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The Role of Rainfall Intensity and Soil in Determining Rates of Flow Through Cryoturbated Chalk

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Abstract. The effects of different water application rates (3, 10, 15 and 30 mm/h) and of topsoil removal on the rate of downward water movement through the cryoturbated chalk zone in southern England were investigated *in situ.* During and after each application of water, changes in water content and matric potential of the profile were monitored and percolate was collected in troughs. The measured water breakthrough time showed that water moved to 1.2 m depth quickly (in 8.2 h) even with application rate as low as 3 mm/h and that the time was only 3 h when water was applied at a rate of 15 mm/ h. These breakthrough times were about 150 and 422 fold shorter, respectively, than those expected if the water had been conducted by the matrix alone. Percolate was collected in troughs within 3.5 h at 1.2 m depth when water was applied at 30 mm/h and the quantity collected indicated that a significant amount of the surface applied water moved downward through inter-aggregate pores. The small increase in volumetric water content (about 3%) in excess of matrix water content resulted in a large increase in pore water velocities, from 0.20 to 5.3 m/d. The presence of soil layer had effect on the time taken for water to travel through the cryoturbated chalk layer and in the soil layer, water took about 1-2 h to pass thorough, depending on the intensity.

Keywords: breakthrough time, cryoturbated chalk; rainfall intensity; water flow

Introduction

Chalk has a dichotomous porous system consisting of intra- and inter-granular porosity. The intra-granular porosity is referred to as the matrix porosity and is usually 35 to 50% of the volume (Burnham, 1990). It has a large air entry suction and small, but relatively uniform pores, 0.1 to 1 μm in diameter (Price, 1987) that result in a low matrix conductivity that is typically 5 to 8 mm/d (Mahmood-ul-Hassan and Gregory, 2002; Cooper *et al*., 1990). The pores of the matrix are sufficiently small to store water against the force of gravity so that, even in the unsaturated zone, most of the pores remain virtually saturated during winter (Price *et al*., 1976).

The inter-granular porosity is referred to as the fissure porosity and constitutes about 4% (Reeves, 1979) to 14% (Mahmood-ul-Hassan, 1998) of the total porosity. The fissures range in diameter from 20 mm to 10 mm (Reeves, 1979) and provide the pathways for rapid flow. Water and solutes moving by this pathway do not move downward with the horizontally-uniform wetting front found in the matrix but, instead, travel rapidly through a small fraction of the total volume bypassing much of the matrix (Arnon *et al.*, 2005; Nativ *et al.*, 2003; Geake and Foster, 1989).

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In most of the chalklands of England, a shallow layer of well-drained and structured soil directly overlies cryoturbated, fractured chalk. This soil can affect the route and rate of water flow through the underlying chalk. In well-structured soils, preferential flow of water can occur at application rates lower than that required for saturated flow (Radulovich *et al*., 1992), so that there is the potential for the overlying soil to augment the preferential flow through the underlying chalk. In such soils, vertical transmission of water through preferred paths can occur during rainfall by either saturating the lower levels of the soil and then passing through the fissures between the chalk fragments, or directly entering into the fissures if they are, by chance, directly connected to the preferred path. However, there are also suggestions that the overlying soil may reduce the opportunity for preferential flow through the underlying chalk. For example, Gardner *et al*. (1990) reported that rainfall intensity rarely exceeds a few millimetres per hour in south-east England and that this can be stored temporarily in the overlying soil and weathered chalk materials near the surface; this buffering capacity allows water to be released to deeper layers at rates low enough to be conducted by the matrix alone.

The underlying fractured chalk has a very small water storage capacity at potentials > -100 kPa (Cooper *et al*., 1990), which means that steady state conditions are established quickly

during rain. Hence, if the hydraulic conductivity of the matrix exceeds the flux from the soil into the chalk, flow will be through the matrix. But, if the flux is larger than the matrix conductivity, the excess will be carried by the fissure system. The flux out of the overlying soil into the fractured chalk and the hydraulic conductivity of the chalk matrix are, then, key factors that determine the partitioning of flow between the chalk matrix and the fissures.

This paper reports the results of a series of field experiments in which known rates of water were applied to chalk with the soil present or not. The objectives of this study were first, to determine the effect of water application rate on preferential flow through the chalk zone and second, to investigate the role of the overlying shallow soil in controlling the pathways of water flow through the underlying chalk. Selected results are presented in this paper from a larger study (Mahmood-ul-Hassan, 1998).

Materials and Methods

Experimental site and instrumentation. The experiments were conducted in Ohio field at Bridgets Experimental Husbandry Farm, Winchester, Hampshire, UK (National Grid Reference SU 517337). The soil was mapped as an Andover series rendzina by Moffat (1985)-thin silty drift over chalk, comprising of about 26 cm calcareous silty clay loam (well structured moderate to fine subangular blocky overlying fractured upper chalk). The underlying fractured upper chalk material, down to about 1.5 m, was rubbly in nature and loose with many voids. Details of the soil and site are given in Mahmood-ul-Hassan and Gregory (2002). The experiments were conducted within a large (20 m x 60 m), long-term (> 5 years) grass plot. Two experimental plots, each 3 m long and 1.5 m wide were established on either side of a pit, approximately 1.4 m wide and 1.5 m deep dug for installation of instruments to observe rapid hydraulic changes at different depths (Fig. 1). Another smaller plot (1.5 m x 1.4 m) alongside the pit was also established and used to collect water draining from different depths.

Theta probe type ML1, moisture sensors (Delta-T Devices, Burwell, Cambridge, UK), were installed in duplicate on opposite sides of the pit on a diagonal line (Mahmood-ul-Hassan and Gregory, 2002), about 0.25 m apart laterally and at 0.2, 0.4, 0.6, 0.8, 1.0 and 1.2 m below the ground level to monitor changes in soil water content. The calibration graph for silty clay loam soil and upper chalk (Mahmood-ul-Hassan, 1998) was used to convert the output of the theta probe to volumetric water content. Changes in soil matric potential were monitored simultaneously using pressure transducer tensiometers (SWT3, Delta-T devices). In both the plots, pressure transducer tensiometers were installed 0.5 m away from the theta

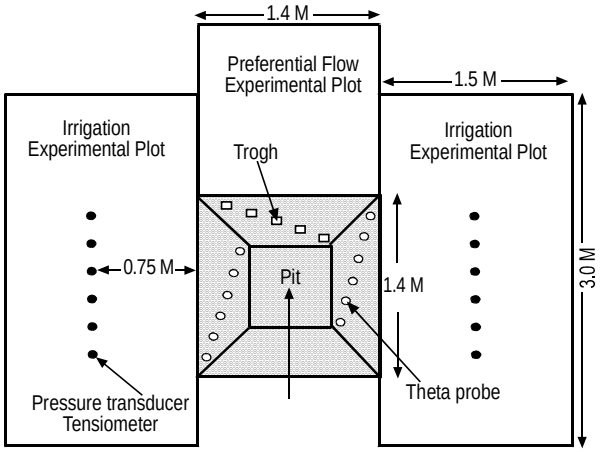

Fig. 1. Layout of experimental plots showing the position of pressure transducer tensiometers (PTT) and the installation of theta probes and troughs to collect percolate in the pit walls.

probe at 0.2, 0.4, 0.6, 0.8, 1.0 and 1.2 m depths and spaced at 0.25 m intervals. Data from the theta probes and pressure transducer tensiometers were logged using a Delta-T logger.

Rainfall simulator. Water was applied to the plots with a rainfall simulator consisting of a rectangular frame with six parallel aluminium pipes each spaced 30 cm apart. Water jets were mounted at 30 cm intervals along each aluminium pipe and the whole system was mounted on a 1.5 m x 3 m frame. The frame was moved across the plots on rails. Water from the jets came out as fine spray and for that reason the sides of the frame were covered with plastic sheets to prevent drift. Water was supplied to the simulator by a water pump at one end and pressure was maintained, for a constant rain intensity, with a gate valve fixed on the other end of the simulator. Surplus water was drained to a water tank. Uniformity of application was tested before and after the experiments for the three different water application rates (10, 15 and 30 mm/h) by computing uniformity coefficient (C_u) expressed as Christian's coefficient (James, 1988);

$$C_u = 100 \left[1 - \frac{D}{Mn}\right]$$

where:

D is the deviation of the individual observation from the mean value, M is the mean values of the observations and n is the number of observations.

The coefficient ranged from 89 to 92% indicating an acceptable uniformity of application.

Irrigation treatments. Irrigation was applied either with intensities of 10, 15 and 30 mm/h in pulses using the simulator

or with an intensity of 3 mm/h using a 1 m long dribble bar attached to a watering can. Irrigation was continued until steady state conditions had been achieved.

Irrigation with an intensity of 3 mm/h was achieved by delivering a total of about 24 mm water in 8.0 h. To achieve an intensity of 10 mm/h, a total of 60 mm water was applied in a period of 6 h. Similarly, 60 mm and 120 mm water was applied in a period of 4 h for the 15 and 30 mm/h treatments, respectively. After every irrigation, the plots were covered with plastic sheeting to prevent evaporation and the profile allowed to drain for one week. Soil water content and matric potential of the profile were monitored throughout.

The overlying soil (0.25 m layer) was then removed from both experimental plots. After removing the soil, a thin layer of fine sand was applied on the surface of the fractured chalk to even out the distribution of water as well as to avoid the direct entrance of water into the inter-granular pore spaces. Water was applied manually at a rate of 3 mm/h using a dribble bar and a total of about 24 mm of water was applied in 8.0 h. The profile was allowed to drain overnight and the next day the experiment was repeated to observe the effects of antecedent water content.

Collection of preferential flow. Preferential flow was collected at depths of 0.4, 0.6, 0.8, 1.0, and 1.2 m in 80 cm long steel channels (Fig. 2). The channels were 4.5 cm wide and 2 cm deep with a perforated steel sheet attached inside the channels about 0.5 cm above the bottom surface to facilitate water flow. A sharpened chisel was fixed on the fore-end of the channel to ease insertion into the chalk and a steel lid was fixed on the end of the channel to stop water dropping into the pit. Plastic tubing was used to run drainage from the steel channels to plastic collectors. The channels were very carefully inserted into the chalk using a jack acting against the opposite wall of the pit. The channels extended 65 cm into chalk and were inclined slightly towards the pit to aid collection.

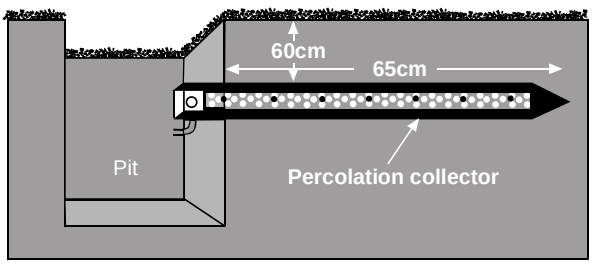

Fig. 2. Schematic diagram of a percolate collector at a depth of 60 cm, lateral view.

Calculation of drainage, unsaturated hydraulic conductivity and first breakthrough time. Drainage flux, D, from each depth was estimated using the water balance equation, assuming vertical flow only and no surface run-off;

$$D = R - E_p + \int_0^z \theta(t_1)dz - \int_0^z \theta(t_2)dz$$

where:

R is the rainfall, E_p is the potential evaporation during the period from t_1 to t_2 and θ is the water content of the profile at time t_1 and t_2 at depth z. (during winter, the potential and actual evaporation are same: Cooper *et al*., 1990).

The hydraulic conductivity was calculated from the drainage fluxes and the measured gradient of hydraulic potential using the instantaneous profile method (Hillel *et al*., 1972). The mean water flow velocity was calculated by dividing drainage flux by the volumetric water content of respective depth. Saturated hydraulic conductivity of overlying soil and of underlying chalk (of different depths) was measured with a constant head using 10 cm long and 10 cm diameter undisturbed cores.

The measured first breakthrough time (MFBT) for a depth was determined as the time from start of the water application to the first appearance of water at the given depth, i.e., the time at which the theta probe at that depth started to show an increase in water content. The expected first breakthrough time (EFBT) for each depth was calculated by dividing interval of the distance by the mean water flow velocity of that depth.

Results and Discussion

Hydraulic properties. The relationship between matric potential and water content describes the hydraulic properties and pore size distribution of a porous medium. Fig. 3 shows that

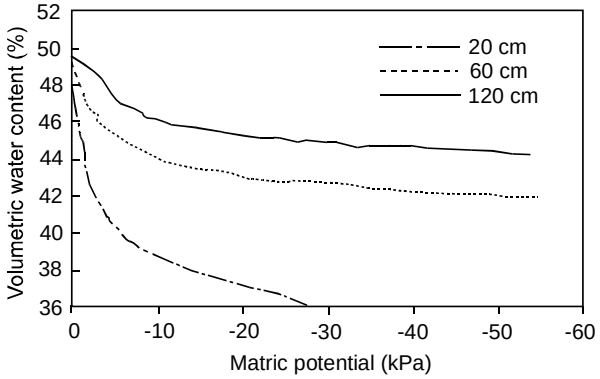

Fig. 3. Water release curves measured *in situ* using theta probes and pressure transducer tensiometers.

the soil overlying chalk released a large amount of water (≈0.10 m^3/m^3) as potential decreased from 0 to -10 kPa; this water represents the macroporosity. Less water was released from the greater depths (0.6 and 1.2 m). The saturated hydraulic conductivity of soil (5.2 ± 0.42 m/day) was greater than that of the underlying cryoturbated chalk (4.65 ± 0.24 and 3.35 ± 0.49 m/day at 0.4 and 0.6 m, respectively). Hydraulic conductivity as function of matric potential measured *in situ* decreased from ≈102 mm/d near saturation to ≈8 mm/d at -10 kPa.

Effect of rate of irrigation on water flow. The measured first breakthrough times at different rain intensities were shorter than the expected first breakthrough times calculated as if only the matrix were conducting (Table 1). This suggests that water flow through the overlying soil and underlying fractured chalk was not piston type flow with a uniform wetting front even at an application rate as low as 3 mm/h. The measured first breakthrough times for applications of 3mm/h were, as expected, longer than those for 10, 15, and 30 mm/h at all depths. The cumulative differences between breakthrough times at 3, 10 and 15 mm/h increased with depth and were greatest at 1.2 m depth but the difference between 15 and 30 mm/h rain was small. There was no difference in the breakthrough time at 0.2 and 0.4 m for applications of 10, 15, and 30 mm/h. At 0.8, 1.0 and 1.2 m depth, the differences in measured times between applications of 10 and 15 mm/h were about two fold, while at application of 15 and 30 mm/h there was no difference except at 0.8 m depth.

The measured breakthrough times at different depths were about 134 to 215 fold (at 3 mm/h intensity) and 430 to 872 times (at 30 mm intensity) smaller than the EFBTs. The decrease in breakthrough times as the rate of application increased was most likely due to the more rapid increase in volumetric water content resulting in a large increase in hydraulic conductivity (Fig. 4). At 1.2 m depth, for example, a 0.03 m^3/m^3 increase in volumetric water content (from 0.46 to 0.49 m^3/m^3) increased the conductivity from about 8 mm/d to 110 mm/d. Similar increases in conductivity for only small increases in water content were also observed at other depths (e.g., 0.6 m, Fig. 4).

Table 1. Measured first breakthrough time (MFBT) compared with the expected first breakthrough time (EFBT) if the matrix only were conducting water; times (h) for each 0.2 m depth are shown

Depth (m)	MFBT (h) Rain intensities (mm/h)				EFBT (h)
	3	10	15	30	
0 - 0.2	2.0	1.0	1.0	1.0	-
0.2 - 0.4	1.0	0.5	0.5	0.5	216
0.4 - 0.6	1.5	0.5	0.5	0.25	211
0.6 - 0.8	1.5	1.0	0.5	0.25	211
0.8 - 1.0	1.5	0.5	0.25	0.25	221
1.0 - 1.2	1.0	0.5	0.25	0.25	214

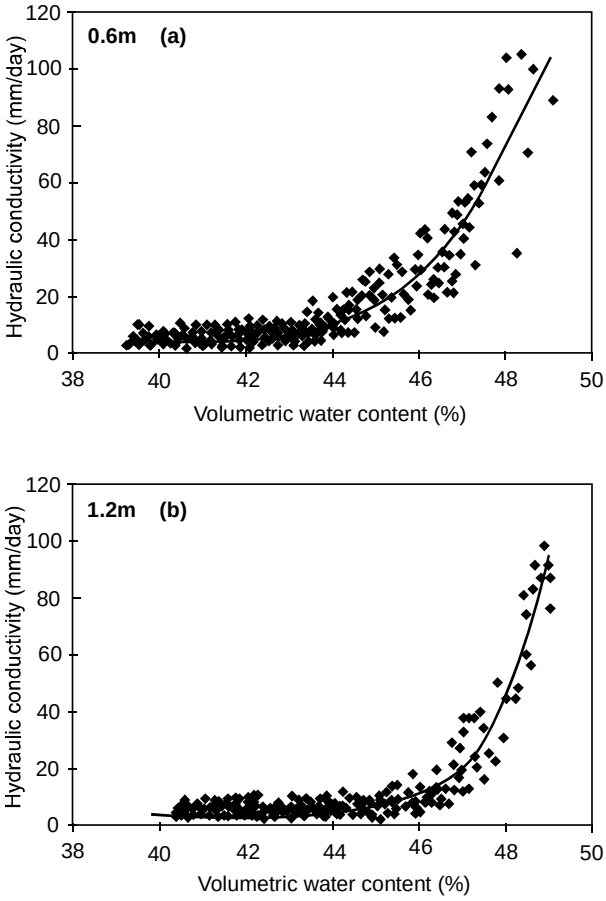

Fig. 4. Unsaturated hydraulic conductivity as a function of water content at (a) 0.6 m and (b) 1.2 m depth.

As additional pores between fragments of chalk become water filled, so the cross-sectional area available for flow increases as does hydraulic conductivity (Hodnett and Bell, 1990). For example, at 1.2 m depth, increasing the cross sectional conducting area by 0.02 and 0.03 m^3/m^3 (i.e., increasing water content from 0.46 to 0.48 to 0.49 m^3/m^3) would increase hydraulic conductivity from 8 mm/d to 57 and 110 mm/d thereby increasing water flow from 17.4 mm/d to 118.7 and 224.5 mm/d, respectively. This 7 and 13 fold increase is a direct consequence of water filling the pores between chalk fragments. The additional flux resulting from the additional wetted area as water content increased would raise the mean flow velocity to 2.45 m/d at water content of 0.48 and to 5.3 m/d at 0.49 m^3/m^3. A similar calculation for 0.6 m depth shows that the flow rate was also similar (5.0 m/d) as water content increased from 0.47 to 0.48 m^3/m^3. These calculated flow rates

are in accordance with the very short measured breakthrough times observed and suggest that during rain storms, most of the flux could pass through this part of the chalk profile very rapidly.

Effect of overlying soil on water flow. The time taken for water to travel through fractured chalk (0.2 to 1.2 m depth) was almost the same with and without the overlying soil but the soil layer took about 2 h to pass through (Table 2). It was also found in other experiments (unpublished) that water took slightly more time to move through the top layer, 0-0.2 m, than through underlying layers of the same thickness. At low rain intensity, 3 mm/h, the initial breakthrough time for the soil layer was 2 h, whereas, the time for all succeeding layers was 1.5 h or less. At higher intensities, the time was 1 h for the top 0.2 m layer and 0.5 h or less for all deeper layers. The longer time taken to pass through the top 0.2 m may have been due to the surface conditions. In the case of overlying soil, there was a layer of moss of about 2 cm thickness on the soil surface under the grass. This may have reduced the infiltration rate into the soil and hence delayed the initial breakthrough time. Another possible factor that can reduce the flux is that at the start of rain, loose soil material may move down with water and plug the macropores, thereby increasing the breakthrough time. The longer breakthrough time through the top layer of the chalk even without the overlying soil might be due to the trampling of the surface during the removal of overlying soil in addition to the downward movement of soil particles. It is clear that once water had passed through the overlying soil, it moved quickly through the underlying chalk.

During winter, there were small changes in water content of the chalk profile (Mahmood-ul-Hassan and Gregory, 2002). Table 3 shows that the effect of water content at the start of irrigation on the measured first breakthrough time was small. In the upper 0.4 m, water content had a negligible effect on breakthrough time but below 0.6 m, the time was reduced by 1 to 1.5 h as water content increased.

Field measurement of preferential flow. Table 4 shows the rate of percolation at different depths as affected by the rate of irrigation. The rate was calculated by dividing the volume of water collected by the percolation collectors by the time in which it was collected. About 90% of the total percolate was collected within 7 h when water was applied at 15 mm/h and within 6 h when applied at 30 mm/h. Percolation started after about 1.5, 2.0. 2.5, 3.0 and 3.5 h at 0.4, 0.6, 0.8, 1.0 and 1.2 m depth when water was applied at 30 mm/h. The quantity of percolate collected suggests that at high rate of application, a significant amount of the surface applied water moved down through the inter-aggregate pores. At 30 mm/h rate, about half

Table 2. Effect of the overlying soil on the measured first breakthrough time at different depths with an irrigation applied at 3 mm/h

Depth (m)	First breakthrough time (h)	
	with soil	without soil
0.2	2.0	-
0.4	3.0	1.5
0.6	4.5	2.5
0.8	6.0	4.0
1.0	7.5	5.5
1.2	8.5	6.0

Table 3. Effect of the initial water content on the measured first breakthrough time (MFBT) at different depths with 3 mm/h application

Depth (m)	Initial water (%)	MFBT (h)	Initial water (%)	MFBT (h)
0.2	37.70	2.0	41.31	1.5
0.4	44.20	3.0	46.19	3.0
0.6	41.28	4.5	43.62	4.0
0.8	43.40	6.0	45.16	5.0
1.0	44.62	7.5	46.75	6.0
1.2	43.10	8.5	46.10	7.0

Table 4. The rate of percolation into troughs at different depths at different rates of water application

Depth (cm)	Rain intensity (mm/h)					
	3		15		30	
	Percolation rate (mm/h)	Percolate (% of applied)	Percolation rate (mm/h)	Percolate (% of applied)	Percolation rate (mm/h)	Percolate (% of applied)
40	-	2.85	1.7	21.7	9.2	47.3
60	-	1.75	0.7	9.6	4.0	20.6
80	-	-	0.3	4.1	1.8	9.2
100	-	-	0.2	2.9	1.6	8.3
120	-	-	0.2	2.7	1.7	8.5

of the applied water moved down freely to 0.4 m depth, one fifth to 0.6 m and one tenth to 1.2 m depth at rates of 9.2, 4.0 and 1.6 mm/h, respectively. With 15 mm/h rain, the percentage of applied water that percolated to 0.8 m was about half of that at 30 mm/h and the rate was about 6 times lower. At 3 mm/h, only a small fraction, 1.7%, of the applied water drained into the trough at 0.6 m. The increase in the quantity of percolated water was not proportional to the increase in irrigation intensity from 3 to 15mm/h. However, a proportional increase (2 fold) in percolation to a depth of 0.8 m occurred as rain intensity increased from 15 to 30 mm/h.

Rainfall intensity or water application rate affects water movement through a profile having macropores (Radulovich *et al*., 1992). Beven and Germann (1982) stated that when the same volume of water is applied at higher intensity, it may run deeper into the profile through the macropores. Results obtained from a column experiment by Edwards *et al*. (1992) support the above statement. In this irrigation experiment, no visible ponding on the soil surface was observed, even with an application rate of 30 mm/h. The water was applied in pulses, each of 5 min duration with 10 min between pulses so that for a mean application rate of 30 mm/h, the actual intensity during the period of application was three times greater. It is widely thought that the soil must be close to or fully saturated, with water ponded or nearly ponded on its surface, before preferential flow can occur (Seyfried and Rao, 1987; Watson and Luxmoore, 1986). Nevertheless, it is also well established that well-structured materials like the over-lying soil and the underlying fractured chalk at the experimental site have dual porosity. In a dual porosity medium, the intra-aggregate porosity has low infiltrability (in case of chalk it is usually 5-8 mm/d) while the inter-aggregate porosity has high infiltrability (14 m/d). In a well-structured soil, inter-aggregate pores are abundant and macropores, such as earthworm burrows and dead root channels may also be present. These pores and channels were especially abundant in the soil under study because it was under long-term grass that had not been tilled for a long period. The pulsed application of water used in these experiments exceeded, by a wide margin, the near saturated hydraulic conductivity of the soil and the chalk matrix. For example, the hydraulic conductivity of the overlying soil and fractured chalk measured *in situ* decreased from ≈110 mm/d near saturation to about <8 mm/d at a matric potential ≈-50 cm water (Mahmood-ul-Hassan and Gregory, 2002). Once the hydraulic conductivity of the soil aggregate/chalk fragments is exceeded, the surface of those aggregates/ fragments becomes saturated and water flows around them and moves downward through the inter-aggregate pore network. Once the inter-aggregate pore network has started to conduct water, a very small increase in water content can then result in very large increase in hydraulic conductivity. Thus, the overlying soil and underlying fractured chalk need not be saturated for water to flow rapidly through the profile.

Freely draining water collected by the troughs at application rates of 15 and 30 mm/h confirms the preferential flow phenomenon in the fractured chalk profile. However, insertion of the trough may have disturbed the soil water pressure profile (boundary conditions) and therefore soil water movement. For water to drop into the trough, the boundary must be saturated and, therefore, matric potential may be different to that in undisturbed chalk. Physical disruption and compaction (due to insertion) may also affect flow in the preferential flow path. For example, local compaction may divert the preferential movement laterally rather than into the trough. Total avoidance of the disturbance of flow *in situ* is very difficult if not impossible. So the impact of the presence of the troughs and particularly the effect on boundary conditions requires further investigation.

Extensive inter-aggregate, non-capillary porosity is demonstrated by the large and rapid decrease in water content (from ≈0.48 to 0.38 m^3/m^3 at 0.2 m, ≈0.5 to 0.46 m^3/m^3 at 0.6 m and ≈0.50 to 0.46 m^3/m^3 at 1.2 m (Fig. 3) and in hydraulic conductivity as matric potential decreased from 0 to -10 kPa (Mahmood-ul-Hassan and Gregory, 2002). The application rates of water used in the experiments exceeded the matrix saturated hydraulic conductivity of the soil overlying the cryoturbated chalk. In another study on the same site, Mahmood-ul-Hassan and Gregory (2002) observed that most of the cryoturbated chalk matrix remained close to saturation during winter and hence had only a small additional ability to store water. A small increase in water content (1-3%, in excess of the usual matrix water content) results in an exponential increase in hydraulic conductivity (Mahmood-ul-Hassan and Gregory, 2002; Radulovich *et al*., 1992; Hodnett and Bell, 1990). This implies that any application of water that exceeds the capacity of the matrix to conduct water would have to pass through the preferred pathways.

The measurements show that preferential flow in macro-pores occurred even at the lowest rate of 3 mm/h but that this was for an effective duration of 8 h and a total application of 24 mm. While events of this intensity and duration are not very common, they do occur in southern England especially in winter and summer storms. For example, in the two winters studied by Mahmood-ul-Hassan (1998), rainfall intensity exceeded 3 mm/h on several occasions and on at least two occasions in each season, the total rainfall was >24 mm/6 h. In winter the matrix remains close to saturation for prolonged periods so

that whenever rain intensity exceeds the saturated hydraulic conductivity of the chalk matrix (0.4 mm/h) early breakthrough and preferential flow is likely.

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References

Arnon, S., Ronen, Z, Adar, E., Yakirevich, A., Nativ, R. 2005. Two-dimensional distribution of microbial activity and flow patterns within naturally fractured chalk. *J. Contaminated Hyydrol*. **79:** 165-186.

Beven, K., Germann, P. 1982. Macropores and water flow in soils. *Water Resour. Res*. **18:** 1311-1325.

Burnham, C.P. 1990. Chalk and chalk debris as a medium for plant growth, with particular reference to Channel Tunnel spoil. *Soil Use Manag*. **6:** 131-136.

Cooper, J.D., Gardner, C.M.K., Mackenzie, N. 1990. Soil controls on recharge to aquifers. *Eur. J. Soil Sci*. **41:** 613-630.

Edwards, W.M., Shipitalo, W.A., Owens, L.B. 1992. Rainfall intensity affects transport of water and chemicals through macropores in no-till soil. *Soil Sci. Soc. Am. J*. **56:** 52-58.

Gardner, C.M.K., Cooper, J.D., Wellings, S.R., Bell, J.P., Hodnett, M.G., Boyle, S.A., Howard, M.J. 1990. Hydrology of the unsaturated zone of the chalk of southeast England. In: *Proc. Int. Chalk Symposium*, J. B. Burland, B. O. Corbett, D. C. Jones, R. N. Motimore and T. S. Roberts (eds.), pp. 611-618, Thomas Telford, London, UK.

Geake, A.K., Foster, S.D. 1989. Sequential isotope and solute profiling in the unsaturated zone of British Chalk. *Hydrol. Sci. J*. **34:** 79-95.

Hillel, D., Krentos, V.D., Stylliannou, Y. 1972. Procedure and test of an internal drainage method for measuring soil hydraulic characteristics in situ. *Soil Sci*. **114:** 395-400.

Hodnett, M.G., Bell, J.P. 1990. Processes of water movement through a chalk coombe deposit in southeast England. *Hydrol. Process*. **4:** 361-372.

James, L.G. 1988. *Principles of Farm Irrigation System Design*, Washington State University, John Wiley and Sons Inc., USA.

Mahmood-ul-Hassan, M., Gregory, P.J. 2002. Dynamics of water movement on chalkland. *J. Hydrol*. **257:** 27-41.

Mahmood-ul-Hassan, M. 1998. Water Movement Through the Unsaturated Zone. *Ph.D. Thesis,* 156 p. The University of Reading, UK.

Moffat, A.J. 1985. *Soil of Bridget Experimental Husbandry Farm, Martyr Worthy, Winchester*, Soil Survey of England and Wales, Rothamsted Experimental Station, Harpenden, Herts, UK.

Nativ, R., Adar, E., Assaf, L., Nygaard, E. 2003. Characterization of the hydraulic properties of fractures in chalk. *Ground Water* **41:** 532-543.

Price, M., Bird, M.J., Foster, S.S.D. 1976. Chalk pore-size measurements and their significance. *Water Serv.* **80:** 596-600.

Price, M. 1987. Fluid flow in the Chalk of England. In: *Fluid Flow in Sedimentary Basins and Aquifers*, J. C. Goff and B. P. J. Williams (eds.), Geological Society Special Publication No. 34, pp. 141-156, Blackwell Sci. Publ., Oxford, UK.

Radulovich, R., Baveye, P., Sollin, P., Solorzano, E. 1992. Bypass water flow through unsaturated microaggregated tropical soils. *Soil Sci. Soc. Am. J*. **56:** 721-726.

Reeves, M.J. 1979. Recharge and pollution of the English Chalk: some possible mechanisms. *Eng. Geol.* **14:** 231-240.

Seyfried, M.S., Rao, P.S.C. 1987. Solute transport in undisturbed column of an aggregated tropical soil: preferential flow effects. *Soil Sci. Soc. Am. J*. **51:** 1434-1444.

Watson, K.W., Luxmoore, R.J. 1986. Estimating macroporosity in a forest watershed by use of a tension infiltrometer. *Soil Sci. Soc. Am. J*. **50:** 578-582.

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Studies on Amylase Activity of Pancreatin Obtained from Bovine Pancreas

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Abstract. The main objective of this study was to prepare pancreatin in liquid and powder form and to determine its amylase activity in crude homogenate of animal tissue. Different conditions were optimized for estimation of maximal activity including pH, temperature and substrate concentration. The optimum pH was found to be 6.8. The enzyme was optimally active at 50 °C. The effect of substrate concentration on enzyme activity was also studied and Km was found to be 0.5%.

Keywords: pancreatin, bovine pancreas, amylase activity

Introduction

Pancreatic α-amylase is an important enzyme needed for starch hydrolysis in the small intestine of both nonruminants and ruminants. Alpha amylase, an endoenzyme, hydrolyzes α-1,4-glucosidic bonds in polyglucosans. The location of the bonds in the molecule to be hydrolyzed is selected at random; each cleavage by α-amylase produces a reducing end (Walker and Harmon, 1996).

The enzymes of amylase family have great significance due to the wide area of their potential application. The spectrum of amylase application has widened in many other fields, such as clinical, medicinal and analytical chemistry. Interestingly, the first enzyme produced industrially in 1894 was an amylase from fungal source, which was used as pharmaceutical aid for treatment of digestive disorders (Pandey *et al*., 2000; Crueger and Crueger, 1989). Amylases constitute a class of industrial enzymes covering approximately 25% of the enzyme market (Sidhu *et al*., 1997). Amylase has also significant role in baking industry, brewing industry, papermaking industry etc. (Wojciechowsski *et al.*, 2001; Dauter *et al*., 1991; Fogarty and Kelly, 1980).

Pancreatin contains pancreatic enzymes that function in the digestion of starch, proteins and fats. It principally contains amylase, lipase and protease obtained from bovine pancreas. The main objective of this study was to prepare pancreatin in liquid and powder form. The extract was prepared from certified disease-free bovine pancreas without the addition of other animal enzymes or fermentation enzyme products. This preparation could be of value in the event of faulty digestion due to deficiency of pancreatic secretion and can be used in different formulations of medicinal products in pharmaceutical industry.

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The current study also deals with determination of amylase activity in crude homogenate of animal tissue and optimization of different conditions for estimation of maximal activity including pH, temperature and substrate concentration.

Materials and Methods

Extraction. Fresh bovine pancreas was purchased from local market. It was washed, cut into small pieces, kneaded thoroughly with water and the liquid was strained. Eighty grams of pancreas was placed in a flask of about 500 ml capacity and 200 ml of phosphate buffer (0.2 N, pH 6.8) was added. The mixture was kept for two h with mixing by rotating at frequent intervals and then was homogenized in 200 ml of the same buffer. After filtration of tissue homogenate, it was centrifuged at 6000 rpm in order to remove remaining residue. Extraction was repeated with another 50 ml phosphate buffer. This filtrate was prepared as enzyme source and amylase activity was determined by dinitrosalicylic acid reagent.

Drying techniques. Various drying techniques have been employed to concentrate extract from bovine pancreas like vacuum oven drying, freeze-drying. It was found that vacuum oven drying is time consuming than the freeze-drying method which is suitable as regards stability of the extract.

Enzyme assay. Amylase activity was assayed by the Bernfield method with slight modification. Assay was performed using starch as substrate. The reaction mixture, containing 1.5 ml substrate, 1 ml acetate buffer (pH 6.8), was preincubated at 37 °C for three min. Enzyme (1:1000 diluted) was added and then the tubes were incubated at 40 °C for 5 min. The reaction was stopped by addition of 0.5 ml NaOH. After addition of 1 ml dinitrosalicylic acid, the tubes were placed in boiling water bath for 5 min. After cooling to room temperature, 9 ml of distilled water was added and the absorbance was recorded

at 540 nm. The level of amylase activity was determined by measuring the reducing sugar released from soluble starch (Bernfield, 1951).

Amylase activity unit was expressed as the micromoles of maltose released per min under specified conditions of assay. Optimal conditions for the enzyme activity were studied at temperature, 20-70 °C, substrate concentration, 0.1% - 1.5% and pH, 4.0 - 8.5.

Results and Discussion

The amylase enzyme activity was studied in the crude extract of bovine pancreas and was found to be 9575 U/mg using modified method of Bernfield. Enzyme activity was found to be directly related to the substrate concentration (Fig. 1). There was gradual rise of activity with the increase in substrate concentration, up to 1% with no further increase in the activity. According to Fig. 2, Km was found to be 0.5%. A straight line is obtained when reciprocals of initial velocity are plotted against reciprocals of substrate concentration.

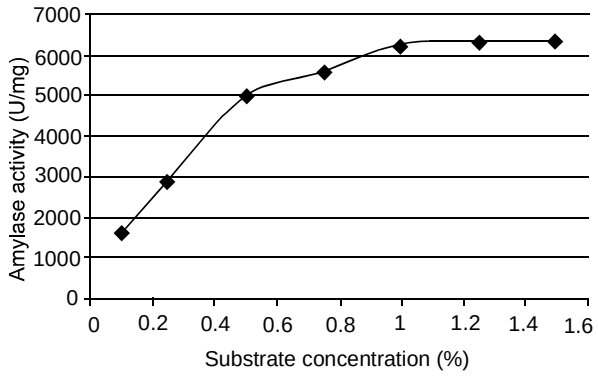

Fig. 1. Effect of substrate concentration on amylase activity of bovine pancreatin.

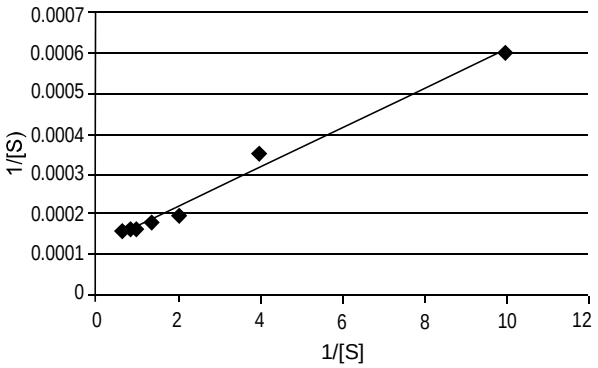

Fig. 2. Lineweaver -Burk plot showing Km value.

The effect of temperature on amylase activity was determined at different temperatures ranging from 20 to 70 °C. Enzyme activity gradually increased with increase in temperature. The maximum activity was achieved at 50 °C which may correspond to the native confirmation, after which there is sharp decline in activity with increase in temperature (Fig. 3). Comparable study showed that as the temperature is increased beyond the optimum temperature, the vibration energy of entire amylase molecule also increases. This puts a strain on the weak interactions that hold the enzyme together and change the native confirmation of enzyme so the activity lessens (Borgstrom *et al*., 1993). Another study explained the phenomenon of denaturation concluding that at higher temperature all hydrophobic and hydrophilic bonds are broken and the three dimensional structure of protein destabilizes (Yamasaki, 2003).

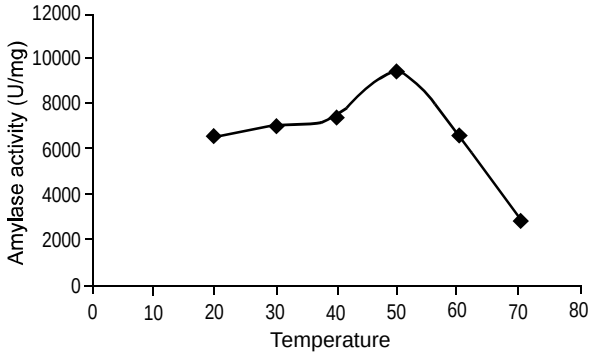

Fig. 3. Effect of temperature on amylase activity of bovine pancreatin.

Kinetic studies of amylase showed that enzyme is highly active in pH range of 6-7. The optimum pH was found to be 6.8 (Fig. 4). Suitable hydrogen concentration is attributed to the formation of enzyme substrate complex. The activity of enzyme falls on either side of the hydrogen ion concentration. The evidence shows that the range of pH of amylase varies from source to source (Teles *et al*., 2004).

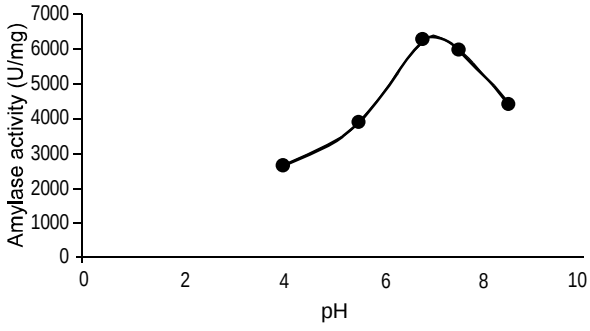

Fig. 4. Effect of pH on amylase activity of bovine pancreatin.

Extracted pancreatins have wide applications in foods, biotechnology and pharmaceutical industries. Use of the enzyme reported in this study may have several advantages over the existing state of the art, the most important being that it does not require such cost intensive filtrations to obtain microbe-free preparations, thereby making it economically feasible. Although bacterial and fungal amylases have already been used as detergent additives, their main drawback is that they require cost-intensive techniques to obtain microbe-free preparations (Phadtare *et al*., 1993). Moreover bovine pancreatin can be easily available for commercial use.

References

Bernfield, P. 1951. Enzymes of starch degradation and synthesis. In: *Advances in Enzymology*, F. F. Nord (ed.), vol. **12,** pp. 379-428, Inter Science Publ. Inc., New York, USA.

Borgstrom, A., Erlanson - Albertsson, C., Borgstrom, B. 1993. Human pancreatic proenzymes are activated at different rates *in vitro*. *Scand. J. Gastroenterol*. **28:** 455-459.

Crueger, W., Crueger, A. 1989. *Biotechnlogy*: *A Textbook of Industrial Microbiology*, Sinauer Associates Inc., Sunderland, Miami, USA.

Dauter, Z., Dauter, M., Brzozowski, A.M., Christensen, S., Brochert, T.V., Beier, L., Wilson, K.S., Davies, G.J. 1991. X-ray structure of Novamyl, the five-domain "maltogenic" alpha-amylase from *Bacillus stearothermophilus*: maltose and acarbose complexes at 1.7 Å resolution. *Biochemistry* **38:** 8385-8392.

Fogarty, W.M., Kelly, C.T. 1980. Amylases, amyloglycosidases and related gluconases. In: *Economic Microbiology, Microbial Enzymes and Bioconversions*, A. H. Rose, (ed.), vol. **5,** pp. 115-170, Academic Press, London, UK.

Phadtare, S.U., Desphpande, V.V., Srinvasan, M.C. 1993. High activity alkaline protease for *Conidiobolus coronatus* (NCL 86.8.20): enzyme production and compatibility with commercial detergents. *Enzyme Microbial Technol*. **15:** 72-76.

Pandey, A., Nigam, P., Soccol, C.R., Soccol, V.T., Singh, D., Mohan, R. 2000. Review: advances in microbial amylases. *Biotechnol. Appl. Biochem.* **31:** 135-152.

Sidhu, G.S., Sharma, P., Chakrabarti, T., Gupta, J.K. 1997. Strain improvement for the production of a thermostable alpha-amylase. *Enzyme Microbial. Technol.* **21:** 525-530.

Teles, R.C., deSouza, E.M., Calderon, A., De Fretas, S.M. 2004. Purification and pH stability characterization of a chymotrypsin inhibitor from *Schizolobium parahyba* seeds. *Phytochemistry* **65:** 793-799.

Walker, J.A., Harmon D.L.1996. Technical Note: A simple, rapid assay for α-amylase in bovine pancreatic juice. *J. Anim. Sci.* **74:** 658-662.

Wojciechowsski, P., Koziol, M., Noworyta, A. 2001. Alteration model of starch hydrolysis by amylolytic enzymes. *Biotechnnol. Bioeng*. **75:** 530-539.

Yamasaki, Y. 2003. Beta-amylase in germinating millet seeds. *Phytochemistry* **64:** 935-939.

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Metabolic Inhibitors as Stimulating Factors for Citric Acid Production

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Abstract. The effect of some metabolic inhibitors on citric acid (CA) production by *Aspergillus niger* in cane molasses medium was investigated. Addition of 0.01-0.1 mM iodoacetic acid and sodium arsenate, 0.05-1.0 mM sodium malonate, 0.01 mM sodium azide, 0.01-0.05 mM sodium fluoride, 0.1-1.0 mM EDTA stimulated CA production (5-49%). Higher concentrations (10 mM) of iodoacetic acid, sodium malonate and 0.5 mM sodium azide caused a complete inhibition of fungal growth. Iodoacetic acid, sodium arsenate and sodium fluoride (0.2 mM) caused a remarkable inhibition of CA production. The implications of those preliminary functions was discussed.

Keywords: citric acid, cane molasses, surface culture, metabolic inhibitor, *Aspergillus niger*

Introduction

Many strains of *Aspergillus niger* are well known for their capacity to produce citric acid under suitable conditions. By careful selection of strains and improving conditions, 80 to 85% of initial sugar substrate can be converted into citric acid (Kiel *et al*., 1981). Citric acid (CA) is a chemical commodity, widely used in different industries. Inexpensive and readily available raw materials remain in demand in industrial processes. Molasses is a desirable raw material for citric acid fermentation due to its availability and relatively low price (Lotfy *et al*., 2007; Haq *et al*., 2001; Pazouki *et al*., 2000).

Many investigators have tried to improve the production of CA by various additives. Moyer (1953) found that methanol, ethanol and isopropanol decreased growth but increased CA production from cane and beet molasses. Since that time, a lot of work had been done to study the effect of alcohol on citric acid fermentation (Lotfy *et al*., 2007; Roukas, 1999; El-Batal *et al*., 1995; Maddox *et al*., 1986; Szczodark and Ilczuk, 1975; Hamissa, 1966).

Millis *et al*. (1963) increased CA yield by about 20-50% when some natural oils with a high content of unsaturated fatty acids were added. Also, supplementation of surface culture with some oil increases CA yield, using molasses medium (Adham, 2002). Moreover, Barrington and Kim (2008) showed statistically that olive oil has significant positive effect on citric acid production.

Specific inhibitors such as fluoroacetate and iodoacetate, are particularly useful (Peters, 1957; Racher and Krimsky, 1952). Addition of some metabolic inhibitor to synthetic medium stimulated citric acid production (Agrawal *et al*., 1983). Ali and Haq (2005) discussed the role of different additives and metabolic microminerals on the enhancement of citric acid production by *A. niger* using different carbohydrate materials. They found that both ethanol and coconut oil in 3.0% (v/w) concentration increased citric acid production. Fluoracetate at a concentration of 1.0 mg/ml bagass increased the yield of citric acid significantly. Also, addition of copper sulphate and molybdenum sulphate remarkably enhanced the production of citric acid using molasses medium.

The present study was undertaken mainly to determine the effect of some metabolic inhibitors on citric acid production by *A. niger* in cane molasses medium.

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Materials and Methods

Microorganisms and culture conditions. Strains of *A. niger* Van Tieghem 595, 599 were provided by the Centre of Culture Collection of Northern Regional Research Laboratory (NRRL), USA. *A. niger* A10 and A20 were provided by the Center of Culture Collection National Research Center (NRC), Egypt. *A. niger* EMCC III, EMCC103 and EMCC 147 were obtained from the Cairo Mercen (CAIM), Egypt.

The slants of *A. niger* were incubated in potato dextrose agar (PDA) at 30 °C for 7 days. Inoculum was prepared from spore suspension (10^5-10^6 spore/ml) in 0.01% v/v tween 80.

Cane molasses. The cane molasses samples used in the present study were kindly supplied by the cane sugar factory of Egypt.

Citric acid fermentation. Fermentation media were prepared by diluting cane molasses (CM) with tap water to approximately 15% sugar concentration. Preparation of molasses was undertaken according to Mohamed and Adham (2003). Stationary cultures were grown on cane molasses media containing different concentrations of the inhibitors and incubated in slanting position (surface fermentation) at 30 °C

for 15 days. Aliquots of the fermentation medium were withdrawn on 4th, 8th and 12th day then analyzed for the total titratable acidity and citric acid content. Maximum citric acid content was reached on the 12th day of incubation.

Analytical techniques. Citric acid was photometerically determined at 420 nm as described by Lowenstein (1969). Citric acid content was calculated as mg/ml sample with reference to the standard solution. Total titratable acidity was estimated by titrating 1 ml aliquots of the fermentation media against 0.1 M of NaOH solution and calculated as anhydrous citric acid; 1 ml of 0.1 M NaOH is equivalent to 6.4 mg anhydrous citric acid.

Growth was measured in terms of grams of dry weight of mycelium per flask. At the end of the incubation period, on the 15th day, mycelial pads were separated, washed and dried at 60 °C for 24 h.

Results and Discussion

Seven different strains of *A. niger* were screened for citric acid production (*A. niger* NRRL 599 and 595; *A. niger* EMCCIII, EMCC102 and EMCC147; *A. niger* A10 and 20) using cane molasses. *A. niger* NRRL 599 exhibited the highest production capacity and was selected for this study to get a preliminary idea of the metabolic reactions involved in the accumulation of citric acid in cane molasses medium.

It should be reported that addition of 0.2 mM iodoacetate inhibited fungal growth, total titrable acidity and citric acid production, while 1.0 mM concentration strongly inhibited citric acid production and total titrable acidity by 85 and 67%, respectively (Table 1). On the other hand, presence of 10 mM iodoacetic acid completely inhibited fungal growth. Lower concentrations (0.01 and 0.1 mM), however, stimulated citric acid production by 34 and 41%, respectively, without markedly affecting fungal growth and titratable acids.

Agrawal *et al*. (1983) demonstrated that addition of 0.001 to 0.1 mM iodoacetate to stationary cultures of *A. niger* grown on a synthetic medium stimulated citric acid production but not total titratable acids and noted that the reason for the enhancement of citric acid production at lower concentration of iodoacetate is not clear. Iodoacetate has been reported to be a rather specific inhibitor of glyceraldehyde-3-phosphate dehydrogenase, especially at concentration ≤ 1 mM. At higher concentrations, other enzymes with sulphydryl groups at the active sites are also affected (Webb, 1966). Hence, it is likely that the interruption of the glycolytic cycle due to iodoacetate inhibition might be responsible for inhibition of fungal growth and consequently, of citiric acid production (Agrawal *et al*., 1983).

Addition of sodium malonate (Table 2) to the fermentation medium inhibited the mycelial growth which in turn was completely inhibited at 10 mM concentration and stimulated citric acid production (up to 27%) without affecting the total titratable acidity. Berk *et al*. (1957) demonstrated that *A. niger* possessed the ability to metabolize malonate. It is thus possible that at low concentrations, malonate is completely metabolized during the early period of fungal growth without adversely affecting citric acid production. At these levels precisely, malonate has been shown to inhibit succinate dehydrogenase, specifically in case of *A. niger* (Tissieres, 1951). Because of this interruption in the tricarboxylic acid cycle, further metabolism of the produced citric acid is probably reduced, thereby leading to an increase in citric acid accumulation in the medium (Agrawal *et al*., 1983). Barron and Ghiretti (1953) reported 73% inhibition of citric acid accumulation by yeast upon addition of high concentration of malonate. This could be due to depression of succinate oxidation and reduction in the rate of acetyl CoA entry into the tricarboxylic acid cycle (Webb, 1966).

Table 1. Effect of iodoacetic acid on mycelial growth and citric acid production

Inhibitor concentration (mM)	Maximum total titratable acids (mg /ml)	Maximum citric acid content (mg/ml)	Change in citric acid content (%)	Dry weight after 15 day growth (g)
0.00*	117.3	45.24	0	3.63
0.01	120.96	60.50	+33.73	3.23
0.10	119.04	63.80	+41.02	3.76
0.20	109.90	39.00	-13.80	2.90
0.50	80.12	15.90	-64.85	2.50
1.00	38.40	7.00	-84.52	2.32
10.0	0.0	0.0	-100.0	no growth

* = control; change in citric acid (CA) content (%) =

$$\frac{\text{CA in cotrol (mg)} - \text{CA in treated sample (mg)}}{\text{CA in control (mg)}} \times 100$$

Table 2. Effect of sodium malonate on mycelial growth and citric acid production

Inhibitor concentration (mM)	Maximum total titratable acids (mg /ml)	Maximum citric acid content (mg/ml)	Change in citric acid content (%)	Dry weight after 15 day growth (g)
0.00*	117.30	45.24	0.00	3.63
0.05	102.99	53.58	+18.43	3.37
0.10	116.73	57.67	+27.47	3.20
1.00	117.00	47.70	+5.43	3.05
10.0	0.00	0.00	-100.0	no growth

* = control

The presence of 0.5 mM sodium azide (Table 3) in the molasses medium completely inhibited fungal growth. On the other hand, 0.1 mM, slightly inhibited citric acid production but inhibited total titratable acidity significantly. The least concentration of sodium azide (0.01 mM) slightly stimulated citric acid production (16%) and inhibited total titratable acid (11%) and dry weight (17%). It is noticeable that the citric acid secretion by *A. niger* is not always parallel to the biomass formation (Franz *et al*., 1993). Tissieres (1951) and Case and Mcllwain (1951) observed inhibitory effects of sodium azide at 1.0 M concentration. This may be attributed to the inhibitory effect of sodium azide on oxidative phosphorylation.

Table 3. Effect of sodium azide on mycelial growth and citric acid production

Inhibitor concentration (mM)	Maximum total titratable acids (mg /ml)	Maximum citric acid content (mg/ml)	Change in citric acid content (%)	Dry weight after 15 day growth (g)
0.00*	117.3	45.24	0.00	3.63
0.01	104.4	52.68	+16.41	3.01
0.10	68.43	44.77	-1.04	3.22
0.50	0.00	0.00	-100.0	no growth
1.00	0.00	0.00	-100.0	no growth

* = control

Lower levels of sodium arsenate (0.01-0.1 mM) stimulated the production of the acid up to 32%. On the other hand higher levels (0.2-1.0 mM) inhibited citric acid production, fungal growth and the total titratable acidity markedly (Table 4). This inhibition may be due to the fact that sodium arsenate is an uncoupler of substrate-linked phosphorylation– for example, during the oxidation of D-glyceraldehyde-3-phosphate and alpha-ketoglutarate– leading to decreased energy production in the cell and, hence, decreased growth (Sanadi *et al*., 1954; Crane and Lipman, 1953). Glutarate-semialdehyde dehydrogenase (EC 1.2.1.20) is more sensitive to sodium arsenate; this could explain the stimulation of citric acid accumulation in the medium at lower concentration levels of sodium arsenate (Agrawal *et al*., 1983).

Addition of sodium fluoride to the molasses significantly stimulated citric acid production at 0.01 mM (49%) but was inhibitory at concentrations ≥ 0.2 mM (16-58%) (Table 5). It should be noted that the same concentration also suppressed the total titratable acidity. However, fungal growth was stimulated up to the concentration of 0.5 mM. At the highest concentration of 10 mM, there was marked inhibition of fungal growth, total titratable acidity and citric acid production (56, 61 and 58%, respectively). Agrawal *et al*. (1983) recorded 100% fungal inhibition in the presence of 10 mM sodium fluoride. This inhibition has been proposed to be due to the fluoride ions and their ability to form complexes with several metalloenzyme system.

Table 4. Effect of sodium arsenate on mycelial growth and citric acid production

Inhibitor concentration (mM)	Maximum total titratable acids (mg /ml)	Maximum citric acid content (mg/ml)	Change in citric acid content (%)	Dry weight after 15 day growth (g)
0.00*	117.3	45.24	0.00	3.63
0.01	112.35	59.79	+32.16	3.50
0.02	128.61	58.74	+29.84	3.92
0.05	87.90	52.89	+16.90	3.79
0.10	71.40	50.58	+11.80	3.65
0.20	68.70	28.21	-37.64	2.90
0.50	66.30	18.84	-58.35	2.50
1.00	66.90	12.00	-73.47	2.15

* = control

Table 5. Effect of sodium fluoride on mycelial growth and citric acid production

Inhibitor concentration (mM)	Maximum total titratable acids (mg /ml)	Maximum citric acid content (mg/ml)	Change in citric acid content (%)	Dry weight after 15 day growth (g)
0.00*	117.3	45.24	0.00	3.63
0.01	127.2	67.38	+48.93	3.87
0.05	109.2	63.37	+40.07	4.00
0.20	76.50	30.0	-33.68	3.77
0.50	94.86	37.8	-16.44	3.80
1.00	61.20	23.85	-47.28	2.19
10.00	45.90	18.75	-58.55	1.60

* = control

Addition of (0.1, 1.0 mM) of the sodium salt of ethylene diaminetetracetic acid (EDTA) stimulated citric acid production, dry weight and total titratable acidity (Table 6). We have came to a conclusion that any increase in the fungal growth may lead to parallel increase in the total titratable acids but at higher concentration (10 mM) inspite of the increase in the fungal growth (15%) there is considerable inhibition in the production of the total titratable acids. Since more of the glycolytic and tricarboxylic acid cycle enzymes are dependent on metal ions (particularly Mg^{2+}) for their activity, it is likely that at higher concentration (10 mM) EDTA chelate certain metals ions essential for the activity of enzymes (directly or indirectly) related to the synthesis and accumulation of citric acid in the medium. Compared with other fungi, *A. niger* can be affected, to a greater extent, by the presence of metal ions in the media (Franz *et al*., 1993).

Table 6. Effect of EDTA sodium salt on mycelial growth and citric acid production

Inhibitor concentration (mM)	Maximum total titratable acids (mg /ml)	Maximum citric acid content (mg/ml)	Change in citric acid content (%)	Dry weight after 15 day growth (g)
0.00*	117.3	45.24	0.00	3.63
0.01	90.71	39.00	-13.79	3.50
0.10	133.45	48.00	+6.2	3.78
1.00	173.47	56.00	+23.78	3.75
10.0	96.77	38.00	-16.00	4.16

* = control

As discussed above, most of the studied inhibitors caused inhibition of growth at concentrations, 1 to 10 mM, with the exception of EDTA. It is likely that some of these inhibitors may affect the growth of the fungus and citric acid production indirectly. Stimulation of fungal growth using different concentration of EDTA, may be due to the removal of excess mineral impurities from molasses by EDTA yielding more suitable molasses for fungal growth and citric acid production. However, 0.2 to 0.5 mM sodium fluoride could inhibit citric acid production slightly without adversely affecting the fungal growth. Also, there appeared to be a poor correlation between the inhibitory effects on total titratable acidity and on citric acid production, total titratable acidity being more or less susceptible to the inhibition.

In addition to citric acid, oxalic acid is a major contributor to total titratable acidity. At lower levels, most of the studied inhibitors stimulated citric acid accumulation in the medium, but not the production of total titratable acidity, thus increasing the proportion of citric acid among other organic acids formed. Perhaps lower levels of these inhibitors suppress the activity of enzyme more closely associated with the synthesis of other organic acids, rather than activity of those concerned with citric acid formation and accumulation in the medium. These findings with the great viability of cane molasses could have important implications in industrial processes.

References

Adham, N.Z. 2002. Attempts at improving citric acid fermentation by *Aspergillus niger* in beet molasses medium. *Bioresour. Technol.* **84:** 11, 97-100.

Agrawal, P.K., Bhatt, C.S., Viswanathan, L. 1983. Effect of some metabolic inhibitors on citric acid production by *Aspergillus niger*. *Enzyme Microb. Technol*. **5:** 369-372.

Ali, S., Haq, I. 2005. Role of different additives and metallic microminerals on the enhanced citric acid production by *Aspergillus niger* MNNG-115 using different carbohydrate materials. *J. Basic Micobiol*. **45:** 3-11.

Barron, E., Ghiretti, F. 1953. The pathway of acetate oxidation. *Biochem. Biophys. Acta*. **12:** 239-249.

Barrington, S., Kim, J.W. 2008. Response surface optimization of medium components for citric acid production by *Aspergillus niger* NRRL 567 grown in peat moss. *Bioresour. Technol.* **99:** 368-377.

Berk, S., Ebert, H., Teitell, L. 1957. Utilization of plasticizers and related organic compounds by fungi. *Indust. Eng. Chem*. **49:** 1115-1124.

Case, E.M., Mcllwain, H. 1951. Respiration and phosphorylation in preparation from mammalian brain. *Biochem. J.* **48:** 1-11.

Crane, R.K., Lipman, F. 1953. The effect of arsenate on aerobic phosphorylation. *J. Biol. Chem*. **201:** 235-243.

El-Batal, A.I., Khalil, A.H., Mostafa, M.M. 1995. Citric acid production by gamma irradiated *Aspergillus niger* from treated beet molasses under different fermentation conditions. *Egypt. J. Rad. Sci. Appl*. **8:** 253-266.

Franz, A., Burgstaller, W., Muller, B., Schinner, F. 1993. Influence of medium components and metabolic inhibitors on citric acid production by *Penicillium simplicissimum*. *J. Gen. Microbiol*. **139:** 2101-2107.

Hamissa, F.A. 1966. Chemical Studies on Citric Acid Fermentation. *M.Sc. Thesis*, Cairo University, Egypt.

Haq, I., Khurshid, S., Ali, S., Ashraf, H., Qadeer, M.A., Rajaka, M.I. 2001. Mutation of *Aspergillus niger* strain for enhanced citric acid production by blacks-strap molasses. *World J. Microbiol. Biotechnol.* **17:** 35-37.

Kiel, H., Guvrin, R., Henis, Y. 1981. Citric acid fermentation by *Aspergillus niger* on low sugar concentrations and cotton waste. *Appl. Environ. Microbiol*. **42:** 1-4.

Lotfy, W.A., Ghanem, K.M., El-Helow, E.R. 2007. Citric acid production by a novel *Aspergillus niger* isolate: I. Mutagenesis and cost reduction studies. *Bioresour. Technol.* **98:** 3464-3469.

Lowenstein, J.M. 1969. Chemical methods for citrate and aconitate. In: *Methods in Enzymol*ogy, J.M. Lowenstein (ed.), vol. **13,** pp. 573-576, Academic Press Inc., New York, USA.

Maddox, I.S., Hossain, M., Brooks, J.D. 1986. The effect of methanol on citric acid production from galactose by *A. niger*. *Appl. Microbiol. Biotechnol*. **23:** 203-205.

Millis, N.F., Trumpy, B.H., Palmer, B.M. 1963. The effect of lipids on citric acid production by an *Aspergillus niger* mutant. *J. Gen. Microbiol*. **30:** 365-379.

Mohamed, K.A., Adham, N.Z. 2003. Formations of autobiploid *Aspergillus niger* strains by collagen calcium treatment and their application to citric acid production from beet molasses. *Egypt. J. Biomedi. Sci*. **12:** 184-198.

Moyer, A.J. 1953. Effect of alcohols on the mycological production of citric acid in surface and submerged culture. I. The nature of the alcohol effects. *Appl. Microbiol*. **1:** 1-7.

Pazouki, M., Felse, P.A., Sinha, J., Panda, T. 2000. Comparative studies on citric aicd production by *Aspergillus niger* and *Candida lipolytica* using molasses and glucose. *Bioprocess Engineering* **22:** 353-361.

Peters, R.A. 1957. Mechanism of the toxicity of the active constituent of *Dichapetalum cymosum* and related compounds. *Adv. Enzymol. Rela. Sub. Biochem*. **18:** 113-159.

Racker, E., Krimsky, I. 1952. The mechanisms of oxidation of aldehydes by glyceraldehydes-3-phosphate dehydrogenase. *J. Biol. Chem*. **198:** 731-743.

Roukas, T. 1999. Citric acid production from carob pod by solid state fermentation. *Enzyme Microb. Technol*. **24:** 54-59.

Sanadi, D.R., Gibson, D.M., Ayengar, P., Ouellet, L. 1954. Evidence for a new intermediate in the phosphorylation coupled to α-ketoglutarate oxidation. *Biochem. Biophys. Acta*. **13:** 146-148.

Szczodark, J., Ilczuk, Z. 1975. Effect of alcohol on the synthesis of citric acid by the fungus *Aspergillus niger*. *Przem Ferment. Rolny*. **19:** 20-21.

Tissieres, A. 1951. A study of the cytochrome system and some other aspects of the respiration of *Aerobacter aerogenes*. *Biochem. J*. **50:** 279-288.

Webb, J.L. 1966. *Enzyme and Metabolic Inhibitors*, vol. **2,** Academic Press, New York, USA.

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Chemical Variability of Fatty Acid Composition of Seabuckthorn Berries Oil from Different Locations by GC-FID

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Abstract. For determining the chemical composition of seabuckthorn oil of different origins, samples of seabuckthorn berries (red and yellow varieties) were collected from different locations of northern areas of Pakistan. Among eight different fatty acids, palmitoleic acid (32.4%) and palmitic acid (36.52 %) were found to be the major fatty acids present along with other important fatty acids i.e., oleic acid (37.07%), linoleic acid (12.36%) and linolenic acid (0.73%). Quantities of unsaturated fatty acids were higher than that of saturated analogues.

Keywords: seabuckthorn berry oil, fatty acid composition, pharmacology

Introduction

Seabuckthorn (*Hippophae rhamnoides*) is a thorny shrub, found widely near streams, sandy places, mountainous and coastal areas of many Asian and European countries (Risto and Baoru, 2003). Natural habitat of seabuckthorn extends widely in China, Mongolia, Russia, India, Nepal, Pakistan, Ukraine, Great Britain, France and most parts of the northern Europe (Li and Shroeder, 1999). In Pakistan it is naturally growing in the northern areas of Gilgit and Chitral.

Scientists have carried out extensive research on seabuckthorn which has resulted in an improved understanding of the health effects and chemical composition of the berry (Yang and Kallio, 2002; 2001). The fruits of seabuckthorn are rich in carotenoids, lipids, ascorbic acid, tocopherols, sterols, flavonoids and triterpenes (Zhemin, 1990; Loskutova *et al*., 1989) and are a rich source of both aqueous and lipophilic antioxidants due to the presence of vitamins D and E, as well as enzymes such as various superoxide dismutase isoenzymes (Eccleston *et al*., 2002).

Seabuckthorn is rich in oil both in seeds (seed oil) and in the fruit soft parts i.e., flesh and peel (pulp oil). The observations reveal that the seeds contain around 10% of oil, whereas the oil content in the soft parts like pulp varies over a much wider range from 0.5 to 21%, largely depending on the varieties and the origin. The biological properties of fruits and oils of *H. rhamnoides* have been widely investigated. Oil extracted from fruit has regenerating antiinflammatory and photoprotective activity with promising applications in dermatology and cosmetics (Pintea *et al*., 2001). Seabuckthorn oil is known to regulate immune functions and antagonize the effects of immune suppressants (Yang and Kallio, 2001). In clinical cancer treatment, seabuckthorn has been used to reduce the immune suppressive and hematoxic effect of chemotherapy and radiation therapy.

Seabuckthorn seed and pulp oils combined have high level of beneficial fatty acids. The fatty acid profile of the oil showed that it contained nearly 90% unsaturated fat. It is high in both linolenic and linoleic acids. The high level of unsaturated fats makes seabuckthorn oil appropriate for decreasing the risk of heart diseases (Yang, 1995). Berry oil of seabuckthorn contains 35-40% of a 16 carbon monosaturated fatty acid called palmitoleic acid also known as omega-7. Palmitoleic acid is believed to possess potent antiviral, antibacterial and healing effects in humans (Parimelazhagan *et al*., 2005).

In medicinal industry about ten varieties of seabuckthorn drugs have been developed and are available in the form of liquid, powder, plasters, pills, liniments and aerosols etc. These drugs are used for treating burns, gastric ulcer, scales, oral mucositis, rectal mucositis, cervical erosion, radiation damage, skin ulcers caused by malnutrition and other damages relating to the skin (Ge, 1992). The most important pharmacological function of seabuckthorn oil is in diminishing inflammation, disinfecting bacteria, relieving pain and promoting regeneration of tissues. The chemical and phytochemical composition of seabuckthorn varies with the origin, climate and method of extraction. (Zeb, 2004; Beveridge *et al*., 1999). Present studies were carried out to analyze the physicochemical composition and fatty acid profile of oil of seabuckthorn berries collected from different altitudes of the northern areas of Pakistan by gas chromatography techniques because data in this respect is not available in the literature.

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Materials and Methods

Extraction of oil. Red and yellow varieties of berries of *H. rhamnoides* were collected from six different locations i.e., Nomal, Bagrote, Ghizar, Skardu Khas, Shigar and Khaplu and stored at -20 °C. Seeds were separated from frozen berries by pressing the juice and the residue was dried at room temperature. The pulp portion was also dried and oil was extracted by the cold press method yielding pure, chemical and solvent free oil.

The extracted oil was analyzed for refractive index, specific gravity, saponification value, unsaponifiable matter, acid value, iodine value and peroxide value according to standard methods (AOAC, 2000). Methyl esters of fatty acids were also prepared by standard methods (AOCS, 2000). Fatty acids were saponified by treating with 0.5M methanolic NaOH solution. After acidification with 2M HCl to pH 4-5, fatty acids were extracted with chloroform and the solvent was then evaporated under reduced pressure and temperature. The extracts were applied to TLC plates. Areas of silica gel corresponding to fatty acids were extracted from the plates with chloroform and then *trans*-esterified with boron trifluoride-methanol for 30 min at 80 °C. Methyl esters of fatty acids were dissolved in *n*-hexane and analysed by GC on a Perkin Elmer gas chromatograph (8410 series) using temperature programme with FID. Fused silica capillary column (25 m x 0.32 mm id, 0.15 µm film thickness) was used. Oven temperature was held at 65 °C for 5 min, then programmed at 5 °C/min up to 220 °C. The carrier gas was nitrogen with 1.5 ml/min flow rate. Injection and detector temperature was 250 °C. Split ratio was 1:20 and injection volume was 0.5 µl. Identification of the fatty acid methyl esters was carried out from the retention times compared to standards.

Results and Discussion

Physicochemical characteristics of oil of yellow and red varieties of seabuckthorn are given in Table 1. The values are mean of three independent readings.

Refractive index and optical rotation are very stable parameters and can be used for checking the identity of oils. Refractive indices of all the samples analyzed were almost the same, with insignificant differences. Maximum specific gravity of yellow variety was observed in samples collected from Nomal (0.951) while lowest value was observed in the samples from Skardu khas (0.855). The highest specific gravity of red variety (0.913) was observed in samples from Khaplu area while the lowest value (0.846) was observed in the samples collected from Ghizar. Acid values and peroxide values of the samples were quantified which were in accordance with the reported parameters and were within the range of reported data (Morsel and Steen, 2003).

Table 1. Physicochemical characteristics of pulp oil of seabuckthorn taken from six different locations

Variety	Nomal	Bagrote	Ghizar	Skardu Khas	Shigar	Khaplu
Refractive index						
Yellow	1.465	1.457	1.543	1.472	1.472	1.526
Red	1.469	1.458	1.543	1.473	1.523	1.531
Specific gravity						
Yellow	0.951	0.868	0.921	0.855	0.859	0.923
Red	0.871	0.860	0.846	0.851	0.905	0.913
Peroxide value						
Yellow	0.88	0.26	0.91	0.23	0.54	0.16
Red	0.81	0.60	0.63	0.23	0.50	0.37
Unsaponifiable matter						
Yellow	1.82	2.26	1.96	2.82	2.44	0.6
Red	3.30	0.80	2.20	2.40	3.60	1.6
Saponification value						
Yellow	192.20	229.20	210.0	186.20	186.90	189.0
Red	188.4	205.0	198.60	202.60	191.60	149.5
Iodine value						
Yellow	70	65	69	60	60	67
Red	71	68	70	61	64	65
Acid value						
Yellow	9.3	8.6	9.6	13.4	10.5	11.2
Red	9.25	9.5	8.00	11.0	12.35	11.35

Saponification value is the index of mean molecular weight of triglycerides comprising of fats. The highest saponification value was observed in oil from Bagrote (229.20), while the lowest was observed in the oil from Skardu Khas sample (186.20). Unsaponifiable matter mainly consists of soluble vitamins, pigments (i.e., carotene and lycopene), steroids, alcohols and hydrocarbons. Oil from Shigar had maximum value of unsaponifiable matter (3.60), while that from Khaplu had minimum value (0.6). It means that the former contained the highest amount of bioactive substances as compared to other samples. Iodine value is the measure of degree of unsaturation in oil or fat. Samples from Nomal had maximum Iodine value (70,71).

Fatty acid composition of seeds and berries of *H. rhamnoides* growing in different regions of the world has been extensively studied (Pintea *et al*., 2001; Kallio *et al*., 2000; Loskutova *et al*., 1989; Stanescu *et al*., 1989). In general, fatty acid composition of the oil from berries has been reported to be rich in palmitic and palmitoleic acids as well as in oleic, linoleic and linolenic acids (Cakir, 2004; Qibikeva, 1989). Our results are in agreement

Table 2. Fatty acid composition of seabuckthorn berries oil

Fatty Acid	Short hand designation	Sample name/amount (%)						
		Skardu red	Skardu yellow	Normal red	Bagrote yellow	Bagrote red	Shigar yellow	Shigar red
Myristic acid	$C_{14:0}$	0.31	0.21	0.21	----	0.21	0.21	0.11
Palmitic acid	$C_{16:0}$	29.76	28.85	28.53	36.52	17.83	33.23	17.62
Palmitoleic acid	$C_{16:1}$	26.60	25.67	24.31	32.41	13.24	29.12	12.41
Stearic acid	$C_{18:0}$	1.44	1.64	1.44	0.51	3.02	----	3.44
Oleic acid	$C_{18:1}$	22.25	22.79	22.14	22.63	30.03	21.30	37.07
Lenoleic acid	$C_{18:2}$	8.03	9.55	7.83	5.86	10.01	7.31	12.36
Lenolenic acid	$C_{18:3}$	0.30	0.21	0.21	0.10	----	0.10	0.73
Arachidic acid	$C_{20:0}$	0.31	0.31	0.14	0.10	0.28	0.10	1.42

with the reported values. Oil samples collected from Shigar were characterized by higher content of linoleic (12.36%), oleic (37.07%) and linolenic acids (0.73%) and the lowest percentage of palmitoleic acid (12.41%) as compared to other locations (Table 2). Yang and Kallio (2002; 2001) have reported similar observations. According to them, in the oil of whole berries, the proportion of palmitoleic acid correlates negatively with the proportion of linoleic and linolenic acids. It is clear from Table 2 that Bagrote oil sample has the highest percentage of palmitoleic acid (32.41%) and the lowest percentage of linolenic (0.1%) and linoleic acids (5.86%). These essential fatty acids play an important role in the prevention of heart diseases and cancer and improve overall immune system (Chai *et al*., 1989). Because of their effect on immunity, linoleic acid may have a useful role in treating disorders relating to hyper-stimulation of immune response (such as rheumatoid arthritis, psoriasis, multiple sclerosis, system lupus etc.).

The results showed that all samples were rich in high amounts of polyunsaturated fatty acids as compared to the saturated analogues.

Conclusion

Seabuckthorn is known as unique source of high valued oils. The oil of seabuckthorn has general nourishing, revitalizing, and restorative action. Its oil is a rich source of unsaturated fatty acids, phytosterols, carotenoids and flavonoides. Oil samples of seabuckthorn berries, collected from different altitudes of Skardu and Gilgit, have shown high concentration of palmitoleic acid, which differentiates it from most other oils of plant origin. In addition the oil of whole berries contains especially high level of caroteniods. The percentage of fatty acid varies depending on the area from where samples had been collected. All oil samples were rich in polyunsaturated fatty acids. The oil of berries collected from Shigar had high percentage of omega-3 and omega-6, while that from Bagrote area was high in palmitoleic acid. The results showed that concentration of polmitoleic acid and palmitic acid of pure pulp oil was greater than that of the saturated fatty acids. Density and refractive index are very stable parameters and may be used for checking the identity of oils. Triglyceride contents ensure the quality of oils. It is believed that imbalance in these vital essential fatty acids in our diet is the major reason for high incidence of heart diseases, hypertension, diabetes, obesity, colitis, premature aging and some types of Cancer (Eccleston *et al*., 2002).

References

AOAC, 2000. *Official Methods of Analysis of AOAC International*, H. William (ed.), vol. **I,** 17th edition, Gaithersburg, Maryland, USA.

AOCS, 2000. Method Ce 1-62. In: *Official and Tentative Methods of the American Oil Chemists Society*; AOCS, Champaign, Illinoise, USA.

Beveridge, T., Li, T.S.C., Oomah, B.D., Smith, A. 1999. Seabuckthorn products: manufacture and composition. *J. Agric. Food Chem*. **47:** 3480-3488.

Cakir, A. 2004. Essential oil and fatty acid composition of the fruits of *Hippophae rhamnoides* L. (seabuckthorn) and *Myrtus communis* L. from Turkey. *Biochem. Syst. Ecol*. **32:** 809-816.

Chai, Q., Xiayan, G., Zhao, M., Wemmin, H., Giang, Y. 1989. The experimental studies on the cardiovascular pharmacology of seabuckthorn extract from *Hippophae rhamnoides* L. In: *Proc. Int. Symp. Seabuckthorn*, pp. 392-397, Xi'an, China.

Eccleston, C., Baoru, Y., Tahvonen, R., Kallio, H., Rimbach, G.H, Minhane, A.M. 2002. Effects of an antioxidant rich juice (seabuckthorn) on risk factors for coronary heart disease in humans. *J. Nut. Biochem*. **13:** 346-354.

Ge, H. 1992. GC-MS analysis on chemical composition of sterols from seabuckthorn fruit oil. *Hippolae* **5:** 7-15.

Kallio, H., Yang, B., Tahvonen, R., Hakala, M. 2000. Composition of seabuckthorn berries of various origin. In: *Proc. Int. Workshop on Seabuckthorn*, Beijing, China.

Li, T.S.C., Schroeder, W.R. 1999. *A Grower's Guide to Seabuckthorn*, 70 p. Agriculture and Agri-Food Canada Publication.

Loskutova, G.A., Baikov, V.G., Starkov, A.V., Medvedev, F.A. 1989. Lipid fatty acid composition in the fruits of *Hippophae rhamnoides. Rast. Resursi*. **25:** 97-103.

Morsel, J-T., Steen, S. 2003. Analysis and identification of seabuckthorn oil of different origin. In: *1st Congress of the ISA-International Seabuckthorn Association*, Berlin, Germany.

Parimelazhagan, T., Chaurasia, O.P., Ahmed, Z. 2005. Seabuckthorn oil with promising medicinal value. *Current Sci.* **88:** 8-9.

Pintea, A., Marpeau, A., Faye, M., Socaciu, C., Gleizes, M., 2001. Polar Iipid and fatty acid distribution in carotenolipoprotien complexes extracted from seabuckthorn fruits. *Phytochem. Anal*. **12:** 293-298.

Qibikeva, D.C. (translated by Zhang Zhemin *et al*.) 1989. Study on fatty acid components of seabuckthorn. In: *Proc. Seabuckthorn Biochemistry and Breeding*, pp. 180-182.

Risto, E., Baoru, Y. 2003. Seabuckthorn oils. Towards healthy mucous membranes. *Women's Health, AGRO Food Industry Hi-tech*., May/June 2003.

Stanescu, U., Morar, R., Neamtu, G., Morar, E. 1989. Phytochemical research on *Hippophae rhamnoides* L. (seabuckthorn) fatty acid contents in certain morphological types of seabuckthorn. *Bull. IACN-ZMV* **43:** 41-44.

Yang, C. 1995. A clinical study of reducing fat and antioxidation of dried *Hippophae* emulsion. *Hippophae* **8:** 33-35.

Yang, B., Kallio, H. 2002. Composition and physiological effects of seabuckthorn (Hippophae)lipids. *Trends Food Sci. Technol*. **13:** 160-167.

Yang, B., Kallio, H. 2001. Fatty acid composition of lipids in seabuckthorn (*Hippophae rhamnoides* L.) berries of different origins. *J. Agric. Food. Chem*. **49:** 1939-1947.

Zeb, A. 2004. Chemical and nutritional constituents of seabuckthorn juice. *Pak. J. Nutr*. **3:** 99-106.

Zhemin, Z. 1990. Advance and counter-measure on research and use of Seabuckthorn in Russia. *Hippophae* **3:** 42-46.

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Applications of Lac Dye Using Different Mordants on Leather

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Abstract. Lac dye aleuritic acid extracted from seed lac, when applied onto leather, using post-mordanting process showed good results. Among acetic acid, formic acid and alum, acetic acid at 0.1 M concentration proved to be the best mordant and gave excellent colour fastness to washing, light and rubbing and good tensile strength.

Keywords: lac dye, mordant, leather, aleuritic acid.

Introduction

Lac (*Laccifer lacca*) are small insects attached in large numbers to the plants and the trees and drain sap from the bark of host tree and secret lac resin (Kongkachuichay *et al.*, 2002a, 2002b). The resin is scrapped off and separated into shellac, wax and lac colour. Ovaries of the insect contain crimson fluid called lac dye which has two major components: laccaic acid A and B whose structures are shown in Figs. 1 and 2, respectively (Chairat *et al.*, 2008; Kamel *et al.*, 2005; Janhom *et al.*, 2004). Laccaic acid represent approximately 0.5-0.75 % by weight of stick lac (Lili *et al.*, 1999).

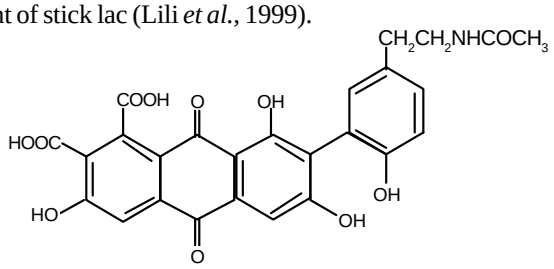

Fig. 1. Laccaic acid A

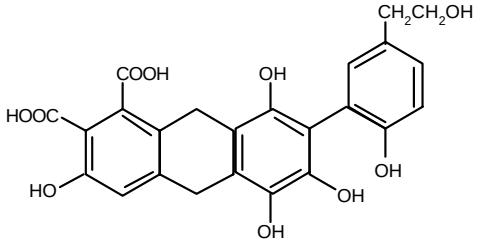

Fig. 2. Laccaic acid B

The deep interest of Pakistan leather industry in environment friendly leather processing techniques, has led to increased efforts to develop chrome-free tanning agents and to find ways to use natural dyestuffs, which previously had applications in the textile industry (Kamel *et al.*, 2007; Haroun, 2005; Kim *et al.*, 2003).

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Natural dyes have been used in textile processing since long but their application to natural leather has been extremely rare (Kumar and Sinha, 2004). Recent observations of the hazardous effects of benzidine compounds on mammals, even in very low amounts, have boosted the interest in natural dyestuffs.

Most of the natural red colour dyes have high solubility in water, therefore, the colour fastness to washing of the dyed leather is quite low (Rattanaphani *et al.*, 2007). In order to improve the colour fastness quality of the dyes, most dyeing processes are conducted using different mordants such as acetic acid, formic acid and alum. Depending on the type of dye and mordant used the colour fastness to washing is reported to be in the range of 3 to 5 for colour change, and 3-4 to 5 for staining (Waheed and Alam, 2004).

Materials and Methods

General procedure. Goat wet blue leather was used for the experiment and was dyed using the microdrum assembly with programmes to control temperature, time and speed of circulation of solution. The dyed leather samples were tested for colour fastness to washing using Launderometer, fastness to light using Xenon weatherometer, fastness to circular rubbing using rub fastness tester, for tensile strength material testing machine was used. Standard test methods for leather of the Society of Leather Technologists and Chemists (SLTC) were followed in all cases.

Leather, dyes and mordants. The leather used was collected from a local tannery of Sialkot. One kg of stick lac was ground into coarse powder. Ten litres of water were added and the solution was stirred and left standing for 24 h. After filtration, a reddish solution of lac dye was obtained. The lac dye solution was kept cold for further use. Three different types of mordants were used as follows: 0.1 M acetic acid, 0.1 M formic acid and 0.1 M alum.

Dyeing with lac dye using different mordants. Different mordants including 0.1 M acetic acid, 0.1 M formic acid and 0.1 M alum were used for lac dyeing of leather by the postmordanting method. The leather sample was first dyed with a solution of lac dye at 100 °C with a motor speed of 750 rpm per h, then the solution of mordant was added to lac dye solution in the ratio of 1:3 v/v for 30-50 min.

The concentration of the remaining dye solution and the amount of dye absorbed on the leather were calculated from the measured absorbance of dye solution at 520 nm (UV-VIS spectrophotometer, Spectronic Unicam) before and after dyeing of leather. The dyed samples were next tested for colour fastness to light, to washing and to rubbing and the tensile strength.

Dyeing by changing temperature and time. Leather (20 g) was dyed in a 2 litre solution of lac dye and 0.1 M acetic acid as previously described. The dyeing temperature was 60 °, 80 ° and 100 °C and the time was varied from 30 to 240 min. The dyed samples were then tested for colour fastness to washing, light, rubbing and the tensile strength.

Results and Discussion

Effect of mordants on dye absorption: Effects of different types of mordants on absorbance of dye by leather at different temperatures are shown in Table 1. Highest absorbance of dye was observed when acetic acid was used as mordant. The amount was approximately 15% and 30% higher than values obtained with formic acid and alum, respectively. The use of acetic acid as mordant offers an environment friendly alternative to the metal mordanted natural dyeing process (Vankar *et al.,* 2007).

Effect of mordants on physical properties. Comparison of dyeing techniques showed that post-mordanting method gave the highest depth of shade (Deo and Desai, 1999). Using this technique acetic acid, formic acid and alum were applied for the lac dyeing of leather. It was observed that type of mordants influenced the quality level and tensile strength of the dyed leather samples (Table 2). Use of acetic acid yielded the greatest tensile strength while 0.1 M alum showed the lowest overall tensile strength. Colour quality such as fastness to washing, fastness to light and fastness to rubbing of finished leather sample was excellent when acetic acid was used as mordant and poor with 0.1 M alum (Janhom *et al.,* 2006).

Table 1. Effect of mordants on the absorption of dye at different temperatures

Mordant	Absorbance		
	60 °C	80 °C	100 °C
Without mordant	1.992	1.868	1.743
0.1 M Acetic acid	0.772	0.697	0.498
0.1 M Formic acid	1.170	1.071	0.871
0.1 M Alum	1.619	1.494	1.245

Effect of dyeing conditions on quality of leather. Dyeing temperature and time are important parameters which influence the quality of dyed samples. Since acetic acid was observed to be the best mordant, the effect of temperature ranging from 60 ° to 100 °C for 30 to 200 min on dyeing with post-mordanting method using acetic acid as mordant was studied (Table 3). Almost all leather samples had excellent quality of staining on standard cloth and felt pad (level 5) except in case of rubbing, where the level decreased to 4-5. Neither dyeing temperature nor dyeing time had any effect on quality of staining; however, the dyeing time seemed to affect the colour change and fastness to light to some extent (Akalin *et al.,* 2004). Dyeing leather at 60 ° to 100 °C for longer than 80 min showed no further improvement in colour change and fastness to light, as the quality level slightly decreased after 80 minutes of dyeing.

Table 2. Effect of mordants on the quality and the tensile strength of leather at 100 °C for 60 min

Mordant	Quality level						Fastness to light	Tensile strength (MPa)
	Fastness to washing		Fastness to rubbing					
			Dry		Wet			
	Staining	Colour change	Staining	Colour change	Staining	Colour change	Colour change	
Without mordant	2 – 3	3	3 – 4	3	2	1 – 2	2 – 3	210.4
0.1M Acetic acid	5	5	5	5	5	5	4	290.5
0.1 M Formic acid	4 – 5	4	4 – 5	4	3 – 4	3	3 – 4	240.8
0.1 M Alum	4	4	4	3 – 4	3	2 – 3	3	235.4

Table 3. Effect of temperature and time of lac dye using acetic acid as mordant on the quality of leather

Dyeing temperature (°C)	Time (min)	Fastness to washing		Fastness to rubbing				Fastness to light
				Dry		Wet		
		Staining	Colour change	Staining	Colour change	Staining	Colour change	Colour change
60	30	5	4 – 5	5	4 – 5	4	4	3 – 4
	60	5	4 – 5	5	4 – 5	4	4	3 – 4
	80	5	4 – 5	5	4 – 5	4	4	3 – 4
	110	5	4	5	4	4	3 – 4	3
	150	5	4	5	4	4	3 – 4	3
	200	5	4	5	4	4	3 – 4	3
80	30	5	4 – 5	5	4 – 5	4	3 – 4	3 – 4
	60	5	4 – 5	5	4 – 5	4	3 – 4	3 – 4
	80	5	4 – 5	5	4 – 5	4	3 – 4	3 – 4
	110	5	4	5	4	4	4	3
	150	5	4	5	4	4	4	3
	200	5	4	5	4	4	4	3
100	30	4 – 5	5	5	5	4 – 5	5	4 – 5
	60	5	5	5	5	5	5	4
	80	5	5	5	5	5	5	4
	110	5	5	5	5	4 – 5	5	4
	150	5	5	5	5	4 – 5	4	4
	200	5	5	5	5	4 – 5	4	3

Effect of dyeing conditions on tensile strength. In the experiments performed to study the effect of dyeing temperature and time on tensile strength of leather using acetic acid as mordant it was found that dyeing temperature in the range of 60 °-100 °C and dyeing time of 30-90 min had little effect on tensile strength (Table 4).

Table 4. Effect of dyeing temperature and time on tensile strength of leather using acetic acid as mordant

Parameter	Tensile strength (MPa)
Time (min)	
30	275.4
40	278.2
60	290.5
70	280.6
90	279.4
Temperature (°C)	
60	265.4
70	270.3
100	290.5

Conclusion

The results indicate that acetic acid used as post mordant in dyeing with lac dye influence the properties of leather *viz.* as colour fastness to washing, fastness to light, fastness to rubbing and the tensile strength.

References

Akalin, M., Merdan N., Kocak, D., Usta, I. 2004. Effects of ultrasonic energy on the wash fastness of reactive dyes. *Ultrasonics* **42**: 161-164.

Chairat, M., Rattanaphani, S., Bremner, J. B., Rattanaphani, V. 2008. Adsorption kinetic study of lac dyeing on cotton. *Dyes Pigments* **76**: 435-439.

Deo, H.T., Desai, B.K. 1999. Dyeing of cotton and jute with tea as a natural dye. *Coloration Technol.* **115**: 224-227.

Haroun, A. A. 2005. Evaluation of modified leather dyeing technique using black dyestuffs from the economical view. *Dyes Pigments* **67**: 215-221.

Janhom, S., Watanesk R., Watanesk, S., Griffiths P., Arquero, O.A., Naksata, W. 2006. Comparative study of lac dye adsorption on cotton fibre surface modified by synthetic and natural polymer. *Dyes Pigments* **71**: 188-193.

Janhom, S., Griffiths, P., Watanesk, R., Watanesk, S. 2004. Enhancement of lac dye absorption on cotton fibres by poly (ethyleneimine). *Dyes Pigments* **63**: 231-237.

Kamel, M. M., El-Shishtawy, R. M., Youssef, B. M., Mashaly, H. 2007. Ultrasonic assisted dyeing: IV. Dyeing of cationised cotton with lac natural dye. *Dyes Pigments* **73**: 279-284.

Kamel, M. M., El-Shishtawy, R. M., Youssef, B. M., Mashaly, H. 2005. Ultrasonic assisted dyeing: III. Dyeing of wool with lac as natural dye. *Dyes Pigments* **65**: 103-110.

Kim, W. J., Heo, J. S., Shin, S. B., KO, J. Y. 2003. Measurement of colour difference in the leather dyeing process with natural dyes. *J. Soc. Leather Technol. Chem*. **87**: 25-29.

Kongkachuichay, P., Shitangkoon, A., Chinwongamorn, N. 2002a. Thermodyamics of adsorption of laccaic acid on silk. *Dyes Pigments* **53**: 179-185.

Kongkachuichay, P., Shitangkoon, A., Chinwangamorn, M. 2002b. Studies on dyeing of silk yarn with lac dye: effects of mordants and dyeing conditions. *Sci. Asia*. **28**:161-166.

Kumar, J. K., Sinha, A. K. 2004. Resurgence of natural colourants: a historic view. *Nat. Prod. Res.* **18**: 59-84.

Lili, W., Ishida, Y., Ohtani, H., Shin, T., Nakayamat, T. 1999. Characterization of natural resin shellac by reactive pyrolysis – gas chromatography in the presence of organic alkali. *Anal. Chem.* **71**: 1316-1322.

Rattanaphani, S., Chairat, M., Bremner, J. B., Rattanaphani, V. 2007. An adsorption and thermodynamic study of lac dyeing on cotton pretreated with chitosan. *Dyes Pigments* **72**: 88-96.

Vankar, P.S., Shanker, R., Verma, A. 2007. Enzymatic natural dyeing of cotton and silk fabrics without metal mordants. *J. Cleaner Production* **15**: 1441-1450.

Waheed, S., Alam, A. 2004. Studies of some natural dyes. *J. Chem. Soc. Pak.* **26**: 255-263.

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Studies on Textile Sludge Treatment Options

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Abstract. Analysis of sludge samples of a textile processing factory revealed that the BOD and COD as well as the levels of total solids, nitrogen and phosphorus contents of sludge liquor were high needing treatment before disposal or reuse. Detention time of 60 days was established for aerobic treatment of the sludge. Optimum dosage for physicochemical methods were established at 4 g/l, using alum and iron III chloride each and 15.5 g/l and 550 mg/l, for lime and polyelectrolyte each. Solids were reduced by 67%, through aerobic and 61% through anaerobic digestion, while the sludge treated by physicochemical method had higher solid content, recording the highest increase with lime.

Keywords: textile sludge treatment, pollution, aerobic sludge treatment, anaerobic sludge treatment

Introduction

Environmental pollution is causing widespread concern and has become an important area of interest in the field of modern research. The costs of pollution control are extremely high. Steward and Towse, back in 1984, estimated the annual cost in the United States alone, to be close to 5 billion dollars, in addition to occurrence of thousands of deaths and diseases due to pollution. Industries produce huge amounts of wastes. Careless disposal of these wastes to the environment without treatment threatens natural habitat and poses real dangers to humans, flora and fauna. Thus it is highly necessary to know the composition of individual wastes and develop the most economical way to treat all wastes (wastewaters and sludge), recycle the water, reclaim the waste chemicals and find possible applications of the treated wastes so as to ensure a safe environment.

The present research work is aimed at characterizing sludge of selected industries, assessing the degree of pollution caused by the sludge, developing simple and efficient treatment methods helpful in determining the efficiency of treatment of the sludge in terms of solid reduction, BOD and COD reduction, nitrification and denitrification, reuse or applicability of the treated sludge, the disposal option(s) most convenient for the unused waste and assessing the efficiency of the treatment methods adopted for particular sludge and for end-use applications.

Materials and Methods

Industrial sludges. The sludge used in this study was obtained from a textile processing factory located in Isolo, Lagos which manufactures mostly cotton fabrics. Main products of the factory are superprint, guarantee-superprint and minibrocade. The factory consists of various departments, which carry out different operations and produce different types of wastewater, containing acids used in desizing and dyeing and bases like caustic soda used in scouring and mercerization. They also contain inorganic chlorine compounds and other oxidants e.g., hypochlorite of sodium, hydrogen peroxide and peracetic acid used for bleaching and other oxidative applications. Organic compounds were also present e.g., dyestuff, optical bleachers, finishing chemicals, starch and related synthetic polymers used for sizing and thickening, surface active chemicals used as wetting and dispersing agents, and enzymes for desizing and degumming. Heavy metal salts present included e.g., copper, zinc salt and iron (III) chloride used as printing ingredients. All these wastes are passed into an effluent tank and then into a drainage system as shown in the flow chart (Fig.1).

Sampling of sludge. Composite samples of the sludge were obtained from primary sedimentation tanks of the factory

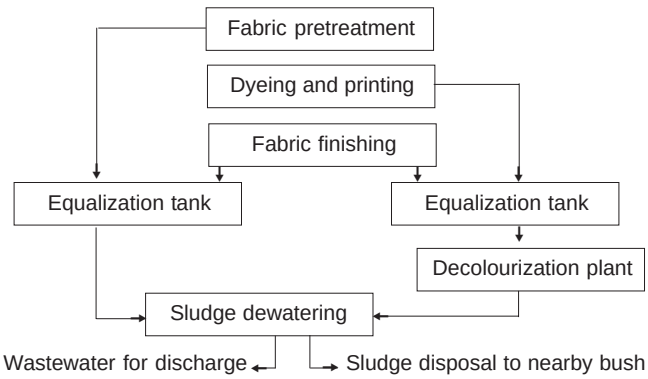

Fig. 1. Flowchart: Processing of a textile factory at Isolo Lagos, Nigeria.

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Seven plastic bowls of one litre capacity each were used to collect samples manually over 12 h sampling period, at 2 h intervals during 7. 00 a.m. to 7.00 p.m- this being the peak (optimum) period for work and sampling was the most convenient during this period.

Composite samples were collected from the factory once a week for seven weeks and analyzed. If analysis could not be carried out immediately, samples were preserved in a refrigerator maintained at 4 °C. At this temperature, biodegradation is inhibited.

Samples were collected on different week days to account for the cyclic and intermittent variations occurring at the work site.

Treatment of sludge. Sludge samples were treated employing different methods *viz.*, aerobic, anaerobic, physicochemical, combined aerobic/physicochemical and combined anaerobic/physicochemical methods. (Asia *et al.*, 2006 ; Asia and Ademoroti, 2005, 2004, 2002; Asia and Oladoja, 2003; Asia, 2000).

Methods of analysis. All samples were analyzed as described by Ademoroti (1996) and APHA (1995). When immediate analysis was not possible, the samples were preserved at 4 °C to inhibit biodegradation.

All the reagents used for the analysis were of analytical grade and obtained from BDH Chemicals Ltd., Poole, England.

Results and Discussion

Characteristics of fresh sludge are shown in Table 1 and 2. Fresh sludge was alkaline with the pH of 8.91 and had specific gravity of 1.01, showing high moisture content (97%) i.e., 3% solid concentration. The turbidity of 2460 NTU indicates large amount of colloidal matter or high solid concentration. The high turbidity may be due to colour impact of various dyes used for fabric dyeing. Also, the total, suspended and volatile solids were relatively high. The sludge contained 6500 mg/kg TS, 3158 mg/kg SS and 4150 mg/kg VS; BOD and COD were 550 and 1694 mg/l, respectively; COD:BOD ratio was 3.08, This indicates that the sludge is capable of undergoing about 50-90% substrate biodegradation. Organic matter can undergo about 50-90% substrate biodegradation if its COD**:** BOD ratio ranges between 2 and 3.5 (Quano *et al.*, 1978); thus, aerobic and anaerobic digestion of the sludge by biological methods is possible. The conductivity values of 148 S/cm indicates presence of mobile ions suggesting that physicochemical method can be as well used for treatment of the sludge.

Nitrogen present in the sludge is more in ammoniacal form (Table 1). Consequently, if such a sludge is discharged to the environment, depletion in the oxygen resources of the

Table 1. Characteristics of fresh sludge liquor of textile processing factory

Sludge liquor characteristics	Unit	Range of values	Mean
pH	-	8.10-9.85	8.91
Temperature	°C	27.0-29.5	28
Conductivity	S/cm	140-160	148
Specific gravity	-	1.0-1.02	1.01
Turbidity	NTU	2100-2650	2460
DO	mg/1	1.50-3.50	2.8
BOD	mg/1	412-669	550
COD	mg/1	954-1932	1694
Total alkalinity	mg/1	720-1050	920
Hydrogen carbonate alkalinity	mg/1	58-80	70
Ammonia nitrogen	mg/1	25.6 -40.1	31.5
Nitrate nitrogen	mg/1	22.4-32.1	27.4
Organic nitrogen	mg/1	14.3-21.7	19.4
Chloride	mg/1	112-134	121
Sulphate	mg/1	108-130	123.4
ABS	mg/1	76.3-109.7	90
Total bacterial count	-	(3.5-4.0) x 10^6	37 x 10^6

Table 2. Characteristics of fresh settled sludge of textile processing factory

Sludge liquor characteristics	Unit	Range of values	Mean
Settleable solids	mg/kg	2990-3350	3158
Moisture	%	95-98	97
Volatile solids	mg/kg	3700-5400	4150
Total solids	mg/kg	4500-8700	6500
Ash	%	17.8-37.8	36.2
Total nitrogen	mg/kg	39.9-61.8	50.9
Phosphorous	mg/kg	3.8-6.2	5.14
Potassium	mg/kg	4.2-6.7	4.7
Oil and grease	mg/kg	160-240.3	198.7
Iron	mg/kg	0.68-1.06	0.90
Calcium	mg/kg	18.0-26.30	23.5
Magnesium	mg/kg	18.6-23.4	20.3
Manganese	mg/kg	nil	nil
Copper	mg/kg	0.2-2.5	1.2
Cadmium	mg/kg	nil	nil
Chromium	mg/kg	nil	nil
Lead	mg/kg	nil	nil
Zinc	mg/kg	2.5-5.0	3.7

receiving water may occur through oxidization of ammonia to nitrate by some groups of bacteria. This necessitates appropriate treatment of sludge before its discharge. Thus the resulting nitrates and phosphates (inorganic nutrients) could be a boost to the plant and the algal growth. Concentrations as low as 0.01 mg/l of phosphorous and 0.1 mg/l of nitrate may be sufficient for eutrophication when other elements are in excess (Henry and Heinke, 1989).

Effect of sludge age on pH during aerobic digestion. The effect of age of sludge on its pH during aerobic digestion is shown in Fig. 2. The sludge detention time for textile processing sludge was 60 days. The steady pH during this period was due to complete stabilization during aerobic biodegradation. Sludge detention time is dependent on temperature and hence on the climatic conditions prevailing in the area.

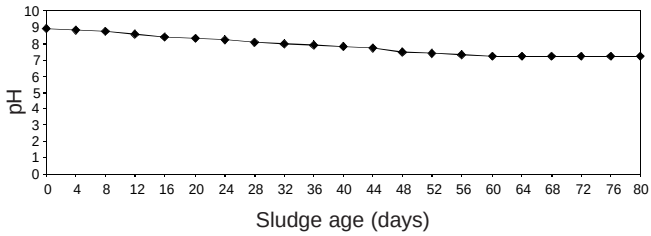

Fig. 2. Sludge age determination.

Characteristics of aerobic and anaerobic digested sludge. Some parameters that affect biological processes are shown in Table 2. A pH drop was observed for the sludge treated by aerobic and anaerobic biological methods. After digestion, it was found that pH of the sludge dropped from 8.91 to 5.81 and from 8.91 to 5.60 for aerobic and anaerobic systems, respectively. The drop in pH could have occurred due to the digestion products. Two products of aerobic digestion suspected for lowering the digester pH are carbon (IV) oxide and hydrogen ions.

$$C_5H_7NO_2 + 7O_2 + \text{bacteria} \rightarrow 5CO_2 + 3H_2O + NO_3 + H^+$$

In the case of anaerobic system, the depression in pH was due to the volatile acids released into the digestion medium. The microbes in the digester convert the organic content of the sludge to lower molecular weight organic acids principally ethanoic and propanoic acids and consequently to carbon (IV) oxides, which dissolve in water to give trioxocarbonate (IV) acid (H_2CO_3).

The reaction products (acids and CO_2) raise the acidic levels in the digester thereby lowering the pH.

Optimum coagulants/flocculant dosages for sludge treatment. Fig. 3a-d show the optimum coagulant/flocculant dosages needed for the treatment of the sludge. For quick

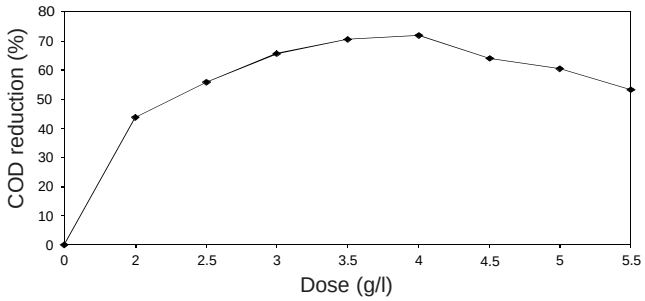

Fig. 3a. Optimum dosage determination using alum.

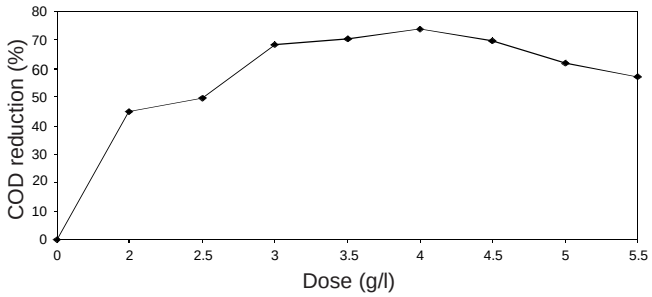

Fig. 3b. Optimum dosage determination using iron (III) chloride.

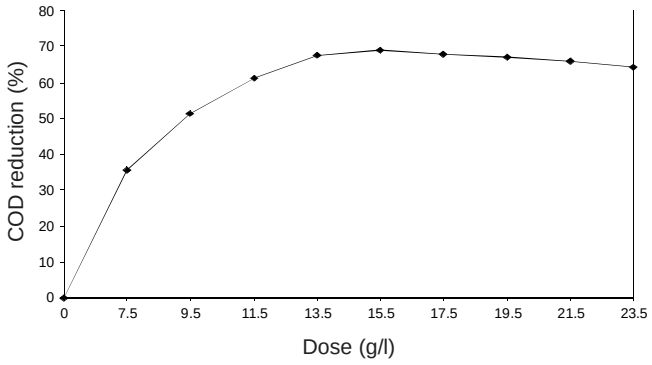

Fig. 3c. Optimum dosage determination using lime.

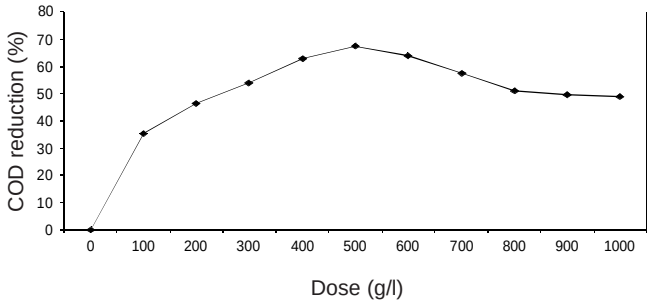

Fig. 3d. Optimum dosage determination using polyelectrolyte.

analysis, COD was chosen to assess the degree of treatment as there is a correlation between COD on one hand and BOD, SS and TS on the other hand (Ademoroti, 1980). The optimum coagulant dosages established were 4g/l for alum and iron (III) chloride, each, 13.5 g/l for lime and 550 mg/l for polyelectrolyte.

The solids. The amount of solids present in raw sludge and sludge treated aerobically, anaerobically, physicochemically and by combined methods are shown in Fig. 4.

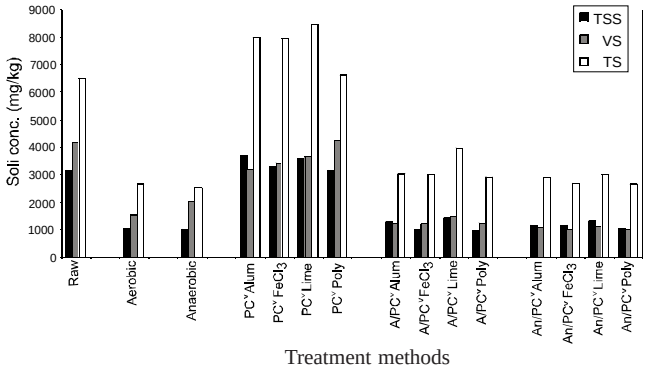

Fig. 4. Amount of solids in raw and treated textile sludge.

Solid reduction of about 59% TS, 67% SS and 62% VS were achieved for aerobic system and 61% TS, 68% SS and 51% VS, for anaerobic system. The ash produced here was 38% and 33% of total solids for aerobic and anaerobic processes, respectively.

The products of the anaerobic digestion process are methane gas and carbon (IV) oxide; both are burnable gases termed biogas which, if harnessed, may be used as useful fuel for heating purpose and for powering industrial plants.

Oxygen demand values. The results of the oxygen demand concentrations of raw and treated sludge are shown in Fig. 5. The BOD and COD values of fresh sludge of textile processing industry were 550 and 1694 mg/l, respectively. However, after aerobic treatment, BOD and COD reduction of about 87 and 90%, was respectively achieved.

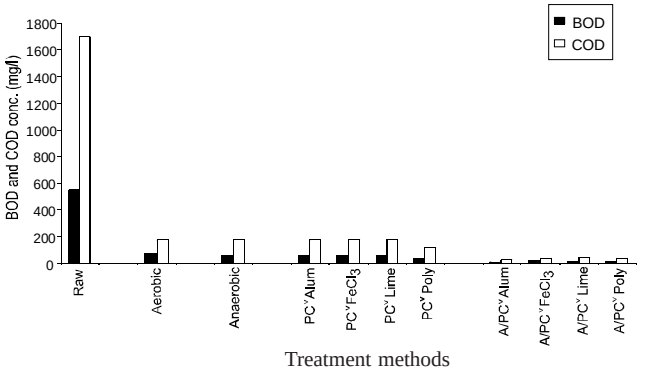

Fig. 5. BOD and COD of raw and treated textile sludges (mg/l).

The anaerobic systems yielded a BOD and COD reduction of 89 and 90 percent, respectively.

Nitrogen amount. Nitrogen content of raw and treated textile sludge was 66.8 and 50.9 mg/l, respectively (Fig.6). Aerobic treatment of the sludge resulted in about 33-49% reduction. This could have resulted from oxidation of some ammonium nitrogen (which contributed substantially to the total nitrogen) to nitrate through nitrification, raising the nitrate level of aerobically treated sludge.

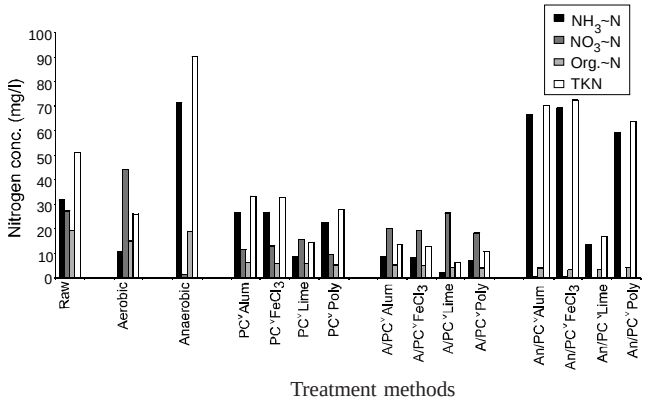

Fig. 6. Nitrogen concentration of raw and treated textile sludge (mg/l).

The anaerobic treatment method increased the total nitrogen content of the sludge. The increase in total nitrogen in the case of the anaerobic system may be attributed to the addition of (urea), a nutrient supplement also for the microbial population in the processed sludge enabling completion of the digestion process.

Phosphorus concentration. Results for phosphorus concentrations of treated sludge (liquor and cakes) are shown in Fig. 7. Sludge treated through biological aerobic systems had higher phosphorous concentrations while the concentration in the liquor decreased to the tune of 5-12 percent. On the other hand, the anaerobically treated sludge had significant reduction of about 37-53 percent in the sludge cake while the liquor was heavily laden with phosphorous with an increase of about 55-64 percent. This may be attributed to the solubilization of phosphorus during anaerobic digestion process. Pitman (1992) and Pitman *et al.* (1991) confirmed that sludge handling liquors from biological nutrient removal plants with anaerobic digesters contained high phosphorus concentrations.

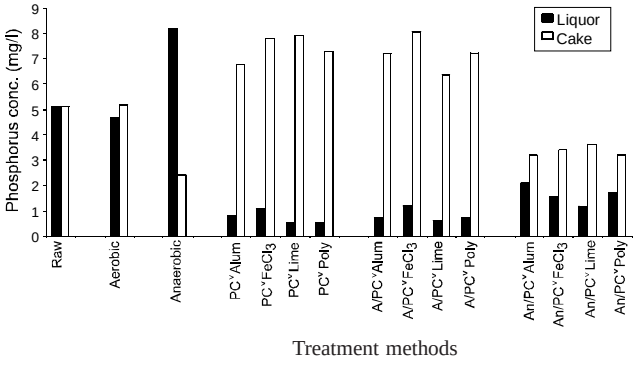

Fig. 7. Phosphorus concentration of raw and treated textile sludge (mg/l).

NPK (fertilizer) values. The levels of nitrogen, phosphorus and potassium have to be critical if the sludge is to be used for agricultural purposes (Sommers, 1977). The fertilizer value of sludge is detected by the percentage concentration of nitrogen, phosphorus and potassium (NPK); typical NPK fertilizer has a composition of 8% N, 8% P and 8% K. It may be difficult to achieve these levels of nutrients in sludge. It was observed that the studied sludge had definite fertilizer values (Fig. 8). It had a concentration of 2.6% N, 0.5% P and 0.6% K. The anaerobically digested sludge had N,P,K concentrations of 9%, 0.2% and 0.5%, respectively. If these nutrient concentrations are improved through some suitable complementary method such as composting, the sludge could be used to fertilize and condition soil. Humid material in the sludge improves the physical properties and cation exchange capacity of the soil.

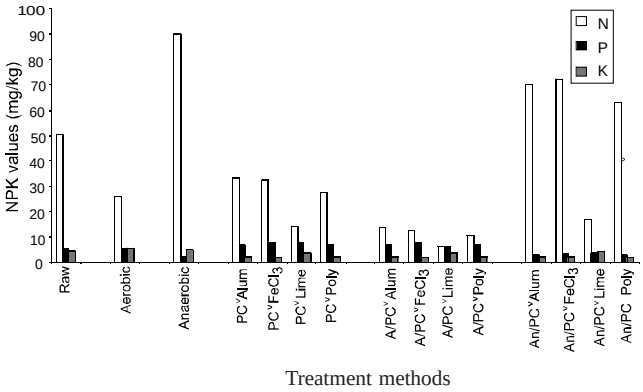

Fig. 8. NPK concentration of raw and treated textile sludges (mg/l).

Physicochemical method of treatment. *The solids.* The quantities of sludge were found to increase for all coagulants used in the treatment. A common trend noticed was that the lime treated sludge had more quantity of solids than the alum treated one, which in turn had more solids than iron (III) chloride treated sludge. Polyelectrolyte treated sludge had only a marginal increase in the sludge solids. The increase in total solids (TS) was in the range of 14-23.7 percent for iron (III) chloride treated sludge, 21-25 percent for alum and 25-29.5 percent for lime treated one. Suspended solids (SS) also increased in the range of 4.2-19.7 percent for iron (III) chloride, 17.5-24.3 percent for alum and 14-25.9 percent for lime treated sludge. Volatile solids (VS), however, decreased in all types of sludge treated with chemical coagulants. Polyelectrolyte flocculant, however, improved the volatile content of sludge, thereby enhancing the biogas (fuel value) production of the sludge.

***Oxygen demand values*.** The percentage BOD and COD reduction achieved in the physicochemical method was in the range of 88-92.6 and 89-92.8 percent, respectively. The phy-sicochemical methods were more effective in terms of BOD and COD removal than the biological systems (Fig.5).

Nitrogens amount. The total Kjeldahl nitrogen (TKN), ammonia nitrogen (NH_3-N), nitrate nitrogen (NO_3-N) and organic nitrogen (org.-N) were reduced in all the samples, more being in the sludge treated with lime.

***Phosphorus concentration*.** The sludge solids contained higher concentration of phosphorus than the fresh sludge while the liquors were almost free of phosphorus (Fig. 7). About 90-93 percent phosphorus was removed from the liquor.

***NPK values*.** Physicochemically treated sludge had little fertilizer values. However, the fertilizer values of raw sludge were higher than the physicochemically treated one.

Combined biological and physicochemical methods of treatment: *The solids*. It was found that the combined aerobic/physicochemically treated sludge had solid reduction ranging from 19.4-27.8 percent total solids, 27-35 percent suspended solids and 36-49 percent volatile solids.

Percentage solid reduction achieved using combined aerobic/physicochemical treatment of sludge was not as high as either the aerobic or the anaerobic method and so, for reduction in sludge quantity, either of the biological methods is more promising than the combined one.

The anaerobic/physicochemical method resulted in better solid reduction comparable to the combined aerobic/physicochemical method, the former being in the range of 26.2-29.4 percent TS, 24-28.4 percent SS and 31.3-37 percent VS; however, this method may not be preferred to any of the biological methods in terms of solid reduction. The lime treated biological sludge had the least amount of solids reduced.

***Oxygen demand values*.** The combined biological and physicochemical treatment methods proved more efficient

than either the biological or the physicochemical method individually in terms of BOD and COD reduction. About 96.1-98.2 percent BOD and 96.8-98.4 percent COD reduction was achieved for aerobic/physicochemically treated sludge, while 96.8-98.8 percent BOD and 96.8-98.9 percent COD reduction was achieved by the combined method.

Nitrogen. There was considerable reduction in the nitrogen content of the treated sludge. The highest reduction was noticed in lime treated aerobic sludge. Ammonia nitrogen reduction of this sludge was about 85.2-93.3 percent. This may be due to the nitrification process during the digestion stage while ammonia was further reduced by the physicochemical treatment. Total nitrogen was also reduced in the sludge using the combined aerobic/physicochemical method, in the range of 74.5-87.6 percent. The highest reduction occured in the lime treated aerobic sludge thus lime is a better coagulant for nitrogen removal from aerobically digested sludge.

In combined anaerobic/physicochemically treated sludge, ammonia nitrogen concentration increased considerably with other coagulants and was reduced in lime treated anaerobic sludge whereas nitrate nitrogen was reduced to zero.

Phosphorus. The results revealed that, the combined anaerobic/physicochemical method can be used to reduce phosphorus both in the sludge cake and in the liquor. This method is the best choice if complete disposal of sludge is intended with removal of nitrates and phosphates.

NPK values. Sludge treated by combined anaerobic/physicochemical method had reduced NPK concentration, (Fig. 8) lower even than the raw sludge. Therefore, for improvement in fertilizer value, this method is not recommended.

Conclusion

Proper sludge management requires an understanding of the place of origin of the sludge within the wastewater treatment process, the age and characteristics of the sludge, various methods available for sludge treatment, the economic potential of treated and untreated sludge and the disposal options available for the unused waste.

The final destination of the sludge will determine degree of the treatment required and the effect on the environment.

For landfill, composting, brick making and incineration, it is unnecessary and undesirable to biologically digest the sludge as heat value of the sludge might be lost as biogas.

For land application of sludge, physicochemical treatment offers a very promising result as sludge solids contain substantial amount of adsorbed phosphate as apatite e.g., calcium hydroxyl apatite, $Ca_5OH\ (PO_4)_3$ in the lime treated sludge.

Biological treatment methods (aerobic and anaerobic) can be used to reduce the quantity of solids in the sludge generated by industrial processes before disposal.

Aerobic biological method and the combined aerobic/physicochemical method are good options for ammonia reduction. The anaerobic method is the best if phosphorous removal from sludge solids is intended before disposal of the solids.

The basic nutrients in sludge, nitrates and phosphates are best removed by the combined anaerobic/physicochemical method. Thus, for prevention of eutrophication of water bodies, this method of treatment is more promising.

References

Ademoroti, C.M.A. 1996. *Standard Method For Water and Effluents Analysis*, Foludex Press Ltd., Ibadan, Nigeria.

Ademoroti, C.M.A. 1980. The effect of pH on wastewater purification. *Effl. Wat. Treat. J.* **20:** 541-549.

APHA 1995. *Standard Methods for the Examination of Water and Wastewaters*, 19th edition, American Public Health Association, Washington DC., USA.

Asia, I.O., Oladoja, N.A., Bamuza-Pemu, E.E. 2006. Treatment of textile sludge using anaerobic technology. *African J. Biotechnol.* **5:** 1678-1683.

Asia, I.O., Ademoroti, C.M.A. 2005. Combined aerobic/physicochemical treatment of pharmaceutical sludge. *Pak. J. Sci. Ind. Res.* **48:** 322-328.

Asia, I.O., Ademoroti, C.M.A. 2004. Integrated biological/chemical treatment of brewery sludge. *Pak. J. Sci. Ind. Res.* **47:** 281-291.

Asia, I.O., Oladoja, N.A. 2003. Determination of optimum dosage of chemical coagulants/flocculants needed for sludge treatment. *J. Sci. Eng. Technol.* **10:** 5031-5043.

Asia, I.O., Ademoroti, C. M. A. 2002. Mesophilic anaerobic treatment of brewery sludge. *World J. Biotechnol.* **3:** 568-577.

Asia, I.O. 2000. Studies on Industrial Sludge Treatment Options. *Ph.D. Thesis*. University of Benin, Nigeria.

Henry, J.G., Heinke, W.G. 1989. *Environmental Science and Engineering*, Eaglewood Cliffs, New Jersey, USA.

Pitman, A.R. 1992. *The Handling of Sludge Liquors*, Paper Presented at the IAWPRC Study for Biological Nutrient Removal and Anaerobic Digestion. Johannesburg, S. Africa.

Pitman, A.R., Deacon, S.L., Alexander, W.V. 1991. The thickening and treatment of sewage sludges to minimize phosphorous release. *Water Res.* **25:** 1285-1294.

Quano, E.A.R., Lohani, B.N., Thanh, N.C. 1978. *Water Pollution Control in Developing Countries*, 567 p, AIT.

Sommers, L.E. 1977. Chemical composition of sewage sludge and analysis of their potential use as fertilizers. *J. Environ. Qual.* **6:** 225-232.

Steward, J.W., Towse, P.J. 1984. *Chemical Technology in Africa: A Resource Book*, I st edition, Cambridge University Press, London, UK.

Biological Sciences

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Antifungal Activity of the Liquid Obtained by Oxidative Cracking of Waste Paper

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Abstract. The composition of the product formed by burning of paper under controlled conditions in an indigenously prepared reactor was determined by GC/MS, while its antifungal activity was assessed qualitatively and quantitatively by comparison with the standard and determination of the MIC. The product was found to be effective against six fungal strains. Amount of the product has been improved upto 25%.

Keywords: antifungal activity, oxidative cracking, waste paper

Introduction

The incidence of fungal infections has lately increased due to the resistance of the causative microbes to the antifungal medicines (Turan-Zitouni *et al*., 2005) as well as the increased number of immune persons taking chemotherapy for various types of cancer or HIV infection (Sionov *et al*., 2005). Hence, there is a need to explore and develop new antifungal reagents of higher activity or having minimum adverse effects (Schmourlo *et al*., 2005; Silver and Bostain, 1993).

Folk medicines have played important role in the treatment of many diseases for quite a long time. In the present work an effective folk medicine used for the treatment of scabies and ringworm has been investigated. This medicine is used in various parts of NWFP, Pakistan for treatment of these diseases for long. Traditionally, when a sheet of paper rolled into the form of a pipe, with its upper end closed is burnt, a small quantity of liquid product is obtained at the lower end of the pipe. This liquid of reddish colour, obtained from the combustion of paper, is known as paper oil.

The author has observed that very chronic cases of these diseases were cured through use of the said oil, twice a day in very small doses for three to seven days. In the present paper, investigations carried out on the composition of the product, its antimicrobial activity as well as measures to improve the yield, have been reported.

Materials and Methods

The process of the oxidative cracking of the paper was carried out in a quick fit pyrex reactor of the Buckner funnel type. The external diameter of the funnel was 60 mm, while its length was 100 mm. Sintered glass disc bed reactor of quick fit type was used. The reaction was carried out by igniting 100 g of fine paper cut into small pieces. The assembly for reaction includes, the pyrex reactor, Buckner type funnel fitted in the suction flask, which was connected with a series of three receiving flasks and the aspirator. The product was sucked into the suction flask followed by its condensation into a reddish colour liquid product with the maximum yield of 25%.

GC/MS analysis. General profile for the liquid product of the oxidative cracking of paper was obtained using EI-MS. Analysis of the liquid product was conducted on a mass spectrometer JMS 600 H Jeol and the product was separated into its components using Agilent 6890N gas chromatograph equipped with a fused capillary column (HP.5 l.; 30 m x 0.32 mm i.d., film thickness, 0.25 μm) with polydimethylsiloxane as the stationary phase. The carrier gas was helium at a flow rate of 1.8 ml/min.

Injection mode was split at a split ratio of 35. The column was initially kept at 50 °C for 5 min and then the temperature was raised to 240 °C at the rate of 5 °C/min. The injector temperature was 250 °C and the amount of sample was 1 μl.

Identification of major components of the product was confirmed using total ion chromatogram analysis (TIC) as well as fragmentation pattern and library matching by NIST Mass Spectral Library, while the quantification was carried out using the peak area.

Antifungal activity. Antifungal activity of the sample was determined using agar tube dilution method. The activity of the sample was investigated against *Trychophyton longifusus*,

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Candida albicans, *Candida glabarata*, *Fusarium solan*i, *Aspergillus flavus* and *Microsporum canis*. Sabourad dextrose agar (SDA) was used for the growth of fungi; 0.4 ml of SDA, for each fungus species, was taken in six separate tubes and autoclaved. 24 mg of the sample was dissolved in 1 ml dimethylsulphoxide and 66.6 µl of this solution was dispensed in each of the six tubes. The tubes were placed in slanting position. These were allowed to solidify for 24 h. Each of the tube was inoculated with 4 mm of fungal stock and incubated at 27 °C for 7 days. The media containing only DMSO was used as blank and Micanozole, as reference, for the samples. The reference for *Aspergillus flavus* was Ampotericin B. All the tests were carried out in triplicate.

Results and Discussion

The liquid obtained by the oxidative cracking of paper was analyzed for composition using GC/MS. It was composed of acids, aldehydes, ketones and furans. The compounds present in the liquid sample are mentioned in Table 1.

The product also contained large amount of water and other undetectable compounds. The activity of the product was significant for all the tested fungal strains in terms of percent inhibition (Table 2).

Based on the presence of oxygenated compounds, it can be inferred that the sample inhibits the growth of fungi through inhibiting the growth of mycelia, interference with the SH enzymes and disruption of the chitinous cell wall. Antifungal activity of the oxygenated compounds has been reported by various workers (Turchetti *et al.*, 2005; Karmen *et al.*, 2003; Stange *et al.*, 1999). The sample contains aldehydes, which are cell wall rupturing compounds (Knobloch *et al*., 1989). It also contains phenolic compounds, which are antioxidant, and interfere with enzymes (Karmen *et al.*, 2003). Presence of acid in the sample supports its antifungal activity as reported by Turchetti *et al*. (2005). Antifungal activity of the sample also gets support from the work of Pour *et al.* (2000) who reported derivatives of furan. However, the activity of our sample is the result of combined action of these compounds and is greater than simple addition.

Table 1. Composition of product of oxidative cracking of paper

Compounds	Retention time (min)	Concentration (%)
Propenoic acid methoxy ester	1.53	11.4888
1-Hydroxypropanone	1.60	7.4038
2,5-Dimethyl furan	2.60	3.8295
2-Methanol-5-hydroxy furan	3.48	2.2339
4-Hydroxy-but-2-enoic acid lactone	5.05	2.0637
4-Methanol cyclohexanol	6.52	0.9361
2-Ethenylfuranone	7.05	0.9701
Hydroquinone	7.52	0.6808
4-Hydroxy-3-methyl-1-one-2-cyclopentene	8.85	1.6169
2-Ethenyl-3-methyl-2-(5*H*)-furanone	9.28	0.3574
2-Propenal-4-methyl phenol	10.7	1.0852
Unknown	11.6	0.3643
1,4:3,6-Dianhydro-α-D-glucopyranose	14.95	0.4144
Unknown	15.77	1.7871
Unknown	23.43	1.0930
Octadecanoic acid	32.6	1.9573

Table 2. Antifungal activity of the product of oxidative cracking of paper

Fungus	Linear growth (mm)		Inhibition	MIC
	Sample	Control	(%)	(µg/ml)
Trichophyton longifusus	0	100	100	370
Candida albicans	0	100	100	390
Aspergillus flavus	0	100	100	340
Microsporum canis	0	100	100	275
Fusarium solani	0	100	100	320
Candida glabarata	0	100	100	375

Conclusion

Based on the traditional knowledge, through oxidative cracking of paper, significant yield of a product was obtained using suction reactor. The product contained furans, furanones, carboxylic acids, aldehydes, ketones and phenols along with water. These compounds are reportedly antimicrobial, acting through enzyme inhibition, antioxidant, membrnane rupture and mycelial growth inhibition. The product displayed antifungal activity against six fungal species. This study helps to formulate a broad spectrum antifungal agent.

References

Karmen, V., Bojana, B., Margareta, V., Franc, P. 2003. Effect of the antifungal activity of the oxygenated aromatic essential oil compounds on the white-rot *Trametes versicolor* and the brown-rot *Coniophora puteana*. *Int. Biodeterio. Biodegrad.* **51:** 51-59.

Knobloch, K., Pauli, A., Iberl, B., Weigand, H., Weis, N. 1989. Antibacterial and antifungal properties of essential oil components. *J. Essent. Oil Res.* **1:** 119-128.

Pour, M., Spulak, M., Balsanek, V., Kunes, J., Buchta, V., Waisser, K. 2000. 3-Phenyl-5-methyl-2*H*, 5*H*-furan-2-ones: tuning antifungal activity by varying substituents on

the phenyl ring. *Bioorg. Med. Chem. Lett*. **10:** 1893-1895.

Schmourlo, G., Mendonca-Filho, R.R., Alviano, C.S., Costa, S.S. 2005. Screening of antifungal agents using ethanol precipitation and bioautography of medicinal and foods plants. *J. Ethnopharmacol.* **96:** 563-568.

Silver, L.L., Bostain, K.A. 1993. Discovery and development of new antibiotics: the problem of antibiotic resistance. *Antimicrob. Agents Chemother.* **37:** 377-383.

Sionov, E., Roth, D., Sandovsky-Losica, H., Kashman, Y., Rudi, A., Chill, L., Berdicevsky, I., Segal, E. 2005. Antifungal effect and possible mode of activity of a compound from the marine sponge *Dysidea herbacea. J. Infection* **50:** 453-460.

Stange, R.R., Sims, J.J., Midland, S.L., McDonald, R.E. 1999. Isolation of a phytoalexin, *trans-p*-coumaryl aldehyde, from *Cucurbita maxima* Cucurbitaceae. *Phytochemistry* **52:** 41-43.

Turan-Zitouni, G., Kaplancikli, Z.A., Yildiz, M.T., Chevallet, P., Kaya, D. 2005. Synthesis and antimicrobial activity of 4-phenyl/cyclohexyl-5-(1-phenoxyethyl)-3-[*N*-(2-thiazolyl)acetamido]thio-4H-1,2,4-triazole derivatives. *Eur. J. Med. Chem*. **40:** 607-613.

Turchetti, B., Pinelli, P., Bzzini P., Romani, A., Heimler, D., Franconi, F., Martini, A. 2005. *In vitro* antimycotic activity of some plant extracts towards yeast and yeast-like strains. *Phytotherapy Res.* **19:** 44-49.

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Development of an Unconventional Method to Control the Ectoparasites in Backyard Poultry

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Abstract. Dust of *Nicotiana tabacum*, *Azadirachta indica* and *Polygonum hydropiper* when applied in the poultry sheds as bedding for control of six species of lice, one species of fly and two species of mites, highest efficacy (96.67%) was shown by tobacco at 15% concentration followed by neem at the same concentration (efficacy, 77.52%) and tobacco at 10% concentration. Tobacco at 15% concentration significantly ($p<0.05$) reduced the ectoparasitic burden within 12 days with maximum mean body weight gain by poultry, being 232.30 g.

Keywords: *Nicotiana tabacum*, *Azadirachta indica*, *Polygonum hydropiper*, ectoparasites, poultry parasites

Introduction

In rural areas, of Bangladesh, poultry are commonly reared in semi-scavenging system. This backyard poultry is generally infested with various type of ectoparasites including different species of lice, mites etc. having various adverse effects specially on body weight gain and egg production (Islam *et al*.,1999; DeVaney, 1979; 1976) and acting as vectors of many microbial pathogens including *Erysipelothrix rhusiopathiae* and *Borrelia anserina* (Chirico *et al.*, 2003; Urquhart *et al*., 1996; Kettle, 1995).

Ectoparasitic infestation of poultry is the most common problem worldwide and utmost attempts have been made to control them by using several chemical compounds. Although some of the chemicals are efficient enough to kill ectoparasites but most of them are unsuitable in terms of food safety and environmental problems as they have residual and cumulative effects (Lee *et al.*, 2002). Moreover, some of them are toxic to mammals including humans. These are also not readily available especially in rural areas. Besides, resistance frequently develops in ectoparasites against chemical insecticides (Szczypel *et al*., 2003). Various species of plants and herbs of medicinal value are available in Bangladesh which are commonly used by the village people to treat many diseases. These herbal products are usually safe, easily available, cost effective and environment friendly. Nahar *et al*. (2005) found acaricidal efficacy of ata, durba, neem, bishkatali and sharifa. Acaricidal and insecticidal activities have been widely evaluated in different countries of the world, such as in India (Rahman *et al*., 2005), South Korea (Kim *et al*., 2004), Pakistan (Khan *et al.*, 2003) etc. The present research work was undertaken to determine the efficacy of three different plants *viz*. *Nicotiana tabacum* (tobacco), *Azadirachta indica* (neem) and *Polygonum hydropiper* (bishkatali) against ectoparasites of poultry so as to develop an unconventional control method which would be easy to use, cost effective and applicable everywhere.

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Materials and Methods

Dust prepared from leaves and/or stems of tobacco, neem and bishkatali were applied in backyard poultry, naturally infested with lice, mites and flies, during July 2005 to May 2006 in Patuakhali district in Bangladesh, covered by the smallholder livestock development project- 2 (SLDP-2). Identification of the parasites, preparation of plant products and other relevant work were done in the Department of Parasitology, Faculty of Veterinary Science (FVS), Bangladesh Agricultural University (BAU), Mymensingh.

Three common plants namely neem (*Azadirachta indica*) bishkatali (*Polygonum hydropiper*) and tobacco (*Nicotiana tabacum*) were selected based on their ethno-veterinary use among the rural people of Bangladesh. Neem and bishkatali were collected from the surrounding area of BAU campus, and tobacco leaves were collected from the local markets. For neem and tobacco only leaves were used, whereas for bishkatali both the stems and leaves were used.

After collection, plants were brought to the laboratory. All the fresh leaves and stems were washed in running tap water and cut into small pieces. The plant materials were dried in

sunlight, and then in the oven at 55-60 °C to constant weight. Dusts of different plants were prepared primarily by manual crushing of dried leaves and then by pulverizing the crushed leaves and stems with the help of pulverizer and preserved in airtight plastic contaners, till their use.

For the study of ectoparasiticidal efficacy, birds were selected from the farmers households in the research area which were regularly vaccinated and dewormed. Thirty families were selected from the said areas, each having at least five birds and all infested with ectoparasites. These families were divided into ten groups containing three families in each. In all the families, almost similar type of management practices such as housing, feeding, cleaning of shed etc. were confirmed by regular monitoring. Birds were identified by leg bands.

Pretreatment observations. One square inch of the body surface area of poultry in which the ectoparasites were densely populated were selected and counted. Body weight of each bird was taken by digital balance and recorded before treatment.

Treatment. Treated groups were marked as GT5, GT10, GT15, GN5, GN10, GN15, GB5, GB10 and GB15. Powdered form of tobacco leaves were given admixture with ashes as litter at 5%, 10% and 15% concentration in GT5, GT10 and GT15 groups, respectively. Another three groups viz. GN5, GN10 and GN15 were treated with neem leaves at 5%, 10% and 15% concentration, respectively. Last groups, GB5, GB10 and GB15 were treated with bishkatali leaves in the same concentrtions. Bedding with only ash (500 g for each poultry shed) was given to the control group. The treatment was given 5 times, at 4 days intervals, to each group (21 days).

Posttreatment observations. Regular monitoring of each poultry house, with counting and recording of ectoparasites was carried out at 4 days intervals in the selected birds to detect the efficacy of the sample substances, up to the end of the experiment. Efficacy was determined in terms of reduction of load of ectoparasites. At the end of the experiment, body weight of each bird was taken and recorded.

Statistical analysis. Odds ratio with its confidence interval was computed for comparing different concentrations (Schlesselman, 1982). Repeated analysis of variance was performed for testing the effectiveness of tobacco, neem and bishkatali using the software Systat 6.0 for Windows (SPSS, 1996). Analysis of Covariance (ANCOVA) was conducted to compute the mean body weight gain per bird as there existed a heterogeneity in age and sex of the experimental birds (Gomez and Gomez, 1984). The benefit cost ratio (BCR), a simple calculation that depicts the total financial return for each taka (Tk, local currency) invested in the programme was computed by the following formula:

$$\text{BCR} = \frac{\text{total benefit}}{\text{total cost}} \text{ (in Tk)}$$

Results and Discussion

In the present study, birds were found to be infested with the following lice species: *Menacanthus stramineus* (74%), *Menopon gallinae* (63%), *Lipeurus caponis* (48%), *Cuclotogaster heterographus* (25%), *Goniodes gigas* (18%) and *Goniocotes gallinae* (14%), one species of fly, namely *Simulium* sp. (3.7%), and two species of mites namely *Dermanyssus gallinae* (57%) and *Knemidocoptes mutans* (43%). The study revealed that regular use of tobacco, neem and bishkatali plant's pulv gradually reduced the mean ectoparasitic load in backyard poultry (Table 1). The highest ectoparasiticidal efficacy was exerted by tobacco at 15% concentration (96.91%) followed by neem at the same concentration (77.52%) and tobacco at 10% concentration (76.92%). Odds ratio of the plants at different concentrations showing highest efficacy was also calculated. Tobacco at 15% concentration was 9.09 and 9.40 times more effective against ectoparasites than neem at 15% concentration and tobacco at 10% concentration, respectively. On the other hand, odds ratio between neem at 15% concentration and tobacco at 10% was 1.03, which indicated that they were almost equally effective for removing ectoparasites (Table 2). Tobacco at 15% concentration drastically reduced the parasitic burden in GT15 group. The changes in the number of ectoparasites were significant up to 3rd observation in that group but tobacco at 10% concentration rapidly reduced ectoparasites on the 1st treatment, then it acted slowly up to the 5th observation. On the other hand, neem at 15% concentration reduced ectoparasitic load slowly throughout the five observations (Table 1).

Efficacy of tobacco was studied by other workers against different types of ectoparasites. Vasanthi *et al.* (2004) treated *Sarcoptes scabiei*-infested rabbit with tobacco decoction and found that it was highly effective with no side effect and re-infestation was not observed even after one and a half month of the treatment. Burkhart and Burkhart (2000) reported that nicotine (active ingredient in tobacco) facilitated removal of adult lice by inducing muscle twitching that may affect the insects' normal grip on hair follicles. Potenza *et al.* (1999) reported more than 80% efficacy of aqueous extract of tobacco against spider mite. Though there are some variations between the present and previous results, it can undoubtedly be concluded that these plant products are effective in removing ectoparasites. However, there can be differences in

Table 1. Effectiveness of tobacco, neem and bishkatali in terms of reduction of mean ectoparasitic load per bird in each square inch of highly affected body region

Groups	Pretreatment observation	Post treatment observation (96 h interval)				
		1	2	3	4	5
Control	7.33^{a1} ±1.46	7.47^{a} ± 1.53	7.73^{a} ± 1.65	8.33^{a} ± 1.64	8.73^{a} ± 1.68	8.87^{a} ± 1.74
GT-5	4.60^{a} ± 1.02	4.13^{b} ± 1.03	3.40^{c} ± 0.82	3.13^{cd} ± 0.82	2.67^{d} ± 0.72	2.40^{e} ± 0.77
GT-10	6.07^{a} ± 1.58	3.33^{b} ± 0.67	2.53^{c} ± 0.49	1.87^{d} ± 0.48	1.60^{ed} ± 0.51	1.33^{e} ± 0.44
GT-15	6.47^{a} ±1.63	2.13^{b} ± 0.39	0.73^{c} ± 0.15	0.27^{d} ± 0.12	0.20^{d} ± 0.11	0.20^{d} ± 0.11
GN-5	6.67^{a} ± 2.00	6.60^{a} ± 2.00	5.80^{b} ± 1.77	5.47^{bc} ± 1.77	5.13^{c} ± 1.68	4.80^{d} ± 1.67
GN-10	3.93^{a} ± 0.57	3.47^{b} ± 0.52	3.00^{c} ± 0.48	2.27^{d} ± 0.36	1.73^{e} ± 0.35	1.60^{e} ± 0.29
GN-15	8.60^{a} ± 1.89	6.73^{b} ± 1.28	5.33^{c} ± 1.06	3.87^{d} ± 0.80	2.80^{e} ± 0.56	1.93^{f} ± 0.33
GB-5	4.67^{a} ± 0.62	4.33^{ab} ± 0.62	4.13^{b} ± 0.60	4.00^{bc} ± 0.64	4.00^{bc} ± 0.64	3.73^{c} ± 0.61
GB-10	6.20^{a} ± 0.95	5.47^{b} ± 0.84	5.27^{b} ± 0.83	5.07^{c} ± 0.77	5.00^{c} ± 0.77	4.40^{c} ± 0.74
GB-15	9.13^{a} ± 1.78	7.93^{b} ±1.58	7.60^{c} ± 1.48	7.40^{c} ± 1.42	7.33^{c} ± 1.41	5.33^{d} ± 1.30

values of different superscripts were statistically significant (P< 0. 5)

Table 2. Efficacy of tobacco, neem and bishkatali against ectoparasites of poultry

Plants	Concentration (%)	Efficacy (%)	Odds ratio		95% CI
Neem	5	28	GT15 vs GN15	9.09*	2.68-30.84
	10	59.32			
	15	77.52			
Tobacco	5	47.83	GN15 vs GT10	1.03	2.68-30.84
	10	76.92			
	15	96.91	GT15 vs GT10	9.40*	0.54-1.95
Bishkatali	5	20	-	-	-
	10	29.03	-	-	-
	15	41.61	-	-	-
Control	-	-	-	-	-

* = indicates significant (p<0.05); GT15 = tobacco 15%; GT10 = tobacco 10%; GN15 = neem 15%

the rate of efficacy depending on the method of application and the type of parasites.

In tobacco, active ingredient is alkaloid which is known as nicotine (Burrows and Tyrl, 2001). Nicotine is a contact poison which is highly toxic and is absorbed through spiracles and integument of ectoparasites. It acts directly on the ganglia of the insects' central nervous system and produces excitation at low concentration but paralysis at high concentration due to a direct action on the synopses (Gillott, 1995). Perhaps, in this manner, they cause paralysis of ectoparasites rapidly. Besides, nicotine sulphate has a fumigant action against *Dermanyssus gallinae* when it is painted on the perches (Soulsby, 1982). Lapage (1962) reported killing of lice without disturbing poultry by painting the perches with strong tobacco extract containing 40% nicotine and keeping the birds in that house for two nights; the warmth of the bird bodies causes the nicotine to evaporate and kill the lice and eggs. He also suggested repeating the treatment after 10 days. In this experiment, tobacco leaves were applied as pulv mixed with ashes as bedding. So, pulv of tobacco leaves came in contact with ectoparasites throughout the night. Furthermore, the said evaporating effect might be also exerted on the ectoparasites. No toxic effect was observed in poultry with tobacco dose used in the study.

Neem at 15% concentration was also effective (77.52%), but it took a little longer time for exerting its effect on the parasites. Efficacy of bishkatali was insignificant in all the three concentrations applied. Pathak *et al.* (2004) recorded that methanol extract of neem was highly effective against tick. Kumar *et al.* (2002) found that neem leaves caused 80% mortality to adult lice. Nahar *et al.* (2005) carried out an *in vitro* trial with neem, bishkatali, durba, ata and sharifa against cattle tick *Boophilus microplus* and observed 100% efficacy of ethanol extract of bishkatali at 2% concentration. But the efficacy of neem was 86.67% when applied as aqueous extract. Probably active ingredients of neem plants, having ectoparasiticidal efficacy, are water soluble. It could be said that contents of neem leaves have some ectoparasitcidal effect in dry preparations but required high concentration. On the other hand, the ingredients present in bishkatali may not be effective against ectoparasites prevalent in poultry or those ingredients are not active in dry preparation. Pulv is a crude product which possibly contains active ingredients in

relatively lower concentration than the soluble form and do not get proper contact with the ectoparasites.

In study of the effect of ectoparasites in terms of body weight gain, among the treated groups, the highest mean body weight (232.3 g) had been achieved by poultry in GT15 group (treated with 15%) followed by that in GT10 (178.26 g) and GN15 (159.76 g) groups that were treated by 10% tobacco and 15% neem leaves, respectively (Table 3).

Table 3. Mean body weight gain per bird (adjusted for heterogeneity in age and sex) at different concentrations of plants after applying them five times during 4 days

Treatment group	Body weight in (gm) (Mean ± SE)		Mean body weight gain (gm)
	Pre-treatment	Post-treatment	
GT-5	810.00±124.62	965.24±111.05	155.24
GT-10	1070.00±115.76	1248.26±111.91	178.26
GT-15	1113.57±102.43	1345.87±67.36	232.30
GN-5	996.67±76.92	1077.51±63.89	80.84
GN-10	1115.00±76.39	1251.21±77.94	136.21
GN-15	1165.33±106.20	1325.09±64.06	159.76
GB-5	1066.25±119.81	924.08±87.23	-142.17
GB-10	1017.33±92.98	981.70±63.85	-35.63
GB-15	956.33±50.17	978.58±63.81	22.25
Control	1215.56±119.29	919.17±94.80	-296.39

It is evident that the birds of each treatment group (except GB15 and GB10) had the advantage of gaining body weight due to the removal of ectoparasites. This finding directly conforms with the result of Devaney (1979) who evaluated the effect of *Ornythonyssus sylviarum* (northern fowl mite) on poultry. He observed that the mean body weight of White Leghorn roosters was approximately 100 g less than that of the uninfested roosters. Devaney (1976) also studied such an effect on lice, (*Menacanthus stramineus*) in White Leghorn hens and found that average body weight of hens decreased (85, 300, and 450 grams in 23, 35, and 49 weeks old birds) in comparison with the uninfested control group. Due to treatment, parasitic load of birds gradually decreased, and simultaneously restlessness was also reduced. So, their feed consumption and utilization increased resulting in improvement of health.

Tobacco is cost effective in the treatment of ectoparasites in backyard poultry. Although, some chemical agents (malathion, dichlorvos, permethrin, pyrethrin etc.) are effective in very low concentration and very small amount of insecticide is required but these may be suitable in the treatment of large poultry farm birds. In small farms or in backyard poultry farms, these may not be equally suitable since farmers need some extra care in applying these chemical insecticides. Besides, tobacco is available in local markets of rural areas all the year round and requires less care and attention. Moreover the effective doses (15% and 10%) are not toxic for the poultry. On the contrary, chemical agents are toxic to the poultry and are not environmentally friendly. In the present experiment, the benefit cost ratio (BCR) per bird was found to be Tk.7.5 (Takka or Tk is local currency of Bangladesh) depicts the investment of Tk. 1 would make return of Tk. 7.5 (taking Tk 130/kg poultry meat and Tk. 50/kg tobacco, mean body weight gain in best treatment group was 232.30 g). Therefore, use of tobacco is recommended at 15% concentration, mixed with ashes as bedding, for 12 days for the control of ectoparasitic infestation in backyard poultry.

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References

Burkhart, C.G., Burkhart, C.N. 2000. Use of topical nicotine for treatment of *Pediculus humanus capitis* (Anoplura: Pediculidae). *J. Medical Entomol.* **37:** 170-171.

Burrows, G.E., Tyrl, R.J. 2001. *Toxic Palnt of North America*, 1124 p, 1st edition, Iowa State University Press, Iowa, USA.

Chirico, J., Eriksson, H., Fossum, O., Jansson, D. 2003. The poultry red mite, *Dermanyssus gallinae* a potential vector of *Erysipelothrix rhusiopathiae* causing erysipelas in hens. *Med. Vet. Entomol.* **17:** 232-234.

DeVaney, J.A. 1979. The effects of the northern fowl mite, *Ornithonyssus sylivarium* on egg production and body weight of caged White Leghorn hens. *Poul. Sci.* **58:** 191-194.

DeVaney, J.A. 1976. Effects of the chicken body louse, *Menacanthus stramineus*, on caged layers. *Poul. Sci.* **55:** 430-435.

Gillott, C. 1995. *Entomology*, pp. 691-744, 2nd edition, Plenum Press. New York, USA.

Gomez, K.A., Gomez, A.A. 1984. *Statistical Procedures for Agricultural Research*, pp. 424-437, 2nd edition, John Wiley and Sons, New York, USA.

Islam, M.K., Mondal, M.M.H., Rahman, M.M., Haque, A.K.M.F., Chowdhury, M.A.A. 1999. Effects of *Lipeurus caponis*, Linnaeus, 1758, (*Mallophaga : Philopteridae*) on laying hens. *Vet. Rev.* **14:** 32-33.

Kettle, D.S. 1995. *Medical and Veterinary Entomology*, pp.

3-6, 2nd edition, CAB International, Cambridge, UK.

Khan, M.N., Nadeem, M., Iqbal, Z., Sajid, M.S., Abbas, R.Z. 2003. Lice infestation in poultry. *Int. J. Agric. Biol.* **5:** 213-216.

Kim, S.I., Yi, J.H., Tak, J., Ahn, Y.J. 2004. Acaricidal activity of plant essential oils against *Dermanyssus gallinae* (Acari: Dermanyssidae). *Vet. Parasitol.* **120:** 297-304.

Kumar, S., Sijngh, S.K., Baslas, R.K., Glildiyal, J.C., Saxena, A.K. 2002. Lousicidal properties of few aqueous plant extracts. *Indian Vet. J.* **79:** 1136-1140.

Lapage, G. 1962. *Monning's Veterinary Helminthology and Entomology,* pp. 446-813, 5th edition, Baillière Tindall and Cox, London, UK.

Lee, S.S., Kim, J.W., Jee, C.H. 2002. Immune effects on the somatic antigens against *Dermanyssus gallinae* and *Dermatophagoides pteronyssinus* in chicken. *Korean J. Vet. Res.* **42:** 253-266.

Nahar, L., Anisuzzaman, Alim, M.A., Karim, M.J., Islam, K.I., Mondal, M.M.H. 2005. *In vitro* acaricidal effects of some indigenous plants against *Boophilus microplus* (Arachnida : Ixodidae). *Bangladesh J. Vet. Med.* **3:** 118-123.

Pathak, D., Mathur, V.C., Latha, B.R., John, L. 2004. *In vitro* effect of indigenous plant extracts on ixodid ticks of small ruminants. *Indian J. Animal Sci.* **74:** 616-617.

Potenza, M.R., Takematsu, A.P., Sivieri, A.P., Sato, M.E., Passerotti, C.M. 1999. Acaricide effect of some plant extracts on *Tetranychus urticae* (Koch, 1836) (Acari: Tetranychidae) in the laboratory. *Arquivos Institute Biologico* **66:** 31-37.

Rahman, I., Gogoi, I, Dolui, A.K., Handique, R. 2005. Toxicological study of plant extracts on termite and laboratory animals. *J. Environ. Biol.* **26:** 239-241.

Schlesselman, J.J. 1982. *Case-Control Studies Design, Conduct, Analysis,* pp. 174-177, Oxford University Press, New York, USA.

Soulsby, E.J.L. 1982. *Helminths, Arthropods and Protozoa of Domesticated Animals*, pp.359-504, 7th edition, Baillière Tindall and Cox, London, UK.

SPSS. 1996. Systat G.O. for Windows: Command Reference, SPSS Inc. Publ., Chicago, USA.

Szczypel, B., Larramendy, R., Hernandez, M. 2003. Evaluation of different insecticides against external parasites of hens. *Rev. Cubana de Ciencia Avicola* **27:** 121-124 (Abstract in English).

Urquhart, G.M., Armour, J., Duncan, J.L., Dunn, A.M., Jennings, F.W. 1996. *Veterinary Parasitology,* 2nd edition, Blackwell Science Inc., London, UK.

Vasanthi, C., Choudhary, R.K., Latha, B.R., John, L. 2004. Effect of *Nicotiana tabacum* decoction on sarcoptic mange infestation in rabbits. *Indian J. Animal Sci.* **74:** 732-733.

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Surveillance of Drinking Water of Karachi City: Microbiological Quality

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Abstract. In the analysis of 329 treated (tap) water samples and 30 well water samples of Karachi city, 94 (28.5%) tap water samples were found contaminated with faecal coliforms and had high heterotrophic plate count (HPC), 153 (46.5%) had very high heterotrophic plate count and only 82 samples were fit for human consumption. Out of 30 well water samples only 2 samples were fit for human consumption; 23 (76%) had high faecal coliform count and HPC, whereas, 5 (16.67%) were rejected owing to extremely high bacterial count.

Keywords: faecal coliforms, heterotrophic plate count, drinking water quality, Karachi

Introduction

In developing countries drinking water and sanitation related diseases are the major contributors of the disease burden (Pruss *et al*., 2002). The potential of drinking water to transport microbial pathogens to consumers causing subsequent illness is well documented in the countries throughout the world. One third of intestinal infections worldwide are caused by water-borne pathogens (Mead *et al*., 1999; Hunter, 1997). In France, most of the gastrointestinal illnesses are associated with the consumption of contaminated tap water (Collin *et al*., 1981). Several water-borne pathogens cause diseases of high mortality rate. Due to these reasons, faecal bacteria are selected as indicators of faecal pollution of drinking water (WHO, 2004).

Enumeration of the total coliform bacterial population as indicator of water quality has been in use for some 60 years (Geldreich, 1966). All the standards for drinking water quality do not allow presence of faecal indicators in drinking water (WHO, 1993). The cases of outbreaks of gastroenteritis in 2000 in Ontario, Canada involved *E. coli* 0157:H7 (BGOSHU, 2000). Mainly faecally derived coliforms, thermotolerant coliforms and *E.coli* are taken as indicators of faecal contamination and this criterion has led to significant improvement in drinking water quality worldwide (WHO, 2006).

The demand of water for more than 16 million population of Karachi city, the industrial hub of Pakistan, (Rahman *et al*., 1997) is presently 601 million gallons per day, whereas studies have shown that due to various reasons bulk supply to the town by the municipal authorities is 293 mgd; thus there is a shortfall of between 260 and 308 mgd potable water for the citizens (Rahman, 2007). Water supply system of Karachi city is 40 years old resulting in corroded and leaking pipes, whereas, wastewater treatment is almost non-existent in the city (Bridges, 2007). Due to shortage of water the supply is mostly intermittent in urban areas. Creation of vacuum in the water supply pipes due to intermittent water supply together with leaky pipes, lead to infiltration of sewage and industrial waste, into drinking water supply system and the drinking water become polluted (Mughal, 2008). The situation is further aggravated during rains and frequent epidemics of gastrointestinal diseases in the city are blamed on to consumption of polluted water. Estimates indicated that annually more then three million Pakistanis become infected with water borne diseases (UNDP-WSP, 2005). The water mainly contains *E.coli*, which is a faecal contaminant, besides the industrial, agrochemical and even radiological population.

In view of this situation of potable water of the Karachi city, microbiological analysis of the samples of consumable water from different localities is important for keeping a vigil on the possible contamination of water, whether at the water treatment plants of the city or elsewhere for taking timely action. Keeping this factor in mind, the present study was undertaken in which a survey of water samples from different sources and localities of Karachi were analyzed to determine the quality of water and present some solutions.

Materials and Methods

In the survey of water, a total of 359 samples of water were randomly collected from different localities of Karachi which included 329 samples of treated (tap) water supplied by municipal authorities and 30 well water samples which were collected from the open wells, usually used by the consumers within the housing localities for drinking purpose.

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Bacteriological analysis of water samples. ***Standard plate count.*** One ml and 0.1 ml of water was assayed, using pour plate procedure with standard agar medium (PCA; Merck) for heterotrophic plate count. A direct colony count was performed after the plate had been incubated for 48 h at 37 °C.

Total coliform count. Water sample (100 ml) was passed through a 0.45 µm cellulose ester nitrate membrane filter and carefully transferred to the surface of lactose triphenyl tetrazolium chloride (LTTC) agar plate and incubated at 36 °C for 24 h.

Orange to yellow colonies with yellow hallow on the back of the plate were counted as thermotolerant coliforms and faecal coliforms which were further streaked on PCA plates. The isolated colonies were examined for oxidase activity.

Thermotolerant coliform count. The same medium used to enumerate total coliforms was used, for the growth of thermotolerant coliforms except that incubation was carried out at 44 °C; after 24 h of incubation, characteristic colonies were counted and inoculated in E.C. medium for thermotolerant coliforms.

WHO standard for drinking water was used for the absence or presence of total coliforms, thermotolerant faecal coliforms and for heterotrophic plate count.

Results and Discussion

The results of the microbiological analysis of 329 treated water and 30 well water samples are given in Fig. 1.

Treated water samples. Out of 329 tap water samples, 94 (28.57%) indicated presence of faecal contaminants and high heterotrophic plate count; 153 (46.5%) had very high HPC and only 82 samples (24.92%) had low HPC with no faecal coliform or their number was within the permissible limit (Fig. 1) as per WHO Guidelines (1993).

Heterotrophic plate count (HPC) is important in assessing the efficiency of water treatment process and also helps in estimation of general hygienic quality of the water (WHO, 2003).

All the organisms that grow best at 37 °C are usually less frequently present in water and their presence in water system indicates their possible access from external source and is a sign of severe pollution (WHO, 2003; Report, 1982). In general, water utilities can achieve heterotrophic bacterial concentration of 10 colony forming units (CFU) per millilitre or less in finished water (Fox and Reasoner, 1999). Heavy fluctuations in HPC in the treated water samples indicate

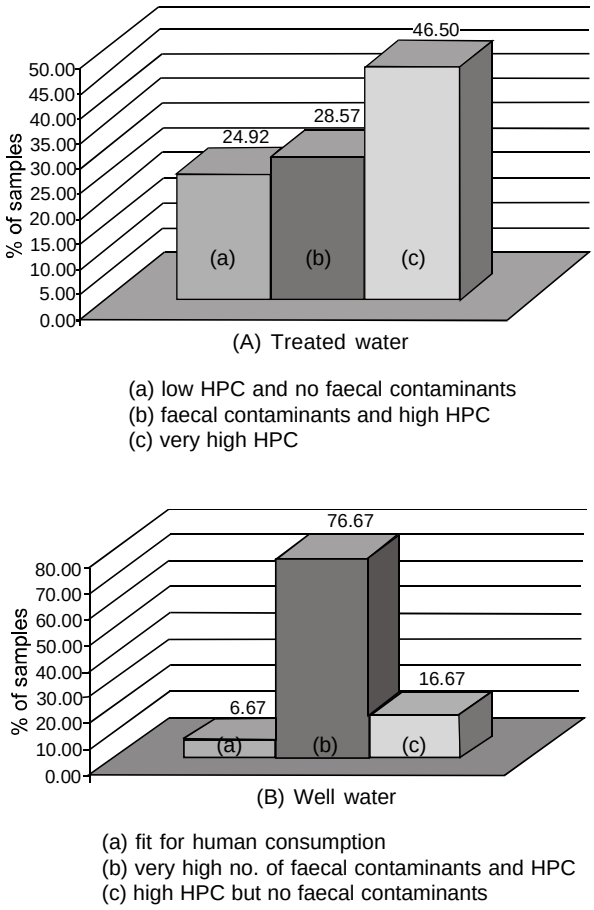

Fig. 1. Microbiological analysis.

severe ingress of faecal pollution in the treatment process (WHO, 2003).

Several species of pathogenic bacteria form part of HPC such as *Flavobacterium*, *Pseudomonas*, *Citrobacter*, *Staphylococcus*, *Aeromonas*, *Alcaligenes*, *Acinetobacter*, *Klebsiella*, *Chromobacterium*, and many others in addition to a range of unidentifiable microorganisms (Bitton, 1994). HPC tests recover a broad range of bacterial species, some of which may be opportunistic bacterial pathogens.

Pseudomonas aeruginosa is most frequently associated with many pathologies, such as urinary tract infections, respiratory diseases, ear and eye infections as well as a range of systemic diseases, such as bacterimia, osteomylites and meningitis (Pollack, 2000).

Aeromonas hydrophila, an additional heterotrophic bacterium that is capable of growing in a distribution system, has been placed on the U.S. Environmental Protection Agency's Contaminant Candidate List. Microorganisms listed on the Contaminant Candidate List are those that are potential health

risks through drinking water and need to be evaluated for possible regulation (US EPA, 2005).

In the present study, 46% samples of treated water had high HPC indicating inadequate treatment of water at the treatment plants. It is therefore, important that such sources of contamination should be located and proper precautionary measures should be taken to avoid such heavy contamination of the drinking water supply.

The drinking water should be suitably treated to follow the accepted standards. Essential health standards do not allow *E. coli* or thermotolerant coliform bacteria to be detectable in 100 ml sample of drinking water (Adams *et al*., 2008). The presence of faecal pollutants in water indicates its inadequate treatment requiring immediate action (Dufour *et al*., 2005).

Microbial and other indicator analysis will be a major source of evidence for qualitative risk assessment. Enumeration of several specific pathogens such as detection of *E. coIi*, total coliforms in either source water or treated water indicates that the water is subject to contamination from human or animal faeces.

In the present study, analysis of treated (tap) water of Karachi city indicates the drinking water to be highly polluted. The presence of faecal coliforms in drinking water may be due to insufficien treatment of water at the plants or their ingress in the supply lines due to out-lived pipe system as indicated by the UNDP-WSP (2005). In any case the situation requires immediate investigation and corrective action.

A regular survey for the source of contamination and relevant monitoring programme should not only cover regular sampling but also be designed to examine high risk factors such as rainfall or the presence of vulnerable sites within the ground water abstracting system. As it is reported non-microbial parameters can also be used for ground water contamination monitoring and give information about the possible potential risk (Beaudeau, 2002; Dangendorf *et al*., 2002).

Well water samples. In the present study, well water samples were collected from different localities of the city, where people are using drinking water of wells due to nonavailability or insufficient supply of treated water. From the presented data in Fig. 1(B), it can be observed that among 30 well water samples only 2 (6.67%) were found fit for human consumption while 23 samples (76.67%) were rejected due to very high faecal pollution and high HPC. Five samples (16.67%) were rejected due to the high HPC only but in which faecal coliforms were not present.

There can be several reasons for groundwater contamination. In USA the most frequent reported source of ground water contamination in 1984, was the septic tank system which had high volume of untreated waste water, discharged into ground water (Hagedorn, 1984). Another active source of ground water contamination is the use of wastewater for crop irrigation. The potential risk of contamination of water source from sewage sludge disposal is well recognized (Mara and Cairncross, 1989). According to Rolland *et al*. (1983), the main source of ground water pollution are manures which mixed with surface water percolate to the ground water. These all may be the possible reasons for faecal contamination of ground water. Rejection of 76.6% samples of water due to high contamination with total coliforms and thermotolerant coliforms (faecal coliforms) and elevated HPC indicates the possible leakage of sewage in ground water.

The data of well water samples with such a heavy faecal contamination of ground water when correlated with the bacteriological analysis of treated water reflects the inadequate treatment given to the sourced water and also possible ingress of contaminated water into the drinking water at the site of treatment plant or into the supply system due to faulty lines. Such heavy contamination of drinking water poses severe health hazards due to consumption of highly polluted water especially by immunocompromised population (Aziz, 2005).

Mismanagement of water is also an important issue. It has been reported that in the third world countries, farmers are using more then required water for irrigation purpose resulting in loss of thousands of gallons of water in the irrigation process, further endangering the situation (PARC, 1982). It is further reported that there is an annual loss of 35 MAF in ground water seepage leading to the rise of under-ground water table and its mixing with the surface water and untreated water due to which several water related diseases have become very prevalent in the Sindh province of Pakistan. The meager health sector spending by the government is worsening the state of affairs which is bound to create havoc in terms of public health and safety.

Conclusion

On the basis of the data of bacteriological analysis of the treated water and well water samples of Karachi city, it can be concluded that the situation is alarming requiring immediate attention and investigation, pinpointing the causes and providing the solution. Adequate treatment should be given to drinking water in treatment plants; contamination of water during the treatment process should be checked and ingress of pollution in the system should be closely monitored and curative measures be introduced. Moreover, policies may be devised for proper management of water.

References

Adams, J., Bartram, J., Chartier, Y. 2008. *Essential Environmental Health Standards in Health Care*, 57 p., WHO Press, Geneva, Switzerland.

Aziz, J.A. 2005. Management of source and drinking-water quality in Pakistan. *East Mediterr. Health J.* **1:** 1087-1098.

Beaudeau, P. 2002. Time series analysis. In: *Drinking Water and Infectious Disease*: *Establishing the Links*. P. R. Hunter, M. Waite and E. Ronchi (eds.), pp. 155-163. CRC Press, Boca Raton, Florida, USA.

BGOSHU, 2000. *The Investigation Report on Walkerton Outbreak of Waterborne Gastroenteritis*, 58 p. Bruce-Grey-Owen Sound Health Unit, Ontario, Canada.

Bitton, G. 1994. *Wastewater Microbiology*, Wiley-Liss Pub., New York, USA.

Bridges, G. 2007. *Asian Water Development Outlook 2007*, Country Paper, Pakistan. Asian Development Bank (ADB).

Collin, J.F., Milet, J.J., Morlot, M., Foliguet, J.M. 1981. Eau d'adduction et gastroenterites en Meurthe-e-moselle. *J. Franc. Hydrologe* **12:** 155-174.

Dangendorf, F., Herbst, S., Exner, M., Kristemann, T. 2002. Geographical information system. In: *Drinking Water and Infectious Disease*: *Establishing the Links*, P. R. Hunter, M. Waite and E. Ronchi (eds.), pp. 143-153. CRC Press, Boca Raton, Florida, USA.

Dufour, A.I., Snozzi, M., Koster, W., Bertran, J., Ronchi, E., Fewtrell, L. (eds.), 2005. *Assessing Microbial Safely of Drinking Water*: *Improving Approaches and Methods*, IWA, Publishing, London, UK.

Fox, K.R., Reasoner, D.J. 1999. Water quality in source water, treatment and distribution system. In: *AWWA Manual of Water Supply and Practices. AWWA M48, Water Borne Pathogens*, American Water Works Association, Denver, CO., USA.

Geldreich, E.E. 1966. *Sanitary Significance of Fecal Coliforms in the Environment*, Water Pollution Control Research Publ. No. WP-20-3. US. Department of the Interior, Federal Water Pollution Control Administration, Washington DC., USA.

Hagedorn, C. 1984. Microbiological aspects of groundwater pollution due to septic tanks. In: *Groundwater Pollution Microbiology*, G. Bitton and C. P. Gerba (eds.), pp. 181-195, Jhon Wiley and Sons, New York, USA.

Hunter, P.R. 1997. *Waterborne Diseases*: *Epidemiology and Ecology*, Jhon Wiley and Sons, New York, USA.

Mara, D.D., Cairncross, S. 1989. *Guidelines for the Safe Use of Wastewater and Excreta in Agriculture*: *Measures for Public Health Protection*, World Health Organization, Geneva, Switzerland.

Mead, P.S., Slutsker, L., Dietz, V., McCraig, L.F., Bresee, J.S., Shapiro, C., Griffen, P.M., Tauxe, R.V. 1999. Food-related illness and death in the United States. *Emerg. Infect. Dis.* **5:** 607-625.

Mughal, F.H. 2008. Opinion: Not a drop to drink. *The Daily Dawn, Karachi*, August 20, 2008.

PARC, 1982. *National Action Programme to Combat Desertification in Pakistan*, PARC, MELGRD, UNEP and ESCAP, August, 1982.

Pollack, M. 2000. *Pseudomonas aeuroginosa*. In: *Principles and Practice of Infectious Diseases*, G. L. Mandel, J. E. Bennett and R. Dolin (eds.), pp. 2310-2335, 5th edition, Churchill Livingstone, Philadelphia, USA.

Pruss, A., Kay, D., Fewtrell, L., Bartram, J. 2002. Estimating the burden of disease from water sanitation and hygiene at a global level: *Environ. Health Persp*. **110:** 537-542.

Rahman, P. 2007. *Water Supply in Karachi*: *Situation, Priority Issues and Solutions*, A Report by Orangi Pilot Project, Karachi, Pakistan.

Rahman, A., Lee, H.K., Khan, M.A. 1997. Domestic water contamination in rapidly growing mega cities of Asia: Case of Karachi, Pakistan. *Environ. Monit. Asses.* **44:** 339-360.

Report, 1982. *The Bacteriological Examination of Drinking Water Supplies*, Reports on Public Health and Medical Subjects No.71, HMSO, London, UK.

Rolland, D., Hartemann, P., Joret, J.C., Hassen, A., Foliguet, J. M. 1983. Evaluation of the load of enteroviruses in a biological wastewater treatment plant. *Water Sci. Tech.* **15:** 115-121.

UNDP-WSP, 2005. *Managing Karachi's Water Supply and Sanitation Services*: *Lessons from a Workshop*, Water and Sanitation Program, Working Paper Series, Report No. 34443.

US EPA 2005. *Drinking Water Contaminant Candidate List 2 and Regulatory Determinations*. US EPA, Office of Ground Water and Drinking Water, Washington, DC., USA.

WHO, 2006. *WHO Drinking Water Quality Series*, IWA Publishing, London, UK.

WHO, 2004. *Guidelines for Drinking Water Qualily*, vol. **I,** *Recommendations*. 3rd edition, WHO, Geneva, Switzerland.

WHO, 2003. *Heterotrophic Plate Count and Drinking Water Safety*: *The Significance of HPCs for Water Quality and Human Health*, J. Bartram, J. Cotruvo, M. Exner, C. Fricker and A. Glasmacher (eds.), WHO, IWA Publishing, London, UK.

WHO, 1993. *Guidelines for Drinking Water Quality*, *Recommendations*, vol. **1,** 2nd edition, World Health Organization, Geneva, Switzerland.

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Respiration, Quantum Yield of Photosynthesis and Transpiration of Two Mungbean Genotypes Differing in Salt Tolerance

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Abstract. The mechanism of salt tolerance in relation to individual leaf growth, transpiration, dark respiration rate, and quantum yield was studied at 0, 50 and 100 mM NaCl solution. At high salt concentration the leaves of BM21 showed higher reduction in growth than those of BM 01. The relative reduction (% of control) of Tr and Dr were less in BM01 than in BM21. The dark respiration rates were less than 1 µmol (CO_2)/m^2/s and accounted for 19 and 32% increase (% of control) at the highest PFD in BM01, while it was 31 and 44% in BM21, respectively at 50 and 100 mM NaCl. More reduced quantum yield in BM21 than BM01 reflects more maintenance costs of energy in repairing injured tissue, enzyme reactions and ion movement.

Keywords: salt tolerance, mungbean, respiration, quantum yield, transpiration

Introduction

The basis of decline in plant growth under saline conditions is poorly understood. It has been suggested that decrease in growth with salinity may be due to increased respiration rate resulting from higher energy requirement (Orcutt and Nilsen 2000; Schwarz and Gale, 1981; Gale, 1975) and reduction of quantum yield of photosynthesis (Seemann and Critchley 1985; Yeo, 1983). Some persons have attributed the depression in growth rate to the combined effects of salinity on photosynthesis and the pattern of carbon allocation (Masojidek and Hall, 1992; Brugnoli and Lauteri, 1991). Another possibility is that increased salinity reduces the photosynthetic surface area available for CO_2 assimilation, i.e., the salinity may reduce the expansion of the leaf surface (Orcutt and Nilsen, 2000). There is increasing evidence that salinity changes photosynthetic parameters, including osmotic and leaf water potential, transpiration rate and leaf temperature (Faruqui, 2002; Kabir, 2002; Islam, 2001; Sultana *et al.*, 1999). In the present study, we compare the effect of NaCl salinity on the components of carbon accumulation in two mungbean genotypes, BM01 and BM21, which differ in their salinity tolerance: dark respiration, quantum yield, transpiration rate and the extension growth of individual leaves.

Materials and Methods

Mungbean seeds of two genotypes, BM01 (tolerant) and BM21 (susceptible), were sown in earthen pots in the vinylhouse at the Environmental Stress Research Site of the Bangabandhu Sheikh Mujibur Rahman Agricultural University, Gazipur. Each pot was filled with 12 kg air-dried soil. Compost (1/4th of the soil volume) and 0.27, 0.28, 0.20 g urea, TSP and MP, respectively, per pot were uniformly incorporated into the soil.The pots were irrigated with tap water until the seedlings were well established. Afterwards tap water in control group and 12.5 mM NaCl solution in salt-treated groups were applied up to three days and 25 mM for the next three days for hardening of seedlings before applying actual treatments. When the trifoliate appeared i.e., ten days after emergence (DAE), the required amount of respective salt solutions (50 and 100 mM) per treatments were applied to 25 pots till harvest.

Dark respiration (0 µmol/m^2/s) was measured using a portable photosynthesis system (LICOR-6200) assembled with an Infrared Gas Analyzer (IRGA) and data logger following the procedure described by Kubota and Hamid (1992) and the leaf chamber was covered with black cloth. The quantum yield for net CO_2 exchange was derermined at intercellular CO_2 concentration which were saturating for photosynthesis and over a photon flux density (PFD) range of 0 and 50 µ mol/m^2/s (photosynthetically active radiation). Each measurement was replicated 3 times. The light source was artificial halogen lamp (OSRAM, HQI-TS 150/NDL). Leaf area was measured with a leaf area meter (Model AAM-7, Hayashi Dehnco Co Ltd., Tokyo, Japan). Specific leaf area (SLA) for individual leaf was calculated as the dry weight per unit leaf area (Leidi and Saiz, 1997).

Results and Discussion

Leaf growth. Salinity reduced the growth of leaves in both the genotypes (Fig.1) and the reduction was more in BM21 as

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compared to BM01. However, the genotypes exhibited striking difference in leaf appearance in response to the salt stress. Leaf appearance rate of BM01was insensitive to salt stress, while the appearance rate of BM21 decreased due to salinity. Genotype BM21 failed to produce 8th leaf under saline condition. Green leaf area followed the same trend as leaf fresh and dry weight (Fig. 1). Leaf area per plant was higher in BM21 than in BM01 at all the treatments but the reduction due to salinity was higher in BM21 than that in BM01. Green leaf area per plant was reduced by 43% and 56% over the control in BM01 and BM21, at 100 mM NaCl, respectively. It indicated that leaves of BM21 were more sensitive to salinity than those of BM01. BM01 had a higher SLA than genotype BM21 except under control conditions (Fig. 1). The reduction of SLA at 50 mM was 8 and 34% in BM01 and BM21, while it was 24 and 40% at 100 mM NaCl, respectively. Leaf expansion was inhibited less in BM01 as shown by higher SLA, leaf area and total leaf number at harvest. Beadle (1993) and Leidi and Saiz (1997) found higher relative growth rate in genotypes with leaves of higher SLA (less carbon invested per unit

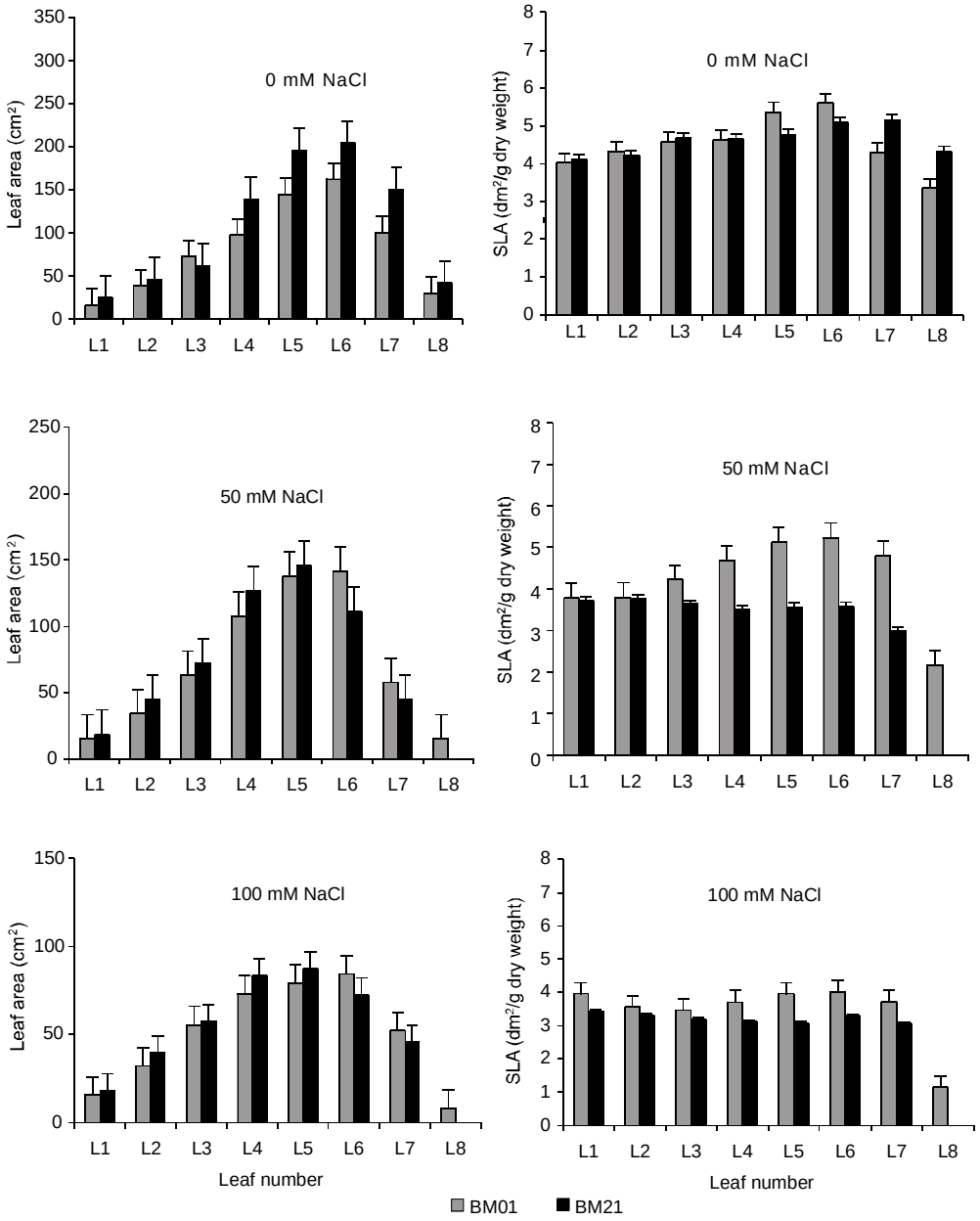

Fig. 1. Leaf area and specific leaf area of individual leaf of BM01 and BM21 as affected by salinity. Error bars represent standard error. Error bars fit within the plot symbol if not shown. SLA = specific leaf area.

of area) under saline condition. This seemed the case of BM01, BM21 under salt stress, as plants of this genotype showed expanding L6, L7 and L8. Genotype BM21 showed a delay in the generation of new leaves with only half of the plants reaching the 7th leaf stage. Sharma (1998) concluded from his study that salinity led to increased leaf diffusive resistance and consequently a decrease in transpiration and increase in leaf temperature. The shift in temperature may play a vital role for tissue desiccation. In addition, increased ionic concentrations in the leaves further aggravated the leaf growth along with tissue dehydration (Leidi and Saiz, 1997; Mangal and Lal, 1988).

Transpiration rate and diffusive resistance. In general, the transpiration rate (Tr) decreased and diffusive resistance (Dr) of both the genotypes increased with increasing levels of salinity (Fig. 2). A weak negative linear relationship ($y = -0.2627x + 3.2283$, $R^2 = 0.4112$) was observed between Tr and Dr. The genotype BM01 showed higher Tr rate and lower Dr than that of BM21 under salt stress condition. The relative reduction (% of control) of Tr and Dr was less in BM01 than that in BM21. Higher transpiration rate of BM01 than that of BM21 indicates that BM01 plants maintained a better water relation than BM21 under salt stress conditions (Orcutt and Nilsen, 2000; Hagemeyer, 1997;). Subbarao *et al.* (1990) observed higher Tr rate in tolerant pigeonpea genotype than salt sensitive one. He reported that the tolerant genotype was able to maintain high transpiration rates, possibly because of the high ability of root system to uptake soil moisture to meet the demands of water required for transpiration under salinity stress. Reduced transpiration rates under salt stress were also observed (Flowers and Yeo, 1995; Munns, 1993; Waisel, 1991).

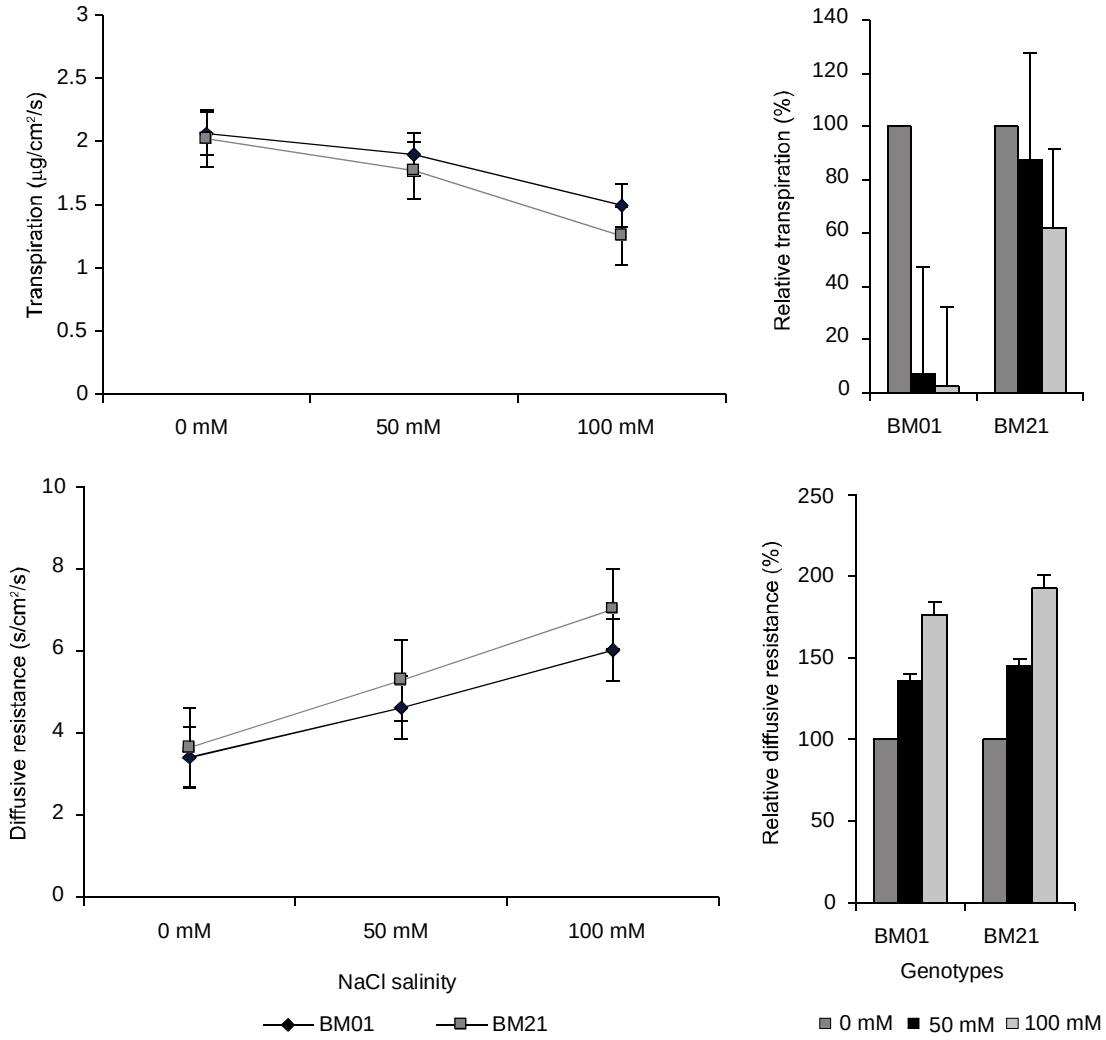

Fig. 2. Transpiration rate, diffusive resistance and their relative values of BM01 an BM21 as affected by salinity. Error bars represent standard error. Error bars fit within the plot symbol if not shown.

Dark respiration rate. The dark respiration rate measured at 0 PFD increased with increasing salinity levels in both the genotypes (Table 1). This increased respiration is generally refered to as salt respiration (Islam, 2001; Datta and Sharma, 1990; Schwarz and Gale, 1981). The respiratory energy may be consumed in repairing the injured tissues and in conducting the active transport processes. Therefore, it has been proposed (Ahmed *et al.*, 1989) that growth inhibition under saline conditions as compared with non-saline condition is partly due to shortage of energy since a considerable amount of energy is consumed in osmotic adjustments. BM21 showed higher relative respiration than BM01. The dark respiration rate was less than 1 µmol $(CO_2)/m^2/s$ and accounted for 19 and 32% increase (% of control) in BM01, while it was 31 and 44% in BM21 at 50 and 100 mM NaCl, respectively. Higher relative respiration in BM21 under salt stress indicates more consumption of respiratory energy in repairing the injured tissues and in conducting the active transport processes. Earlier reports indicated that salinity has little effect on dark respiration of salt tolerant plant (Ahmed *et al.*, 1989; Schwarz and Gale, 1981).

Quantum yield. Quantum yield was estimated from the slope of the PFD response curves in the linear regression between 0 and 50 µmol/m^2/s PFD. A reduction in photosynthetic efficiency with salt stress was observed in the quantum yield for CO_2 fixation (µmol CO_2 fixed/mol absorbed quanta, photosynthetically active radiation) for BM01 and BM21 (Table 1). The quantum yield of leaves from BM01 grown at 100 mM NaCl was approximately 50% below that of control plants, while it was 74% in BM21. Determination of quantum yields at high CO_2 concentration precluded interference from stomatal closure resulting from salinity stress (Seemann and Critchley, 1985; Yeo, 1983). The reduction in quantum yield of photosynthesis with salt stress may constitute at least a partial basis for the reduction in the rate of photosynthesis over the CO_2 saturtion portion of the Pn curve. Salt might have a direct effect upon processes involved in light harvesting, electron transport and/or photophosphorylation resulting in decrease in the quantum efficiency of photosynthesis (Seemann and Critchley, 1985). Another possibility is that the reduced quantum yield reflects energy utilization associated with ion movement. Energy costs of ion movement have been estimated by Yeo (1983), and in some cases it is calculated that ion transport in glycophyte, is in fact, more costly than that in halophyte (Gale *et al.*, 1967). Therefore, more reduced quantum yield in BM21 than BM01 reflects more maintenance costs of energy in repairing injured tissue, enzyme reactions and ion movement (Cheeseman, 1988).

Table 1. Quantum yield (µmol CO_2 fixed/mol absorbed quanta, photosynthetically active radiation (PAR)) and dark respiration of BM01 and BM21 under saline conditions.

NaCl salinity	Quantum yield*		Dark respiration	
(mM)	BM01	BM21	BM01	BM21
0	0.0972	0.1160	-2.82	-3.02
	(100)	(100)	(100)	(100)
50	0.0646	0.0510	-2.28	-2.06
	(66)	(55)	(119)	(131)
100	0.0532	0.0298	-1.97	-1.69
	(50)	(26)	(133)	(144)

* = quantum yield was determined between 0 and 50 µmol/m^2/s at 30 °C and saturating CO_2 (Seemann and Critchley, 1985)

References

Ahmed, A. M., Radi, F. A., Heikal, M. D., Abdel-Basset, R. 1989. Effect of Na-Ca combination on photosynthesis and some related processes of *Chlorella vulgaris. J. Plant Physiol.* **135:** 175-178.

Beadle, C.L. 1993. Growth analysis. In: *Photosynthesis and Productin in a Changing Environment,* D. O. Hall, J. M. O. Scurlock, H. R. Bolhard-Nordenkampf, R. C. Leegood and S. P. Long (eds.), pp. 36-45, Chapman and Hall, London, UK.

Brugnoli, E., Lauteri, M. 1991. Effects of salinity on stomatal conductance, photosynthetic capacity, and carbon isotope discrimination of salt-tolerant (*Gossypium hirsutum* L.) and salt sensitive (*Phaseolus valgaris* L.) C_3 non-halophytes. *Plant Physiol.* **95:** 628-635.

Cheeseman, J. M. 1988. Mechanisms of salinity tolerance in plants. *Plant Physiol.* **87:** 547-550.

Datta, K.S., Sharma, K.D. 1990. Effect of chloride and sulphate types salinity on characteristics of chlorophyll content, photosynthesis and respiration of chickpea (*Cicer arietinum* L.). *Biol. Plant.* **32:** 391-395.

Faruqui, M.A.B. 2002. Effect of Water Stress on Morpho-physiological Changes in *Vigna radiata* L. Wilezek Grown Under Saline Conditions. *M.S. Thesis*, pp. 65-68, Shake Muzibur Rahman Agricultural University, Gazipur, Bangladesh.

Flowers, T.J., Yeo, A. R. 1995. Breeding for salinity resistance in crop plants: Where next? *Aust. J. Plant Physiol.* **22:** 875-884.

Gale, J. 1975. Water balance and gas exchange of plants under saline condition. In*: Plants in Saline Environments*, A. Poljakoff-Mayer and J. Gale (eds.), pp. 168-185, Springer-Verlag, Berlin, Germany.

Gale, J., Kohl, H.C., Hagan, R.M. 1967. Changes in the water balance and photosynthesis of onion, bean and cotton

plants under saline conditions. *Physiol. Plantarum* **20:** 408-420.

Hagemeyer, J. 1997. Salt. In: *Plant Ecophysiology*, M. N. V. Prasad (ed.), pp. 174-205, John Wiley & Sons. Inc., New York, USA.

Islam, M.S. 2001. Morpho-Physiology of Blackgram and Mungbean as Influenced by Salinity. *M. S. Thesis*, pp. 87-92, Shake Muzibur Rahman Agricultural University, Gazipur, Bangladesh.

Kabir, M.E. 2002. Role of Nutrients (N.P and K) on Salinity Tolerance of Mungbean (*Vigna radiata* L Wilczek). *M.S. Thesis*, pp. 75-87, Shake Muzibur Rahman Agricultural University, Gazipur, Bangladesh.

Kubota, F., Hamid, A. 1992. Comparative analysis of the dry matter production and photosynthesis between mungbean (*Vigna radiate* (L.) Wilczek) and black gram (*Vigna mungo* (L.) Hepper) grown in different light intensities. *J. Fac. Agr. Kyushu Univ.* **37**: 71-80.

Leidi, E.O., Saiz, J.F. 1997. Is salinity tolerance related to Na accumulation in upland cotton (*Gossypium hirsutum*) seedlings? *Plant Soil* **190:** 67-75.

Mangal, J.L., Lal, S. 1988. Salt tolerance behaviour of Kharif onion variety N-53. *Haryana J. Hort. Sci*. **17:** 78-82.

Masojidek, J., Hall, D.O. 1992. Salinity and drought stresses are amplified by high irradiance in sorghum. *Photosynthetica* **27:** 159-171.

Munns, R. 1993. Physiological processes limiting plant growth in saline soils: some dogmas and hypotheses. *Plant Cell Environ.* **16: 1**5-24.

Orcutt, D. M., Nilsen, E.T. 2000. Salinity. In: *The Physiology of Plants Under Stress*: *Soil and Biotic Factors*, pp. 177-234, John Wiley & Sons Inc., New York, USA.

Schwarz, M., Gale, J. 1981. Maintenance respiration and carbon balance of plants at low levels of sodium chloride salinity. *J. Exp. Bot.* **32:** 933-941.

Seemann, J.R., Critchley, C. 1985. Effects of salt stress on the growth, ion content, stomatal behaviour and photosynthetic capacity of a salt-sensitive species, *Phaseolus vulgaris* L. *Planta.* **164:** 151-162.

Sharma, S.K. 1997. Plant growth, photosynthesis and ion uptake in chickpea as influenced by salinity. *Indian J. Plant Physiol.* **2:** 171-173.

Subbarao, G.V., Johansen, C., Kumar-Rao, J.V.D.K., Jana, M.K. 1990. Salinity tolerance in F_1 hybrids of pigeonpea and a tolerant wild relative. *Crop Sci*. **30:** 785-788.

Sultana, N., Ikeda, T., Itoh, R. 1999. Effect of NaCl salinity on photosynthesis and dry matter accumulation in developing rice grains. *Environ. Exp. Botany* **42:** 211-220.

Waisel, Y. 1991. Adaptation to salinity. In: *Physiology of Tress*, A.S. Raghavendra (ed.), pp. 375-381, John Wiley and Sons Inc., New York, USA.

Yeo, A.R. 1983. Salinity resistance: physiologies and prices. *Physiol. Plant.* **58:** 214-222.

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Review

Pesticide Immunoassays: Experience and Future Perspective in Pakistan

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Abstract. An overview on the use of immunochemical technology for pesticide residue analysis has been described. Pioneering work on the development of pesticide immunoassays (IA) using ELISA technique in Pakistan has been carried out and described in detail. Dieldrin, atrazine and DDT IAs were performed using Enzyme-Linked Immunosorbent Assay (ELISA) kits to determine residual levels of these pesticides in soil and water. ELISA was found to be successful for dieldrin, atrazine and DDT residue analysis. A highly sensitive in-house ELISA has now been developed for atrazine residues determination. Polyclonal antibodies were raised in rabbits by immunization with an atrazine-BSA conjugate and optimized with atrazine-peroxidase (POD) conjugate. It showed middle of the test (50% B/Bo) at 90 ng/l and lowest detection limit (LDL) at 1ng/l in water. For environmental samples, 50% B/Bo was at 75 ng/l and LDL at 4 ng/l. No cross-reactivities were shown by terbutryn, ametryn, des-isopropylatrazine, de-ethylatrazine except terbuthylazine (17%) and hydroxyatrazine (30%). Data obtained by ELISA, significantly correlated with those obtained by HPLC (r = 0.990). It required no clean-up for water samples and less clean-up steps (than HPLC) in soil/coloured extracts, but showed matrix effect. Validation showed good accuracy and precision thereby suggesting that this test can be applied accurately for atrazine detection in water. This IA experience demands future IAs development in Pakistan for commonly used pesticides.

Keywords: pesticides, immunoassay, HPLC

Introduction

Environmental contamination is recognized as a worldwide problem. Part of this problem is caused by the application of pesticides that are being used in agriculture, public health, forestry and animal husbandry. Pesticides are important in modern farming and remain inevitable for the foreseeable future in order to feed such a large world population applying safe agricultural practices throughout the food chain (Dankwardt, 1999; FAO/IAEA Training and Reference Centre). However, food quality is of equal importance to food quantity. Inherently, pesticides show a certain degree of toxicity and especially the less degradable, more persistent compounds such as organochlorine pesticides pose hazardous problems in the environment (Meastroni *et al*., 2001; 1998; Hock *et al*., 1994; Hussain *et al*., 1994a, b, c, 1995; Dreher and Podratzki, 1988; Fenske and Sternback, 1987; Louis and Kisselbach, 1987; Brook, 1974). Over the past few years, there has been an increasing concern about their pollution potential because of their effects on non-target organisms (Moore and Waring, 2001; Dankwardt *et al*., 1998a; Taguchi and Yakushiji, 1988). This concern is expressed in established regulations, norms, standards, monitoring programmes, acts, etc. (EPA, USA, 2008; GEMS/Food, 1997). The concern seems valid since the huge amount of data, generated over the recent years, showed significant influences on the whole ecosystem including commodities like water, soil, plant and animal tissues and more specifically food (EPA, USA, 2008; Ibitayo and Monosson, 2007; Frank and Wilson, 2004; Dankwardt and Hock, 1997, 1993; Jourdan *et al*., 1996; Lawrence *et al*., 1996; Leavitt *et al*., 1991; Chamberlain, 1990; Bushway *et al*., 1989). Widespread occurrence of pesticides in the evironment requires periodic monitoring during seasonal applications (Greenfield *et al*., 2004; WHO, 1997). However, in Pakistan, which is an agricultural country and a variety of pesticides are being continuously sprayed on a variety of crops to maintain agricultural productivity, no documentation regarding routine environmental monitoring for pesticide residues is made. Only pesticide dosages are standardized according to the targeted pests attack and recommended to the end-users (Saleem and Haq, 2003).

Need to develop an ELISA technique. Keeping in view the persistence, toxicity and common use of pesticides, an efficient methodology for their routine monitoring in water, soil, agricultural produce and other samples, indirectly related to these commodities, is of utmost importance. Much effort is therefore, required in the research and investigation concerning development of the new and improvement of the existing methods for pesticide analysis. Conventional analytical methods used by pesticide residue analysts worldwide involve multi-step sample clean-up procedures followed by

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gas chromatography (GC), gas chromatography-mass spectrometry (GC/MS) or high-performance liquid chromatography-mass spectrometry (HPLC-MS) (Aysal *et al*., 2007; Giorgi *et al*., 2005; Aguera *et al*., 2002; Brady *et al*., 1995; Schews *et al*., 1993; Chamberlain, 1990; Ferris and Haigh, 1987; Vermeulen *et al*., 1982). However, these methods are expensive, sophisticated, time consuming, require highly purified gasses and relatively large volume of solvents involving extraction of large volumes of water, expensive instrumentation, extensive purification, and often derivatization is required. For a developing country like Pakistan, these factors become the major problem for routine environmental monitoring, which involves large number of samples. These methods do not cope with the volume of work if strict check on permissible maximum residual limits (MRL) in the environment is required (Dankwardt, 1999). For this purpose, a sensitive, rapid and most important cost-effective technology should be explored. As a consequence, attention has been directed to newer methods and immunoassay (IA) is likely the best option (Kim *et al*., 2007; Ahn *et al*., 2007a, 2004; Hennion and Barcelo, 1998; Jones *et al*., 1997; Knopp, 1995; Meulenberg *et al*., 1995; Aga *et al*., 1994; Sherry, 1992; Hammock *et al*., 1990).

Immunoassay is not a new technique. In the field of clinical chemistry, it was developed for the measurement of insulin in blood in 1960. Since that time, it had been used for many years in clinical chemistry as a reliable, sensitive, and selective method to determine low concentration of organic compounds, for example, in diabetes, HIV, hepatitis, for analyzing blood, urine, cerebrospinal fluid, saliva, and tissue extracts. Rapid field tests are required to achieve semi quantitative or qualitative results and ELISA offers applicability because immunoassay methods of analysis need equipment that are significantly less complex and less expensive than the chromatographic methods. It is also being used for diagnosis of different animal diseases (e.g., haemorrheagic septicemia) and plants diseases e.g., curl viruses of cotton leaf (CLCV) and potato leaf (PLCV).

As IAs are based on the selective and sensitive antibody-antigen (Ab-Ag) reaction, these have become a valuable tool in the field of environmental analysis; especially for screening a large number of samples within a short time (Hall *et al*., 1997; Jones *et al*., 1997; Kramer and Schmid, 1991; Hammock *et al*., 1990, 1987; Hammock and Mumma, 1980; Kemney and Challacombe, 1989). These assays may require less sample clean-up steps and scale because these are specific tests. IAs are being used for determination of different environmental pollutants like xenostrogens, pesticides or mercury (Schollhorn *et al*., 2000; Marx *et al*., 1998; Marx and Hock, 1998; Oosterkamp *et al*., 1997; Mapes *et al*., 1992). The possibility to adapt IAs for environmental studies was recognized more than a decade ago. Since that time, numerous assays have been developed, of which a great deal refers to pesticides. IAs are becoming more acceptable monitoring tools to monitor pesticide contamination. (Ahn *et al*., 2007b, 2006; Meastroni *et al*., 2001, 1998; Matsui *et al*., 2000; Yazynina *et al*., 1999; Haupt *et al*., 1998; Hennion and Barcelo, 1998; Dankwardt *et al*., 1998a, b; Dankwardt and Hock, 1997).

Principle of ELISA methods. Mostly, pesticide immunoassays are based on competitive ELISA where, antibodies (Abs) are adsorbed to a solid phase (e.g., the cavities of a microtiter plate), in a coating step. Immobilization in this case is based on the passive adsorption of the Abs to a plastic surface e.g., polystyrol. After washing in order to remove unbound molecules, hapten (standard or sample) and enzyme tracer are added and incubated for the immune reaction. Hapten and enzyme tracer both compete for the available Abs-binding sites. Therefore, the less hapten is available in the assay, the more enzyme tracer is bound by the Abs. This competing situation becomes apparent after the washing step that follows. After the subsequent addition of enzyme substrate, the rate of substrate conversion is determined, which is proportional to the concentration of the bound tracer and therefore, inversely proportional to the applied hapten concentration. The concentration of the hapten of unknown samples, therefore, can be determined by means of the calibration curve (Maqbool *et al*., 2008, 2002).

For pesticide immunoassays, polyclonal antibodies (pAb), monoclonal antibodies (mAb) and recombinant antibodies (rAb) have been used so far. Polyclonal antibodies (pAb) are obtained from the serum and comprise a mixture of different Ab populations (Garrett *et al*., 1997; Kramer and Hock, 1996a, b; Hock *et al*., 1994). Monoclonal antibodies consist of a single monospecific Ab population. These Abs are produced in cell culture by single hybridoma cell derived from fusion of B-lymphocytes with myeloma cells (Bryne *et al*., 1996; Kohler and Milestein, 1975). The hydridoma cells can then be propagated almost indefinitely in culture and will continue to produce the Ab of the lymphocyte parent. Since an individual lymphocyte produces only a single Ab type, all of the Ab molecules produced by a hybridoma cell line derived from a single hybrid cell, are identical and have same binding properties. Therefore, the hybridoma technology guarantees the unlimited production of mAb with constant characteristics (Hock *et al*., 1995). Due to great labour required for mAb production, many IA are still employing pAb. During the last years a third possibility to create Ab has emerged, i.e. recombinant Ab (rAb) techniques (Kramer and Hock, 1996a, b;

Chaudhary *et al*., 1995). Here, immunoglobulin genes can be cloned, introduced and expressed in expensive and relatively simple host systems. Although several non-mammalian host systems (yeast, plant and insect cells) have been used to produce rAb, the most common vehicle is *E. coli* (Hall *et al*., 1997; Lee *et al*., 1995).

Nevertheless, ELISA technique has not been used widely for pesticide residue analysis. The reasons include, concerns about interference from substances co-extracted with the pesticide (the so-called matrix effects), cross-reactivity to compounds within a group of related chemicals and doubts that it gives sufficiently quantitative results. But immunoassays (IA) offer advantages over chromatographic methods in terms of cost and simplicity and speed where large number of similar samples are involved. Very sensitive competitive immunoassays have also been developed with detection limits between 1 and 50 mg/l for example for the triazine and urea herbicides (Pichon *et al*., 1995; Schneider *et al*., 1994; Giersch, 1993).

Development of ELISA technique for pesticides. ***Pesticide immunoassays*****.** Commercial ELISA kits are relatively expensive (US $ 300-500 per kit). If single rather than triplicate analyses are carried out, this would give up to 90 samples at a cost of about US $ 5 per sample. But very careful pipetting has to be carried out in order to obtain valid data with single replicates. The current cost of a GC analysis is about US $ 150 per sample. However, the price of a kit may still be quite expensive for developing countries like Pakistan (Dankwardt, 1999). If no kits are commercially available or sample numbers are too great to be screened using costly commercial kits, other sources of antibodies and immuno-reagents have to be found. In this regard, these reagents can be produced at the local laboratory scale. A variety of IA has been developed for pesticides.

Commercial as well as in-house developed assays, had been tested in field as well as in laboratory experiments, by several groups. Different groups working in different parts of the world have used ELISA for monitoring of different classes of pesticides like organochlorinates, organophosphates, carbamates, and pyrethyroid (Kim *et al*., 2007; Gao *et al*., 2006; Park *et al*., 2004; Lee *et al*., 2003, 2002; Watanabe *et al*., 2001; Schneider *et al*., 1994; Francis and Craston, 1994; Itak *et al*., 1993; Gee *et al*., 1988). There may be research groups from universities, which have produced surplus antibodies. An example of this approach is one group in Germany (Hock *et al*., 1994; Kramer *et al*., 1994) which has explored immunoassays for pesticides monitoring. Similarly, an American group of scientists has also developed a variety of ELISAs for a variety of pesticides (Van Emon *et al*., 2008; Ahn *et al*., 2007a, b, 2006, 2004; Mak *et al*., 2005; Shan *et al*., 2004; 2000; Lucas *et al*., 1995; 1993; Schneider *et al*., 1992; Hammock *et al*., 1990, 1987, 1980; Gee *et al*., 1988). Even, in India, a group has developed antibodies for cyclodienes, DDT, DDA, carbendazin/benomyl and others (Skerritt and Rani, 1996).

s-Triazine immunoassays**.** Extensive work on ELISA development for detection/quantification of triazine group of herbicides has also been done (Wittmann and Schmid, 1994; Wittmann and Hock, 1993, 1990; Wust and Hock, 1992; Goh *et al*., 1992). In all of these studies, they have used ELISA using polyclonal or monoclonal antibodies with varying detection limits ranging from 1 µg/l to a maximum of 40 µg/l. Field samples were analyzed for cross-reactivity measurements. Many of the developed ELISAs are also commercially available (Dankwardt *et al*., 1998a; Hennion and Barcelo, 1998).

Atrazine immunoassays**.** Typical laboratory analysis for atrazine involves lengthy solvent extraction followed by gas-liquid chromatography (GLC) (Lee and Chau 1983; Sirons *et al*., 1973) or high performance liquid chromatography (HPLC) analysis (Dicorcia *et al*., 1987; Ferris and Haigh, 1987; Vickery *et al*., 1980). Immunoassay techniques have been developed for atrazine, which are relatively inexpensive and can be quickly conducted in the laboratory or at field locations (Dankwardt *et al*., 1998b; Jiang *et al*., 1995; Muldoon *et al*., 1993; Wust and Hock, 1992; Weller *et al*., 1992; Schneider and Hammock, 1992; Van Beveren and Noji, 1991; Wittmann and Hock, 1990, 1989; Bushway *et al*., 1989, 1988; Schlaeppi *et al*., 1989).

Present status in Pakistan. At present, herbicides are extensively used to control weeds in crops. For these herbicides, many ELISA tests have been developed in the environmental monitoring programme. Interest in the use and development of IA techniques for pesticide residue analysis has grown worldwide over the years, but in Pakistan a little work has been done and IAs have yet to be utilized to their full potential (Maqbool *et al*., 2008, 2002, 1998a, b; Maqbool, 2003; Maqbool and Qureshi, 1994). Pioneer studies were initiated to use commercial ELISA kits for pesticide residue analysis. These kits performed successful standardization and residue analysis of dieldrin, DDT/DDE and atrazine in soil and water (Maqbool *et al*., 1998a, b; Maqbool and Qureshi, 1994). These studies lead to thinking that this cost-effective technique should be developed under local environmental conditions, so that all the virtues of this technology can be fully utilized. Although, commercial ELISA tests are cheaper than GC or HPLC, they are still quite costly for developing countries. Production of polyclonal antibodies against pesticides in a developing country like Pakistan can remarkably reduce the price of ELISA kits (Skerritt and Rani, 1996; Dankwardt *et al*., 1995).

For this purpose, studies were initiated to develop and standardize an in-house ELISA kit for *s*-triazine (specifically atrazine) detection and determination at the residue levels in different environmental samples (Maqbool *et al*., 2002). Atrazine (2-chloro-4-ethylamino-6-isopropylamino-*s*-triazine) is a *s*-triazine herbicide which prevents the growth of susceptible weed species by inhibition of photosynthesis. Depending on the weed infestation, atrazine may be used alone or mixed with other herbicides to control *s*-triazine resistant weeds or grasses. In Pakistan, atrazine is used in combination with different other herbicides like ametryn [2,4-diamine, N-ethyl- N'-(1-methylethyl)-6-(methylthio)-1,3,5, triazine], cyanazine 2-[(4-chloro-6(ethylamino)-1,3,5-triazine-2yl) amino)-methyl-] and metolachlor [2-chloro-N-(2-ethyl-6-methylphenyl)-N-(2-methoxy-1-methylethyl) (Saleem and Haq, 2003). Atrazine is degraded by different mechanisms in water, soil, plant, food and environment and immunoassay has been used for its determination in different types of matrices (Dankwardt *et al*., 1998b, 1994; Hardy and Hurburg, 1994; Wittmann and Hock, 1993; 1990; Winklemann and Klain, 1991; Dunbar *et al*., 1985).

The need for validation of newly developed ELISAs either by HPLC or GC/MS is recognized (Dankwardt *et al*., 1997; Brady *et al*., 1995; Dankwardt *et al*., 1995; Mountfort *et al*., 1994; Thurman *et al*., 1990; Penegelley, 1985). This in-house developed ELISA was also validated by comparing it with ELISA itself, (internal validation) and by HPLC (external validation) for its sensitivity, robustness and reproducibility with HPLC for atrazine residue determination in different environmental samples. The details of these studies are as under:

In-house development of ELISA for atrazine residue determination in Pakistan. A highly sensitive enzyme immunoassay has been developed, optimized and evaluated at the laboratory scale for the detection of atrazine residues. The hapten (atrazine derivative) was conjugated to bovine serum albumin (BSA) to obtain an immunizing antigen and to horseradish peroxidase enzyme (POD) to have enzyme tracer. The formation of these conjugations was confirmed by UV spectroscopy as well as by gel-electrophoresis. Coupling was evaluated by the change in UV absorbance i.e., in conjugate, shifting of the peak to a different position as compared with atrazine derivative and BSA. The absorbance increase depends upon the hapten density after coupling. The molar ratios of the conjugate were determined by UV spectra, assuming that the absorbances of BSA and modified hapten were additive. This conjugation procedure yielded 29-atrazine residues/BSA molecule. With this atrazine-BSA conjugate, polyclonal antibodies were raised in rabbits by immunization. An ELISA on microtitration plates was optimized with peroxidase-atrazine conjugate. The middle of the test (50% B/Bo) was found to be at 90 ng/l, which is well below the maximum concentration permitted by the EC guidelines for drinking water. Detection limits for atrazine of about 1 ng/l could be reached. The assay did not require concentration or clean up steps for drinking or ground water samples. Validation experiments showed a good accuracy and precision. No cross-reactivities were shown by other *s*-triazines like terbutryn, ametryn, terbuthylazine, desisopropylatrazine, de-ethylatrazine except terbuthylazine (17%) and hydroxyatrazine (30%) (Maqbool *et al*., 2002).

Internal (by ELISA itself) as well as external validations (by HPLC) of this developed ELISA showed good accuracy and precision for reproducibility. In-house developed ELISA was standardized to monitor atrazine residues in different environmental samples. The standard curve was linear, indicating an increase in log concentration with decrease in absorbance (%B/B0 = 1.075-0.042 Log C; r = -0.966). The middle of the test was at 75 ng/l and the lowest detection limit at 4 ng/l. ELISA significantly correlated with the high performance liquid chromatography (HPLC) (r = 0.990). Internal validation showed good accuracy and precision. Maximum atrazine residues were present in Jehlum River water/sediments and maize/sugarcane plant roots. Most of the food samples were found contaminated. ELISA required less clean-up steps than HPLC, but showed matrix effect in soil/coloured extracts (Maqbool *et al*., 2008).

It is concluded that the newly developed ELISA is sensitive, reproducible, cheaper and require fewer clean-up steps than HPLC for atrazine monitoring in water and low-coloured sample extracts. It significantly correlated with HPLC estimates and detected appreciable amounts of atrazine residues in water, sediment, soil, plant portions and food samples. Water samples were analyzed directly. For low-coloured extract, an increase in settling period, and one-step filtration improved ELISA performance, but for highly coloured matrices, repeated clean-up steps and matrix effect calibrations have to be applied. In contrast, HPLC required filtration, centrifugation, and solid-phase extraction for all types of matrices including water. The running cost of in-house developed ELISA is US$ 3 per sample for triplicate analysis. On this cost/benefit basis, an integrated approach is suggested to use ELISA first for screening and only positive samples should be subjected to instrumental techniques like GC, HPLC or GC/MS. Based on these results, it is suggested that this test can be applied to obtain fairly accurate results for atrazine concentration in water samples from different sources.

Recommendations for future. The strength of immunochemical methods lies in the screening of a large number of samples

within a short time at low costs. Therefore, they can be valuable supplements to conventional analytical methods. Important applications are seen in the analysis of ground and drinking water, where matrix effects are seldom observed. Also, food commodities that turn over quickly are ideal targets for IA measurements. Due to the low costs of one analysis, more replicates from one site can be measured or special sites can be sampled more often, enabling more information about variations of analyte concentrations over sites and seasons (Dankwardt, 1999).

Some restrictions are imposed by the fact that IA is single analyte method. However, new approaches for multianalyte measurements are being undertaken, such as the integration of IA with liquid chromatography (LC). Here, Abs are used in conjunction with LC, e.g., to concentrate an analyte from a large volume of sample and separate it from an interfering matrix (Katmeh *et al*., 1997; Lawrence *et al*., 1996; Kramer *et al*., 1994). In this case, LC uses an immunoadsorbent column before analysis. The immunoadsorbent column contains immobilized specific Abs, which bind the analyte, while interfering substances pass through. The analyte can be eluted by using a pH gradient or an organic solvent (Marx and Geirsch, 1995; Pichon *et al*., 1995). Therefore, large sample volumes with low concentrations of the analyte can be reduced to small volumes with sufficiently high concentrations without co-extracting interfering substances like humic acids or food compounds. This raises the effective sensitivity of the analysis. Antibody mixtures can be used to bind substances from different compound classes, e.g., the phenyl urea herbicides and the triazines. Immunoadsorbents are now commercially available.

When cross-reacting Abs are applied in IA, the obtained signal is not only related to the analyte, but also to similar compounds. This problem can be circumvented by the use of LC prior to the IA. LC-IA was applied by Kramer *et al*. (1994), to determine 4-nitrophenols. The nitrophenols were separated with different LC-systems and determined by IA. LC-IA was about 8-10 times more sensitive than LC with UV detection. Therefore, the integration of LC with IA combines the high separation quality of the LC and the sensitivity of an IA (Lucas *et al*., 1995; Frutos and Regnier, 1993). Another approach is seen in the performance of a homogeneous IA without tracer followed by the separation of the bound and the free reaction partners and the quantification of the bound and the free Ab fraction immuno-LC (Dankwardt, 1999; Hock *et al*., 1995). Hybrid LC/MS systems allow the subsequent identification of the bound analyte. The problem of cross-reactivities inherent to all IA is therefore, resolved by the structural analysis of any cross-reacting compound.

Furthermore, multi-analyte systems are under development. One concept is the microspot IA (Ekins *et al*., 1990), which uses many microspots with fluorescence-labelled Abs of different selectivity immobilized on a ship. After incubation with the analyte (antigen or hapten) a fluorescence-labelled tracer Ab is added. The tracer Ab is either directed against the antigen or consists of an anti-idiotype Ab directed against the binding site of the capture antibody. Sensor and tracer Ab carry different fluorescence labels. Therefore, it is possible to determine the amount of analyte bound to the sensor Ab with optical scanning methods by measuring the signal ratio (radiometric assay). Lately, a variety of non-competitive and competitive microspot analysis systems have been developed, mainly related to the medical field (Chu *et al*., 1997), but are clearly of particular importance in areas such as environmental monitoring. Another possibility is the use of cross-reacting Abs for multianalyte detection. Known cross-reactivities of different Abs can be used to calculate various concentrations of different analytes in a sample containing several contaminants (Muldoon *et al*., 1993). The estimation of the individual concentration is carried out by complex calculating procedures, e.g. by neural networks or iterative procedures (Jones *et al*., 1997; Wittmann *et al*., 1997).

Immunochemical analysis is a fast developing field with numerous possibilities for further improvement. Much effort is put into the development of continuous measurements, such a flow injection immunoanalysis (FIIA) and immunosensors (Rigo and Hock, 1997; Kramer, 1996). A quasi-continuous FIIA of pesticides was developed by Kramer and Schmid (1991) on the basis of a competitive IA. Here, the Abs are immobilized on a membrane. The reaction takes place in the membrane reactor, the central part of the flow injection system. All reagents are sequentially added to the reactor and the product is assayed with the aid of a flow fluorimeter. The measuring range of the flow injection analysis almost equals that of the EIA. Wittmann and Schmid (1994) used an Ab column reactor filled with polystyrene or glass beads with the Ab, immobilized via the avidin/biotin system. This system showed a stable Ab activity for a minimum of 500 measuring cycles. Detection limits for atrazine of about 1 ng/l with pAb and 30 ng/l with mAb could be reached.

Important progress is to be expected in the field of immunosensors where the detectors are based on Ab (Rigo and Hock, 1997). Some relatively simple devices are dipsticks or dot-blots and immunofiltration test. The test principle is the same as for the microtiter plate tests but the reaction time is much shorter due to the high surface area of the membrane and the short distance between reaction partners. Application of remission measurements yields a proportional relationship between

analyte and remitted light. By using a pocket reflectometer, this set-up is ideally suited for field-monitoring purposes (Niessner, 1994). An interesting development is liposome-amplified immunomigration strips (Seibert *et al*., 1995; Reeves *et al*., 1995a). They employed liposome-encapsulated markers which act as single-enhancers of the competitive binding reaction instead of enzymes. These devices have been used for the determination of alachlor (Wittman and Schmid, 1994). If a pesticide of interest is conjugated to a lipid, it can also be incorporated into the liposome structure, leading to a competitive liposome IA (Reeves *et al*., 1995b).

In more complicated systems, the immunological recognition system is immobilized in the direct vicinity of a transducer, an electrochemical, and optical or gravimetric device. They respond to chemical compounds or ions and yield electrical signals, which depend on the concentration of the analyte. Immunosensors with piezoelectric crystals as physical sensors are in a relatively advanced state of development (Minnunni *et al*., 1995). They function as microbalances on to which Ab are immobilized. Other physical sensors use optical systems such as surface plasmon resonance (SPR), interferometry or grating couplers (Brecht *et al*., 1995; Bier and Schmid, 1994). A biosensor employing SPR was used for the determination of atrazine. Bier and Schmid used a grating coupler immunosensor for the determination of terbutryn, a triazine herbicide. A detection limit of 15 nmol/l was established.

Also new strategies for Ab production are being developed. Genetically engineered monoclonal Abs appear very attractive because their selectivity and affinity can be tailored by site directed mutations without requiring new immunizations (Barbas and Burton, 1996). Methods are now provided to rapidly isolate desired clones from Ab libraries and to manipulate individual recombinant antibodies (rAb) to match specific demands of environmental analysis. Binding proteins derived from Ab but consisting only of a part of their light or heavy chain and recombinant Ab fragments (Fab) directed against different *s*-triazines, diuron and parathion have been produced (Bryne *et al*., 1996; Ward *et al*., 1993). In several cases, the detection limit of the rAb was the same as with the parent mAb (Garrett *et al*., 1997; Kramer and Hock, 1996a, b; Karu *et al*., 1994). A promising goal is completely synthetic production of binding proteins or other synthetic receptors, which are fitted to the structure of the analyte by molecular design. The use of libraries guarantees to overcome the bottleneck in Ab production. Also, Ab with special properties such as resistance to matrix effects or organic solvent stability can be selected from the libraries, providing an important contribution to the analysis of water, soil and food samples.

All this review of literature strongly recommends that immunoassays have great potential to play a vital role in environmental protection issues. Such new trends are direly needed in a developing country like Pakistan, where the persons directly engaged with practical pesticide use, are commonly illiterate. They are using these toxicants continuously without any proper training. Sometimes, their illiteracy results in misuse of these toxicants ultimately polluting the whole ecosystem. ELISA development studies have great potential to be explored for most commonly used pesticides such as pyrethroids (extensively used for cotton crop production) and herbicides in drinking water. This type of research has been done extensively in the developed countries (Van Emon *et al*., 2008; Ahn *et al*., 2007a, b, 2006, 2004; Mak *et al*., 2005; Shan *et al*., 2004; 2000; Lucas *et al*., 1995, 1993; Schneider *et al*., 1992; Hammock *et al*., 1990, 1987, Gee *et al*., 1988; Hammock and Mumma, 1980) but no work has been done in Pakistan. This is an area to be explored and researched in future.

References

Aga, D.S., Thurman, E.M., Pomes, M.L. 1994. Determination of alachlor and its sulfonic acid metabolite in water by solid-phase extraction and enzyme-linked immunosorbent assay. *Anal. Chem*. **66:** 1495-1499.

Aguera, A., Contreras, M., Crespo, J., Fernandez-Alba, A.R. 2002. Multiresidue method for the analysis of multiclass pesticides in agricultural products by gas chromatography-tandem mass spectrometry. *Analyst* **127:** 347-354.

Ahn, K.C., Ma, S.J., Tsai, H.J., Gee, S.J., Hammock, B.D. 2006. An immunoassay for a urinary metabolite as biomarker of human exposure to the pyrethroid insecticide permethrin. *Anal. Bioanal. Chem*. **384:** 713-722.

Ahn, K.C., Gee, S.J., Kim, H.J., Nichkova, M., Lee, N.A. Hammock, B.D. 2007a. Progress in the development of biosensors for environmental and human monitoring of agrochemicals. In: *Rational Environmental Management of Agrochemicals*: *Risk Assessment, Monitoring and Remedial Action*: *Proceedings of the American Chemical Society Pacifichem 2005, Honolulu, HI, ACS Symposium Series No. 966*, I. R. Kennedy, K. Solomon, S. J. Gee, A. Crossan, S. Wang and F. Sanchez-Bayo (eds.), vol. **966,** pp. 138-154, American Chemical Society, Washington DC., USA.

Ahn, K.C., Lohstroh, P., Gee, S.J., Gee, N.A., Lasley, B., Hammock, B.D. 2007b. High-throughput automated luminescent magnetic particle-based immunoassay to monitor human exposure to pyrethroid insecticides. *Anal. Chem*. **79:** 8883-8890.

Ahn, K.C., Watanabe, T., Gee, S.J., Hammock, B.D. 2004. Hapten and antibody production for a sensitive immunoassay

determining a human urinary metabolite of the pyrethroid insecticide permethrin. *J. Agric. Food Chem.* **52:** 4583-4594.

Aysal, P., Ambrus, A., Lehotay, S.J., Cannavan, A. 2007. Validation of an efficient method for the determination of pesticide residues in fruits and vegetables using ethyl acetate for extraction. *J. Environ. Sci. Health - B*, **42:** 481-490.

Barbas, C.F., Burton, D.R. 1996. Selection and evolution of high-affinity human antiviral antibodies. *Trends. Biotechnol*. **14:** 230-234.

Bier, F.F., Schmid, R.D. 1994. Real time analysis of competitive binding using grating coupler immunosensors for pesticide detection. *Biosens. Bioelectron.* **9:** 125-130.

Brady, J.F., LeMasters, G.S., Williams, R.K., Pittman, J.H., Daubert, J.P., Cheung, M.W., Skinner, D.H., Turner, J.L., Rowland, M.A., Lange, J., Sobek, S.M. 1995. Immunoassay Analysis and gas chromatography confirmation of atrazine residues in water samples from a field study conducted in State of Wisconsin. *J. Agric. Food Chem.* **43:** 268-274.

Brecht A., Piehler, J., Lang, G., Gauglitz, G. 1995. A direct optical immunosensor for atrazine detection. *Anal. Chim. Acta* **311:** 33, 289-299.

Brook, G.T. 1974. Action of chlorinated insecticides In: *Chlorinated Insecticides. Biological & Environmental Aspects*, G. Zweig (ed.), vol. **II,** pp. 63-144, CRC Press Inc., Cleveland, Ohio, USA.

Bushway, R.J., Perkins, B., Savage, S.A., Lekousi, S.J., Fergusson, B.S. 1988. Determination of atrazine residues in water and soil by enzyme immunoassay. *Bull. Environ. Contam. Toxicol*. **40:** 647-654.

Bushway, R.J., Perkins, B., Savage, S.A., Lekousi, S.L., Ferguson, B.S. 1989. Determination of atrazine residues in food by enzyme immunoassay. *Bull. Environ. Contam. Toxicol*. **42:** 899-904.

Bryne, F.R., Grant, S.D., Porter, A.J., Harris, W.J. 1996. Cloning, expression and characterisation of a single-chain antibody specific for the herbicide atrazine. *Food Agric. Immunol*. **8:** 19-29.

Chamberlain, S.J. 1990. Determination of multi-residues in cereal products and animal feed using gel-permeation chromatography. *Analyst* **115:** 1161-1165.

Chaudhary, P.V., Lee, H.A., Hammock, B.D., Morgan, M. 1995. Recombinant antibodies: New tools for immunoassay. In: *New Frontiers in Agrochemical Immunoassay*, D. A. Kurtz, J. H. Skerritt and L. Stanker (eds.), pp. 171-185, AOAC International, Arlington, Viana, USA.

Chu, F.W., Edwards, P.R., Ekins, R.P., Berger, H., Finckh, P., Krause, F. 1997. Microarray-based immunoassays. In: *Immunochemical Technology for Environmental Applications, ACS Symposium Series No. 657*, D. S. Aga and E. M. Thurman (eds.), vol. **657,** pp. 170-184, American Chemical Society, Washington DC., USA.

Dankwardt, A. 1999. *Recommendations About the Use of Immunochemical Methods for the FAO/IAEA Training and References Centre for Food and Pesticide Control*, Report by Sension GmbH, UTG-Centre for Environmental Technologies Am Mittleren Moos 48, 86167 Augsburg, Germany.

Dankwardt, A., Pullen, A.S., Hock. B. 1998a. Immunoassays. Applications for the aquatic environment. In: *Microscale Testing in Aquatic Toxicology-Advances, Techniques and Practice*, P. G. Wells, K. Lee and C. Blaise (eds.), pp. 13-29, CRC Press, Boca Raton, Florida, USA.

Dankwardt, A., Muller, J., Hock, B. 1998b. Stabilization of enzyme immunoassays for atrazine. *Anal. Chim. Acta*. **362:** 35-45.

Dankwardt, A., Hock, B. 1997. Enzyme immunoassays for the analysis of pesticides in water and food. *Food Technol. Biotechnol*. **35:** 165-174.

Dankwardt, A., Thurman, E.M., Hock, B. 1997. Terbuthylazine and deethylterbutylazine in rain and surface water-Determination by enzyme immunassay and gas chromatography/mass spectrometry. *Acta Hydrochim. Hydrobiol*. **25:** 5-10.

Dankwardt, A., Pullen, S., Rauchalles, S., Kramer, K., Just, F., Hock, B., Hofmann, R., Schewes, R., Maidl, F.X. 1995. Atrazine residues in soil two years after the atrazine ban: A comparison of enzyme immunoassay with HPLC. *Anal. Lett*. **28:** 621-634.

Dankwardt, A., Wust, S., Elling, W., Thurman, E.M., Hock, B. 1994. Determination of atrazine in rainfall and surface water in Germany by enzyme immunoassay. *Environ. Sci. Pollut. Res*. **1:** 196-204.

Dankwardt, A., Hock, B. 1993. Rapid immunofiltration assay for the detection of atrazine in water and soil samples. *Biosens. Bioelectron.* **8:** 20-21.

De Frutos, M., Regnier F.E. 1993. Tandam chromatographic-immunological analyses. *Anal. Chem*. **65:** 17A-25A.

Dicorcia, A., Marchetti, M., Samperi, R. 1987. Rapid determination of simazine and atrazine in water at the parts per trillion level. *J. Chromatogr.* **405:** 357-367.

Dreher, R.M., Podratzki, B. 1988. Development of an enzyme-immunoassay for endosulfan and its degradation products. *J. Agric. Food Chem*. **36:** 1072-1075.

Dunbar, B.D., Niswender, G.D., Hudson, J.M. 1985. Antibody for the Detection and Quantification of Atrazine. US Patent 4, 530, 786, 23rd July 1985.

Ekins, P.R., Chu, F.W., Biggart, E.M. 1990. Multianalyte immunoassay: The immunological “compact disk” of the

future. *J. Clin. Immunoassay* **13:** 169-181.

EPA, USA 2008. *Risk Assessment Guidance for Superfund: Human Health Evaluation Manual (Part-A)*, vol. **1,** Office of Emergency and Remedial Response, US. Environmental Protection Agency, Washington DC., 20450, USA.

FAO/IAEA Training and Reference Centre for Food and Pesticide Control (together with Food and Environmental Protection), Vienna, Austria. http://www-naweb.iaea.org/nafa/fep/index.html

Fenske, R.A., Sternback, T. 1987. Indoor air levels of chlordane in residences in New Jersey. *Bull. Environ. Contam. Toxicol*. **39:** 903-910.

Ferris, I.G., Haigh, B.M. 1987. A rapid and sensitive HPLC procedure for the determination of atrazine residues in soil water extracts. *J. Chromatogr. Sci*. **25:** 170-173.

Francis, J.M., Craston, D.H. 1994. Immunoassay for parathion without its removal from solution in hexane. *Analyst* **119:** 1801-1805.

Frank, R., Wilson, K.R. 2004. Pesticide Contamination of Farm Water Supplies: Recommendations on Avoidance, Cleanup and Responsibilities. OMAF Factsheet Order No. 00-099, Replaces OMAF Factsheet, Pesticide Contamination of Farm Water Supplies, Order No. 86-077.

Gao, H., Ling, Y., Xu, T., Zhu, W., Jing, H., Sheng, W., Li, Q.X., Li, J. 2006. Development of an enzyme-linked immunosorbent assay (ELISA) for pyrethroid insecticide cyhalothrin. *J. Agric. Food Chem.* **54:** 5284-5291.

Garrett, S.D., Appleford, D.J.A., Wyatt, G.M., Lee, H.A., Morgan, M.R.A. 1997. Production of a recombinant anti-parathion antibody (Sc-V); stability in methanolic food extracts and comparison of an anti-parathion monoclonal antibody. *J. Agric. Food Chem*. **45:** 4183-4189.

Gee, S.J., Miyamoto, T., Goodrow, M.H., Buster, D., Hammock, B.D. 1988. Development of an enzyme-linked immunosorbent assay for the analysis of thiocarbamate herbicide molinate. *J. Agric. Food Chem.* **36:** 863-870.

GEMS/Food, 1997. Guidelines for predicting dietary intake of pesticide residues (revised), prepared by the Global Environment Monitoring System - Food Contamination Monitoring and Assessment Programme (GEMS/Food), in collaboration with the Codex Committee on Pesticide Residues. Programme of Food Safety and Food Aid, (WHO/FSF/FOS/97.7).

Giersch, T. 1993. A new monoclonal antibody for the sensitive detection of atrazine with immunoassay in microtitre plate and dipstick format. *J. Agric. Food Chem*. **41:** 1006-1011.

Giorgi, M., Cugini, I., Meucci, V., Bestini, S., Giuliani, M., Soldani, G. 2005. New HPLC and GC-MS methods for the investigation of cypermethrin in edible portions of fish: development, validation and comparison. *Vet. Res. Commun*. **29:** 293-295.

Goh, K.S., Richman, S.J., Troiano, J., Garretson, C.L., Hernandez, J., Hsu, J., White, J., Barry, T.A., Ray, M., Tran, D., Miller, N.K. 1992. ELISA of simazine in soil: applications for a field leaching study. *Bull. Envirn. Contam. Toxicol*. **48:** 554-560.

Greenfield, B.K., Wittner, E., David, N., Shonkoff, S., Davis. J.A. 2004. *Monitoring Trace Organic Contamination in Central Valley Fish: Current Data and Future Steps*. Report to the Central Valley Regional Water Quality Control Board. Oakland, CA, SFEI Contribution No. 99, 63 p., San Francisco Estuary Institute, Oakland, CA, USA.

Hall, J.C., O'Brien, G.M., Webb, S.R. 1997. Phage-display technology for environmental analysis. In: *Immunochemical Technology for Environmental Applications, ACS Symposium Series No.657*, D. S. Aga and E. M. Thurman (eds.), vol. **657,** pp. 22-27, American Chemical Society, Washington DC., USA.

Hammock, B.D., Gee, S.J., Harrison, R.O., Jung, F., Goodrow, M.H., Li, Q.X., Lucas, A.D., Szekacs, A., Sundaram, K.M.S. 1990. Immunochemical technology in environmental analysis. Addressing critical problem. In: *Immunochemical Methods for Environmental Analysis, ACS Symposium Series No. 442*, J. M. van Emon and R. O. Mumma (eds.), American Chemical Society, Washington DC., USA.

Hammock, B.D., Gee, S.J., Cheung, P.Y.K., Miyamoto, T., Goodrow, M.H., van Emon, J., Seiber, J.N. 1987. Utility of Immunoassays in pesticide trace analysis. In: *Pesticide Science and Biotechnology*, R. Greenhalgh and T. R. Roberts (eds.), pp. 309-316, Blackwell Scientific Publications, Oxford, UK.

Hammock, B.D., Mumma, R.O. 1980. In: *Pesticide Analytical Methodology, ACS Symposium Series No. 136*, J. Harvey Jr. and G. Zweig (eds.), vol. **136,** pp. 321-352, American Chemical Society, Washington DC., USA.

Hardy, C.L., Hurburg Jr. C.R. 1994. Immunoassay detection of herbicide residues in corn. *Cereal Chem.* **71:** 107-111.

Haupt, K., Dzgoev, A., Mosbach, K. 1998. Assay system for the herbicide 2,4-dichlorophenoxyacetic acid using a molecularly imprinted polymer as an artificial recognition element. *Anal. Chem*. **70:** 628-631.

Hennion, M.C., Barcelo, D. 1998. Strengths and limitations of immunoassays for effective and efficient use for the pesticide analysis in water samples: a review. *Anal. Chim. Acta*. **362:** 3-34.

Hock, B., Dankwardt, A., Kramer, K., Marx, A. 1995. Immunochemical techniques: Antibody production for pesticide

analysis: a review. *Anal. Chim. Acta*. **311:** 393-405.

Hock, B., Giersch, T., Dankwardt, A., Kramer, K., Pullen, S. 1994. Toxicity assessment and on-line monitoring: Immunoassays. *Environ. Toxicol. Water Quality* **9:** 243-262.

Hussain, A., Maqbool, U., Asi, M.R., Mahmood, S.T., Ali, S.W., Naqvi, S.H.M. 1995. Effects of simulated commercial processing procedures on 14C-Dimethoate residues in cottonseed oil. *IAEA TECDOC* **818:** 73-79.

Hussain, A., Maqbool, U., Asi, M. 1994a. Studies on dissipation and degradation of ^{14}C-DDT and ^{14}C-DDE in Pakistani soils under field conditions. *J. Environ. Sci. Health Part B: Pesticides, Food Contaminants and Agricultural Wastes* **B29:** 1-15.

Hussain, A., Tirmazi, S.H., Maqbool, U., Asi, M., Chaughtai, F.A. 1994b. Studies of the effect of temperatures and solar radiation on volatilization, mineralization and binding of ^{14}C-DDT in soil under laboratory conditions. *J. Environ. Sci. Health Part B: Pesticides, Food Contaminants and Agricultural Wastes* **B20:** 141-151.

Hussain, A., Maqbool, U., Asi, M. 1994c. Studies on the dissipation of ^{14}C-DDT from water and solid surfaces. *J. Environ. Sci. Health Part B: Pesticides, Food Contaminants and Agricultural Wastes* **B29:** 177-184.

Ibitayo, O., Monosson. E. 2007. Agricultural pesticide contamination. In: *Encyclopedia of Earth*, C. J. Cleveland (ed.), Environmental Information Coalition, National Council for Science and the Environment, Washington DC,. USA.

Itak, J.A.E.G., Olson, J.R., Fleeker, Herzog, D.P. 1993. Validation of paramagnetic particle-based ELISA for the quantitative determination of carbaryl in water. *Bull. Environ. Contam. Toxicol.* **51:** 260-267.

Jiang, T., Halsall, H.B., Heineman, W.R., Giersch T., Hock, B. 1995. Capillary enzyme immunoassay with electrochemical detection for the determination of atrazine in water. *J. Agric. Food Chem*. **43:** 1098-1104.

Jones, G., Wortberg, M., Rocke, D.M., Hammock, B.D. 1997. Immunoassay of cross reacting analytes. In: *Immunochemical Technology for Environmental Applications, ACS Symposium Series No. 657*, D. S. Aga and E. M. Thurman (eds.), vol. **657,** pp. 331-342, American Chemical Society, Washington DC., USA.

Jourdan, S.W., Scutellaro, A.M., Hayes, M.C., Herzog, D.P. 1996. Adapting immunoassays for the analysis of food samples. In: *Immunoassay for Residues Analysis*: *Food Safety, ACS Symposium Series No. 621*, R. C. Beier and L. H. Stanker (eds.), vol. **621,** pp. 82-98, American Chemical Society, Washington DC., USA.

Karu, A.E., Scholthof, K.B.G., Zhang, G., Bell, C.W. 1994. Recombinant antibodies to small analytes and prospects for deriving them from synthetic combinatorial libraries. *Food Agric. Immunol*. **6:** 277-286.

Katmeh, M.F., Godfrey, A.J.M., Stevenson, D., Aherne, G.W. 1997. Enzyme immunoaffinity chromatography - a rapid semi - quantitative immunoassay technique for screening the presence of isoproturon in water samples. *Analyst*. **122:** 481-486.

Kemeny, D.M., Challacombe, S.J. (eds.) 1989. In: *ELISA and Other Solid Phase Immunoassays*: *Theoretical and Practical Aspects*, John Wiley and Sons Inc., New York, USA.

Kim, H.J., Ahn, K.C., Ma, S.J., Gee, S.J., Hammock, B.D. 2007. Development of sensitive immunoassays for the detection of the glucuronide conjugate of 3-phenoxybenzyl alcohol, a putative human urinary biomarker for pyrethroid exposure. *J. Agric. Food Chem*. **55:** 3750-3757.

Knopp, D. 1995. Application of immunological methods for the determination of environmental pollutants in human biomonitoring. A review. *Anal. Chim. Acta*. **311:** 383-392.

Kohler, G., Milstein, C. 1975. Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* **256:** 495-497.

Kramer, P. 1996. Biosensors for measuring pesticide residues in the environment: Past, Present and Future. *J. Assoc. Off. Anal. Chem.* **79:** 1245-1254.

Kramer, K., Hock, B. 1996a. Recombinant antibodies for pesticide analysis. In: *Residue Analysis in Food Safety, ACS Symposium Series No. 621*, R. C. Beier and L. H. Stanker, (eds.), vol. **621,** pp. 471-484, American Chemical Society, Washington DC., USA.

Kramer, K., Hock, B. 1996b. Recombinant single-chain antibodies against *s*-triazines. *Food Agric. Immunol*. **8:** 97-109.

Kramer, P.M., Li, Q.X., Hammock, B.D. 1994. Integration of liquid chromatography with immunoassay. An approach combining the strength of both methods. *J. Assoc. Off. Anal. Chem*. **77:** 1275-1287.

Kramer, P.M., Schmid, R.D. 1991. Automated quasi-continuous immunoanalysis of pesticides with flow injection system. *Pest. Sci*. **32:** 451-462.

Lawrence, J.F., Menard, C., Hennion, M.C., Pichon, V., Le Goffic, F., Durand, N. 1996. Use of immunoaffinity chromatography as a simplified cleanup technique for liquid chromatographic determination of phenylurea herbicides in plant material. *J. Chromatogr.* **A-732:** 277-281.

Leavitt, R.A., Kells, J.J., Bunkelmann, J.R., Hollingworth, R.M. 1991. Assessing atrazine persistence in soil following a severe drought. *Bull. Environ. Contam. Toxicol*. **46:** 22-29.

Lee, H.J., Shan, G., Ahn, K., Park, E-K., Watanabe, T., Gee, S.J., Hammock, B.D. 2004. Development of an enzyme-linked

immunosorbent assay for the pyrethroid cypermethrin. *J. Agric. Food Chem*. **52:** 1039-1043.

Lee, H.J., Watanabe, T., Gee, S.J., Hammock, B.D. 2003. Application of an enzyme-linked Immunosorbent Assay (ELISA) to determine deltamethrin residues in milk. *Bull. Environ. Contam. Toxicol*. **71:** 14-20.

Lee, H.J., Shan, G., Watanabe, T., Stoutamire, D.W., Gee, S.J., Hammock, B.D. 2002. Enzyme-linked immunosorbent assay for the pyrethroid deltamethrin. *J. Agric. Food Chem*. **50:** 5526-5532.

Lee, H.A., Alcocer, M.J.C., Lacarra, T.G., Jeenes, D.J., Morgan, M.A. 1995. Recombinant antibodies: Expression in *Escherchia coli* using plasmid and phagemid vectors and application to food analytes. In: *New Frontiers in Agrochemical Immunoassay*, D. A. Kurtz, J. H. Skerritt and L. Stanker, (eds.), pp. 187-196, AOAC International, Arlington, Viana, USA.

Lee, H., Chau, A.S.Y. 1983. Determination of trifluralin, diallate, triallate, atrazine, barban, diclofop-methyl, and bezoyl-propyl-ethyl in natural waters at parts per trillion levels. *J. Assoc. Off. Anal. Chem*. **66:** 651-658.

Louis, J.B., Kisselbach, K.C.Jr. 1987. Indoor air levels of chlordane and heptachlor following termiticide application. *Bull. Environ. Contam. Toxicol*. **39:** 911-918.

Lucas, A.D., Gee, S.J., Hammock, B.D., Seiber, J.N. 1995. Integration of immunochemical methods with other analytical techniques for pesticide residue determination. *J. Assoc. Off. Anal. Chem*. **78:** 585-591.

Lucas, A.D., Bekheit, H.K.M., Goodrow, M.H., Jones, A.D., Kullman, S., Matsumura, F., Woodrow, J.E., Seiber, J.N., Hammock, B.D. 1993. Development of antibodies against hydroxyatrazine and hydroxysimazine: applications to environmental samples. *J. Agric. Food Chem*. **41:** 1523-1529.

Maestroni, B.M., Skerritt, J.H., Ferris I.G., Ambrus, A. 2001. Analysis of DDT residues in soil by ELISA: an international inter-laboratory study. *J. Assoc. Off. Anal. Chem*. **84:** 134-142.

Maestroni, B.M., Skerritt, J.H., Ferris, I.G. 1998. Application of competitive enzyme immunoassay for screening DDT and DDE in food and environmental samples: initial collaborative study. In: *Seeking Agriculture Produce Free of Pesticide Residues*, *ACIAR Proceedings No. 85*, I. R. Kennedy, J. H. Skerritt, G. I. Johnson and E. Highly (eds.), vol. **85,** pp. 270-277, Australian Center for International Agricultural Research, Canberra, Australia.

Mak, S.K., Shan, G., Lee,H-J., Watanabe, T., Stoutamire, D.W., Gee, S.J., Hammock, B.D. 2005. Development of a class-selective immunoassay for the type II pyrethroid insecticides. *Anal. Chim. Acta*. **534:** 109-120.

Mapes, J.P., McKenzie, K.D., McClelland, L.R., Movassaghi, S., Reddy, R.A., Allen, R.L., Friedman, S.B. 1992. Penta RISC ™ Soil-A rapid, on-site screening test for pentachlorphenol in soil. *Bull. Environ. Contam. Toxicol*. **49:** 334-341.

Maqbool, U. 2003. Development of ELISA Technique for the Determination of Agrochemical Residues in Pakistani Environment, *Ph.D Thesis*, pp. 1-170, Punjab University, Lahore, Pakistan.

Maqbool, U., Haq, A., Qureshi, M.J. 2008. Comparison of in-house developed ELISA with HPLC technique for the analysis of atrazine residues. *J. Environ. Sci. Health*. Part-B, **43-B:** 224-230.

Maqbool, U., Haq, A., Qureshi, M.J., Iqbal, M.Z., Hock, B. Kramer, K. 2002. Development of ELISA technique for the analysis of atrazine residues in water. *J. Environ. Sci. Health*, Part-B **37:** 307-322.

Maqbool, U., Qureshi, M.J., Haq, A., Iqbal, M.Z. 1998a. Enzyme-Linked Immunosorbent Assay for the determination of dieldrin and atrazine residue in different environmental samples. In: *Seeking Agriculture Produce Free of Pesticide Residues, ACIAR Proceedings No. 85*, I. R. Kennedy, J. H. Skerritt, G. I. Johnson and E. Highly (eds.), vol. **85:** pp. 241-253, Australian Center for International Agricultural Research, Canberra, Australia.

Maqbool, U., Qureshi, M.J., Haq, A., Iqbal, M.Z. 1998b. Immunochemical technology for pesticide residues analysis. In: *Seeking Agriculture Produce Free of Pesticide Residues, ACIAR Proceedings No.85*, I. R. Kennedy, J. H. Skerritt, G. I. Johnson, E. Highly (eds.), vol. **85:** pp. 380-385, Australian Center for International Agricultural Research, Canberra, Australia.

Maqbool, U., Qureshi. M.J. 1994. Evaluation of immunoassay kits for the analysis of pesticide dieldrin residues in soil and water. In: *Recent Trends in Biochemical Research in Pakistan*, Qasim, Hasnain, Ishaq and Azhar (eds.), pp. 207-212, BCCT Press, University of Karachi, Karachi, Pakistan.

Marx, A., Hock. B. 1998. Characterization of a new monoclonal antibody against Mercury (II). *Anal. Lett*. **31:** 1663-1650.

Marx, A., Kroetz, E., Hock, B. 1998. Improvement of an enzyme immunoassay for the determination of Mercury (II). *Anal. Lett*. **31:** 1651-1661.

Marx, A., Geirsch, T. 1995. Immunoaffinity chromatography of *s*-triazines. *Anal. Lett*. **28:** 267-278.

Matsui, J., Fujiwara, K., Takeuchi, T. 2000. Atrazine-selective polymers prepared by molecular imprinting of trialkylmelamines as dummy template species of atrazine. *Anal. Chem*. **72:** 1810-1813.

Meulenberg, E.P., Mudler, W.H., Stocks, P. G. 1995. Immunoassay for pesticides. *Environ. Sci. Technol*. **29:** 553-561.

Minnunni, M., Mascini, M., Guilbault, G.G., Hock, B. 1995. The quartz crystal microbalance as biosensor. A status report on its future. *Anal. Lett*. **28:** 749-764.

Moore, A., Waring, C.P. 2001. The effects of a synthetic pyrethroids pesticide on some aspects of reproduction in Atlantic salmon (*Salmo salar* L.). *Aquat. Toxicol*. **52:** 1-12.

Mountfort, K.A., Reynolds, S.L., Thorpe, S.A., White, S.N. 1994. Comparison of ELISA with HPLC techniques for the analysis of carbendazim and thiabendazole residues in fruits and vegetables. *Food Agric. Immunol*. **6:** 17-22.

Muldoon, M.T., Fries, G.F., Nelson, J.O. 1993. Evaluation of an ELISA for the multianalyte analysis of s-triazines in pesticide waste and rinsate. *J. Agric. Food Chem*. **41:** 322-328.

Niessner, R. 1994. Immunoassays in environmental analytical Chemistry: Some thoughts on trends and status. *Anal. Meth. Instr*. **1:** 134-144.

Oosterkamp, A.J., Hock, B., Seifert, M., Irth, H. 1997. Novel monitoring strategies for xenoestrogens. *Trends Anal. Chem*. **16:** 544-553.

Park, E.K. Kim, H.J., Gee, S.J., Watanabe, T., Ahn, K.C., Hammock, B.D. 2004. Determination of pyrethroids residues in agricultural products by an enzyme-linked immunosorbent assay. *J. Agric. Food Chem*. **52:** 5572-5576.

Pengelley, W.L. 1985. Validation of immunoassay. In: *Plant Growth Substances*, M. Bopp (ed.), pp. 35-43, Springer Verlag, Berlin, Germany.

Pichon, V., Chen, L., Hennion, M.C. 1995. On-line preconcentration and liquid chromatographic analysis of phenylurea herbicides in environmental water using a silica-based immunosorbent. *Anal. Chim. Acta*. **311:** 429-436.

Reeves, S.G., Seibert, S.T.A., Roberts, M.A., Durst, R.A. 1995a. Liposome immunosensing devices for environmental contaminant screening. *Trends Anal. Chem*. **14:** 351-355.

Reeves, S.G., Seibert, S.T.A., Durst, R.A. 1995b. Liposome-amplified immunoanalysis for pesticides. In: *Immunoanalysis of Agrochemicals, Emerging Technologies, ACS Symposium Series No. 586*, J. O. Nelson, A., Kar and R. B. Won (eds.), vol. **586,** pp. 210-222, American Chemical Society, Washington DC., USA.

Rigo, A., Hock, B. 1997. On-line analysis with immunosensing devices. *GIT Lab. J*. **1:** 22-24.

Saleem, A., Haq, I. 2003. *Pesticides Standardized in Punjab: Dose(s), Crop(s) and Target Pests(s)*, vol. **1,** pp. 7-18, Agriculture Department, Government of Punjab, Pakistan.

Schlaeppi, J.M., Fory, W., Ramsteiner, K. 1989. Hydroxyatrazine and atrazine determination in soil and water by enzyme-linked immunosorbent assay using specific monoclonal antibodies. *J. Agric. Food Chem*. **37:** 1532-1538.

Schews, R., Maidl, F.X., Fischbeck, G., Gleissenthall, J.L., Suss, A. 1993. Trace determination of weathered atrazine and terbuthylazine and their degradation products in soil by high performance liquid chromatography-diode-array detection. *J. Chromatogr.* **641:** 89-93.

Schneider, P., Goodrow, M.H., Gee, S.J., Hammock, B.D. 1994. A highly sensitive and rapid ELISA for the aryl urea herbicides diuron, monouron and liuron. *J. Agric. Food Chem*. **42:** 413-422.

Schneider, P., Hammock, B.D. 1992. Influence of the ELISA format and the hapten enzyme conjugate on the sensitivity of immunoassay for *s*-triazine herbicides using monoclonal antibodies. *J. Agric. Food Chem.* **40:** 525-530.

Schollhorn, B., Maurice, C., Flohic, G., Limoges, B. 2000. Competitive assay of 2,4-dichlorophenoxyacetic acid using a polymer imprinted with an electrochemically active tracer closely related to the analyte. *Analyst*. **125:** 665-667.

Seibert, S.A., Reeves, S.G., Roberts, M.A., Durst, R.A. 1995. Improved liposome immunomigration strip assay for alachlor determination. *Anal. Chim. Acta* **311:** 309-318.

Shan, G., Huang, H., Stoutamire, D.W., Gee, S.J., Leng, G., Hammock, B.D. 2004. A sensitive class-specific immunoassay for the detection of pyrethroids metabolites in human urine. *Chem. Res. Toxicol*. **17:** 218-225.

Shan, G., Leeman, W.R., Stoutamire, D.W., Gee, S.J., Chang, D.P.Y., Hammock, B.D. 2000. Enzyme-linked immunosorbent assay for the pyrethroid permethrin. *J. Agric. Food Chem*. **48:** 4032-4040.

Sherry, J. 1992. Environmental chemistry: The immunoassay option. *Crit. Rev. Anal. Chem*. **23:** 217-300.

Sirons, G.J., Frank, R., Sawyer, T. 1973. Residues of atrazine, Cyanazine and their phytotoxic metabolite in a clay loam soil. *J. Agric. Food Chem*. **21:** 1016-1020.

Skerritt, J., Rani, B.E.A. 1996. Detection and removal of sample matrix effects in agro chemicals immunoassays. In: *Residue Analysis in Food Safety, ACS Symposium Series No. 621*, R. C. Beier and L. H. Stanker (eds.), vol. **621,** pp. 29-43, American Chemical Society, Washington DC., USA.

Taguchi, S., Yakushiji, T. 1988. Influence of termite treatment in the home on chlordane concentration in human milk. *Arch. Environ. Contam. Toxicol*. **17:** 65-71.

Thurman, E.M., Meyer, M.T., Pomes, M., Perry, C. A., Schwab, A.P. 1990. Enzyme-Linked Immunosorbent assay compared with gas chromatography/mass spectrometry for the determination of triazine herbicides in water. *Anal. Chem*. **62:** 2043-2048.

Van Beveren, J., Noji, T. 1991. Preliminary investigations regarding immunoassays for triazines in water. *KIWA Rep*. **91:** 137.

Van Emon, J.M., Chuang, J.C., Lordo, R.A., Schrock, M.E., Nichkova, M. Gee, S.J, Hammock, B.D. 2008. An enzyme-linked immunosorbent assay for the determination of dioxins in contaminated sediment and soil samples. *Chemosphere* **72:** 95-103.

Vermeulen, N.M.J., Apostolides, Z., Potgieter, D.J.J., Nel, P.C., Smith, N.S.H. 1982. Separation of atrazine and some of its degradation products by high performance liquid chromatography. *J. Chromatogr*. **240:** 247-253.

Vickery, T.M., Karlesky, D.L., Blachmer, G.L. 1980. Colorimetry and high performance liquid chromatography of atrazine residues in soil: comparison of methods. *J. Assoc. Off. Anal. Chem*. **63:** 506-510.

Ward, V.K., Schneider, P.G., Kreissig, S.B., Hammock, B.D., Choudhry, P.V. 1993. Cloning, sequencing and expression of the Fab fragment of a monoclonal antibody to the herbicide atrazine. *Protein Eng*. **6:** 981-988.

Watanabe, T., Shan, G., Stoutamire, D.W., Gee, S.J., Hammock, B.D. 2001. Development of a class-specific immunoassay for type I pyrethroid insecticides. *Anal. Chim. Acta*. **444:** 119-129.

Weller, M.G., Weil, L., Niessner, R. 1992. Increased sensitivity of an enzyme immunoassay (ELISA) for the determination of triazine herbicides by variation of tracer incubation time. *Microchim. Acta* **108:** 29-40.

WHO, 1997. Guidelines for predicting dietary intake of pesticide residues (revised). In: *World Health Organization, Programme of Food Safety and Food Aid*. https://www.who.int/foodsafety/publications/chem/en/pesticide_en.pdf

Winklemann, D.A., Klain, S.J. 1991. Degradation and bound residue formation of atrazine in western Tennessee soil. *Environ. Toxicol. Chem*. **10:** 335-345.

Wittmann, C., Schmid, R.D., Loffler, S., Zell, A. 1997. Application of a neutral network for pattern recognition of pesticides in water samples by different immunochemical techniques. In: *Immunochemical Technology for Environmental Applications, ACS Symposium Series No. 657*, D. S. Aga and E. M. Thurman, (eds.), vol. **657,** pp. 343-360, American Chemical Society, Washington DC., USA.

Wittmann, C., Schmid, R.D. 1994. Development and application of an automated quasi-continuous immunoflow injection system for the analysis of pesticide residues in water and soil. *J. Agric. Food Chem*. **42:** 1041-1047.

Wittmann, C., Hock, B. 1993. Analysis of atrazine residues in food by enzyme immunoassay. *J. Agric. Food Chem*. **41:** 1421-1425.

Wittmann, C., Hock, B. 1990. Evaluation and performance characteristics of a novel ELISA for the quantitative analysis of atrazine residues in water, plant and soil. *Food Agric. Immunol*. **2:** 65-74.

Wittmann, C., B. Hock. 1989. Improved enzyme immuunoassay for the analysis of *s*-triazines in water samples. *Food Agric. Immunol*. **1:** 211-224.

Wust, S., Hock, B., 1992. A sensitive enzyme immunoassay for the detection of atrazine, based upon sheep antibodies. *Anal. Lett.* **25:** 1025-1037.

Yazynina, E.V., Zherdev, A.V., Dzantiev, B.B., Izumrudov, V.A., Gee, S.J., Hammock, B.D. 1999. Immunoassay techniques for the detection of the herbicide simazine based on use of oppositely charged water-soluble polyelectrolytes. *Anal. Chem*. **71:** 3538-3543.