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Physical Sciences

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Synthesis and Spectral Studies of Some Novel Coumarin Based Disperse Azo Dyes

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Abstract. Synthesis of some novel coumarin based azo dyes was carried out by diazotization of heterocyclic amines using nitrosyl sulphuric acid and then coupling them with 7-hydroxy-4-methyl Coumarin. The synthesized dyes when applied on polyester fibers showed moderate to good light fastness and very good to excellent fastness to washing, rubbing, perspiration and sublimation.

Keywords: disperse azo dyes, coumarin-based-azo dyes, heterocyclic amines

Introduction

Azo dyes are the most widely used class of the dyes due to their versatile application in various fields such as the dyeing of textile fibers, coloring of plastics and advanced applications in the organic synthesis. Many patents and papers describing the synthesis and dyeing properties of azo compounds have been previously presented (Huang, 2008; Zhi-Gang *et al.,* 2009; Ramazan *et al.,* 2007; Alaa *et al.,* 2006; Filkret *et al.,* 2006; Saeed *et al.,* 1988).

Coumarin (2*H*-1-benzopyran-2-one) and its derivatives occur widely in nature. Many natural and synthetic derivatives of coumarin have been used in various applications in chemistry, biology, medicine and physics (Mach *et al.,* 1972), including additives in food and cosmetics, optical brightening agents as well as dispersed fluorescent and laser dyes (Christie, 1993; Christie and Lui 1999; Christie and Lui 2000; Siegrist *et al.,* 1991; Harold *et al.,* 1994; Ayyangar *et al.,* 1991; Griffiths and Miller, 1995; Dai and Wu, 1999). Coumarin compounds have attracted great interest in recent years in the field of optoelectronic materials (Avramenbo *et al.,* 1996; El-Ansary *et al.,* 1998; Vijayan, 1998). Due to the lactone skeleton, coumarins produce stronger fluorescence than open chain analogs (Davendra *et al.,* 2008; Fan, 2001). Their strong fluorescence is also closely associated with charge transfer configuration arising from electron transfer from the styryl to the carbonyloxy group (Hinohara *et al.,* 1981). Hue and fluorescence properties of coumarins can be affected strongly by substituting different groups at the 3- or 7-position (Wheelock, 1959). Initially these dyes were developed for colouring synthetic fibers such as polyester; subsequently, its use extended to include daylight fluorescent pigments and functional applications such as dye lasers, solar collector systems, organic light emitting diodes (LED) and numerous biological applications. A few comparable investigations have been made using coumarin based dyes.

In the present work, synthesis of ten heteroaryl azo coumarin dyes **Da-Dj** has been reported through diazotization of hetrocyclic amines using nitrosyl sulphuric acid and then coupling them with 7-hydroxy-4-methyl coumarin. Synthesized dyes were characterized using different elemental techniques and were assessed for their fastness properties using polyester fiber. The dye structures are shown in Scheme 1.

Materials and Methods

All the chemicals used in the synthesis were obtained from Sigma-Aldrich and Acros Organics and were used without further purification. Melting points were taken on a Gallenkemp melting point apparatus and are uncorrected. Elemental analysis was performed on Perkin Elmer CHNS Analyser. IR spectra were recorded on Schimadzu FTIR 8400, mass spectra on Jeol D-3000 Spectrometer and UV absorption spectra on Cecil 2000 UV-VIS spectrometer. ^{1}H-NMR spectra were recorded on Brucker AC-300MHz and chemical shifts were reported in ppm relative to Me_4Si as internal standard.

Synthesis of 7-hydroxy-4-methyl coumarin. Sixteen gram of poly- phosphoric acid was added to a solution of 1.1g (0.1mol) of resorcinol in 13g (0.1 mol) of ethyl acetoacetate. The mixture was stirred and heated at 75-80 °C for about

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20 min and then poured into ice-water. The pale yellow solid was collected by suction filtration, washed with cold water and dried at 60 °C. The yield of 7-hydroxy-4-methyl coumarin was 1.8 g (89%). The compound was recrystalised using ethanol. Its m.p. was found to be 185 °C.

Synthesis of niterosyl sulphuric acid. Nitrosyl sulphuric acid was prepared by mixing sodium nitrite (1g), conc. sulphuric acid (7 ml) at 70 °C for 6-7 h. It was further stirred for an additional 1 h at 0 °C.

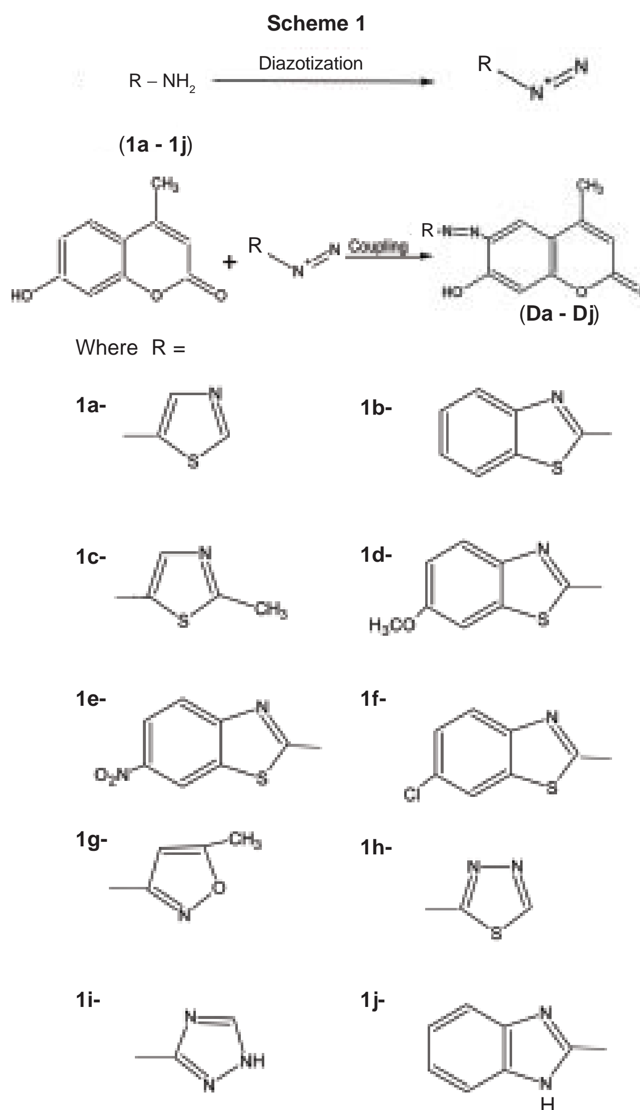
Diazotization of heterocyclic amine (2-amino thiazole). Diazotization of heterocyclic amine (2-amino thiazole) was carried out with nitrosyl sulphuric acid. The procedure for diazotization of 2-amino thiazole (**1a**) was given as follows:

2-amino thiazole (2m mol) was dissolved in hot glacial acetic acid (2.5 ml) and then shifted to an ice bath temperature maintained between 0 to -5 °C. The liquor was then added in portions to cold solution of nitrosyl sulphuric acid in span of 30 min.

Coupling. After completion of diazotization, the azo liquor was slowly added to the vigorously stirred solution of 7-hydroxy-4-methyl coumarin (2m mol) in sodium carbonate solution made by dissolving 2m mol of Na_2CO_3 in 2 ml water. Sodium carbonate was added in portions periodically to maintain the pH of the reaction mixture at 7-8. Stirring was continued for about 1 h maintaining the temperature at 0 to 5 °C and pH at 7-8. The reaction was monitored by TLC using hexan-ethylacetate (1:1) as the solvent mixture. After completion of the reaction, the resulting dye was filtered, washed with cold water and dried in a hot air oven. Recrystallization was carried out using the same hexan-ethylacetate (1:1) mixture. Yield of the dye **Da** was 72 %.

Synthesis of dyes Db-Dj. The same procedure was repeated as described above for synthesis of **1a** but using different amines i.e. **1b-1j** to get the dyes **Db-Dj**. Physical and analytical data of the synthesized dyes are given in Table 1.

Dyeing of polyester fabrics using dyes Da-Dj. A paste of dye (35 mg), dispersing agent (Dodamol 75 mg) and 3-4 drops of the wetting agent (1% Tween-80) was prepared by mixing all the compounds in mortar and pestle. To this mixture, water (100 ml) was added while stirring till dispersion of the dye was achieved. The pH of the solution was maintained at 7-8 by adding sodium carbonate. Dyeing of polyester was carried out at 115 °C for 90 min. The dyed fabric was then treated with a solution of detergent (0.2 g) and Na_2CO_3 (0.1g) in 200 ml of water at 60 °C for 30 min. Later, the fabric was washed several times with water to remove any attached dye and then dried.



Fastness properties of synthesized dyes. Light fastness, wash fastness, fastness to rubbing, perspiration and sublimation of dyes on polyester fabric was determined after dyeing pieces of fabric with all the synthesized dyes (**Da-Dj**), using standard test methods as adopted by Hari *et al.* (2002). Results obtained are shown in Table 2.

Results and Discussion

Dyes **Da**-**Dj** were synthesized by coupling 7-hydroxy-4-methylcoumarin with diazotized heterocyclic amines (**1a**-**1j**) in nitrosyl sulphuric acid. Characteristics of the resultant dyes **Da**-**Dj** are given in Table 1. The dyes were applied to the polyester fiber at a 2% shade. All the dyes gave good coloration of polyester from bright yellow to deep brown hues (Table 2). All the dyes tested showed good to very good light fastness propertis except **Da, Db and Dj**.Washing and

Table 1. Physical and analytical data of the synthesized dyes

Dye	Molecular formula	Mol.Wt	Yield (%)	M.P(°C)	Absorption maximum (λ_{max}/nm)	Exhaustion (%)
Da	$C_{13}H_{11}N_3O_3S$	289.301	72	142	421	77
Db	$C_{17}H_{13}N_3O_3S$	339.368	71	125-126	426	70
Dc	$C_{14}H_{13}N_3O_3S$	303.336	67	234-235	359	77
Dd	$C_{18}H_{15}N_3O_4S$	369.394	73	138	470	73
De	$C_{17}H_{12}N_4O_5S$	384.365	71	153-154	481	81
Df	$C_{17}H_{12}ClN_3O_3S$	373.813	76	211	462	67
Dg	$C_{14}H_{13}N_3O_4$	287.270	74	167	413	71
Dh	$C_{12}H_{10}N_4O_3S$	290.297	69	171	419	80
Di	$C_{12}H_{11}N_5O_3$	273.243	77	232-233	353	78
Dj	$C_{17}H_{14}N_4O_3$	322.318	74	128	355	85

Table 2. Fastness characteristics (score) of the synthesized dyes

Dye	Colour of the dyed fabric	Light fastness	Wash fastness	Rubbing fastness	Perspiration fastness	Sublimation fastness
Da	Orange	4-5	4	4	4	3-4
Db	Orange	4-5	4	4	4	4-5
Dc	Bright yellow	3-4	4	4	4	4
Dd	Reddish brown	4-5	4	3-4	4	3
De	Reddish brown	3	4	4-5	4	4-5
Df	Bright yellow	4	4	5	4	3-4
Dg	Yellowish orange	4-5	4	4-5	4	3
Dh	Yellowish orange	4	4	4-5	4	4-5
Di	Yellow	3-4	3	4	4	3-4
Dj	Yellow	3	4	4	4	4

Table 3. Elemental composition of the synthesized dyes

Dye	C		H		N		O		S	
	Calc. (%)	Found (%)	Calc. (%)	Found (%)	Calc. (%)	Found (%)	Calc. (%)	Found (%)	Calc. (%)	Found (%)
Da	53.98	53.86	3.83	3.81	14.52	14.48	16.58	16.51	11.10	11.03
Db	60.16	60.09	3.86	3.78	12.39	12.32	14.15	14.09	9.44	9.38
Dc	55.42	55.41	4.34	4.31	13.84	13.82	15.86	15.78	10.51	10.47
Dd	58.52	58.48	4.09	4.06	11.38	11.29	17.33	17.28	8.68	8.61
De	53.12	53.09	3.15	3.11	14.57	14.52	20.80	20.79	8.34	8.28
Df	54.62	54.55	3.24	3.18	11.24	11.18	12.84	12.77	8.58	8.49
Dg	58.52	58.50	4.55	4.47	14.62	14.59	22.28	22.21	-	-
Dh	49.65	49.61	3.47	3.44	19.31	19.28	16.52	16.48	11.05	10.98
Di	52.75	52.66	4.06	4.01	25.63	25.60	17.57	17.51	-	-
Dj	63.35	63.22	4.38	4.33	17.38	17.29	14.89	14.77	-	-

Table 4. Structural characterization of the synthesized dyes

Dye	Spectral data
Da	IR (cm^{-1} KBr)1591 (C=C), 1698 (C=O) ^{1}H NMR (DMSO-d^6) 6.32 (s, 1H, -OH), 3.39-383 (m, 2H, Ar-H), 4.31-4.52 (m, 2H, Ar-H), 1.96 (s, 3H, - CH_3) Molecular ion peak (m/z)= 289.012
Db	IR (cm^{-1} KBr) 11665 (C=C), 1688 (C=O) ^{1}H NMR (DMSO-d^6) 5.14 (s, 1H, -OH), 7.50-7.67 (m, 2H, Ar-H), 7.83-8.19 (m, 2H, Ar-H), 2.03 (s, 3H, - CH_3) Molecular ion peak (m/z)= 337.918
Dc	IR (cm^{-1} KBr) 1599 (C=C), 1699 (C=O) ^{1}H NMR (DMSO-d^6) 6.34 (s, 1H, -OH), 7.53-8.47 (m, 2H, Ar-H), 7.79-8.10 (m, 2H, Ar-H), 2.51 (s, 3H, - CH_3) Molecular ion peak (m/z)= 301.325
Dd	IR (cm^{-1} KBr) 1601 (C=C), 1698 (C=O) ^{1}H NMR (DMSO-d^6) 6.12 (s, 1H, -OH), 7.06-7.47 (m, 2H, Ar-H), 7.88-8.14 (m, 2H, Ar-H) , 3.75 (s, 3H, - CH_3) Molecular ion peak (m/z) = 367.881
De	IR (cm^{-1} KBr) 1593 (C=C), 1698 (C=O) ^{1}H NMR (DMSO-d^6) 6.12 (s, 1H, -OH), 7.36-7.87 (m, 2H, Ar-H), 8.08-8.34 (m, 2H, Ar-H) , 2.47 (s, 3H, - CH_3) Molecular ion peak (m/z)= 383.301
Df	IR (cm^{-1} KBr) 1691 (C=C), 1699 (C=O) ^{1}H NMR (DMSO-d^6) 7.12 (s, 1H, -OH), 7.14-7.38 (m, 2H, Ar-H), 7.46-8.14 (m, 2H, Ar-H),2.19 (s, 3H, - CH_3) Molecular ion peak (m/z) = 371.015
Dg	IR (cm^{-1} KBr) 1599 (C=C), 1698 (C=O) ^{1}H NMR (DMSO-d^6) 6.92 (s, 1H, -OH), 7.48-7.68 (m, 2H, Ar-H), 7.86-8.04 (m, 2H, Ar-H), 1.96 (s, 3H, - CH_3) Molecular ion peak (m/z) = 285.124
Dh	IR (cm^{-1} KBr) 1585 (C=C), 1698 (C=O) ^{1}H NMR (DMSO-d^6) 6.22 (s, 1H, -OH), 7.45-7.68 (m, 2H, Ar-H), 7.96-8.04 (m, 2H, Ar-H), 2.49(s, 3H, - CH_3) Molecular ion peak (m/z)= 289.195
Di	IR (cm^{-1} KBr) 1695 (C=C), 1698 (C=O), 3567 (N-H) ^{1}H NMR (DMSO-d^6) 6.81 (s, 1H, -OH), 7.65-7.82 (m, 2H, Ar-H), 7.96-8.04 (m, 2H, Ar-H) , 3.99 (s, 3H, - CH_3) Molecular ion peak (m/z)= 273.011
Dj	IR (cm^{-1} KBr) 3146 (NH), 1699 (C=O) ^{1}H NMR (DMSO-d^6) 6.81 (s, 1H, -OH), 3.82-4.30 (m, 2H, Ar-H), 4.52-5.67 (m, 2H, Ar-H) , 2.17 (s, 3H, - CH_3) Molecular ion peak (m/z)= 321.129

rubbing fastness properties were good to excellent and perspiration and sublimation fastness properties were excellent.

Spectrophotometric properties of the prepared monoazo disperse dye stuffs were determined and the absorption maxima of these dyes and their intensities were obtained (Table 1).

The structure and the purity of the prepared dyes (**Da-Dj**) were confirmed by elemental analysis, UV-VIS. absorption spectra, FT-IR, ^{1}H-NMR, and MS. The structural analysis of the dyes is presented in Table 3&4. IR spectra showed typical aromatic absorption. IR spectra of the dyes showed absorption band at 1550-1575 cm^{-1} (-N=N- group), 1700 cm^{-1} (-C=O group). The ^{1}H-NMR chemical shift data and M^+ values were in consistent with the structure of dye.

Conclusion

The present investigation explores the suitability of coumarin based monoazo dispersed dyes for dyeing polyester. The optical and dyeing properties, light fastness, wash fastness and sublimation fastness of synthesized dye stuffs were proved to be reasonable good.

Thus, it may be concluded that the hydroxy coumarin related compounds could be used as reasonably good monoazo disperse dyes with all round dyeing properties for polyester fabrics.

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Intercorrelation of Amino Acid Quality between Raw, Steeped and Germinated Pearl Millet (*Pennisetum typhoides*) Grains

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Abstract. In the study of amino acids in the pearl millet grains, *Pennisetum typhoides*, steeped sample was best in Arg, Glu, Ser and protein contents, germinated sample was best in His, Lys, Met, Phe, Thr, Val, Ala, Asp, Cys (shared with raw sample), Pro and Tyr whereas raw sample was best in Ile, Leu and Gly. Total amino acid contents in steeped grains were 432 mg/g crude protein (c.p.), in germinated grain 464 mg/g c.p. and in raw grain 439 mg/g c.p. with respective essential amino acids of 210 mg/g c.p., 233 mg/g c.p. and 224 mg/g c.p. Percentage Cys/TSAA trend was 53.1 (raw) > 52.1 (germinated) > 51.2 (steeped). Predicted protein efficiency ratio (P-PER) levels were 1.32 (steeped), 1.66 (raw) and 1.57 (germinated). The Leu/Ile ratio levels were 2.22 (raw) and 2.46 (both steeped and germinated). Amino acid scores based on whole hen's egg had Met as the limiting amino acid for the three samples. The two treatments enhanced the quality of the pearl millet amino acid levels thereby providing high potentials for use in weaning foods and formulations. However, no significant difference was seen between raw/steeped, raw/germinated and steeped/germinated samples at $p < 0.05$.

Keywords: amino acid profile, *Pennisetum typhoides*, processed grains, pearl millet

Introduction

Millet, grown predominantly in the dry areas of the Far East and Africa, is a staple food in African countries. It is used primarily as a grain crop in Nigeria. Its grain is richer in nutritive value than guinea corn and is used for making heavy bread or porridge and alcoholic beverages, *ogi* in Nigeria and is also used in animal feed, particularly for poultry and other birds (Kochhar, 1986).

There is dearth of information on the nutritional quality of flour processed millet. This work reports on the amino acid composition of the raw, steeped and germinated grains of *Pennisetum typhoides* with a view to providing information on the best treatment for enhancing the protein quality for its various food uses.

Materials and Methods

Samples of pearl millet grains were purchased from the main market of Ado-Ekiti in the southern part of Nigeria in Ekiti State. About 1.5 kg of the grains was used for the experiments. After removing stones, damaged grains, glumes and glumela manually, the endosperm was extracted from kernels and divided into three equal parts for use as raw, steeped and germinated pearl millet samples and labelled accordingly.

Sample treatment. Raw sample (0.5 kg) was not specially treated but only dried to constant weight (6.38 g/100 g moisture content). For steeping, 0.5 kg grains were placed in plastic container, covered with distilled water and left in the laboratory at ambient temperature (30.9 °C) at 0.41 Im^2/ft light intensity. After four days, grains were washed with distilled water, dried in the sun to constant weight (6.45% moisture content) and stored in covered plastic container. For germination of samples, 0.5 kg grains were soaked in water at room temperature for 24 h; then spread on a damp fabric, protected from direct sunlight, for approximately 48 h, until 5.04 cm long sprouts developed. Germinated grains were dried in the sun for 3 days until constant weight (7.41% moisture content); the sprouts were manually removed and the desprouted grains were stored in a plastic container (WHO, 1999). Each sample was then homogenised, sieved using 200 mm mesh and kept in the refrigerator (-4 °C). Six replicates of steeped and germinated grains were used for amino acid analysis.

Instrumentation. The Technicon Sequential Multisample Amino Acid Analyzer (TSM) – an automated instrument – was used to separate, detect, and quantitate amino acids.

The column operating conditions for hydrolysate were; for acid–neutral column: flow rate (0.5 ml/min), operating temperature (60 °C), resin bed (23.0–23.5 cm) and resin type (C-3); for basic column: flow rate (0.5 ml/min) operating temperature (60 °C), resin bed (4.5-5.0 cm) and resin type (C-3).

Standard samples for both hydrolysate and physiologic systems were 0.025 µmoles of each amino acid equal to 0.010 ml (10 µl) of the Technicon 2.5 µml amino acid standard. For unknown hydrolysate: hydrolysate samples may be

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taken up in either 0.01 M HCI or the pH 2.0 buffer. The equivalent of 0.05 to 0.10 mg of protein was applied as sample. An internal standard Norleucine, approx. 0.025 µM of each hydrolysate sample and 0.025-0.050 µM with each physiologic sample was included.

Amino acid analysis. Pearl millet flour (2.0 g) of each group was defatted by extraction with some chloroform-methanol (2:1 v/v) using the Soxhlet apparatus as 61.7-65.0 °C (AOAC, 2005). Extraction of lipid continued for 5 h to ensure total lipid extraction. Approximately 30-35 mg of the defatted sample was put in a glass test tube, 7 ml of 6 M HCl was added and oxygen was expelled by flushing with nitrogen gas tube was sealed and was placed in oven at 105±5 °C for 22 h, allowed to cool and content was filtered. The filtrate was evaporated to dryness at 40 °C under vacuum in rotary evaporator. The residue was dissolved in 5 ml acetate buffer (pH 2.0) and stored in plastic specimen bottles, which were kept in the freezer. Approximately 5-10 ml of the sample was dispensed into the cartridge of the analyzer. The method of amino acid analysis was by ion-exchange chromatography (IEC) (Spacman *et al*., 1958) using TSM. Determinations were performed in duplicate and run time was 76 min for each sample. The column flow rate was 0.50 ml/min at 60 °C with reproducibility within ±3% using norleucine as the internal standard.

Method of calculating amino acid values from the chromatogram peaks. The net height of each peak produced by the chart recorder of TSM (each representing an amino acid) was measured. Half-height of the peak on the chart was found and the width of the peak on the half-height was accurately measured and recorded. Approximate area of each peak was then obtained by multiplying the height with the width at half-height.

Norleucine equivalent (NE) for each amino acid in the standard mixture was calculated using the formula:

NE = Area of norleucine peak ÷ area of each amino acid

A constant S was calculated for each amino acid in the standard mixture:

$$S_{std} = NE_{std} \times \text{mol. weight} \times \mu MAA_{std}$$

Finally the amount of each amino acid present in the sample was calculated in g/16N or g/100 g protein using the following formula:

$$\text{Concentration (g/100 g protein)} = NH \times W @ NH/2 \times S_{std} \times C$$

$$C = \frac{\text{Dilution} \times 16}{\text{Sample wt (g)} \times N\% \times 10 \text{ vol. loaded}} \div NH \times W\,(\text{Nleu})$$

Where: NH = net height
W = Width @ half-height and
Nleu = Norleucine

Estimation of isoelectric point (pI). The estimation of the isoelectric point (pI) for a mixture of amino acids was calculated using the equation below:

$$IPm = \sum_{i=1} IPiXi$$

where IPm is the isoelectric point of the mixture of amino acids, IPi is the isoelectric point of the ith amino acid in the mixture and Xi is the mass or mole fraction of the ith amino acid in the mixture (Olaofe and Akintayo, 2000).

Estimation of quality of dietary protein. Total amino acid scores were calculated based on the whole hen's egg amino acid profile (Paul *et al.*, 1976) while the essential amino acid scores were calculated using the following formula (FAO/WHO, 1973):

Amino acid score = Amount of amino acid per test protein [mg/g]/amount of amino acid per protein in reference pattern [mg/g].

Predicted protein efficiency ratio (P-PER) was determined using one of the equations developed by Alsmeyer *et al.* (1974) as follows:

P-PER = -0.468 + 0.454 (Leu) -0.105 (Tyr).

Essential amino acid index was calculated by using the ratio of test protein to the reference protein for each of the eight essential amino acids plus histidine in the equation (Steinke *et al.*, 1980):

$$\text{Essential amino acid index} = \sqrt[9]{\frac{\text{mg lysine in 1 g test protein}}{\text{mg lysine in 1 g reference protein}} \times \text{etc. for all 8 essential amino acids + His}}$$

Determination of the ratio of total essential amino acids (TEAA) to the total amino acids (TAA), i.e. (TEAA/TAA), total sulphur amino acid (TSAA), percentage cystine in TSAA, total aromatic amino acid (TArAA), total neutral amino acid (TNAA), total acidic amino acid (TAAA) and total basic amino acid (TBAA) were estimated from the results of amino acid profile. The leucine/isoleucine ratio was also calculated.

Statistical analysis. All data generated were analysed statistically (Skoog *et al.,* 2004; Dixon and Massey, 1983). Mean, standard deviation and coefficient of variation (%) were

calculated. Amino acid composition of the raw/steeped, raw/germinated, steeped/germinated samples, amino acid differences in composition between raw/steeped and between raw/germinated as well as their essential amino acid scores with respective degrees of freedom of 16 and 8 at $p<0.05$ were subjected to F-test analysis. Calculations were meant to determine the level of variation among the data obtained for raw, steeped and germinated samples.

Results and Discussion

Table 1 shows amino acid composition of the samples. On pair wise basis, most of the values for germinated samples were generally better than the values for raw and steeped samples. Specifically, levels of His, Lys, Met, Phe, Thr, Val, Ala, Asp, and Pro in germinated samples were correspondingly higher than those for raw and steeped ones, indicating that germinated sample was 60.0% best in 9 parameters (9/15) of the amino acids although steeped sample was best in Arg, Glu and Ser or in 3/15 (20.0%) while the amino acid level was best in the raw (or remained unchanged) i.e. Ile, Leu and Gly or in 3/15 (20.0%). Cystine shared similar levels of amino acid (7.6 mg/g) between raw and germinated samples and Tyr shared similar levels of amino acid (18.6 mg/g crude protein c.p.) between raw and steeped samples. The protein level of steeped sample was the highest (8.54 g/100 g) > germinated (7.54 g/ 100 g) > raw (5.11 g/100 g). The results of the pearl millet were in contrast to the observations in guinea corn where the steeped samples had the best levels of amino acids (AA) in His, Arg, Thr, Ser, Pro, Gly, Ala, Met, Cys, Val, Phe and Tyr, almost like the germinated samples of pearl millet (Adeyeye, 2008a). The highest AA was Glu with values of 67.4, 79.7 and 78.2 (mg/g c.p.) for raw, steeped and germinated grains, respectively. Tryptophan was not determined. Guinea corn samples had the highest levels of Glu (Adeyeye, 2008a). Leucine was the highest concentrated essential AA in raw (51.2 mg/g), steeped (43.7 mg/g) and germinated (49.5 mg/g) samples. Ile was the highest concentrated essential AA in guinea corn samples (Adeyeye, 2008a).

The least varied AA was Lys which ranged from 20.0 mg/g c.p. (steeped) to 21.7 mg/g c.p. (germinated) with coefficient of variation of 4.13% while the most varied AA was Gly with values of 17.9 mg/g c.p. (raw), 16.9 mg/g c.p. (steeped) and 11.6 mg/g c.p. (germinated) with CV of 21.9%. F-test results showed $F_{calculated}$ values between raw/steeped, raw/germinated and steeped/germinated to be 1.22, 1.12 and 1.09, respectively, at $p<0.05$ which were all lower than F_{Table} (2.24) which are not significantly different. Results in pearl millet were comparable in His being 15.8-18.1 mg/g c.p. with His (16.5-22.9 mg/g c.p.) in guinea corn but better in Lys (20.0-21.7 mg/g c.p.) in guinea corn (Adeyeye, 2008a). Even though Lys content in the samples (20.0-21.7 mg/g c.p.) was lower than the Lys content of the reference egg protein (62 mg/g c.p.), they can be

Table 1. Amino acid (mg/g crude protein) composition of raw, steeped and germinated millet (on dry weight)

Amino acid	Raw	Steeped	Germinated	Mean	SD	CV(%)
Arginine(Arg)*	29.4	33.6	31.0	31.3	2.12	6.77
Histidine(His)*	15.8	16.2	18.1	16.7	1.23	7.37
Isoleucine(Ile)*	23.1	17.8	20.1	20.3	2.66	13.1
Leucine(Leu)*	51.2	43.7	49.5	48.1	3.93	8.17
Lysine(Lys)*	20.6	20.0	21.7	20.8	0.86	4.13
Methionine(Met)*	6.7	5.9	7.0	6.53	0.57	8.73
Phenylalanine(Phe)*	27.7	26.0	29.4	27.7	1.70	6.14
Threonine(Thr)*	20.1	17.7	22.1	20.0	2.20	11.0
Tryptophan(Try)*	-	-	-	-	-	-
Valine(Val)*	29.6	29.0	33.9	30.8	2.67	8.67
Alanine(Ala)	18.5	21.0	23.0	20.8	2.25	10.8
Aspartic acid(Asp)	57.8	50.0	60.1	56.0	5.29	9.45
Cystine(Cys)	7.6	6.2	7.6	7.13	0.81	11.4
Glutamine(Glu)	67.4	79.7	78.2	75.1	6.71	8.93
Glycine(Gly)	17.9	16.9	11.6	15.5	3.39	21.9
Proline(Pro)	8.8	7.7	9.9	8.80	1.10	12.5
Serine(Ser)	17.8	22.0	20.0	19.9	2.10	10.6
Tyrosine(Tyr)	18.6	18.6	20.3	19.2	0.98	5.10
Protein(g/100g)	5.11	8.54	7.54	7.06	1.76	24.9

*Essential amino acid; SD = standard deviation; CV = coefficient of variation; - = not determined

enhanced by mixing with legumes, which are high in Lys. The increase in AA content of germinated pearl millet may be due to increase in the protease activity of enzymes which break down the protein to release amino acids needed for the activity.

Table 2 shows several quality parameters of proteins in the samples. The essential amino acids (EAA) ranged between 214 mg /g c.p. to 231 mg /g c.p. These values were far from the value of 566 mg/g c.p. of the egg reference protein (Paul *et al.*, 1976) but slightly close to 453 mg/g c.p. for peanut meal (Lusas, 1979) and better than 190 mg/g c.p. for *Colocynthis citrullus* (Akobundu *et al.*, 1982) flours. Total sulphur AA (TSAA)of the samples was 14.3, 12.1 and 14.6 mg/g c.p. for raw, steeped and germinated, respectively; these values were individually approx. 25% of the value (58 mg/g c.p.) recommended for infants (FAO/WHO/UNU, 1985). Aromatic amino acid (ArAA) range suggested for ideal infant protein (68-118 mg/g c.p.) (FAO/WHO/UNU, 1985) is much higher than the current report (46.3-49.7 mg/g c.p.) indicating, pearl millet when used as the weaning food, should be complemented with ArAA rich foods particularly when raw or steeped pearl millet is used. The percentage ratio of EAA to TAA in the flour ranged from 48.6-51.1. These values were well above the 39% considered to be adequate for ideal protein food for infants, 26% for children and 11% for adults (FAO/WHO/UNU, 1985). The percentages of EAA/TAA for the pearl millet samples could be favourably compared with that of egg (50%) (FAO/WHO, 1990), pigeon pea flour (43.6%) (Oshodi *et al.*, 1993) and beach pea protein isolate (43.8-44.4%) (Chavan *et al.*, 2001). The predicted protein efficiency ratio (P-PER) as shown in Table 2 ranged from 1.32 to 1.66. The experimentally determined PER usually ranged from 0.0 for very poor protein to the maximum possible of just over 4 (Muller and Tobin, 1980). In the samples (Table 1) it could be seen that the values for Leu and Tyr (from which P-PER were calculated) were almost half of each other: raw (51.2 and 18.6 mg/g c.p., respectively), steeped (43.7 and 18.6 mg/g c.p., respectively) and germinated (49.5 and 20.3 mg/g c.p., respectively). The P-PER in guinea corn were: raw (<0.00), steeped (0.23) and germinated (0.29) indicating the pearl millet samples to be much better than the guinea corn (Adeyeye, 2008a).

The Leu/Ile values were 2.22 (in raw), 2.46 (in steeped) and 2.46 (in germinated) samples. In all the samples (Table 1) the level of Leu was more than twice the level of Ile. Endemic pellagra in cereal-eating populations was first described by Gopalan Srikantia (1960) particularly in poor agricultural labourers around Hyderabad in Andhra Pradesh (India). It has been suggested that an amino acid imbalance from excess leucine might be a factor in the development of pellagra

Table 2. Total, essential, non-essential, neutral, acid, basic, sulphur, aromatic amino acid (mg/g crude protein). Protein efficiency ratio (P-PER), isoelectric point (pI), Leu/Ile ratio, Leu/Ile difference of millet (on dry weight)

Amino acid	Raw	Steeped	Germinated	Mean	SD	CV (%)
TAA[a]	439	432	464	445	16.8	3.78
TNEAA[b]	214	222	231	222	8.50	3.83
TEAA[c]						
-with His	224	210	233	222	11.6	5.23
-no His	219	194	215	209	13.4	6.41
%TNEAA	48.9	51.4	49.8	50.0	1.27	2.54
%TEAA						
-with His	51.1	48.6	50.2	50.0	1.27	2.54
-no His	49.9	44.8	46.3	47.0	2.62	5
TNAA[d]	248	233	254	245	10.8	4.41
%TNAA	56.5	53.8	54.9	55.1	1.36	2.47
TAAA[e]	125	130	138	131	6.56	5.01
%TAAA	28.5	30.0	29.8	29.4	0.81	2.76
TBAA[f]	65.8	69.8	70.8	68.8	2.65	3.85
%TBAA	15.0	16.2	15.3	15.5	0.62	4.00
TSAA[g]	14.3	12.1	14.6	13.7	1.37	10.0
%TSAA	3.3	2.8	3.1	3.07	0.25	8.14
%Cys in TSAA	53.1	51.2	52.1	52.1	0.95	1
TArAA[h]	46.3	44.6	49.7	46.9	2.60	5.54
%TArAA	10.6	10.3	10.7	10.5	0.21	2.00
P-PER	1.66	1.32	1.57	1.52	0.18	11.8
Leu/Ile	2.22	2.46	2.46	2.38	0.14	5.88
Leu-Ile(diff.)	28.1	25.9	29.4	27.8	1.77	6.37
%Leu- Ile(diff.)	54.9	59.3	59.4	57.9	2.57	4.44
pI(calculated)	2.4	2.4	2.6	2.47	0.12	4.86
pI(expt.)	4.0	4.0	3.0	3.67	0.58	15.8
pI difference	1.6 (40%)	1.6 (40%)	0.4 (13.3%)	1.20	0.69	57.5
EAAI[i]	0.67	0.61	0.70	0.66	0.05	7.58

[a] = total acid; [b] = total non-essential amino acid; [c] = total essential amino acid; [d] = total neutral amino acid; [e] = total acidic amino acid; [f] = total basic amino acid; [g] = total sulphur amino acid; [h] = total aromatic amino acid; [i] = essential amino acid index

(FAO, 1995). High Leu in the diet impairs tryptophan and niacin metabolism and is responsible for niacin deficiency in sorghum eaters (Belavady *et al.*, 1963) and hence, the hypothesis that excess Leu in sorghum is aetiologically related to pellagra in sorghum-eating populations (FAO, 1995). The study of Krishnaswamy and Gopalan (1971) had suggested that the Leu/Ile balance is more important than dietary excess of Leu alone in regulating the metabolism of Try and niacin and hence the disease process. However, it has also been suggested that factors other than excess Leu and poor Leu/Ile balance in cereal proteins are responsible for the development of the disease. Krishnaswamy *et al*. (1976) have shown that vitamin B_6 is involved in the metabolism of Leu as well as that of Try and niacin suggesting that regional

differences in the prevalence of pellagra might be related to the nutritional status of the population in terms of vitamin B_6. Experiments in dogs fed with sorghum have shown that animals proteins with less than 11g percent (110 mg/g c.p.) Leu did not suffer from nicotinic acid deficiency (Belavady and Udayasekhara Rao, 1979). The current report shows Leu to range from 43.7-51.2 mg/g c.p. which was far less than 110 mg/g c.p., therefore, considered safe and could be beneficially exploited to prevent pellagra in endemic areas (Deosthale, 1980).

Table 2 shows that the Cys (%) in TSAA ranged from 51.2-53.1. Cys can improve protein quality and has positive effects on mineral absorption, particularly that of zinc (Mendoza, 2002). Cys/TSAA (percentage) values obtained in this study were comparable to the value of 62.9 reported for coconut endosperm (Adeyeye, 2004). The Cys in TSAA in guinea corn ranged from 58.9-72%, and it is 50.5% in cashew nut (Adeyeye *et al*., 2007); but 40.7% in *Triticum durum* (Adeyeye, 2007), 44.4% in *Parkia biglobosa* seeds (Adeyeye, 2006), 44.3% in *Cola acuminata* and 37.8% in *Garcinia kola* (Adeyeye *et al*., 2007). Most animal proteins are low in cystine e.g. 36.3 in *Macrotermes bellicosus* (Adeyeye, 2005a); 25.6 in *Zonocerus variegatus* (Adeyeye, 2005b); 35.3 in *Archachatina marginata*, 38.8 in *Archatina archatina* and 21.0 in *Limicolaria* sp.; (Adeyeye and Afolabi, 2004); 26.5 in turkey muscle and 26.0 in turkey skin (Adeyeye and Ayejuyo, 2007); 29.8 in *Gymnarchus niloticus* (trunk fish) (Adeyeye and Adamu, 2005); 13.3-15.9 in various parts of West African fresh water male crab (Adeyeye and Kenni, 2008); 26.9 in the flesh of raffia palm tree grub (Adeyeye and Aye, 2008); 23.8-30.1 in three different fresh water fish samples (Adeyeye, 2008b). Thus for animal protein, Cys is unlikely to contribute up to 50% of the TSAA (FAO/WHO, 1991). The Cys (%)/TSAA had been set at 50% in rat, chick and pig diets (FAO/WHO, 1991). The calculated isoelectric point (pI) ranged between 2.4-2.6 whereas pI experimental (not yet reported) was 3.0-4.0. The information on pI is a good starting point in predicting the pI for proteins in order to enhance a quick precipitation of protein isolate from biological samples (Olaofe and Akintayo, 2000). The low pI values could be a function of the TAAA (125-138 mg/g c.p. or 28.5-30.0%) which were much higher than the TBAA (65.8-70.8 mg/g c.p. or 15.0-16.2%) (Table 2). The calculated pI values were close to the experimental values with $pI_{calc.} - pI_{expt.}$ range of 0.4-1.6 or 13.3-40%. The essential amino acid index (EAAI) ranged from 0.61-0.70 which were all lower than the value of 1.26 in a defatted soya flour (Cavins *et al*., 1972). The EAAI method can be useful as a rapid tool for evaluating protein quality in food formulations (Nielsen, 2002).

Table 3 contains a summary of the differences between raw/steeped and between raw/germinated samples. The highest CV(%) was observed in Tyr with a value of 133 whereas Pro and Phe had CV(%) value of 0.00. Table 3 gave all the differences in amino acid parameters.

Table 4 shows that Met was the limiting amino acid in all the samples based on whole hen's egg (Paul *et al.*, 1976); values ranged from 0.18 (18%)-0.22 (22%). Most of the results have

Table 3. Differences in amino acid composition between raw and steeped, and between raw and germinated millet samples

Amino acid	Raw-steeped	Raw-germinated	Mean	SD	CV(%)
Arg	-4.2(14.3)**	1.6(5.40)*	2.90	1.80	62.1
His	-0.4(2.50)	2.3(14.6)	1.40	1.30	92.9
Ile	5.3(22.9)	3.0(13.0)	4.20	1.60	38.1
Leu	7.5(14.6)	1.7(3.30)	4.60	4.10	89.1
Lys	0.6(2.90)	-1.1(5.30)	0.85	0.35	41.2
Met	0.8(11.9)	-0.3(4.50)	0.60	0.40	66.7
Phe	1.7(6.10)	1.7(6.10)	1.70	0.00	0.00
Thr	2.4(11.9)	2.0(10.0)	2.20	0.30	13.6
Try	-	-	-	-	-
Val	0.6(2.00)	-4.3(14.5)	2.50	2.60	104
Ala	-2.5(13.5)	-4.5(24.3)	3.50	1.40	40.0
Asp	7.8(13.5)	-2.3(4.00)	5.10	3.90	76.5
Cys	1.4(18.4)	0.0(0.0)	0.7	1.0	14.3
Glu	-12.3(18.2)	-10.8(16.0)	11.6	1.10	9.48
Gly	1.0(5.60)	6.3(35.2)	3.70	3.70	100
Pro	1.1(12.5)	-1.1(12.5)	1.1	0.00	0.00
Ser	-4.2(23.6)	-2.2(12.4)	3.20	1.40	43.8
Tyr	0.0(0.00)	-1.7(9.10)	0.90	1.20	133
Protein	-3.43(67.1)	-2.43(47.6)	2.93	0.71	24.2

* = figures in brackets are percentage values; ** = a figure preceded by '-` means both the figure and the figure in bracket carry t

Table 4. Amino acid scores of samples based on whole hen's egg amino acid profile

Amino acid	Raw	Steeped	Germinated	Mean	SD	CV(%)
Arg	0.46	0.55	0.51	0.51	0.04	7.84
His	0.66	0.68	0.75	0.70	0.05	
Ile	0.41	0.32	0.36	0.36	0.05	13.9
Leu	0.62	0.53	0.60	0.58	0.05	8.62
Lys	0.33	0.32	0.35	0.33	0.02	6.06
Met	0.21	0.18	0.22	0.20	0.02	10.0
Phe	0.54	0.51	0.58	0.54	0.04	7.41
Thr	0.39	0.35	0.43	0.39	0.04	10.3
Try	-	-	-	-	-	-
Val	0.39	0.39	0.45	0.41	0.03	7.32
Ala	0.34	0.39	0.43	0.39	0.05	12.8
Asp	0.54	0.47	0.56	0.52	0.05	9.62
Cys	0.42	0.34	0.42	0.39	0.05	12.8
Glu	0.56	0.66	0.65	0.62	0.06	9.68
Gly	0.60	0.56	0.39	0.52	0.11	21.2
Pro	0.23	0.20	0.26	0.23	0.03	13.0
Ser	0.23	0.28	0.25	0.25	0.03	12.0
Tyr	0.47	0.47	0.51	0.48	0.02	4.17

scores less than 50% except His, Leu, Phe and Glu in all the three pearl millet samples. In order to correct for the whole amino acid profile in the samples 100/21, 100/18 and 100/22, 4.76, 5.56 and 4.55 times respectively as much raw, steeped and germinated grain flour would have to be eaten when they serve as sole protein source in the diet.

Table 5. Amino acid scores of millet samples based on provisional amino acid scoring pattern

Amino acid	Raw	Steeped	Germinated	Mean	SD	CV (%)
Ile	0.58	0.45	0.50	0.51	0.07	13.7
Leu	0.73	0.62	0.71	0.69	0.06	8.70
Lys	0.37	0.36	0.39	0.37	0.02	5.41
Met+Cys (TSAA)	0.41	0.35	0.42	0.39	0.04	10.3
Phe+Tyr	0.77	0.74	0.83	0.78	0.05	6.41
Thr	0.50	0.44	0.55	0.50	0.06	12.0
Try	_	_	_	_	_	_
Val	0.59	0.58	0.68	0.62	0.06	9.68
Total	0.59	0.53	0.60	0.57	0.04	

Table 6. Summary of the amino acid profiles into factors A and B

	Millet samples (Factor A)			Factor B
	Raw	Steeped	Germinated	means
Amino acid composition (Factor B)				
Total essential amino acid	224	210	233	222
Total non-essential amino acid	214	222	231	222
Factor A means	219	216	232	222

Table 7. Amino acid profiles of pearl millet and guinea corn compared

Amino	Raw		Steeped		Germinated	
acid	Millet	Sorghum	Millet	Sorghum	Millet	Sorghum
Arg	29.4	31.9	33.6	42.8	31.0	39.4
His	15.8	16.5	16.2	22.9	18.1	18.1
Ile	23.1	46.2	17.8	50.9	20.1	56.0
Leu	51.2	10.9	43.7	21.3	49.5	21.4
Lys	20.6	14.8	20.0	18.8	21.7	20.0
Met	6.7	6.2	5.9	8.0	7.0	7.0
Phe	27.7	21.6	26.0	26.0	29.4	23.4
Thr	20.1	22.4	17.7	32.3	22.1	30.2
Try	-	-	-	-	-	-
Val	29.6	18.5	29.0	38.1	33.9	30.3
Ala	18.5	12.9	21.0	43.9	23.0	20.1
Asp	57.8	37.4	50.0	41.9	60.1	55.2
Cys	7.6	8.9	6.2	20.6	7.6	17.2
Glu	67.4	60.5	79.7	82.0	78.2	91.2
Gly	17.9	13.6	16.9	29.3	11.6	23.0
Pro	8.8	9.9	7.7	32.9	9.9	21.9
Ser	17.8	30.0	22.0	40.1	20.0	39.0
Tyr	18.6	16.9	18.6	25.3	20.3	20.3
Protein (g/100g)	5.11	5.11	8.54	8.24	7.54	4.93

Table 5 contains amino acid scores of the pearl millet samples based on essential amino acid scoring pattern (FAO/WHO, 1973) which shows that Lys (0.37 or 37%) was the limiting AA in raw, Met+Cys (0.35 or 35%) was the limiting AA in steeped while Lys (0.39 or 39%) was also the limiting AA in germinated samples. Therefore, in order to fulfil the day's needs for all the EAA in pearl millet sample flours, 100/37 (raw), 100/35 (steeped) and 100/39 (germinated) or 2.70 times as much raw *P. typhoides* protein; or 2.86 times as much steeped *P. typhoides* protein, or 2.56 times as much germinated *P. typhoides* protein respectively would have to be eaten when it is the only protein source in the diet.

The data obtained for the TNEAA, TEAA and EAA scores as well as the scores based on whole hen's egg were all subjected to the F-test. In raw, the TEAA/TNEAA value was F_c(1.39) but F_T(3.50), not significantly different; in steeped, the F_c (4.69) but F_T (3.73), was significantly different; in germinated the F_c (4.66) but F_T (3.73), was significantly different. For TAA/TAA scores (based on whole hen's egg), F_c raw/steeped and raw/germinated was 1.45 in each case while F_c steeped/germinated was 1.0 and F_T was 2.35 showing that all the results were not significantly different in the compared samples. For scores based on essential amino acid scoring pattern, germinated/steeped had F_c of 1.25, raw/germinated, F_c was 1.15 and raw/steeped, F_c was 1.08 whereas F_T was 4.28 showing that none of the pairs was significantly different from each other. Level of significance was at $p<0.05$.

In conclusion, it is seen (Table 6) that TEAA trend was germinated > raw > steeped whereas TNEAA trend was germinated > steeped > raw in *Pennisetum typhoides.* Mean factor A had the trend: germinated > raw > steeped whereas Factor B mean values (Table 6), which relate to all the samples for their TEAA and TNEAA, give us similar values of 222 in each case. This is an interesting revelation. The germination technique is recommended for improvement of TEAA before *P. typhoides* is processed for food consumption or formulation. However, it should be noted that while germinated pearl millet had the highest TEAA, the steeped sample had the highest TEAA in the guinea corn; this comparison is easily seen in Table 7.

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Chemical and Amino Acid Composition of Cooked Walnut (*Juglans regia*) Flour

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Abstract. The proximate analysis of cooked walnut (*Juglans regia*) flour revealed the composition as protein (14.18%), moisture (11.01%), ash (3.14%), crude fibre (3.03%), crude fat (10.22%), carbohydrate (58.42%), phytate (20.18 mg/g), oxalate (1.13 g/g) and tannin (2.33%). Glutamic and aspartic acids were the most predominant amino acids in the sample with values of 151.6 mg/g and 89.5 mg/g, respectively.

Keywords: *Juglans regia*, nutritional composition, amino acids; antinutritional factors

Introduction

Walnut (*Juglans regia*), a deciduous tree, produces fine quality nuts from which considerable quantities of edible oil can be extracted. Walnuts are important food of people of the tropical countries, some parts of France and southern Nigeria. People belonging to the developing countries depend heavily on starchy foods and cereals. These people suffer from shortage of protein rich foods. Edible protein rich seeds, grown abundantly in tropical countries of the world are underutilized due to the lack of attention on the part of food scientists.

The present paper deals with the determination of nutritional and antinutritional factors and the amino acids of cooked walnut (*Juglans regia*) flour and its suitability for human consumption.

Materials and Methods

English walnuts (*Juglans regia*) were obtained from Idoka village near Ilesa, Osun State, Nigeria. The nuts were screened for quality, cooked and cooled. After removing the shells kernels were dried and milled into fine powder. The flour was kept in a rubber container and stored in freezer until use.

Moisture, ash, crude fat, crude fibre and nitrogen contents of the sample were determined according to the method of the Association of Official Analytical Chemists (1990). Crude protein was calculated by multiplying the total nitrogen content by a factor of 6.25. Carbohydrates were determined by the method of difference.

Oxalates and tannins were determined using the methods of Day and Underwood (1986). 1.0 g of sample was taken in 100 ml conical flask, 75 ml of 1.5 N H_2SO_4 was added and the mixture was stirred for 1 h and filtered. The filtrate (25 ml) was titrated against 0.1 N $KMnO_4$ until faint pink colour persisted for 30 sec.

Phytate was determined on Spectronic 20 colorimeter (Gallenkamp, UK) using the method described by Harland and Oberleas (1986). The amount of phytate in the sample was calculated as hexaphosphate equivalent using the formula:

Phytate (mg/g) sample = $K \times A \times 20/0.282 \times 1000$ where A is absorbance, mean K = standard P. Phytate = 28.2 % P

For determination of tannins, 10 ml of 70% acetone was added to 200 mg of the sample. The mixture was put in an ice bath and shaken for two h at 30 °C. The mixture was later centrifuged at 3,600 rpm; the filtrate was pipetted out into test tube to which 0.8 ml of distilled water was added. Standard tannic acid solution was prepared from 0.5 mg/ml stock and the solution was made up to 1 ml with distilled water. Folin reagent, (0.5 ml), was added to the sample and the standard solutions both, followed by addition of 2.5 ml of 20% Na_2CO_3. The solutions were vortexed, allowed to incubate for 40 min at room temperature and the absorbance was measured at 725 nm.

The amino acid profile of the sample was determined using the method described by Spackman *et al.* (1958). The defatted sample was dried to constant weight, hydrolysed, evaporated under vacuum and then loaded into the Technicon Sequential Multisample Amino Acid Analyser (TSM, Taryton, NY).

Results and Discussion

Proximate composition of cooked walnut flour is given in Table 1. Protein content is 14.18%. This value is low as compared to that of soybean, 40.8%, reported by Oyenuga (1968) and 32.5% reported for soybean by Paul and Southgate

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(1985). The value is also lower than those of crude protein content of cowpea varieties (20.6 g/100 g DM in TVX, 24.2 g/ 100 g DM in Ife brown) (Aletor and Aladetimi, 1989), of *Sphenostylis stenocarpa* (20.63% DM) reported by Abulude (2005) and of lima bean varieties (21.8-26.2%), of *Bombax buonopozence* (13.37% DM) (Oshodi and Adeladun, 1993) but higher than that of *Irvingia gabonensis* (12.78% DM) reported by Abulude (2005).

Moisture and fat contents were 11.01% and 10.22%, respectively. The fat content is lower than that of oil seeds, reported by Olaofe *et al*. (1994). The fat content of cooked walnut is higher than that of Kersting's groundnut (4.9-5.9%) and scarlet runner beans (5.3-6.9%) reported by Aremu *et al*. (2005). Fibre, carbohydrate and ash values were 3.03%, 58.42% and 3.14%, respectively. The fibre content is low when compared with the values reported by Ogungbenle (2006) for benniseed but higher than those of gourd seed (2.80%), white melon (2.00%) and yellow melon (2.60%) reported by Ogungbenle (2006). However, the value compares favourably with that of pigeon pea flour (3.82%) reported by Oshodi and Ekperigin (1989).

Table 2 shows the level of antinutrients in the sample. The antinutrients studied were: phytate, tannin and oxalates. The level of phytate in walnut seems to be much higher than that of moth bean cultivars (8.52-8.99 mg/kg) reported by Khokhar and Chauhan (1986) but is still higher than that of whole grain maize (539 mg/100 g) reported by Oke (1969) and phytate level of 530 mg/100 g for cassava, yellow yam (452 mg/100 g) and white yam (694 mg/100 g) reported by Adeyeye *et al*. (2000). This further implies that the phytate level is relatively high when compared with other grains. The value of tannin, 2.33%, is very close to 2.6% reported for faba beans (Marguardt, 1989), rapeseed (Yapar and Clandinin, 1972) and sorghum (Price *et al*. 1979). The value of oxalate is 1.13% which falls in the range of 1.7-6.5% reported for some oil seeds by Enujiugha and Ayodele (2003). Dietary phytic and oxalic acids have shown to disturb efficient utilization of certain minerals such as calcium, zinc and magnesium and led to development of rickets when certain legumes and cereals are consumed (Aletor, 1987; Liener, 1976). Therefore, consumption of oxalate may require dietary supplementation of the divalent minerals. The amino acid composition of cooked walnut flour is shown in Tables 3, 4 and 5. Glutamic and aspartic acids had the highest values of 151.6 mg/g protein and 89.5 mg/g protein, respectively. This observation was in close agreement with that of Olaofe *et al*. (1994). Total amino acid (TAA) content was 757.1 mg/g protein shared by the total non-essential amino acids (TNEAA), 312.5 mg and the total essential amino acid (TEAA), 444.6; TEAA was 58.7% with histidine and 55.5% (without histidine) (Table 3). These TAA values of walnut are within the range of 705-918 mg/g for African yam bean reported by Adeyeye (1997). Total essential amino acid (TEAA) with histidine was 444.6 mg/g and without histidine, was 420.1 mg/g; these values compare favourably with those reported for soybean by Altschul (1958) as 444 mg/g and for dehulled and hulled African yam beans (376-518 mg/g) reported by Adeyeye (1997). TEAA percentage is also comparable

Table 1. Proximate composition of the sample

Component	Composition (%)
Moisture	11.01
Ash	3.14
Crude protein	14.18
Crude fat	10.22
Crude fibre	3.03
Carbohydrate (by difference)	58.42

Table 2. Anti-nutritional factors of the sample

Component	Content
Phytate (mg/g)	20.18
Tannin (%)	2.33
Oxalate (%)	1.13

Table 3. Amino acid profile

Amino acid	(mg/g crude protein)
Lysine*	42.1
Histidine*	24.5
Arginine*	46.1
Aspartic acid	89.5
Threonine*	29.6
Serine*	30.0
Glutamic acid	151.6
Proline	30.0
Glycine	26.6
Alanine	37.1
Cystine	14.0
Valine*	39.2
Methionine*	15.9
Isoleucine*	30.6
Leucine*	87.5
Tyrosine	23.2
Phyenylalanine*	39.6

*Essential amino acids

with that of cow milk being 490 mg/g protein with histidine but without tyrosine, 463 mg/g protein without histidine or tyrosine and egg, 495 mg/g with histidine but without tyrosine and 473 mg/g, without histidine and tyrosine (FAO/WHO/UNU, 1985). Percentage composition of TNEAA was 41.3 and that of TEAA was 58.7 with histidine and 55.5, without histidine. This indicates that the sample had less TNEAA thus making walnut a good source of plant protein for children and adults (Table 4).

Table 4. Essential and non-essential amino acids of the sample

Amino acid	(mg/g protein)
Total amino acids (TAA)	757.1
Total non-essential amino acids (TNEAA)	312.5
Total essential amino acids (TEAA)	
– With histidine	444.6
– Without histidine	420.1
TNEAA %	41.3
TEAA %	
– With histidine	58.7
– Without histidine	55.5

Comparison between the amino acid profile of walnut and the amino acid reference values quoted by FAO/WHO/UNU (1985) demonstrates that most of the amino acids in cooked walnut meet the range of amino acid requirements recommended for infants, pre-school children and school going children as well as for adults (FAO, 1970). Both histidine and arginine are particularly essential for children (FAO/WHO/UNU, 1985; Harper, 1984; Muller and Tobin, 1980). The present results show that walnut is a good source of essential amino acids (Table 3). The quality of dietary protein can be estimated in various ways but basically the ratio of amino acids available in the food to the needs gives a good comparison (Bender, 1992; Orr and Watt, 1957) (Table 5).

Table 5. Amino acid score of the sample

Amino acid	Score
Isoleucine	0.8
Leucine	1.3
Lysine	0.8
Methionine + cystine	0.9
Phenylalanine + tyrosine	1.0
Threonine	0.8
Valine	0.8

Conclusion

It can be concluded from the study of cooked walnut flour that walnut has good nutritive value which is favourably comparable with that of other conventional sources of proteins. The anti-nutrient values were within the recommended range and walnut contained essential amino acids required by pre-school children, school going children and adults.

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Comparative Study of Heavy Metals in Selected Vegetables Collected from Different Sources

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Abstract. In present study two types of vegetables, one irrigated with tap and other with sewage water, were analyzed with respect to heavy metals. The concentration of heavy metals was high in sewage, soil, and vegetables than in house source. All metals were not detected except iron (0.07 mg/l) in tap water. The accumulation of Cr in sewage ranged 0.1- 14.1 mg/kg that was maximum in Carrot and minimum in Reddish, while Cd was not detected in Carrot. The concentrations of Mn, Fe and Zn in sewage-irrigated vegetables were more than house holds samples.

Keywords: heavy metals, vegetables, sewage water

Introduction

Presence of heavy metals in ecosystem particularly their accumulation in biosystem including soil has far–reaching implications for the humans. However their impact depends upon the chemical composition of contaminated soil, its physical characteristics, cultivated vegetables, irrigation water and the consumption rate (Morton-Bermea *et al*., 2002; Govil *et al*., 2001; Cobb *et al*., 2000). Soil acts as temporary storage for heavy metals and as their source under certain conditions.

Untreated municipal sewage is generally used for irrigation of urban agricultural soil in many parts of the world, particularly in developing countries (Qadir *et al.* 1999), owing to low sewage disposal cost and reclamation of nutrients and water from it (Wang, 1984).

Use of polluted water for irrigation is a matter of major concern due to the presence of toxic metals and other pollutants in it, which contaminate the soil and plants growing in the effluent receiving areas (Singh *et al.,* 2004; Oudeh *et al*., 2002; Armienta *et al*., 2001; Samantaray *et al*., 2001).

Present study was carried out to compare the concentration of heavy metals in selected vegetables namely radish, turnip, carrot, and beetroot growing in home gardens and sewage-water-irrigated areas of Lahore city.

Materials and Methods

Samples of selected vegetables (radish, turnip, carrot, and beetroot) were collected from two different sites; home gardens of Lahore and site of Rohinallha in Lahore, using random sampling technique with the hand sorting method. Vinyl gloves were used for picking the vegetables and samples were carefully packed in polythene bags.

A total of three hundred samples, (including tap water, sewage water, soil at 0-30 cm depth and vegetables) were collected. Each sample of vegetable and soil weighed 1.5 kg on fresh weight basis and those of tap water and sewage water measured 1.0 litre. Five replica of each vegetable along with the soil samples were taken and sampling was performed four times during the study period. Water samples were also taken on the same pattern.

Vegetables were washed thrice with distilled water, finally with deionized water and dried in oven at 65-70 °C; samples were then ground using a ceramic-coated grinder.

Soil samples were air dried at room temperature, powdered and sieved through 2 mm mesh to remove large stones, sand, pebbles etc. For metal analysis soil samples were dried at 105 °C for 2 h and ground to pass through 60 mesh sieve and homogenized.

The representative samples of tap and sewage water were collected in plastic containers previously rinsed with distilled water, and stored at 4 °C in icebox.

Digestion of samples. For metal estimation, nitric-perchloric acid digestion was performed, following the procedure recommended by AOAC (1990). Soil sample (1 g), was taken in 250 ml digestion flask and 10 ml of con. HNO_3 was added. The mixture was boiled gently for 30-45 min and then cooled. After cooling, 5 ml of 70% perchloric acid was added and the mixture was boiled gently until dense white fumes appeared.

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After cooling, 20 ml of distilled water was added and the mixture was boiled further to remove any remaining fumes. The solution was again cooled, filtered through Whatman No.42 filter paper and < 0.45 um millipore filter paper and transferred to 25 ml volumetric flask by adding double distilled water.

Concentrated nitric acid (4 ml) and 2 ml of conc. hydrogen peroxide were added twice to the vegetables samples (1.0 g). The mixture was heated until a clear solution was obtained. It was made up to 5 ml with 1M HNO_3. (Tokalioglu and Kartal, 2006).

Concentrations of metals (Zn, Cd, Mn, Fe, Cr) in the solution were determined using atomic absorption spectrometer (AAS, Varian-240, Australia).

All reagents were of analytical reagent grade (Merck, Germany). Deionized and double distilled water was used for preparing solutions and dilutions. Standard solutions, prepared for the study of elements, were stored in polyethylene containers.

Results and Discussion

Heavy metals were not detected in tap water except iron (0.7 mg/litre), while in sewage water all the metals were present within the permissible limits of National Environmental Quality Standards (NEQS) except Cr and Cd whose amount ranged between 0.9-1.3 and 0.012-1.06 mg/litre, respectively (Table 1).

Table 1. Elemental analysis of tap and sewage water in (mg/litre)

Metals	Tap water	WHO standard	Sewage water	NEQS standard
Cr	N.D	0.05	0.9-1.3	1.0
Mn	N.D	0.1	0.3-1.0	1.5
Fe	0.7	0.3	1.1-5.3	8.0
Zn	N.D	3.0	0.3-4.07	5.0
Cd	ND	0.003	0.012-1.06	0.1

Total concentrations of heavy metals Cr, Mn, Fe, Zn and Cd in the home garden soil, irrigated with tap water and Rohinullah soil irrigated with sewage water are represented in Table 2. Two important heavy metals Cd and Cr were not detected in home garden soil. The range of concentration of zinc, manganese and iron in home garden soil was 0.07-0.9 mg/kg (x = 0.242 mg/kg), 0.01-0.03 mg/kg (x = 0.01 mg/kg) and 2.54-4.0 mg/kg (x = 1.635 mg/kg), respectively. In Rohinullah soil, the range of the concentration of heavy metals Cr, Mn, Fe, Zn and Cd was 2.59-17.5 mg/kg soil (x = 5.022 mg/kg), 35-106 mg/kg soil (x = 35.25 mg/kg), 230-455 mg/kg (x = 171.25 mg/kg), 54-135 mg/kg (x = 47.25 mg/kg), and 0.02-1.09 mg/kg (x = 0.28 mg/kg), respectively. The concentration of heavy metals in Rohinullah site soil samples was elevated due to the use of sewage water for irrigating the vegetation which affected the heavy metal distribution in soil (Rattan *et al*., 2005).

Table 2. Elemental analysis of home garden and Rohinullah soil samples

Metals	Metals (mg/kg)			
	Home garden soil		Rohinullah soil (mg/kg)	
	Range	x	Range	x
Cr	N.D	N.D	2.59-17.5	5.022
Mn	0.01-0.03	0.01	35-106	35.25
Fe	2.54-4.0	1.635	230-455	171.25
Zn	0.07-0.9	0.242	54-135	47.25
Cd	N.D	N.D	0.02-1.09	0.28

Comparative study of the presence of heavy metals in vegetables collected from different sites (Table 3) indicates that the concentration of heavy metals in Rohinullah samples was high as compared to home garden samples (irrigated LDA supply). Cr was not detected in all home garden samples, while in Rohinullah samples, level of Cr in beet root was 1.3-12.7 mg/kg, in carrot 1.1-14.1 mg/kg, in radish 0.1-0.9 mg/kg and in turnip1.1-11 mg/kg; the concentration of Cr varied between vegetables according to their own absorption capacity.

Mn contents in vegetables growing at different Rohinullah sites were higher than those of home garden. Maximum quantity was in beetroot (12-95 mg/kg), while in the soil samples of these sites, average value of Mn was 35.25 mg/kg.

Maximum accumulation of Fe was found in Rohinullah vegetable samples, the order being 105-210 mg/kg in beetroot > 71-110 mg/kg in turnip > 55-97 mg/kg in radish > 25-40 mg/kg in carrot; the sequence in tap water–irrigated vegetable samples was different.

Zn is essential element, required in small quantity for the growth and development of the plant, however, excessive concentration could be toxic (UNEP/FAO/WHO, 1988). Concentration of Zn ranged between 9-110 mg/kg in all the Rohinullah vegetable samples, while in home garden vegetable samples, amount of Zn was much less, the range being 0.2-11 mg/kg.

Contribution of a crop to dietary intake of Cd in human body depends on the quantity of crop consumed as food and on Cd concentration in the consumed foods. In sewage water–irrigated samples, the concentration of Cd and Cr was higher than the tap water-irrigated samples.

Table 3. Concentration of heavy metals in different vegetables from different sources

Metals	Beet Root (mg/kg)		Carrot (mg/kg)		Reddish (mg/kg)		Turnip (mg/kg)	
	Sewage	Tape	Sewage	Tape	Sewage	Tape	Sewage	Tape
Cr	1.3-12.7	N.D	1.1-14.1	N.D	0.1-0.9	N.D	1.1-11	N.D
Mn	12-95	5-7.1	15-48	3-4.5	7-78	4-53	10-78	5-11
Fe	105-210	11-75	25-40	6-18	55-97	27-81	71-110	17-21
Zn	15-110	0.2-1.5	9-60	2-11	9-55	7-11	21-49	1-7
Cd	0.01-1.01	N.D	N.D	N.D	0.1-0.09	N.D	0.01-0.12	N.D.

Concentration of different heavy metals was different in vegetables but Cd and Cr was maximum in sewage water irrigated vegetables as compared to home garden ones because these were easier to absorb and accumulate as compared to other metals (Run Sheng *et al.* 2007). This seems to imply that different types of vegetables have different abilities to accumulate the metals. In spite of the mechanism involved in the elemental uptake by roots, plant are known to respond to the amount of readily mobile type of metals in soil (Madrid *et al*., 2002).

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Antimicrobial Screening of Some Derivatives of Methyl α-D-Glucopyranoside

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Abstract. *In vitro* antimicrobial functionality test of methyl 4,6-*O*-cyclohexylidene-α-D-glucopyranoside and its twelve acylated derivatives against ten human pathogenic bacteria and six phytopathogenic fungi comparative to Ampicillin and Nystatin revealed the tested chemicals to possess moderate to good antibacterial and antifungal activity and to be more effective against fungal phytopathogens. Many of these chemicals exhibited better antimicrobial activity than the standard antibiotics. Minimum Inhibition Concentration (MIC) of methyl 4,6-*O*-cyclohexylidene-2-*O*-myristoyl-3-*O*-palmitoyl-α-D-glucopyranoside against *Bacillus cereus, Bacillus subtilis* and *Staphylococcus aureus* was 25, 12.5 and 25 µg/disc, respectively.

Keywords: antibacterial activities, antifungal activities, methyl glucopyranoside derivatives

Introduction

Carbohydrates, especially acylated glycosides, are very important due to their effective biological activity. A large number of biological compounds possess aromatic, heteroaromatic and acyl substituents. Nitrogen, sulphur and halogen containing substituents enhance the biological activity of the parent compound (Ghorab *et al*., 1988).

Over the last few years, a wide variety of acylated monosaccharide derivatives were prepared and their biological evaluation was carried out at the laboratory of the University of Chittagong (Kabir *et al*., 2005; 2004; 2003; 2002). It was found that the combination of two or more acyl substituents in a single molecular framework enhances the biological activity manifold than their parent compounds. For example, some acylated derivatives of D-glucopyranose were found more active than those of the standard antibiotics (Kabir *et al*., 2007; 2005).

Later, some derivatives of methyl α-D-glucopyranoside containing a cyclohexane moiety and various acyl groups (e.g. octanoyl, decanoyl, lauroyl, myristoyl, palmitoyl, acetyl, benzoyl, 2-chlorobenzoyl, 4-chlorobenzoyl, mesyl, brosyl and pivaloyl) were synthesized in a single molecular framework, and their antimicrobial aganist bactria and fungal screening was carried out using a variety of bacterial and fungal strains. The synthetic part of this piece of work has been reported earlier (Kabir *et al*., 2006); the results of *in vitro* antimicrobial screening experiments are reported here.

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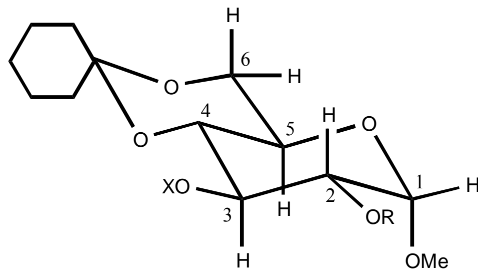
Materials and Methods

Methyl 4,6-*O*-cyclohexylidene-α-D-glucopyranoside (**1**) and its acylated derivatives (**2-13**) were used as test chemicals for the determination of antimicrobial activity. The test chemicals **1-13** (Fig. 1) were synthesized, isolated and purified at the Organic Research Laboratory of the Department of Chemistry, University of Chittagong. Test tube cultures of bacterial and fungal pathogens were collected from the Microbiology Research Laboratory, Department of Microbiology, University of Chittagong, Bangladesh.

Test bacteria. Test chemicals **1-13** were subjected to antibacterial screening against four gram-positive and six gram-negative bacterial strains viz., *Bacillus subtilis* BTCC 17, *Bacillus cereus* BTCC 19, *Bacillus megaterium* BTCC 18, *Staphylococcus aureus* ATCC 653, *Escherichia coli* ATCC 25922, *Salmonella typhi* AE 14612, *Shigella dysenteriae* AE 14396, *Salmonella paratyphi*, *Shigella sonnei* CRL (ICDDR,B) and INABA ET (Vibrio).

Test fungi. Test compounds **1-13** were screened for their antifungal activities against six phytopathogenic fungi viz., *Colletotrichum corchori* (Ikata Yoshida), *Macrophomina phaseolina* (Tassi) Goid, *Curvularia lunata* (Wakker Becdijin), Fusarim *equiseti (Corda) Sacc., Alternaria alternata* (Fr.) Kedissler and *Botryodiplodia theobromae* (pat).

Antibacterial studies. *In vitro* antibacterial activities of the test chemicals were studied by disc diffusion method (Bauer *et al.,* 1966) and nutrient agar (NA) was used for culture of



1. R = X = H
2. R = Myristoyl; X = H
3. R = Myristoyl; X = Ac
4. R = Myristoyl; X = Bz
5. R = Myristoyl; X = 2-Cl.Bz
6. R = Myristoyl; X = 4-Cl.Bz
7. R = Myristoyl; X = Ms
8. R = Myristoyl; X = Bs
9. R = Myristoyl; X = Pv
10. R = Myristoyl; X = Octanoyl
11. R = Myristoyl; X = Decanoyl
12. R = Myristoyl; X= Lauroyl
13. R = Myristoyl; X = Palmitoyl

Fig. 1. Structure of compounds.

bacteria. Throughout the experiment, paper disc of 4 mm diameter and petridish of 70 mm diameter were used which were sterilized in autoclave and dried at 60 °C in an oven. Pour plates were made with sterilized melted NA (45 °C). After solidification of pour plates, the test organisms (suspension) were seeded uniformly over them. Paper discs after soaking with test chemicals (2% in $CHCl_3$) were placed at the centre of the inoculated pour plates. A control plate was also maintained in each case with chloroform. The plates were kept for 4 h at low temperature (4 °C). Test chemicals diffused from discs to the surrounding medium by this time. The plates were then incubated at 35± 2 °C and the growth of test organisms was observed at 24 h intervals for two days. The activity was expressed in terms of inhibition zone diameter in mm. Each experiment was repeated thrice. Standard antibiotic, Ampicillin (for bacteria) from FISONS (Bangladesh) Ltd. was used as a positive control and compared with test chemicals under identical conditions.

Antifungal studies. *In vitro* antifungal activities were determined by poisoned food technique (Grover and Moore, 1962) in some modified conditions (Miah et al., 1990) and Potato Dextrose Agar (PDA) medium was used for culture of fungi. Specific amount of medium was taken in conical flasks separately and was sterilized in autoclave at 120 °C and 15 psi. Weighed amount of test chemical was added to the medium in conical flask at the time of pouring to obtain the desired concentration. The flask was shaken thoroughly for uniform mixing of the chemical with the medium. The medium with definite amount of chemical (100 mg) was then poured into separate sterilized petridishes. Proper control was maintained separately with sterilized PDA medium without chemical and three replicates were prepared for each treatment. The media were allowed to solidify.

Mycelial blocks (5 mm approx.) of individual test fungus were cut out from the outer margin of the cultures growing on PDA plates. The blocks were then placed at the center in all document of each petridish in an inverted position. All the plates were inoculated at 25±2 °C and incubated for 3-5 days.

The linear mycelial growth of fungal colony was measured, in two directions at right angle to each other after 3-5 days of incubation and average of three replicates was taken as the diameter of the colony in mm. The percentage inhibition of mycelial growth of test fungi was calculated as follows:

$$I = \{ \frac{C - T}{C} \} \times 100$$

Where, I = percentage of inhibition, C = diameter of the fungal colony on control ($CHCl_3$) dish and T = diameter of the fungal colony on the test dish.

Results were compared with that of the standard antibiotic, Nystatin.

Results and Discussion

Aim of the present study is directed towards investigation of the antibacterial and the antifungal activities of the synthesized D-glucose derivatives (**1-13**). Test chemicals (**2-13**) were prepared from a common precursor, namely, methyl 4,6-*O*-cyclohexylidene-α-D-glucopyranoside (**1**) and contain a wide variety of substituents. The test chemicals (**1-13**) were screened for their antimicrobial activity against ten human pathogenic bacteria and six phytopathogenic fungi. For

Table 1. Zone of inhibition of the test chemicals against gram-positive test organism

Chemical	Diameter of inhibition zone in mm (100 μg dw./disc)			
no.	*B. subtilis*	*B. cereus*	*B. megaterium*	*S. aureus*
1	00	01	02	04
2	-	-	08	08
3	-	-	-	-
4	-	-	-	-
5	-	-	-	-
6	-	-	-	-
7	-	-	08	18
8	06	10	10	18
9	-	-	-	-
10	15	09	12	-
11	08	12	*22	15
12	-	-	-	-
13	08	*25	11	*25
Ampicillin	25	22	19	20

N.B.: * =Marked inhibition; - = No inhibition; dw = Dry weight

Table 2. Zone of inhibition of the test chemicals against gramnegative test organism

Chemical	Diameter of zone of inhibition in mm (100 µg dw./ disc)					
no.	*E. coli*	*S. typhi*	*S. paratyphi*	*S.dysenteriae*	*S. sonnei*	*Vibrio* (Inaba et)
1	01	02	-	01	05	03
2	10	-	-	-	10	-
3	-	-	-	-	-	-
4	-	-	-	08	-	-
5	-	-	-	-	-	-
6	-	-	10	-	-	-
7	10	-	-	-	15	10
8	-	-	-	06	10	10
9	-	-	-	-	-	-
10	10	08	-	10	12	15
11	12	10	11	08	16	14
12	-	-	-	-	-	-
13	*14	06	-	-	08	-
Ampicillin	13	24	17	35	35	25

* = marked inhibition; - = no inhibition; dw = dry weight

comparative study, the antimicrobial activity of two standard antibiotics, viz. Ampicillin and Nystatin, were also evaluated against these microorganisms. The results of antibacterial activity of the test chemicals (**1-13**) were expressed in terms of zone of inhibition in mm (Table 1 and 2).

The test chemicals exhibited promising inhibitory activity against a number of gram-positive as well as gram-negative bacterial strains. The inhibition data indicated that the chemical **11** was more active (zone of inhibition, 22 mm) against *B. megaterium* and chemical **13** was more active against *B. cereus* (T=25 mm) and *S. aureus* (T=25 mm) than the standard antibiotic, Ampicillin. On the other hand, chemical **13** was more effective against *E. coli,* than the standard drug wheras, chemicals **1-6** displayed little inhibition against the bacterial strains. It was also observed that chemical **13** was very effective against *B. cereus, B. subtilis* and *S. aureus* as compared to Ampicillin; result of minimum inhibition concentration (MIC) test of chemical **13** against these bacterial strains are presented in Table 3.

It was found that selectively acylated derivatives **8, 10, 11** and **13** showed moderate to marked inhibition against gram-positive bacteria while chemicals **7, 10, 11** and **13** were very active against gram-negative bacteria. It was also observed that the acylated derivative **11** was reasonably effective against both the gram-positive and gram-negative organisms.

MIC is the minimum concentration of the antibacterial agent in a given culture medium below which bacterial growth is not inhibited. MIC methods are widely used in the comparative testing of new agents. In clinical laboratories MIC method is used to establish the susceptibility of organisms that give equivocal results in disc tests, in cases where disc tests are

Table 3. Minimum inhibition concentration (MIC) values of test chemical **13**

Test chemical	Bacteria	Sample concentration (µg/ disc)	Zone of Inhibition (mm)	MIC (µg/disc)
13	*B. cereus*	100	25	25
		50	18	
		25	12	
		12.5	-	
		6.25	-	
	B. subtilis	100	27	12.5
		50	*19*	
		25	14	
		12.5	07	
		6.25	-	
	S. aureus	100	25	25
		50	17	
		25	10	
		12.5	-	
		6.25	-	

Table 4. Antifungal activity of the test chemicals and Nystatin

Chemical no.	% Inhibition of fungal mycelial growth, 100 µg (dw) sample/ ml PDA					
	F. equiseti	*M. phaseolina*	*C. corchori*	*B. theobromae*	*Curvularia Iunata*	*Alternaria alternata*
1	8.15	5.01	7.11	3.26	1.5	6.35
2	30.7	34.2	*44.4	20.0	-	15.4
3	30.7	28.5	33.3	32.5	35.7	+6.6
4	38.4	50.0	33.3	+6.6	40.0	+4.7
5	35.3	35.7	33.3	20.6	38.5	20.0
6	35.3	31.4	37.7	28.8	38.5	21.4
7	35.3	50.0	*66.6	40.0	42.8	23.8
8	30.7	*71.4	*66.6	15.0	42.8	28.5
9	30.7	42.8	33.3	15.3	-	*64.2
10	*46.1	42.8	55.5	35.6	48.5	16.6
11	*45.0	28.5	37.7	35.6	44.2	+4.7
12	33.8	35.7	37.7	10.6	37.1	*59.0
13	38.4	35.7	*51.1	+4.4	55.1	40.4
Nystatin	44.70	70.78	40.51	70.0	70.0	51.0

* = marked inhibition; - = no inhibition; dw = dry weight; + = stimulation

unreliable and when a more accurate result is required for clinical management. The MIC value of the chemical **13** was 25, 12.5 and 25 mg /disc against *B. cereus, B. subtilis* and *S. aureus*, respectively. Considering the remarkable inhibitory activity of compound **13** among the tested chemicals against three potential pathogenic bacteria, further experiments are required for evaluating the efficacy of this compound.

The antifungal activity of the test chemicals were evaluated against six phytopathogenic fungi and compared with the similar activity of the antifungal antibiotic, Nystatin. Results of the inhibition of fungal mycelial growth are presented in Table 4.

***Fusarium equiseti*.** Among the chemicals tested, chemicals **10** (46.1%) and **11** (45.0%) showed the highest inhibition, which were more than the standard antibiotic Nystatin employed (44.7%). The rest of the chemicals were, however, less toxic to this fungus as compared to Nystatin.

***Macrophomina phaseolina*.** Most of the test chemicals displayed moderate to marked toxicity towards plant pathogenic fungus *M. phaseolina*. Chemical **8** showed relatively higher inhibition (71.4%) than the standard antifungal drug Nystatin. (70.78%). The rest of the chemicals were either less effective than Nystatin or did not show any inhibition or stimulation.

***Colletotrichum corchori*.** Most of the D-glucose derivatives displayed marked toxicity towards *C. corchori*. Among these, chemicals **2** (44.4%), **7** (66.6%), **8** (66.6%), **10** (55.5%) and **13** (51.1%) showed relatively higher inhibition than the standard antibiotic, Nystatin (40.51%).

***Botryodiplodia theobromae*.** Chemicals **3** (32.5%), **7** (40.0%), **10** (35.6%) and **11** (35.6%) showed reasonable toxicity against *B. theobromae*, but were not as toxic as the antifungal drug Nystatin (70.0%). Here again, chemicals **4** (+6.6%) and **13** (+4.4%) showed stimulation rather than inhibition against this fungus.

***Curvularia lunata*.** Chemicals **4-8** and **10-13** were very effective in the inhibition of mycelial growth of *C. lunata* though not as effective as Nystatin (75.00%); whereas chemicals **2** and **9** were inactive against this plant pathogenic fungus.

***Alternaria alternata*.** Chemicals **9** (64.2%) and **12** (59.0%) displayed very effective inhibition, though not as effective as Nystatin (51.55%). Chemicals **3** (+6.6%), **4** (+4.7%) and **11** (+4.7%), showed stimulation whereas the rest of the chemicals displayed moderate to poor inhibition against this phytopathogen.

Conclusion

The synthesised chemicals (**1-13**) had not been tested as yet against the selected bacterial and fungal pathogens; this is the first report on the subject. The antimicrobial screening data indicated the tested compounds to possess promising biologi-

cal activities and can be used as good source of antimicrobial agents at least in the field of agriculture. It is concluded that some of the synthesised acylated derivatives of methyl 4,6-*O*-cyclohexyli-dene-α-D-glucopyranoside (**1**) may be further tested against a wide range of bacteria and phytopathogenic fungi. Afterwards, these could be sent to pesticide manufacturers for further tests. It is also expected that this piece of work employing carbohydrate derivatives as test chemicals will contribute towards the development of pesticides and medicines for human and plant disease control with minimum environmental hazards.

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Antibacterial Activity of Some Commonly Used Food Commodities Against *Escherichia coli, Salmonella typhi* and *Staphylococcus aureus*

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Abstract. The activity of commonly used spices and salt, sugar and pickles against *Escherichia coli, Salmonella typhi*, and *Staphylococcus aureus* was tested. The antibacterial activity was found to be in descending order like coriander>pickles>salt and sugar>clove>turmeric>black pepper>red chilli against *S. typhi* and garlic>clove>onion>ginger against *S. aureus.*

Keywords: spices, antibacterial activity, food commodities

Introduction

Due to the increasing microbial resistance to drugs, use of phytochemicals for pharmaceutical purposes has gradually increased (Erdogrul, 2002). According to World Health Organization (WHO) more than 80% of the world's population, at present, relies on botanical preparations to meet their health needs (Diallo *et al.,* 1999), usually in rural areas of many developing countries (Sandhu and Heinrich, 2005; Gupta *et al.,* 2005).

Among various diarrhoeagenic serotypes of *Escherichia coli*, enterohemorrhagic *E. coli* O157:H7 has been implicated in a large number of food-borne outbreaks in many parts of the world including developed nations and has low infective dose. Worldwide, annually 1.3 billion cases of human salmonellosis are reported with three million deaths. *Staphylococcus aureus* is a toxigenic pathogen capable of initiating clinical symptoms in humans, consumd through many foods commonly contaminated during handling; thus it is an indicator of unsanitary conditions during handling and or processing of foods (Indu *et al*., 2006).

Herbs and spices are generally considered safe and have proved to be effective against certain ailments. At least 35000 plant species are used in traditional herbal medicine throughout the world (Kong *et al*., 2003). Medicinal value of plants lies in the chemical substances having a definite physiological action on the human body (Edeoga *et al.,* 2005). During the last few years, a number of studies were conducted in different countries to find out the medicinal value of different plants (Nascimento *et al*., 2000; Fabio *et al*., 2003; Nanasombat and Lahasupthawee, 2005).

Flavonoids, such as quercetin are health supportive compounds produced by onions; they are active against a wide array of microorganisms. In humans, ginger is considered to act directly on the gastrointestinal system to reduce nausea. Traditionally, ginger has been used to treat intestinal infections, especially related to digestive problems. Ginger has the capacity to eliminate harmful bacteria, such as *E. coli*, responsible for most types of diarrhoea, especially in children (Azu *et al.*, 2007). Garlic also possesses antibacterial activity and is used as a remedy for many infections, digestive disorders, and fungal infections such as thrush (Sofia *et al.*, 2007). Clove possesses antibacterial activity; it contains eugenol and is usually more bacteriostatic than other spices (Frazier and Westhoff, 1978). Cumin has moderate and red-pepper has a weak inhibitory activity against microbes (Agaoglu *et al.*, 2007). Antimicrobial effect of turmeric is well documented (Marthi, 1999). Coriander seed oil has antibacterial properties and is used for treating colic, neuralgia and rheumatism. Salt and sugar tend to tie up moisture and thus have an adverse effect on microorganisms (Frazier Westhoff, 1978). The antibacterial effects of spices differ with the kind of spice, the microorganisms being tested, and the medium used (Frazier and Westhoff, 1978).

In the present study, the antibacterial effect of widely used spices such as turmeric, clove, black pepper, red chilli, coriander, cumin seed, garlic, ginger, onion and some other food commodities such as salt, sugar and pickles has been evaluated against the bacteria, *E. coli, S. typhi* and *S. aureus*.

Materials and Methods

Procurement of samples. Commonly used spices and foods, namely turmeric, clove, black pepper, red chilli, coriander, cumin seed, garlic, ginger, onion, salt, sugar and pickle were purchased from the local markets in whole form.

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Preparation of samples. Spices were powdered in a grinder whereas garlic, ginger, onion and pickles were blended using Waring Blender (Westpoint, Model TSK-242). 50 g of both powdered and blended samples were mixed with 450 ml of diluent (Butterfield phosphate buffer) to make 1:10 dilution, from which 10 fold serial dilutions were prepared (1:10, 1:100 and 1: 1000).

Sterilization of samples. Whatman filter paper was used to remove large particles from the 1:10 dilutions of all samples and then they were sterilized before preparation of further dilutions using membrane filter (cellulose nitrate, 0.45 μm).

Preparation of bacterial cultures. All the cultures were revived befor every experiment by inoculating nutrient broth with working cultures and then incubating at 35 °C for 18 h. *E.coli* was grown in nutrient broth for 2 h before use.

Agar well diffusion method. Antimicrobial activity was determined by agar well diffusion method (Zaika, 1988). Plates of Baird Parker agar, MacConkey's agar and Bismith sulphite agar were spreaded with 0.1 ml culture of *S. aureus*, *E. coli* and *S. typhi,* respectively. Wells were filled by adding 100 μl of food extracts of varying dilutions while sterile diluent was used as control. Plates were kept at room temperature for diffusion of sample dilution in the media then incubated at 35 °C. After 24 h, the diameter of inhibition zones were measured (including well) in mm with the help of antibiotic zone measuring scale.

Results and Discussion

Most of the commodities showed good inhibitory activity against *S. typhi*. These included turmeric, coriander, pickle, salt and sugar at the conc. of 0.1%, whereas black pepper and red chilli showed inhibitory activity only at a concentration of 10%. Coriander and pickle gave strong antibacterial activity against *S. typhi* with 42 mm and 40 mm zones of inhibition, respectively, at a concentration of 10 %. Cumin seeds, garlic, ginger and onion had no antibacterial activity against *S. typhi* (Fig. 1). In a study carried out by Suresh *et al.* (2004), garlic exhibited very good antibacterial activity even in very low concentrations against *Salmonella* species whereas onion and ginger showed only moderate levels of inhibitory effect.

Clove and garlic exhibited good inhibitory activity at 1% concentration; garlic, clove, ginger, turmeric and onion also showed inhibitory effect against *E. coli* but at 10% concentration (Fig. 2). In a similar study of Adler and Beuchate (2002), addition of garlic to a food substance enhanced the inactivation of *E. coli* at varying temperatures including 35 ºC. On the contrary, black pepper, red chili, coriander, cumin seeds, pickle, salt and sugar did not show any antibacterial activity against *E. coli*.

Garlic displayed strong inhibitory activity against *S. aureus* with 25 mm zone of inhibition at a concentration of 10%. Clove, ginger and onion showed less inhibitory activity whereas turmeric, black pepper, red chili, coriander, cumin seed, pickle, salt and sugar did not show any antibacterial activity against *S. aureus*. (Fig. 3).

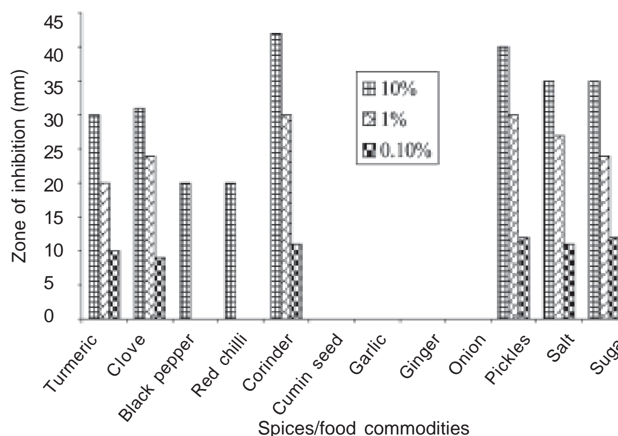

Fig. 1. Antibacterial activity of spices/food commodities against *S. typhi*.

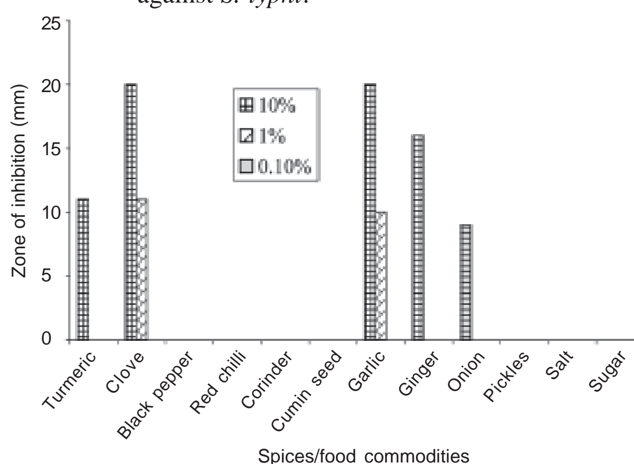

Fig. 2. Antibacterial activity of spices/food commodities against *E. coli.*

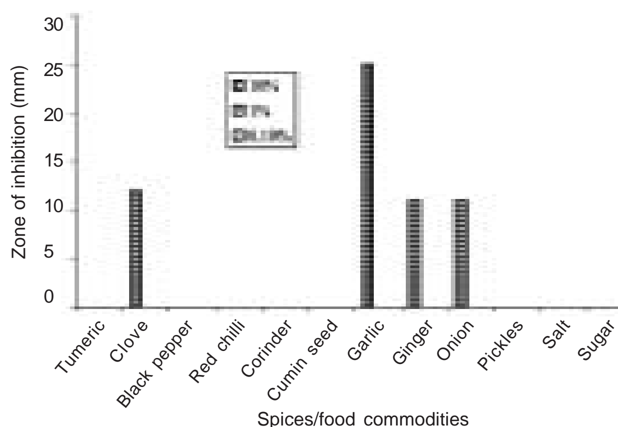

Fig. 3. Antibacterial activity of spices/food commodities against *S. aureus.*

Conclusion

The results confirm that tested spices and other commodities possess antimicrobial activity in varying degrees and they can act as natural food preservatives and natural therapeutics. In developing countries like India and Pakistan, where spices are produced and used as food additives, their use as antimicrobial agents and potential preservatives can be extremely useful.

The standard method for testing of *Salmonella,* described in the *Bacteriological Analytical Manual* (an Online USFDA Publication), recommends using higher dilutions for plating i.e. dilution of 1:100 in case of cinnamon, oregano, all spice and even dilution of 1:1000 in case of clove instead of regular 1:10 dilution for most of the food commodities other than these spices. The present study has shown that there are some other commodities as well, such as coriander, turmeric, pickles, salt and sugar which also require further dilutions to neutralize the effect of natural antagonism against *Salmonella* which was found to be the most sensitive organism among the tested organisms. Other organisms tested did not show sensitivity to these spices and food commodities to that extent.

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Effect of Modified Water Chestnut *(Trapa bispinosa)* Starch on Physical and Sensory Properties of Sponge Cakes

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Abstract. Study of the effect of chemically and physically modified water chestnut (*Trapa bispinosa*) starch on volume index, symmetry index and uniformity index of sponge cake revealed that addition of acetylated starch (3%) increased the volume index of control sponge cake to a greater extent. In case of acid-thinned starch, increase in symmetry index was not very significant except that of concentration of 1%. At 4% - 5% concentration, pregelatinized starch and acid-thinned starch showed excellent uniformity index. Acetylated starch at 1% and 5% concentration significantly increased the grain structure.

Keywords: water chestnut starch, symmetry index of cakes, uniformity index of cakes, volume index of cakes, starch, cakes

Introduction

Native and modified starch obtained from a number of plants sources, such as corn and other cereal grains and roots, are used in food, paper and textile industries. Modified starch can be obtained through chemical, physical or enzymatic process. (Singh *et al*., 2007; Jobling, 2004; Thomas and Atwell, 1999).

Uses of native (unmodified) starch have certain shortcomings which sometimes limit their wider application and industrial use. Addition of modified starch to bakery goods yields benefits that may not be achieved by the use of wheat flour alone. Primary benefits of adding modified starch are moisture retention and texture improvement. It also improves cell structure, increases volume, enhances shelf life and prevents particles from setting. Pregelatinized starch with swelling ability in cold water have been used in products such as thin muffin batters containing fruit particles (e.g. blueberries) that would otherwise settle to the bottom before the thickening effect of the wheat starch sets in during baking (Thomas and Atwell, 1999).

Modified starch, particularly lightly cross linked and substituted pregelatinized starch, help in binding the moisture present in the baked foods thus providing improved tenderness in the final product and contributing to the development of a fine, uniform cell structure, better paste clarity and stability, increased resistance to retrogradation and freeze-thaw-stability (BeMiller, 1997; Glover *et al*., 1986). They also extend shelf life and enhance the textural properties of cakes, cookies and other sweet goods.

Natural and modified starch are also the most economic fillers. In many products, they are used as neutral raw material to prevent aggregation of small particles and provide dispersion of viscous and non-viscous compounds by penetrating between the surfaces. Starch also prevent the products from cracking, crumbling and water exudation (Karaoglu *et al*., 2001). Several studies have been carried out showing the potential use of hydrocolloids in microwave baked cakes (Gomez *et al*., 2007; Seyhun *et al*., 2005).

The objective of the present study is to overcome the structural and textural problems encountered in cake making by addition of modified starch in the formulations.

Materials and Methods

Dried water chestnut, what flour, sugar, fresh eggs and dried milk powder were purchased from local markets of Karachi, Pakistan.

Starch was extracted from dried water chestnut and modified physically and chemically. Six different types of starch were used in cake formulations i.e. native (nWCS), acid-thinned (atWCS), acetylated (aWCS) and pregelatinized (pgWCS), as well as double modified i.e. pregelatinization followed by acid-thinning (pgatWCS)and pregelatinization followed by acetylation.

Isolation of starch. The selected dehydrated water chestnuts were comminuted and 1 kg of sample was stirred for 30 min. with 2 litres of water, while maintaining pH at 9.0 by adding 0.2% sodium hydroxide solution. The slurry was filtered through 100 and 170 mesh sieves and consequently centrifuged at 3000 rpm using a Beckman Coulter Allegra^{TM} X-22 centrifuge. The residue was washed with tap water to remove colour pigments. The starch was then air dried in oven at 45 ± 1 °C to 13% moisture content (Tulyathan *et al*., 2004).

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Pregelatinization of starch. Pregelatinization method as described by Waliszewski *et al*. (2003) was used. A sample of 300 g starch was suspended in 1 litre of distilled water and heated to 80 °C for 15 min with slow stirring. Pregelatinized starch was placed on stainless steel tray in the form of a thin film (1-2 mm) and dried in a convection oven at 40 °C for 48 h, ground in mortar to pass through a 100 mesh screen and stored at room temperature in air tight glass jar.

Acetylation. The method of Sathe and Salumkhe *et al*. (1981) was used for acetylation. 100 g of starch were dispersed in 500 ml of distilled water and stirred for 20 min. The pH of the slurry was adjusted to 8.0 using 1 M NaOH. Acetic anhydride (10.2 g) was added over a period of 1 h, while maintaining the pH range of 8.0-8.5 and thereafter the reaction was allowed to proceed for 5 min. pH of the slurry was adjusted to 4.5 using 0.5 M HCl. It was then filtered and washed four times with distilled water before drying at 30 ± 2 °C for 48 h.

Acid-thinning. The method of Lawal (2004), was employed with some modifications for acid-thinning. 100 g of native water chestnut starch was slurried in 500 ml of 0.05 M HCl. The mixture was stirred for 8 h, while maintaining the temperature at 50 °C. The acid-modified starch was filtered and the residue was washed four times with distilled water. It was air dried for 48 h at 30 ± 2 °C.

Pregelatinization followed by acid thinning. Pregelatinization was carried out by the method described by Waliszewski *et al*. (2003) followed by acid-thinning (Lawal, 2004).

Pregelatinization followed by acetylation. Pregelatinization (Waliszewski *et al*., 2003) was followed by acetylation (Sathe and Salunkhe, 1981).

Preparation of baking powder. Baking powder was prepared according to the American Association of Cereal Chemists Approved Methods 10-90 (AACC, 2000).

Preparation of sponge cake. Cakes were made according to the American Association of Cereal Chemists Approved Methods 10-90 (AACC, 2000). All the cakes, except control, were supplemented with 1%, 2%, 3%, 4%, 5% and 6% (flour weight basis) of each modified and unmodified starch.

Physical measurements. Uniformity, volume and symmetry indices were determined by the approved method of AACC (2000). Digital caliber was used to measure the cake height in mm; density of cakes was also determined. Each baked product was allowed to cool to room temperature (left for about 1 h after baking) prior to quality measurement.

Sensory evaluation of sponge cakes. The samples were labelled randomly with three digit numerical codes and the method of AACC (2000) was followed for the evaluation of cake quality on the basis of taste, aroma, colour, mouth feel, surface appearance and texture. During the taste panel session, the members were instructed to rinse their mouths with water and eat unsalted crackers before tasting each sample (Celik *et al*., 2006). The control cake was presented simultaneously with the rest of samples and was evaluated in random order by the panelists.

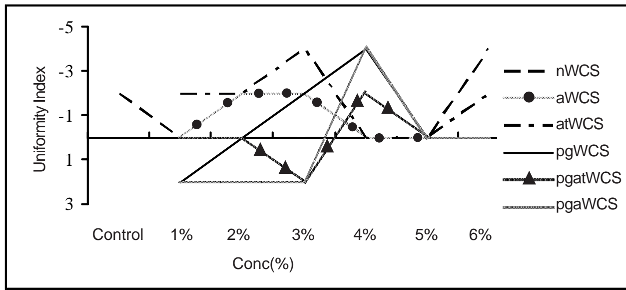
Results and Discussion

Effect of modified and unmodified starch on physical properties of cakes. Physical parameters of sponge cakes i.e. volume index, symmetry index and uniformity index, after the addition of unmodified and modified water chestnut starch at varying concentration are shown in Fig. 1.1 - 1.5. Volume index is an indicator of the volume of the cake, whereas symmetry index indicates the difference in height between the central zone and the lateral zone, giving an idea of the gas retention in the final baking phase. Thus, high symmetry suggests that cakes mainly rise in their central part, while a negative symmetry indicates that cake volume falls down at the end of the baking process.

The differences in the symmetry, uniformity and volume index for all sponge cakes were less apparent; however, addition of acetylated water chestnut starch (3%) increased the volume index of control sponge cake to a greater extent. Addition of atWCS, pgWCS and pgaWCS at all concentrations decreased volume index of sponge cakes, whereas pgatWCS increased the index at 6% concentration.

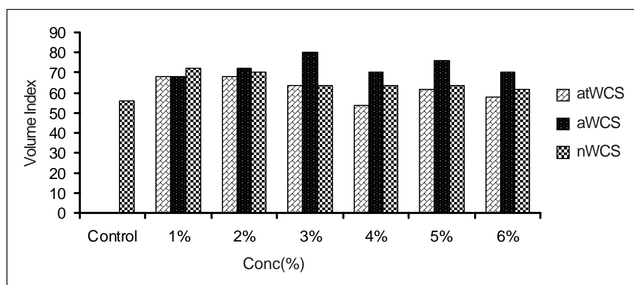
Better symmetry of sponge cakes was noticed after the addition of modified starch as compared to the control cake (Fig. 1.4-1.5). By the addition of aWCS, symmetry index increased at almost all concentrations, however, the maximum increase was observed at 3%-5%. The only decrease was notable at 2% concentration. In case of acid-thinned starch, increasing pattern of symmetry index was observed but was not very significant except at concentration of 1%. Double modified starch (pgatWCS) also markedly increased the symmetry index, while at 2% concentration no significant change was observed, where as double modified starch (pgaWCS) increased at only 5% concentration. Better symmetry index was observed at 4%-5% concentration by the addition of pgWCS.

The highest level of addition (6%) of the modified (acetylated) starch caused the greatest increase in uniformity index while with 2-3% addition, a decrease in uniformity index was observed. At 4%-5% addition, atWCS showed excellent uniformity index whereas with 1%-2% addition, the value was



* control = without starch

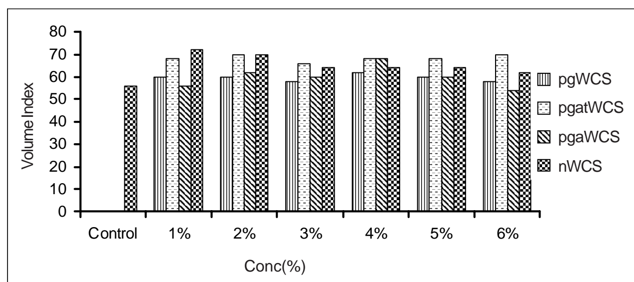
Fig. 1.1. Effect on uniformity index of sponge cakes by the addition of nWCS, aWCS, atWCS, pgWCS, pgatWCS and pgaWCS at varying concentrations.



* control = without starch

atWCS
pgWCS
pgatWCS
pgaWCS

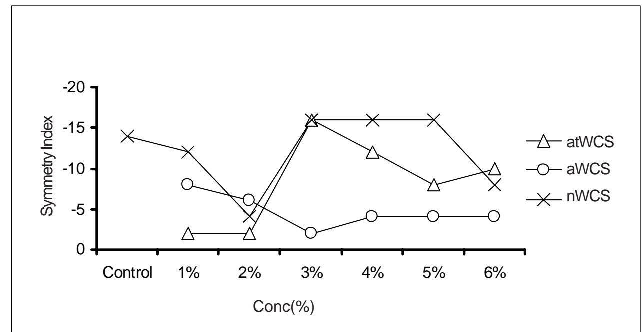
Fig. 1.2. Effect on volume index of sponge cakes by the addition of nWCS, aWCS, and atWCS at varying concentrations.



* control = without starch

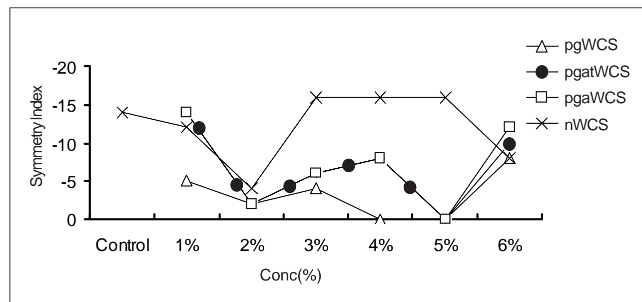
Fig. 1.3. Effect on volume index of sponge cakes by the addition of nWCS, pgWCS, pgatWCS and pgaWCS at varying concentrations.

almost zero. nWCS gave better results as compared to atWCS but with 6% addition, modified starch gave better results than that of native starch. At 3% concentration nWCS showed better uniformity index as compared to the same percentage of the double modified starch (pgatWCS). Uniformity index of sponge cake was significantly increased by the addition of



* control = without starch

Fig. 1.4. Effect on symmetry index of sponge cakes by the addition of nWCS, aWCS, and atWCS at varying concentrations.



* control = without starch

Fig. 1.5. Effect on symmetry index of sponge cakes by the addition of nWCS, pgWCS, pgatWCS and pgaWCS at varying concentrations.

Key to Figs. nWCS (native water chestnut starch); aWCS (acetylaed water chestnut starch), atWCS (acid thinned water chestnut starch), pgWCS (pregelatinized water chestnuts tarch), pgatWCS (pregelatinization followed by acid thinning, water chestnut starch), pgaWCS (pregelatinization followed by acetylation, water chestnut starch).

pgWCS as well as double modified starch (pgaWCS) at 6% concentration.

Effect of modified and unmodified starch on sensory evaluation. The effect of unmodified and modified water chestnut starch on the sensory attributes of sponge cakes (Table 1) was significant. By the addition of native starch at 1%, 2% and 6% concentration, there was enhancement of cells structure as compared to the sponge cake without the addition of starch. However, the bad grain structure was observed at all concentrations. An enhancement in the texture was observed at 1% concentration but there was no significant effect on crumb colour and flavour of the sponge cake by the addition of

Table. 1. Effect of varying concentrations of nWCS, aWCS, at WCS, pgWCS, pgatWCS and pgaWCS on sensory properties of sponge cake

Starches	Concentrations					
	1%	2%	3%	4%	5%	6%
	Cells					
Control* = 22.4						
nWCS	21	21	15.5	15	15	22
aWCS	20.4	23.6	19.2	17.6	20	14.8
atWCS	19.6	18	12.8	10.4	10	10
pgWCS	20	16	18	22	20	21
pgatWCS	19	21	16	6	20	22
pgaWCS	25.33	25.3	21.3	25.3	24.6	25.3
	Grains					
Contol* =12						
nWCS	12	12	10	10.5	11	11
aWCS	13.6	11.6	12.4	12.4	13.6	11.2
atWCS	10.8	10.8	10	8.4	8.8	10.4
pgWCS	11	11	10	8.4	8.8	10.4
pgatWCS	12	10	10	9	12	10
pgaWCS	10.6	8.6	11.3	13.3	8.6	8.6
	Texture					
Control* = 22.8						
nWCS	31.5	30	28.5	27.5	27.5	28.5
aWCS	30	30.4	30	31.6	29.6	29.6
atWCS	25.2	26.4	22.4	22.4	20.8	26
pgWCS	26	28	23	22.4	21	26
pgatWCS	30	30	27	26	27	28
pgaWCS	30	30	31.33	29.3	29.3	29.3
	Crumb colour					
Control* = 6						
nWCS	7	6	7	6	9	7
aWCS	7.2	6.8	7.2	6.8	7.2	6
atWCS	5.6	6.4	4.8	7.2	7.2	7.2
pgWCS	7	6.4	5	8	7.2	8.5
pgatWCS	8	7	8	7	8	7
pgaWCS	8	7.33	7.3	7.3	7.33	7.3
	Flavour					
Control* = 10						
nWCS	10	10	5	10	5	5
aWCS	10	10	10	10	8	8
atWCS	10	10	4	8	6	6
pgWCS	10	10	10	10	10	10
pgatWCS	10	10	10	10	10	10
pgaWCS	10	10	10	10	10	10

* Control = without starch

Key: nWCS (native water chestnut starch); aWCS (acetylaed water chestnut starch); atWCS (acid thinned water chestnut starch); pgWCS (pregelatinized water chestnuts tarch); pgatWCS (pregelatinization followed by acid thinning water chestnut starch); pgaWCS (pregelatinization followed by acetylation water chestnut starch)

native starch. Addition of aWCS at 1% and 5% concentration, significantly increased the grain structure comparative to the addition of native starch.

Acid-thinned starch showed overall decreasing effect on the sensory properties of sponge cake. Sensory panelist gave low score to the acid-thinned cake. Double modified starch (pgatWCS) showed significant decrease in cell structure whereas other sensory properties were quite similar to the other cakes. Double modified starch (pgaWCS) increased the overall sensory properties of sponge cakes.

Thus, it was noticed that addition of unmodified and modified starch at different concentrations to the sponge cake increased its sensory properties.

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Short Communication

Efficacy of Copxykil Against Some Pathogenic and Non-Pathogenic Microorganisms

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Abstract. Efficacy of copxykil – with copper oxychloride as active ingredient – as fungicide and bactericide was evaluated against *Alternaria alternata*, *Fusarium oxysporum*, *F. solani, Aspergillus flavus*, *A. fumigatus*, *A. niger* and *Pencillium expansum* as well as against *Escherichia coli*, *Shigella dysentriae* and compared with a standard imported commercial product 'COBOX'. The test fungicide proved to be more effective than the commercial one.

Keywords: copxykil, fungicidal effect, bactericidal effect, copper oxychloride

Copper oxychloride is a fungicide and has been effective in controlling a number of plant diseases such as early and late blight caused by *Alternaria solani and Phytophthora infestants*, respectively, potato diseases in different parts of India (Paharia, 1961a, b; Chowdhury, 1954; Chattopadhya, 1953), fusarium root rot caused by *Fusarium solani* (Crop Profile for Dry Beans in Kansas, 2003), altenaria brown spot due to *Alternaria alternata* on citrus fruits etc. (Rangaswami and Madhawan, 2005). Swart *et al*. (2008) stated that higher percentage of export quality fruits was obtained by the use of copper oxychloride as compared to that of other fungicides, specially Mancozeb.

Copper products showed longer residual activity and higher rain fastness than did the brands Mancozeb, Difenoconazole, Iprodione, Famoxadone and Pyraclostrobin. Cuprous oxide and copper oxychloride provided satisfactory control of fruit diseases through 28 days and withstood 71 mm of rain fall in the orchard. Thus these chemicals saved the numbers of sprays by approx. half per season for fruit protection (Vincent *et al*., 2007).

In Pakistan about 25-30% of the crop yield is damaged due to diseases. Keeping in view the agricultural importance of copper oxychloride as a fungicide and its extensive use for protecting plants from fungal diseases, this fungicide was synthesized in the laboratory as wettable powder following a new economical method and the product was registered as 'Copxykil'. In the present communication, preparation and efficacy of this fungicide has been discussed as compared to that of the imported fungicide, 'Cobox' and against some pathogenic and non-pathogenic fungi as well as some water borne pathogenic bacteria *viz. Escherichia coli* and *Shigella dysentriae* (Todar, 2008).

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Copper oxychloride was prepared employing a modified method by the action of air on scrap copper in hydrochloric acid and sodium chloride salt solution (Qaimkhani *et al*., 2008). The product has copper content of 56-58% and apparent density of 420-520 g/litre. It is a bluish green powder, insoluble in water, but soluble in ammonium hydroxide solution. Its composition varies according to the conditions of manufacture but generally approaches the formula $CuCl_2.3Cu(OH)_2$. The product is in the form of wettable powder having approx. 80% active ingredients and 20% inert material. Its fungicidal efficacy has been tested versus that of the commercial fungicide Cobox BASF (Brasileria S.A. Industries Quimicas, Sao Paulo-Brasil) which is a wettable powder having approx. 84% copper oxychloride as active ingredient and 16% inert material.

As copper oxychloride is insoluble in water, therefore, its emulsification was made using the emulsifier polyethylene glycol which has no fungicidal activity of its own (Leven *et al*., 1979). A series of different concentrations of both fungicides were made as 0.1%, 0.5%, 1%, 5% and 10% with the above emulsifier.

Eight fungi were taken as test organism; four of them were plant pathogens *viz*., *Alternia alternata, Fusarium solani, F. oxysporum and Helminthosporium sp.* and four were saprophytes viz. *Aspergillus niger*, *A. flavus*, *A. fumigatus* and *Pencillium expansum*. These fungi were isolated from infected fruits. The fungi were grown and maintained on Czapek's Dox agar and the bacteria were cultivated on nutrient agar.

Bactericidial activity of Copxykil was also tested against water borne human pathogenic bacteria *viz*., *Escherecia coli* and *Shigella dysentriae*. Results are tabulated in Table 1.

Table 1. Fungicidal effect of Copxykil on plant pathogenic and non-pathogenic fungi and pathogenic bacteria at different concentrations

Microbial species	Test fungicide (Copxykil) Concentration					Standard fungicide (Cobox) Concentration				
	0.1 %	0.5%	1%	5%	10%	0.1%	0.5%	1%	5%	10%
	Zone of inhibition (dia. in cm)					Zone of inhibition (dia. in cm.)				
Pathogenic fungi										
Alternaria alternata	–	1.0	1.5	2.7	3.0	–	–	1.3	2.2	2.7
Fusarium oxysporum	1.0	1.5	1.8	2.5	3.2	–	1.1	1.5	2.3	2.8
F. solani	–	1.5	2.0	2.5	3	–	–	1.5	2.0	2.5
Helminthosporium sp.	–	1.0	2.5	2.8	3.5	–	1.0	1.5	2.2	2.9
Non-pathogenic fungi										
Aspergillus flavus	–	–	1.0	1.1	1.3	–	–	–	1.1	1.2
A. fumigatus	–	–	1.0	1.1	1.3	–	–	–	1.1	1.3
A. niger	–	1.5	2.0	2.5	3.0	–	1.5	2.0	2.4	2.8
Penicillium expansum	–	–	1.1	1.5	1.7	–	–	1.0	1.5	1.7
Pathogenic bacteria										
E. coli	--	1	1.5	2.5	3	1	1.5	2.5	3	3.5
Shigella dysentriae	--	1	1.6	2	2.5	1	1.5	2	2.8	3

For testing fungicidal activities diffusion plate method (Reddish, 1950) was used for the product as well as the standard commercial fungicide 'Cobox' whereas agar diffusion method was used for testing antimicrobial activity. All these experiments were performed in duplicate. The culture plates were examined for zone of inhibition for a period of 2 weeks. Same technique was used for the control.

The zones of inhibition of microorganisms (Table 1) indicate that the efficacy of the formulation 'Copxykil' as fungicide is equivalent to that of the standard one i.e. 'Cobox'. The lowest inhibition concentration of 'Copxykil' was generally 0.5%, whereas that of 'Cobox' was 1.0%.

Comparatively, pathogenic fungi displayed more sensitivity to the formulated fungicide than the non-pathogenic fungi. Maximum activity was shown by *Helminthosporium* sp. This confirms the findings of Gupta *et al.* (1980) relating to *H. oryzae*. Also *A. alternata* showed results approximately close to *Helminthosporium* sp. The next pathogenic fungal species which showed sensitivity to this fungicide were *F. oxysporum* and *F. solani*. Upadhyay and Roy (1987) reported earlier that *F. moniliform* was checked completely by 10 ppm concentration of six fungicides, including copper oxychloride.

Least inhibition was exhibited by *A. flavus* and *A. fumigatus*. This reaction could be due to the presence of toxin in the fungus clashing with the fungicidal power of copper oxychloride. Other saprophytic fungi showed high sensitivity to this fungicide. *Penicillium expansum* showed less activity than *A. niger*.

Bactericidal efficacy of 'Copxykil' was found to be comparable with that of 'Cobox' against the bacteria, *E. coli* and *S. dysenteriae*.

From these studies, it is clear that Copxykil can be used as a fungicide as well as a bactericide at the pre- and post- harvest stages for protection of plants against fungal and bacterial pathogens.

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Technology

Quantification of Methotrexate by Liquid Chromatography Ultraviolet Detection for Routine Monitoring of Plasma Levels

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Abstract. A high-performance liquid chromatographic (HPLC) technique with ultraviolet detection incorporating solid phase extraction (SPE) was developed to meet analytical and metrological requirements for routine serum level monitoring of methotrexate (MTX), with several parameters optimised such as temperature, flow rate, composition of the mobile phase and pH of the buffer solution. Two standard curves were constructed to cover the high and low levels of the calibrator range (0.02-600 µmol/litre). Reproducibility (precision) of the method for intra assay was 2.7; 2.10; 1.38% at the lowest level and 2.11; 3.4; 2.01% at the highest level and for inter assay was 2.8; 2.2; 2.94% at the lowest level and 2.4; 2.74; 2.68% at the highest level; recovery was between 90.47 and 98.53 percent. Response was found linear over the whole range of the calibrator set with a correlation coefficient of 0.999. The limit of quantification and the limit of detection were 0.02µmol/litre and 0.0063µmol/litre, respectively. The method is suitable for quantification of methotrexate with good accuracy and precision

Keywords: methotrexate, liquid chromatography, solid-phase extraction, plasma level monitoring

Introduction

Methotrexate (MTX) is a competitive inhibitor of dihydrofolate reductase (Balloy *et al*., 2007), a key enzyme of nucleic acid biosynthesis (Albertioni *et al*., 1995). Thus, it can block tumoral cell growth and is widely used as a cytostatic agent (Albertioni *et al*., 1995). In cancer treatment, high-dose MTX therapy is followed by leucovorin (folinic acid) rescue. Serious toxicity might be detected by monitoring serum methotrexate concentrations (Balloy *et al*., 2007; Aboleneen *et al*., 1996; Albertioni *et al*., 1995).

The aim of the study is to describe a simple, fast and precise method for determination of methotrexate in plasma for pharmacokinetic studies and for use in routine therapeutic drug monitoring in cases of high-dose intravenous infusion of this drug.

Materials and Methods

Chemicals and reagents. Methotrexate, hydroxymethotrexate and 8-chlorotheophylline (used as internal standard) were purchased from Sigma, potassium dihydrogen phosphate (KH_2PO_4) was purchased from Carlo Erba whereas methanol, and acetonitrile, HPLC grade, were obtained from Merck. All other chemicals and solvents used were of analytical grade. The reference of reversed phase cartridge for solid phase extraction (SPE) was Chromsystems Ref: 7008 lot 212.

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Instrumentation. The LC system, used for method development and validation, was obtained from Merck and consisted of L-6000 pump, L-5025 oven and L-4250 spectra absorbance detector.

Sample preparation. Stock solutions used in this work were MTX, 0.75 mg/ml (1651 µmol/litre) and OH-MTX 0.05 mg/ml (106.15 µmol/litre). They were serially diluted in 14% $CaCO_3$. Further stock solutions of MTX and OH-MTX were prepared in different concentrations as follow: MTX (0.02 to 0.8 µmol/litre and 5 to 500 µmol/litre) and OH-MTX (0.2 to 0.77 µmol/litre and 4.8 to 480 µmol/litre).

8-Chlorotheophylline was used as internal standard (IS) because it is a synthetic molecule that cannot be administered to patients. Stock solution of internal standard was prepared in water at a concentration of 200 µg/ml.

MTX, OH-MTX and IS stock solutions were stored at -30 °C.

Chromatographic conditions. The chromatographic column used was 250 x 4 mm (Lichrospher®) with 5 µm particle size and 1 cm long guard column. Mobile phase was phosphate buffer, pH = 6.1: methanol (80/20, v/v); flow rate of mobile phase was 1 ml/min. Column was maintained at 25 °C, detection wavelength was 297 nm and injection volume was 50 µl. These parameters were used at the beginning of the experiment and the chromatographic conditions were optimized for the required analysis.

Extraction procedure. The coextractive cleanup procedure was performed according to the method reported by Aboleneen *et al.* (1996) with slight modification.

Heparinised plasma (300 µl) containing 20 µl of internal standard, 40 µl of MTX and methanol, twice its volume, were mixed thoroughly. After centrifugation (10 min, 3000 g at 4 °C), the deproteinized supernatant mixture was transferred to the extraction cartridge. Cartridges were prepared with 9 ml methanol followed by 3 ml of 0.04 M phosphate buffer (pH 6), drawn through centrifugation or suction, discarding the effluent. The eluate, containing analyte and IS, was dried under nitrogen stream at 60 °C. The residue was reconstituted in 200 µl HCl 0.005 M. A 50 µl aliquot of the reconstituted sample was injected on to the HPLC column.

Patients and treatment. The study was approved by the local ethics committee and informed consent was obtained from all the children and their parents. The study group comprised of 34 children with acute lymphocytic leukaemia (ALL). They were enrolled at the time of diagnosis in the European Organization for Research and Treatment of Cancer (EORTC). MTX infusion (5 g/m^2) was administered at 24 h interval. For MTX monitoring, blood samples were collected from all the patients at intervals of 24 h, 48 h and 72 h from the time of injection/infusion and adjusted until MTX concentration was below 0.2 µmol/litre.

Results and Discussion

Optimized chromatographic conditions. For optimizing separation of the drugs, several mobile phase compositions were investigated. Initially, phosphate buffer pH=6.1: methanol (80/20, v/v) was used; however, MTX, OH-MTX and IS cannot be separated under this condition. Subsequently, phosphate buffer pH=4: methanol (80/20, v/v) was used, wherein all the drugs were separated but the retention time was as long as 25 min. When acetonitrile was added to the mobile phase, with adjustment ratio of phosphate buffer pH=4: acetonitrile: methanol (82:12:6, v/v/v), the retention time of three drugs decreased. This mobile phase was found the most suitable and gave a good baseline.

In the study of effects of different temperatures in the range of 25 °C to 70 °C and flow rate of 0.7 to 1.2 ml/min, good separation was obtained at 40 °C and flow rate of 1 ml/min (resolution > 1.5). Other chromatographic conditions included the ratio of mobile phase of phosphate buffer pH=4: acetonitrile: methanol to be 82:12:6, v/v/v, flow rate 1, ml/min and temperature, 40 °C. The retention time for MTX, OH-MTX and IS was 8.77, 10.5 and 12.88 min, respectively. Chromatograms are shown in Fig. 1.

Precision. For precision, six assays per concentration were made. Within- and between-day accuracy and precision values are given in Table 1.

The inter-assay and intra-assay variation of the measured concentration was assessed by the relative standard error (% RSD) which was calculated as the standard deviation of the measured concentrations divided by the mean and multiplied by 100. Batches, for which the recovery did not fall within 71-125%, and/or having RSD value greater than 5%, were reanalyzed. For assessing the accuracy of the method,

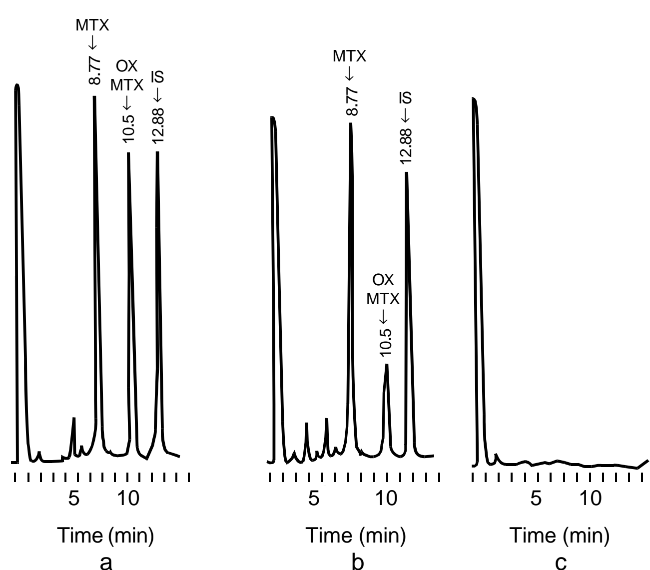

Fig. 1. Representative chromatograms of spiked plasma with MTX and OH MTX and IS (8-chlorotheophylline) (a), patient sample (b) and blank plasma (c)

Table 1. Precision data for methotrexate

Theoretical value (µmol/litre)	Measured value (mean ± SD) (µmol/litre)	RSD (%)	Accuracy (%)
Intra-assay (n = 6)			
0.02	0.018 ± 0.0005	2.77	90
0.4	0.38 ± 0.008	2.10	95
0.8	0.72 ± 0.010	1.38	90
5	4.55 ± 0.096	2.11	90
50	35.88 ± 1.22	3.4	71.76
500	531.83 + 10.72	2.01	106.36
Inter-assay (n = 6)			
0.02	0.025 ± 0.0007	2.8	125
0.4	0.36 ± 0.008	2.2	90
0.8	0.68 ± 0.02	2.94	85
5	4.91 ± 0.12	2.4	98.2
50	44.46 ± 1.22	2.74	88
500	526.25 + 14.12	2.68	105

recovery of methotrexate from plasma samples of known concentrations was compared with the solutions of methotrexate at the same concentrations. Mean recoveries were between 90.47% and 98.53% for inter- and intra-assay, respectively.

Linearity. Calibration curves for plasma samples were constructed within the concentration ranges of (0.02-500 µmol/litre) using linear least-square regression. The MTX calibrators were divided in two concentration ranges: the low levels (0.02; 0.4; 0.8 µmol/litre) and the high levels (5; 50; 500 µmol/litre). Correlation coefficient, slope and intercept for six standard curves constructed for each range, were 0.99, 0.85 and 0.34, respectively. The specificity was determined by comparing 15 plasma samples collected from patients who did not receive MTX. Each sample was analysed and any detectable signal corresponding to the retention times of MTX and IS was not found.

Limit of detection and limit of quantification. For estimating the limit of detection (LOD) and the limit of quantification (LOQ), 10 replicates of control plasma samples (blanks) were analyzed by the described procedure. LOD was calculated as mean blank response plus three times the standard deviation and LOQ was calculated as mean blank response plus 10 times the standard deviation (Vassault *et al*., 1999). The LOD and LOQ of the described method for MTX in plasma samples were 0.0063 and 0.02 µmol/litre, respectively.

Application. The method has been applied successfully for determination of MTX concentration in 234 dosages (Fig. 1). The average age was 18 years (8 to 21 years). Mean plasma concentration at 24 h, 48 h and 72 h intervals was 1.90; 0.18 and 0.03 µmol/litre, respectively. Statistic analysis showed no correlation between dose and plasmatic level of MTX at 24 h, 48 h and 72 h.

Considerable individual variability has been observed and regular monitoring is required in clinical use. Moreover, further investigations are necessary to assess the clinical utility of monitoring MTX concentrations and to identify the relation between the pharmacokinetic parameters of MTX and clinical efficacy and toxicity in Tunisian population.

The present study describes a highly sensitive, accurate, and reproduceable HPLC method for determination of MTX in human plasma. The procedure of sample preparation is rapid; it uses a small plasma volume (300 µl) and is less expensive.

In order to optimise a method, several parameters were modified. Solid-phase extraction (SPE) was essential for plasma samples; in fact, without SPE, a larger solvent front interferes with the two analyte peaks. This extraction was the preferred method by Albertioni *et al*. (1995). This extraction required, a procedure for denaturing proteins, which was achieved by the precipitation of proteins in the samples with methanol (Floridia *et al*., 1999). The other method uses acetonitrile, trichloroacetic acid or centrifugation (Fotoohi *et al*., 2005, Nadège *et al*., 2003; Albertioni *et al*., 1995).

The procedure was based on the internal standard method, as reported in several studies (Albertioni *et al*., 1995; Cociglio *et al*., 1995). The mobile phase reported in the present study uses phosphate buffer at pH=4: methanol: acetonitril in the ratio 82:12:6, v/v/v). This phase was described in other studies but in different proportions (Fotoohi *et al*., 2005, Turci *et al*., 2000, Vassault *et al*., 1999; Aboleneen *et al*., 1996).

Calibration curves were constructed using linear least-square regression. Determination was based on the internal standard method, (Fotoohi *et al*., 2005; Aboleneen *et al*., 1996). The coefficient correlation of the method was R = 0.999 and the overall RSD < 5%. Within- and between-day accuracy was 3.65 and 3%, respectively. In other studies, these values varied between 3 and 4% (Aboleneen *et al*., 1996; Albertioni *et al*., 1995; Cociglio *et al*., 1995). Values of quantification reported in the literature ranged from 0.03 µmol/litre to 0.08 µmol/litre. In the present assay, the limit of quantification was 0.02 µmol/litre and the limit of detection was 0.0063 µmol/litre.

Conclusion

A technique for rapid and sensitive assay of methotrexate in the serum was developed. This method was free from plasma matrix interferences and determined methotrexate concentrations at the lowest and the highest levels. The utility of the method was further demonstrated by measuring methotrexate levels in patient samples. The method can be used to estimate OH-MTX in plasma sample.

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Experimental Investigation of VOCs Emitted from a DI-CI Engine Fuelled with Biodiesel, Diesel and Biodiesel-Diesel Blend

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Abstract: Experimental investigation of volatile organic compounds (VOCs) emitted by a turbocharged direct injection compression ignition (DI-CI) engine, alternatively fuelled with biodiesel and its 20% blend with diesel, revealed dominancy of diesel and biodiesel in aromatic hydrocarbons, esters other oxides, respectively, in total volatile organic compounds (TVOCs). The overall brake specific emission of VOCs increased at rated speed compared to maximum torque speed. The VOCs exhibited their maxima at low load, and minima at medium load for diesel and B100. Engines with a speed of 2300 r/min and 100% load showed a reduction in BTX emissions from B20 and B100, as compared to diesel. The sum of VOC-components of B20 and B100 reduced as compared to that of the diesel, for all the engine conditions. The mean BSE of BTX-components taken from all the engine conditions decreased with B20 and B100, relative to fossil diesel.

Keywords: compression ignition engine, biodiesel, unregulated emissions, volatile organic compounds

Introduction

In the face of depleting reservoirs of conventional fossil fuels and stringent emission regulations for compression ignition (CI) engines, biofuels are promising alternative fuels, which could resolve not only the energy security problems but the environmental pollution issues as well.

Biodiesel (methyl esters of animal and plant origin) as an alternative fuel has already been investigated for performance, combustion, injection and emission in diesel engines. Many studies regarding regulated emissions have reported that biodiesel reduces the particulate matter (PM), carbon monoxide (CO), hydrocarbons (HC) and oxides of sulfur (SO_x) (Sinha and Agarwal, 2005; Senda *et al.,* 2004; Turrio-Baldassarry 2004; Monyem and Gerpen, 2001). Unregulated emissions comprise a wide range of pollutants such as individual non-methane hydrocarbons (NMHC), polycyclic aromatic hydrocarbons (PAHs), medium diameter of the PM (D_m), soluble organic fraction (SOF) of the PM, and volatile organic compounds (VOCs) (Ballesteros *et al.,* 2008). Some studies, focused on unregulated emissions, have revealed that biodiesel and its blends reduce the polycyclic aromatic hydrocarbons (PAHs) (Shah *et al.,* 2009a; Lin *et al.,* 2006). It has also been reported that mutagenicity of biodiesel particulate emissions is much lower than that of petroleum-based diesel fuel (McDonald *et al.,* 1995). Although some work on VOCs has been reported on the exhaust of diesel engines, however, biodiesel and its blends with diesel fuel still need to be addressed comprehensively. This should be done particularly for VOC-emissions at maximum torque speed and rated speed for varying loads, particularly when engine is unmodified. In this work, it has been attempted to determine the VOC-components emitted from a direct injection compression ignition engine fuelled with diesel, biodiesel and 20% biodiesel-diesel blend. Results are drawn on comparison in terms of brake specific emissions (BSE) for different engine conditions (loads and speeds).

The VOC-components are an important class of unregulated emissions from the exhaust of diesel engines. The World Health Organization (WHO) has defined the VOCs as organic compounds that has boiling point with in the ranges of 50-100 °C and 240-260 °C. There are two major reasons to select VOCs for the current study. In the first place, some volatile organic compounds are toxic and thus directly influence the human health. According to international chemical safety cards published by The National Institute for Occupational Safety and Health (NIOSH), U.S.A., benzene is carcinogenic to humans and may affect the blood forming organs, liver and immune system; toluene may affect the central nervous system, resulting in decrease in learning ability and psychological disorders; *p,m*-xylene may affect the central nervous system and human reproduction; and *o*-xylene may cause damage to central nervous and hearing systems (Haupt *et al.,* 2004). Furthermore, it has also been reported that among the non-methane volatile organic compounds (NMVOCs), the aromatic

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compounds like benzene, toluene and xylene isomers are suspected carcinogens and their prolonged exposure may lead to leukemia (US EPA, 1990).

Secondly, VOCs contain reactive compounds which are capable of participating in the formation of photochemical smog or ozone (Schulz, 1999), and thus pose a serious air pollution problem under direct sunlight. Xylene isomers, the important components of VOCs, have been blamed for converting significant amounts of NO to NO_2 (Simpson, 1995).

Materials and Methods

Test engine, fuels, and working conditions. The engine used in this study is a heavy duty, turbocharged, direct injection, intercooled, with 4 cylinders (China made 4CK series), which was run on an electric dynamometer (SCHENCK HT 350) as shown in Fig. 1. No modification or alteration was made in the engine. Its specifications (Shah *et al.,* 2009a) are listed in Table 1.

Three fuels were used in the study namely commercial diesel (D), biodiesel (B100) and its 20% blend by volume with diesel (B20) using commercial diesel as a reference fuel. Biodiesel was obtained from waste cooking oil, provided by Zhenghe Bioenergy Co., Ltd., Hainan, China, (Shah *et al.,* 2009a,b). Main properties of the test fuels are given in Table 2. The experiments were performed in accordance with the engine conditions given in Table 3. The maximum torque speed at full load and maximum power speed (rated speed) at 10%, 50%, and 100% loads were selected for the study. The engine load (torque) was measured by torque flange and was read, along with engine speed, directly on monitor supported by software "Automation system STARS Rev. 1.5" in the control room. Fuel flow rate was measured by PLU-V2 (Pier Berg); crank angle was found with the help of a sensor (2613A) by Kistler Corporation and Dewetron (DEWE-5000); instantaneous pressure in the cylinder was determined using Piezo-electric sensor (Kistler 6125B) and combustion analyzer (Dewetron, DEWE-5000); ignition delay was estimated using the needle lift curve, traced by needle lift transducer (sensor) and cylinder pressure curve (Shah *et al.,* 2009b). Engine oil and coolant temperatures were measured using Pt-100 (sensor) and exhaust temperature was measured using thermocouple (K-series).

Sampling methodology. An ejector diluter is used in order to get emissions directly from the exhaust pipe, (Dekati Ltd. Finland). Its J-shaped stainless-steel sampler probe was inserted into the exhaust pipe as shown in Fig. 1. The specifications of the ejector-diluter (Shah *et al.,* 2009a), in brief, are as follows. It consists of a set of filters, a dryer, a temperature controller, a pressurized air heater and two diluters. The dry, particle-free and pressurized air was introduced into the primary diluter and was heated up to the exhaust gaseous

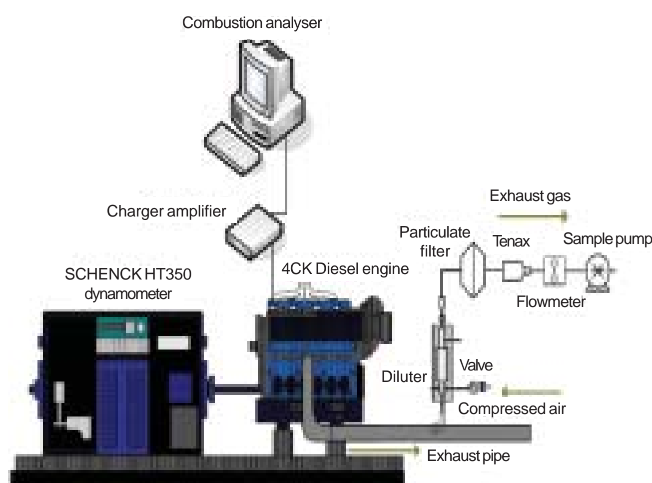

Fig. 1. Experimental setup

Table 1. Engine specifications.

Number of cylinders	4
Bore (mm)	110
Stroke (mm)	125
Displacement (litre)	4.752
Compression ratio	16.8
Rated power (kW@ r/min)	117/2300
Maximum torque (N.m@ r/min)	580/1400
Nozzle hole diameter (mm)	0.23
Number of nozzle holes	6

Table 2. Properties of fuels.

Properties	B100	B20	D	Standards
Density (kg/m^3)	886.4	845.1	834.8	SH/T 0604
Viscosity (mm^2/s) at 20 ºC	8.067	4.020	3.393	GB/T 265
Lower heating value (MJ/kg)	37.3	41.57	42.8	GB/T 384
Sulfur content (mg/L)	25	n/a	264	SH/T 0253-92
Cetane number	60.1	n/a	51.1	GB/T 386-91
Carbon content (%)	76.83	n/a	86.92	SH/T 0656-98
Hydrogen content (%)	11.91	n/a	13.08	SH/T 0656-98
Oxygen content (%)	11.33	n/a	0	Element analysis

Table 3. Engine conditions.

	Speed (r/min)	Load (%)
Engine condition 1	1400	100
Engine condition 2	2300	10
Engine condition 3	2300	50
Engine condition 4	2300	100

temperature. The diluted sample was then introduced into the secondary diluter for further dilution. The dilution ratio of the primary and the secondary diluters was 8, so the over all dilution ratio of the instrument was about 64. Before sampling, the calibration was made by using two concentrations of CO_2 which were measured before and after the dilution. Total residence time was about 0.1s for primary and secondary diluters as discussed by Dekati Ltd. The exhaust gas, filtered from the fiber glass filter, was taken in sampling tubes Tenax TA® (Markes U.K.) shown in Fig. 2a, and all the samples were collected at a temperature less than 58 °C. Sampling pumps used are constant volume pumps (SKC USA, Air Chek 2000), (Fig. 2b). Sampling volume was 220 ml and it took 10 minutes to sample at every mode. After sampling the Tenax tubes were sealed with aluminum foil and were refrigerated at -10 °C.

Extraction of sampled material and analysis of VOC-components. After sampling, tubes were analyzed in the laboratory for VOCs according to the Environment Protection Agency (EPA) standard method TO-17 (US EPA, 1999). For thermal desorption, automatic thermal desorber (TD), UNITY (Markes UK) was used. Tenax tubes were first blown by dry inert gases and then heated. The desorbed compounds were cryogenically focused in a cold trap at -10 °C. After focusing, the trap underwent rapid heating to 280 °C to volatilize the compounds into the gas chromatograph (GC) (Agilent 6890N/5975) capillary column (HP-5MS, 30m × 0.25 mm × 0.25 μm) through a fused silica line heated at 280 °C. The temperature programme was 35 °C (10 min)-5° C/min–280 °C. The equipment and its specifications are given in Fig. 2c and Table 4, respectively.

Materials used for the identification and quantification of the compounds were purchased from Sino-Japan Friendship Center for Environment Protection. Six different concentrations of the standard solution were used for the calibration curve. In this way benzene, toluene, butyl acetate, ethyl benzene, *p,m*-xylene, styrene, *o*-xylene, and undecane were identified by comparing retention times of chromatographic peaks of emission samples with those from standard mixtures and by comparing mass spectra with those contained in U.S. National Institute of Standards and Technology (NIST) library (the grade of similarity was more than 85%).

After identification, the compounds were quantified by using external standard method to make the linear standard curve. The purchased standard liquids were measured in quantities 1, 2, 4, 10, 20 and 40 μl, using micro- sampler. The standard gas from the gas carrier was injected into the Tenax tube and then material was put into GC/MS for analysis to get the target compounds (Table 5) by the regression method of their peak areas. In this case, peak areas of the relative standard deviation (RSD) of each compound were recorded.

(a)

(b)

(c)

Fig. 2a-c. (a) Tenax TA sampling tubes, (b) constant volume sampling pumps and (c) TD-GC/MS.

Results and Discussion

Combustion parameters. Even though it is not the purpose of this study to discuss combustion parameters of

the engine at different engine conditions (ECs) for different fuels because authors have already studied them for their performance and combustion (Shah *et al.,* 2009b), some basic data is mentioned for the discussed engine conditions in order to provide a background for the study. As presented in Fig. 3a, maximum combustion pressure (MCP) is higher compared with fossil diesel in the cases of B20 and B100. This is due to the difference in physio-chemical properties of the test fuels as illustrated in Table 2. Properties like higher cetane number, faster flame propagation speed, better fuel atomization and simple chemical structure of the biodiesel and its blends advance the combustion process when they burn in an unmodified diesel engine. Moreover, biodiesel is an alkyl ester containing internal oxygen atoms which promote the burning of the impurities under the same condition of use (Kegl, 2008).

As shown in Fig. 3b, B20 and B100 have larger start of injection (SOI) angle relative to diesel for all the engine conditions. This is attributed to different densities and bulk modulus or compressibility of the test fuels. Biodiesel is less compressible than fossil diesel, so, faster pressure develops in the fuel injection system; consequently, the propagation of pressure wave is faster in biodiesel than diesel fuel even at the same nominal pump timing, resulting in earlier injection of biodiesel with higher pressure and rate. Moreover, higher viscosity of biodiesel and its blends is helpful in reducing the fuel losses during the injection process as compared to lower viscosity of diesel. The reduction in fuel losses results in quicker development of pressure which ultimately improves the injection timing (Shah *et al.,* 2009b).

From Fig. 3c, it is clear that biodiesel has shorter ignition delay (ID) as compared to the commercial diesel. This shorter ID angle of biodiesel is ascribed to the difference in cetane numbers of the test fuels. Relative to fossil diesel fuel, biodiesel has larger cetane number, hence resulting in earlier start of combustion in the combustion chamber of the engine. Furthermore, shorter ignition delay results in complete

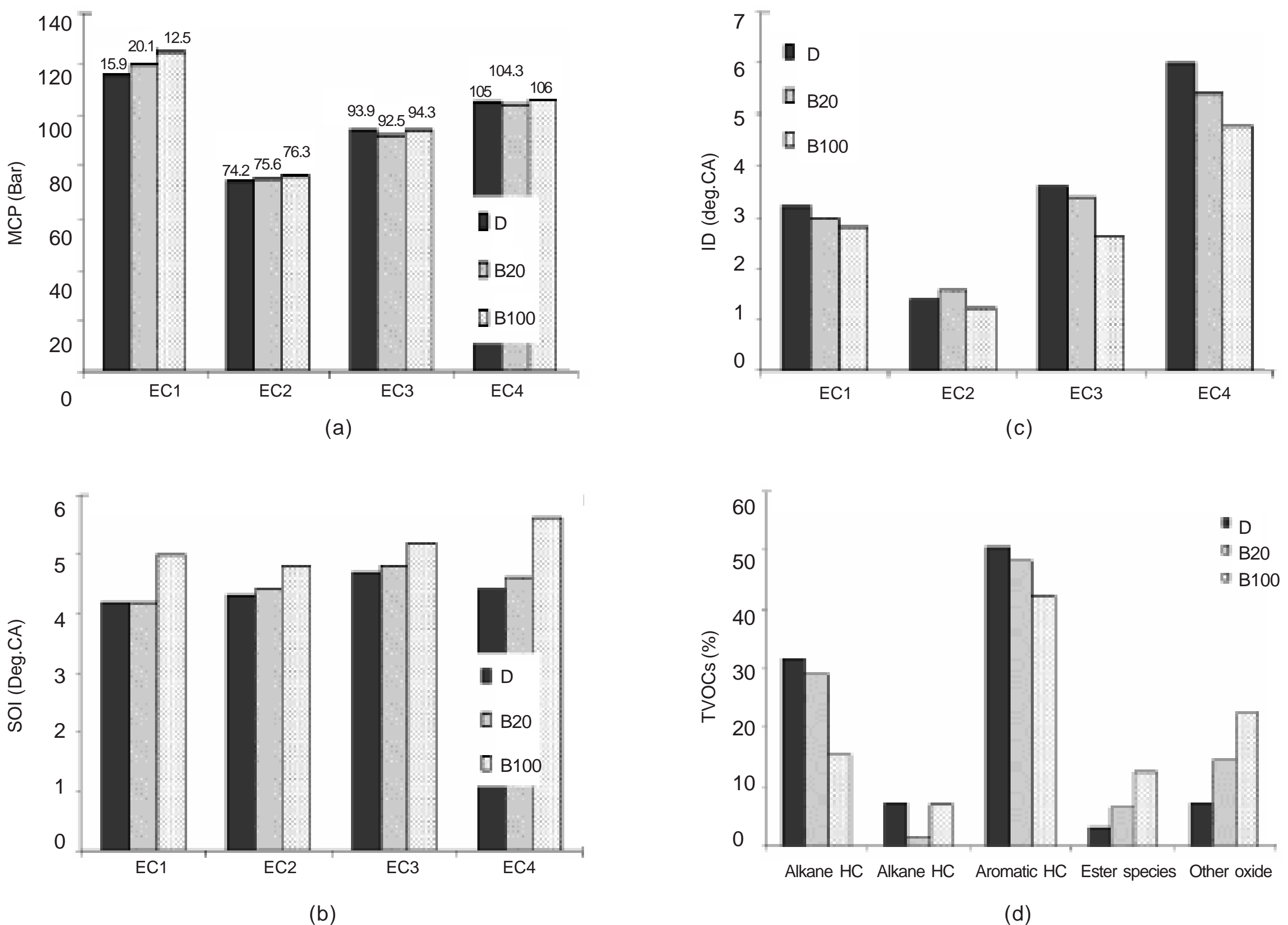

Fig. 3a-d. (a) Maximum combustion pressure, (b) angle of start of injection, (c) ignition delay, and (d) Percentage distribution of total volatile organic compounds of the test fuels

volatilization and hence clean burning of the fuel with the reduction of pollutants (Sureshkumara *et al.,* 2008).

Total volatile organic compounds. Total volatile organic compounds (TVOCs), based on the mean of the four values taken at four working conditions, have been discussed in term of percentage distribution of the detected species including alkanes, alkenes, aromatics, esters and some other species containing oxygen, as shown in the Fig. 3d. Among the detected species, aromatics and alkanes are more prominent in their percentage for all the test fuels. Aromatic hydrocarbons in diesel, B20 and B100 are 50.6%, 48.3% and 42.2%, respectively, and alkanes are 31.6%, 29.1% and 15.5%, respectively. Esters and other oxides show their dominancy in B100 and B20 as compared to diesel, however, alkenes of diesel and biodiesel are almost equal in their percentage distribution. Relative to B20 and B100, higher percentage of aromatic species in commercial diesel is quite understandable and is due to the aromatic content inherently possessed by the diesel fuel. The possible reason for higher percentage of esters and other oxides in B20 and B100, compared with diesel fuel is the higher oxygen content possessed by them; since biodiesel has been produced by the esterification of the waste cooking oil, so it is inherently oxygen enriched. Considering their maximum contribution to TVOCs and health hazards, especially associated with benzene, toluene, *p,m*-xylene, and *o*-xylene (BTX), aromatic species commonly known as VOCs have been selected for this study.

Effect of engine conditions on VOCs. The VOC-components have been discussed in term of their brake specific emissions (BSE), defined as the mass of the pollutants emitted per kilo-watt power developed in the engine in 1 h. Fig. 4 shows emissions of the eight confirmed aromatic compounds, designated as VOCs, for four different engine conditions. Experimental results show that overall BSE of VOCs increase at engine condition 4 (rated speed) relative to the condition 1 (maximum torque speed). This increase is 67%, and varies from 16% to 90% for different species. The increase in BSE at condition 4 relative to condition 1 is attributed to the decrease in combustion temperature and the increase in ignition delay caused by the increase in engine speed, as illustrated in Fig. 3, since engine speed can affect the swirl characteristics, injection timing and combustion temperature of the engine (Shah *et al.,* 2009a). At higher speed (2300 r/min), turbulence in the combustion chamber increases which increases the heat loss to the combustion chamber walls hence decreases the combustion temperature (Gill *et al.,* 1959). Higher temperature is prone to decomposition of VOC-components, especially the BTX compounds which decrease in amount at higher temperatures (Di *et al.,* 2009; Cheung *et al.,* 2008).

A positive correlation has been found between engine load and VOC-components for all the test fuels. The VOCs show their maxima at low load (10%), and minima at medium load (50%) for the diesel and B100 fuels; however, B20 exhibits its minima at full load (100%). This anomaly may be attributed to different stoichiometric air/ fuel ratios of the test fuels.

The reason for maximum VOCs pollutants at low load is the large excessive air/fuel ratio and increase in over-lean mixture area, which results in incomplete combustion of the fuels, and hence high VOC emissions. Furthermore, low engine load gives rise to emissions by quenching of the flame front in the clearance between the piston top and the cylinder head near top dead center (Shah *et al.,* 2009a). This argument is further strengthened by the statement of Kittelson (1998) that volatile particles originate from unburned or partly combusted fuel, oil fumes, water etc. Minimum VOC emissions of diesel and B100 at medium load indicates the load level at which optimum air/fuel ratio develops in the combustion chamber, and thus both of the fuels combust completely with minimum emissions. The increase in VOC emissions of diesel and B100 at full load (condition 4) relative to medium load (condition 3) may be due to their incomplete combustion or due to decrease in higher air/fuel ratio, resulting in rich mixture formation thus reducing the oxidation rate of the fuels.

When VOC-components are treated individually, results show that emissions of benzene, toluene, butyl acetate, ethyl benzene, *p,m*-xylene, *o*-xylene and undecane decrease for all the test fuels at engine condition 4, relative to condition 2; styrene on the other hand, shows an opposite trend. BTX-components, the most carcinogenic in the VOCs, decrease appreciably at engine condition 4 (high load) compared to engine conditions 2 and 3, particularly in cases of B20 and B100. In the case of B20, the decrease in benzene, toluene, and *o*-xylene at condition 4 is 66% and 80%, 20% and 23%, and 39% and 81%, respectively, for the engine conditions 2 and 3, respectively. In case of B100, the decrease in benzene, toluene, and *o*-xylene at condition 4 is 38% and 100%, 14% and 100%, and 0% and 13%, respectively, compared with conditions 2 and 3.

Although it is difficult to explain the exact behaviour of individual species at different load conditions because VOCs emitted from a diesel engine are complex mixtures (Ballesteros *et al.,* 2008), the reduction in BTX-components of B20 and B100 at condition 4 relative to conditions 2 and 3 is, however, attributed to the development of comparatively higher combustion temperature because of rich mixture formation. This is further enhanced in the presence of B20 and B100 due to their oxygen enrichment, and hence decomposes the

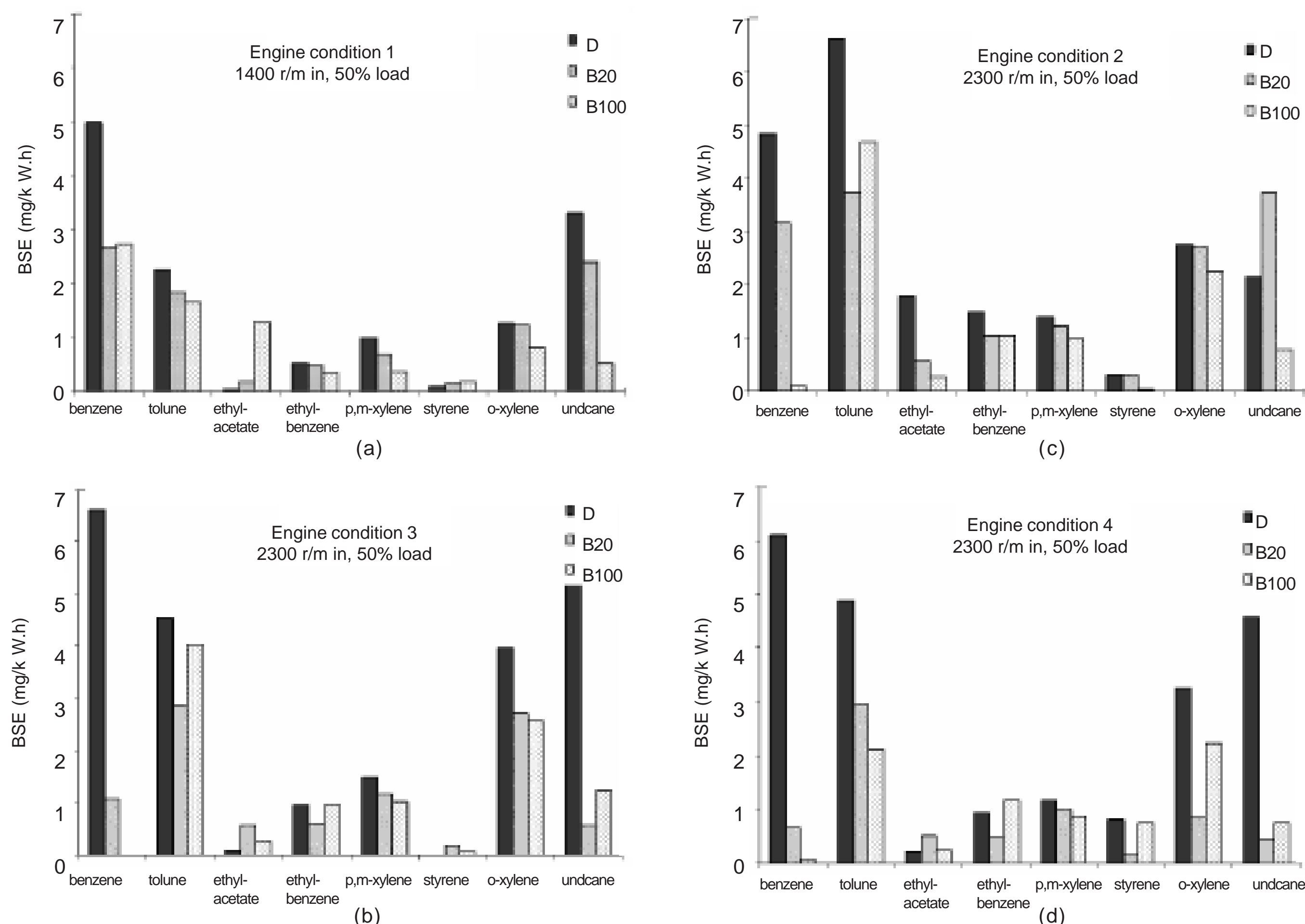

Fig. 4a-d. Brake specific emission of VOCs at different engine conditions.

BTX-components. This finding is in good agreement with that of Di *et al.* (2009) that BTX reduction efficiency is higher at higher engine load because of the higher combustion temperature and exhaust gas temperature.

Effect of biodiesel on the VOC-components. Relative to B20 and B100, diesel VOCs are all high at all the engine conditions discussed in this study when VOCs are treated collectively. In case of B20, the reduction in VOCs emission relative to diesel is 26.9%, 14.5%, 48.1%, and 73.9% for engine condition 1, 2, 3 and 4, respectively. Similarly, relative to diesel, B100 shows a reduction of 41.7%, 45.8%, 57%, and 39.6% in VOC-components for engine condition 1, 2, 3 and 4, respectively. The reduction in VOC-components in cases of B20 and B100 relative to diesel fuel is ascribed to oxygen enrichment, maximum cylinder pressure and temperature, shorter ignition delay, higher flash point, earlier fuel injection, higher cetane number, higher viscosity and faster flame propagation speed, as discussed above regarding combustion parameters.

Table 4. TD- GC/MS specifications.

Thermal desorber (TD)	Tube: 280 °C (5 min); purge: 1 min; column pressure: 8.5 psi; split ratio: (75:1); cryotrap: from -10 °C at 40 °C/s to 280 °C (3 min)
Gas chromatograph (GC)	Capillary column: HP-5MS (30m × 0.25 mm × 0.25 µm); column flux: 1 ml/min; carrier gas: helium (99.999%); oven temperature program: from 35 ºC (10 min) at 5 ºC/min to 280 ºC
Mass spectrometer (MS)	Transfer line to MS: 250 ºC; ion source: electron impact (EI) 70 eV; ion source temperature: 200 ºC; solvent cut time: 2.5 min; acquisition mode: SCAN; range of scan: 35-450 amu; electron multiplier voltage: 1.0 kV; NIST05 library

Table 5. Compounds with their equations of standardization curve, correlation coefficients and RSD.

Compound	Quantitative ion	Reference ion	Standard curve	Correlation coefficient	RSD (%)
Benzene	78	77, 52	y =3413356x	0.9952	2.93
Toluene	91	92, 65	y =4234547x	0.9873	3.04
Butyl acetate	43	56, 73	y =4137719x	0.9933	4.32
Ethyl benzene	91	106, 51	y =5453847x	0.9918	2.08
p,m-Xylene	91	106, 105	y =10143114x	0.9919	2.08
Styrene	91	78, 104	y =3129981x	0.9995	5.44
o-Xylene	91	106, 105	y =5397768x	0.9996	1.62
Undecane	57	43, 71	y =2813398x	0.9996	4.81

The most important parameter which is deemed to be responsible for the reduction of emissions, particularly VOC-components in case of biodiesel is its simple chemical structure, compared with diesel fuel. Fossil diesel consists of straight chain alkanes which have to be converted into carbon dioxide and water during the combustion process. This conversion of diesel to CO_2 and water is not direct, rather through a series of processes from alcohols, to carbonyl compounds, to carboxylic acids, to esters and finally to CO_2 (Guarieiro *et al.,* 2008). But, for the burning of biodiesel and its blends, conditions are more favourable for conversion of the esters directly to CO_2, particularly when biodiesel is obtained from waste cooking oil. Waste cooking oil, normally, comes from soybean oil which has already undergone repeated thermal oxidative process before using it for the biodiesel production. So, this residual oil consists, mainly, of minor short-chain compounds originating from the breakdown of unsaturated fatty acids during cooking (Peng *et al.,* 2006). This is the major reason for better oxidation, complete combustion, and hence reduced VOC emissions in cases of B20 and B100, compared with commercial diesel.

In order to discuss the effect of biodiesel on the individual species of VOCs, components having serious impact on human life and environment have been considered in terms of their mean values taken from four different engine conditions. The results show that the mean BSE of benzene, toluene, *p,m*-xylene, and *o*-xylene decreases by 68.8% and 87%, 42.7% and 4.1%, 28.8% and 33.5%, and 14.1% and 30.1%, respectively, for B20 and B100 respectively, compared with diesel fuel. This decrease in BTX emissions is consistent with the previous studies (Di *et al.,* 2009; Correa and Arbilla, 2006; Turrio-Baldassarri *et al.,* 2004). The important point to consider from this discussion is the reduction of mean BSE of benzene, toluene and xylene isomers in cases of B20 and B100, which indicates the superiority of biodiesel and its blends over diesel in terms of toxic emissions.

Conclusion

The VOC-components emitted from biodiesel, diesel, and 20% biodiesel-diesel blend have been investigated in this study. Followings are the significant findings:

- In the total volatile organic compounds, diesel, B20 and B100 contribute 50.6%, 48.3% and 42.2%, respectively to aromatic hydrocarbons, and 31.6%, 29.1% and 15.5%, respectively, to alkanes. Relative to diesel, B20 and B100 show their dominancy in esters and other oxides, however, alkenes of diesel and biodiesel are almost equal in their percentage distribution.
- The overall BSE of VOCs is 67% higher at engine condition 4, relative to condition 1. The VOCs show their maxima at low load, and minima at medium load for diesel and B100, however, B20 reflects its minima at full load.
- Benzene, toluene, butyl acetate, ethyl benzene, xylene isomers, and undecane decrease for all the test fuels at engine condition 4, relative to condition 2; however, styrene shows an opposite trend.
- In case of B20, the decrease in benzene, toluene, and *o*-xylene at condition 4 is 66% and 80%, 20% and 23%, and 39% and 81%, respectively, compared with the engine conditions 2 and 3, respectively. In case of B100, the decrease in benzene, toluene, and *o*-xylene at condition 4 is 38% and 100%, 14% and 100%, and 0% and 13%, respectively, compared with conditions 2 and 3, respectively.
- In case of B20, the reduction in VOC emissions relative to diesel is 26.9%, 14.5%, 48.1%, and 73.9% for engine condition 1, 2, 3, and 4, respectively. Relative to diesel, B100 shows a reduction of 41.7%, 45.8%, 57%, and 39.6% in VOC-components for engine condition 1, 2, 3, and 4, respectively.
- Mean BSE of benzene, toluene, *p,m*-xylene, and *o*-xylene decreases by 68.8% and 87%, 42.7% and 4.1%, 28.8% and 33.5%, and 14.1% and 30.1%, respectively, for B20 and B100, respectively, compared with diesel fuel.

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Noise Characteristics of Pumps at Tehran's Oil Refinery and Control Module Design

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Abstract. Considering industrial excessive noise exposure, sound pressure level of 4 pumps with different applications installed in Isomax Unit of Oil Refinery Centre of Tehran, Iran was studied. The A-weighted sound pressure level and maximum sound pressure level showed that the emitted noise is far above the permissible limits. Installing enclosure around the noise source was found to be the best noise control measure. Results of operational calculating transmission loss of the designed module with a sandwich layer showed that it is possible to provide 19.7 dB (A) reduction in overall sound pressure level and 20 dB reduction in dominant frequency. Designing the module with given specifications and probable leak estimation and prevention gives remarkable and effective results in the studied field.

Keywords: pump noise, noise pollution, noise exposure

Introduction

Exposure to excessive noise is associated with high risk of hearing loss (Golmohammadi, 2007; Esmaeelzadeh *et al*., 2006). National Institute for Occupational Safety and Health (NIOSH) categorized hearing loss as one of the ten most important work related illnesses and estimated that 25% of workers of more than 55 years, who were exposed to excessive noise (higher than 90 dB) suffered from different levels of hearing loss. Workers in petrochemical industries also suffered from noise exposure problems (Cheremisinoff Paul and Allen Ernest, 1977).

An investigation for evaluation of noise pollution in oil refinery fields in Iran was undertaken by Nassiri and Ahmadi (2004). It was found that exposure of workers to noise, in most cases, was far above the permissible limits provided by American Conference of Governmental Industrial Hygienists (ACGIH).

In another study of Iranian petrochemical industry, it was reported that the noise level of the studied sources was so high that the exposed workers could hardly work in that condition and in all cases control measures were required (Gholshah, 1997).

Reduction of industrial and environmental noise pollution has been the subject of many different studies. (Monazzam and Nassiri, 2009; Monazzam and Lam, 2008). Hansen (2005) in a study demonstrated that applying control methods, such as installing enclosures, effectively reduced noise to considerable levels. In another study, Joseph *et al*. (1991) found that ignoring the structural path that caused sound leak from module reduced the effectiveness of the control measure. It was also reported that the exact recognition of noise source and surface specifications surrounding the module, plays crucial role in precise acoustic efficiency assessment. The results demonstrated that the application of a module, with complex layers proportionate to the design requirement of the module, produced 12 to 19 dB reduction in the noise level (Min and Ying, 2008). Another study of noise control showed that use of a multiporous enclosure reduced the sound pressure level by more than 40 dB. Hakimi *et al*. (2006) estimated a 20 dB reduction in the sound level by applying a module in the air outlet.

In this paper, results of an extensive theoretical and experimental study on noise propagation character of pumps along with application of control measures in a unit of Tehran oil refinery are presented.

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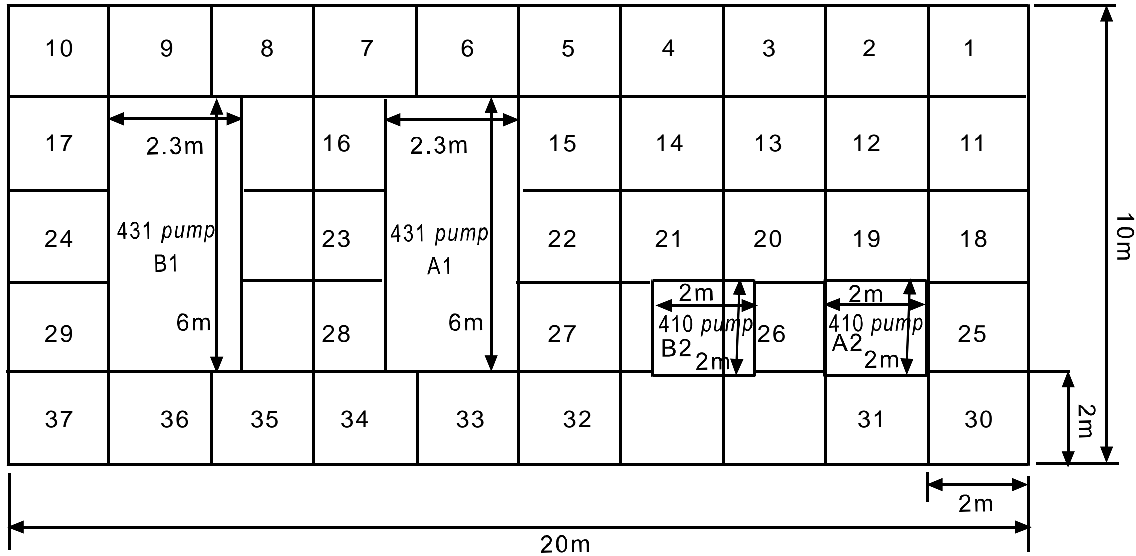
Materials and Methods

A study for sand pressure levels was conducted at Isomax unit of the Oil Refinery Centre of Tehran (ORCT), that had 4 pumps for different applications. Field measurements and calculations were carried out for evaluation and prediction of noise pollution at the site along with the characterization of the noise sources.

Field measurements. The study field comprised of a 20x10 m open site having 4 pumps installed on a rigid floor. Fig. 1 presents a simple plan showing type and location of the pumps



Power of motors: A_1 = 200 KW: A_2 = 75 KW; B_1 = 55 KW; B_2 = 230 KW

Fig. 1. Sound plan of Isomax pumps.

and 37 measurement points. The workers at this site were carrying out different tasks. Motors of power 200, 75, 55 and 230 KW were distributed over the site, which are labelled as A1, A2, B1 and B2, respectively. There were no control measures for the noise sources at the site.

For field measurement, first, the A-weighted sound pressure level ($L_{p(rms)}$) was measured using calibrated TES-1385 sound level meter; then the maximum sound pressure level ($L_{p(max)}$) at 37 zones of 2x2 meter was measured according to the lattice method. Finally, the crest factor was determined by calculating the difference between $L_{p(max)}$ and $L_{p(rms)}$. For the measurement of sound, standard method ISO 9612 (1997) was followed.

Sound frequency analysis and evaluation were carried out using analyzer sound level meter TES-1385 and standard calibrator B&K 4231 was applied for calibrating the device. The sound level meter time constant was set on slow mode; microphone position was set at 1.5 m above the ground, pointing in the direction of the workers.

Calculations. Applying RPM meter (RPM indicator Model No.RM-20), the RPM of pumps was specified and using the following equation, dominant frequency (f) of the sources was predicted.

$$f = \frac{N_b \text{ x RPM}}{60} \quad (1)$$

where N_b is the number of pump blades.

Results and Discussion

The A-weighted sound pressure level and maximum sound pressure level along with their relevant crest factor in Isomax unit of Tehran Oil Refinery Centre are shown in Table 1.

Using surfer software, a plan for the sound field was drawn (Fig. 2). It can be seen that the highest sound pressure levels are located in the mid-field where two pumps with the highest noise pressure levels (A1 and B2) are placed side by side.

Table 1. A-weighted sound pressure level $Lp_{(rms)}$, maximum sound pressure level $Lp_{(max)}$ and crest factor (CF) of Isomax pumps area

Station	$Lp_{(rms)}$ (dB)	$Lp_{(max)}$ (dB)	CF	Station	$Lp_{(rms)}$ (dB)	$Lp_{(max)}$ (dB)	CF
1	87.8	104	16.2	20	93.1	109	16.9
2	87.7	101.9	14.2	21	94	108	16.7
3	88.5	106.2	17.7	22	94.5	112	17.9
4	88.4	106	17.6	23	93.7	112	18.3
5	88.6	105	16.4	24	87.8	105	17.2
6	88	105.6	17.6	25	90.2	105.6	15.4
7	87.5	104	16.5	26	92.3	108	15.7
8	88.2	105.8	17.6	27	94.6	111	17
9	87.5	103.6	16.1	28	92.5	109	17
10	85.8	101.1	16.3	29	86.4	102	15.6
11	88.5	106.2	17.7	30	88.3	105	16.7
12	89.8	107.7	17.9	31	88.8	106	17.7
13	90	106	16	32	92.3	108	15.7
14	89.5	105.8	16.3	33	92.6	108.4	15.8
15	91	109.2	18.2	34	90.2	106	15.8
16	90.4	106	15.6	35	85.5	105.7	16.2
17	85.5	102.6	17.1	36	88.4	104.5	16.1
18	89.7	107	17.3	37	88.2	103.1	16.9
19	90	106.5	16.5				

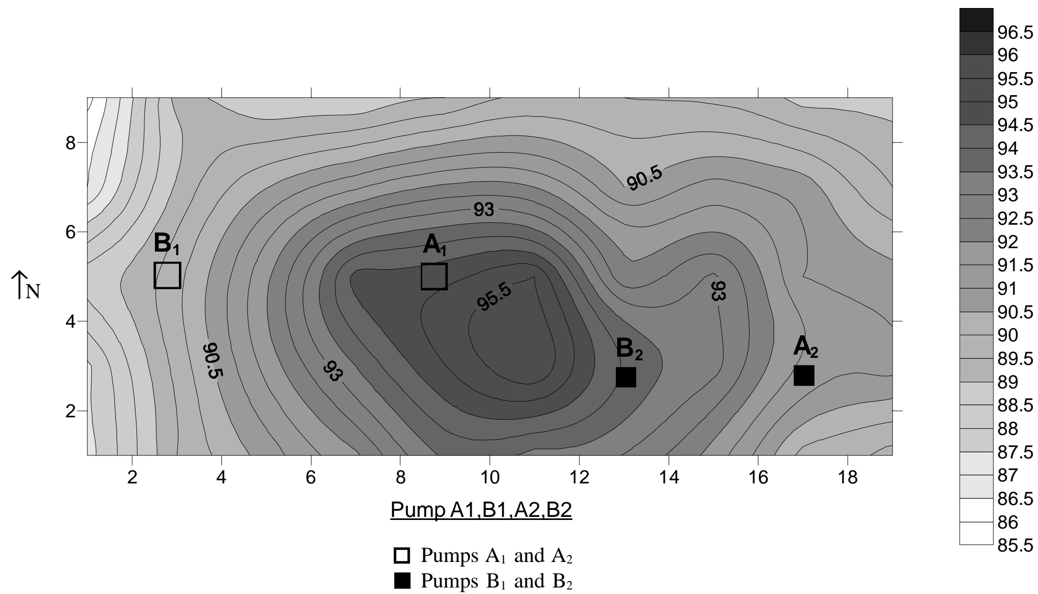


Fig. 2. Noise contours around pump positions.

It may be noted that at all stations, the sound pressure level is well above the standard level (85 dBA). The frequency analysis was carried out in octave band of 63 to 8000 Hz by dividing the site into four station; each station having one pump and other devices at the specified and measurable distance (Fig. 1). The results are given in Table 2.

Table 2. The octave band frequency analysis and dominant frequency of the studied noise sources

Measuring station frequency (Hz)	Station 9	19	21	23
63	83.2	87.3	83.2	81.7
125	77.1	82.4	81.0	84.2
250	76.7	86.6	83.0	81.5
500	77.4	87.4	80.8	86.2
1000	82.8	88.0	87.0	85.0
2000	80.4	89.8	89.6	87.7
4000	83.9	84.0	87.8	89.6
8000	78.6	77.4	84.0	81.3

Prediction procedure. In order to calculate the dominant frequencies of the pumps, their rotation speed was measured using RPM meter. Fluctuations in frequency of devices around the pumps were also accounted for. Rotation speed of pumps A2 and B2 was 2935 and 3000, respectively, and of pumps A1 and B1, 4500 and 5600, respectively. Applying equation (1), the dominant frequency of each source in the studied field was calculated.

The number of blades of pumps A2 and B2 is 50 and so the dominant frequency of these pumps is between 2445 to 2500 Hz; number of blades of pump A1 and B1 is 49 and so the dominant frequency of these pumps ranges between 3675 to 4573 Hz. So, the dominant frequency of pumps with index 2 (A2, B2) is found to be in 2000 Hz and that of the pumps with index 1 (A1, B1) is predicted to be in the range 4000 Hz in octave band scale.

By comparison of the field measurement and the estimation approach, a perfect agreement between these two methods is found. In this case the dominant frequency of noise in stations 9 and 23, which are adjacent to pumps with index 1, is 4000 Hz and the dominant frequency of noise in stations 19 and 21, which are close to pumps with index 2, is 2000 Hz. These results uphold precision of the field measurement.

Control module. ***Enclosure design*****.** For designing enclosure, it is important to determine the critical frequency of the main insulator, (2 mm steel). By applying the well known equation 6.17 for calculating the critical frequency (Lewis and Douglas, 1994), its frequency is predicted to be 8978 Hz which is far above the dominant frequency of our main noise source.

Layout and specification of the module sandwich layers. ***Absorbent*****.** In the design, a layer of slag wool with 2.5 kg/m^2 surface density and 25 mm thickness was applied as an absorbent for the considered frequency. Reflective surfaces around the noise source increase the sound pressure level due to multiple reflection of sound. So, applying an absorbent for the sound, particularly in the dominant frequency range, is one of the principal actions in the module and the enclosure design. In this study, slag wool is applied as the appropriate absorbent.

Frame. The sandwich panel was fixed by a wooden frame of 15 mm thickness and surface density of 7 kg/m^2.

Insulator. For insulating the structure - borne noise passing from the panel, 2 mm steel with surface density of 17 kg/m^2 was applied in the centre-line of the panels. Considering the dominant frequency of pumps in the range 2000 to 4000 Hz, minimum surface density of the insulator should be 12 kg/m^2 (Lewis and Douglas, 1994).

External surfaces. For prevention of sound reflection from the external surface of the modules, chipboard of 9 mm thickness and 7 kg/m^2, surface density was applied on the external surface of the module.

Door. A common gash door of dimensions 1.8 x 0.7 m, 43 mm thickness and surface density of 9 kg/m^2 was used for the entrance of the enclosure.

Windows. For giving perfect view of the pumps to the operators, three windows of dimension 1x1 m were designed which were vacuumed, double glazed with 9 mm thickness and 7 kg/m^2, surface density.

The dimension of the designed enclosure was 3×3×3 m. By the application of sandwich layers and using the following equation, the overall panel surface density ($\overline{w}$) of the enclosure was found to be 16.25 kg/m^2:

$$\overline{w} = \Sigma\, w_i \times s_i / \Sigma\, s_i \qquad (2)$$

where w_i and s_i are, the surface density (kg/m^2) and the area (m^2) respectively, of each panel component (Fig. 3).

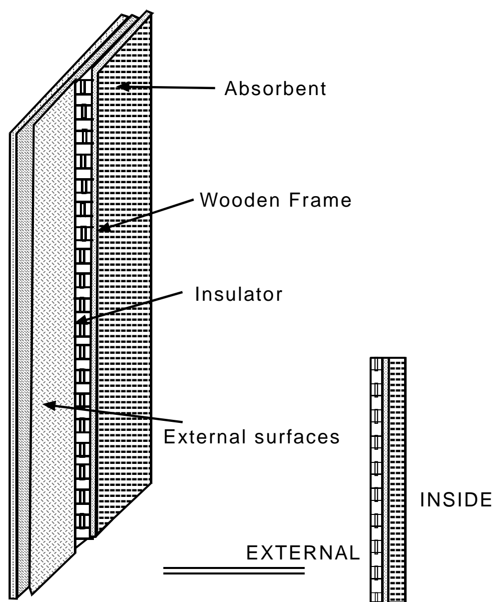


Fig. 3. Detailed structure of the main enclosure panel.

Frequency analysis, TL and NR calculation. Using the above mentioned field measurement results (95 dB (A)) and Iranian noise exposure limit (85 dB (A)), it is easily found that the total noise reduction required is 15 dB (95 dB (A) - 85 dB (A) +5 dB (A)); 5 dB (A) is added to arrive at the practical results. The noise reduction level for dominant frequency (according to the above method) was found to be 20 dB (A).

Total noise reduction achieved by installing the designed enclosure is calculated to be 19.7 dB (by the difference of total outdoor and indoor noise levels). In this case, the overall noise level inside the enclosure was measured to be 95 dB while outside, it was estimated to be 75.3 dB. Fig. 4 provides sound pressure level variations before and after installing the enclosure in octave band centre frequencies.

The architectural plans and related details are designed by AutoCAD software and a cross section of the design enclosure is shown in Fig. 5.

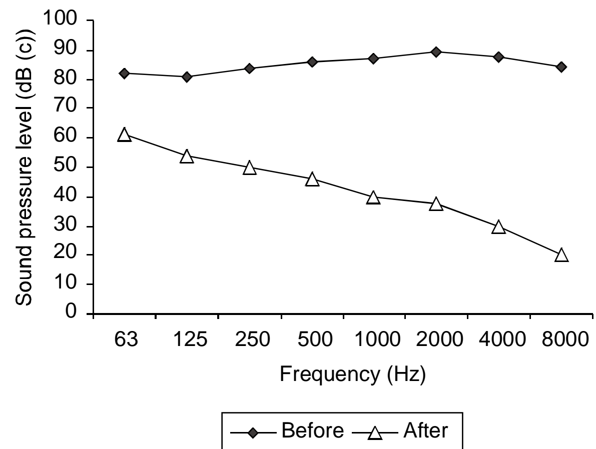


Fig. 4. Comparison between sound pressure level before and after installing the enclosure.

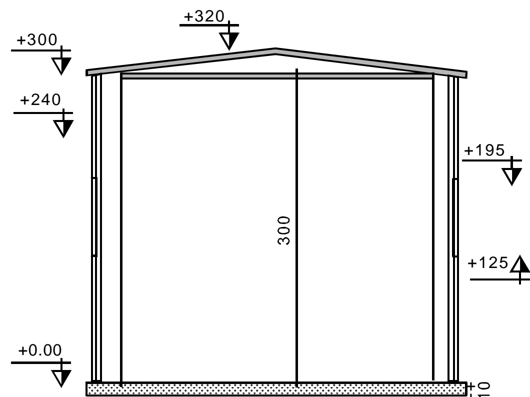


Fig. 5. A cross section of the designed enclosure for Isomax unit.

Isomax unit contains four pumps which operate to feed and lubricate the compressors. Results of field evaluation (Table 1) demonstrated that at all the tested stations, the sound pressure level was far above 85 dB(A). The results of evaluation of sound contour plan also showed that the Iso-sonic contour in the field between pumps A2 (90 dB(A) and A1 with 95 dB (A) is the highest (Fig. 2).

Frequency analysis results of octave band of pumps reveal the dominant frequency of pumps A2 and B2 to be 2000 Hz and that of pumps A1 and B1, 4000 Hz (Table 2). Evaluation of the rotation speed of pumps and determination of the dominant frequency of the pumps due to their technical specifications and calculating procedure, uphold me asurement procedure well. In this case the results of prediction method showed that the dominant frequency of pumps A2 and B2 was in the range of 2445 to 2500 Hz and that of pumps A1 and B1 pumps was in the range of 3675 to 4573 Hz.

It is thus concluded that designing and installation of an acoustic enclosure is the right choice for controlling noise of the pumps.

It was found that use of a layer of steel of 2 mm thickness and critical frequency of 8978 Hz − which is well above the dominant frequency of the noise sources − as insulator is a suitable control measure. However, applying steel as a layer in the module causes multiple reflective surfaces around the source and sound pressure level rises accordingly. Therefore, using absorbent material at the source side of the enclosure is necessary. It is worth adding that the results of evaluation of the average absorbent coefficient of the module, room factor and sound transmission loss in dominant frequencies showed that application of a single layer does not provide the expected transmission loss. Hence, multiple layers were used in the design of the enclosure.

Slag wool with surface density of 2.5 kg/m^2, was found to be the best absorbent. For providing enough distances between the two layers of the absorbent material, a wooden frame of 15 mm thickness was used. This improved the performance of the enclosure in 2000 Hz, which is the dominant frequency of some of the pumps. In order to reduce the reflection from external surfaces of the enclosure, the sandwich panel was finished by a layer of chipboard of 9 mm thickness. To provide straight vision to the pump operators, three vacuumed doubled glazed windows were also designed. Comparison of surface density of combined panel (16.25 kg/m^2) and minimum surface density needed for dominant frequency (12 kg/m^2), it was demonstrated that the designed module is much effective.

Results of the operational calculation of the transmission loss of the designed module with multiple layers showed that by close recognition of sound source and applying the above module, it is possible to provide 19.7 dB (A) reduction in overall sound pressure level and 20 dB reduction in dominant frequency. Lastly, it is concluded that designing the module with the given specifications and probable leak estimation and prevention gives remarkable and effective results in the field of study.

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