plant; Akbar *et al*. (2009) for spike length; Chowdhry *et al*. (2005) for spike density, Moneam (2009) for grain yield per plant and Akbar *et al*. (2009) for seed index.

Conclusion

The overall findings revealed the importance of both additive and non-additive genetic variances and effects which suggested that the use of integrated breeding strategies can efficiently utilize both gene actions. Among the parents, TD-1, Mehran, Moomal and Marvi were found as best general combiners hence can be regarded potential parents for hybridization and selection programmes to improve yield traits under study. Furthermore, if hybrid wheat becomes feasible, the better parent heterosis could be of practical value and hybrids like SKD-1 × Mehran, Marvi × Mehran, Marvi × Moomal and TD-1 × SKD-1 may be more meaningful for improving yield in wheat.

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Effect of Various Levels of N in Combination with FYM on the Growth and Establishment of Date Palm (Dhakki) Cultivar

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Abstract. This study was carried out during the year 2008-2009 at AZRI farm, D.I. Khan to investigate optimum dose of nitrogen (N) fertilizer and Farm Yard Manure (FYM kg/plant) for improving fruit bearing growth parameters of date palm cv. Dhakki. Four treatments consisted of (1) control (2) 0.5 kg N + 15 kg FYM (3) 1 kg N + 15 kg FYM (4) 1 kg N + 0 kg FYM/plant were applied. The results showed that 1 kg (N) + 15 kg FYM had number of fronds (38)/plant, plant spread (511.7 cm), plant girth (211.7 cm), frond length (253 cm), number of fruit (9.3)/stain, fruit weight (4.3 kg)/bunch and total fruit yield (26.58 kg)/plant at 5 year stage among all other treatments. However, the maximum values for these parameters including fruit yield (11.32 kg/tree) were recorded in control where no fertilizer or manure was applied.

Keywords: nitrogen; farm yard manure; growth parameters, fruit yield

Introduction

Date palm (*Phoenix dactylifera* L.) belongs to Palmaceae family. It is one of the most ancient fruits of the arid, tropical and subtropical regions of the world. The yield of Dhakki dates in Dera Ismail Khan is not up to its potential due to lack of suitable cultural practices including application of proper fertilizer to the soils which are deficient in micro and macro nutrients and organic matter. Determination of optimum dose of fertilizers for this plant may lead to more yield production, higher quality of fruits and considerable reduction in the farm expenses. Earlier researchers like Kassem *et al.* (1997) and Abdalla *et al*. (1987) quoted that the yield, fruit quality and leaf mineral have been improved with the application of nitrogen and potassium fertilization in date palm. Sabbah (1993) reported that using 750 g N and 750 g P_2O_5 for each fruitful tree caused the highest yield production. El-Hammady *et al.* (1991) studied the effect of potash fertilization on yield and fruit quality of date palm and found that the potash fertilizer has a considerable effect on physiological parameters of date palm plants. Harhash (2000) studied the effect of potassium fertilization on fruit thinning of date palm and observed that potassium fertilizer caused the best yield and fruit quality. Bilsborough and Blackpool (2000) and Carpenter (1981) reported that nutrients deficiency in young date palm has apparent symptoms that pose bad impact on yield and quality of fruit. Saleh (2008) and Dong (2005) depicted that using 2.5 kg NPK along with micro-nutrient fertilizers caused the highest yield and best quality fruit.

Keeping in view the desirable effects of macronutrients application on yield and fruit quality, it was necessary to determine the optimum levels of fertilizers and manure in order. This study was therefore, conducted to determine the optimum dose of nitrogen and FYM to obtain maximum yield and desirable fruit quality of cultivar 'Dhakki' under the ecological zone of Dera Ismail Khan, KPK, Pakistan.

Materials and Methods

The experiment was conducted at AZRI farm, D.I. Khan during 2008-2009 to investigate the optimum dose of nitrogen (N) with Farm Yard Manure (FYM). The study was carried out on 5 years old plants of Dhakki cultivar which were planted in RCBD at 8 meters apart, three palm trees about 5 years old as uniform as possible were selected for data recording. Soil samples were taken before applying the treatments and analyzed for various physico-chemical properties. Results are presented in Table 1. Treatments were consisted of 4 levels of nitrogen + FYM (i.e., 0, 0.5 kg N + 15 kg FYM, 1 kg N + 15 kg FYM and 1 kg N + 0 kg FYM g/tree). These nitrogen (N) and farm yard manure levels were applied with basal dose of 1 kg P_2O_5 and 0.5 kg K_2O_5 tree during the month of December. The fertilizer was applied in bands about 4 feet wide and 18 inches away from the trunk. After applying the fertilizer the

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plants were irrigated immediately. The data were recorded on plant height, number of fronds/plant, plant spread, plant girth, frond length, number of pineae/frond, number of suckers/plant, number of bunches/plant, number of strains/bunch, number of fruits/stain, fruit weight/bunch (kg) and total fruit yield/plant. The data were analyzed statistically using analysis of variance (ANOVA) procedure and LSD ($P < 0.05$) values calculated for comparing means (Steal and Torrie, 1980). All other cultural practices were performed uniformly throughout the growing season.

Table 1. Physico-chemical properties of the experimental site during 2008-2009

Properties	Parameters	Value
Chemical properties	pH	8.1
	ECe (dS/m)	4.5
	Organic matter (%)	0.71
	Available K(mg/kg)	110
Physical properties	Clay	56.0
	Silt	36.0
	Sand	8.0
	Texture	Clay

Results and Discussion

Data presented in Table 2 revealed that all the growth parameters were significant at 5% probability level ≤ 0.05 affected with various levels of nitrogen (N) in combination with Farm Yard Manure (FYM) except branches/plant.

Plant height (cm). Plant height ranged between 298.3 to 359.5 cm. Minimum plant height of 298.3 cm was observed in control plot followed by receiving of only 1 kg N with FYM appeared with a plant height of 308 cm. Both the said treatments were at par statistically (Table 3). Plant height of date palm increased with nitrogen and farm yard manure application. However, 1 kg N and 15 kg farm yard manure/tree caused the highest plant height i.e., 359 cm followed by at par value 356.7 cm plant height with the combination of 0.5 kg N + 15 kg FYM. Both of these combinations showed their positive effect due to receiving of proper rain fall and suitable temperature during February to April (Table 4). Increase in plant height by using nitrogen fertilizer and farm yard manure (FYM) in date palm was also reported by Harhash (2000); Kassem *et al*. (1997); Sabbah (1993); Furr *et al.* 1951.

Table 2. Effect of various levels of N in combination with FYM on the growth of date palm c.v. "Dhakki" during 2008-2009

Treatments	Fertilizer rate N+FYM kg/ha	No. of suckers/plant	No. of bunches/plant	No. of strands/bunch	No. of fruit/strand	Fruit/ brunch kg	Total fruit yield/plant
T1	Control	4.06b	5	30.67b	6.0c	2.83c	11.32c
T2	0.5-15	4.6b	5	34.33a	7.0bc	3.7b	18.65b
T3	1.0-15	7.3a	6	35.33a	9.3a	4.43a	26.58a
T4	1.0-0	7.3a	5	31.00b	8.3ab	3.76b	18.80b
LSD 0.05	–	2.1	NS	29.92	1.9	0.38	5.22

a, b, ab and c letters are indicating Duncun multiple range test. Means within a column followed by the same letter are not significantly different at the 5% level.

Table 3. Effect of various levels of N in combination with FYM on the growth of date palm c.v. "Dhakki" during 2008-2009

Treatments	Fertilizer rate N+FYM kg/ha	Plant height (cm)	No. of fronds/plant	Plant spread	Plant girth	Fronds length (cm)	No. of pinnae/frond
T1	Control	298.3b	29.67b	440.7c	170.0c	208.3d	142.7b
T2	0.5-15	356.0a	30.67b	476.7b	180.0c	237.b	147.0ab
T3	1.0-15	359.0a	38.00a	511.7a	211.7a	253.0a	151.7a
T4	1.0-0	308.0b	26.00c	463.3b	195.0b	235.0c	142.7b
LSD 0.05		19.9	1.85	18.5	11.5	10.4	5.3

a, b, ab, c and d letters are showing Duncun multiple range test. Means within a column followed by the same letter are not significantly different at the 5% level.

Table 4. Mean agro-meteorological data of the experimental site during 2008-2009

Month	Air temperature		Relative humidity (%)		Rain fall (mm)
	Max	Min	0800 h	1400 h	
July-08	39	25	83	56	90
August-08	37	23	83	66	54
September-08	35	22	78	61	58
October-08	32	20	76	55	-
November-08	29	9	71	38	-
December-08	23	5	81	56	9
January-09	21	5	76	57	3.5
February-09	23	9	81	54	15.5
March-09	28	13	79	60	48.0
April-09	33	17	71	54	15.5
May-09	39	23	70	43	1.0
June-09	39	25	70	48	-
July-09	37	27	83	62	30.0
August-09	37	26	88	71	51.5
Total	–	–	–	–	376

Number of fronds/plant. Number of fronds/plant ranged from 29.6 to 38.0/plant. The application of 1.0 kg and 15 kg FYM produced maximum fronds; 38/plant among the treatments, while the lowest number of fronds 26/plant observed in plots receiving only 1 kg N with out FYM as well as control appeared with fronds (20)/plant (Table 2). Positive effects of nitrogen fertilizer and FYM on number of fronds plant of date palm has been reported by Al-Kharusi *et al*. (2007); Harhash (2000); Montasser *et al*. (1991); El-Hammady *et al*. (1991).

Plant spread. Plant spread differed significantly with various levels of N and FYM application ranging from 440.7 cm to 511.7 cm. Among the treatments receiving of 1 kg N in combination with 15 kg FYM plant, produced maximum 511.7 cm plant spread, while lower level 0.5 kg N along with 15 kg FYM plant and only receiving 1 kg N plant appeared statistically the same producing 476.7 and 463.3 cm plant spread. Moreover, the control treatment produced the lowest plant spread 440.7 cm (Table 3). The results are in line with the researchers like Rajaie *et al.* (2009); Harhash (2000) and Kassem *et al.* (1997), who reported the positive effects of N and FYM fertilization on date palm.

Plant girth. Plant girth was also significantly affected with different levels of N and FYM ranging from 170 to 211.7 cm. The highest plant girth (211.7 cm) was noted among the treatment with the application of 1 kg N along with 15 kg FYM/palnt while the lowest 170 cm plant girths in control plot. Application of 1 kg N + 15 kg FYM/plant appeared the optimum combination for obtaining maximum plant girth (Table 3).

Frond length. The frond length of various treatments varied from 208.3 cm to 253 cm. The maximum frond length (253 cm) was obtained in treatment receiving 1 kg with 15 kg FYM/plant followed by 0.5 kg N with 15 kg FYM/plant produced frond length of 273.3 cm. However, control and nitrogen applied alone showed the lowest 208.3 and 225 cm frond length. This reduction in frond length with alone application of N showed the importance of FYM added treatments (Table 3). Optimum fertilizer in date palm improves quantitative and qualitative properties of production. In the present experiment the increase in frond length could be due to nitrogen and FYM's effect as reported by Harhash (2000); Broschat (1999) and Kassem *et al.* (1997).

Number of pinnea/frond. The data regarding number of pinnaea frond showed significant effect with N and FYM combination ranging from 147 to 151.7/frond although non-significant with both combinations (i.e., 0.5 or 1 kg N with 15 kg FYM/plant). But the lowest number of pinnea 142.7/frond were seen in the control as well as in the treatment where only N was applied (Table 3). This reduction in pinea/frond might be the results of non application of FYM with nitrogen fertilizer.

Number of suckers/plant. Number of suckers ranged from 4 to 7. The maximum suckers 7.3/plant were obtained in the treatment where 1 kg N in combination with 15 kg FYM/plant was applied which appeared at par with treatment receiving only 1 kg N/plant. But the lowest suckers 4.06/plant values were in control as well as in the treatment received 0.5 kg N along with 15 kg FYM/plant (Table 2).

Number of bunches/plant. The number of bunches/plant appeared for the first time at 5 years age as the date palm tree got its fruits but did not differ significantly with the application of organic and inorganic fertilizer. But its combined effect was improving as 1 kg N plus 15 kg FYM/plant generally produced highest 6 number of bunches/plant as compared to control i.e. 4 bunches/plant (Table 2).

Number of strand/bunch. The number of strand varied from 30.67 to 35.33/bunch. The highest number of

strand 35.33 were obtained in treatment receiving 1 kg N in combination with15 kg FYM/plant among the treatments apart from the treatment of 0.5 kg N in combination with the same rate of 15 kg FYM/plant produced 34.33 number of strand/bunch. But both the treatments receiving of N alone and control produced significantly the lowest numbers of strand/bunch showing the importance of FYM which increased the efficiency of nitrogen (Table 2).

Number of fruit/strand. The number of fruit/strand with various levels of N and FYM significantly differed, ranging from 6 to 9.3. But the application of 1 kg N with 15 kg FYM/plant produced the highest fruit i.e., 9.3/strand followed by treatment that received only 1 kg N/plant and gave 8.3 fruit/strand. The lowest number of fruit 6/strand was recorded in the control where fertilizer and manure was not applied (Table 2).

Fruit weight/bunch. Fruit weight/bunch was also significantly affected with different levels of fertilizer ranging from 2.83 to 4.43 kg/bunch. Similar to most other of the other characters of the highest fruit 4.43 kg/bunch was recorded in the treatment received 1 kg N with 15 kg FYM/plant while 3.7 kg/bunch was recorded in both treatments i.e. applied 0.5 kg N with 15 kg FYM or 1 kg N/plant, but the lowest fruit 2.83 kg/bunch was observed in control treatment (Table 2).

Total fruit yield/plant. Significant differences were observed in fruit yield/plant with various levels of N and FYM application ranging from 11.32 kg to 26.58 kg. The said fruit data was recorded initially for the first time as date palm tree usually bearing fruit after 5 years of age. The fertilizer combined treatment like 1 kg N with 15 kg FYM/plant significantly produced the highest fruit 26.58 kg plant among the treatment followed by treatment receiving 0.5 kg N in combination with 15 kg FYM and 1 kg N alone/plant yielded fruit 18.65 and 18.80 kg plant and appeared at par statistically with each other. The lowest fruit yield 11.32 kg/plant was recorded in control plot. The application of 1 kg N with 15 kg FYM/plant appeared the optimum combination for obtaining maximum fruit yield/plant with an increase of 135% over control plot (Table 2). The present results are in line with Bashir *et al.* (2009) who reported that maximum yield 64 kg/plant of guava was recorded with the application of 20 kg FYM + 1 kg each of N-P_2O_5-K_2O/plant.

Conclusion

It can be concluded from the results that most of the physiological fruit bearing parameters were maximum with the combined application of 1 kg N + 15 kg FYM as compared to control as well as other levels of organic and inorganic fertilizer application. Therefore, the said rates of N with FYM appeared the most appropriate combination for obtaining maximum fruit setting parameters towards fruit/yield/tree.

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Growth and Yield Characteristics of Chilli as Affected by Nitrogen in Presence and Absence of Phosphorus and Potassium

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Abstract. An effect of nitrogen (N) in presence and absence of phosphorus (P) and potassium (K) on growth and yield characteristics of chilli (*Capsicum annum* L.) was studied at Coastal Agricultural Research Station, Southern Zone Agricultural Research Centre, PARC, Karachi, during 2008-2009. The crop under investigation was fertilized with a total of six treatments i.e. 90-0-0, 90-60-75, 0-60-75, 120-0-0, 120-90-105 and 0-90-105 kg/ha of NPK. The analyses of data revealed that it was possible to harvest a satisfactory crop yield without addition of P or K, but it would not be possible to get desired crop yields without application of N, because an adverse effect on fruit yield was noted in absence of N. On the other hand stoppage of P and K did not show any economically adverse effects. However, combined application of NPK positively enhanced growth and yield characters. It was concluded that N in presence of P and K (120-90-105 kg/ha) proved best for better production of chilli var. Malir local under agro climatic conditions of Malir district, Sindh.

Keywords: chilli, growth, yield, fertilizer, nitrogen, phosphorus, potassium

Introduction

Chilli (*Capsicum annum* L.) belongs to the family Solanaceae. Chilli has a wide range of cultivation, being grown under both tropical and subtropical conditions (Malik, 1994). In Pakistan chilli is grown on an area of 38.4 thousand hectares with production of 90.4 thousand tonnes, with an average yield of 1.7 tonnes per hectare with 1.5% share in the GDP. Sindh is the major producer of chilli (70%) followed by Punjab (25%) and remaining 5% is produced by Balochistan and NWFP provinces (Mahmood *et al.,* 2002). Chilli is not only an important ingredient in food but is also used for essence production. It is used in foods for pungency and red colour while, it also contributes in part to the flavour of ginger ales. Chilli is an excellent source of vitamins A, B, C, E, P and high in antioxidants. Pakistan earned Rs.1.127 billion during 2003-2004 by exporting red chilli powder, whereas, export earnings from all fruits were Rs. 5.912 billion during the same period. This reveals the potential of this non-staple crop. There are various factors responsible for the low yield of chilli in Sindh province as compared to other advanced agricultural countries of the world. Among them one of the most important cause is the under dosage of major essential nutrients such as N, P and K in the cultivating soils.

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Crops respond differently to different fertilizer elements, and proper fertilizer management for a plant species is important for increasing yield and quality. Nitrogen, phosphorus and potassium are the three major nutrients, which individually and/or together maintain growth, yield and quality of plants (Kodali, 2006). Nitrogen is involved in chlorophyll formation and it influences stomatal conductance and photosynthetic efficiency (Ivonyi *et al*., 1997). Phosphorus stimulates root development and promotes flower formation and fruit production (Malik *et al*., 2006). Particularly, it is considered essential for seed formation. It hastens maturity of crops grown on soils low in phosphorus. Phosphorus indirectly promotes plant growth and absorption of K as well as other nutrients (McKenzie *et al*., 2002). Potassium plays catalytic role in the plant rather than becoming an integral part of plant components (Malvi, 2011). It regulates the permeability of cell walls and activities of various mineral elements as well as neutralizes physiologically important organic acids (Britto and Kronzucker, 2008). Plants with an inadequate supply of K show poor fruit or seed formation (Bartall and Pressman, 1996), yellowing of the leaves, poor growth, and low resistance to coldness and drought (Asgharipour and Heidari, 2011). A sufficient supply of K promotes N uptake efficiency of plants due to its stimulant effect on plant growth (Malik *et al*., 1989).

In earlier work, conducted by Naeem *et al*. (2002) reported that different doses of NPK were significantly different for days to flowering, fruit characters and total yield in chilli. Vadhana (2003) revealed that the maximum number of fruits per plant (78.73/plant), highest green chilli yield (175.43 g/plant) and higher fruit yield (5957 kg/ha) was recorded in the higher fertilizer level (250:125:125 NPK kg/ha) than fertilizer level (150:75:75 NPK kg/ha).The results obtained by Miano *et al.* (2004) regarding effect of different doses of N and K_2O on chilli indicated that application of 120 kg/ha N and 125 kg/ha of K_2O proved best for vigorous growth and fruit yield than all other treatments. In a field trial, Baloch *et al.* (2004) found NPK level of 83-78-73 kg/ha highly significant on the growth, yield and yield components of chilli describing the importance of balanced supply of nutrients. The results obtained by Ramakrishna and Palled (2005) revealed that fruit yield and total dry matter production of chilli was significantly higher with the application of 150:75: 75 kg/ha of N, P_2O_5 and K_2O (2:1:1) over other fertilizer levels. Din *et al.* (2007) reported that NPK levels of 120-90-60 kg/ha significantly performed better with growth, marketable yields and head yield of cabbage. Jilani *et al.* (2008) reported that nitrogen application at 100 kg/ha produced significantly maximum survival percentage, fruit characters and yield per hectare in brinjal. While, Yahaya (2008) reported that productivity in chilli pepper could be enhanced with efficient fertilization. The non-significant response exhibited by some parameters could be due to their inherent character that is genetically controlled rather than being influenced by management (Ahmad *et al.,* 2009). Keeping in view the importance of fertilizer management, it is necessary to educate our farmers regarding balanced use of fertilizers. The present experiment was conducted to evaluate the growth and yield characteristics of chilli as affected by nitrogen in presence and absence of phosphorus and potassium under agro-climatic conditions of Malir district, Karachi, Pakistan.

Materials and Methods

The experiment was conducted to find out the optimum dose of N, P and K and to evaluate the impact of nitrogen in presence and absence of phosphorus and potassium on the growth and yield characteristics of chilli during 2008-2009. Seed of Malir local variety was sown in well-prepared and manured plot in the experimental field of Coastal Agricultural Research Station, Southern Zone Agricultural Research Centre, PARC, Karachi. Germination was found satisfactory. The experiment was laid out in RCBD replicated four times maintaining plot size of 2.5 m × 3 m for all treatments. The NPK treatments tested during the study were comprised of T1 90-00-00, T2 90-60-75, T3 00-60-75, T4 120-00-00, T5 120-90-105 and T6 00-90-105 NPK kg/ha. At least six weeks old seedlings were transplanted in main field on both sides of ridges with plant to plant and row to row distance of 60 cm. Before transplanting the seedlings, land was prepared and at that time all P and K fertilizers along with half dose of N were added and mixed well in the soil before irrigating the ridges for transplanting. The remaining N was applied subsequently in three equal doses at 15, 30 and 45 days after planting (DAP) due to the light texture of the soil. Nitrogen was applied in the form of Urea (46% N), while phosphorus and potassium were used in the form of single super phosphate (18% P_2O_5) and potassium sulphate (50% K_2O), respectively to get the required doses according to recommended treatments schedule. All the cultural operations like weeding, hoeing and irrigation were carried out uniformly in all the treatments as and when needed for better growth and development of the crop.

Soil analysis. The composite soil samples from experimental site were taken at two depths (0-15 and 15-30 cm). These samples were air-dried, ground, sieved and then analyzed for various physical and chemical characteristics of soil. The soil texture was determined with hydrometer as per method described by Bouyoucos (1962) and pH in soil water suspension (1:5) was determined with glass electrode pH meter (SP-34 Suntex) by Richards (1954). EC was determined with digital (HI-8333) conductivity meter (Kanwar and Chopra, 1959). Organic matter was determined by Walkely and Black procedure as suggested by Jackson (1958). Nitrogen was determined by Macro-Kjeldahl method (Paul and Berry, 1921). Available phosphorus was determined by the method described by Soltanpur and Schwabe (1977) and potassium by AB-DTPA method as suggested by Soltanpur and Schwabe (1985).

Data collection and statistical analysis. Five plants in each bed were selected at random and labeled for recording observations on germination percentage (%), plant height (cm), number of branches/plant, days to flowering, days to fruiting, fruits/plant, fruit diameter (cm), fruit length (cm), fruit weight/plant (g) and total fruit yield (kg/ha) during the growth and after harvest of the crop. The data thus collected were subjected to statistical analysis to analyze the treatments variance.

L.S.D test was also applied to observe the statistical differences according to the method developed by Gomez and Gomez (1984).

Results and Discussion

Physico-chemical characteristics of experimental site. Physical as well as chemical characteristics data of the experimental area soil is presented in Table 1. The data revealed that the soil was sandy clay loam in texture, low in organic matter content (%), deficient in nitrogen and phosphorus but contained sufficient amount of available potassium. The pH of the surface and sub soil layers was of 7.5 and 7.7, respectively.

Table 1. Physico-chemical analyses of soil of the experimental field

Soil characteristics	Depth (cm)	
	0-15	15-30
EC (dS/m)	4.73	1.92
pH	7.5	7.7
O.M (%)	0.619	0.222
N (%)	0.030	0.011
P (ppm)	4.0	1.9
K (ppm)	100	110
Texture	Sandy clay loam	Sandy clay loam

Data regarding the effect of nitrogen in presence and absence of P and K on the growth and yield characteristics of chilli as recorded are given:

Plant height (cm). Mean plant height (75.4 cm) was significantly greater in the plots fertilized with 120-90-105 NPK kg/ha, followed by 120-0-0 and 90-0-0 NPK kg/ha with mean plant height of 73.53 cm and 71.66 cm, respectively (Table 2). The plots treated with 0-60-75 NPK kg/ha had shorter plants (64.66 cm) as compared to plots where N was applied alone (90-0-0) or N was applied with P and K 90-60-75 kg/ha. The height of chilli plants progressively increased from 64.66 cm up to 75.64 cm as crop received increasing fertilizer dose. The minimum plant height might be due to the unavailability of essential nutrients required by the plants for their proper growth. On the other hand increasing plant height with N was probably because of the role of N in cell division and cell enlargement, which ultimately affected the vegetative growth particularly height of the plant. These results resemble with that of Olanrewaju and Showemino (2003) who found an improvement in plant height with increasing nitrogen application.

Number of branches/plant. Comparatively more number of branches (3.42) recorded when N, P and K were applied at the rate of 120-60-105 NPK kg/ha. The results further demonstrated that with the combination of NPK levels, an increase in number of branches was observed. While, minimum number of branches (2.52) were obtained in plots which received P and K only at the rate of 00-60-75 NPK kg/ha. Earlier studies conducted regarding effect of nitrogen state that it has significant effect on the vegetative growth which ultimately results in an increase in number of branches per plant (Sajid *et al*., 2001; Singh *et al.,* 1999). The results are in support with the findings of Suresh (2000) who concluded that combined application of 100:50:75 N, P_2O_5 and K_2O kg/ha produced significantly higher number of branches/plant over other fertilizer levels.

Days to flowering. Data on days to flowering showed that minimum days to flowering (48.66 days) were recorded in plots where fertilizer level was at 120-60-105 NPK kg/ha, followed by (50.00 days) at fertilizer level of 00-90-105 NPK kg/ha, while maximum days to flowering (53.33 days) were recorded where N was applied without P and K at the rate of 90-00-00 NPK kg/ha. This might be due to the fact that high doses of nitrogen delayed flowering and enhanced vegetative growth in early stages while, phosphorus helps in flowering. Similarly Satpal and Saimbhi (2003) observed that nitrogen significantly affected flowering in two brinjal hybrids. Phosphorus enhances flowering and fruiting and an early flowering was observed where nitrogen was less and phosphorus was greater, as more nitrogen applied alone delayed flowering.

Table 2. Growth characteristics of chilli (*Capsicum annum,* L.) as affected by N in presence and absence of P and K

Treatment NPK (kg/ha)	Plant height (cm)	Branches/ plant	Days to flowering	Days to fruiting
T1 90-00-00	71.66	2.90	52.62	6.30
T2 90-60-75	69.20	3.08	50.33	8.24
T3 00-60-75	64.66	2.52	51.33	5.93
T4 120-00-00	73.53	3.35	53.33	8.71
T5 120-90-105	75.64	3.42	48.66	9.25
T6 00-90-105	66.00	2.62	50.00	7.56
LSD (P<0.05)	2.02	0.22	1.33	0.14

Days to fruiting. The data regarding days to fruiting has been presented in Table 2. It indicates that the plants received fertilizer at the rate of 120-90-105 NPK kg/ha took maximum (9.25) days to fruiting, followed by (8.71) days to fruiting where only N was applied at the rate of 120-00-00 NPK kg/ha, while minimum days (5.93) were taken by the plants fertilized with a dose of 00-60-75 NPK kg/ha. This is because of nitrogen as the application of nitrogen delays flowering that results in delayed fruiting. Due to high nitrogen the days to flowering and days to fruiting were delayed. These results are in close conformity to those of Sutagundi (2000) who reported that early fruiting (43.66 days) was recorded in plants received FYM (10 t/ha) as compared to NPK 100:50:50 kg/ha for the same parameter (43.75 days) in chilli.

Number of fruits/plant. The plots which had received NPK (120-90-105 kg/ha) had maximum number of fruits (111.62) /plant, followed by 120-0-0 NPK levels (99.65) fruits/plant (Table 3). While, the least number of fruits/plant (60.66) were obtained under 0-60-75 NPK levels. From the data presented in Table 3, it is clear that the combination of nutrients i.e., 120-90-105 NPK kg/ha gave significantly higher number of fruits (111.62) with greater weight/plant (381.82 g) under agro climatic conditions of Malir. It might be due to the effect of nitrogen in presence of phosphorus and potassium applied in sufficient and balanced amount that increased vegetative and overall growth of plants; this might increase number of fruits/plant. These results agree with the findings of Akanbi *et al*. (2010) who obtained maximum fruits/plant when nitrogen was applied along with phosphorus and potassium.

Table 3. Yield characteristics of chilli (*Capsicum annum,* L.) as affected by N in presence and absence of P and K

Treatment NPK (kg/ha)	No. of fruits/ plant	Fruit diameter (cm)	Fruit length (cm)	Days to fruiting	Fruit yield (kg/ha)
T1 90-00-00	71.96	3.33	7.56	191.98	5620.66
T2 90-60-75	82.63	3.43	8.24	219.95	5743.33
T3 00-60-75	60.66	3.00	5.93	176.33	5255.22
T4 120-00-00	99.36	3.66	8.71	230.30	7386.33
T5 120-90-105	111.62	3.86	9.25	381.82	7679.66
T6 00-90-105	62.10	3.16	6.30	185.20	5310.33
LSD (P<0.05)	4.65	0.66	1.02	6.55	312.03

Fruit diameter (cm). Mean fruit diameter was significantly greater (3.86 cm) in the plots fertilized with 120-90-105 NPK kg/ha, followed by 120-0-0 and 90-60-75 NPK kg/ha with mean fruit diameter of 3.66 cm and 3.43 cm, respectively. The plots treated with 00-60-75 NPK kg/ha had fruit diameter of 3.00 cm. The minimum fruit diameter might be due to the unavailability of essential nutrients required by the plants for their proper growth. An improvement in fruit diameter under balanced amount of nitrogen along with phosphorus and potassium elements was mainly linked with better plant growth that caused healthier fruits with better diameter. These results are in agreement with the results of Islam *et al.* (2008), who observed that application of higher amount of nutrients increased vigorous plant growth, number and size of capsule as compared to farmers' practice who apply little or no fertilizer.

Fruit length (cm). The data with regard to the effect of N in presence and absence of P and K on size of the fruit has been expressed as length in cm. The effect of NPK on this parameter was found positive. The size of fruit harvested/picked from plots where NPK was applied with 120-90-105 kg/ha was larger (9.25 cm) in length as compared to those where N was applied alone at the rate of 90-0-0 NPK kg/ha had fruits of 6.30 cm in length. However, fruits obtained from the plots received P and K alone in absence of N with 0-60-75 NPK kg/ha had minimum length (5.93 cm). These results are in line with that of Yahaya (2008), who observed improvement in fruit size in response of balanced amount of fertilizers. It means that recommended fertilization would affect the fruit size and other growth parameters as well.

Fruit weight/plant (g). Yield in terms of weight of fruits/plant has been presented in Table 3. The maximum yield (381.82 g/plant) was harvested from the plots which had received nitrogen 120 kg/ha with phosphorus and potassium at 90 and 105 kg/ha (T5) followed by 120-0-0 NPK levels (T4), where N was applied alone having 230.30 g of fruits/plant. The weight of fruits (219.95 g) harvested from plots treated with NPK with 90-60-75 kg/ha ranked at third. While, the least fruit weight (176.33 g/plant) was obtained from plots which were fertilized with P and K in absence of N (0-60-75 kg/ha). The combined application of P and K showed antagonistic effects on vegetative growth. P and K without N probably created nutrient imbalance, which resulted in an antagonistic effect on vegetative growth. Malawadi *et al.* (2004) reported that the application of

FYM at 10 t/ha along with major nutrients (100:50:50 NPK kg/ha) recorded higher fruit weight and 100 fruit weight in chilli crop.

Total yield (kg/ha). The results exhibited that the maximum fruit yield (7679.66 kg) was obtained where nitrogen was applied along with phosphorus and potassium with a dose of 120-90-60 kg/ha (T5), followed by 120-0-0 kg NPK levels (7386.33 kg). While, the least fruit yield (5255.66 kg) was obtained under 0-60-75 kg/ha NPK levels. The high yield might be due to high nitrogen, phosphorus and potassium rates. Nitrogen helps in vegetative growth, due to which branches increased and if there are more branches there may be more fruits and in turn yield will be high. Phosphorus along with nitrogen and potash also helps in improving fruit quality and enhancing flowering due to which yield is increased. These results are in agreement with the findings of Naeem *et al.* (2002), who reported that different dozes of NPK behaved significantly different for total yield. Likewise, Jilani *et al.* (2008) reported that N application at 100 kg/ha significantly increased brinjal yield. It is concluded from the study that fertilizers level of 120-90-75 NPK kg/ha had significantly affected the yield and growth characteristics of chilli. The above level of fertilizers had positive effect on most of the parameters therefore, yield was increased significantly.

Conclusion

The combined application of NPK fertilizers positively affected growth and yield characteristics of chilli as compared to nitrogen applied alone. It was concluded that, N in presence of P and K with 120-90-105 kg/ha proved best for better production of chilli var. Malir Local under agro climatic conditions of Malir district, Sindh, Pakistan.

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Pharmacokinetic Modelling of Methotrexate from Routine Clinical Data in Patients with Acute Lymphoblastic Leukemia

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Abstract. Pharmacokinetic modelling was performed in NONMEM (version 6.1) using a dataset including 273 patients (aged 2 to 23 years) who received high-dose MTX (5 g/m^2 per course) in long-term treatment. Total 2582 methotrexate plasma concentrations were performed by fluorescence polarisation immunoassay (FPIA). A three compartment open model with elimination from the central compartment described the pharmacokinetics of methotrexate. The most important covariates affecting the disposition of methotrexate were age (age, year), body weight (BW, kg), and creatinine clearance (CLR, lh^{-1}). The final model with exponential disposition of MTX was clearance (CL, lh^{-1}) = (6.11 + WT*6.7310^{-2}) + (1.0810^{-4}* CLR)* EXP(1.9510^{-1}), (V, l) = 10,8+(AGE* 9.310^{-2}) *EXP(9.110^{-1}), Q(lh^{-1}) = 2.0410^{-3} *WT. Pharmacokinetic parameters (%CV) in this study were CL, 8.72 lh^{-1} (44 %); V1, 17.49 l (95%); V2, 6.048 l (56%); V3, 0.015 l (52%) . The model predictions in the qualification group were found to have no bias and satisfactory precision

Keywords: population pharmacokinetics, methotrexate, acute lymphoblastic leukaemia, NONMEM

Introduction

Methotrexate (MTX) with folinic acid rescue is widely used in the treatment of leukaemia. However, high dose MTX has been proven to cause substantial toxicity and have high intra-and inter-patient variability.

The MTX is characterized by a narrow therapeutic window combined with high-and inter-patient variability (Dupuis *et al.,* 2006). It has been demonstrated that HDMTX toxicity is directly linked to its exposure time and plasma concentration (Balloy *et al.,* 2007). Elimination of MTX is prolonged in patients with renal impairment or third space fluid collections due to a slow redistribution from this extravascular fluid accumulation.

Population pharmacokinetic analysis is a useful tool for identification of sources of pharmacokinetic variability during anticancer drug development and can aid the design of alternative dosing regimens to enhance their efficacy and safety.

Objective of this study was to develope and validate a population pharmacokinetics model of MTX in Tunisian patients with acute lymphoblastic leukaemia (ALL) (aged from 2 to 23 years). Clinical covariates have been described that influence MTX pharmacokinetic for predicting optimal dose to reduce MTX toxicity.

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Materials and Methods

Patients. Two hundred and seventy three patients (aged 2 - 23 years, median 13 years) who received high-dose MTX therapy (1-4 courses of chemotherapy) have been studied. The disease treated was ALL. They were enrolled at the time of diagnosis in the European Organization for Research and Treatment of Cancer (EORTC) protocol 58951 (Falkenrodt *et al.,* 1994) approved by an institutional review committee for clinical trials.

Data collection. Blood samples were collected by prospective chart review including: age, gender, weight, body surface area (BSA), significant delay in MTX elimination defined as MTX concentration at 24 h after the end of infusion ($Cp_{24\ h}$) > 5 µmol/L, and/or MTX concentration 48 h after the end of the infusion ($Cp_{48\ h}$) > 0.5 µmol/L.

MTX administration. Urine alkalinisation was performed by administration of 1.4 % sodium bicarbonate, 10 mL/kg, 2 h prior to the start of MTX infusion. Intravenous hydration (1.4 % sodium bicarbonate (1/3) and 5 % glucose solution with 2 g/L KCl (2/3), 24 mL/m^2/24 h) was administered from the start of the infusion up to 72 h. The dose MTX (5000 mg/m^2) was given as a loading dose (1600 mg/m^2 over 0.5 h in 250 mL of 5% glucose), followed by the rest of the dose (6400 mg/m^2 over 23.5 h in 500-1000 mL of 5% glucose).

Urine pH was checked after every 6 h and a dose of 1.4% sodium bicarbonate, 6 mL/kg IV, was administered if pH < 7. Folinic acid, at the dose of 12 mg/m^2 IVwas administered to adjust to MTX concentrations according to protocol guidelines, at 36 h after the initiation of MTX infusion and repeated every 6 h until MTX concentration was < 0.02 μmol/L.

Sample analysis. For each patient, the blood samples were collected in dry tubes protected from light 24, 48, 72, 96 h at the end of infusion and rapidly centrifuged at 2500g and stored at -20°C until simultaneous analysis. MTX concentrations were measured with ABBOTT TD_X fluorescence polarization immunoassay (FPIA). The detection limit of the assay was 0.01 μmol/L. Cross reactivity with the main circulating metabolite, i.e. 7-hydroxy MTX (7-OH-MTX), was less than 1.5%. Cross reactivity with 2.4 diamino-N-methyl-pteroic acid was as high as 44%; but this metabolite produced in the gut is usually undetectable in plasma samples and therefore could not interfere with the assay (Colom *et al.,* 2009). The assay by FPIA was highly sensitive and rapid enough to measure plasma MTX concentration.

Pharmacokinetics analysis. Population pharmacokinetic modelling was applied using NONMEM (version 6.1) to estimate pharmacokinetic parameters, intersubject and residual variability (random effects) in terms of patients specific information such as age or body weight (fixed effects). All analyses were performed with the first-order conditional estimation method with interaction. Graphical diagnostics and comparison of competing models using the objective function values (OFV) in the likelihood ratio test guided the model development.

Statistical model. The choice of the structural (or pharmacokinetic) model was based on a comparison of two and three compartment models with first order input and first order elimination from the central compartment. Analysis of model diagnostics favoured selection of the three compartment model. Therefore, a three open compartmental model disposition of methotrexate was described in terms of clearance (Cl), volume V1 of central compartment, volume V2 and V3 of the peripheral compartments, the intercompartmental clearance Q_2, Q_3 between the central and peripheral compartments, and the elimination constant K_{10} from the central compartment. (PREDPP subroutine ADVAN11 TRANS4 in NONMEM).

To estimate the pharmacokinetic parameters of methotrexate in the investigated population, the following models were used to describe the intersubject variability in cleareance:

$$Cl_i = Clpop * e^{\eta_{JCL,i}}$$

Where, Cl_i is the plasma clearance of MTX from the 'j'th patient; Cl pop is the population mean value of Cl or a known function that describes the expected value of Cl_j as a function of individual specific covariates, such as age, body weight, gender, etc, and the vector of population average parameters. $\eta_{JCL,i}$ is between-patient variability (BPV) and inter-occasion variability were modelled exponentially assuming a log-normal distribution. Residual error (intra individual variability) in the concentration was modelled with exponential error structure as follows:

$$Cij = F*\exp(\varepsilon_{1,i,j})$$

Where, Cij is the jth methotrexate serum concentration observation in the ith individual, Fij is the jth model predicted concentration in the ith individual, and $\varepsilon_{i,j}$ are the residual variability error terms and are assumed normally distributed with mean 0 and variance σ^2 which is sometimes written in shorthand as $\varepsilon = N(0,\sigma^2)$.

Development of covariate model. The regression model describes the relationship between a pharmacokinetic parameter and a covariate. A graphical approach to exploratory data analysis can be useful tool in elucidating the presence of these relationships. An initial screening with Excel© software was performed. The initial screening gives a first impression of the relative importance of several covariates and pharmacokinetic parameters. To carry out this preliminary step, individual estimates of clearance were first obtained and subsequently the significance of each possible covariate in affecting the parameter was evaluated. After the initial screening step, with the estimates of the individual Cl values treated as 'data', a regression model was derived with stepwise regression. This step corresponds to the classical regression problem of variable selection. The influence of age (AGE), body weight (BW), gender (GEN, 0= male, 1= female), creatinine clearance (CLR) was observed.

Development of a regression model is an attempt to maximize the predictive ability of the model without sacrificing parsimony. To achieve this objective,

covariates were first screened by individually testing each covariate alone in the base pharmacostatistical model to determine which ones significantly improves the ability of the model to predict the observed concentration-time profile. The difference in the objective function value (- 2*log likelihood) between two hierarchical models, defined as the log likelihood difference (LLD), is asymptomatically chi squared (χ^2) distributed with degrees of freedom equal to the difference in the number of parameters between the two models. During the covariate screening step, addition of one parameter to the model had to effect a LLD of at least 3.84 to achieve the desired level of significance of α= 0.05 ($p<0.05$) from chi squared distribution with one degree of freedom. Traditional methods of model building reported in the literature assemble all significant covariates into an intermediate full model that is then subjected to backwards elimination procedure to produce a parsimonious model (Combe *et al.,* 1995).

Qualification of the model. To evaluate the performance of the final model in predicting MTX concentrations, a second group of 50 patients treated with MTX was studied. The demographic data of the evaluation group is shown in Table 1. The measured concentrations of MTX in these patients were compared with the corresponding predicted values obtained using the final population pharmacokinetic model, patients' covariates and dosing information. Predictive performance of the model was assessed by calculating the mean error (predicted-observed concentration) and its 95% confidence interval (CI) as an estimate of bias, and the root mean squared prediction error and 95% CI as an estimate of precision. CIs including the value zero were considered unbiased (Sheiner and Ludden, 1992).

The final model was subjected to a bootstrap analysis (1,000 replicates) using R software-package (version 2.9.2). Table 2 summarizes the results presented as medians and 95% confidence intervals (2.5^{th} and 97.5^{th} percentiles). Bootstrap medians and parameter estimates from the original dataset were reasonably similar and indicated acceptable precision.

Results and Discussion

Patients. Pharmacokinetic data were available for 273 children and young adults with ALL receiving high dose of MTX (5 g/m^2). Patient characteristics are outlined in Table 1.

Pharmacokinetic model. Analyses of residual plots from the two and three compartment model fits were compared. The three compartment model provides a superior fit to the data compared to the two compartment model fit (LLD increased by 128, statistically significant $p< 0.05$). Potential explanatory covariables might include patient's age, weight, gender, height, and creatinine clearance. The change in the NONMEM objective function produced by the inclusion of a covariate term was used to compare alternative models. Finally, accepted covariates were added to the model and the population pharmacokinetic parameters were estimated. To

Table 1. Patient characteristics

Parameters	Population analysis (Mean ± SD)	Qualification analysis (Mean ± SD)	Abbreviation
Age (years)	13.74 ± 8.57	10.59 ± 5.12	Age
Body weight (kg)	42.40 ± 18.9	35.52 ± 19.31	WT
Body surface (m^2)	1.27 ± 0.38	1.5 ± 0.75	SC
Serum creatinine (mg/L)	108.16 ± 58.8	110.55 ± 45.9	CLR
Gender (Female/ Male)	138/135	35/15	GEN
MTX dosage (g/m^2)	5	5	

Table 2. Expected pharmacokinetic parameter estimates

Parameter	Estimates	Inter-individual variability (CV %)	Inter-occasion variability (CV %)	Relative standard error (RSE %)	Confidence intervals (95%)
CL (l/h)	8.72	44	13.8	13	[0.14-0.24]
V1 (l)	17.49	95	_	3.7	[0.85-0.98]
V2 (l)	6.048	56	_	7.5	[0.27-0.36]
V3 (l)	0.015	53	_	7.19	[0.23-0.31]
Residual variability	σ	75	_	_	_

demonstrate that retained covariates contributed to an improvement of the fit of the population pharmacokinetic model, each covariate was deleted sequentially from the proposed final model (backward elimination) in order to confirm statistical significance (χ^2 test). If the objective function did not vary significantly, the relationship between the covariate and pharmacokinetic parameter was ignored. (Table 3) summarizes the data of the covariates.

Inter-occasion variability was investigated on CL, V1, and V2 and was only significant on CL.

Plots of model-population predicted versus population observed concentrations obtained from the final model based on individual and population parameter estimates are shown in Figs. 1a and 1b. Various statistical tests were carried out with a no significant outcome difference when the regression line of individual predicted concentrations versus observed concentration (slope= 0.63, SE=0.03; intercept =1.56) was compared to the reference line slope = 1 and intercept = 0); and the frequency of the distribution histogram of the normalized residuals was as expected (normal with zero mean and unitary variance). The vast majority of the weighted residuals laid within two units of perfect agreement and was symmetrically distributed around the zero ordinate (Fig. 1b).

Table 3. Forward selection model building summary

No.	Model*	OBJ	LLD**	Significance***
1	Base Model	-919	-	No
2	1+Age~Cl	-919	0	No
3	2+WT~Cl	-1041	122	Yes****
4	3+GEN~Cl	-742	-177	No
5	4+CLR~Cl	-1079	160	Yes****
6	5+Age~V1	-956	37	Yes****
7	6+WT~V1	-919	0	No
8	7+GEN~V1	-919	0	No
9	8+CLR~V1	-919	0	No
10	9+Age~Q2	-736.34	-183	No
11	10+WT~Q2	-897.22	-22	No
12	11+GEN~Q2	-919	0	No
13	12+Age~Q3	-919	0	No
14	13+WT~Q3	-926	7	Yes****
15	14+GEN~Q3	-919	0	No

*=Model indicates sequential steps taken to achieve a full model. '~' symbolizes 'as a linear function of'. Intercept terms are implicit; **=LLD is the change in objective function value obtained for the modelling step; ***=Significance is at the P<0.05 level (LLD>3.84); ****= Covariate dropped in final model as the 95%; confidence interval enclosed zero.

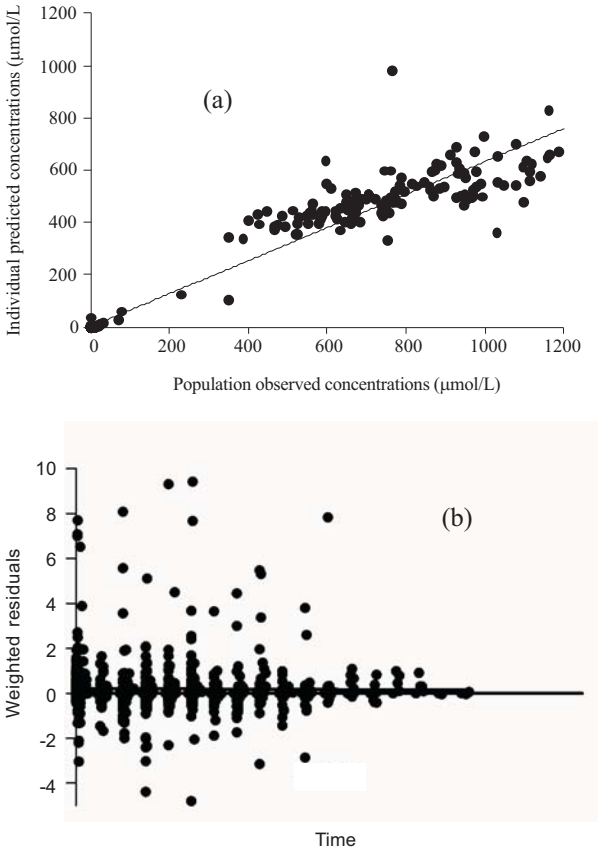

Fig. 1a-b. Model performance and diagnostic plots (n = 273 patients; 2582 MTX concentrations) a- Model population-individual predicted versus population-observed concentrations obtained with the final model based on population parameter estimates. b- Weighted residuals versus time.

Development of the full model. The final regression model was determined as follows;

Clearance:
CL (l h^{-1}) = (θ1+WT* θ 7)+(θ 9*CLR))*EXP(ETA(1))
Volume of distribution:
V_C = θ2 + (AGE*θ8) * EXP (ETA (2))
Intercompartmental clearance:
Q (l h^{-1}) =θ 6*WT

Table 4 displays the expected pharmacokinetic parameters estimates, additionally as estimated by NONMEM, intersubject coefficient of variation (CV) for the final model is presented.

To assess its predictive performances, the final model was used to predict concentrations in a new group of

Table 4. Predictive performance of final model established in the validation group

Final model	Error
4.88 (2.73- 7.02)	Mean prediction error (MPE)
2.21 (2.17-2.26)	Mean squared prediction error (MSPE)
1.48 (0.39-1.53)	Root mean squared prediction error (RMSE)

patients. Table 4 summarizes the prediction error in the qualification group. The results show no bias, a good precision and indicate acceptable predictive performance.

Previous reports in the literature suggest that the disposition of methotrexate could be characterized by a two or three compartment model with first order elimination (Zhang *et al.,* 2010; Colom *et al.,* 2009; Aumente *et al.* 2006; Faltaos *et al.,* 2006). In our population model was fitted to the present data. Model diagnostics in the form of residual plots and the log likelihood difference suggested that the two compartment model offered no improvement in the fit to the data while the three compartment model best fitted ours data. Therefore, subsequent modeling was implemented with a three compartment model.

In this analysis, mean values for clearance, and central volume of distribution, values CL, 8.72 lh^{-1}; V1, 17, 49 l estimated by NONMEM were close to values reported in other population pharmacokinetic study as values reported by Plard *et al.* (2007) CL, 8.8 lh^{-1}; V1, 17,3 and by Rousseau *et al.* (2002) CL, 7.4 lh^{-1}; V1, 18,2 l.

Clearance increased with increasing renal filtration as assessed by creatinine clearance and increased with increasing body weight, presumably representing non-filtration elimination. Statistically significant correlation was reported between creatinine clearance and total body clearance of methotrexate (Zhang *et al.,* 2010; Oudoul *et al.,* 1999; Monjanel *et al.,* 1979). However, these relations were for one variable only and of poor predictive value. Combe *et al.* (1995) and Lafforgue *et al.* (1995) also reported a statistically significant correlation between methotrexate clearance and creatinine clearance.

More recently, Faltaos *et al*. (2006) in a population pharmacokinetic analysis reported a statistically significant positive correlation between creatinine clearance and methotrexate clearance. It was also observed that methotrexate clearance was inversely correlated to patient age, although the relationship was not as strong as with creatinine clearance (Colom *et al.,* 2009; Aumente *et al.,* 2006; Faltaos *et al.,* 2006)., Lafforgue *et al*. (1995) reported similar findings on the relationship between age, creatinine clearance and methotrexate clearance. However, in the full model, the parameter relating methotrexate clearance to age was not precisely estimated as evidenced by a 95% confidence interval enclosing zero. The clinical interpretation of such results might be that age related decreases in methotrexate clearance are adequately accounted for by incorporation of creatinine clearance assessments. Therefore, renal function monitoring is necessary in patients receiving methotrexate and adjusting appropriate dosage.

Volume of the central compartment was approximately 17.49 l in this study population. This value is somewhat lower than that reported by Bressolle *et al*. 1996 (34.8 l) but similar (22.2 l) to that published by Zhang *et al.* (2010); Plard *et al.* (2007); Oudoul *et al.* (1999) and Sabot *et al.* 1995). Volume of the peripheral compartment increased with increasing body weight.

The population pharmacokinetics of methotrexate in children with ALL was determined using an extended least squares approach to nonlinear mixed effects modelling. Estimates of mean pharmacokinetic parameters correspond well with values previously reported in the literature. However, the population approach has provided estimates of intersubject variability and a method by which to explain some of this variability in terms of subject specific covariates. This can be seen in Table 4. The population pharmacokinetics of methotrexate in children with ALL was well described by this investigation. Substantial interpatient variability was explained by incorporating patient specific data into regression equations predicting pharmacokinetic parameters and the clinical relevance and implications of the relationships have been presented.

Conclusion

The pharmacokinetic parameters of high dose methotrexate were accurately estimated. Creatinine clearance and weight influenced the high methotrexate clearance and age influenced volume distribution. Care should be taken in the choice of the dose regimen.

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A Novel pH-Responsive Superabsorbent Hydrogel Based on Collagen for Ephedrine Controlled Release

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Abstract. A novel family of pH-responsive polymeric hydrogel based on collagen was prepared for controlled delivery of ephedrine. Acrylic monomers, acrylic acid (AA) and itaconic acid (IA) were simultaneously graft copolymerized onto collagen backbones by a free radical polymerization technique using ammonium persulphate (APS) as initiator and methylene bisacrylamide (MBA) as a crosslinker. Hydrogel formation was confirmed by FTIR spectroscopy. Thermogravimetric analysis showed the thermal stabilities of the hydrogels. Results from scanning electron microscopy (SEM) observation also showed a porous structure with smooth surface morphology of the hydrogel. Swelling profiles obtained indicated clearly that these hydrogels swell slightly in a simulated gastric fluid (SGF) and strongly in a simulated intestinal fluid (SIF). The model drug, ephedrine, was successfully loaded into the hydrogels and *in vitro* release studies were performed in SGF for the initial 122 min, followed by SIF until complete dissolution. The release of ephedrine was continued up to 215 min. The release mechanism of the hydrogels was also studied using the Ritger-Peppas model.

Keywords: collagen, hydrogel, acrylic acid, itaconic acid, ephedrine

Introduction

Drug delivery systems (DDSs) are regarded as a promising means to control post-operative inflammation (Kranz and Bodmeier, 2008), although design improvements are needed to increase biocompatibility and effectiveness, as well to prolong controlled release of the drug (Oh *et al.,* 2008). Interest in biodegradable polymers, and specifically in a DDS matrix is growing day by day. The main reason for this is that delivery systems based on biodegradable polymers do not require removal of the polymers from the body at the end of the treatment period, as they degrade into physiologically occurring compounds that can be readily excreted from the body (Kakinoki *et al.,* 2007).

In recent years, much interest has been shown in the development of synthesis of natural-based super-absorbent hydrogels (Bagheri *et al.,* 2011; Sadeghi and Hosseinzadeh, 2010; Hua and Wang, 2009; Zheng and Wang, 2009). These biopolymer materials are crosslinked hydrophilic polymers, capable of absorbing large quantities of water, saline or physiological solutions (Buchholz and Graham, 1997; Peppas and Harland, 1990). Because of their non-toxicity, biocompatibility and biodegradability, natural-based hydrogels have attracted in many fields such as hygiene, cosmetics, and agriculture (Zhou *et al.,* 2011; Sokker *et al.,* 2011; Raghavendra *et al.,* 2010).

Stimuli-responsive smart hydrogels that can respond to environmental physical and chemical stimuli, such as magnetic field (Wang *et al.,* 2009), temperature (Chu *et al.,* 2007), light (Tatsuma *et al.,* 2007), pH (Kim *et al.,* 2006) and electric field (Kwon *et al.,* 1991), have attracted great interests in recent years due to their versatile applications such as controlled drug and gene delivery systems (Cheng *et al.,* 2008; Hamidi *et al.,* 2008; Thornton *et al.,* 2007; Chu *et al.,* 2002; Soppimath *et al.,* 2002), chemical-/bio-separations (Yang *et al.,* 2008), and sensors and/or actuators (Eddington and Beebe, 2004). Among those smart hydrogels, pH-responsive hydrogels have been extensively investigated for potential use in site-specific delivery of drugs to specific regions of the gastrointestinal tract and have been prepared for delivery of low molecular weight drugs.

Proteins are widely distributed in nature and are synthesized mainly in animals, i.e. collagen, keratin, and etc., and in a few plants such as Soya. In general, proteins are high molecular weight polymers and their solubility

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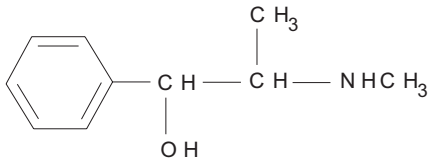
in aqueous solutions is difficult. Two efficient methods for preparation of aqueous soluble proteins are alkaline and enzymatic hydrolysis. According to the literature survey based on Chemical Abstract Service, a few studies have been reported in the case of protein-based hydrogels (Branco *et al.,* 2010; Zohuriaan-Mehr *et al.,* 2009; Yin *et al.,* 2008; Pourjavadi and Kurdtabar, 2007; Rathna and Damodaran, 2002).

Ephedrine, L-erythro-1-phenyl-2-methylaminopropanol-1 (Scheme 1), is an alkaloid that is present in various forms of the ephedrine family, extracted from *Ephedra sinica* and *Ephedra equisetina*. The pharmacological action of ephedrine is typical of noncatecholamine sympathomimetics of mixed action. It is mainly used for bronchial asthma, allergic illnesses, as an antiedemic for mucous membranes in rhinitis, and also as a drug to increase blood pressure during surgical interventions. It is used locally in ophthalmology as a vasoconstricting agent for dilating pupils.



Scheme 1. Chemical structure of drug ephedrine.

Hence, the target of the current study was to exploit novel pH-sensitive collagen-based hydrogels for the effective ephedrine controlled release system. Drug absorption and release capacities of hydrogel systems were also examined.

Materials and Methods

Materials. Hydrolyzed collagen (Parvar Novin-E Tehran Co.) is industrial grade which is available in market and has nearly 25% insoluble phosphate salt. Acrylic acid (AA, Merck) was used after vacuum distillation. Itaconic acid (IA, Merck) and ammonium persulphate (APS, Merck) was used without purification. Methylene bisacrylamide (MBA, Fluka) was used as received. All other chemicals were of analytical grade. The drug, ephedrine was obtained from Jaberebne Hayan Pharmaceutical Co. (Tehran, Iran). Double distilled water was used for the hydrogel preparation and swelling measurements.

Preparation of hydrogel. A general procedure for chemically crosslinking graft copolymerization of AA and IA onto collagen backbones was conducted as follows. Hydrolyzed collagen (1.33 g) was dissolved in 50 mL distilled water and filtered to remove its insoluble phosphate salt. Then the solution was added to a three-neck reactor equipped with a mechanical stirrer (Heidolph RZR 2021, three blade propeller type, 300 rpm). The reactor was immersed in a thermostated water bath preset at a desired temperature (80 °C). Then a definite amount of APS solution (0.1 g in 5 mL H_2O) was added to collagen solution and was allowed to stir for 10 min. After adding APS, certain amounts of AA and IA (AA 1.20 g, IA 0.80) were added simultaneously to the collagen solution. MBA solution (0.05 g in 5 mL H_2O) was added to the reaction mixture after the addition of monomers and the mixture was continuously stirred. After 60 min, the reaction product was allowed to cool to ambient temperature and neutralized to pH 8 by addition of 1N sodium hydroxide solution. The hydrogel was poured to excess non solvent ethanol (200 mL) and kept for 3 h to dewater. Then ethanol was decanted and the product scissored to small pieces. Again, 100 mL fresh ethanol was added and the hydrogel was kept for 24 h. Finally, the filtered hydrogel was dried in oven at 60 °C for 10 h. After grinding (using mortar) the powdered superabsorbent was stored away from moisture, heat and light.

FTIR analysis. Fourier transform infrared (FTIR) spectra of samples were taken in KBr pellets, using an ABB Bomem MB-100 FTIR spectrophotometer (Quebec, Canada), at room temperature.

Surface morphology. The surface morphology of the gel was examined using scanning electron microscopy (SEM). After Soxhlet extraction with methanol for 24 h and drying in an oven, superabsorbent powder was coated with a thin layer of gold and imaged in a SEM instrument (Leo, 1455 VP). Brunauer-Emmett-Teller (BET) analysis was used to determine the pore size of the hydrogels.

Thermogravimetric analysis. Thermogravimetric analyses (TGA) were performed on a Universal V4.1D TA Instruments (SDT Q600) with 8-10 mg samples on a platinum pan under nitrogen atmosphere. Experiments were performed at a heating rate of -20 °C/min until 600 °C.

Swelling measurements. Hydrogel (0.25 g) were immersed in 250 mL solution with various pH values (pH 1.2 and pH 7.4) at 37 °C to reach swelling equilibrium. Swollen samples were then separated from unabsorbed water by filtering through a 100-mesh screen under gravity for 30 min without blotting

the samples. The equilibrium swelling (ES) capacity in buffer solution was measured triple according to the following equation:

$$ES(g/g) = \frac{\text{Weight of swollen gel} - \text{Weight of dried gel}}{\text{Weight of dried gel}} \quad (1)$$

The accuracy of the measurements was ± 3%. The standard deviation (s) for a sample of data that is of limited size is given by the following equation:

$$S = \sqrt{\frac{\sum_{i=1Q}^{N} (X_i - \overline{X})^2}{N-1}} \quad (2)$$

where, $(X_i - \overline{X})$ is deviation from average of *i*th measurement and N is number of replicates of each measurement (here N=3).

Encapsulation of model drug. Loading of ephedrine (20% w/w, based on the total weight of the hydrogel) was carried out by swelling of dried polymeric hydrogel sample in phosphate buffer solution (pH 7.4) at 37 °C. After immersing the vacuum dried powdered samples (0.1 g) for 24 h, it was taken out, dried and accurately reweighed. The increase in the weight of the hydrogel was taken as the amount of drug loaded i.e. ephedrine encapsulation efficiency percentage, (EE%). The swollen hydrogels loaded with drug were placed in a vacuum oven, dried under vacuum at 37 °C and stored until further investigation.

The entrapped drug exhibited the same λ_{max} as free drug. This clearly indicates that the entrapped drugs have not undergone any possible chemical reaction during the matrix formation. The difference in between initial drug and the drug content in the washing solutions is taken as an indication of the amount of entrapped drug:

$$\text{Drug entrappment (\%)} = \frac{\text{Amount of drug present in hydrogel}}{\text{Theoretical amount of drug}} \times 100 \quad (3)$$

It should be pointed out that the phosphate may be lightly absorbed into the hydrogel along with the model drug. But the absorbed phosphate was very little. The anionic phosphate can not be absorbed largely by anionic hydrogel. However, the loaded hydrogel was washed by distilled water for removing the residues.

Spectrophotometric analysis of model drug. A UV/visible spectrophotometer (Shimadzu, UV-2550) was used to determine the maximum spectra of the drug. Model drug in aqueous solution was prepared for determining the maximum absorption wavelength. The characteristic peak was observed. The absorbance value at the maximum wavelength of 276 nm of the model drug was read and the corresponding model drug concentrations were calculated from the calibration curve.

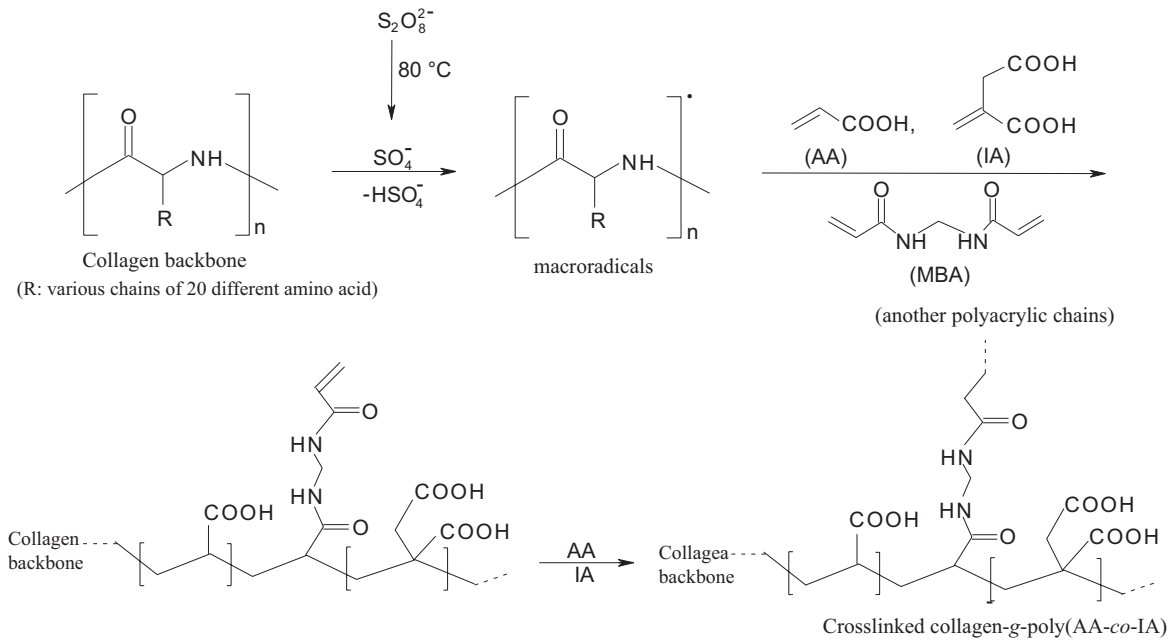
Determination of the amount of drug entrapped. The amount of ephedrine entrapped into the hydrogels was calculated by measuring the absorbance of the gelling medium at 276 nm. The amount of ephedrine entrapped was estimated by the difference between the initial and the final amount of drug in gelling media. Encapsulation efficiency percentage was expressed as the weight of drug entrapped in the polymeric hydrogel divided by the initial weight of ephedrine in solution. Moreover, it is important to notice that the drug exhibited the same λ_{max} for whatever the release medium used in this study, as the free drug in water and the presence of dissolved polymers did not interfere with the absorbance of the drug at this wavelength.

Release studies. *In vitro* release studies were performed in simulated gastric fluid (SGF) and strongly in a simulated intestinal fluid (SIF) at 37 °C. Accurately weighed amounts of dried drug-loaded polymeric hydrogel (ranging from 0.1 to 0.2 g) were placed in beakers containing 1 L of the release medium at 37 °C. At periodic intervals 5 mL of aliquots were collected from the release medium, and the ephedrine concentrations were measured using a spectrophotometer at λ_{max} 276 nm. The percentage of cumulative amount of released ephedrine, obtained from three experiments, was calculated and plotted against time.

Results and Discussion

Synthesis of hydrogels. A general reaction mechanism for collagen-based hydrogel formation is shown in Scheme 2. At the first step, the thermally dissociating initiator, i.e. APS, is decomposed under heating to produce sulphate anion-radical. Then, the anion-radical abstracts hydrogen from one of the functional groups in side chains (i.e. COOH, SH, OH, and NH_2) of the substrate to form corresponding radical. So, these macroradicals initiated monomers grafting onto collagen backbones led to a graft copolymer. In addition, cross-linking reaction was carried out in the presence of a crosslinker, i.e., MBA, so that a three dimensional network was obtained.

FTIR spectroscopy. The grafting was confirmed by comparing the FTIR spectra of the collagen substrate



Scheme 2. Proposed mechanistic pathway for synthesis of the collagen-based hydrogels.

with that of the grafted products. The band observed at 1644 cm^{-1} can be attributed to C=O stretching in carboxamide functional groups of substrate backbone (Fig. 1a). The superabsorbent hydrogel product comprises a collagen backbone with side chains that carry sodium carboxylate functional groups that are evidenced by peak at 1561 cm^{-1} (Fig. 1b). This characteristic band is due to asymmetric stretching in carboxylate anion that is reconfirmed by another peak at 1422 cm^{-1} which is related to the symmetric stretching mode of the carboxylate anion.

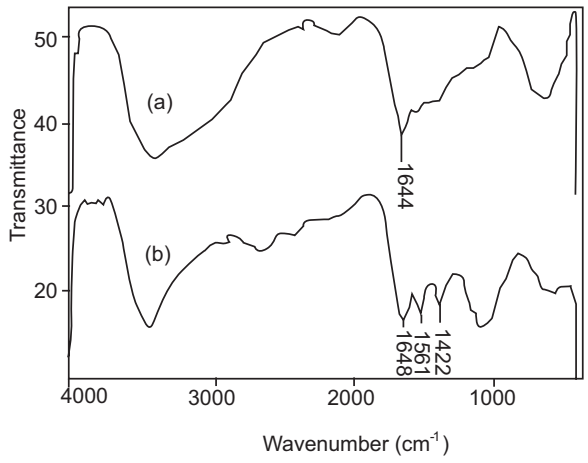


Fig. 1a-b. FTIR spectra of collagen (a) and collagen-*g*-poly(AA-*co*-IA) hydrogel (b).

Morphology of hydrogel. The morphology of the crosslinked hydrogel was observed by scanning electron microscope (SEM). Although the water inside the hydrogel was sublimed to make cavities, the structure of the hydrogel was preserved. Figure 2 shows the scanning electron microscope (SEM) photographs of the surface (Fig. 2a) and the cross-sectional area (Fig. 2b) of the hydrogel with interconnected pores. The hydrogel has a porous structure. It is supposed that these pores are the regions of water permeation and interaction sites of external stimuli with the hydrophilic groups of the graft copolymers. The cross-sectional view of hydrogels (Fig. 2b) also exhibited large, open, channel-like structure.

The results of BET analysis showed that the average pore diameter of the synthesized hydrogel was 7.4 nm. This proves that the structure of the hydrogel is very porous, and it could help to form a high-water-content hydrogel, as is generally shown in other hydrogels (Koo *et al.,* 2009; Crescenzi *et al.,* 2007).

Thermogravimetric analysis. TGA curves for collagen and collagen-based hydrogel are shown in Fig. 3a-b. The grafted collagen has shown improvement in thermal stability as clear from TGA curve. The initial decomposition temperature of the collagen on grafting was increased from 168 to 402 °C with maximum decomposition rate at 523 °C, in comparison to original decomposition tem-

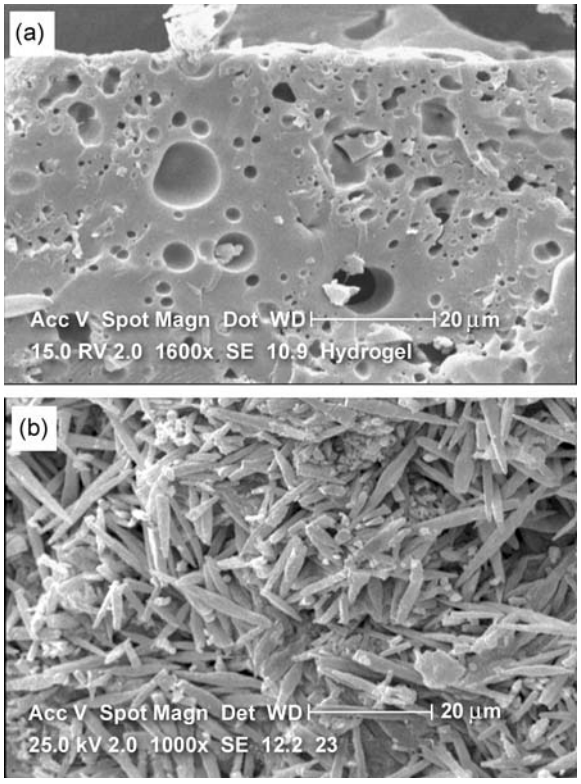


Fig. 2a-b. SEM photograph of the hydrogel. (a) Surface of porous hydrogel; (b) cross-sectional area of porous hydrogel. Surfaces were taken at a magnification of 1000, and the scale bar is 20 µm.

perature of 325 °C of collagen. These observations have clearly indicated that grafting of poly(AA-*co*-IA) has improved the thermal stability of collagen.

Amount of drug encapsulated. The amount of ephedrine encapsulated in the polymeric hydrogels increased with increasing drug concentration. The ephedrine encapsulation efficiency percentages (%EE) are 63, 74 and 88% according to the concentrations 0.15, 0.75 and 1.2 % of ephedrine, respectively.

***In vitro* release behaviour of hydrogels.** In order to simulate the possible effect of pH on drug release rate, a swelling study was conducted in simulated gastric fluid (pH 1.2) and simulated intestinal fluid (pH 7.4) at physiological temperature of 37 °C (Fig. 4). It should be pointed out that there are no differences between SGF (pH 1.2) and SIF (pH 7.4) besides the pH. At pH 7.4, the hydrogel swells due to anion-anion repulsive electrostatic forces, while at pH 1.2, it shrinks within a few minutes due to protonation of the carboxylate

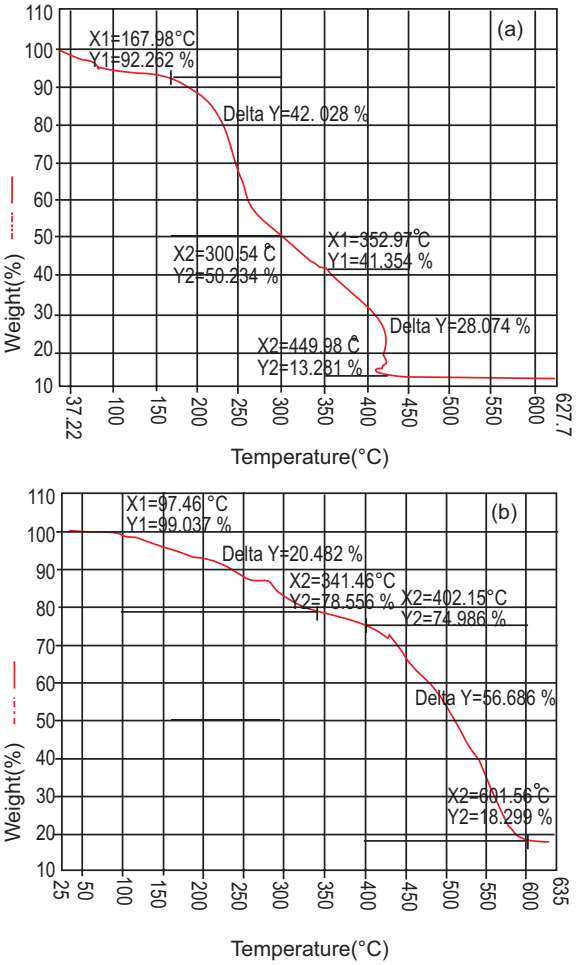


Fig. 3a-b. TGA thermograms of (a) collagen and (b) collagen-*g*-Poly(AA-co-IA) superabsorbent. Heating rate 20 °C/min, under N_2.

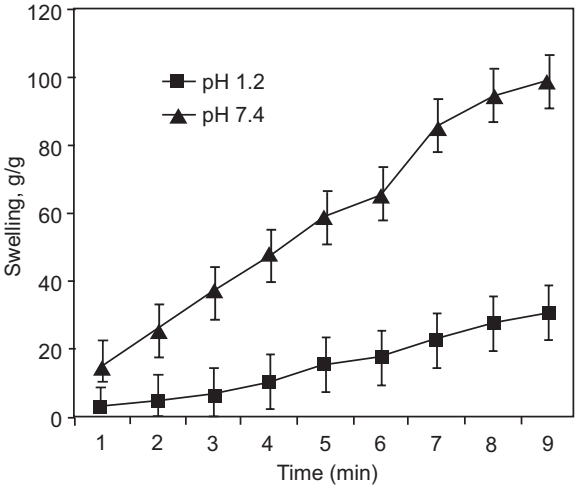


Fig. 4. Effect of pH of solution on swelling of collagen-*g*-poly(AA-*co*-IA) hydrogel.

anions. This swelling behaviour of the hydrogels makes them as suitable candidate for designing drug delivery systems.

The most challenging task in the development of drug pharmaceuticals is to deal with instabilities of drugs in the harsh environment of the stomach. Drug encapsulation processes that require the use of organic solvents or heating might potentially physically modify or denature the therapeutic proteins. Encapsulation processes that require chemical bond formation among the encapsulation reagents might unintentionally chemically modify the therapeutic proteins. However, drug loading process in the present study was desirable as the encapsulation of ephedrine was performed avoiding any organic solvent, high temperature, unfavourable pH and other harsh environmental conditions. The conditions were benign sufficiently as the resulting hydrogel physically entrapped the ephedrine drug. Figure 5 shows the ephedrine release profile of the test hydrogels at pH 1.2 and subsequently at pH 7.4. The time point at which the samples were collected for analysis was 2 min. The amount of ephedrine released at pH 1.2 was low; only about 15% ephedrine was released from the test hydrogel, whereas that released at pH 7.4 increased significantly (94%). The favourable ephedrine release performance could be attributed to the pH-sensitivity of the hydrogel. Swelling of such hydrogel in the stomach was minimal and thus the drug release was also minimal. Due to increase in pH, the extent of swelling increased as the hydrogel passed down the intestinal tract, the hydrogel swelled and the controlled release of ephedrine was affected. Figure 6 shows the schematic diagramof actuation at a distance and resultant squeezing effect for the pH-responsive collagen-based system. Because of the high matrix porosity of the hydrogel (Fig. 2), the capillary forces could reinforce the diffusion of solvent into the hydrogel; thereby the ephedrine release from the hydrogel matrix occurred mainly due to the diffusion of the drug through the pores of the swelled matrix in the intestinal pH.

The dependence of the extent of crosslinking on *in vitro* release was also displayed in Fig. 7. It is observed that release rates depend upon the amount of MBA used as crosslinking agent. The cumulative drug release of ephedrine from the hydrogels was decreased with increasing MBA content. This could be due to the fact that at higher crosslinking, free volume of the matrix

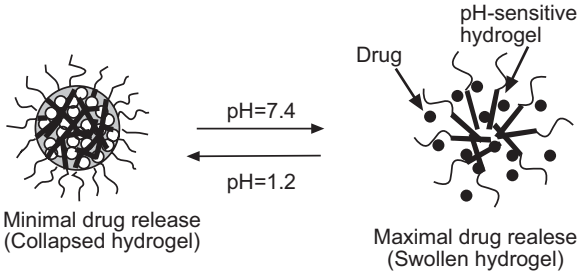


Fig. 6. Schematic diagram showing the effect of ON–OFF cycles of pH on swelling behaviour. It shows the pH triggered collapse and resultant burst release due to squeezing effect.

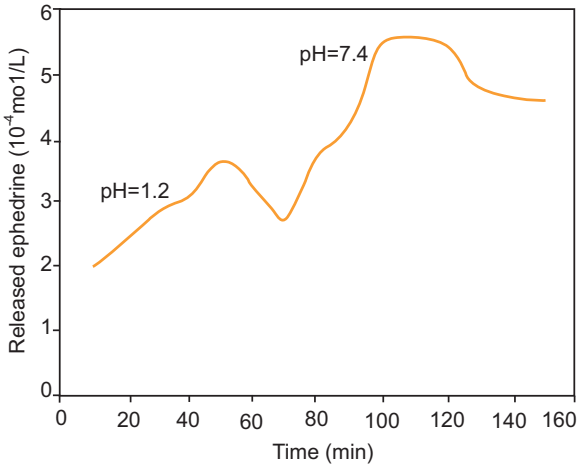


Fig. 5. Ephedrine release profile in SGF (pH 1.2) and subsequently in SIF (pH 7.4) at 37 °C. The time point that the samples are collected for analysis was 2 min.

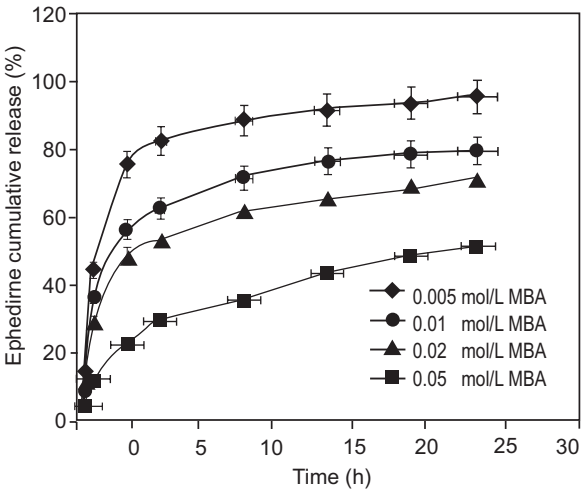


Fig. 7. *In vitro* cumulative release of ephedrine from the hydrogel with different crosslinker content at pH 7.4 and 37 °C.

will decrease, thereby hindering the transport of drug molecules through the matrix.

Drug release mechanism. A simple semi-empirical equation has been introduced to express general drug release behaviour depending on the geometry of a system (Siepmann and Peppas, 2001):

$$\frac{M_t}{M_\infty} = kt^n \quad (4)$$

where M_t M_∞ are the absolute cumulative amounts of drug released at time t and after the finish of release respectively, k is a diffusional kinetic constant for the characteristics of a polymer network system, and n is a diffusional exponent representing the release mechanism.

When log M_t/M_∞ is plotted against log t the value of n is obtained. The case of n=0.5 is for purely diffusion-controlled drug release (Fickian release) and the case of n=1 is for a drug release rate independent of time, corresponding to zero-order release kinetics (Case II transport). Other values for n are for anomalous transport kinetics and combined mechanisms of pure diffusion and Case II transport. In the present experiments, the diffusion coefficients were calculated based on fitting 20% and 60% of drug release, respectively. When 20% of drug had been released n was 9.74, indicating Case II transport close to zero order release. In the 60% case n was 0.71, related to non-Fickian or anomalous transport.

Conclusion

A new pH-responsive drug delivery system based on collagen hydrogel was developed for oral drug delivery of a poorly water-soluble drug to the intestinal environment. Ephedrine was encapsulated as a model drug and *in vitro* release studies were carried out in SGF and SIF. These studies indicated that the model drug encapsulation efficiency was increased with increase in the concentration of ephedrine. It was also evidenced that the release of ephedrine from these systems was influenced not only by the pH of swelling medium, but also by crosslinking content. The release value of ephedrine from hydrogels at pH 7.4 was higher than that at pH 1.2 due to the electrostatic repulsion between carboxylate groups. Moreover, the drug release from the hydrogels was decreased with increasing MBA content. Overall, it is possible to conclude that by varying crosslinking density or especially by changing the pH of solution, the drug release rate can be controlled and modulated.

Overall, the synthesized network is a novel hydrogel according to the literature survey based on Chemical Abstract Service. The main application of this DDS hydrogel is *in vitro* controlled release model drugs. As an extension of *in vivo* releases of this work, however, the hydrogels are being subjected to further investigation. In comparison to other pH-responsive natural-based hydrogel DDS, presently synthesized hydrogel has a good drug encapsulation and loading efficiency. The release value of drug from hydrogels at basic pHs was also considerable. The main limitation of this product is low release rate of drugs at acidic pHs.

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Simultaneous Spectrophotometric Determination of Lycopene and Beta-Carotene Concentrations in Carotenoid Mixtures of the Extracts from Tomatoes, Papaya and Orange Juice

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Abstract. A simple and inexpensive spectrophotometric equation model for the simultaneous determination of lycopene and β-carotene concentrations in a mixture of carotenoids is proposed. Lycopene could be exclusively determined (with the relative accuracy of more than 95%) using the absorbance data at 502 nm. Because quantifying the β-carotene concentration in a ccarotenoid minture using the sole absorbance at 450 nm is prone to error, an equation to determine the concentration of this compound from the absorbances data at two wavelengths was modeled. Using the modeled equations to re-check the molar absorptivity of lycopene at 472 nm, the value obtained was about 98% close to the value reported in literature. The relative accuracy of the predicted concentrations of two carotenoids using the modeled equations is a function of the ratio of these carotenoids in the samples.

Keywords: lycopene, β-carotene, spectrophotometry, absorptivities, tomato, papaya, orange, isoprene

Introduction

Carotenoids, the C_{40} tetraterpenoids derived from head-to-tail condensation of eight isoprenoid units, are notable for their wide distribution, structural diversity, and various functions. More than 600 carotenoids, excluding *cis* and *trans* isomers, have been isolated and characterized from natural sources (Pfander, 1987). Carotenoids have been credited with several beneficial effects on human health ranging from provitamin A activity to the enhancement of the immune response and reduction of the risk of degenerative diseases such as cancer, cardiovascular diseases, cataract, and macular degeneration (Olson 1999a; Astrog, 1997; Burri 1997; Mayne, 1996; Olson and Krinsky, 1995; Bendich, 1994; Krinsky, 1994; Gaziano and Hennekens, 1993). The action of carotenoids against chronic diseases has been mainly attributed to an antioxidant property, specifically, their ability to quench singlet oxygen and interact with free radicals (Palozza and Krinsky, 1992). However, other mechanisms, such as modulation of carcinogen metabolism, inhibition of cell proliferation, enhancement of cell differentiation and stimulation of cell-to-cell communication have been reported (Olson, 1999a; 1999b). The ability of carotenoids to quench singlet oxygen has been linked to the conjugated double bond system, the maximum efficiency being shown by carotenoids with nine or more conjugated double bonds (Foote *et al.*, 1970). Lycopene was found to be twice more efficient than the dicyclic β-carotene (Di Mascio *et al.,* 1989), despite of both compounds possessing 11conjugated double bonds. The effects of lycopene on human health as an antioxidant protector against lung, stomach, and prostate cancers have attracted considerable interest (Giovannucci, 1999; Clinton, 1998; Sies and Stahl, 1998; Gerster, 1997; Stahl and Sies, 1996). Average daily dietary lycopene intake levels (assessed by means of food frequency questionnaire) were estimated to be 25.2 and 33.39 mg/day in the Canadian and Nigerian diets, respectively (Olajire *et al*., 2007; Rao *et al*., 1998).

Carotenoid analysis is inherently difficult and error prone. In developed countries, separation and quantification of carotenoids are usually carried out using high-performance liquid chromatography (HPLC) technique (Monge-Rojas and Campos, 2011; Barba *et al.,* 2006). This method, although is reliable, accurate and time-consuming it requires extensive sample preparation, the use and disposal of hazardous organic solvents. It also requires highly trained personnel and expensive equipments. Novel chemical techniques that have been evaluated for direct determination of lycopene

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and other nutritionally important carotenoids include the application of optothermal and photothermal methods (Bicanic, 2011; Bicanic *et al*., 2005; 2004). These methods are gaining the popularity because of their simplicity. Other commonly used methods include resonance Raman spectroscopy (Bhosale *et al*., 2004), infrared (De Nardo *et al*., 2009; Halim *et al*., 2006) and near infrared spectroscopy (Baranska *et al*., 2006; Pedro and Ferreiram, 2005). Fourier transform infrared (FTIR) in combination with multivariate analysis offers a powerful and rapid technique for the analysis of agricultural and food products (Jha and Matsuoka, 2000). Conventional UV-visible spectrophotometric assays have also been shown to be simple, rapid, and inexpensive methods for measuring lycopene content in tomato and tomato products (Biswas *et al*., 2011; Davis *et al*., 2003), but absorbance interference from lycopene provides poor accuracy and overestimation of β-carotene levels (Olives-Barba *et al*., 2006). However, in some developing nations with limited accessibility to HPLC, the quantification of lycopene and β-carotene is often accomplished by means of the traditional ultraviolet-visible spectrophotometry. This method should be accurate enough, in that it has been shown that the content of lycopene in samples determined by the use of both experimental (calibration curve) and theoretical data (Beer-Lamberts law) shows a good agreement, with a relative error below 3% (Ravelo-Pérez *et al*., 2008). Some published absorption coefficients values may contain significant levels of error or uncertainty (Britton, 1995), likewise different authors choose different absorption coefficients for same carotenoids (in the same solvents), thus accounting for a good part of the variations in analytical results. In some studies the spectrophotometer detector was set at 472 nm to quantify lycopene (Moraru and Lee, 2005; Sharma and Le Maguer, 1996) and at 436 nm (Lumpkin, 2005) while in others it was determined at 503 nm (Ravelo-Perez *et al*., 2008). Although the absorbance of lycopene at 503 nm is not the highest, it was so selected to avoid interferences from other carotenoids present in the samples. The values of λ_{max} for carotenoids in hexane and petroleum ether are practically the same for diethyl ether, methanol, ethanol and acetonitrile, and 2-6 nm, 10-20 nm, 10-20 nm and 18-24 nm higher in acetone, chloroform, and *n* dichloromethane as well as in toluene, respectively (Britton, 1995). However, in a carotenoid mixture containing lycopene and β-carotene, the choice of absorption wavelengths of 450 nm and 470 nm to quantify β-carotene and lycopene, respectively may not reflect the true concentrations of each of the two carotenoids, since both absorb significantly at those two wavelength ranges, while the choice of 502 nm is perfect for lycopene determination. In the present work, the model equations for simultaneous determination of beta-carotene and lycopene concentrations in hexane layer extract of lycopene/β-carotene mixtures have been derived. This was then used to quantify β-carotene and lycopene in separate hexane extracts of the carotenoid mixtures from tomatoes, papaya and orange juice samples.

Materials and Methods

Tomato fruit samples (Ibadan-local cultivar bought from a farmland near Wazobia Market, Ogbomoso, Nigeria) were randomly selected and packed into nylon bags and quickly taken into the laboratory, then they were rinsed with distilled water and left to drain for some minutes. About 500 g (15-25 fruits) of fresh tomatoes was chopped, blended and then homogenized in a laboratory homogenizer. Conventional solvent extraction methods (Perkins-Veazie *et al.,* 2001; Sadler *et al.*, 1990) were employed for carotenoid extraction. Approximately 10g of the tomato serum was subjected to extraction with hexane, methanol and acetone (2:1:1) containing 2.5% butylated hydroxytoluene (BHT). The extract was treated with distilled water, methanol and 20 % KOH/methanol (1:1:1) to saponify any triglyceride present. The extract was then washed with distilled water and re-dissolved in hexane. The hexane extracts were scanned in the visible light wavelength range of 400-750 nm using HEλI0Sα UV-visible spectrophotometer (in a 1 cm path length quartz cuvette blanked with *n*-hexane) and the maximum absorbances were observed at 450, 472 and 502 nm, respectively for the lycopene - β-carotene hexane layer mixture. The mixture was diluted with *n*-hexane using dilution factors of 2, 3, 6, 8 and 16, respectively to check for result consistencies and subsequent absorbances were measured. Samples of papaya and orange juice extracted in hexane were also subjected to carotenoid analysis. The molar extinction coefficient of 172,000 L/mol/cm at 502 nm was used to estimate lycopene concentration, using the Beer-Lamberts law (Ravelo-Perez *et al*., 2008; Zechmeister and Polgar, 1943).

Results and Discussion

Figure 1A-B represent typical overlap spectra of the hexane extract of the lycopene/β-carotenoid mixture in tomatoes with absorption maxima at 450 nm, 472 nm

and 502 nm. Most carotenoids exhibit absorbance maxima at three wavelengths, resulting in a three-peak spectrum. As the number of conjugated double bonds increases, the λ_{max} shifts to longer wavelengths. Thus, the most unsaturated acyclic carotenoid, lycopene, with 11 conjugated double bonds is red and absorbs at the longest wavelengths (λ_{max} at 443, 471, 502 nm) (Rodriguez-Amaya and Kimura, 2004). Cyclization results in steric hindrance between the methyl group at C-5 of the ring and the hydrogen atom at C-8 of the polyene chain. This hindrance takes the π electrons of the ring double bond out of plane with respect to those of the chain, causing a hypsochromic shift (displacement of λ_{max} to shorter wavelength), a hypochromic effect (decrease in absorbance) and loss of fine structure (spectrum with less defined peaks). Thus, the dicyclic molecule, β-carotene, is yellow-orange and despite possessing the same number of conjugated double bonds as lycopene, exhibits absorption peaks at 450 and 472 nm and a mere shoulder at 425 nm (Rodriguez-Amaya and Kimura, 2004). This is so because both carotenoids absorb substantially in the overlapping wavelength ranges. As already stated that the choice of 502 nm to quantify lycopene is alright despite the fact that this wavelength value is not equal to λ_{max} (Ravelo-Perez *et al*., 2008). The correlations of the absorbances of the mixture at 450 nm and 502 nm relative to those at 472 nm, respectively (Fig. 2A-B) is very high ($R^2 = 0.999$ in both cases). This makes quantification of these carotenoids in mixture more ambiguous. Concentrations of lycopene and β-carotene in the mixture were calculated (Table 1) based on the assumption that the absorbances at 450 nm and at 502 nm are exclusively, for β-carotene and lycopene respectively. This is to be re-calculated using the proposed model and will then be compared henceforth. However, analytes such as

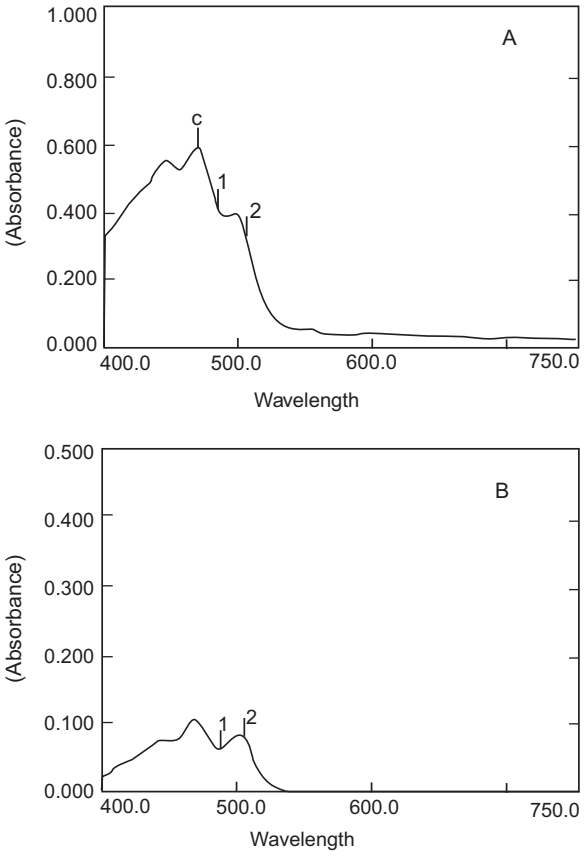

Fig. 1A-B. UV-visible spectra of *n*-hexane extract of the lycopene/β-carotene mixture of tomato samples at dilution factors of (A) 1 (B) 16.

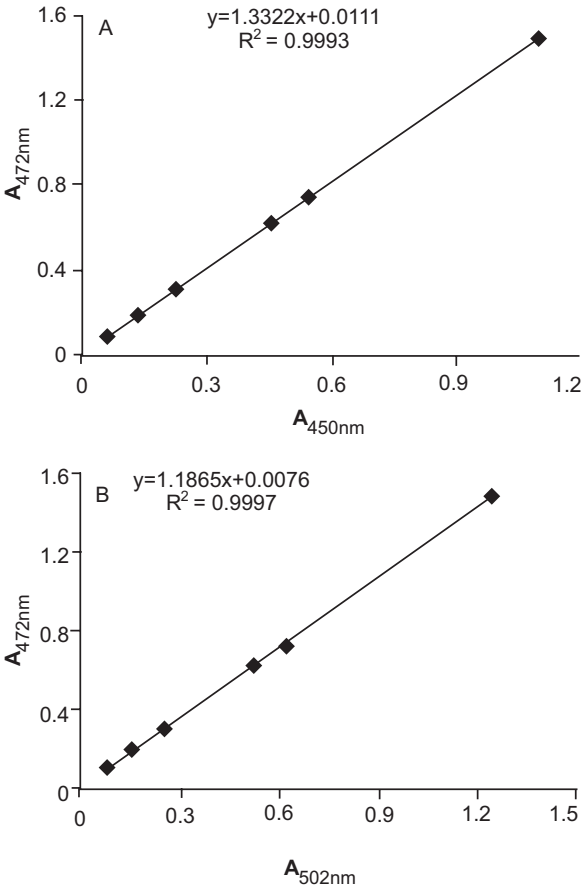

Fig. 2A-B. The correlations between the absorbances measured at 450nm (A) and 502 (B) nm to that measured at 472 nm for the hexane extract of the lycopene/β-carotene mixture of tomato samples.

Table 1. The ideal molar concentrations of lycopene and β-carotene in tomatoes, (Ibadan- local cultivars) in case that absorbances at 450nm and 502 nm are exclusively due to β-carotene and lycopene, respectively

Dilution factors	Absorbances (nm)			[a] β-Carotene concentration ± SD (μM)	[b] Lycopene concentration ± SD (μM)
	450 nm	472	502		
1	1.100	1.487	1.240	7.929 ± 0.202	7.209 ± 0.175
2	0.550	0.720	0.615	3.965 ± 0.156	3.576 ± 0.129
3	0.453	0.620	0.516	3.265 ± 0.162	3.000 ± 0.173
6	0.225	0.305	0.250	1.622 ± 0.224	1.453 ± 0.032
8	0.133	0.191	0.152	0.959 ± 0.102	0.884 ± 0.205
16	0.068	0.109	0.080	0.490 ± 0.126	0.465 ± 0.092

[a] = Calculated from Beer-Lamberts Law using the absorbances at 450 nm and molar absorptivity of 1.39×10^5 L mol/cm/ (Du *et al*., 1998); [b] = Calculated from Beer-Lamberts Law using the absorbances at 502nm and molar absorptivity of 1.72×10^5 L mol/cm (Markovic *et al*., 2006); The results are the means of triplicate analysis with reported standard deviation (SD).

norfloxacin, ofloxacin and lomerfloxacin in their mixture have been determined simultaneously using chemometric method (Huang *et al*., 2009) and other analytes such as chlorpromazine, perphenazine and acetopromazine were quantified by kinetic wavelength-pair method (Carreto *et al*., 1997). Here, lycopene and β-carotene concentrations are determined by partial least squares calibration (Skoog *et al*., 2000). This involves determination of the absorbances of several solutions at the wavelength at which analytes, lycopene and β-carotene, absorb. The wavelengths were carefully chosen such that the molar absorptivity of one component is much larger than that of the second component. Therefore, absorbances at 450 nm and 502 nm were used where the molar absorptivities of β-carotene are 1.39×10^5 and 2.63×10^4 L/mol/cm, respectively and those of lycopene being 1.16×10^5 and 1.72×10^5 L/mol/cm, respectively. According to the law of Lamberts and Beer, the absorbance at 450 nm and 502 nm (in a 1 cm path-length quartz cuvette) of the carotenoid mixture of lycopene and β-carotene can be expre as follows:

$$A_{450} = \varepsilon^{450}_{lycopene}[Lycopene] + \varepsilon^{450}_{\beta-carotene}[\beta-carotene] \quad \text{..... (1)}$$

$$A_{502} = \varepsilon^{502}_{lycopene}[Lycopene] + \varepsilon^{502}_{\beta-carotene}[\beta-carotene] \quad \text{..... (2)}$$

where, A_{450} and A_{502} are the absorbances (at 1 cm cell path-length) of the lycopene/ β-carotene mixture in the hexane extract at 450 nm and 502 nm, respectively; [lycopene] and [β-carotene] are molar concentrations of lycopene and β-carotene, resspectively; $\varepsilon^{450}_{\beta\text{-}carotene}$ and $\varepsilon^{450}_{lycopene}$ are the molar absorptivities of lycopene and β-carotene at 450 nm while $\varepsilon^{502}_{\beta\text{-}carotene}$ and $\varepsilon^{502}_{lycopene}$ represent the respective molar absorptivities of lycopene and β-carotene at 502 nm.

Solving equations (1) and (2) simultaneously, the molar concentration of lycopene from equation (1) could be expressed as:

$$[Lycopene] = \{A_{450} - \varepsilon^{450}_{\beta-carotene}[\beta\text{-}carotene]\} \Big/ \varepsilon^{450}_{lycopene} \quad \text{..... (3)}$$

Substituting equation (3) into equation (2), the concentration of β-carotene can be calculated as:

$$[\beta-carotene] = \frac{A_{450}\dfrac{\varepsilon^{502}_{lycopene}}{\varepsilon^{450}_{lycopene}} - A_{502}}{\dfrac{\varepsilon^{502}_{lycopene}}{\varepsilon^{450}_{lycopene}}\varepsilon^{450}_{\beta-carotene} - \varepsilon^{502}_{\beta-carotene}} \quad \text{..... (4)}$$

But the values $\varepsilon^{450}_{\beta\text{-}carotene}$, $\varepsilon^{450}_{lycopene}$, $\varepsilon^{502}_{\beta\text{-}carotene}$ and $\varepsilon^{502}_{lycopene}$ are known to be 1.39×10^5, 1.16×10^5, 2.63×10^4 and 1.72×10^5 L/mol/cm, respectively (Clinton, 1998; Du *et al.,* 1998; Krinsky *et al.,* 1990; Zechmeister and Polgar, 1943), therefore:

$$[\beta\text{-}carotene] = \frac{1.483\,A_{450} - A_{502}}{1.798\times10^5} \quad \text{..... (5)}$$

$$[Lycopene] = \frac{A_{450} - 1.39\times10^5[\beta\text{-}carotene]}{1.16\times10^5} \approx \frac{A_{502}}{1.72\times10^5} \quad \text{... (6)}$$

Equations (5) and (6) were employed to calculate the concentrations of lycopene and β-carotene, respectively

and the results are as presented in Table 2. It was clearly shown that the use of the absorbances at 450 nm to exclusively determine β-carotene concentration is extremely prone to error. This is so because the relative accuracy of the predicted β-carotene concentration using the modeled equations compared with what could have been reported when its concentration was calculated from their absorbance values at 450 nm, is extremely low (an average of 26.78%). However, it could be inferred that absorbance at 502 nm could be exclusively attributed to lycopene, since an average relative accuracy of more than 95% was obtained. This is in agreement with other study (Fish *et al*., 2002) which suggests that constituent carotenoids other than lycopene will contribute to the absorbance at 503 nm up to about 4% for fresh red tomatoes, 2% for red-fleshed watermelon and 6% for pink grapefruit. This also corroborated the findings that the lycopene contents in different varieties of tomatoes, analyzed by UV-visible spectrophotometry at 502 nm and HPLC methods, are quite similar (Laleye *et al*., 2010).

To check for the correctness of the lycopene concentration at 472 nm, equation 1 may be re-written as:

$$A_{472} = \varepsilon^{472}_{lycopene}[Lycopene] + \varepsilon^{472}_{\beta\text{-}carotene}[\beta\text{–}carotene] \quad \text{..... (7)}$$

Assuming $\varepsilon^{472}_{lycopene}$ to be unknown, it can be calculated thus:

$$\varepsilon^{472}_{lycopene} = \frac{A_{472} - \varepsilon^{472}_{\beta\text{-}carotene}[\beta\text{–}carotene]}{[Lycopene]} \quad \text{..... (8)}$$

Substituting the value of A_{472} and $\varepsilon^{472}_{\beta\text{-}carotene} = 1.09 \times 10^5$ L/mol/cm (Du *et al.,* 1998) as well as the concentrations of lycopene and β-carotene of the stock solution (solution of dilution factor of 1, that is 6.876 and 2.175 μM $\varepsilon^{472}_{\beta\text{-}lycopene}$ value of 1.82 x105 L/mol/cm was obtained as against a value of 1.86 x 105 L/mol/cm previously reported by Clinton (1998). This gives a relative accuracy (ratio of predicted value to the true value) of about 98%.

The absorption spectra of the hexane extracts samples of papaya and orange juice Fig. 3A-B are shown in Equations (5) and (6) were then tested on the absorbance

Table 2. Predicted concentrations of lycopene and β-carotene in the hexane extract from tomatoes (Ibadan-local cultivar)

Dilution factors	Absorbances (nm)			[a] β-Carotene concentration ± SD (μM)	[b] Lycopene concentration ± SD (μM)	[c] Relative accuracy	
	450	472	502			β-carotene at 450 nm	Lycopene at 502 nm
1	1.100	1.487	1.240	2.175 ± 0.127	6.876 ± 0.212	27.431	95.377
2	0.550	0.720	0.615	1.116 ± 0.062	3.404 ± 0.156	28.150	95.201
3	0.453	0.620	0.516	0.870 ± 0.152	2.866 ± 0.124	26.644	95.533
6	0.225	0.305	0.250	0.466 ± 0.094	1.381 ± 0.136	28.733	95.013
8	0.133	0.191	0.152	0.250 ± 0.022	0.847 ± 0.087	26.057	95.845
16	0.068	0.109	0.080	0.116 ± 0.013	0.448 ± 0.056	23.666	96.320
Average percentages	-	-	-	-	-	26.780	95.548

[a] = Calculated using equation (5); [b] = Calculated using equation (6); The results are the means of triplicate analysis with reported standard deviation (SD); [c] = Relative accuracy means the ratio of the predicted concentrations of lycopene and β-carotene when the modeled equations were used relative to what could have been reported when their concentrations were calculated from their absorbance values at 502 nm and 450 nm, the wavelengths assumed to be attributed exclusively to the respective carotenoids in their mixtures.

Table 3. Predicted concentrations of lycopene and β-carotene in papaya and orange juice

[a] Sample	Absorbances (nm)		[b] β-Carotene concentration (μM)	[c] Lycopene concentration (μM)	β-Carotene concentration (μg/g)	Lycopene concentration (μg/g)
	450	502				
Papaya	0.585	0.401	2.590 ± 0.132	1.934 ± 0.124	27.765 ± 1.415	20.732 ±1.329
Orange juice	0.123	0.051	0.731 ± 0.092	0.185 ± 0.022	7.836 ± 0.986	1.983 ±0.236

[a] = Sample weight is 10 g and the volume of hexane extract is 200 mL; [b] = calculated using equation (5); [c] = calculated using equation (6); The results are the means of triplicate analysis with reported standard deviation (SD).

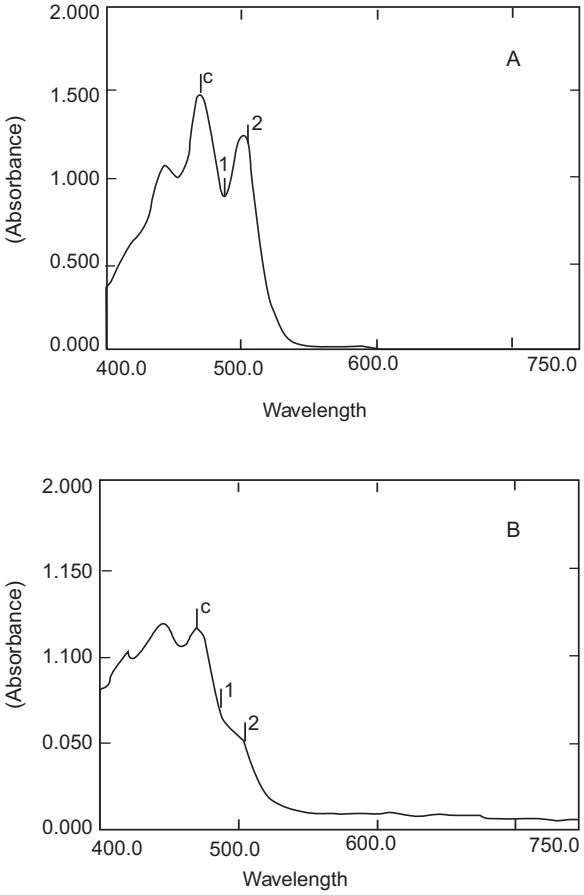

Fig. 3A-B. UV-visible spectra of *n*-hexane extract of the lycopene/β-carotene mixture in samples of (A) orange juice (B) papaya.

results of the hexane extract of papaya and orange juice samples to calculate the respective amounts of β-carotene and lycopene in the lycopene/β-carotene mixture. The results are presented in Table 3. The results with other fruit or fruit product apart from tomatoes confirm that the β-carotene concentrations are higher than that of lycopene. This is evident from the spectra shown in Fig. 3A-B. High relative accuracy values of the predicted β-carotene concentration relative to that computed exclusively at 450 nm of 61.42% and 82.45% were observed for papaya and orange juice, respectively in contrast with an average of 26.78% observed for tomato fruits. Furthermore, the relative accuracies (82.95% and 62.39%) of the predicted lycopene relative to that computed exclusively at 502 nm observed for papaya and orange juice, respectively are lower than an average of 95.55% obtained for tomatoes. This may be attributed to lycopene/ β-carotene concentration ratio in the sample under investigation or/and the presence of appreciable amounts of other hydrocarbon carotenoids in papaya and orange. It is hereby recommended that the predicted equations in this work be employed in the determination of concentrations of lycopene and β-carotene in the hexane extract of lycopene/ β-carotene mixture and the results can be compared with those from HPLC analysis, to check for precision and accuracy.

Conclusion

A simple and inexpensive spectrophotometric equation model for the simultaneous determination of lycopene and β-carotene concentrations in a carotenoid mixture is proposed. This could be useful to analysis in some developing nations where UV-visible spectrophotometry is available but accessibility to HPLC is minimal. The equations proposed here worked best if the percentage composition of other hydrocarbon carotenoids (relative to lycopene and β-carotene) in the hexane extract is minimal.

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Quantification and Detoxification of Aflatoxin in Food Items

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Abstract. The present study was conducted to quantify and detoxify the aflatoxins in food items. For this purpose, total 30 samples of food were collected. The samples were quantified using thin layer chromatography (TLC) for the presence of aflatoxin level in food items. Out of them aflatoxins were not found in 10 samples. Remaining 20 aflatoxins +ve samples were treated with various chemical solutions i.e. 0.1% HCl, 0.3% HCl, 0.5% HCl, 10% citric acid, 30% citric acid, 50% calcium hydroxide, 0.2 and 0.3% NaOCl, 96% ethanol and 99% acetone for detoxification. The aflatoxins were reduced to 55.1%, 90.9%, 28.08% and 80.0% in Super Sella rice, Super Basmati rice, Brown rice and White rice, respectively. The aflatoxin level was reduced in maize grain, damaged wheat, peanut, figs and dates upto 31.3 %, 64.3 %, 63.6%, 42.7% and 19.8%, respectively. Aflatoxins were detoxified in cereals Dal Chana, Dal Mash, Dal Masoor), turmeric (Haldi) and *Nigela* seeds (Kalwangi) upto 70.5%, 83.0%, 46.2%, 82.09% and 36.9%, respectively. Reduction of aflatoxins was carried out 39.7 %,7.1 % 39.5% 82.0% and 62.0% in red chilli, makhana, corn flakes, desert (Kheer Mix) and pistachio. The significant results (p =0.042) of detoxification of aflatoxins in food items were obtained from present study.

Keywords: pepsin extraction, enzyme activity, stomach mucosa, buffalo

Introduction

Aflatoxins are toxic and carcinogenic metabolites produced by species of *Aspergillus,* especially *Aspergillus flavus* and *Aspergillus parasiticus.* The toxic effects include acute hepatitis, immune-suppression. In humans, the risks associated with aflatoxin consumption are well documented and the International Agency for Research on Cancer (IARC) has designated aflatoxin as a human liver carcinogen. Because of these toxic effects, the Food and Drug Administration regulates the aflatoxin concentration in food with aflatoxin. Commodities or food with aflatoxin exceeding 20 ppb (μg/kg) cannot move in trade (Wogan, 1999). Very little was known about mould metabolites prior to 1961. In that year some alarming reports of a mysterious disease of Turkey poults came from South East of England, tentatively named as Turkey X disease. In 500 such outbreaks about 1, 00,000 poults, mostly between three to six weeks of ages, died. Similarly 5000 partridges and pheasants from one farm and 14000 ducklings from another farm died (Asplin and Carnaghan, 1961). Reports from other places also accumulated on outbreak of acute poisoning of farm animals such as pigs (Loosmore and Harding, 1961) and calves (Loosmore and Markson, 1961). Bioassay test on duckling also helped in furthering and establishing the toxic factor (Sargeant *et al.,* 1961). Aflatoxins producing ability is confined to *A. flavus* and *A. parasiticus.* Strains of these two species are common and wide-spread and have been isolated from a number of different host materials. Colonies of *A. flavus* are green-yellow to yellow-green and that of *A. parasiticus* are dark green. The toxin is produced by mycelium and secreted into the medium or substrate, spores contain very little aflatoxin. Different strains of *A. flavus* produce varying amount of aflatoxin and same strains also produce varying amount of aflatoxins. Aflatoxin production is a genetical process depending on specific nutrient and environmental factors (Patterson, 1973). Investigation carried out by various researchers at Tropical Development and Research Institute, London indicated that *A. flavus* is found in the soil and air throughout the world. Both *A. flavus* and *A. parasiticus* are more prevalent in warmer climate and these moulds can be isolated from stores, dried stuff and tropical soil (Christensen, 1957). Aflatoxins naturally occur in rice (including brown, white, black, red and basmati) of

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different countries, including United State, Canada, Pakistan, India and Thailand (Bansal *et al.,* 2011). Aflatoxins (AFB_1, AFB_2, AFG_1 and AFG_2) also occur in freshly harvested corn grains in different regions of Brazil (Liliana *et al.,* 2009). The presence of aflatoxins in red chillis may be a great threat to the health of populations. Total 183 samples of red chilli were screened out for aflatoxin determination. 48 samples were positive for Aflatoxins B_1 with the range from 1.2 ppb to 968.3 ppb. Aflatoxin B_2 was detected only in 3 samples with the range of 0.3 ppb - 159.8 ppb. Aflatoxin G_1 and G_2 were absent in all chilli samples (Nisa *et al.,* 2012). Chronic poisoning of aflatoxin results in cancer (hepatocellular carcinoma) because liver is the target organ of aflatoxins. Acute intoxication of aflatoxins in human body is also lethal (Milita *et al.,* 2010). Many countries regulate aflatoxin levels in their foods. USA and EU (Europe Union) permit level lower than 20 ppb and Korea and Japan 10 ppb (Chiavaro *et al.,* 2001). Due to its importance different food items were selected for this study and different chemicals were used for detoxification of aflatoxins in these food samples.

Materials and Methods

This study was conducted in Food and Biotechnology Research Centre of PCSIR Laboratories Lahore. The food samples were prepared for aflatoxin analysis (Begum *et al.,* 1985). Aflatoxins were detected by Romers' method (Romer, 1975). Estimation of aflatoxins in toxic extracts was made by comparison with standard technique (AOAC, 2005). In this study, TLC technique was used for the determination of Aflatoxin in all samples.

Nature of samples. Samples of food such as Corn, Wheat, Wheat Flour, White Rice, Brown Rice, Super Basmati, Super Kernal Rice, Red Chilli, Pistachio, Cornflakes, Figs, Haldi, Garam Masala, Peanut, Kalwangi, Makhana and Dates were selected for the present study.

Sample collection. During research work food samples were collected from local market of the city and brought to the Laboratory for quantitative determination and detoxification.

Sampling. Since the aflatoxins are not uniformly distributed in commodities, grains were likely to have pockets of high aflatoxin concentration, firstly due to highly heterogeneous distribution of aflatoxins and secondly due to marketing in lumps of various sizes. To obtain most representative sample, a suitable sampling plan was adopted. These commodities were found stacked in jute bags and stored in house type godown. In order to obtain a more representative portion of these samples, 500 g were collected through a sample probe directly in plastic bags piercing jute bags diagonally from 2 to 3 places. They were passed through sample divider and reduced to approximately 200 g for the purpose of analysis and thus a greater homogeneity of contaminated portion was achieved. Each sample was then thoroughly mixed, ground and made into fine powder for experimental analysis.

Extraction. Extraction procedures and analytical methods vary from one commodity to another because of diverse chemical composition, preventing the development of any one method which could be applied uniformly to all products. However, extraction with chloroform is most suitable method for aflatoxins (B_1, B_2, G_1, G_2, M_1 and M_2).

Then test portion was taken from mixture. 50 g of ground sample was kept into 500 mL conical flask and 25 mL water and 150 mL chloroform was added into flask. Conical flask was shaken on wrist action shaker for 30 min and sample was filtered through filter paper. 50 mL chloroform was taken into beaker and put on steam bath for evaporation.

Chromatographic tank. The dilutions for spotting were got in micro liter. The 25 µL spot of test solution was applied on thin layer chromatography plate with micro syringe. Spot of 5 or 10 µL of aflatoxins (B_1, B_2, G_1, G_2, M_1 and M_2) standard was spotted on same plate as an internal standard. The plate was developed with anhydrous ether in thin layer chromatographic tank upto half then removed and dried. Then plate was redeveloped in the same direction in thin layer chromatographic tank with acetone-chloroform (1:9). Plate was removed and test solution spot was observed for presence or absence of aflatoxins under UV light. If preliminary plate would show that new concentration of test solution required then new concentration were prepared for spotting. Different 1 to 25 µL spots of test solution (3.5, 10.5, 24.9 µL) were spotted on new thin layer chromatographic plate and on the same plate 1 to 25 µL aflatoxins standard was spotted (Braicu *et al.,* 2008).

Detection and Estimation. Fluorescing intensities of sample spots were compared with those of standard aflatoxin spots. In case, fluorescing spot of sample lied between the standard spots, the average value of two standard spots was taken into consideration.

Confirmation. Another very important step in the aflatoxins analysis was the fluorescing sample spots. This was carried out by spraying, evenly the thin layer chromatographic plate with aqueous sulphuric acid (50/50 v/v). After the spraying, thin layer chromatographic plate was allowed to dry and then viewed under UV light (365 nm).

Calculation. Concentration of aflatoxins (µg/kg) present in sample was calculated as follows.

$$\text{Aflatoxins (µg/kg)} = \frac{S \times Y \times V}{Z \times W}$$

Where:

S = volume in µL of aflatoxins standard of equivalent intensity to Z (µL of sample)
Y = concentration of aflatoxins standard in µg/ mL
V = volume in µL of solvents required to dilute final extract
Z = volume in µL of sample extract required to give fluorescence intensity comparable to that of S = µL of aflatoxins standard
W = weight in g of original sample contained in final extract

Treatment for detoxification. Fifty grams of grinded samples in which aflatoxins had been detected were kept in separate 500 mL conical flasks. Chemical solutions of 0.1% HCl, 0.3% HCl, 0.5% HCl, 10% citric acid, 30% citric acid, 05% calcium hydroxide, 0.2 and 0.3% NaOCl, 96% ethanol and 99% acetone were added into different flasks (Table 1). Conical flasks were shaken on wrist action shaker for 2 h and sample was filtered through filter paper and dried for two days.

Quantification after detoxification. Quantification of detoxified sample for aflatoxins was carried out by same method such as chloroform extraction, detection by thin layer chromatography, estimation through UV light and calculation by formula.

Statistical analysis. The statistical significance of the data was analyzed (p=0.042) using pair t-test (Steel *et al*., 1997).

Results and Discussion

Aflatoxins were detoxified by the treatment of different chemical solutions. For this purpose, total 30 samples were collected. These 30 samples were quantified using thin layer chromatography (TLC) for the presence of aflatoxins level in food items (Table 2). The aflatoxins were not found in 10 samples of food product, remaining 20 samples of food in which aflatoxins had been found were treated with chemical solutions.

Chemical solutions 0.1%, 0.3% and 0.5% of hydrochloric acid reduced aflatoxins to 39.7%, 55.1%, 90.9%, 39.5%, 62.0% and 82.0% in food items (Table 4) which are in line with work of Aly and Hathout (2011) who reduced aflatoxins 27.6%, 42.5% and 90% in food with concentrations of hydrochloric acid at different hours. Aflatoxins also reduced to 49.3%, 86.5% and 71.39% with concentration of 0.1%, 0.3% and 0.5% of hydrochloric acid which is same work as Aly and Hathout (2011) who did reduction of food items.

Aflatoxins reduced to 31.3%, 64.3%, 19.8%, 28.08%, 70.5% and 83.05% with treatment of 10% citric acid, 30% citric acid, 1% sodium bisulphate, 2% sodium bisulphate, 0.2% sodium hypochlorite and 0.3% sodium hypochlorite in food items and Aflatoxins reduced to 63.0%, 70.0%, 69.16%, 53.9%, 10.0% and 35.05% with treatment of 10% citric acid, 30 % citric acid, 1% sodium bisulphate, 2% sodium bisulphate 0.2% sodium hypochlorite and 0.3% sodium Hypochlorite in food items which are in line with work of Mukendi *et al.* (1991). They had detoxified

Table 1. Solutions for detoxification of Aflatoxins in food items

Food product	Chemical solution for detoxification
Red chilli	0.1 % HCl
	0.3% HCl
Red chilli	5 % NaOH
Super Sella rice	0.3 % HCl
Super Basmati rice	0.5 % HCl
White rice	5% $Ca(OH)_2$
Maize grain	10 % Citric acid
Wheat damage	30 % Citric acid
Peanut	99 % Acetone
Figs	96 % Ethanol
Dates	1 % Sodium bisulphate
Brown rice	2 % Sodium bisulphate
Makhana	5 % KOH
Dal chana	0.2 % NaOCl
Dal mash	0.3 % NaOCl
Corn flakes	0.1 % HCl
Kheer mix	0.3 % HCl
Pistachio	0.5 % HCl
Haldi	5 % $Ca(OH)_2$
Kalwangi	10 % Citric acid
Dal masoor	99 % Acetone

Table 2. Detection and estimation of Aflatoxins in food products

Sample No.	Food product	Aflatoxin	S	Y	V	Z	W	ppb
1.	Red chilli	B_1	0.5	2.02	0.5 (1000)	4.7	15.04	7.14
2.	Red chilli	B_1	2	2.02	0.51 (1000)	4.9	16.72	25.15
3.	Super Sella rice	B_1	2	2.02	0.51 (1000)	2.9	16.78	42.
4.	Super Basmati rice	B_1	5	2.02	0.99 (1000)	0.9	16.92	656.9
5.	Dal mash	Absent	_	_	_	_	_	_
6.	White rice	B_1	8.5	2.02	0.51 (1000)	24.9	16.70	125.53
7.	Pistachio	Absent	_	_	_	_	_	_
8.	Haldi	Absent	_	_	_	_	_	_
9.	Maize grain	G_1	0.5	2.03	0.51 (1000)	0.9	16.65	34.48
10.	Wheat damage	B_1	0.9	2.02	1470	0.9	16.67	174.32
11.	Peanut	B_1	8.5	2.02	0.51 (1000)	4.9	16.70	21.08
12.	Figs	B_1	1.0	2.02	0.50 (1000)	1.0	16.77	14.9
13.	Dates	B_1	0.5	2.02	1980	3.9	16.68	30.76
14.	Brown rice	B_1	2	2.02	0.51 (1000)	1.0	16.76	123.58
15.	Makhana	B_1	0.5	2.02	0.51 (1000)	14.9	16.73	2.09
16.	Dal chana	B_1	0.5	2.02	0.99 (1000)	14.9	16.72	8.02
17.	Figs	Absent	_	_	_	_	_	_
18.	Peanut	Absent	_	_	_	_	_	_
19.	Dal mash	B_1	0.5	2.02	0.5 (1000)	4.7	15.04	7.14
20.	Corn flakes	B_1	1.0	2.02	1 (1000)	9.5	16.27	13.0
21.	White rice	Absent	_	_	_	_	_	_
22.	Kheer mix	B_1	0.9	2.02	0.51 (1000)	4.9	16.70	11.32
23.	Pistachio	B_1	1.0	2.02	3.0 (1000)	2.9	16.51	167.17
24.	Haldi	B_1	1	2.02	1980	2.9	16.68	82.68
25.	Peanut	Absent	_	_	_	_	_	_
26.	Brown rice	Absent	_	_	_	_	_	_
27.	Kalwangi	B_1	4.0	2.02	0.51 (1000)	5.0	16.72	49.2
28.	Super Sella rice	Absent	_	_	_	_	_	_
29.	Super Basmati rice	Absent	_	_	_	_	_	_
30.	Dal masoor	B_1	2.0	2.02	0.91 (1000)	2.9	16.67	76.05

aflatoxins by comparing chemicals citric acid, sodium bisulphate, sodium hypochlorite (Table 3 and Table 4).

The present study showed significant detoxification in aflatoxins ($p = 0.042$) of food items when pair T-test was applied to quantified aflatoxins of food items before and after detoxification. It means that aflatoxins had been reduced statistically when aflatoxins were compared before and after detoxification.

Present study also showed that thin layer chromatography is a reliable method for detection and quantification of aflatoxins in food items before and after detoxification. (Okwu *et al.,* 2010, Olufunmilayo and Oyefolu, 2010).

Table 3. Detection and estimation in Aflatoxins in food products after detoxification

Sample No.	Food product	Aflatoxin	S	Y	V	Z	W	ppb
1.	Red chilli	B_1	0.9	2.02	0.99 (1000)	24.9	16.67	4.3
2.	Red chilli	B_1	0.9	2.02	0.51 (1000)	4.9	16.71	11.34
3.	Super Sella rice	B_1	0.5	2.02	0.91 (1000)	2.9	16.67	19.01
4.	Super Basmati rice	B_1	2.5	2.02	1975 (1000)	2.0	16.70	59.68
6.	White rice	B_1	2.0	2.02	0.51	4.9	16.72	25.51
9.	Maize grain	G_1	9.5	2.03	0.51 (1000)	24.9	16.71	23.67
10.	Wheat damage	B_1	1.0	2.02	0.515 (1000)	1.0	16.69	62.31
11.	Peanut	B_1	1.5	2.02	0.99 (1000)	24.9	16.77	7.2
12.	Figs	B_2	0.5	0.5	0.48 (1000)	0.9	16.56	8.54
13.	Dates	B_1	2	2.02	0.50 (1000)	4.9	16.72	24.66
14.	Brown rice	B_1	8.5	2.02	0.51 (1000)	5.9	16.71	88.87
15.	Makhana	B_1	0.5	2.02	0.51 (1000)	5.9	16.72	1.94
16.	Dal chana	B_1	1	2.02	487	24.9	16.68	2.36
19.	Dal mash	B_1	0.5	2.02	0.50 (1000)	24.9	16.69	1.21
20.	Corn flakes	B_1	0.5	2.02	0.51 (1000)	3.9	16.69	7.9
22.	Kheer mix	B_1	0.9	2.02	0.99 (1000)	24.9	16.77	4.3
23.	Pistachio	B_1	0.5	2.02	1980	3.9	16.68	30.7
24.	Haldi	B_1	6.0	2.02	0.51 (1000)	24.9	16.69	14.8
27.	Kalwangi	B_1	7.5	2.02	0.51 (1000)	14.9	16.72	31.0
30.	Dal masoor	B_1	3.0	2.02	0.50 (1000)	4.7	15.90	40.9

Table 4. Comparison of Aflatoxins estimation of food products before and after detoxification

Sample No.	Food product	Aflatoxin	Estimation before detoxification (ppb)	Chemical solution for detoxification	ppb after detoxification	Reduction in %age
1.	Red chilli	B_1	7.14	0.1 % HCl	4.3	39.7
2.	Red chilli	B_1	25.15	5% NaOH	11.34	54.9
3.	Super Sella rice	B_1	42.34	0.3% HCl	19.01	55.1
4.	Super Basmati rice	B_1	656.9	0.5% HCl	59.68	90.9
6.	White rice	B_1	125.53	5% Ca(OH)	25.51	80.0
9.	Maize grain	G_1	34.48	10% Citric acid	23.67	31.3
10.	Wheat damage	B_1	174.32	30% Citric acid	62.31	64.3
11.	Peanut	B_1	21.08	99% Acetone	7.2	63.67
12.	Figs	B_2	14.9	96% Ethanol	8.54	42.7
13.	Dates	B_1	30.76	1% Sodium bisulphate	24.66	19.8
14.	Brown rice	B_1	123.58	2 % Sodium bisulphate	88.87	28.08
15.	Makhana	B_1	2.09	5% KOH	1.94	7.1
16.	Dal chana	B_1	8.02	0.2% NaOCl	2.36	70.5
19.	Dal mash	B_1	7.14	0.3% NaOCl	1.21	83.05
20.	Corn flakes	B_1	13.0	0.1% HCl	7.9	39.5
22.	Kheer mix	B_1	11.32	0.3% HCl	4.3	62.0
23.	Pistachio	B_1	167.17	0.5% HCl	30.7	82.0
24.	Haldi	B_1	82.68	50% $Ca(OH)_2$	14.8	82.09
27.	Kalwangi	B_1	49.2	10% Citric acid	31.0	36.9
30.	Dal masoor	B_1	76.05	99% Acetone	40.9	46.2

Ultra-violet (UV) light, a standard procedure was used to differentiate the toxin from non-toxin forms of *A. spergillus* species.

Conclusion

It was concluded that significant detoxification i.e. 90.9% was observed when 0.5% HCl was used as detoxifying agent for aflatoxin B_1 in Super Basmati rice. Similarly, 83.05% detoxification of Aflatoxin B_1 was observed in Dal Mash, 82.09% in Haldi, 82% in Pistachio and 80% in White rice when 0.3% NaOCl, 50% $Ca(OH)_2$, 0.5% HCl and 5% $Ca(OH)_2$ were used, respectively.

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Statistical Modelling of a Facile Process for the Extraction of Crude Constituents of *Curcuma longa*

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Abstract. A preliminary study on a process for the extractions of crude constituents of *Curcuma longa* using statistical modelling is reported. The effects of three independent variables namely; solvent system (ethanol 1:0/water 0:1), temperature °C (30-70) and contact time (min) (5-30) were studied using the central composite rotatable design on the extraction of the crude constituents of the rhizome. Three characteristics of crude extracts, namely; total phenolic content, colourimetric index and relative total soluble solids as responses were studied. Equations for predicting the responses were developed and adequacy confirmed using analysis of variance and residual assessment. The empirical model could find usefulness as a base data for extraction of the crude constituent of *C. longa*.

Keywords: *Curcuma longa,* extraction process, phenolic content, dietary colourant, statistical modelling

Introduction

Ethno medicinal values of *Curcuma longa* have been recognised since pre-historic times. It is used in ethno medicinal formulations for the treatment of spectrum of diseases notably heart disorder, liver problems, asthma, arthritis, gall bladder infections, digestive disorders and dysmenorrhoea (Adeniji, 2003). The herb is endowed with a colourant of superior tinctorial strength in comparison to synthetic dye of similar shade e.g. tartrazine (Henry, 1996) therefore, prefered to use in colouring delicasies such as soup and puddies. The rhizome is a herb of domestic commerce in western Nigeria and could contribute to import substitution therefore, such goal should be prioritised. Enhancing agronomical value essentially involves exploiting upstream and downstream opportunities that abound in agricultural produce. At the entry point of the opportunity chain lies feed stock, a product of primary process usually characterised with low technology process but less effective. It also reduces packaging and transportation cost in comparison to the large sized rhizomes. Using high technology, such feed stock could be processed to yield speciality natural products in nature of dietary colourant or herbal medicine principally inherent to *C. longa*. Catalogue of bio active principle and prospective pharmacological consequence of *C. longa* have been given by Henry (1996). Added to this, the active components of *C. longa* have been modified to enhance its antifungal, antibacterial (Misra *et al.,* 2007) and anticancerous properties (Rojsitthisak *et al.,* 2011). Crude extracts which serve as feed stock for colourant and bioactive components inherent in *C. longa* are of different chemical groups. Therefore, the components could be preliminarily separated using appropriate solvent system enhanced by physical variables such as time and temperature.

The aim of this study was to model a facile process using temperature, contact time and solvent system as independent variables for the extraction of crude constituents of *C. longa* with emphasis on dietary colourant and therapeutic components which could serve as feedstock for the production of specialized natural product with therapeutic (antibiotics) or colourant specificities consequently lending enhancement of agronomical value of *C. longa.*

Materials and Methods

Materials. Wholesome *Curcuma longa* rhizome (red ginger) fingers were obtained from central commercial market in Ado-Ekiti, Nigeria. All chemicals used were of analytical grade: ethanol and distilled water.

Extraction protocol. *Curcuma longa* rhizome without blemish was washed, peeled and dried. The dried product was milled and used in subsequent crude constituent extraction process. The schematic diagram for the

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selected bioactive constituent recovery process using two solvents mixture system, contact time, medium temperature as extraction independent variables is shown in Fig. 1. Details of extraction variables in the experimental design are presented in Table 1. The lowest and highest levels of independent variables were chosen from the results of preliminary investigations.

Experimental design. A central composite rotatable design for k=3 was used (Cochran and Cox, 1957). The 3-factor, 5-level design generated 20 sample combinations comprising eight points peculiar to 2^3 factorial, six star points and six central points for replication. The effects of independent variables namely: solvent-mixture ratio, temperature and contact time were noted for extraction of the crude constituents. The crude constituents markers namely: total phenolic content, tinctorial (colour intensity) index and relative total soluble solids of solution were evaluated. Step-wise regression analyses were performed on the data to yield equations for predicting extraction of crude constituents of *C. longa* with reference to designated functionality.

Analytical Methods. ***Relative total soluble solids determination.*** Refractive index of sample was measured using Abbey Refractometer (ABBE 325, ZUZI) and corresponding soluble solids was determined using a procedure adapted from sugar analysis method and reference to appropriate designated table. Result is reported as relative total soluble solids (Table 2).

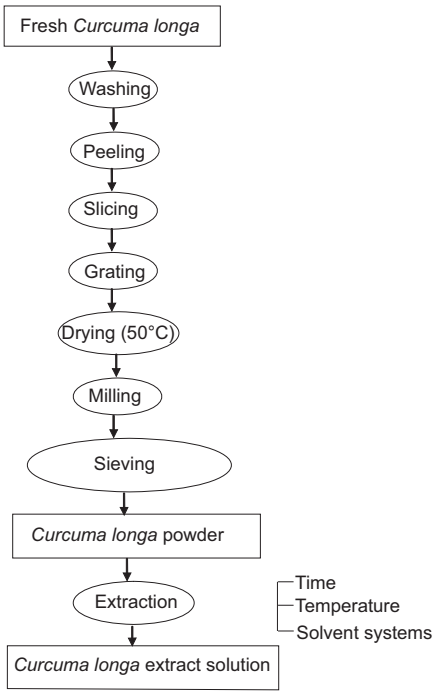

Fig. 1. Flow sheet for the extraction of crude constitutents of *C. longa.*

Determination of colour density/polymeric colour. Colour density was determined according to the method described by Wrolstrad *et al*. (1982). Colour density was calculated as the sum of the absorbances at 420 nm and 510 nm.

Evaluation of total phenolic content. Total phenolic content was evaluated according to the method described by Taga *et al*. (1984). Briefly, a 100µL of Folin - Ciocalteau reagent (2N wrt acid Fluka Chemic AG - Ch-9470 BUCHS) was added to each sample (20µL) and well mixed after addition of 1.58 mL of water. After 30s, 300 µL of 2% sodium carbonate solution was added and the sample tubes were left at room temperature for 2 h.The absorbance(A) of the developed blue colour was measured at 750 nm using Unicam Helios & uv/vis/spectrophotometer. A plot of A_{750} nm against corresponding concentration was used to calculate phenolic content using ascorbic acid as standard and result expressed in mg ascorbic acid equivalent/g sample.

Statistical analysis. The central composite orthogonal designed was analysed as reported by Cochran and Cox (1957). Each of the X-matrix was multiplied by the Y-column (response) to obtain corresponding sums of products that is 0y to 13y for X_O to X_1 X_3.Consequently, the coefficients b_0 to b_{13} were calculate as:

Table 1. Process variables used in the central composite rotatable design (k =3) levels

Independent variable	Code	-1.682	-1	0	1	1.682
Time (min)	X_1	5	10	15	20	30
Temp (°C)	X_2	30	40	50	60	70
Solvent(water/ethanol)*	X_3	1:0	2:1	1:1	1:2	0:1

*0.5g of *C. longa* in 100 mL solvent mixture

Table 2. Range of values for total phenolic content, tinctorial index and relative total soluble solids

Model parameter	Range value
Total phenolic content mg/g	0.001-0.051
Tinctorial index	0.03-0.45
Relative total soluble solids (%)	4-23 -

$b_0 = 0.166338(0y) – 0.056791 \Sigma (iiy)$ ..... (1)
$bi = 0.073224(iy)$ ..... (2)
$bii = 0.062500(iiy) + 0.006889 \Sigma (iiy) – 0.056791(Oy)$ ..... (3)
$bij = 0.125000(ijy)$ ..... (4)

The quadratic model was fitted using the regression coefficients and the predicted response calculated for each of the observed values. The model was observed for adequacy by subject to analysis of variance and residual analysis.

Results and Discussion

Literature suggests that active principle of *C. longa* is insoluble in water. However, it is worth noting that bioactive components of natural colours do not occur in isolation in nature, but usually glycosylated (Oszmianski *et al.,* 2004).Hence, glycosylation is suggested to be the principal factor accounting for aqueous solubility of active constituents of *C. longa* as instantaneous colouration of hot water appeares on addition of *C. longa* powder. Thus, water is used as one of the solvents in this study due to ready availability

Table 3. Regression coefficients for the quadratic model equation for extraction of crude constituent of *C. longa*

Sum of products	Phenolic content	Relative total soluble solids	Tinctometric property	Regression coefficients	Phenolic content	Relative total soluble solid	Tinctometric property
0y	0.473	335	5.46	b0	0.03549	18.1668	0.36412
y	0.1267	54.96	1.6023	b1	0.00928	4.02439	0.11732
2y	0.053	8.77	0.4364	b2	0.00388	0.642	0.03195
3y	0.0976	-5.31	0.61414	b3	0.007150	-0.389	0.04496
11y	0.308	205.35	3.5988	b11	-0.00237	-1.63	-0.01914
32y	0.223	227.98	2.9484	b22	-0.00766	-0.215	-0.05979
33y	0.229	227.98	3.0332	b33	-0.00731	-0.125	-0.05449
12y	-0.005	1.00	0.02	b12	-0.00063	0.125	0.00250
13y	-0.001	7.00	0.2	b13	0.00013	0.875	0.02500
23y	-0.005	-1.00	-0.2	b23	-0.00063	-0.125	-0.02500
Σ (iiy)	0.760	661.31	9.5804				

Table 4. Analysis of Variance (ANOVA) for the Predictive Model Equations

Independent variable	Statistical term	DF	SS	MS	F-Ratio calculated	Tabulated 5%
Phenolic content	First order	3	0.00207953	0.0006931756	21.1198	5.41
	Second order	6	0.00149	0.0002483	7.5933	4.95
	Lack of fit	5	0.0002885	0.0000577	1.76	5.05
	Error	5	0.0001635	0.0000327		
	Total	19				
Tinctorial index	First order	3	0.2295556	0.0765185`	30.125	5.41
	Second order	6	0.097061	0.01617684	6.3688	4.95
	Lack of fit	5	0.0401	0.00802	3.15748	5.05
	Error	5	0.0127	0.00254		
	Total	19				
Relative total soluble solids	First order	3	228.872	76.291	55.8497	5.41
	Second order	6	47.9911	7.998	5.857	4.95
	Lack of fit	5	28.06	5.612	4.1083	5.05
	Error	5	6.831	1.366		
	Total	19				

DF = Degree of Freedom; SS = Sum of Square; MS = Mega Square

Table 5. Residual analysis of assessed parameters

Expt run	Total Phenolic content			Relative total soluble solids			Tinctorial index		
	Observed	Predicted	Residual	Observed	Predicted	Residual	Observed	Predicted	Residual
1	0.003	-0.00355	0.00655	14	12.70441	1.29859	0.11	0.038926	-
2	0.015	0.01653	-0.00153	18.2	18.75319	-0. 55319	0.30	0.21858	0.08142
3	0.006	0.00673	-0.00073	16	13.9884	2.0116	0.18	0.147836	0.032164
4	0.02	0.02429	0.00429	20	20.53719	-0.53719	0.38	0.337489	0.042511
5	0.017	0.01227	0.00473	11	10.42641	0.57359	0.15	0.128866	0.021134
6	0.033	0.03183	0.00117	18	19.97519	-1.97519	0.44	0.4085196	0.03148
7	0.022	0.02003	0.00197	12	13.21041	-1.21041	0.12	0.137745	-0.017745
8	0.031	0.03707	-0.00607	20	21.25919	-1.25919	0.42	0.427428	-0.00743
9	0.006	0.013179	-0.007179	4	6.788136	-2.788136	0.08	0.1126279	-0.03263
10	0.051	0.0444	0.0066	23	20.326184	2.673816	0.45	0.5073153	-0.057315
11	0.001	0.0073	-0.0063	17	16.478936	0.521064	0.05	0.1412667	-0.091267
12	0.026	0.02035	0.00565	18	18.6386	-0.6386	0.25	0.2487616	0.001238
13	0.003	0.00279	0.00021	17	18.213078	-1.213078	0.03	0.134363	-0.104363
14	0.026	0.02684	-0.00084	18	16.90448	1.09552	0.30	0.285641	0.014359
15	0.030	0.03549	-0.00549	18	18.1668	-0.1668	0.35	0.364125	-0.01413
16	0.039	0.03549	0.00351	19	18.1668	0.8332	0.38	0.364125	0.015875
17	0.032	0.03549	-0.00349	20	18.1668	1.8332	0.30	0.364125	-0.064125
18	0.03	0.03549	-0.00549	18	18.1668	-0.1668	0.34	0.364125	-0.024125
19	0.038	0.03549	0.00251	17	18.1668	-1.1668	0.45	0.364125	0.085875
20	0.044	0.03549	0.00851	17	18.1668	-1.1668	0.38	0.364125	0.015875

Residual = Observed – Predicted

and relatively low cost. Basically three technological parameters namely; phenolic content, tinctorial index and relative total soluble solids were evaluated for the extraction of crude constituents of *C. longa.* The constraints (Table 1) explored in this study, exerted varied extraction effects as shown by the three responses (Table 2) evaluated therefore, subsequently modeled for prediction of constituent extraction outcome that is dictated by constituent's functionality.

The central composite orthogonal design to fit the polynomial model for the extraction of the three classes of crude constituents in *C. longa* was accomplished as elicited by Cochran and Cox (1957). The computed sums of products and regression coefficients to fit the model are shown in Table 3.

Total phenolic content. One of the most important groups of natural product with respect to therapeutic or biological value is phenolics. Therefore, total phenolic content of the extracted crude constituents in the *C. longa* solution can give insight to therapeutic potential of the designated rhizome. Thus the quadratic model takes the form:

$$P_t=0.03549+0.00928X_1+0.00388X_2+0.00715X_3-0.00237X_1^2-0.00766X_2^2-0.00731X_3^2-0.00063X_1X_2-0.00013X_1X_3-0.00063X_2X_3 \quad \text{.....(5)}$$

The predicted total phenolic content p_t for each of the experimental run and their respective residual are shown in Table 4. Examination of the residuals suggests that the fitted model was reasonably adequate. The assertion was confirmed on model testing. Added to this, the analysis of variance to test the fitness of the model is presented in Table 5. The first and second order terms were significant as shown by the higher calculated F-ratio in comparison with the tabulated values. However, since the calculated F-ratio for the lack of fit was lower than the tabulated value, adequacy of the fitted model is affirmed. One of the flexibilities affordable by this model is that it can be adapted for extraction of crude constituent of interest in terms of dietary colourant or bioactive (therapeutic) dominate.

Tinctorial index (T_P). Poor tinctorial index is the foremost drawback of colourant of natural origin. However, it is gratifying to note that, such negativity is not the case with

shade intensity of *C. longa*. Colourimetric power (index) of *C. longa* is higher than available synthetic colourants of similar shade (Henry, 1996). Therefore, predictive model for tinctorial index of crude extract of colourant from *C. longa* deserves determination. Thus the quadratic model takes the form:

$$T_I = 0.36412498 + 0.1173268X_1 + 0.03195495X_2 + 0.0449698X_3 - 0.019149X^2_1 - 0.059799X^2_2 - 0.054499X^2_3 + 0.0025X_1X_2 + 0.025X_1X_3 - 0.025X_2X_3 \quad (6)$$

The predicted tinctorial index (T_I) for each of the experimental run and their residual are presented in Table 4. Their residuals suggest that the fitted model is adequate. The claim on adequacy of the model fitness was verified by conducting analysis of variance test (Table 5). Since the first and second order terms were significant as revealed by higher calculated F-ratio in comparison with tabulated values and the calculated F-ratio for the lack of fit were lower than the tabulated value, attesting adequacy of the model.

Relative total soluble solids (RTSS). Relative total soluble solids reflect a composite of colourant of the crude constituents of *C. longa*. Its value is principally dictated by solvent composition (mixture) and bio chemical nature of the crude constituents (i.e. colourant and phenolic compounds). Nevertheless, the other variables influence RTSS of *C. longa* constituents in solution. The quadratic model takes the form:

$$RTSS = 18.1668 + 4.02439X_1 + 0.642X_2 - 0.389X_3 - 1.63X^1_2 - 0.215X^2_2 - 0.215X^2_3 + 0.125X_1X_2 + 0.875X_1X_3 - 0.125X_2X_3 \quad (7)$$

Using same assessment protocol reported for previous model parameters, the fitness of RTSS model was evaluated using analysis of variance (Table 4) and residual analysis (Table 5) and found adequate.

Conclusion

Extraction of crude constituents of *Curcuma longa* using three process variables yielded constituent that could essentially be dietary colourant and antimicrobial bases. The models developed were found adequate to extract crude constituent of interest with respect to colourant or antibiotics from the *C. longa* powder. In addition, the preliminarily separated crude constituents could serve as feedstock for further processing to yield product of high functionality. Bearing in mind that *C. longa* is partly plant food, its constituents can be used as additive in food preparations that are subjected to heat but not necessarily exposed to light. Such foods are: bread, cake, potatoes, mayonnaise, rice, macaroni and frankfurters. The findings of this study also offer possibility for reducing transportation and packaging cost as a result of miniature volume of the crude constituents in comparison to the large size (bulk) of the milled rhizome.

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Variation in Activity of Pepsin Extracted from Buffalo Stomach Mucosa

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Abstract. Pepsin was extracted from the buffalo's mucosa in an acidic medium by incubating at 40 °C for 48 h and dried in an air blanket at 50 °C. Conditions for the maximum yield of pepsin were optimized. Changes in pH, temperature and incubation time affect the yield of pepsin. It has been noted that the time of the year in which extractions were made under optimized conditions was an important factor which affected the yield as well as activity of pepsin. Studies showed that maximum yield 11.5% was in February 2009 and minimum 10.3% in May 2009. It was further studied that the activity of the pepsin extracted in February was higher i.e 110 U/mg as compared to the activity of the enzyme extracted during the month of May which was 102.6 U/mg. The purpose of the study was to consider the conditions of the slaughter houses to attain maximum yield of pepsin with maximum activity.

Keywords: pepsin extraction, enzyme activity, stomach mucosa, buffalo

Introduction

Pepsin is a digestive protease (Dunn and Fink, 1984) which is released by the chief cells in the stomach and degrades food proteins into peptides (Andreeva, 1994). Pepsin was discovered in 1836 by Theodor Schwann who also coined this enzyme's name from the Greek word pepsin, meaning digestion (Fruton, 2002). It was the first animal enzyme to be discovered, and in 1929 it became one of the first enzymes crystallized by Northrop (1946).

Pepsin is expressed as a pro-form zymogen pepsinogen, whose primary structure has additional 44 amino acids. In the stomach, chief cells release pepsinogen. This zymogen is activated by hydrochloric acid (HCl), which is released from parietal cells in the stomach lining. The hormone gastrin and the vagus nerve triggers the release of both pepsinogen and HCl from the stomach lining when food is ingested (Klomklao *et al.,* 2007). Pepsin digests up to 20 % of ingested carbon bonds by cleaving preferentially after N-terminal of aromatic amino acids such as phenylalanine, tryptophan and tyrosine (Bovey and Yanari, 1960). Peptides may be further digested by other proteases (in the duodenum) and eventually absorbed by the body. Pepsin is stored as pepsinogen so it will only be released when needed, and does not digest the body's own proteins in the stomach's lining (Northrop, 1946).

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Pepsin functions best in acidic environments, particularly those with a pH of 1.5 to 2 (Andreeva, 1994) and will denature if the pH rises up to 5 (Dee *et al.,* 2006). When the pH is adjusted back to 6.0 activity returns (Johnston *et al.,* 2007). It should be stored at very cold temperatures (between-20 °C and -80 °C) to prevent autolysis (self-cleavage). Autolysis may also be prevented by storage of pepsins by reductive methylation (Tanji *et al.,* 1988).

Pepsin is a multipurpose enzyme and is of great importance in food industry (Aukkanit and Garnjan-agoonchorn, 2010) and has therapeutic value (Barsky *et al.,* 1984.

Animal tissues like pancreas, liver, small and large intestine are available daily in very large amount from slaughter houses in the cities. These tissues are source of many other enzymes that are of great importance (Dunn and Fink, 1984). The stomach mucosa of buffalo is a rich source of pepsin. In this context, the objective of this study was to analyze the effect of pH, temperature and time of incubation in the activity of pepsin extracted from buffalo stomach mucosa. Its maximum yield and minimum loss in its activity within different months of a year was also estimated.

Materials and Methods

Collection of buffalo stomach. The buffalo stomachs were obtained from animal slaughter house, Lahore immediately after slaughtering the animals, during

February to May 2009. The collected tissues were kept in ice water and transported to the Pakistan Council of Scientific and Industrial Research Laboratories Complex, Lahore, Pakistan as early as possible and were stored at -20 °C for further processing.

Processing. Extraction of pepsin from buffalo stomach mucosa was done according to the method of Zia-ur-Rehman and Shah (1997) with slight modifications. The stomachs were cut opened and washed with ice cold water to clean it from other waste products. The inner lining of mucosal part was obtained by cutting with sharp knife at 10 °C and minced in meat mincing machine. Two batches were set, each batch containing 1 kg wet weight of stomach mucosal lining. The linings were then suspended in 1.5 L of water. Afterward the water was acidified with 2N HCl and homogenized. The pH of homogenate was adjusted to 2.0 with HCl. The tissues of first batch of 1 kg wet weight were incubated at 40 °C for 24 h and the other batch was incubated at 40 °C for 48 h for the conversion of pepsinogen into pepsin. After completion of time, the extract was squeezed using muslin cloth. Saturated NaCl was added to the semitransparent liquid obtained after squeezing the extract. Mixture of each batch was allowed to settle down to extract maximum amount of precipitate that was filtered by using Whatman 40 filter paper. The precipitate obtained was dried in an air blanket at 50°C in tunnel oven so as to collect the pepsin. The dried pepsin was grinded to obtain amorphous powder and was preceded for analysis.

Analysis. The activity of enzyme was estimated according to Anson (1938), using hemoglobin as a substrate. One unit of enzyme can cause an increase in optical density by 0.001 at 280 nm at 40 °C.

$$U/mg = \frac{(A_{280nm} \text{ of sample} - A_{280nm} \text{ of blank}) \times 1000}{(10 \text{ mins}) \text{ (mg. Enz/mL of reaction mixture)}}$$

$A_{280\,nm}$ ---- absorbance at 280 nm
10 min--- incubation time

Results and Discussion

Reproducibility of the extraction method. Reproducibility of the method used for the pepsin extraction from buffalo stomach mucosa was also studied to gain maximum yield with maximum activity.

Effect of pH. The influence of pH on the extraction of pepsin was studied. It was observed that the yield of pepsin greatly influenced by the change in pH (Fig.1). Maximum yield was obtained at pH 2.0 and with the increase in pH from 2.0 to 3.5 a decrease of 13 % in the yield of pepsin was noted and this decrease in yield reached to 40% when pH raised from 2.0 to 4.5.

Effect of temperature and incubation time. Effect of incubating temperature and time is shown in Fig. 2 and 3, respectively. Maximum yield of pepsin was obtained when mucosal lining was incubated at 40 °C for 48 h. Above and below this time and temperature yield of pepsin decreased tremendously. Results are slightly contradictory to the findings of Saeed and Ford (1999), who reported maximum yield at 40 °C for 18 h incubation.

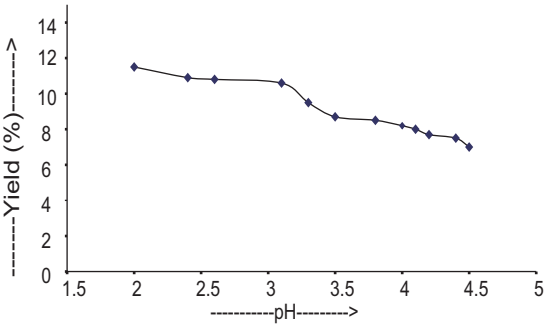

Fig. 1. Effect of pH on the yield of pepsin.

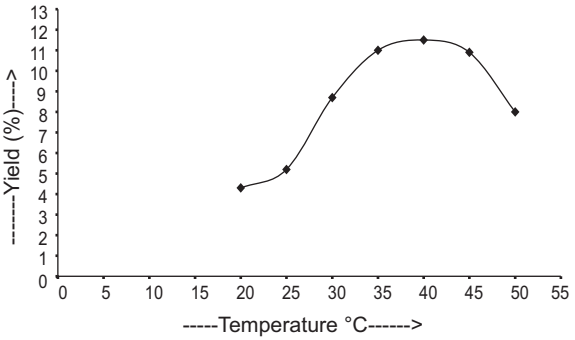

Fig. 2. Effect of incubation temperature on the yield of pepsin.

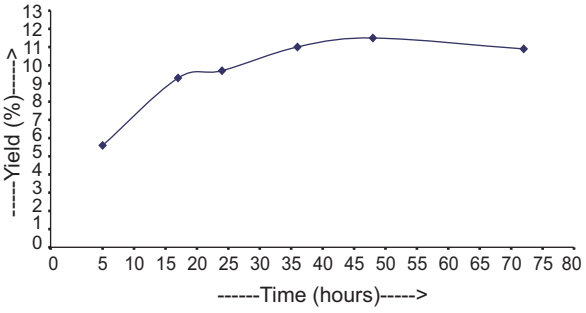

Fig. 3. Effect of incubation time on the yield of pepsin.

Effect of NaCl. Pepsin extracted from buffalo stomach mucosa in an acidic media was precipitated from the filtrate by adding sodium chloride (NaCl). Maximum pepsin was precipitated after the addition of 250 g NaCl to the filtrate (Fig. 4). Beyond this amount the addition of NaCl did not show any increase in the yield or activity of the enzyme.

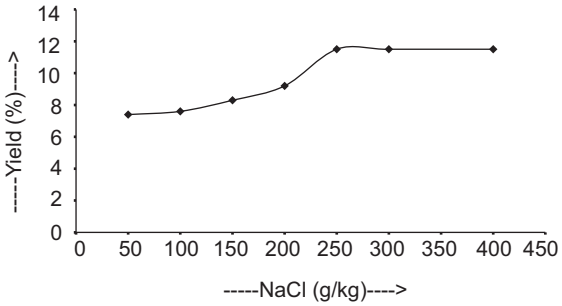

Fig. 4. Effect of NaCl on the yield of pepsin.

Effect of storage time. The minced mucosa of the buffalo was stored at -20 °C for 20 days. It was observed that the yield as well as the activity of the enzyme showed a slight decrease after 20 days of storage Table 1. The pepsin extracted from the fresh mucosa tissues showed maximum activity, while after storage it showed a decrease of activity, approximately 0.9 U/mg in February and March and 3.89 U/mg in May.

Effect of extraction in different months. The lower temperature in February seemed to be favourable for the maximum yield of pepsin. The maximum activity of pepsin was observed in the sample processed in the month of February i.e. to 110 units/mg enzymes (Anson, 1938). Poland and Bloomfield (1929) determined the activity of pepsin by using standard 2% solution of edestin and were found in the range of 100 to 110. These results were in agreement with the findings of Piper and Fenton (1965), who studied the activity of pepsin by radioiodinated serum albumen method. Tanji *et al.* (1988) demonstrated that pepsin activities were appreciable in preparation made from the fundus region of the stomach which was similar to present findings.

The minimum activity of the enzyme pepsin was observed during the month of May i.e. ranged from 102.6 to 87 units/mg in the samples. Niazi *et al.* (1997) studied extraction and isolation of pepsin from the stomach mucosa of buffalo and also noted the activity of this enzyme using hemoglobin as a substrate. Moreover, the precipitation method eliminated expensive and time consuming step of dehydration under vacuum. It was found that the stomach of buffalo slaughtered in big cities, if properly processed in time, would yield large amount of pepsin and save the expenses on its import. By improving the atmospheric conditions of the slaughter houses and controlling the temperature, pepsin yield as well as its activity can be improved.

Conclusion

The study proposes the use of slaughter house waste as a useful source for extraction of pepsin. The extraction

Table 1. Yield and activity of pepsin extracted from buffalo stomach mucosa

Months	Temperature of slaughter house (°C)	Days of storage at -20 °C	Yield (%)	Amount (g/kg)	Activity U/mg
February		1	11.50	115	110
	20	10	11.43	114.3	110
		20	11.20	112	109
March		1	11.30	113	109
	25	10	11.11	111.1	108
		20	10.94	109.4	108
April		1	11.30	113	103
	30	10	10.80	108	102
		20	10.29	105.1	99
May		1	10.30	103	102.6
	35	10	10.26	102.6	100
		20	10.26	102	87

steps are remarkably simple and do not involve costly chemicals and unique instrumentation. Yield as well as activity of enzyme is dependent on the temperature. Not only the temperature at which stomach mucosa stored before processing is important, but atmospheric temperature in which animals are slaughtered is also important to gain maximum yield with increased activity of the enzyme. The results of this study clearly showed the potential and versatility of this method, which could be applied to manage slaughter house waste and save foreign exchange.

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Short Communication

Culture of Earthworm *Lampito mauritii* Kinberg, 1867 in Fish Pond Sludge and Cardboard

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Abstract. The earthworms, *Lampito mauritii* Kinberg, 1867 were treated with fish pond sludge (FPS) and card board (CB) mixture in different feedstock concentrations (5%, 10%, 15%, 20%, 25%, 50%, 75% & 100% FPS) for the period of 63 days in July-September, 2011. The parameters such as initial and final biomass, survival rate of earthworms were noted at constant moisture and pH level. Among the eight treatments; 15% FPS with 85% of CB was found most suitable for vermicomposting.

Keywords: earthworm, fish pond sludge, vermicomposting

Earthworms are considered as an important component of soil ecosystem; they convert organic waste materials to a highly valuable soil amendment (Datar *et al.,* 1997). The worms are also used in vermicompost technology where they process biodegradable materials like leaves, sewage water, municipal waste, household garbage, livestock manure, non-toxic solid and liquid waste of the industries into natural organic fertilizer (Edwards and Fletcher, 1988). Some workers suggested the recycling of fish pond waste as organic fertilizer for agriculture purpose as it is enriched with organic matter, nitrogen, phosphorus, as well as macro and micro-nutrients (Marcet *et al.,* 2010; Mizanur *et al.,* 2004; Mazzarino *et al.,* 1998).

Present study is aimed to process fish pond sludge (FPS) into organic fertilizer for agriculture crop by using earthworms. *Lampito mauritii* were introduced in eight different FPS treatments in association with cardboard (CB) as feedstock showed significant production of earthworm biomass in 15% FPS and 85% CB.

The study was carried out in Aquaculture Laboratory of PCSIR Labs-Karachi, for the period of 63 days in July-September, 2011. The specimens of earthworm were dug out from the garden soil and adult stages were sorted out; identification of species was done using the description of Parshad (1916). Used corrugated CB boxes were shredded in small pieces, FPS was collected from the Tilapia (*Oreochromis* spp.) pond located at PCSIR Labs. and refrigerated. Eight different feedstock treatments were prepared with CB and FPS in ratios ranging from 5%, 10%, 15%, 20%, 25%, 50%, and 75%, whereas 100% contained only FPS, a control setup which comprised of just an ordinary soil was also maintained with same number of earthworms. Circular 1 L glass containers (diameter 14 cm, depth 12 cm) were filled with 500 g each of feedstock material. In each container 25 earthworms were inserted and covered with nylon net cloth to provide aeration, and kept in dark place. Moisture content was maintained from 68-70% in each container during the experiment by sprinkling water with a hand spray when required while pH was also maintained from 7.5 to 8.5. Each container was checked regularly to observe the earthworm survival rate and biomass; the number of cocoons and larvae if present were also noted; organic carbon was estimated from each container by the method of ASTM (2000).

Table 1 shows the observation taken in 8 different treatments (mixture of FPS and CB) and control setup in terms of earthworm survival, biomass, cocoon production and larval numbers. The estimates of organic carbon in each setup are also given in Table 1, which is lowest in control (10.3 g/kg), whereas highest in 100% FPS (27.6 g/kg) treatment. The total biomass of *L. mauritii* in all setups ranged from 9.15-11.97 g initially while 7.35-25.42 g finally. The percentage

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Table 1. Results obtained after using earthworms *Lampito mauritii* (Kinberg, 1867) with different treatments

Feedstock treatment concentration	Organic carbon (g/kg) mean±S.D.	Earthworm biomass (g) (n=25) Mean±S.D.		Percentage increase/decrease in the total biomass	No. of cocoons	No. of larvae
		Initial	Final			
5 % FPS+ 95 % CB	12.2±0.25	10.78±0.13	20.48±0.46	89.98 (22±0.35)	30	14
10 % FPS+ 90 % CB	13.7±0.29	11.97±0.24	21.53±0.52	79.86 (22±0.34)	48	23
15 % FPS+ 85% CB	15.5±0.35	9.83±0.21	25.42±0.55	158.6 (25±0.46)	63	33
20 % FPS+ 80 % CB	16.5±0.63	10.14±0.27	19.13±0.54	88.65 (21±0.64)	56	28
25 % FPS+ 75 % CB	18.8±0.58	9.48±0.34	17.60±0.43	85.6 (20±0.24)	46	24
50 % FPS+ 50 % CB	20.9±0.74	9.88±0.37	16.50±0.37	67.0 (18±0.28)	37	19
75% FPS+ 25% CB	23.7±0.72	9.15±0.31	11.65±0.31	27.32 (13±0.33)	30	9
100 % FPS+ 0 % CB	27.6±0.89	9.35±0.22	7.35±0.21	-21.4 (10±0.22)	28	6
Control	10.3±0.25	10.98±0.15	14.0±0.55	27.5 (15±0.41)	35	16

increase or decrease in the total biomass given in Table 1 shows loss of 21.4% biomass with 100% FPS. The control setup which is of normal soil showed 27.5% increase in biomass of *L. mauritii*. The setup with CB mixtures exhibited increase and the total biomass was highest (158.6%) in the setup with 15% FPS and 85% corrugated cardboard. Number of cocoons and larvae were found abundant in 15% FPS feedstock treatment, whereas decreased gradually in other treatments (Table 1). The results revealed that FPS in combination with CB favours earthworm growth.

Development of earthworm culture in different medium has been studied by various researchers. Datar *et al.* (1997) observed that high percentage of moisture in soil is important for the high survival rate of earthworms. Hand *et al.* (1988) assessed the suitability of cow slurry with paper waste which provided results of greater earthworm growth and cocoon production. Neuhauser *et al.* (1980) used sewage instead of cow slurry for vermicomposting and reported higher growth of earthworm. Some worm grower claim that corrugated cardboard stimulates worm reproduction (Georg, 2004). Muthukumaravel *et al.* (2008) used cow dung and vegetable waste in different experiments for the growth of earthworms. Present study has been carried out for the first time by using fish pond sludge (FPS) in combination of corrugated cardboard. The preliminary observation showed earthworm (*L. mauritii*) encouraging growth rate and high survival rate. It may be suggested that FPS in combination of the CB can be utilized for earthworm culture which eventually can be used for soil treatment. However, it requires further studies focused in this direction before such setup is planned.

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