RNI - No. MAHENG/2017/74063
VOLUME 2 (Issue 1) July - December 2018

ISSN No. 2581-5911
BI-ANNUAL SUBSCRIPTION : Rs. 2000/-

G P GLOBALIZE RESEARCH JOURNAL OF CHEMISTRY

RNI - No. MAHENG/2017/74063

ISSN NO 2581-5911

G P GLOBALIZE RESEARCH JOURNAL OF CHEMISTRY

VOLUME 2 (Issue 1) July - December 2018

BI-ANNUAL 2018

CIN U22130MH2016PTC287238

UAN- MH19D0008178

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RNI No. MAHENG/2017/74063 ISSN No. 2581–5911

Volume 2 Issue 1 ❖ July – December 2018

G P GLOBALIZE RESEARCH JOURNAL OF CHEMISTRY

GAURANG PUBLISHING GLOBALIZE PRIVATE LIMITED, MUMBAI

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UAN - MH19D0008178

GP GLOBALIZE RESEARCH JOURNAL OF CHEMISTRY

An International Peer Reviewed Journal of Chemistry

RNI No: MAHENG/2017/74063 **ISSN (Print) No: 2581-5911**

Printed and Published by Gaurang Rajan Pendurkar on behalf of Gaurang Publishing Globalize Private Limited and printed at NIL CREATION, Shop No. 7, 35/55, Bandu Gokhale Path, Mugbhat Cross Lane, Jivanji Maharaj Chawl (Shree Swami Samarth Nagar), Girgaon, Mumbai 400004 and published at Gaurang Publishing Globalize Private Limited 1, Plot 72, P M M M Marg, Tardeo, Mumbai-400034.
Editor-in-Chief Dr. D.V. Prabhu.

G P GLOBALIZE RESEARCH JOURNAL OF CHEMISTRY

RNI No: MAHENG/2017/74063 **ISSN No (Print): 2581-5911**

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G P GLOBALIZE RESEARCH JOURNAL OF CHEMISTRY

RNI No. MAHENG/2017/74063 ISSN No. (Print) 2581-5911

Volume 2 Issue 1 ❄ July-December 2018

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Papers

RNI No. MAHENG/2017/74063
ISSN No. (Print) 2581-5911

Synthesis of spiroheterocycles derived from 9,10 – phenanthrenequinone and olefin-acetylenic dipolarophiles

Anita, Barkha Shrivastava, Rahul Joshi and Pushpa Pardasani*
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Abstract
[3+2] Cycloaddition of phenanthrenequinone with electron-deficient dipolarophiles in 68-75% yield has been described. Comprehensive molecular orbital calculations at DFT-B3LYP level have been carried out to address the mechanism as well as stereochemical course of the reaction.The products have been characterized by physical and spectroscopic techniques.

Keywords: *azomethineylides, DFT calculations, dipolarophile, 1,3-dipolarcycloaddition, phenanthrenequinone, spiroheterocycles, stereoselectivity.*

Introduction

Heterocycles have achieved a prominent place among various classes of organic compounds for their diverse biological and medicinal activity.[1,2] Amongst the heterocyclic compounds, α-diones have aroused special attention because of their applications as pharmaceuticals, hormones and pigments.[3] Besides, they play a crucial role in photosynthesis and electron transport system. They are versatile building blocks in organic synthesis by virtue of their multiple reactivity profile.[4-8] Phenanthrenequinone derivatives have exhibited various pharmacological activities like antioxidant[9-11], antitumoiur[12], antimalarial[13]and antiviral.[14] Synthetically phenanthrenequinone has been reported to undergo several types of reactions.[15-17] Over the past, one decade we have explored the reactions of indole-2,3-dione[18,19], benzo[b]thiophene-2,3-dione[20,21] and acenaphthylene-1,2-dione[22,23] with various cyclic α-amino acids. However, the 1,3-dipolar cycloaddition reactions of phenanthrene-quinone with several cyclic α-amino acids has remained unexplored and merited detailed investigation. Thus in pursuit of this theme we have examined the 1,3-dipolar cycloaddition reactions with amino acids *viz* thiazolidine-2-carboxylic acid (TCA) and the results are presented below.

Materials and Methods

Experimental

Melting points were determined in an open-glass capillary and are uncorrected.The solvents were purified by standard procedures.[24, 25] The IR spectra were recorded on Nicolet Magna IR TM model 550 in KBr pellets. The ^{1}H and ^{13}C NMR spectra were obtained on a JEOL AL-300 instrument at 300 and 75 MHz using $CDCl_3$ or DMSO-d_6 as solvent and tetramethylsilane (TMS) as an internal standard. Chemical shifts are given in ä ppm. Elemental analyses were performed by Perkin-Elmer series C, H, N, and S analyser-2400. Thin-layer chromatography (TLC) was performed on alumina foil on Merck's Kiesel gel 60 F254 sheets, visualization was

achieved at ultra fluorescence on an Indian Equipment Corporation equipment, IEC-312 at 354 nm.Column chromatography was carried over silica gel 60–120 mesh as absorbent, using solvents of rising polarity.

General procedure for synthesis of (2S, 5S)-Spiro-{ 1-aza-3-oxa-7-thia-bicyclo-[3,3,0]-octane-2, 1'-phenanthrene-4,2'-dione.

In a 100 mL round bottom flask, a mixture of 9,10-phenanthrenequinone**(1)** (0.624 g, 3.0 mmol) and thiazolidinone-2-carboxylic acid **(2)** (0.4g, 3.0 mmol) in equimolar ratio in dry acetonitrile (50 mL) was refluxed under nitrogen atmosphere for 22 h. Reaction was monitored by TLC, unreacted acid was filtered off from the cooled reaction mixture and the filtrate was evaporated in *vaccuo*. The concentrated reaction mixture was then allowed to stand overnight. However, no crystallization occurred and therefore the crude product was subjected to column chromatography over silica gel by elution with solvents of increasing polarity. The compound **(5)** was obtained from petroleum ether/chloroform fraction (1:2) as a brown powder. Yield (68%), m.p.=118°C;IR (KBr) (ν_{max}) 3060(C-H_{aro}), (1700 .>C=O), 1480(C=C), 1260 (C-N), 1120 (C-O), 680 (C-S) cm^{-1}; ^{1}H-NMR ($CDCl_3$, 300 MHz) 2.46 (6-H), 2.85 (8-H), 4.10 (5-H), 7.47 (5'-H + 8'-H), 7.72 (4'-H + 9'-H),8.02 (3'-H + 10'-H), 8.19 (6'-H + 7'-H) in δ; ^{13}C-NMR ($CDCl_3$ 75.47 MHz) δ: 190.87 (2' C=O), 180.30 (lactonic C-4); 136.02-123.94 (C-3' – C-10'); 82.05 (spiroC-2), 59.26 (spiroC-5), 48.29 (C-8), 29.54 (C-6).Elemental Analysis (%) calcd; C, 66.84; H, 4.03; N, 4.30. Found: C, 66.86; H, 4.05; N, 4.33; $C_{18}H_{13}NO_3S$

(5S,7S, 8R)-spiro-{7-methoxycarbonyl-1-aza-4-thiabicyclo-[3,3,0]-6-octane-8,1'-phenanthrene]-2'-one (7). Yield (68%), dark brown solid m.p.=180°C IR (KBr) (ν_{max})3070 (C-Haro), 1700 (lactonic>C=O), 1690 (>C=O), 1490 (C=C), 1290 (C-N) 940 (C-O-C), 680 (C-S)cm^{-1}; ^{1}H-NMR ($CDCl_3$, 300 MHz) δ: 1.92 (3-H), 2.25 (3-H), 3.61 (5-H), 7.12-7.51 (7Ar H), 8.18 (6-H+7-H);^{13}C-NMR ($CDCl_3$) δ: 189.5(2' C=O), 124.9-132.1 (C-3'-C-10')123.8 (C=C), 110.2 (C-7), 65.1 (C-5), 52.03(C-2), 35.1 (C-3), 14.08 (CH_3) ppm; Anal.calcd for $C_{21}H_{19}NO_3S$: C, 68.02; H, 5.23; N, 3.80. Found C, 69.02; H, 5.24; N, 3.83.

(5S,8R)-spiro-{7-phenyl-1-aza-4-thia-bicyclo-[3,3,0]-6-octene-8,1'-phenanathrene}-2'-one(8). Yield (71%), dark brown solid m.p. = 136°C. IR (KBr) (ν_{max}) 3080 (C-Haro), 1670 (>C=O), 1480 (C=C), 1290 (C-N), 930 (C-Haro), 680 (C-S) cm^{-1}; ^{1}H-NMR ($CDCl_3$, 300 MHz) δ: 1.86 (5-H), 2.90 (2-H+6-H), 7.14(9-H), 7.21-7.59 (5 Ar H+3'-Hto 10'-H); ^{13}C-NMR ($CDCl_3$) δ: 180.28 (>C=O), 127-129.1 (C-3'-C-10'), 126-128.4 (C-8-C-10), 114.2 (C-7), 63.9 (C-5), 59.01 (C-2),14.08 (CH_3); Anal,calcd for $C_{25}H_{19}NOS$: C, 78.70; H, 4.02; N, 3.66. Found C, 78.71; H, 5.02; N, 3.67.

(5S,8R)spiro-{6-methyl-7-Phenyl-1-aza-4-thia-bicyclo-[3,3,0]-6-octene-8,1'-phenanathrene}-2'-one (9). Yield (69 %), shiny brown crystalsm.p.= 185°C. IR (KBr) (ν_{max}) 3055 (C-Haro), 1675 (>C=O), 1285 (C-N), 930 (C-Haro), 660 (C-S) cm^{-1}; ^{1}H-NMR ($CDCl_3$, 300 MHz) δ:2.25 (3-H), 2.89 (2-H), 3.86 (5-H), 7.29 (5'-H+8'-H), 7.50 (5 ArH), 7.70 (4'-H+9'-H), 8.01 (3'-H+10'-H),8.18 (6'-H+7'-H); ^{13}C-NMR ($CDCl_3$) δ:180.17 (>C=O), 135.98-129.48 (6 ArC+C-3'-C-10'), 123.88 (C=C); 84.10 (C-8), 62.13 (C-5), 52.03 (C-2),14.08 (CH_3) ppm; Analysis calcd for $C_{31}H_{23}NOS$: C, 81.35; H, 5.06; N, 2.06: Found C, 81.37; H, 5.07; N, 3.06.

(5S, 8R)Spiro-{6-ethoxycarbonyl-7-Phenyl-1-aza-4-thia-bicyclo-[3, 3, 0]-6-octene-8, 1'phenanathrene}-2'-one (10). Yield (72%), coffee brown solid m.p.=178°C. IR (KBr)(ν_{max}): 3060 (C-Haro), 1680 (O-C=O), 1670 (>c=O), 1280 (C-N), 930 (C-Haro), 660 (C-S) cm^{-1}; ^{1}H-NMR ($CDCl_3$, 300 MHz) δ: 1.36 (CH_3,3H), 2.92 (2-H), 3.94 (5-H),4.30 (OCH_2,2H), 7.26-8.18 (13 ArH);^{13}C-NMR (CDCl3) δ: 196.5 (2'>C=O), 145.4 (7-C); 84.1 (C-5), 55.7 (C-8), 35.1 (C-6); Analysis calcd for $C_{28}H_{23}NO_3S$: C, 74.14; H, 5.10; N, 3.09. Found C, 74.15; H, 5.11; N, 3.09.

(5S, 8R) spiro-{6-7-diphenyl-1-aza-4-thia-bicyclo-[3, 3, 0]-6-octene-8, 1'-phenanathrene}-2'-one (11). Yield (65%), coffee brown solid m.p.= 178°C. IR (KBr)(

νmax): 3060 (C-Haro), 1680 (O-C=O), 1670 (>c=O), 1280 (C-N), 930 (C-Haro), 660 (C-S) cm-1; 1H-NMR (CDCl3, 300 MHz) δ: 1.36 (CH3,3H), 2.92 (2-H), 3.94 (5-H),4.30 (OCH2,2H), 7.26-8.18 (13 ArH); 13C-NMR (CDCl3) δ: 196.5 (2'>C=O), 145.4 (7-C); 84.1 (C-5), 55.7 (C-8), 35.1 (C-6); Analysis calcd for $C_{31}H_{23}NOS$: C, 74.14; H, 5.10; N, 3.09. Found C,74.15; H, 5.11; N, 3.09.

Computational details

All computations were performed *via* different theoretical methods by using the Gaussian 03 suite of programs.[26] The optimization of the geometries and population analysis were carried out using generalized gradient approximation Becke3-Lee-Yang-Parr (B3LYP) level of theory with 6–31G basis set. Harmonic vibration frequencies of all stationary points have been computed to characterize them as energy minima (all frequencies are real) or transition states (one and only one imaginary frequency). An imaginary frequency has been obtained for each transition state, which substantiates the actual formation of the transition state. Intrinsic reaction coordinate (IRC) calculation was carried out to confirm the reaction pathway and the transition state.

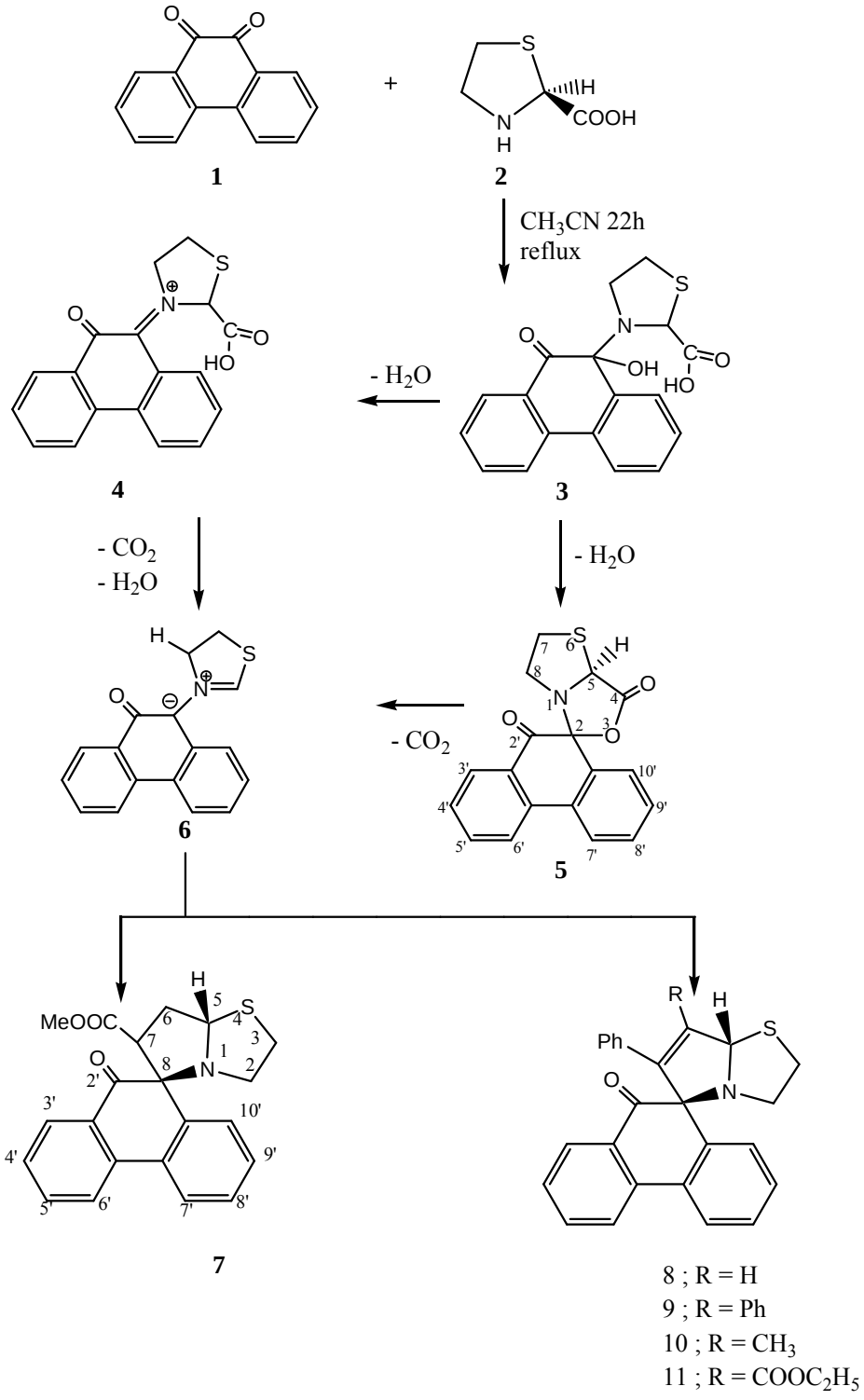
Results and Discussion

The reaction of 9,10-phenanthrenequinone **(1)** with thiazolidine-2-carboxylic acid (TCA) **(2)** was carried out in an equimolar ratio refluxing in acetonitrile for 22 h whereby it afforded (2S,5S)-spiro-{1-aza-3-oxa-6-thia-bicyclo-[3,3,0]-octane-2,1'-phenanthrene-4,2'-dione**(5)** in 64% yield (Scheme 1).

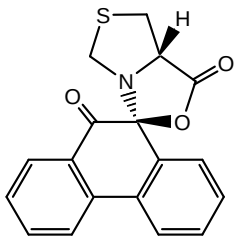
The reaction occurs probably *via* formation of intermediate hydroxyl keto acid **(3)** which loses water molecule to produce the thiazolidinone**(5)**. The structure of thiazolidinone**(5)** has been unambiguously established from its spectral data. In the IR spectrum of **(5)** characteristic absorption bands were observed at 3060, 2925, 1700, 1680, 1480, 1260, 930 and 600 cm^{-1} for C-$H_{aromatic}$, C-$H_{aliphatic}$, >C=O lactonic, >C=O, C-N, C-O-C, C-$H_{aromatic}$ bending and C-S vibrations respectively. Its ^{1}H NMR spectrum showed a doublet at d 2.46 for 7-H, a singlet at d2.85 for 8-H and a triplet at d 4.10 for 5-H. In the aromatic region, two triplets were observed at d 7.47 (J=7.33 Hz) for 5′-H +8′-H and at d 7.72 (J= 8.06 Hz) for 4′-H + 9′-H whereas another two doublets resonated at d 8.02 (J=8.23 Hz) for 3'-H+10'-H and at d 8.19 (J= 7.78 Hz) for 6'-H + 7'-H respectively. Its ^{13}C NMR spectrum showed the C-2' carbonyl carbon at d 190.87 and lactonic carbon C-4 at d 180.30 ppm. The benzenoid carbons C-3′- C-10′ appeared in the region d 136.02-123.94 ppm. The two chiral carbons C-2 and C-5 were observed at d 82.05 and d 59.26 ppm. Methylene carbons C-8 and C-7 appeared at d 48.29 and d 29.54 ppm respectively.

When the reaction of phenanthrenequinone**(1)** with thiazolidine-2-carboxylic acid (TCA) **(2)** in equimolar ratio was carried out under refluxing condition for 22h in the presence of methyl acrylate dipolarophile, it afforded (5S, 7S, 8R)-spiro-{7-methoxycarbonyl-1-aza-4-thia-bicyclo-[3,3,0]-octane-8,1'-phenanthrene}-2'-one**(7)** in 73% yield. Analogous reaction in presence of other dipolarophiles*viz* phenyl acetylene, 1-phenyl-1-propyne and ethyl phenyl propiolate,diphenyl acetylene afforded (5S, 8R) spiro-{7-phenyl-1-aza-4-thia-bicyclo-[3,3,0]-6-octene-8,1'-phenanthrene}-2'-one **(8)**, (5S, 8R) spiro-{6-methyl-7-phenyl-1-aza-4-thia-bicyclo-[3,3,0]-6-octene-8,1'-phenan-threne}-2'-one **(9),** (5S, 8R) spiro-{6-ethoxycarbonyl-7-phenyl-1-aza-4-thia-bicyclo-[3,3,0]-6-octene-8,1'-phenanthrene}-2'-one **(10),** (5S, 8R) spiro-{6,7-diphenyl-1-aza-4-thia-bicyclo-[3,3,0]-6-octene-8,1'-phenanthrene}-2'-one**(11)** in 70%-76% yields. Several attempts to purify the products by crystallization failed and hence all the cycloadducts were purified by column chromatography over silica gel by solvents of rising polarity.

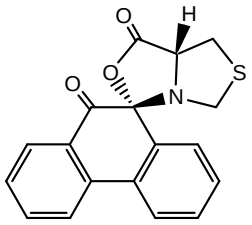
The thiazolidinone derivative **(5)** has two chiral centres and therefore a total of $2^2 = 4$ **(5i-5iv)** stereoisomer are possible. Since the product **(5)** is formed upon dehydration of ketocarboxylic acid **(3),** the stereochemistry at C-5 should remain unchanged with regard to the TCA.



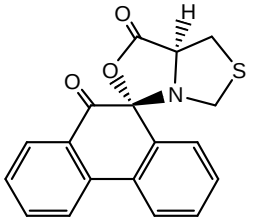
Scheme1: Synthesis of thiazolidinone derivatives



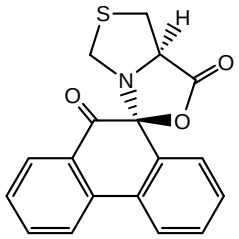
5(i)



5(ii)



5(iii)



5(iv)

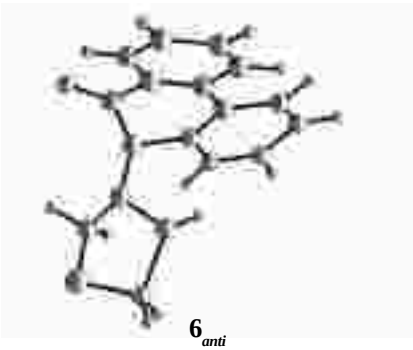
On this basis, the possibility of stereoisomer **5(i)** and **5(ii)** have been ruled out. Out of the remaining two isomers, **5(iv**) would be formed predominantly as it has lower ΔH_f than **5(iii).**

Table 1. Comparison of heat of formation (ΔH_f) of thiazolidinones 5(iv) v/s 5 (iii)

Cycloadduct	5 (iv)		5 (iii)	
	5a (iv)	5b (iv)	5a (iii)	5b (iii)
ΔH_f (kcal/mol)	62.53	30.42	72.45	53.40

In presence of a dipolarophile, the intermediate pyrolidinium species **(4)** may undergo decarboxylation to produce azomethineylide(*amy*) **(6)** which on 1,3-dipolar cycloaddition produces cycloadducts **(7-11)**. Geometry optimization of *amy* **(6)** indicated that it has a planar structure and can exist in two isomeric forms, one in which the >C=O group of the phenanthrenequinone ring and -C-H of the dipole are on the same side *i.e.* the $\mathbf{6}_{syn}$ and the other in which these two groups are on the opposite side *i.e.*$\mathbf{6}_{anti}$.

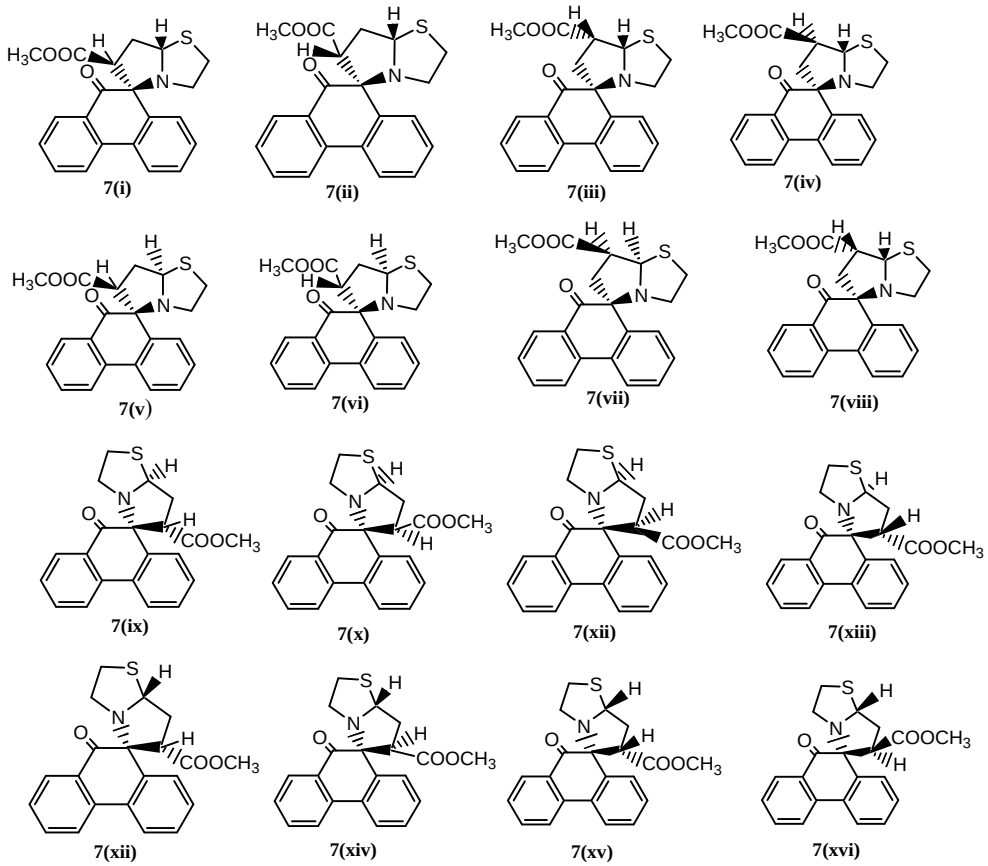
$\mathbf{6}_{syn}$



$\mathbf{6}_{anti}$

Fig 1. Optimized geometries of azomethineylide (6)

Methyl acrylate may approach either 6_{syn} or 6_{anti} with the formation of products having 3 chiral center. Therefore, a total of 8+8 =16 isomers 7(i)-7(xvi) **are possible.**



Attack of methyl acrylate on $\mathbf{6}_{anti}$ (Figure 2) results in the inward movement of the thiazolidinering towards phenanthrenequinone nucleus and may lead to steric crowding between both rings making the system unstable and hence failing to produce transition state geometry.

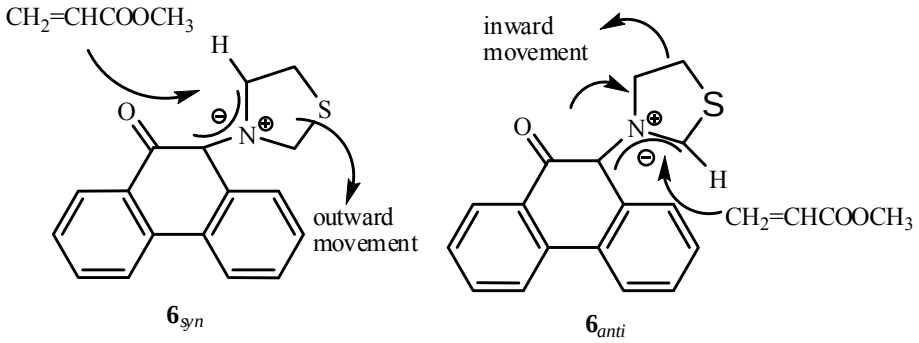


Fig 2. Mode of attack of methyl acrylate on amy (6)

Thus, only possibility is the attack on $\mathbf{6}_{syn}$ leaving only 8 isomers **7(i)-7(viii)** for consideration. Out of these, only 4 isomers have concerted mechanism and we have been able to locate the transition state in case of **(7i)** and **(7ii)** only. Between these two possibilities, the major isomer can be predicted on the basis of FMO approach along with the *endo* approach. The transition state of the concerted 1,3-dipolar cycloadditions are usually controlled by Frontier Molecular Orbitals (FMOs) of dipolarophiles and dipole (*amy*). The $\ddot{A}H_f$, HOMO and LUMO energies and HOMO-LUMO and LUMO-HOMO energy gaps of *amy***(6)** with various dipolarophiles are given in Table 2. From the Table 2, it is evident that $HOMO_{dipole}$-$LUMO_{dipolarophile}$ energy gap is lower than the $LUMO_{dipole}$-$HOMO_{dipolarophile}$ energy gap and therefore the dominant FMO approach is $HOMO_{dipole}$-$LUMO_{dipolarophile}$.

Table 2. ΔH_f, HOMO, LUMO, energies and H-L and L-H energy gaps

	ΔH_f(kcal/mol)	HOMO (eV)	LUMO(eV)	Energy gaps (eV)	
				H-L	L-H
Dipole *amy* (6)	153011.11	0.215	0.212	-	-
Dipolarophiles					
Meac	212312.10	0.478	0.444	7.65	10.09
Phac	163370.32	-0.415	0.222	7.64	8.42
Diph	378042.54	0.416	0.220	7.27	7.78
Phpr	208240.65	0.417	0.225	7.68	8.01
Etph	401480.86	0.434	0.254	7.05	8.74

Amy = azomethineylide; meac = methyl acrylate; phac = phenyl acetylene;
diph =diphenylacetylene; phpr = phenyl propyne; etph = ethyl phenyl propiolate

In the HOMO of the *amy* (dipole), the orbital coeffecient is larger at C_1 (0.0248) than at C_2 (-0.0167). Similarly in the LUMO of methyl acrylate the orbital coefficient on carbon atom bearing the -$COOCH_3$ group is large (0.052) than that away from it (-0.065).

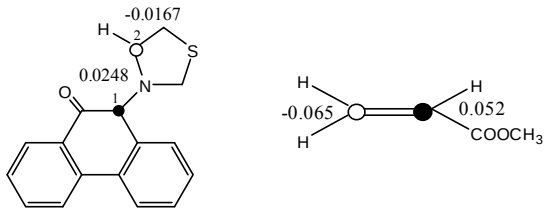


Fig 3. Atomic orbital coefficients of $HOMO_{amy}$ and $LUMO_{dipolarophile}$

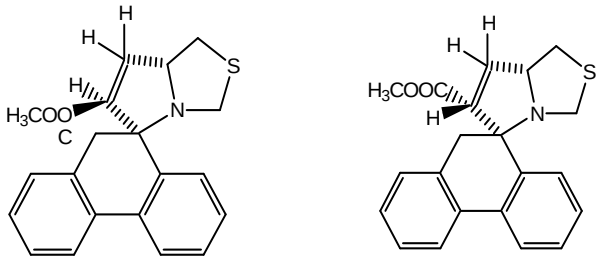


Fig 4. Transition state for isomer 7(i) and 7 (ii)

An efficient orbital overlap between C_1 of *amy* and the C-atom bearing the -$COOCH_3$ group results in the formation of product **7(i)**, ruling out the possibility of **7(ii)**. Thus cycloadduct**7(i)** is formed in diastereomeric excess due to the *endo*approach of -$COOCH_3$ group.

Table 3. ΔH_f-R, ΔH_f-TS, ΔH_f-P, Ea and stabilization energy of Amy with different dipolarophiles

Product	ΔH_f (kcal/mol)			Ea (kcal/mol)	Stabilization energy (kcal/mol)
	R	**TS**	***P***		
Amy**(6)**+ meac	806683.91	827014.08	794615.70	20330.17	12068.21
Amy**(6)** + phac	859020.70	867170.35	840112.52	8149.65	18908.18
Amy **(6)** + diph	961295.04	992551.92	957172.54	31256.88	4122.5
Amy **(6)** + phpr	92150.03	96134.94	90112.69	3984.91	2037.34
Amy **(6)** + etph	816584.92	847085.63	804413.70	30500.71	12171.22

Amy = azomethineylide; meac = methyl acrylate; phac = phenyl acetylene; dpa = diphenyl acetylene; phpr = phenyl propyne; etph = ethylphenylpropiolate.

Parallel to methyl acrylate, phenyl acetylene, 1-phenyl-1propyne and ethyl phenyl propiolate can also attack $\mathbf{6}_{syn}$to produceaproductwithtwo chiral centres and therefore 4+4=8 regioisomersare possible in each case.

Out of these, we could optimize the transition state for isomer **8-11(i)** only in each case and their formation can also be explained on the basis of FMO approach along with the *endo* approach of the phenyl rings. Atomic orbital coefficients of the $HOMO_{amy}$ and $LUMO_{dipolarophiles}$ are given in Figure 5.

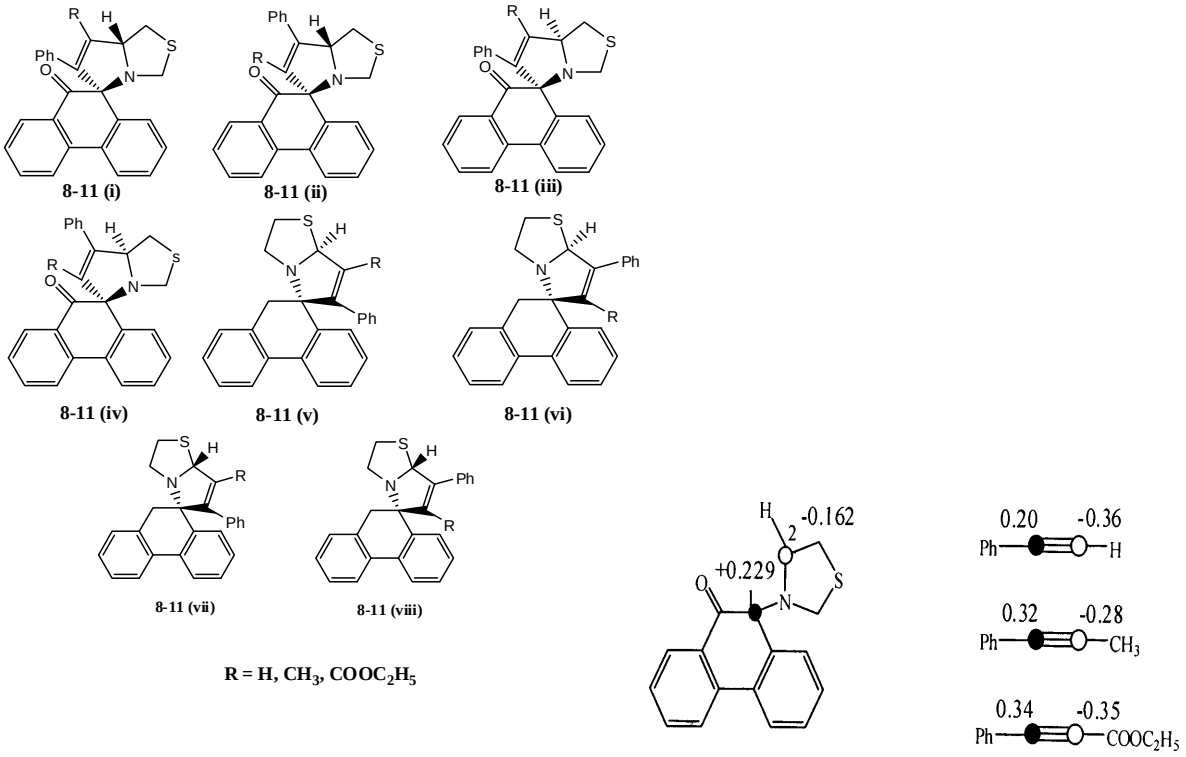
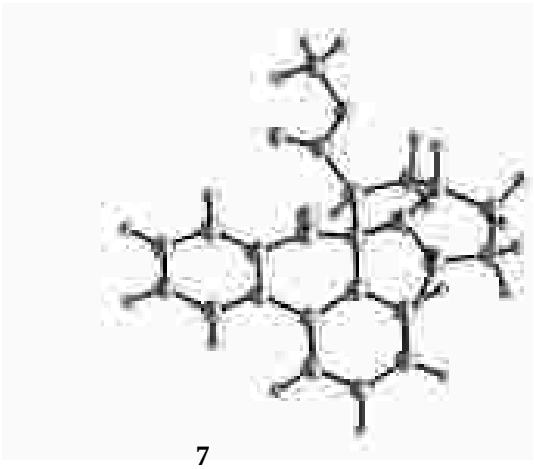


Fig 5. Atomic orbital coefficients of $HOMO_{amy}$ and $LUMO_{dipolarophile}$

Parallel calculations have been performed on other cycloadducts also. The optimized geometries of cycloadducts **(7-11)** are shown in figure 6.



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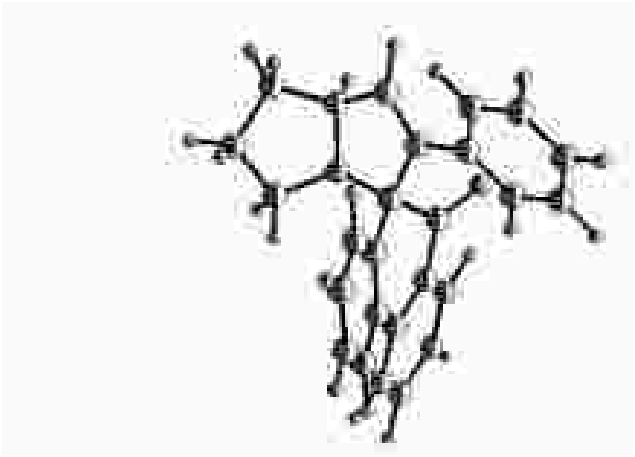

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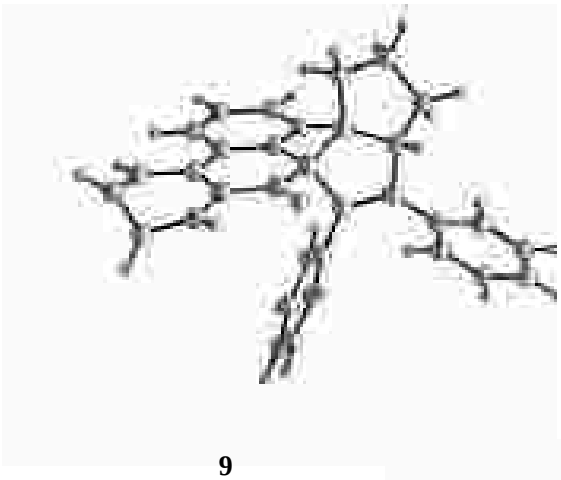

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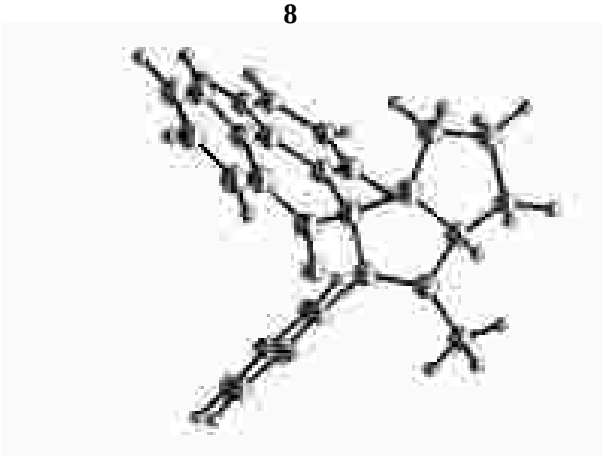

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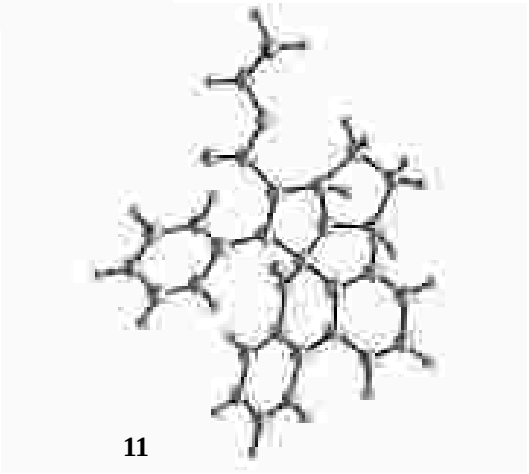

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Fig 6. Optimized geometry of cycloadducts (7-11)

Conclusions

An expedient methodology giving higher yield for cycloadducts **(7-11)** has been reported. We believe this protocol will be of relevance to the pharmaceutical industry. On the basis of the calculation of the heat of formation, it may be concluded that **5(iv)** would be formed predominantly than the other corresponding thiazolidinone intermediate **5(iii)**. It is also concluded that the cycloadduct **7(i)** is formed in diastereomeric excess due to the *endo* approach of $-COOCH_3$ Group and its transition state is usually controlled by Frontier Molecular Orbital's (FMOs) of dipolarophile and dipole.

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RNI No. MAHENG/2017/74063
ISSN No. (Print) 2581-5911

Impact of Lean Six Sigma on Speciality Chemical Manufacturers

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Abstract
Quality management has become a key strategy for finding solutions for improvements that ensure competition and continual improvement.
This paper brings out the successful implementation of quality improvements in Speciality Chemical manufacturers in Maharashtra, India as part of the author's doctoral study.
Tools such as Lean Six Sigma have been found to help in finding solutions, bringing about improvements and cost saving. Cost saving helps in organization become cost competitive globally especially in a highly-customized scenario for manufacturing Speciality Chemicals.

Keywords: *Lean, Six Sigma, Global Competition*

Introduction

Lean Six Sigma (LSS) is a popular quality tool for improvements in Speciality Chemical manufacturing in Maharashtra, India. This helps organizations achieve breakthrough results. LSS help organizations stay ahead of competition and to be globally competitive. LSS uses both tools of Lean and Six Sigma and they complement each other for industries to adopt.

Lean ensures value addition by removal of wastes and deletion of waste also leads to value addition. An easy understanding of Lean is removal of extra fat in an organization as in a body. Lean consists of four principles:

1. Waste removal
2. Faster speed or reduction in cycle time
3. Quality improvement by defect elimination.
4. Cost reduction.

There are many Lean tools but the most popular are 5S (Sort, Set in Order, Standardize, Shine and Sustain); Poka-Yoke or mistake proofing or ensuring zero defect, Single Minute Exchange of Dies, Total Productive Maintenance, Just in Time, Kanban, Andon Chart, Visual Management and Value stream mapping.

Six Sigma is a quality standard of maintaining 3.4 defects per million opportunity in manufacturing or for

that matter any activity. This employs statistics wherever required and uses DMAIC process to achieve improvement across industries, functions and trade. Six Sigma employs various belts called Master Black belt, Black belt, Green Belt, Yellow belt and achieves breakthrough results through projects. Essentially an organization will employ one or more black belts who will lead a 5 to 8-member cross functional team. Six Sigma eliminates errors and variations in the process, improves quality and make process robust. Design of experiment (DOE) helps in deciding the right chemical mixtures or % combination of raw materials for final product. Organizations apply PDCA to make continuous improvements.

Speciality chemicals are chemicals based on their performance and effects and are generally applicable for special applications and are customer focused such as Construction chemicals, Electronic chemicals, Textile chemicals, Water Management chemicals, Lubricating additives, Cosmetic additives, Adhesives and sealants, Paper chemicals, Printing chemicals, Plastic additives, Oil field chemicals, water Soluble polymers, Flavours and Fragrance etc. and globally expected to have reached a turnout of Rs.50000 crores by the end 2016.

Research Methodology

The research was based on more than hundred literature studies and on study done through a questionnaire sent to over 500 Speciality Chemical manufacturing organizations in Maharashtra and the feedback received from over 76 of them.

Literature study

Mozammel, Mapa and Scachitti, (2011), in their research on "Application of LSS in Healthcare" concluded that LSS reduces variability, waste, improved patient care, greater patient satisfaction rates that translate to bottom-line improvement.

Muthukumar. N, Anantharaman. N and Nachaippan. R.M,, (2007), in "Achieving World Class Status-Reducing variability in a continuous line manufacturing system- A Case Study" concluded that application of Six Sigma results in reduction in variations in manufacturing, eliminate defects and improves customer satisfaction.

Muthukumaran, Venkatachalapathy and Pajaniradja, (2013) in "Impact on integration of Lean Manufacturing and Six Sigma in various applications - a review" concluded that LSS is a quality improvement tool for betterment and can be applied to any industry for increased performance, reduction in through put time and to improve the quality by reducing the process variability.

Nakhai. B and Neves. J.S., (2008), in "The challenges of Six Sigma in improving service quality" found significant contribution of Six Sigma to reliability of services, responsiveness of service delivery, assurance of service delivery, empathy of service delivery and tangible service delivery.

Ben (2009), in "Lean Six Sigma" says that the key concepts of Lean are the cost reduction through Value Engineering, elimination of seven deadly wastes, usage of 5S System, Visual Workplace, application of TPS, JIT and lead time reduction

Nikolic and Nikolic, (2009), in "LSS in food industry" explored the impact of critical factors in the implementation of LSS in food industry based on interviews with 16 food companies in Europe. They identified ten factors that influence the application of LSS viz.: top driven down management, quality culture, quality driven training, problem solving teams, voice of customer for deciding the projects, and strategic orientation of LSS.

Nunes I.L. (2015), in "Ergonomics and Lean Six Sigma integration, A systems approach" concluded that for global competition, Lean Manufacturing and SS are major approaches to increase performance, by focusing on reduction on production waste, variability and costs.

Lean and SS technologies are frequently used together as LSS and by integrating with implementation of ergonomics, substantial benefits and improvement in working conditions were observed.

Oguz, Kim, Hutchison, and Han, (2014), in "Implementing Lean Six Sigma: A case study in concrete panel production", concluded that Lean Six Sigma method is recognized widely and has been implemented predominately in manufacturing and used on projects to improve the process by eliminating the variations and creating workflow in a process. They also observed that Six Sigma technique can be applied to the construction-based production system along with lean construction techniques. Construction companies applied Lean Six Sigma for improving process capability, promote continuous improvement of processes by both analysing root cause of variation and eliminating waste.

O'Rourke, P.M (2005), in "A multiple-case analysis of Lean Six Sigma deployment and implementation strategies", concluded that LSS is applied by several high-profile companies for continuous improvement and that several factors appeared to significantly contribute to implementation success viz Fusing business strategy with continuous improvement strategy, leadership commitment and involvement, and an organizational model that links the continuous improvement efforts with the performance measurement system and senior leadership.
Patrícia Abreu, Sérgio Sousa, and Isabel Lopes, (2012), in "Using Six Sigma to Improve Complaints Handling" spoke of the Six Sigma Project adopted by auto companies to improve customer satisfaction, loyalty and expectations. Six Sigma application resulted in better response time to customer complaints and improved the process of analysis of defective products or root causes of low performance and solutions for improvements were implemented which substantially increasing productivity.

Pepper M.P.J. and Spedding T.A., (2010), in "The evolution of Lean Six Sigma" examined the integration of Lean with SS as a coherent approach to continuous improvement and if used together are powerful tools that give breakthrough results. LSS has experienced success in a wide-ranging spectrum of industries with huge potential for process improvement and organizational change management.

Pettersen, (2009), in his study "Defining Lean production: some conceptual and practical issues" concluded that Lean should be applied to improve performance of the organisation.

Prasad and Sharma (2014), in their study "Studies linking Lean manufacturing methodology with Green manufacturing" concluded that Lean manufacturing and Green manufacturing are complimentary and adoption of one automatically improves the other and together there can be improvements such as environmental pollution abatiment, optimum utilization of resources, improvement in productivity and profitability.

Gaps in Literature review

Few literature studies are available on impact of Lean Six Sigma on Speciality Chemical Manufacturing industries in Maharashtra. LSS has not been fully explored for implementation in Maharashtra, due to lack of awareness, absence of standard process for application of DMAIC process and projects done as per convenience, lack of knowledge of the top management and support for LSS.

A few case studies are available on Speciality Chemical Manufacturers in Maharashtra, India.

Data Analysis

A Survey Questionnaire on Speciality Chemical Manufacturers in selected zones of Maharashtra was made and was used as a primary data. The survey included demography of the organization, details of the surveyed person, impact of Lean Six Sigma and Impact of Lean Six Sigma on their operations, various tools employed and their rating for usage of various tools and on factors

that impact the manufacturing because of LSS.

Objectives and Hypothesis of the Study

Lean Six Sigma has less or no impact on improvement processes on Speciality Chemical manufacturing as compared to other improvement processes, LSS has no significant impact on Cycle time in Speciality Chemical manufacturing, LSS has significant impact on Cycle time and reduces cycle time in Speciality Chemical manufacturing, Lean Six Sigma has no significant impact on inventory in Speciality Chemical manufacturing, Lean Six Sigma has no significant impact on defects in Speciality Chemical manufacturing. Lean Six Sigma employs DMAIC (Define, Measure, Analyse, Improve and Control) in improvement process with less or no significant results in Speciality Chemical manufacturing and also has less or no great impact on competitiveness in Speciality Chemical manufacturing.

Research findings

Some typical Lean six sigma project application include improvements in inventory reduction, performance, continuous process improvement, reduction in working capital, profit, receivables reduction, customer satisfaction index, export, reliability, quality, facilities, return on invested capital, labour productivity, 5S implementation, pollution, capacity, packaging, costs savings, customer penetration rate, reduction in delivery time, application of SPC, application of DOE and FMEA, etc.

Conclusion

As per analysis done on the surveyed data using SPSS 16.0, it was concluded through hypothesis testing and application of Chi-Square test that —

a) LSS has more impact on manufacturing as compared to other improvement processes
b) LSS has an impact on reduction in cycle time
c) LSS has an impact on inventory reduction
d) LSS has an impact on defect reduction
e) LSS has an impact on improvement processes through DMAIC
f) LSS helps the organization to be competitive.

Recommendations

It is recommended that separate studies be done on each of the sector of Speciality Chemicals. Projects have adopted Lean Six Sigma quality tools for better and systematic approach of improvements and results.

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RNI No. MAHENG/2017/74063
ISSN No. (Print) 2581-5911

Development of Immobilized Enzymatic Trypsin Micro-reactor on Polymer Monolith Column for Biocatalytic Reactions in Capillary Liquid Chromatography

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Abstract
A immobilized trypsin reactor based on sub-micron skeletal polymer monolith has been developed. A monolithic enzymatic microreactor was prepared in fused-silica capillary by in-situ *polymerization of glycidyl methacrylate (GMA) and ethylene dimethacrylate (EDMA) in the presence of ternary porogenic mixture of 1,4-butanediol, decanol, and ethanol, and trypsin was then introduced to form an immobilized reactor (IMER). Enzyme immobilization was smoothly achieved by passing a trypsin solution through the column and kept at 4°C for 24 hours. The performance of the monolithic microreactor column was demonstrated with the digestion of bovine serum albumin (BSA). The result was obtained by passing the protein solution through the microreactor and the tryptic fragments were separated by reversed phase column. For digestion of BSA by the immobilized microcolumn, Tris-HCl buffer pH 7.3-8.3 was found to be the optimum condition for on-line protein digestion. The characterization of the column by using scanning electron microscopy demonstrates that the porous polymer has homogeneous structure across the entire monolith.*

Keywords: *Polymer monoliths, Methacrylate base, Enzyme immobilization, Trypsin, On-line protein digestion, Peptide separation*

Introduction

Proteins regulate many cellular functions and analyzing the presence and abundance of proteins in biological samples is the central focus in proteomics. Within a few years, proteomics has emerged as a new subject to understand the various problems encountered in the identification of proteins in cells, tissues or body fluids. The proteolytical digestion of protein generally takes place with one or more proteases, which usually have their own unique specificities. For example, trypsin is capable of selectively cleaving proteins at arginine and lysine residues, which could provide peptides in a mass range compatible with MS for amino acid sequence determination.

In recent years, several methods have demonstrated the feasibility and have been developed for protein digestion using enzyme immobilized on various supports, such as porous monolithic material, porous silicon matrix, polymer, glass and membrane. Among the methods, monoliths have several advantages such as high permeability and fast mass transfer and hence are supposed to be excellent for enzyme immobilization support. Therefore, the application of a monolithic column as a separation medium for bioseparation has received great

attention in recent years. Three types of monolithic stationary phases have been developed: silica-based monolithic columns, organic polymer-based monolithic columns, and organic–silica hybrid monolithic columns.

The ordinary in-solution based protein digestion has several difficulties such as time consuming nature for digestion, unavoidable enzyme autodigestion and usually, the sample should be prepared by off-line digestion. Immobilization of the enzyme is a promising strategy for high throughout digestion as it reduces the digestion time and for on-line digestion system, it connects the immobilized enzyme reactors (IMERs) and separation column. A large number of IMERs have been prepared in capillaries and applied because the monolith has high binding capacity for enzyme, low back pressure, biological inertia, and mechanical stability. Because of easy modification, the porous poly(glycidyl methacrylate-co-ethylene dimethacrylate) monolith is included as one of the popular materials for enzymatic reactor. *In situ* prepared monoliths are chemically attached to the inner wall of capillaries, and retaining frits are not required to support the monolithic matrices.

This study describes the preparation and characterization of a poly(glycidyl methacrylate-co-ethylene dimethacrylate) monolith column for development of a bioreactor based on the immobilized trypsin. Trypsin was covalently immobilized on the epoxy-modified monolith support with a single reaction. Several effects on enzyme activity were studied, such as flow rate, buffer type, and pH. After the digestion, the separation and detection of the peptides were conducted by capillary liquid chromatography.

Materials and Methods

Apparatus

The chromatographic measurement was conducted by using a capillary LC system constructed by an L. TEX-8301 microfeeder (L. TEX Corporation, Tokyo, Japan) equipped with an MS-GAN 050 gas-tight syringe (0.5 mL, Ito, Fuji, Japan) as a pump, a model M435 microinjection valve (Upchurch Scientific, Oak Harbor, WA, USA) with injection volume of 0.3 µL, a 100 mm x 0.32 mm i.d. of microcolumn and a UV-1575 intelligent UV/Vis detector (JASCO, Tokyo, Japan) and a data processor (CDS-Lite ver 5.0; LA soft, Chiba, Japan). The morphology of the column was characterized by an S-4800 scanning electron microscope (Hitachi, Tokyo, Japan).

Reagents and materials

Glycidyl methacrylate (GMA) (97%) and ethylene dimethacrylate (EDMA) (97%) were obtained from Wako Osaka, Japan. 3-(Trimethoxysilyl)propyl methacrylate (γ-Maps, 98%) and 2,2'-azobisisobutyronitrile (AIBN) were obtained from TCI Tokyo, Japan. Trypsin, 0.1 mol/L phosphate buffer, 1 mol/LTris-HCl buffer, 1,4-butanediol, decanol, ethanol, sodium borohydride were obtained from Nacalai Tesque Kyoto, Japan. Benzamidine was obtained from Sigma-Aldrich Saint Louis, USA. All solutions used in this study were prepared using ultrapure water prepared in the laboratory using a simplicity UV waterpurification system (Millipore, Molsheim, France).

Pre-treatment of the capillary

Initially, the fused silica capillary was flushed with 1M NaOH solution, deionized water and 1M HCl respectively for approximately 30 min. 30% (v/v) of γ-MAPS in acetone was poured into the capillary; thereafter the capillary was sealed and allowed to react thermally in water bath at 60°C for 24 hours. It was used for introducing methacrylate groups on the inner side of the capillary tube, and rinsed with acetone (30 min) and then dried using nitrogen gas for atleast 30 min. With this procedure, the monolith support column could be covalently anchored to the activated inner surface wall and the silanization reaction started by introducing methacrylic anchoring groups.

Preparation of the monolith column

Mixture solutions of monomer, cross-linker, and porogen were prepared. The appropriate solvent was directly added to the porogen mixture. As shown in Table 1

monomer, cross-linker and porogen were added in various ratios. As the solution for the fabrication of the monolith column, a mixture of GMA (v/v), EDMA (v/v), 1,4-butanediol(v/v), decanol (v/v), ethanol (v/v), and AIBN (1% with respect to the monomers) was added, followed by sonication for 5 min. Subsequently, the solution was manually introduced into the pretreated capillary using a syringe. Both ends of the capillary were sealed and heated at 60°C for 24 h in a water bath for polycondensation and polymerization. After polymerization was completed, the capillary column was flushed with methanol to wash porogenic solvents and other compounds.

Immobilization of the enzyme

The capillary was first rinsed with water for 30 min and then by trypsin for one-step immobilization through the epoxy groups, because these groups readily reacted with amino groups of the enzyme, as shown in Fig 1. The trypsin solution was prepared by mixing 2.5 mg/mL trypsin in phosphate buffer pH 7.6 in the presence of benzamidine (trypsin inhibitor) to prevent autodigestion. Trypsin solution was pumped through the columns for 12 h at 4°C. Later, the enzyme-immobilized monolith column was rinsed with 5 mg/mL sodium borohydride solution overnight. It was filled with 50 mM Tris-HCl buffer pH 7.0 containing 10 mM $CaCl_2$ and 0.02 % NaN_3 and stored in refrigerator at 4°C before use.

Digestion of the protein

The protein sample (bovine serum albumin) was dissolved in 2 mL Tris-HCl buffer solution (10 mmol/L) containing 8 mol/L urea. The protein denaturation was performed at 4°C for 24 h. Then, 14 mL Tris-HCl buffer (10 mmol/L) was added and the denaturation protein solution was introduced into the immobilized-enzyme column.

Characterization

The morphology of the monolith surface was also observed by using scanning electron microscopy for characterization of the internal surface of the monolith.

Results and Discussion

The preparation of the monolith microreactor

The preparation of enzymatic microreactor was carried out in two steps. The preparation procedure is outlined in Fig 1. Thermally initiated *in situ* polymerization was first conducted. In this study a mixture of solutions of monomer, cross-linker, and porogens was prepared and mixed with AIBN. Thermal polymerization was carried out in a water bath at 60°C for 24 h. The capillary was rinsed with methanol after polymerization to remove unreacted reagents and porogenic solvents. Secondly, trypsin was attached *via* covalent immobilization onto the functionalities of the support. The epoxy-activated monoliths are the most often used enzyme supports because of easy fabrication and modification as well as relatively low cost. Due to the direct coupling, the enzyme *via* the epoxy groups can be modified with various reagents.

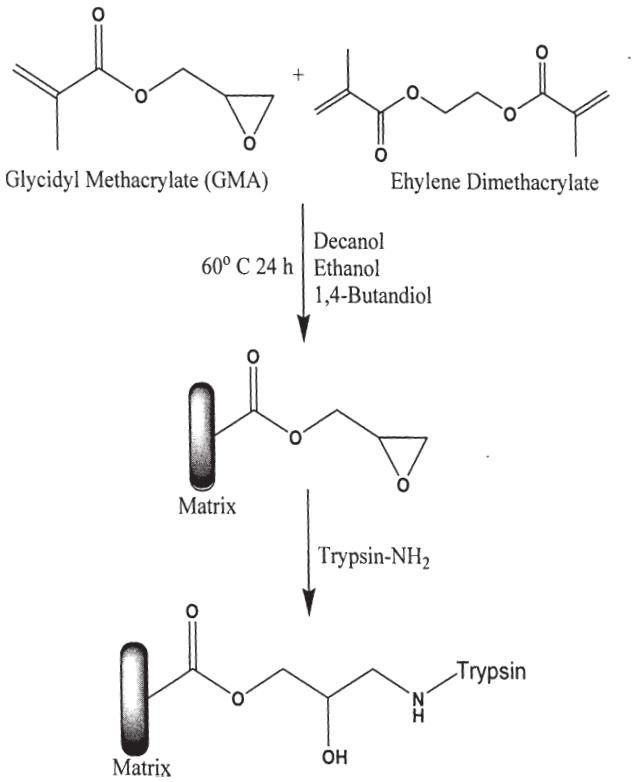

Fig. 1 Schematic diagram for IMER preparation

To increase the amount of the enzyme immobilized on the support, low-temperature (4°C) and long reaction time (24 h) have to be used to increase the enzyme activity. The reactions with an amino group from protein could be performed with high speed even under ambient conditions. However, in this study the reaction was conducted for a long time, with slow flow rate and under low temperature. Sodium borohydride was passed through to improve the stability of the enzyme immobilization and reduce the C=N bonds.

The D3 monolith column in Table 1 was chosen as microreactor after several mixtures had been tested. First, the choice of the cross-linker was made but the important factor was to avoid failure during polymerization. The length of the polymerization chain was too long, resulting in very small pores and the polymer structure was too weak to resist high flow. Addition of a cross-linker had an impact to the strength of the monolith. The ratio of monomer to cross-linker was fixed and polymerization time was fixed for 24 h.

Table 1 Various polymerization conditions

Column	Monomer[a] % (v/v)	Crosslinker[b] % (v/v)	Porogen[c] % (v/v)	Mixture ratio
D1	7.5	2.5	90	10/90
D2	15	5	80	20/80
D3	22.5	7.5	70	30/70
D4	30	10	60	40/60

[a]Glycidyl methacrylate as a monomer
[b]Ethylenedimethacrylate (EDMA) as a cross-linker
[c]Ternary porogens*i. e.* 1,4-butanediol, decanol, and ethanol (1:0.5:0.1)

Characterization of the monolith

The SEM images of the monolithic supports in capillaries are shown in Fig. 2. The monomer/porogen ratio was 30/70, for the homogeneous porous polymerisation across the entire monolith. The introduction of trypsin had little effect on the porosity properties of the monolithic column. The macroporous and microporous structure resulted in high throughput and low backpressure.

The small size and uniform particles of the monolith column achieved a high surface area for enzyme immobilization and the monolith was attached to the inner wall of the capillary. Less amount of porogen can also cause the dense structure of the monolith. The macroporous structure led to low backpressure. Also, the small particles and their uniformity could provide a high surface area for trypsin immobilization. The porosity properties of the monolith support have to adjust to the flow through the pores of the monolith column at a low backpressure. As shown in Fig. 3, the relationship between the flow rate and the back pressure demonstrated that the monolith column (D3) was mechanically stable upto flow rate 6 µL/min.

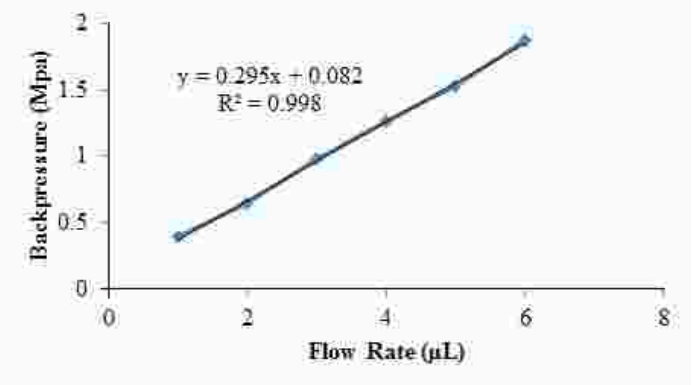

Fig. 3 Effect of the flow rate on backpressure. Condition: mobile phase, Tris-HCl buffer 0.1 mol/L. Immobilized column : 100 x 0.32 mm I.D

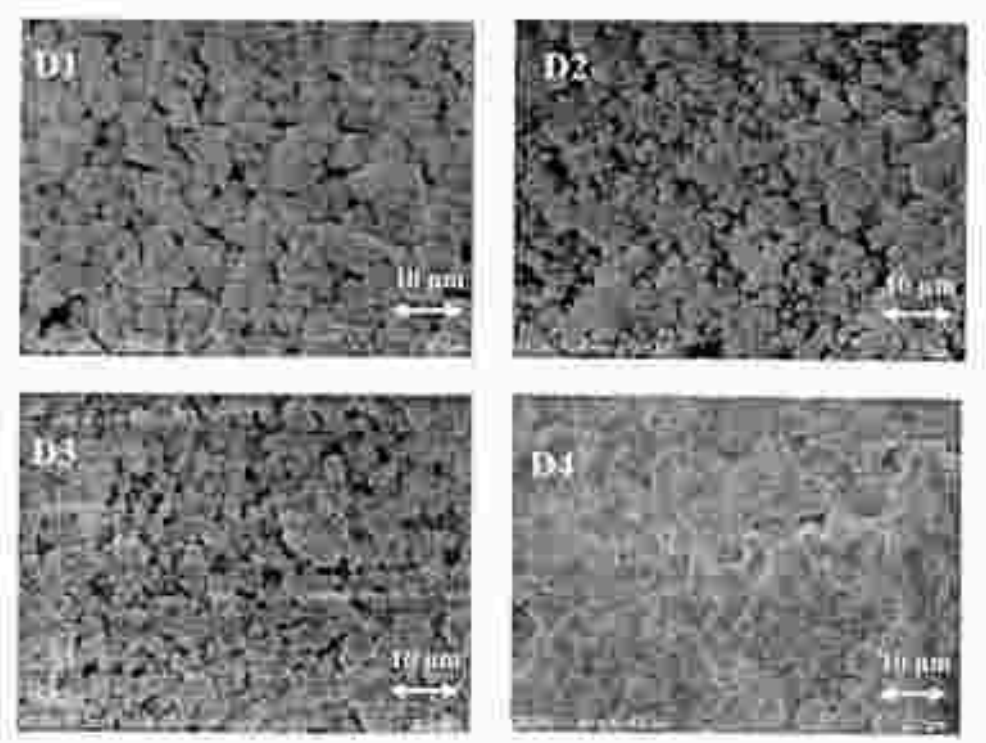

Fig. 2 Scanning electron microscopy of internal structure of monolith in capillary(magnification 2000x).

Denaturation of the protein sample (BSA)

Proteins can be denaturated by direct interaction whereby urea breaks the hydrogen bonds and other chemical bonds of the polar moieties of the protein. Urea also denatures proteins by decreasing the hydrophobic effect and by directly binding to the amide units *via* hydrogen bonds, particularly peptide groups [23]. The BSA sample solution was examined in the pressure and absence of urea before passing through the immobilized enzyme microcolumn.

Fig.4 shows the BSA sample solution treated and untreated with urea. It can be seen that the BSA sample solution treated by urea was required to encourage the tryptic effect of the microcolumn.

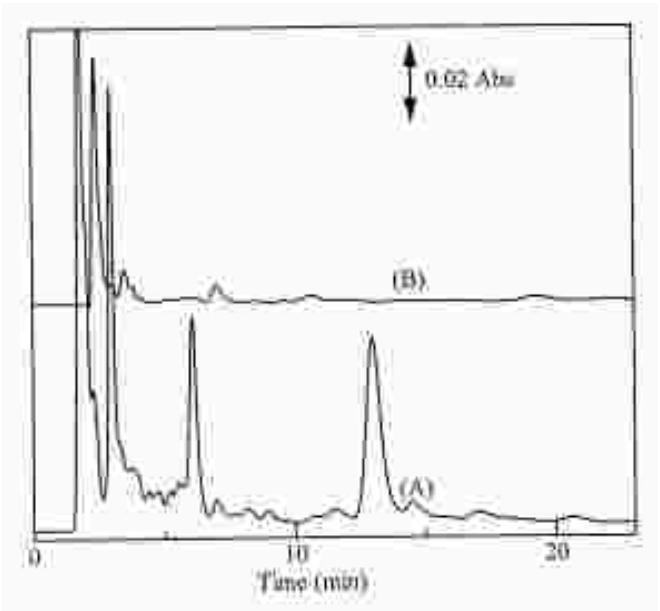

Fig. 4 Effect of urea on on-line BSA digestion in the immobilized-enzyme microcolumn (100 x 0.32 mm I.D) (A) with urea (B) without Urea. Condition: mobile phase, 0.1 mol/L Tris-HCl buffer (pH 8.3) with flow rates 3 µL/min for separation and flow rate 1 µL/min for digestion. Separation column: L-column2 ODS (100 x 0.32 mm I.D). Digestion system monitored at 214 nm.

Application of the monolith enzymatic microreactor for digestion

The important part of the digestion ability of IMERs is the amount of trypsin immobilization onto the monolith column. Theoretically, greater the immobilization of the enzyme, higher the digestion rate. Nevertheless, the immobilization methods and the supporting properties materials could also affect the digestion ability of IMERs. A standard protein bovine serum albumin (BSA) was used to test the performance of the trypsin-immobilized microreactor. The reference monolith D3 was chosen to verify chromatographic separation potential of the microreactor. The immobilized enzyme monolith column was used for on-line protein digestion and then peptide separation by reversed phase column.

The BSA solution was passed through the enzyme micro-reactor column with several variations of flow rate 1, 2, 3 and 4 µL/min and then the tryptic fragments were injected directly onto the reversed phase separation column, as shown in Fig.5.

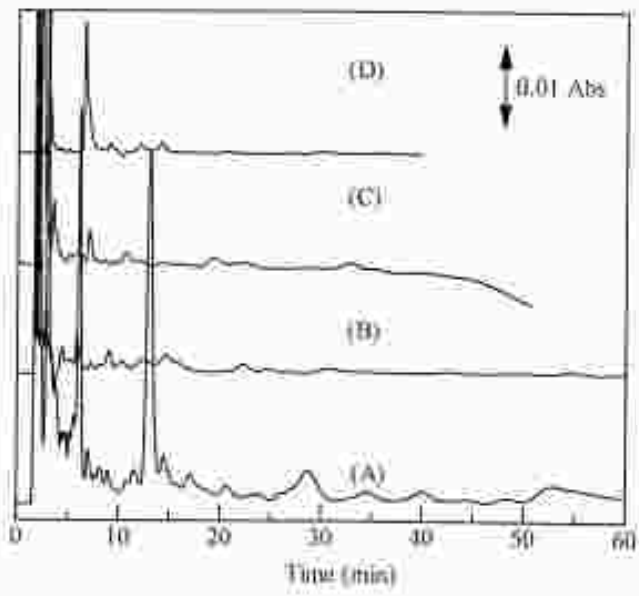

Fig. 5 Effect of the sample flow rate on on-line BSA digestion in the immobilized-enzyme microcolumn (100 x 0.32 mm I.D). Condition: mobile phase, 0.1 mol/L Tris-HCl buffer (pH 8.3) with flow rates of digestions (A) 1 (B) 2 (C) 3 and (D) 4 µL/min and separation flow rate 3 µL/min. Separation column: L-column2 ODS (100 x 0.32 mm I.D). Digestion system monitored at 214 nm.

The factor that could affect the digestion of the protein is the flow rate of the sample solution or the contact time of the protein inside the immobilized enzyme microcolumn. The effect of the flow rate of the protein digestion was examined by varying flow rate when the micropump drained the solution to the immobilized enzyme micro-column from 1 to 4 µL/min. In Fig. 5 it can be shown that the digestion of the protein increases with decreasing flow rate and larger peaks are obtained, indicating the longer contact time of the protein inside the monolith column with the enzyme sites. Since the digestion could be expected with 1 µL/min as the optimum flow rate at lower speed and complete digestion of the protein is observed at 1 µL/min, this flow rate was used for all further experiments.

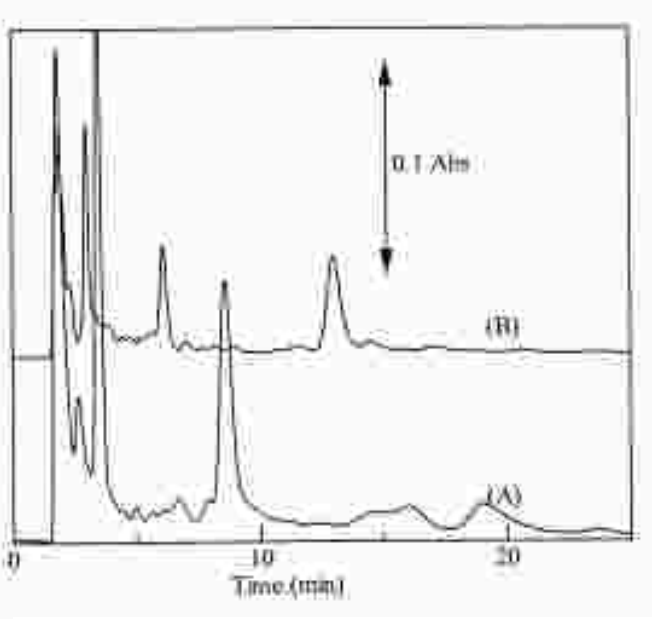

Fig. 6 Chromatograms of the injection of BSA in the immobilized-enzyme microcolumn (100 x 0.32 mm I.D.). Digestion system monitored at 214 nm. The experiment was conducted with (A) 0.1 mol/L Tris-HCl buffer solution (pH 8.3) (B) 0.1 mol/L phosphate buffer solution (pH 8.1) with flow rate for separation 3 µL/min and flow rate for digestion 1 µL/min. Separation column: L-column2 ODS (100 x 0.32 mm I.D.).

In this study, the activity of trypsin for BSA digestion was assessed by using buffer solutions such as phosphate buffer and Tris-HCl buffer solution as the mobile phase. Fig. 6 shows the chromatograms of the sample injected into the immobilized enzyme microcolumn, for (A) Tris-HCl buffer and (B) phosphatebuffer. We found that the digestion in Tris-HCl buffer was more effective than phosphate buffer and also showed much higher rate of digestion. Tris-HCl buffer solution breaks up open cells for use in molecular biology experiments. It is completely denaturated because it contains salts which regulate the acidity and osmolarity and maintain the ionic strength in the buffer solution.

Enzymes activity is affected by the change of pH. Enzymes are effective at specific pH values. Extremely high or low pH values generally result in complete loss of activity for most enzymes. pH is also a factor in the stability of enzymes and there is a region of pH of optimal stability. Theoretically, the optimum pH value of trypsin enzyme is between 7.8 – 8.7. As can be shown in Fig. 7, there are 3 variations of pH of the mobile phases. The experiment was conducted with Tris-HCl buffer solution of pH 8.3, 7.3 and 5.4. At higher pH (8.3 and 7.3) the digestion of the injected BSA leads to the presence of multiple peptide fragments. Further, at lower pH (5.4) the digestion of the injected BSA did not lead to multiple peptide fragments.

Chromatogram for on-line digestion obtained with the capillaries with 0.32 mm I.D. (Fig. 8) shows a blank run in a capillary with no enzyme and on-line digestion for a micro reactor containing the enzyme. The experiment was conducted with a monolith column without trypsin as a blank column and immobilized enzyme microcolumn. The digestion of the injected BSA leads to the presence of multiple peptide fragments for the latter case. As can be seen, the microcolumn is capable of digesting the injected protein resulting in the production of many proteolytic fragments of the peptide.

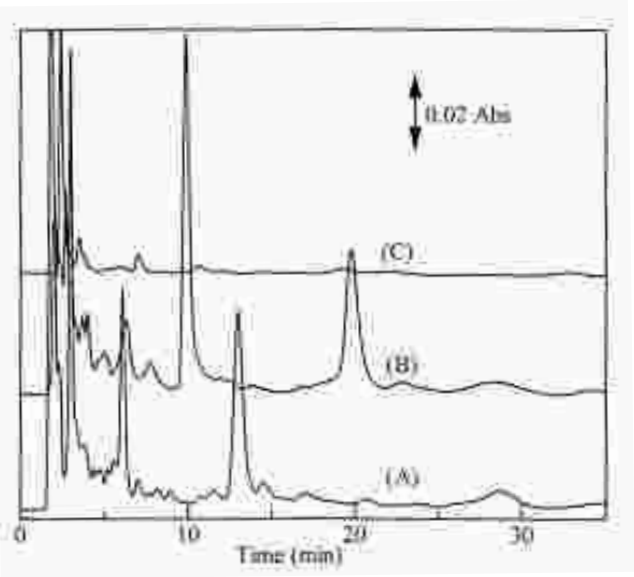

Fig. 7 Chromatograms of the injection of BSA in the immobilized-enzyme microcolumn (100 x 0.32 mm I.D.). Digestion system monitored at 214 nm. The experiment was conducted with 0.1 mol/L Tris-HCl buffer solution pH (A) 8.3 (B) 7.3 (C) 5.4with flow rate for separation 3 µL/min and flow rate for digestion 1 µL/min. Separation column: L-column2 ODS (100 x 0.32 mm I.D.)

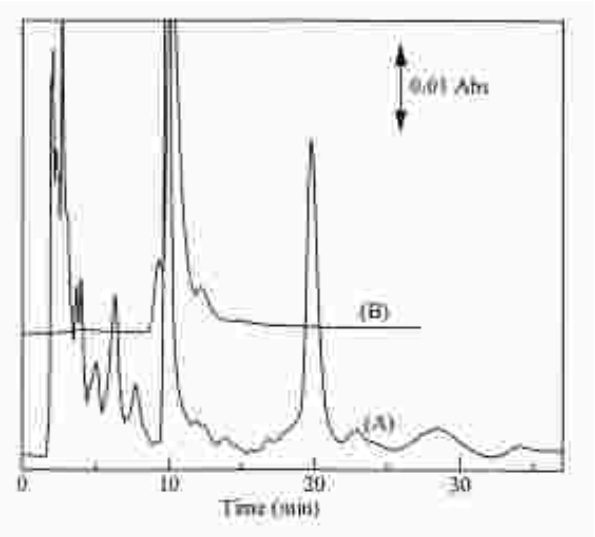

Fig. 8 Chromatograms of the injection of BSA in the immobilized-enzyme microcolumn (100 x 0.32 mm I.D.). Digestion system monitored at 214 nm. The experiment was conducted with (A) an immobilized enzyme microcolumn (B) a monolith column containing no trypsin (blank) with flow rate for separation 3 µL/min and flow rate for digestion 1 µL/min. Buffer solution: 0.1 mol/L Tris-HCl buffer solution (pH 8.3). Separation column: L-column2 ODS (100 x 0.32 mm I.D.).

Conclusions

Monolithic polymer column was successfully used as immobilized enzyme microcolumn and prepared *via* thermally initiated *in situ* polymerization and immobilization of trypsin. Denaturation of the protein (BSA) with urea increased the digestion in the microcolumn. This microreactor was applied to digestion of protein (BSA). Demonstration of the digestion of the protein in Tris-HCl buffer solution with pH 7.8 proved to be more compatible for on-line digestion. This system used microcapillary column for digestion of the protein (immobilized enzyme microcolumn) and for separation of the tryptic fragments.

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RNI No. MAHENG/2017/74063
ISSN No. (Print) 2581-5911

New Stability Indicating High Performance Liquid Chromatography Method for the Estimation of Aripiprazole in Bulk and Formulations

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Abstract

A simple, specific, accurate, precise and stability-indicating reverse phase high performance liquid chromatography (RP-HPLC) method was developed for the estimation of Aripiprazole (ARI) in tablet dosage form. For the HPLC method, Symmetry® C-18 5µ column consisting of 4.6 × 250 mm internal diameter in isocratic mode, with mobile phase containing Acetonitrile and methanol in the ratio of 35:65 v/v was used. The flow rate was 1mL min^{-1} and the effluent was monitored at 254 nm. The method was validated in terms of linearity, accuracy, precision, limit of detection (LOD), limit of quantification (LOQ) etc. in accordance with ICH guidelines. Linear regression analysis data for the calibration plot showed that there was good linear relationship between response and concentration in the range of 10 - 100 µg mL^{-1}. The LOD and LOQ values for HPLC method were found to be 0.4 and 10 µg mL^{-1}. No chromatographic interference from tablet excipients was found. The proposed method was successfully used for estimation of ARI in tablet dosage form.

Keywords: *Aripiprazole, development and validation, RP-HPLC, degradation study*

Introduction

Aripiprazole (ARI) is chemically 7-{4-[4-(2,3-dichlorophenyl) piperazin-1-yl]butoxy}-3,4-dihydroquinolin-2(1H)-one and is a typical antipsychotic and antidepressant used in the treatment of schizophrenia, bipolar disorder, and clinical depression[1-2]. Literature survey revealed few analytic methods like HPLC for the estimation of ARI in formulation.. Liquid chromatography/mass spectrometry (LC/MS) method has been reported for the determination of ARI in biological fluids[3- 4] The present work describes a novel stability indicating method for the determination of ARI in tablets using reverse phase HPLC. The proposed method was found to be suitable for routine determination of the drug in formulations.

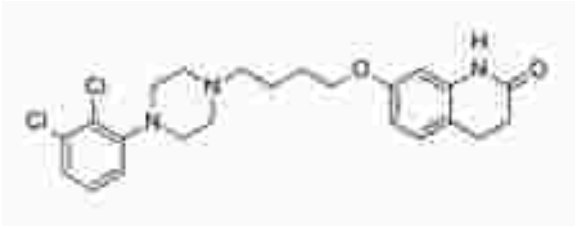


Fig.1: Chemical structure of Aripiprazole

Materials and Methods

Chemicals

Standard bulk drug sample of ARI was provided by Hetero Pharmaceutical Limited Hyderabad, India. Pharmaceutical dosage form used in this study was **ARIP-MT** tablets labeled to contained 5 mg (Torrent Pharmaceutical Limited, India). Acetonitrile (HPLC grade), methanol (HPLC grade) and water (HPLC grade) were obtained from Qualigen Fine Chemicals, Mumbai, India. Sulphuric acid (LR grade), hydrochloride (LR grade) and glacial acetic acid (LR grade) were obtained from Ranbaxy Fine Chemicals Ltd, New Delhi, India. Ammonia solution (LR grade) was obtained from S.D. Fine Chemicals Ltd, Mumbai, India.

Apparatus and chromatographic conditions

HPLC method development and validation was done on a Shimadzu (Japan) Liquid chromatograph equipped with (LC-20 AD pump), LC-20A UV/Vis detector, (rheodyne) 7725i injection with 20µl loop and LC-Solutions software. Stationary phase used was Symmetry® C-18 5µ Column consisting of (4.6 × 250mm) i.d. and the mobile phase used was acetonitrile and methanol in the ratio of 35:65 v/v. The mobile phase was filtered using 0.45µ membrane filter (Rankem Nylon membranes, New Delhi, India). The mobile phase flow rate was 1mL min^{-1} and injection volume was 20µL.

HPLC METHOD

Preparation of standard solution

Standard stock solution of ARI (100 µg mL^{-1}), and PAR (100 µg mL^{-1}) was prepared in Acetonitrile: Methanol (65: 35 % v/v).

Preparation of Calibration graph

Stock solution was diluted with mobile phase to get a series of concentration ranging from 10-100 µg mL^{-1} each containing 10 µg mL^{-1} of Paracetamol as internal standard and analyzed in triplicate. The peaks obtained were integrated, peak areas were noted and calibration graph was plotted using peak area to internal standard peak area ratios vs. concentration of standard solutions.

Preparation of sample solution

Twenty tablets were weighed and the average weight was calculated. Quantity equivalent to 10 mg ARI was weighed, transferred to 100mL standard flask, extracted with acetonitrile and made up to the mark with the same solvent. This solution was filtered through Whatman filter paper and suitable aliquots of formulation solutions were prepared corresponding to concentrations in the linearity range.

Method Validation

The developed method was validated according to ICH guidelines for validation of analytical procedure.

The linearity was evaluated by linear regression analysis. The calibration graph was plotted for the HPLC method (10-100 µg mL^{-1}).

Precision studies were done in terms of repeatability (intra day precision) and intermediate (inter day precision) and was expressed as relative standard deviation (RSD) of a series of measurements. Intraday precision was calculated from six replicate readings at 3 concentration levels within the linearity range. Interday precision was studied by comparing the results on three different days.

To study the accuracy of the method, recovery studies were carried out by addition of standard drug solution to preanalyzed sample at 3 different levels i.e 50, 100 and 150%. The resultant solutions were then reanalyzed by the proposed method.

LOD and LOQ was calculated using single-to-noise (S/N) ratio method. LOD was taken as a concentration of analyte where S/N is 3 and LOQ was taken as a concentration of analyte where S/N is 10.

Robustness was evaluated by studying the influence of small deliberate changes of the analytical parameters on the retention times and peak shapes.

The method should be robust enough with respect to all critical parameters so as to allow routine laboratory use.

Specificity of the method towards the drug was studied by determination of purity for drug peak in a mixture of stress samples using in a UV detector.

Degradation Studies

Procedure

The stress testing was conducted as per ICH[5] guidelines and USP[6]

Forced degradation for the drug substance was carried out under acid/base/neutral hydrolysis, photolytic and oxidative stress conditions.

Drug at a concentration 1 mg/mL was used in all degradation studies.

In each study, blanks and control (zero hour samples) were used to compare and calculate the percentage degradation.

There were four samples prepared in each stress test as indicated below:

1) blank solution stored under normal conditions,
2) blank solution subjected to stress like the drug,
3) zero time sample containing the drug which is stored under normal condition (control) and
4) drug solution subjected to stress.

Hydrolytic Studies

Acidic condition

The solution was prepared by dissolving the drug substance in water and the drug was subjected to accelerated degradation under acidic condition by refluxing with 0.05 N HCl at 100°C and the sampling was done at intervals of five minutes till sufficient degradation was achieved. The resulting solution was neutralized, appropriately diluted and chromatograms were recorded.

Alkaline condition

The drug substance was dissolved in water and the drug was subjected to accelerated degradation under alkaline condition by refluxing with 0.05 N NaOH at 100°C and the sampling was done at intervals of five minutes till sufficient degradation was achieved. The resulting solution was neutralized, appropriately diluted and chromatograms were recorded.

Neutral condition

For forced degradation study in neutral condition, the drug dissolved in water was heated at 100°C and samples were withdrawn at appropriate time intervals and subjected to HPLC analysis after suitable dilution.

Oxidative studies

Initial oxidation studies were performed in 3% H_2O_2 at room temperature for 10 hrs. Subsequently the drug was exposed to 6% H_2O_2 at room temperature and analyzed periodically. The resultant solution was appropriately diluted and chromatograms were recorded.

Photolytic studies

For photolytic stress studies, the samples of drug substance and drug product were exposed to sunlight during daytime for 4 days. Samples were withdrawn at appropriate time intervals and subjected to HPLC analysis after suitable dilution. Appropriate controls were also prepared and injected for each degradation study.

Results and Discussion

For RP-HPLC studies, chromatographic conditions were optimized to get best resolution and peak shape. The selection of mobile phase was based on peak parameters, (symmetry, theoretical plates and capacity factor) ease of preparation and cost. Symmetrical peaks with good separation (retention time for ARI 4.58±0.40 min and for PAR 1.2±0.20 min) was obtained with C_{18} column and mobile phase consisting of acetonitrile and methanol in the ratio of 35:65 v/v was used at a flow rate of 1mL min^{-1}. A typical chromatogram obtained from the analysis of drugs using the developed method is shown in Fig. 2.

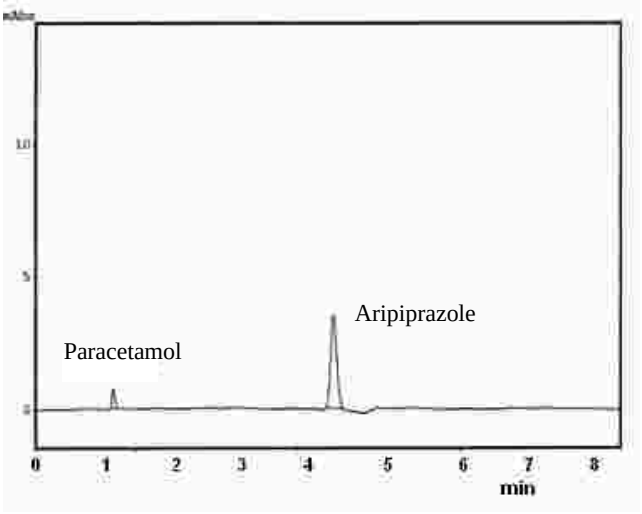


Fig. 2: Optimized Chromatogram of Paracetamol (10 µg mL^{-1}), Aripiprazole (0.2 µg mL^{-1}),

The optimum wave length for detection and quantification was 254 nm, at which good detector response for both drugs were obtained. There was no interference from the diluents and excipients present in the pharmaceutical formulation.

To check the linearity, standard calibration curve for the drug was constructed by plotting ratios of standard drug peak area to internal standard peak area vs. concentration of standard solutions and the curve showed good linearity over a concentration range of 10–100 µg mL^{-1}. The regression equations for the drugs were found by plotting peak area vs. concentration µg mL^{-1}. Table 1 summarizes the linearity range and the linear regression equation for the drug. The LOD and LOQ values of ARI were found to be 0.4 µg mL^{-1} and 10 µg mL^{-1} respectively.

The precision of the method was determined by repeatability (intraday) and intermediate precision (interday). Precision was expressed as the RSD of the results. The values obtained for the precision studies presented in Table I, indicate good repeatability and low interday variability.

The robustness of the proposed method was evaluated by slight modification in the organic composition of mobile phase and flow rate. During these studies, it was found that there was not much change in retention time, area and symmetry of peak.

Table 1: Summary of validation parameters for proposed methods

Parameters	HPLC method
Linearity	10-100 µg mL^{-1}
Linear regression	
Intercept (c)	0.0921
Slope (m)	18.7660
Correlation coefficient(r)	0.9993
Limit of detection (LOD)	0.4 µg mL^{-1}
Limit of quantification (LOQ)	10 µg mL^{-1}
Precision (% RSD)	
Intraday (n=3)	0.2644
Interday (n=3)	0.9443
Repeatability of injection (n=10)	0.5497

The recovery results in the range 97-100% show the accuracy of the method (Table 2).

Table 2: Recovery studies

Accuracy level (%)	% Recovery	% RSD
50	98.50	0.36
	98.52	
	99.12	
100	97.50	0.52
	98.52	
	97.95	
150	99.20	0.51
	98.52	
	98.22	

The developed method was used for the assay of commercially available tablets and six replicate determinations were performed. Experimental values obtained for the determination of tablets are given in Table 3. The interference of excipients was studied by comparing the chromatograms of standards and formulations. Similar

shapes and retention times of peaks showed that there was no interference from the excipients.

Table 3: Analysis of formulation

Labeled amount, mg tablet^{-1}	Amount found, mg tablet^{-1}	% RSD
10	9.899	0.12
	9.922	
	9.911	

The system suitability test of the chromatographic system was performed before each run. Five replicate injections of standards were made and peak asymmetry, theoretical plate number and RSD of peak areas determined. For all system suitability injections, asymmetry was < 1.5, theoretical plate number was > 5000 and RSD of peak areas was < 1%.

Degradation Studies

HPLC studies of samples obtained on stress testing of Aripiprazole under different conditions using Acetonitrile: Methanol (65:35% v/v) as mobile solvent system suggested the following degradation behavior.

Forced degradation studies

Acidic condition

The drug was subjected to 0.05 N HCl for 30 min at 100°C forming degradation products at retention time 1.01, 1.76, 2.71 and 4.5 min (Fig. 3b). The rate of hydrolysis in acid was faster as compared to that in alkali or water.

Alkaline condition

The drug was subjected to 0.05 N NaOH for 3 hrs at 100°C (Fig. 3c).

Neutral Condition

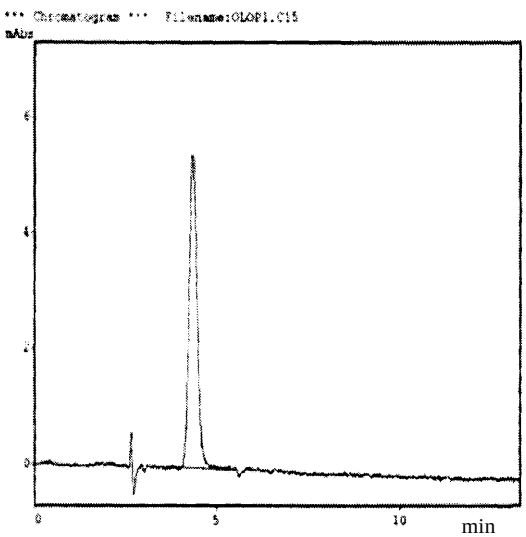
In neutral condition, significant degradation of the drug was achieved after heating the drug at 100°C for 8 hrs (Fig. 3d).

Oxidative studies

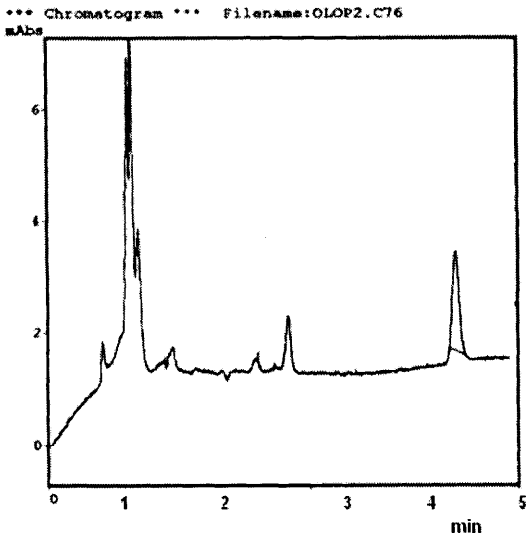
In oxidative condition, significant degradation of the drug was obtained after exposure to 6% H_2O_2 for 24 hrs (Fig. 3e).

Photolytic studies

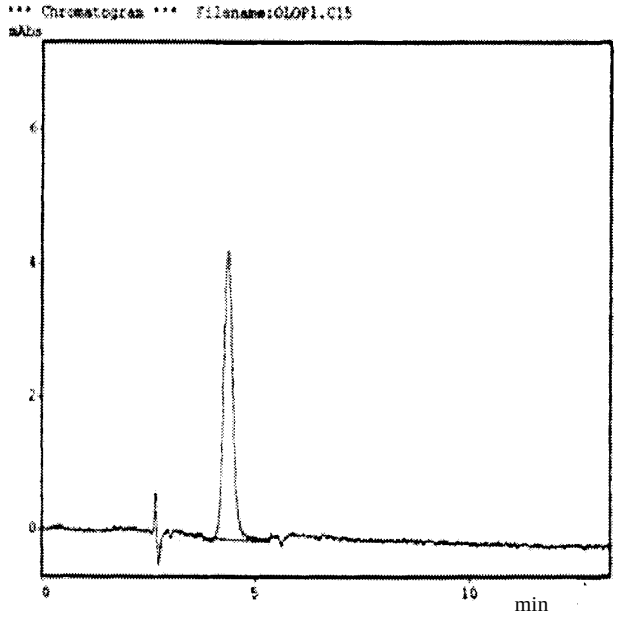
Sufficient degradation was achieved by exposing the drug product to sunlight during day time for 3.5 days (Fig. 3f). Degradation products were formed at retention times 1.4, 1.7 and 4.5 min.



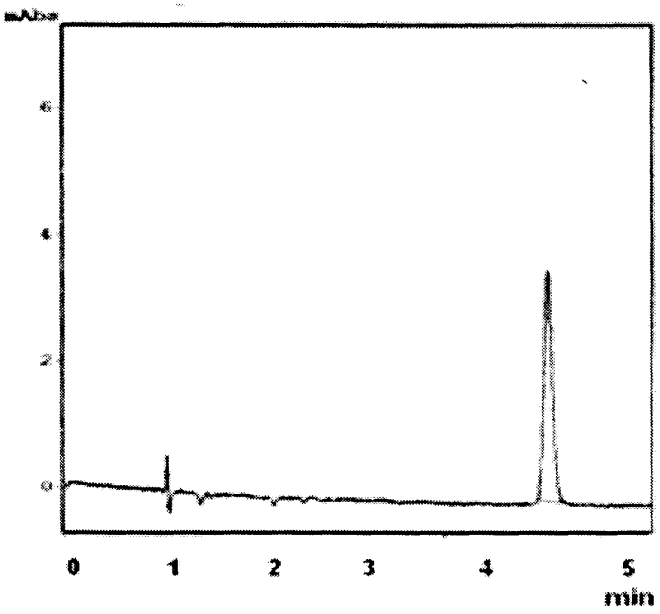
3a. Standard solution of Aripiprazole



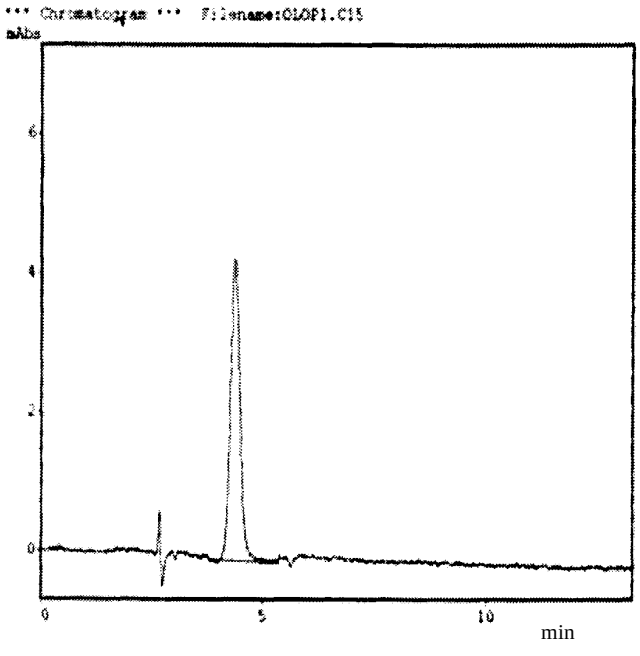
3b. Sample degraded in 3N Hydrochloric acid



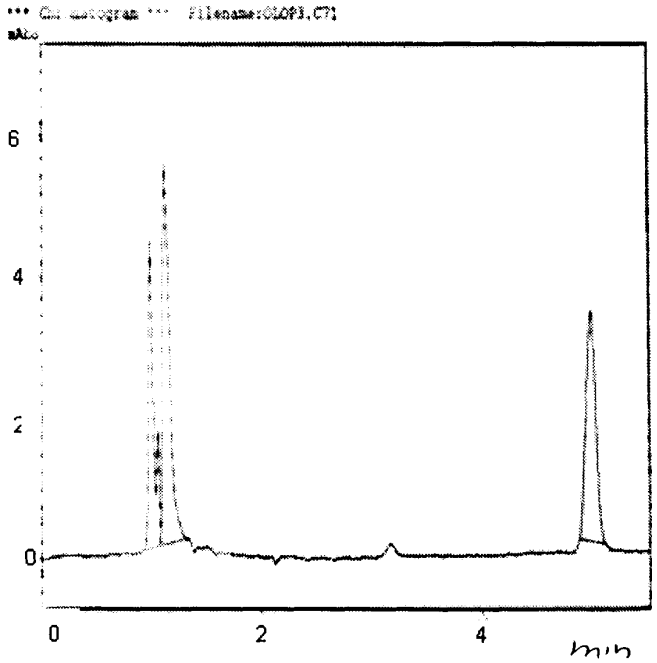
3c. Sample degraded in 3 N Sodium Hydroxide



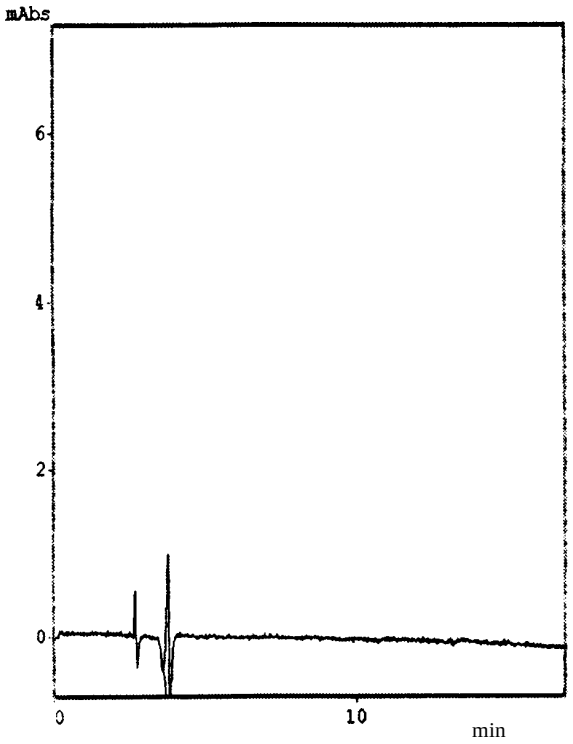
3d. Sample heated in water



3e. Sample subjected to Oxidative degradation



3f. sample subjected to photolytic degradation



3g. Blank

Fig. 3: HPLC Chromatograms of forced degradation samples of Aripiprazole

In all degradation studies except acid hydrolysis and photolytic studies, there was no corresponding formation of degradation products when compared to the standard solution of the drug. This indicated that, may be the drug degraded to low molecular weight non-chromophoric compounds. Singh and Bakshi[7] suggested a target degradation of 20-80 % for establishing the stability indicating nature of the assay method since intermediate degradation product should not interfere with any stage of drug analysis. The drug showed extensive degradation in acid hydrolytic condition. UV/Vis detection was used as an evidence of the specificity of the method and to evaluate the homogeneity of the drug peak. Chromatographic peak purity data was obtained from the spectral analysis report and peak purity index value of 1.0000 indicates a homogeneous peak thus establishing the specificity of the assay method. The results of degradation study are summarized in Table 4. Hence the developed HPLC method is stability indicating and can be used for routine analysis of Aripiprazole.

Table 4: Forced Degradation and Stability

Condition	Aripiprazole	
	% Assay	% Degradation
Acid	81.25	-18.75
Base	86.39	**-13.61**
Peroxide	90.18	-9.82
Photolytic	84.96	-15.04
Hydrolytic	85.15	-14.85

Conclusions

The methods developed showed no interferences from diluents like lactose, mannitol etc. and excipients like talc, magnesium stearate etc., and hence can be used for the routine analysis of Aripiprazole in its dosage forms.

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RNI No. MAHENG/2017/74063
ISSN No. (Print) 2581-5911

Ion selective electrode determination and removal of fluoride ions using starch derivatives as adsorbents from groundwater samples of Barmer District, Rajasthan, India

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Abstract
The evaluation of groundwater quality is a critical element in the assessment of water resources and this analysis becomes even more important when used for determining concentration of toxic anion, like fluoride. In the present investigation, firstly, samples of groundwater were collected from various sites of Barmer (an arid district of western Rajasthan). Fluoride ion concentrations were then determined in these samples using suitable ion electrodes and further the use of quarternary aminised resins from starch as adsorbents was evaluated using Freundlich adsorption isotherm for removal of fluoride.

Keywords: *Fluoride, determination, removal, starch, water analysis*

Introduction

Groundwater contamination by fluoride (F^-) results mainly from the dissolution of minerals from subterranean strata [1, 2].Though small amount of fluoride is vital for humans, it is toxic when present in large amounts. According to World Health Organization, the maximum acceptable concentration of fluoride in drinking water lies below 1.5 mg/L (WHO, 2004). When fluoride present is more than 1.5 mg/L, it causes mottling of teeth and lesions in thyroid gland, liver and other organs [3, 4]. The effect of prolonged use of fluoride containing water on human health is shown in Table-1.

Table 1: Effect of prolonged use of fluoride containing water on human health

F – mg/L	Health outcome
<0.5	Dental caries
0.5-1.5	Optimum dental health
1.5-4.0	Dental fluorosis
4.0-10.0	Dental and skeletal fluorosis
>10.0	Crippling fluorosis

The most common symptoms of chronic fluoride exposures are dental fluorosis and skeletal fluorosis. In dental fluorosis, the structural integrity of enamel is affected and small pits are left in teeth as it breaks away and in skeletal fluorosis accumulation of fluoride occurs in skeletal tissues.

The common fluoride bearing minerals found in soil are fluorspar (CaF_2), cryolite (Na_3AlF_6) and chilotite ($Na_5Al_3F_{14}$) [5].

Fluoride displaces hydroxide ion from hydroxyapatite, $Ca_5(PO_4)_3OH$, the prinicipal mineral constituent of teeth (enamel) and bones to form the harder and tougher fluoroapatite, $Ca_5(PO_4)_3F$. In small amouns, fluoride strengthens the enamel, however, at high concentrations the conversion of a large amount of hydroxyapatite to fluoroapatite makes teeth and bones (after prolonged intake) denser, harder and more brittle. This causes mottling and embrittlement of teeth (dental fluorosis) and after a prolonged exposure of fluoride, this progresses into skeletal fluorosis (Table-1). Fluoride is thus considered beneficial in drinking water at low concentrations (about 0.7 mg/L) but harmful when concentration exceeds 1.5 mg/L[6].

It was estimated (WHO, 1984) that more than 260 million people worldwide are consuming drinking water with fluoride content > 1.0 mg/L. The excess level of fluoride in drinking water is becoming a matter of great concern. This concern is more serious in countries where ground water is used as a main water supply resource.

Several defluoridation methods based on the adsorption, precipitation & ion exchange resins have been developed during last four decades, however, none of these could solve this problem at a completely acceptable level[7].

Considering the potential risks imposed on human health by presence of excess of fluoride ions in drinking water, the present study was carried out to determine fluoride contents in ground water samples from various blocks of Barmer district (Rajasthan, India) potentiometrically using ion selective electrodes and preliminary studies on application of quarternary aminised resins from starch as adsorbents for removal of fluoride were also carried out.

Material and Methods

All the reagents and chemicals used in this study are commercially available and are of high purity (AR grade). Analysis of fluoride was performed using fluoride ion selective electrode. Double distilled water (Millipore) was used throughout the experiments. Fluoride stock solutions (1000 ppm) were prepared from sodium fluoride and stored in polyethylene containers. Total ionic strength adjustment buffer (TISAB) solution containing 58 gm of NaCl, 57 ml of glacial acetic acid, 4 gm of 1,2- cyclohexanediamine –N,N,N',N'-tetraacetic acid (CDTA) and approximately 150 mL of 6 M NaOH in a volume of 1000mL (pH 5.0-5.5)[8,9] was used to regulate the ionic strength of samples and standard solution, to adjust the pH and also avoid interferences by polyvalent cations such as Al(III), Fe(III) and Si(IV) which are able to complex or precipitate with fluoride and reduce the free fluoride concentration in the solution. CDTA forms more stable complexes in solution with polyvalent metal cations than metal-fluoride complexes (AlF_6^{3-}, FeF_6^{3-}, etc.). CDTA forms metal-CDTA complexes in a metal-ligand ratio of 1:1 [10], freeing the fluoride ion. The electrode is selective to fluoride ion over other common anions by several orders of magnitude and only the hydroxide ion appears to offer some interference.

The adsorbents used in this study are cross linked starch derivatives, namely GlyscidylTrimethylAmmonim Chloride (GTMAC) GlyscidylTriethyl Ammonium Chloride (GTEAC) and diethyl amino-ethyl- starch (referred hereafter as GTMAC-, GTMEC- & DEAE- Starch respectively). These were prepared by mixing cross linked starch and GTMAC, GTMEC and DEAE in the ratio of 2:1 in chlorine form. The mixture was kept at 60°C with constant stirring for two hrs. The products were brought to room temperature where they were kept for 6-8 hours and then filtered. The physical properties of starch derivatives viz. degree of substitution, particle size, water regain, salt splitting capacity, exchange capacity, swelling and stability were evaluated by known methods. These parameters are reported in Table 2.

Table 2: Physical properties of starch derivatives

S.No.	Derivatives	Particle Size %	Mesh	Degree of Substitution %	Water Regain g/g	Salt Splitting Capacity Meg/g	Weak Exchange Capacity
1.	GTMAC-S	60	40-60	0.27	2.26	0.62±0.04	0.25+0.05
		40	>60				
2.	GTMEC- S	60	40-60	0.27	2.32	0.34±0.02	0.21+0.05
		40	>60				
3.	DEAE- S	60	40-60	0.23	2.42	0.29±0.03	0.20+0.07
		40	>60				
Stability: 6-8 weeks at pH 3- 10 Swelling in water: slight							

About 27 ground water samples were collected from different blocks/areas of Barmer district Rajasthan, India. All the samples were collected from deep-wells and hand-pumps. These samples were transported to Environmental Laboratory, Department of Chemistry, J.N.V. University, Jodhpur where fluoride analysis was performed immediately.

The development of ion-selective electrodes (ISE) using electrode membranes makes possible the determination of the concentration of ions of choice, greater selectivity and sensitivity. Therefore, ion selective electrodes were used to determine fluoride concentrations in ground water samples. The samples and fluoride standard solutions were diluted 1:1 with TISAB. The solution containing 20 mL of TISAB and 20 mL of sample was stirred using a magnetic stirrer for 5 min.

The batch technique was selected to obtain the equilibrium and kinetic data. The uptake of fluoride on the adsorbent was estimated in terms of distribution coefficient or adsorption capacity, K_d. The derivatives (0.04-0.2 g) were stirred with 100 mL of 0.02 g dm^{-3} aqueous solution of samples at pH 7-8, in skew cap jars until complete equilibrium was obtained. The agitation was continued for 30 min. at ambient temperature (30 ± 2°C). The equilibrated solutions were shaken in a thermostat shaker at speed of 300 rpm. The adsorbent was then removed by filtration through glass wool and the residual [F^-] in the solution was determined using ion selective electrodes.

Results and Discussions

The fluoride concentrations in ground water samples collected from different locations are shown in Table 3. Figure 1 indicates the variation for fluoride ion concentrations at various sampling sites.

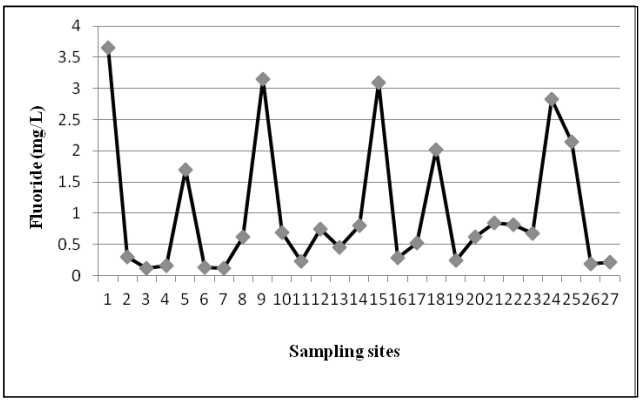

Fig. 1: Variation in Fluoride ion concentration (mg/L) at various sampling sites of Barmer district, Rajasthan, India

Table 3: Fluoride concentrations in ground water samples from various locations in Barmer District, Rajasthan, India

Sample location	Sample location	F^- concentration (mg /L)
S1	Sarwari	3.65
S2	Tarsinghri	0.306
S3	Gharsikabara - 1	0.120
S4	Gharsikabara -2	0.161
S5	Pataukala	1.69
S6	Pataukhurd	0.136
S7	Kuri	0.119
S8	Bhandiyawas	0.622
S9	Pachpadra	3.15
S10	Bithuja	0.692
S11	Bhagundi	0.228
S12	Akarlibakshiram	0.740
S13	Sambhara	0.448
S14	Lakhekiberi	0.802
S15	Khara par	3.10
S16	Tilwara	0.282
S17	Kher temple	0.516
S18	Badu kabara	2.02
S19	Kudi	0.250
S20	Bhandiyawas -1	0.622
S21	Bhandiyawas -2	0.843
S22	Bhanduyawas-3	0.822
S23	Borawas	0.680
S24	Bhagundi	2.83
S25	Khara par -1	2.15
S26	Khara par-2	0.194
S27	Kuri deep well	0.215

The analysis of ground water samples collected from different areas of Barmer district revealed that out of 27 samples, high fluoride level was obtained at Sarwari, Patau Kala, Pachpadra, Khara Par and Bhagundi sampling areas. The water of these regions probably passes through soil or rock layers rich in fluoride in comparison to other regions. The concentration of fluoride in ground water depends on various factors such as geological, chemical, consistency of soil, porosity of rocks, pH, temperature and depth of wells.

Some locations like Tarsingri, Gharsika Bara, PatauKhurd and Kuri had insufficient fluoride content with an average of about of 0.168 mg/L.

The efficiency of starch derivatives for the removal of F^- from water was evaluated using Freundlich isotherm. The linear plot of log Q_e *vs* log C_e indicated that all the starch derivatives had good affinity for fluoride removal and order of adsorption for fluoride ions towards the derivatives was GTMAC-S > GTMEC-S > DEAE-S.

The constant values of K_d and 1/n are a measure of adsorption capacity and adsorption intensity, respectively.

These constants are given in Table 4. Values of 1/n lying between 0 and 1 and values of n lying in the range of 1-10 confirmed the favorable conditions for adsorption [10].

The results also indicate that the K_d values increase with the decrease in pH of solution, which may be attributed to the neutralization of surface negative charge of starch derivatives[11].

Adsorption Isotherms (AI) for fluoride ions on starch derivatives when the system was in equilibrium were found to be regular, positive and convex to the concentration axis followed by a plateau and another rise and formation of plateau. The sharp rise of isotherms in the initial stages of low equilibrium concentration indicated that there are plenty of readily available sites for fluoride ion. Eventually a plateau was reached in all the three cases, which indicated the saturation of adsorbent surface at this stage. Further rise in the adsorption isotherms and the formation of another plateau probably indicates mono dispersity of adsorption accompanied by the formation of small sized ionic micelles on the surface. A similar behavior has been observed in the adsorption of merocynine over silver halides[12, 13].

The experimental data was analyzed in the light of Freundlich equation[14] to predict the nature of adsorption. The plots of log C_e against log x/m were found to be linear yielding an intercept on the ordinate. The Freundlich constants K_d and1/ n were calculated from the intercept and slope of these curves using computerized LRG program and reported in Table 4. The extent of adsorption, n**,** was found in the range of 2.52 to 3.1, which is quite satisfactory.

Table 4: Freundlich constants for the adsorption of fluoride ion on starch derivatives

[[F^-] = 0.02 g dm^{-3}			Time of Contact = 30 min				
Adsorbent = 0.15 g			Temperature = 30°C				
Volume = 0.1 dm^3			pH = 8				
Adsorbent	**Freundlich Constants**						
	Average K_a	**K**	**n**	**1/n**	**SD**	**R^2**	**r**
GTMAC- S	2135±50	3.459×10^{-3}	2.52	0.396	1.06×10^{-2}	0.99	0.99
GTMEC-S	1580±70	2.887×10-3	2.60	0.385	2.15×10-2	0.99	0.97
DEAE-S	1782±50	2.286×10^{-3}	3.10	0.322	0.86×10^{-2}	0.98	0.99

The mechanism of adsorption may be considered as a mixed effect of classical as well as of chromatographic adsorption. The introduction of glycedyltrialkyl ammonium groups in the starch derivatives imparts it a strongly cationic anion exchanger character[15]. Initially all the Cl^- ions of the GTMAC-Starch were exchanged by F^- ions with the formation of the first plateau after the saturation point was reached i.e. the capacity of ion exchanger was exhausted the F^- ions were then held by hydrogen bonding and weak VanderWaal's forces showing the rise in adsorption isotherm and the formation of another plateau.

$[R_1\text{-O-CH}_2\text{-CHOH-CH}_2\text{-N}^+R_3]Cl^- + F^- = [R_1\text{-O-CH}_2\text{-CHOH-CH}_2\text{-N}^+R_3]\ F^- + Cl^-$

where R_1 stands for cross linked starch and R for methyl or ethyl groups.

Starch is basically a storage polysaccharide of plants which acquires a negative surface charge by virtue of primary hydroxyls on each D-glucopyranose units. The alkaline treatment of starch, during the introduction of quaternary ammonium group, causes degradation of about 2% with the loosening of the structure and the introduction of some acidic sites which do contribute to

negative charge[16]. In acidic media, the primary hydroxyls on the D-glucopyranose units may get protonated acquiring a partial positive charge which helps the fluoride ions to move in the vicinity of the starch molecule where hydrogen bonding comes into play and the fluoride ion is held by hydrogen bonding, resulting in the increased adsorption after exhausting the anion exchange capacity.

Conclusions

Salient features of the presented research work are:

1. The research work has provided empirical data about F^- ions in ground water of the Thar desert regions of Barmer district, which indicates that ground water of some areas of Barmer district is not suitable for drinking purpose and therefore proper management plans must be adopted to control F^- ion concentrations.
2. The present study demonstrates that cationic starch derivatives have potential to remove fluoride from drinking water without any other toxic affect.
3. The uptake of fluoride ion is rapid in initial stages and decreases gradually while approaching equilibrium.

Acknowledgement

The author is thankful to UGC New Delhi, India for providing financial grant in the form of Start up Grant project (BSR).

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RNI No. MAHENG/2017/74063
ISSN No. (Print) 2581-5911

Synthesis, characterisation and antimicrobial activities of copperII mixed ligand complexes

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Abstract
A series of six mono nuclear Schiff-base complexes, namely $[CuL1NO_3]NO_3$ 1, $[CuL2NO_3]NO_3$ 2, $[CuL1phen]NO_{32}$ 3, $[CuL1bpy]NO_{32}$ 4, $[CuL1pybiH]NO_{32}$ 5 and $[CuL1dpk]NO_{32}$ 6 where L1 and L2 are tridentate N,N,N Schiff-base ligand; L1 = N,N-dimethyl-N'-[phenylpyridin-2-ylmethylene]ethane-1,2-diamine, L2 = N-[phenylpyridine-2-ylmethylenepyridin-2-yl]methanamine, phen = 1, 10-phenanthroline, bpy = 2, 2'-bipyridinyl, pybim = 2-pyridin-2-yl-1H-benzoimidazole and dpk = di-pyridin-2-yl-methanone were synthesized and characterized by elemental analysis, UV-Vis, IR and EPR spectroscopy. In the complexes 3, 4, 5 and 6 the metal ion is coordinated by three nitrogen atoms from L1 or L2 ligand and two nitrogen atoms from the bidentate auxiliary ligand and have approximately square pyramidal coordinate geometry. In Complexes 1 and 2, the metal ion is coordinated by three nitrogen atoms from the Schiff base ligand and two oxygen atoms from nitrate anion. The in-vitro antimicrobial activity of the complexes were tested against the bacteria Escherichia coli, Staphylococcus aureus, Bacillus subtilis, fungi Candida albicans and Aspergillusflavus by disc diffusion method. All the copper II complexes show moderate antimicrobial activity.

Keywords: *Copper(II), tridentate, Schiff-base ligand, ESR, antimicrobial activity*

Introduction

Schiff bases are well known organic compound which are widely used as dyes, pigments, catalysts, intermediates in organic synthesis and polymer stabilizers[1-3]. Schiff bases and their metal complexes are considered as promising class of compounds due to their physical and chemical properties. Interestingly Schiff base ligands are able to coordinate to different metal ions and are stabilized in various oxidation states[4]. Because of the relative ease of synthesis and flexibility and the special property of azomethine group -HC=N-, Schiff bases are considered as excellent chelating agents and are known to form potentially stable complexes with metal ions. Different types of Schiff base based metal complexes have been extensively studied due to their potential anticancer[5], antiviral[6] and catalytic properties[7]. Metal complexes of Schiff bases derived from heterocyclic compounds containing N, S and O as ligand atoms are of great interest for simple structural models of complicated biological systems[8-10]. Metal complexes with mixed ligands of current interest due to their structural diversity. Further, coordination chemistry is about tuning of properties of metal ions using different ligands[11-13]. This

includes stabilization of different oxidation states, modulation of the solubility and its photophysical properties. In the present work, the structures of synthesized bidentate ligands and its copper complexes have been characterized by various physicochemical techniques including elemental analysis, IR and EPR spectral studies, and were also screened for antimicrobial activities against Gram-positive bacteria: Staphylococcus aureus MTCC 96, Bacillus subtilis MTCC 441, Gram-negative bacteria: Escherichia coli MTCC 119 and Fungai as Candida albicans MTCC 227, Aspergillusflavus MTCC 230.

Materials and Methods

All the chemicals and reagents used were of analytical grade and were used as received without further purification. Solvents were dried and distilled according to standard literature procedures[14]. Phenyl-pyridin-2-yl-methanone, 2-pyridin-2-yl-1H-benzoimidazole, di-pyridin-2-yl-methanone were obtained from Sigma Aldrich. N,N-dimethylethylenediamine, 2-aminomethyl pyridine, 1,10-phenanthroline, 2,2'-bipyridine, copper nitrate trihydratewere purchased from Alfa Aesar. Elemental analyses were carried out with a Perkin-Elmer 2400 II elemental analyzer. The electronic spectra were measured on a Perkin-Elmer Lambda-40 UV-Vis spectrophotometer. Infrared spectra were recorded on a Perkin-Elmer Spectrum RX FTIR instrument in the range of 4000-350 cm^{-1} as KBr pellets. Conductivity measurements wereconducted using a Lab India, PICO conductivity meter.ESR spectra of the copper complexes in DMSO at liquid nitrogen temperature 77 K were recorded on a JEOL JES-TE100 ESR spectrometer operating at X-band frequencies and having a100 kHz field.

Synthesis of Ligand
N,N-dimethyl-N'-[phenyl2-pyridyl methylene]ethane - 1,2-diamine (L1.)

The ligand was synthesized following a reported procedure[15]. A solution of 2-benzoylpyridine 0.183 g, 1.0 mmol in methanol 20 mL was added dropwise to a solution of N,N-dimethylethylenediamine 0.089 g, 1.0 mmol in methanol. After completion of the addition the reaction mixture was refluxed for 3 hours and then allowed to cool. The solvent was removed in a rotary evaporator to give the compound as a yellow oily liquid which was used without further purification.

Phenyl-pyridin-2-yl-methylene-pyridin-2-ylmethyl-amine (L2.)
This ligand was prepared by adopting the procedure used for the isolation of L1 but using 2-aminomethyl pyridine instead of N,N-dimethylethylenediamine.

Synthesis of Complexes

Synthesis of $[CuL1NO_3]$ NO_3 (1.)
A methanolic solution 10 mL of copperII nitrate trihydrate 0.241 g, 1 mmol was added to a solution of L1 0.253 g, 1 mmol in methanol with constant stirring. The blue colored precipitate obtained was washed with cold methanol and diethyl ether and dried under vacuum. Yield: 70 %. Anal.calcd for $CuC_{16}H_{19}N_5O_6$: C, 43.59; H, 4.34; N, 15.88 %. Found: C, 43.50; H, 4.40; N, 15.95 %. ESI-MS m/z: = 378.89 $[M\text{-}NO_3]^+$.

Synthesis of $[CuL2NO_3]$ NO_3 (2.)
The solution of copper nitrate trihydrate 0.241 g, 1.0 mmol in methanol 20 mL was mixed with the solution of ligand L2 0.273g, 1.0 mmol in ethanol20 mL and the mixtures was refluxed for 30 minutes and then cooled. The precipitate were filtered and washed with ethanol and then dried in a vacuum over anhydrous $CaCl_2$. Yield: 68 %. Anal. calcd for $CuC_{18}H_{15}N_5O_6$: C, 46.91; H, 4.28; N, 15.20 %. Found: C, 46.80; H, 4.30; N, 15.25 %. ESI-MS m/z: = 398.10 $[M\text{-}NO_3]^+$.

Synthesis of $[CuL1phen]NO_{32}$ (3.)
A methanolic solution 10 mL of copperII nitrate trihydrate 0.370 g, 1 mmol was added to a solution of L1 0.253 g, 1 mmol and phen 0.198 g, 1 mmol in methanol with constant stirring. The blue colored precipitate obtained was washed with cold methanol and then diethyl ether and dried under vacuum. Yield: 68 %.

Anal. calcd for $CuC_{28}H_{27}N_7O_6$: C, 54.15; H, 4.38; N, 15.79 %. Found: C, 54.10; H, 4.34; N, 15.75 %. ESI-MS m/z: = 558.15 $[M\text{-}NO_3]^+$.

Synthesis of $[CuL1bpy]NO_{32}$(4.)

This complex was prepared by adopting the procedure used for the isolation of 3 but using 2,2'-bipyridineinstead of 1,10-phenanthroline. Yield: 65 %. Anal.calcd for $CuC_{26}H_{27}N_7O_6$: C, 52.30; H, 4.56; N, 16.42 %. Found: C, 52.25; H, 4.40; N, 16.54 %. ESI-MS m/z: = 534.15 $[M\text{-}NO_3]^+$.

Synthesis of $[CuL1pybim]NO_{32}$(5.)

This complex was prepared by adopting the procedure used for the isolation of 3 but using 2,2'-bipyridyl benzimidazoleinstead of 1,10-phenanthroline. Yield: 67 %. Anal. calcd for $CuC_{28}H_{28}N_8O_6$: C, 52.87; H, 4.44; N, 17.62 %. Found: C, 52.92; H, 4.50; N, 17.70 %. ESI-MS m/z: = 573.15 $[M\text{-}NO_3]^+$.

$[CuL1dpk]NO_{32}$(6.)

This complex was prepared by adopting the procedure used for the isolation of 3 but using di-pyridin-2-yl-methanone instead of 1,10-phenanthroline. Yield: 77%. Anal. calcd for $CuC_{27}H_{27}N_7O_7$: C, 51.88; H, 4.35; N, 15.69%. Found: C, 51.70; H, 4.30; N, 15.75 %. ESI-MS m/z: = 562.15 $[M\text{-}NO_3]^+$.

Antimicrobial Screening

The standard microbial stains employed were Gram-positive bacteria: *Staphylococcus aureus* MTCC 96, *Bacillus subtilis* MTCC 441, Gram-negative bacteria: *Escherichia coli* MTCC 119 and Fungai as *Candidaalbicans* MTCC 227, *Aspergillusflavus* MTCC 230 and were obtained from Microbial type culture collection MTCC at the Institute of Microbial Technology IMTECH, Chandigarh, India.The antibacterial activities of all the copper complexes were screened in vitro for growth inhibition against *Staphylococcusaureus, Bacillus subtilis* and *Escherichia coli* using disc diffusion method, at different concentrations compared with Chloramphenical as a positive control. Then the anti-fungal activities of copper complexes **1-6** were investigated against *Candida albicans, Aspergillusflavus* by the same method as stated above but with Fluconazole as a positive control.

Results and Discussion

Synthesis and Characterisation

The ligands L1, L2 were synthesized using the reported procedure with slight modifications, by condensing 2-benzoyl pyridine with N,N-dimethyl ethylenediamine and 2-amino methyl pyridine respectively. The Schiff base ligands readily formed complexes on treating of one equivalent each of copperII nitrate and corresponding co-ligands in methanol at room temperature.Mononuclear and mixed-ligand copperII complexes of the types $[CuLNO_3]NO_3$ and $[CuLdiimine]ClO_{42}$, where L is the tridentate ligand L = N,N-dimethyl-N'-[phenyl2-pyridyl methylene]ethane - 1,2-diamine L1, Phenyl-pyridin-2-yl-methylene-pyridin-2-ylmethyl-amine L2 and dimine is 1,10-phenanthroline phen, 2,2'-bipyridine bpy, 2-pyridin-2-yl-1H-benzoimidazole pybim, di-pyridin-2-yl-methanone dpk, have been isolated as their nitrate salts in good yields. All the complexes were obtained in good yield and characterized by using elemental analysis, UV-Vis, ESI-MS and EPR spectroscopic techniques. The molar conductance 90 W^{-1} cm^2 mol^{-1} of the complexe suggests that it behaves as 1:1 electrolyte in solution[16].

Electronic spectral properties

The electronic spectral data of all the Cu(II) complexes is summarized in Table 1 and the typical electronic absorption spectra of **3-6** are shown in Figure1. An intense band in the range 275-310 nm has been assigned to Np®CuII ligand to metal charge transfer LMCT transition[17] suggesting involvement in coordination of the diimine nitrogen atoms even in solution. Further, the complexes exhibit only one broad band in the visible region 635-690 nm, which is typical of a distorted square-based coordination geometry[18] around copper(II). The broadness associated with the d-d bands is generally taken as an indication of the geometrical distortion of the complex from perfect planar symmetry.

IR Spectra

The important infrared spectral bands and their assignments for the synthesized complexes were recorded as KBr pelletsare shown in Table 2. IR spectrum of Complex 2 is shown in Figure 2. The IR spectra of all the compoundsshow =C–H stretching vibration, due to the aromatic ring around 3040 cm^{-1}, while the –C–H stretching vibration due to the CH_3 group appeared around 2865 cm^{-1}. The peak around 1590-1605 cm^{-1} in the complexes indicate the presence of azomethine C=N stretching frequency. In Complex 5, the peaks observed at 1603 and 1624 cm^{-1} have been assigned to the C=N stretching frequencies of benzimidazole group. The peaks corresponding to the ring stretching frequencies uC=C and uC=N at 1515-1520 cm^{-1} and 1445-1430 cm^{-1} indicate the coordination of the heterocyclic nitrogen atoms of phen and bipy with the metal ion[19].

EPR Spectra

The EPR spectra of complexes **1-6** in DMSO at 70 K were found as anisotropic and their hyperfine features have been resolved into both parallel $g_{\|}$ and perpendicular $g_{¥"}$ regions Figure3, Table 1. The EPR spectra of all the complexes are axial with the range of $g_{\|}$ 2.20 - 2.38 > $g_{\wedge}$ 2.04 - 2.08 suggesting the presence of unpaired electron on $d_{x^2-y^2}$ orbital with $^1B_{2g}$ ground state[20,21]. Thus, highest-energy half-occupied d-orbital is $d_{x^2-y^2}$ because its lobes point directly at the ligands and thus it has the largest repulsive or antibonding interaction with the ligand field[22,23]. All the complexes exhibit $g_{\|} > g_{\wedge}$with f-values of range 120-136 cm^{-1}, which are closer to the range of f-value observed for square-based geometries 105-135 cm^{-1}[24]. This is further supported by the large $A_{4\%}$ values in the range of 163-186 ´ 10^{-4} cm^{-1}. The large hyperfine splitting in the $g_{\|}$ region and $A_{\|}$, 172-193 ´ 10^{-4} cm^{-1}, suggesting the presence of square based geometry[25]. This hyperfine coupling $A_{\|}$ of the unpaired electron to the nuclear spin of the copperII center is contributed from Fermi contact, spin dipolar and orbital dipolar[26]. The observation of larger g and lower A values suggests the coordination of an axial ligand and more delocalization of charge into the ligand orbitals due to the formation of a square pyramidal structure[27]. Interestingly, the present complexes **1-6** exhibit very similar solution EPR parameter $g_{\|}$, 2.20-2.38; $A_{\|,}$ 172-193 10^{-4} cm^{-1} as reported in literature[28].

Antimicrobial Activity

In vitro antibacterial screening, effects of the investigated Copper(II) complexes **1-6** were tested against species *S. aureus, B. subtilis* and *E. coli* by disc diffusion producer[29,30]. The observed activity values for copper II complexes are given in Table 3. These complexes showed more activity with 5-15 mm inhibition at different concentrations. However, the complexes showed similar activity as that of standard drug Chloramphenical. Antifungal activity of the all Copper(II) complexes was tested against *C. albicans* and *A. flavus* (Table 4.) These complexes indicated higher activity with 6 - 14 mm inhibition at different concentrations. All complexes show considerable biological activity at higher concentration 150 µL against microorganisms and the development of the complexes can be explained with the help of Tweedy's chelation theory[31] and Overtone's concept[32]. The result shows that the Copper(II) complexes inhibit the development of microorganism by stopping the formation of protein and thus decelerating the respiration process. This inhibitory growth of bacteria and fungi may be explained on the basis of the structure due to the increase in liposolubility of the molecules across the cell membranes of the microorganisms.

Conclusions

We have successfully synthesized six new mononuclear mixed ligand Copper(II) complexes and characterised them by physicochemical and spectroscopic methods. The analyses confirmed that the copper metal ion coordinated with three nitrogen atoms from the primary tridentate Schiff bases and two nitrogen atoms from the auxiliary ligands. The geometry of the complexes is assigned as square based pyramidal structure. All the complexes show moderate antimicrobial activity.

Acknowledgement

The authors are greatful to the Principal, Department of Chemistry, National College, Tiruchirappalli, India for laboratory facilities and Head, SAIF, IIT Chennai, India for EPR measurements.

Table 1: Electronic absorption and EPR spectral propertiesa of CopperII complexes 1-6.

S.No.	Complex	UV-Vis	EPR spectra in frozen dimethyl sulfoxide glass at 77K		
			$g_{\|}$	$g_{\perp}$	$A_{\|}$
1	[CuL1NO_3] NO_3	648, 315, 228	2.38	2.07	172
2	[CuL2NO_3] NO_3	650, 319, 210	2.20	2.05	180
3	[CuL1phen] NO_{32}	692, 272, 226	2.28	2.08	188
4	[CuL1bpy] NO_{32}	634, 296, 216	2.26	2.06	180
5	[CuL1pbiH] NO_{32}	645, 307, 214	2.29	2.06	193
6	[CuL1dpk] NO_{32}	636, 272, 209	2.21	2.04	187

Table 2: IR spectral properties of Copper(II) complexes 1-6.

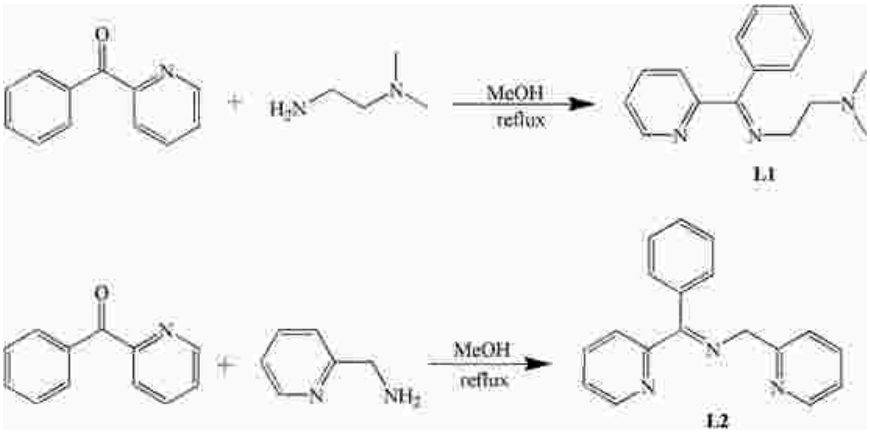
S.No.	$\bar{\upsilon}$ C=N ring	$\bar{\upsilon}$ C=N	$\bar{\upsilon}$ Cu-N	$\bar{\upsilon}$ C-H ring	$\bar{\upsilon}$ C-H	$\bar{\upsilon}$ NO_3
1	1597	1656	540	3061	2979	1364, 943
2	1486	1607	589	3014	2401	1383,946
3	1587	1627	645	3056	2400	1365
4	1442	1599	651	3065	2808	1356
5	1447	1602	570	3063	2910	1300
6	1760	1609	621	3153	2411	1377

Table 3: Antibacterial activity of Copper(II) complexes 1-6.

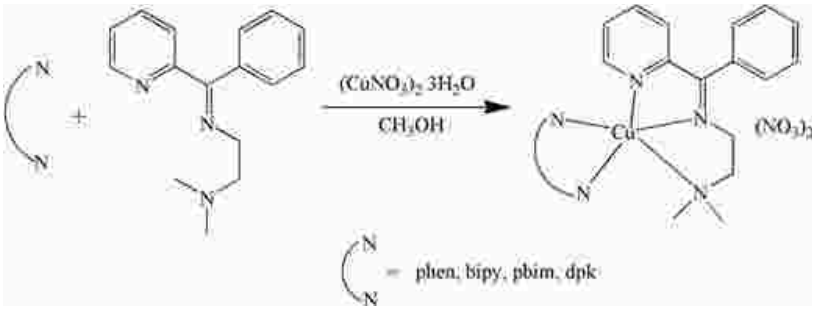
Compound	Zone of inhibition in mm														
	Escherichia coli					*Staphylococcus aureus*					*Bacillus subtilis*				
	Control 30 µL	Standard 30 µL	50 µL	100 µL	150 µL	Control 30 µL	Standard 30 µL	50 µL	100 µL	150 µL	Control 30 µL	Standard 30 µL	50 µL	100 µL	150 µL
1	0	13.4	8.3	9.7	11.2	0	13.8	9.7	11.9	13.3	0	13.5	9.2	11.1	12.4
2	0.7	12.6	7.9	9.7	12.8	0.4	12.3	5.7	8.3	12.5	0.6	12.7	6.4	8.6	10.5
3	0	14.8	9.7	11.4	13.6	0	14.2	10.3	12.8	13.6	0	13.4	10.9	11.6	12.7
4	0	13.6	8.8	10.2	13.7	0	13.4	8.9	10.4	13.5	0	14..1	8.6	10.9	12.6
5	0	14.2	9.1	11.3	13.9	0	12.9	9.6	11.7	12.5	0	14.4	9.3	11.2	13.1
6	0	13.7	9.4	10.8	13.3	0	13.6	8.7	10.9	12.9	0	13.8	8.5	10.7	12.9

Table 4: Antifungal activity of Copper (II) complexes 1-6.

Compound	Zone of inhibition in mm									
	Candida albicans					*Aspwegillusflavus*				
	Control 30 µL	Standard 30 µL	50 µL	100 µL	150 µL	Control 30 µL	Standard 30 µL	50 µL	100 µL	150 µL
1	0	12.9	7.3	10.7	11.4	0	14	9.2	12.4	13.2
2	0.2	13.1	7.9	9.8	12.5	0.3	12.3	8.3	10.5	11.1
3	0	14.7	8.9	11.1	13.2	0	14.1	9.5	12.2	13.8
4	0	13.4	8.5	9.2	11.7	0	13.9	9.1	11.7	12.5
5	0	13.2	8.1	9.6	11.3	0	13.6	8.9	10.4	11.9
6	0	12.3	6.8	8.9	10.7	0	13.5	9.2	11.6	12.3



Scheme 1: Synthesis of ligands



Scheme 2: Synthesis of complexes

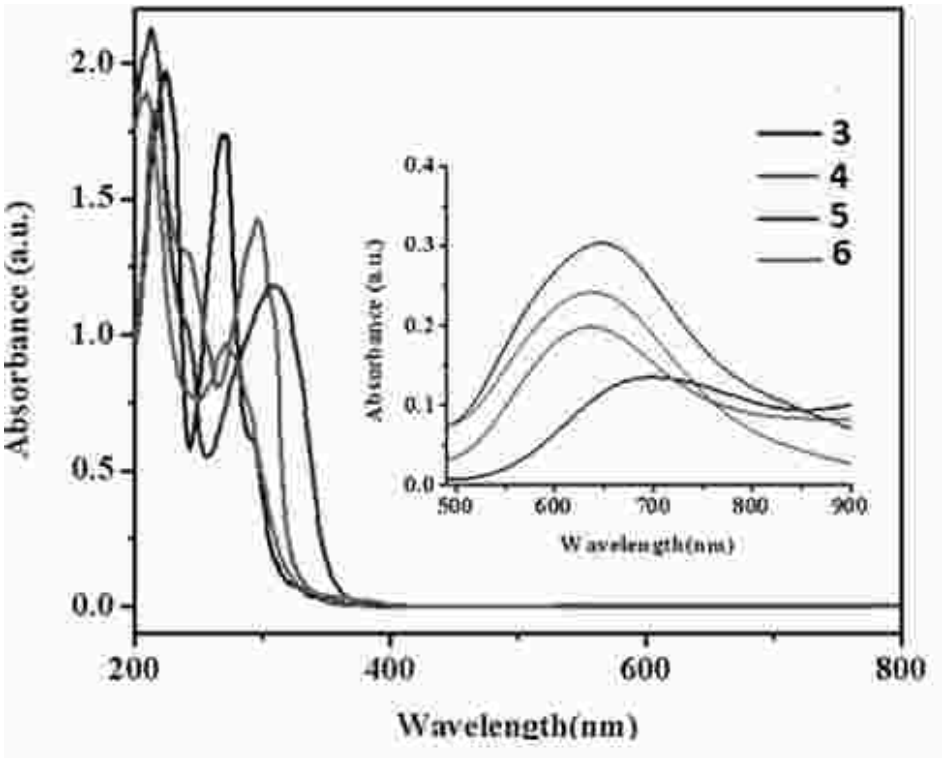


Fig 1: Absorption spectra of Complex 3-6 in acetonitrile.

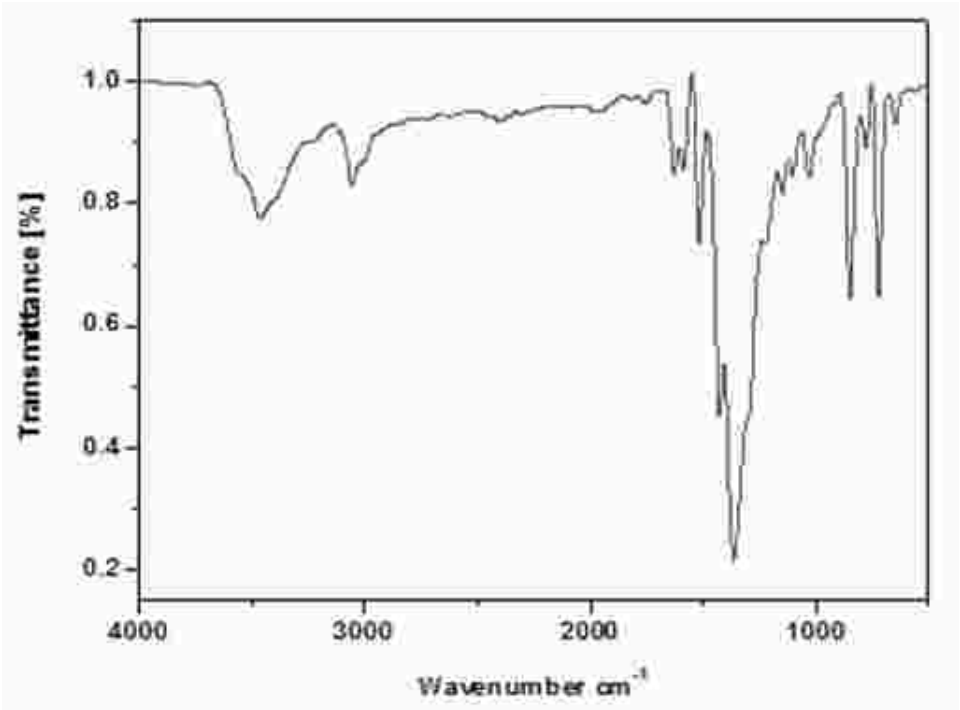


Fig 2: IR spectrum of Complex 2.

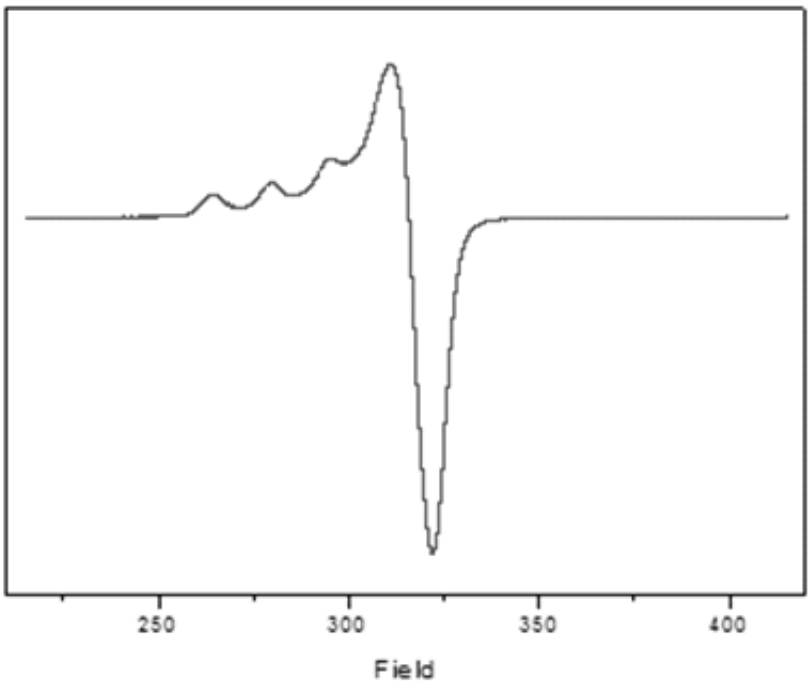


Fig 3: X-band EPR spectrum of Complex 3.

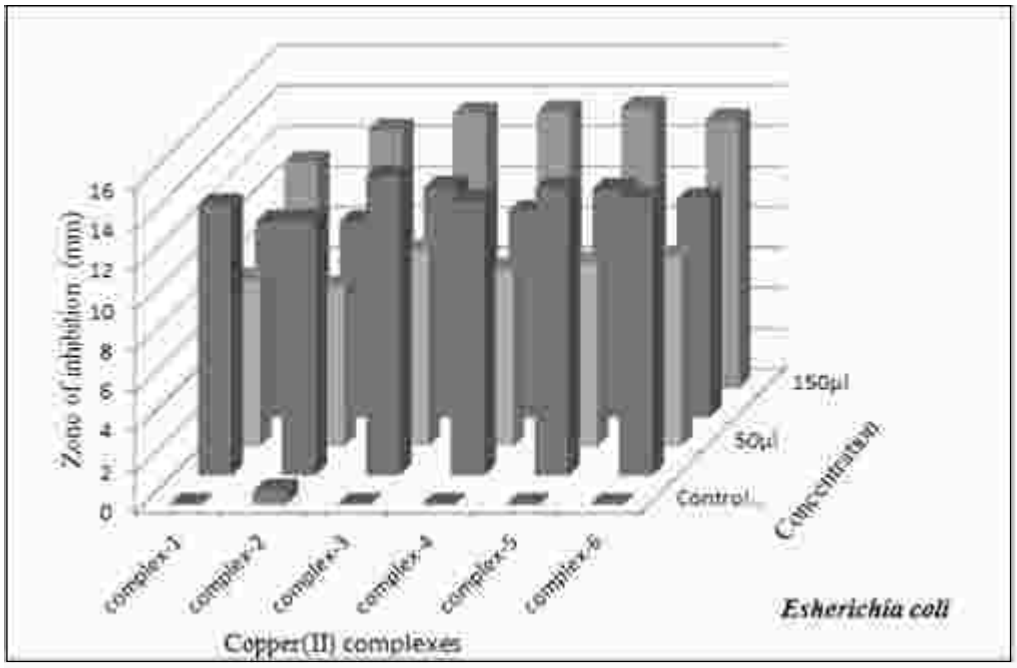


Fig 4: Biological bacterial activity of Copper(II) complexes 1-6.

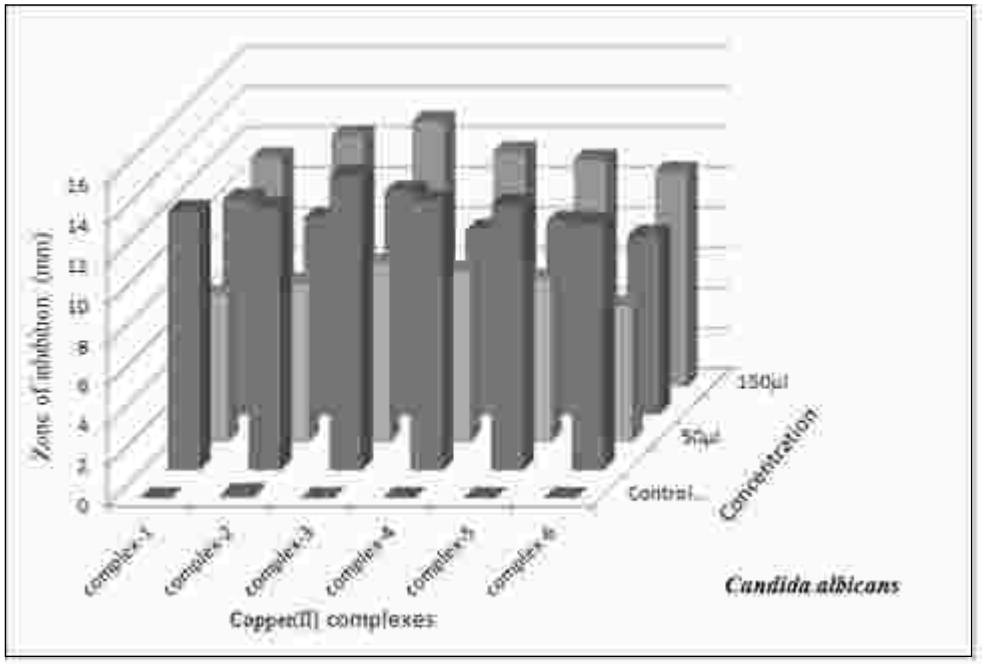


Fig 5: Biological fungal activity of Copper(II) complexes 1-6.

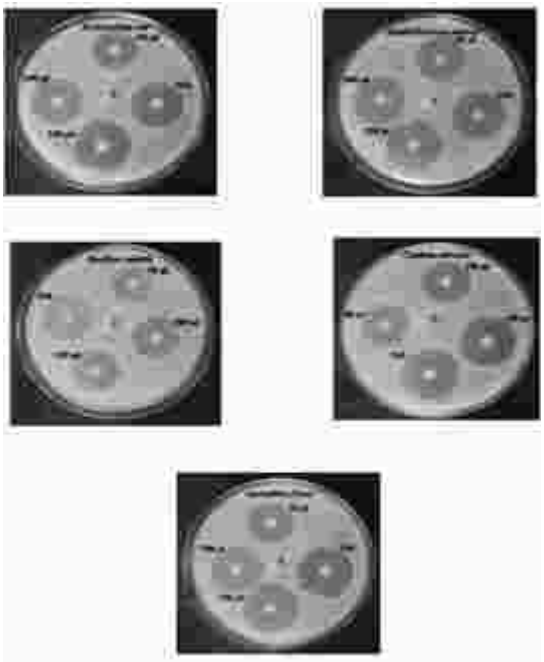


Fig 6: Zone of inhabitation against Gram-positive bacteria: Staphylococcus aureus MTCC 96, Bacillus subtilis MTCC 441, Gram-negative bacteria: Escherichia coli MTCC 119 and Fungai as Candida albicans MTCC 227, Aspergillusflavus MTCC 230.

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RNI No. MAHENG/2017/74063
ISSN No. (Print) 2581-5911

One-Pot multicomponent synthesis of novel heterocyclic frameworks

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Abstract
Cycloaddition reactions of azomethine ylides, generated from acenaphthequinone, isatin and thioisatin with different secondary cyclic amino acids, and electron-deficient dipolarophiles produced novel heterocyclic framework in 63-81% yield. DFT calculations have been performed to understand the stereochemical course of the cycloaddition reactions. The products have been characterized by physical and spectroscopic techniques.

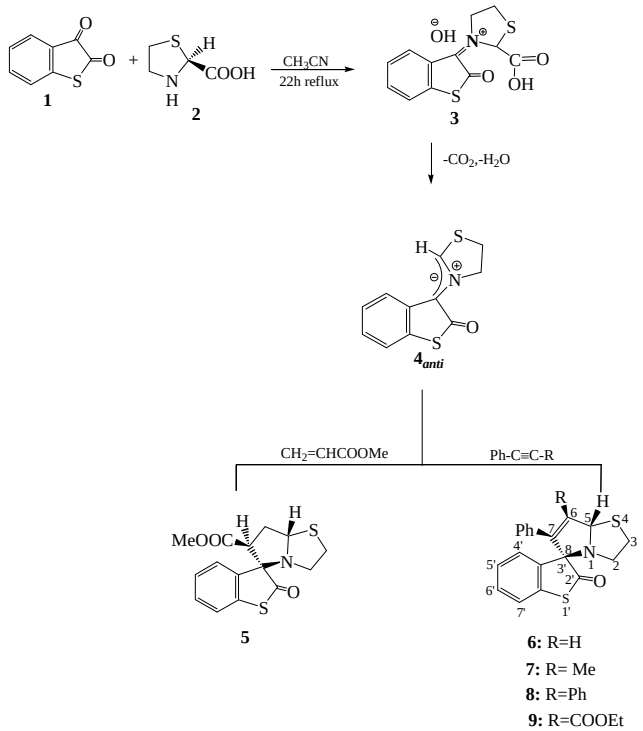
Keywords: *1,3-dipolar cycloaddition, azomethine ylides, dipolarophile, spiroheterocycles, DFT calculations.*

Introduction

1,3-Dipolar cycloaddition[1-3]of azomethine ylides with different dipolarophiles leads to the formation of novel heterocyclic spiro compounds having two or more chiral centers. The importance of azomethine ylide[4-15] chemistry has enhanced, more than ever, as a tool for constructing nitrogen containing five-membered heterocyclic systems which are often central to natural products. Interest in the synthesis of pyrrolidine derivatives *via* azomethine ylides has increased dramatically because these systems have widely been encountered in a number of molecules of interest[16-17].

Results and Discussion

The reaction of thioisatin **1** with (R)-(-)-thiaproline **2** in equimolar ratio in refluxing acetonitrile for 22h generated, *in situ*, azomethine ylides **(Scheme 1)**



1 **2** **3** **4**$_{anti}$ **5**

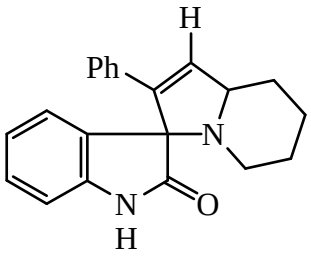
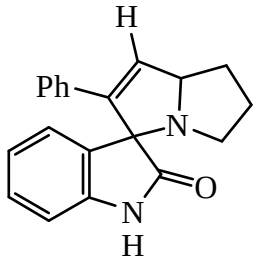
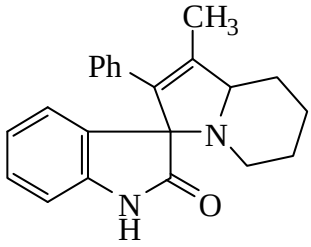
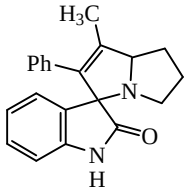
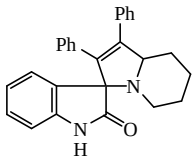
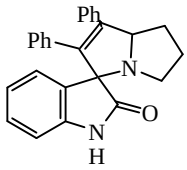
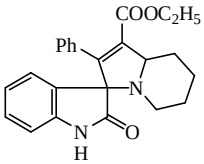
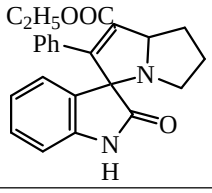
6: R=H
7: R= Me
8: R=Ph
9: R=COOEt

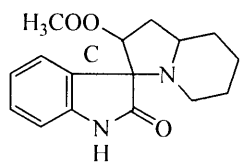
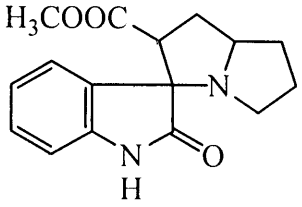
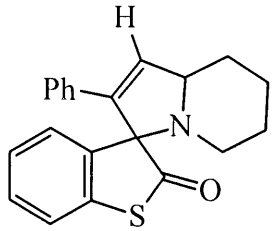
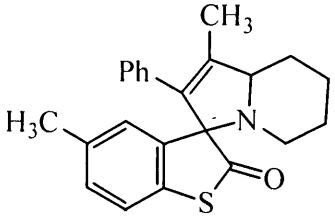
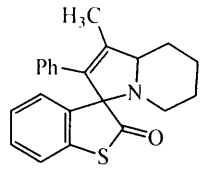
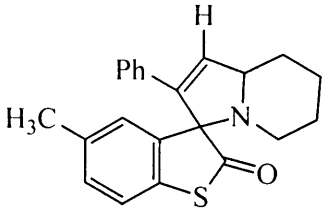
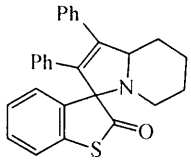
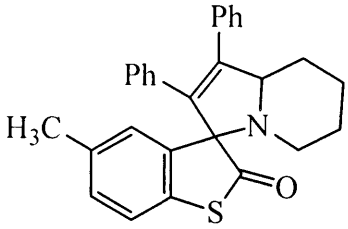
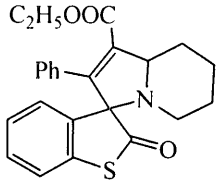
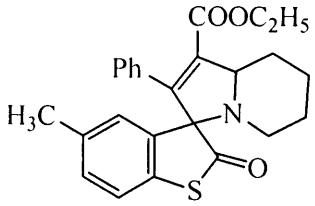
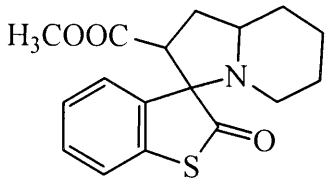
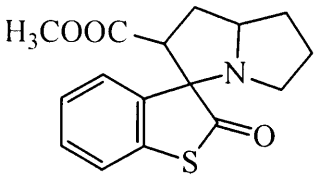
Scheme 1

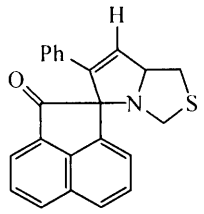
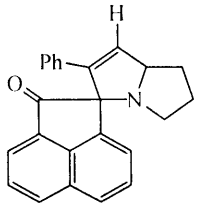
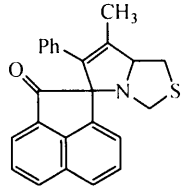
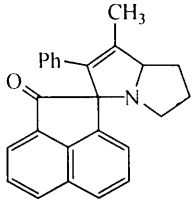
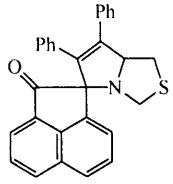
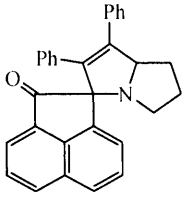
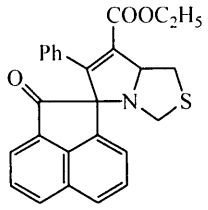
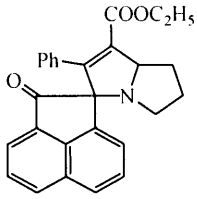
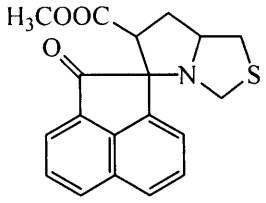
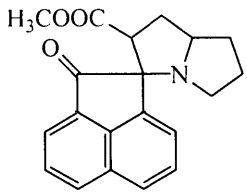
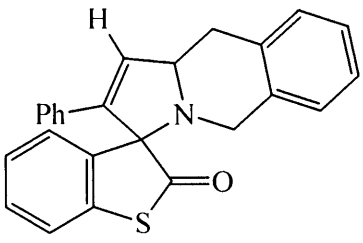
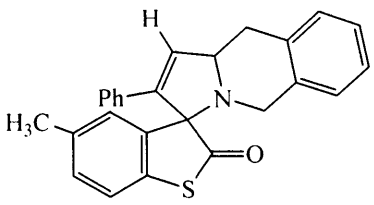
Compound **4** has been trapped as cycloadduct in the presence of various dipolarophiles, *viz* methyl acrylate, phenyl acetylene, 1-phenyl-1- propyne, diphenyl acetylene and ethyl phenyl propiolate thereby producing azabicycloadducts **5-9** in **62-75%** yield. The mechanism for the formation of cycloadducts involves initial formation of intermediate azomethine ylide (amy) **4** by the loss of CO_2 *via* stereo-specific cycloreversion[18] which subsequentiy undergoes 1,3-dipolar cycloaddition reactions with various dipolarophiles to produce spiroazabicycloadducts. The results are in good harmony with the observation of Grigg *et al*[19-20] Over the past 15 years, in our laboratory, we have exponentially exploited the generation of amy's and their 1,3-dipolar cycloaddition reactions. Some of the examples are summarized below:-

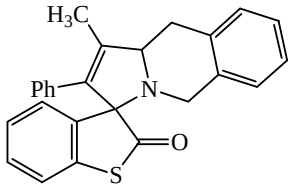
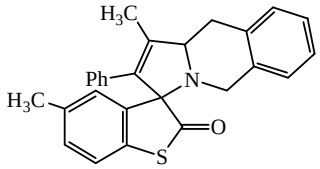
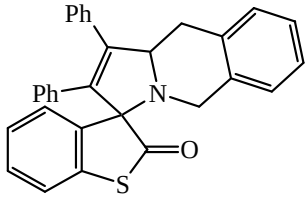
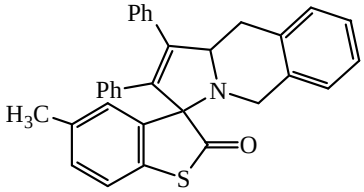
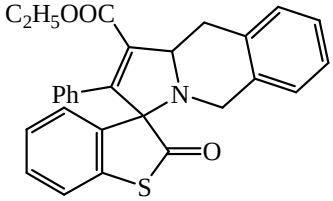
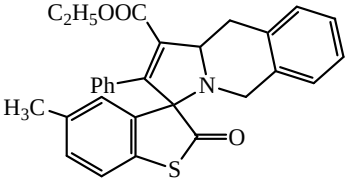
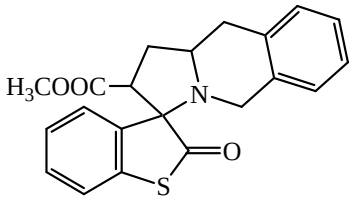
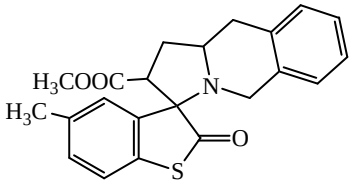
1. Reaction of acenaphthequinone with L-proline and (R)-(-)-Thiaproline[21,22]
2. Reaction of isatin or thioisatin with L-proline and (R)-(-)-thiaproline[23,24]
3. Reaction of isatin or thioisatin with *L* or *R* - piperidine-2-carboxylic acid[25]

Newly synthesized spiroheterocycles are summarised in the following table:

Cycloadduct	% Yield	Cycloadduct	% Yield
	**78**		**79**
	**69**		**73**
	**63**		**76**
	**77**		**78**

Cycloadduct	% Yield	Cycloadduct	% Yield
	75		74
	65		75
	68		68
	64		63
	70		74
	71		70

Cycloadduct	% Yield	Cycloadduct	% Yield
	**68**		**68**
	**77**		**77**
	**71**		**70**
	**73**		**73**
	**80**		**81**
	**62**		**82**

Cycloadduct	% Yield	Cycloadduct	% Yield
	**70**		**75**
	**73**		**85**
	**70**		**80**
	**68**		**80**

Molecular Orbital Analysis

Detailed DFT molecular orbital studies were conducted employing Gaussian 03 program to understand the stereochemical course of the cycloaddition reaction. Geometry optimization of azomethine ylide **4** indicated that —

1. it has an almost planar structure **(Fig. 1).**

2. the thiazolidine ring instead of having enevelpe shape is planar and lies in the same plane as that of istain

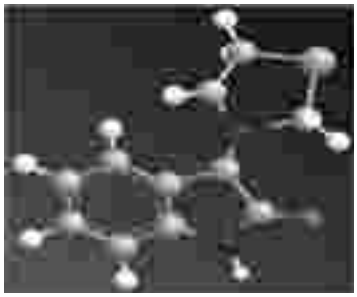


Fig. 1: DFT-optimized geometry of azomethine ylide 4

Azomethine ylide may exist as two conformers.

1. One in which C=O and –CH groups of dipole are *anti* (11_{anti}) and the other in which these are *syn* (11_{syn}). (**Fig 2).**

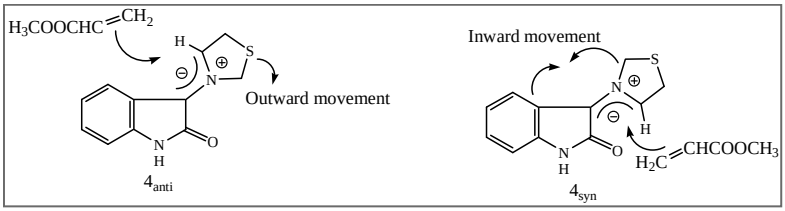
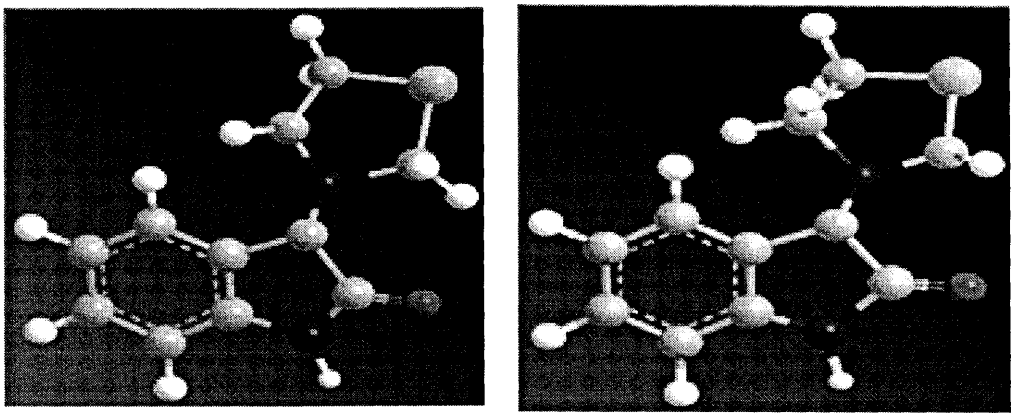


Fig. 2: Mode of attack of dipolarophile (methyl acrylate) on azomethine ylide

1. Both the isomers have almost the same heat of reaction indicating formation of both isomers in solution.
2. *Anti* form is more stable, and
3. Transition state calculations indicated the interconversion of each other and separated by an energy barrier of 16.35 kcal/mol.



anti form *syn* form

Fig. 3: Optimized Geometries of *anti* and *syn* azomethine ylides

Methyl acrylate may approach either of the azomethine ylides (amy) *syn* or *anti* with the formation of products having three chiral centers. Therefore, a total of 8+8= 16 stereoisomers could be possible. **(Fig. 4).**

Attack of methyl acrylate on *syn*-azomethine ylide results in the inward movement of thiapyrolidine ring toward the naphthalene nucleus and the transition state (TS) could not be located even in a single case **(Fig. 2)**. It may be due to the steric hindrance between the quinone nucleus and the thiaproline ring that makes it unstable and hence fails to produce transition state geometry, ruling out the possibility of formation of cycloadducts **[5(i)-5(p)]**. Thus it leaves the possibility of attack on only the *anti* azomethine ylide and hence only four isomers **5(a)-4a(h)** are left for consideration. Out of these four possibilities, only two have concerted mechanism **5(a)** and **5(b)** and we could optimize the transition state in case of **5(a)** only. This can be explained using the frontier molecular orbitals (FMO) approach along with the *endo* approach of phenyl ring.

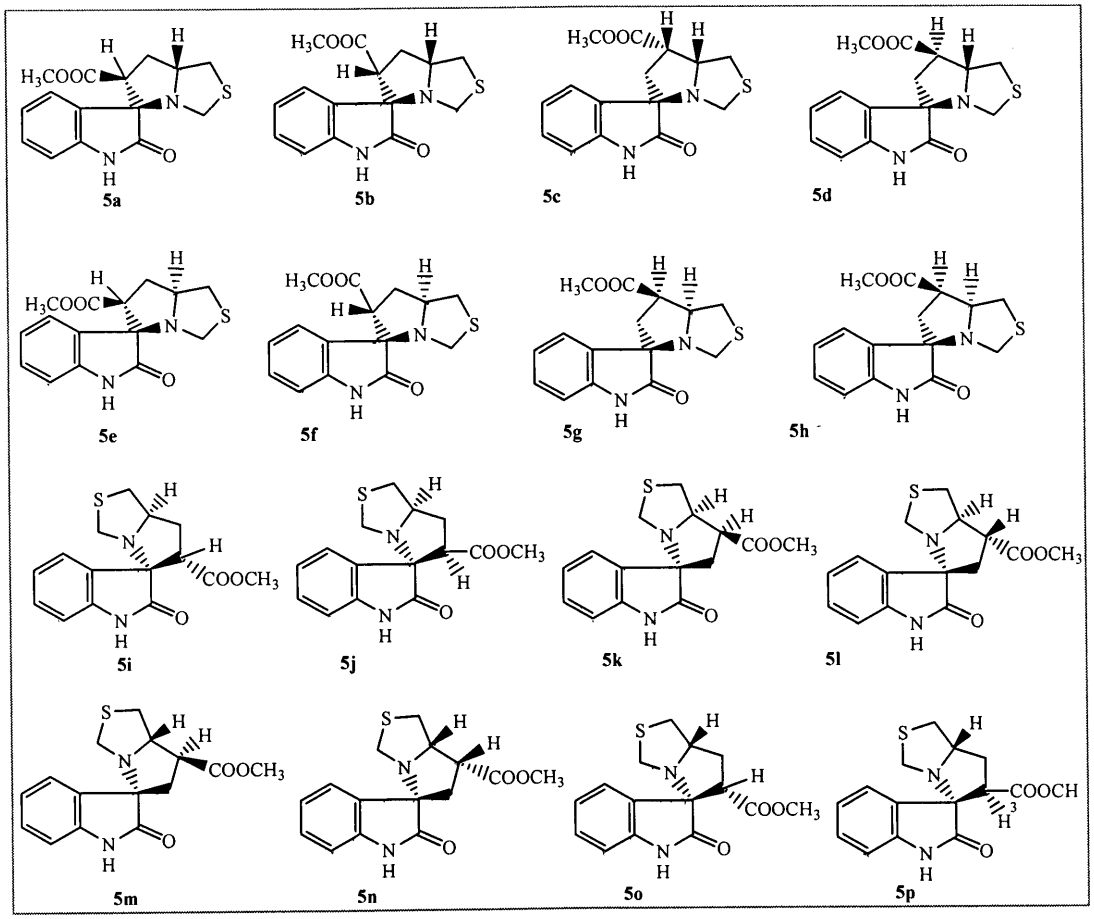


Fig. 4: Possible stereoisomers of the Cycloadduct

1. The favored path involves the $HOMO_{dipole}$ and $LUMO_{dipolarophile.}$ The transition state of the concerted 1,3-dipolar cycloadditions is usually controlled by FMOs of dipolarophiles and dipole (azomethine ylide).

2. The "H_f, HOMO, LUMO energies, and HOMO-LUMO energy gaps of azomethine ylide **4** with dipolarophiles are given in **Table 2.** From the table, it may be concluded that $HOMO_{dipole}$-$LUMO_{dipolarophile}$ energy gap is lower than the $LUMO_{dipole}$-$HOMO_{dipolarophile}$ gap

Table 2: HOMO, LUMO Energies and H-L and L-H Energy Gaps *Energy Gaps(eV)*

Dipole /dipolarophile	5_{anti}		5_{syn}	
	H_{dipole}-$L_{dipolarophile}$	L_{dipole}-$H_{dipolarophile}$	H_{dipole}-$L_{dipolarophile}$	L_{dipole}-$H_{dipolarophile}$
Methyl acrylate	8.06	10.12	7.97	10.16
Phenyl acetylene	8.05	8.45	7.96	8.49
Ethyl phenyl propiolate	7.46	8.77	7.37	8.81

3. The dominant FMO approach is $HOMO_{dipole}$-$LUMO_{dipolarophile.}$ Both the HOMO and the LUMO of the dipole show uneven distribution of the electron density along the C-N-C dipole. Similary in the LUMO of methyl acrylate, the atomic orbital coefficient on the C- atom bearing the phenyl group is larger than that of the atom away from it. Thus, there is a better orbital overlap between C_1 of azomethine ylide and the C-atom bearing the methyl acrylate. **(Fig.5).**

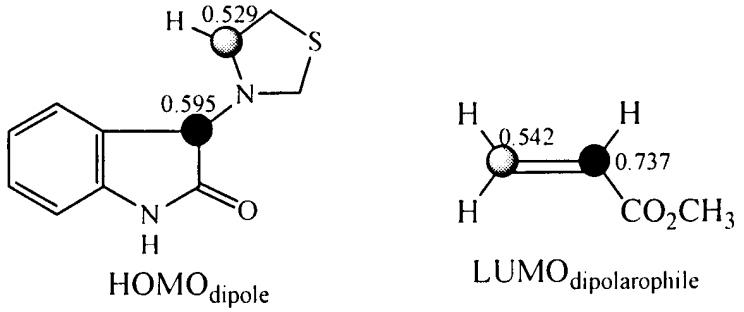
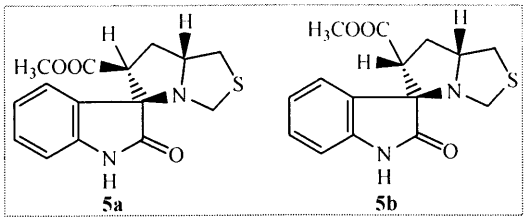


Fig. 5: Atomic orbital coefficients and overlapping of dipole (3a) with dipolarophile (meac)

This results in the formation of product **5(a),** thus ruling out the possibility of **5(b)**, in which case, we could not optimize the transition state. Besides a secondary interaction between the two phenyl rings, the ***endo*** approach also favors the formation of product **5a**.



The energy profile diagrams for the cycloadduct **5a** and **5b** are depicted in **(Fig. 6).**

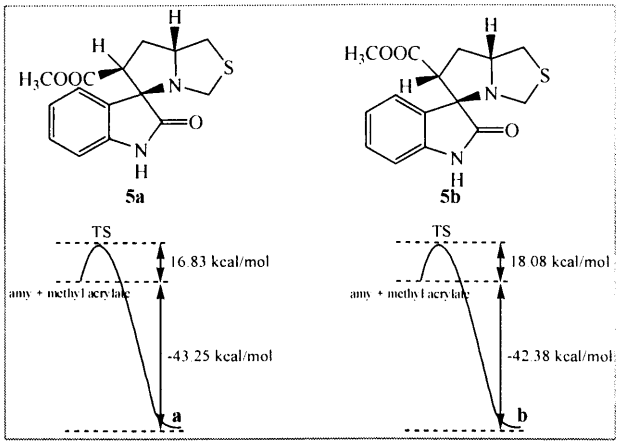


Fig. 6: Energy profile diagram of cycloadducts 5a and 5b

Parallel calculations have been performed on other cycloadducts, and the following conclusions may be drawn:

1. The *amy* is stabilized by delocalization of dipolar charge into the indole nucleus.
2. The dominant FMO approach for cycloaddition is $HOMO_{dipole} - LUMO_{dipolarophil}$ because the energy gap is lower.
3. The *amy* has planar geometry and exists in two conformations.
4. Spiro product **5a** is favoured in enantiomeric excess over **5b** due to *endo* approach of $COOCH_3$ group; it lies near to the isatin nucleus.

Conclusions

Similar calculations have been performed for other dipolarophiles *viz* phenyl acetylene, ethyl phenyl propiolate, etc. and all theoretical predictions are in complete agreement with experimental results and synthesis of novel spiropyrrolidines may serve as precursor for new natural products and synthetic drugs.

Acknowledgement

We authors are thankful to BRNS, CSIR, UGC, UOR and Central University of Rajasthan, India for funding for research work.

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RNI No. MAHENG/2017/74063
ISSN No. (Print) 2581-5911

Separation of Dansyl Amino Acids in Capillary Liquid Chromatography Using Cyclodextrin-Bonded Chiral Monolithic Stationary Phases

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Abstract
Capillary liquid chromatography columns for chiral stationary phase have been prepared using glucuronyl glucosyl β-cyclodextrin (GUG-β-CD. The optimized monolithic column was used for enantio separation of derivatized amino acids. The monolithic columns were prepared via *the copolymerization of β–CD in a one pot approach with glycidyl methacrylate (GMA) as functional monomer and ethylene dimethacrylate (EDMA) as crosslinker. The experiment with polymer monolith is less time consuming and can be easily reproduced. The monomer ratio composition was varied. The enantioselective separation was conducted under reversed phase conditions. The optimal conditions for polymerization of polyGMA-GUG-β–CD-co-EDMA monolithic column was obtained from column A2. The effects of mobile phase pH and content of acetonitrile and buffer on the enantio separation were investigated. The GMA/GUG-β–CD proportion resulted in a homogeneous matrix. Suitable mobile phase was acetonitrile/triethylammonium acetate (pH 4.1) 30/70 (v/v).*

Keywords: *Polymer monoliths, Methacrylate base, β-Cyclodextrin, Chiral separation, Dansyl amino acids*

Introduction

In recent years development of methods for chiral separation has attracted attention due to increasing demands for chiral compound separation and fabrication of enantiomerical compounds. A lot of chromatographic methods have been used and developed for chiral separation such as gas chromatography (GC[1], supercritical fluid chromatography (SFC, high-performance liquid chromatography (HPLC)[2,3], capillary electrophoresis (CE) [4,5], and micro-liquid chromatography [6].

Several kinds of analytical methods for the separation of enantiomers have been developed, the most accepted method among them being HPLC[7]. In recent years, liquid chromatography has been greatly used in the analysis of many biological substances because of its advantages such as high sensitivity, low sample consumption, high enantioselectivity, and quick analysis[6, 8].

Cyclodextrins (CDs and their derivatives as chiral selectors in liquid chromatography still represent a significant tool for the analysis of structurally different compounds in modern analytical chemistry. The wide use of CDs as the chiral stationary phase is due to their natural chirality and ability to form inclusion complexes with molecules *via* hydrophobic cavity [9]. There are two common ways to use CDs in liquid chromatography:

chiral mobile phase additives (CMAs) and chiral stationary phases (CSPs). CDs and their derivatives are useful for chiral selection as running buffer additives for separation of racemic drugs. [10]

A CD-based chiral stationary phase is one of the most widely used chiral stationary phase in recent years. Direct enantio separations by HPLC linked with CDs derivatized and CSPs have emerged as one of the most favored analysis [11]. CDs have many advantages such as stable performance, strong chiral recognition ability, and low cost[12]. Different types of amino acid derivatives have been chirally separated by liquid chromatography. The stereochemistry of amino acids play a significant part in their biological properties. Their increasing use indicates their significant effect in Life sciences [6].
Several studies have described advances in the analysis and chiral separation of amino acids. Tang *et al.* described chiral separation of dansyl amino acids using mono-(3-methyl-imidazolium-β-CDchloride as the selector[4]. Gabriela *et al.* described the enantioseparation of chiral amino acids and dipeptides using sulfobutylether-β-CD[13]. Recently, Li *et al.* used *N*-benzyl-phenethylamine-β-CD for enantioseparation of dansyl amino acids[11].

In this study, the efforts are focused on developing glucuronyl glucosyl β-cyclodextrin (GUG-β-CD based stationary phases to perform the enantio separation of chiral derivatives of amino acids by capillary liquid chromatography. GUG β-CD is a single isomer, mono-substituted β-CD with glucuronyl glucosyl group, which has one carboxylic acid group [10]. Among the chiral staionary phases, CD-based stationary phases have many applications for the analytical separation of different chiral compounds. These chiral stationany phases can be prepared by click chemistry or dynamic coating [14,15]. Indirect optical resolution of amino acids is performed in the chiral environment by the interaction of enantiomers with chiral selectors added to the separation buffer[4].

Materials and Methods

Apparatus

The chromatographic measurement was done by using a capillary LC system constructed by an L. TEX-8301 micro feeder (L. TEX Corporation, Tokyo, Japan) equipped with an MS-GAN 050 gas-tight syringe (0.5 mL, (Ito, Fuji, Japan) as a pump, a model M435 micro-injection valve (Upchurch Scientific, Oak Harbor, WA, USA) with injection volume of 0.3 µL, a 100 mm x 0.32 mm I.D. of microcolumn and a UV-1575 intelligent UV/Vis detector (JASCO, Tokyo, Japan) operated at 254 nm and a data processor (CDS-Lite ver 5.0; LA soft, Chiba, Japan). The morphology of the column was characterized by an S-4800 scanning electron microscope (Hitachi, Tokyo, Japan).

Reagents and materials

Glycidyl methacrylate (GMA (97% and ethylene dimethacrylate (EDMA (97%, GUG-β–CD were obtained from Wako Pure Chemical Industries, Osaka, Japan. 3-(Trimethoxysilylpropyl methacrylate (γ-Maps, 98%, 2,2'-azobisisobutyronitrile (AIBN and acetonitrile (ACN were obtained from TCI Tokyo, Japan. 1,4-Butanediol, decanol, ethanol, triethylamine, and acetic acid were obtained from Nacalai Tesque Kyoto, Japan. Dansyl-DL-amino acids and dansyl-L-amino acids were obtained from Sigma Chemical Company St. Louis, USA. All solutions used in this study were prepared using ultrapure water prepared in the laboratory using a Simplicity UV water purification system (Millipore, Molsheim, France.

Preparation of the monolith columns

Initially, for the preparation of the monolith, the fused silica capillaries were first pre-treated with γ-MAPS. Using this procedure, the monolith support column could be covalently anchored to the activated inner surface wall, and the silanization reaction introduced methacrylic anchoring groups [16]. The mixture solutions of monomer, cross-linker,porogens and GUG-β–CD were prepared. The appropriate solvent was directly added to the porogen mixture. As the solution for the fabrication of

the monolith column, a mixture of GMA, EDMA, GUG-β–CD, 1,4-butanediol, decanol, ethanol, and AIBN (1% with respect to the monomers was added, followed by sonication for 5 min. Subsequently, the solution was manually introduced into the capillary pretreated column using a syringe. Both ends of the capillary were sealed and heated at 60°C for 18 h in a water bath for polycondensation and polymerization. After polymerization was completed, the capillary column was flushed with methanol to remove the residuals. The column was cut to a total length of 10 cm and 2-5 mm of the capillary was used for SEM analysis.

Table 1 Polymer composition mixture used in the preparation of poly(GMA-GUG-β–CD-co-EDMA monolithic column

Column	GMA:GUG-β–CD (mixture ratio	Monomer %(v/v GMA:GUG-β–CD	Crosslinker % (v/v EDMA	Porogen* % (v/v	Monomer:Porogen (Mixture ratio
A1	3:1	40	10	50	50/50
A2	2:1	30	10	60	40/60
A3	1:1	20	10	70	30/70

*Ternary porogens, i. e., 1,4-butanediol, decanol, and ethanol (1:0.8:0.2

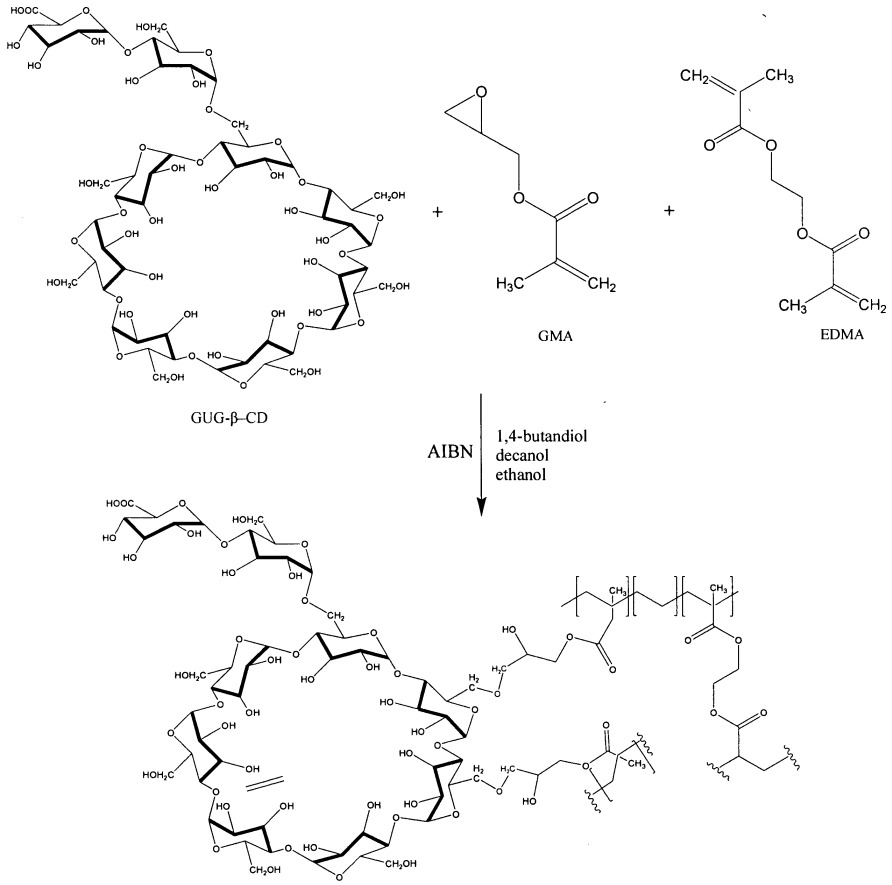

Fig. 1 Schematic diagram for chiral stationary phase preparation

Standard solution and sample preparation

The triethylammonium acetate (TEAA) buffer solution was prepared by dissolving appropriate amount of triethylamine in ultrapure water and stirred under cold conditions. Then, the desired pH value was adjusted with acetic acid, and stored in the refrigerator. Ammonium acetate (AMAC) buffer was prepared by dissolving ammonium acetate in deionized water and adding acetic acid [13]. The mobile phases were prepared by mixing the desired amount of ACN and the buffer solution. GUG-β-CD with concentration 1 mg/mL in methanol (MeOH) was used as chiral selector solution. MeOH was used as a solvent in majority of cases. Stock solutions of dansyl amino acid at concentration 0.5 mg/mL in methanol were prepared. All analytes were detected at 254 nm wavelength.

Results and Discussion

Preparation of poly (GMA-GUG-β–CD-co-EDMA monoliths

The preparation of poly (GMA- GUG-β–CD-co-EDMA monolithic columns was carried out in one step. Three polymer monoliths were prepared *via in-situ* copolymerization. First *in situ* polymerization was thermally initiated. In this study, a mixture of solutions of monomer, GUG-β–CD, cross-linker, and porogens were prepared and then the solution was mixed with AIBN. Thermal polymerization was carried in a water bath at 60°C for 18 h. The capillary was rinsed with methanol after polymerization to remove unreacted reagents and porogenic solvents.

The group to be coupled to epoxy groups was determined for cyclodextrin agent. In this study, a one-pot route integration of the GMA-GUG-β–CD monomer followed by copolymerization inside the same vial was needed to prepare poly(GMA-GUG-β–CD-co-EDMA monolithic column, as shown in Fig. 1. The composition of the reaction mixture has a major effect on the morphology, permeability, and selectivity of the monolith, and the ratio of GMA-GUG-β–CD and the content of the porogens were optimized as shown in Table 1. The ratio GMA/GUG-β–CD in the reaction mixture can influence the amount of the functional monomer and the skeleton. The mixture ratio of GMA/GUG-β–CD of the monolith was varied from 3:1 (A1) to 1:1 (A3) while keeping the other conditions constant. The porous properties of the support of the monolith have to be adjusted for flow through the pores of the monolith column at a low backpressure. As shown in Fig. 2, the relationship between the flow rate and the back pressure demonstrated that the monolith column (A2) was mechanically stable upto the flow rate of 6 μL/min.

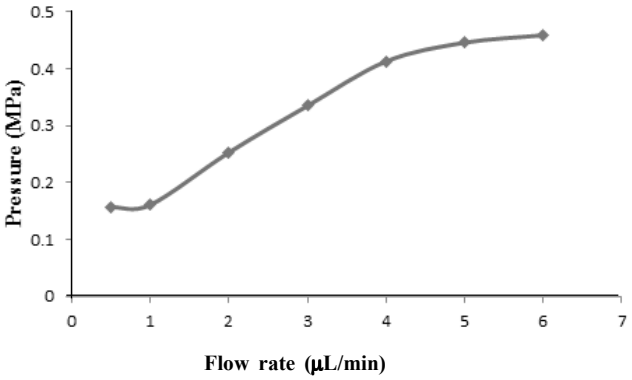

Fig. 2 Effect of the flow rate on backpressure. Condition: mobile phase, ACN/100 mM TEAA (pH4.1 30/70 (v/v. Chiral stationary phase column: 100 x 0.32 mm I.D.

Fig.3 shows the SEM of chiral stationary phase under optimum conditions. The optimal conditions for polymerization of poly(GMA-GUG-β–CD-co-EDMA monolithic column was obtained from Column A2. The GMA/GUG-β–CD proportion resulted in a homogeneous matrix. The A1 column was too dense and the A3 column could not be used because of failure of the polymerization.

Separation of chiral analytes

Enantio separation of dansyl amino acids using columns containing chiral stationary phase based on β–cyclodextrin derivatives was examined at the analytical level. The retention factor (*k)* and separation factor (α) for column for the separation of all compounds using a mixture of ACN/TEAA buffer and ACN/AMAC as the eluents were determined. Examination and optimization

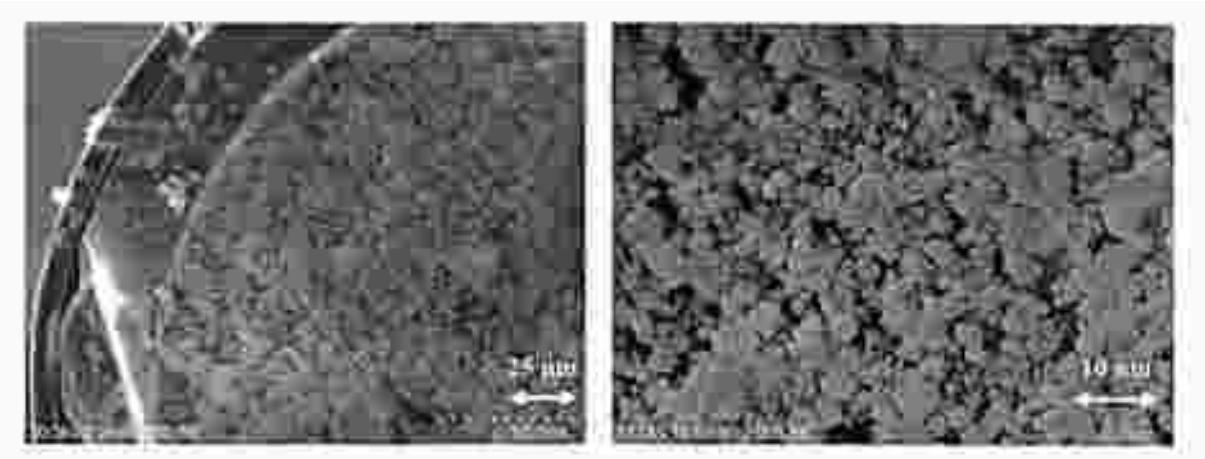

Fig. 3 Scanning electron microscopy of poly(GMA-GUG-β–CD-co-EDMA monolithic column (column: A2, magnification 600x and 2000x, respectively

of the composition of mobile phases for chiral analytes separated on the chiral stationary phases were obtained from derivatized amino acids.

The selection of buffer and operating pH is important due to several factors. The main factor is that the polarity, and charge of acidic and basic analytes are affected by pH which will in turn affect the solute interaction with the chiral stationary phase. Obviously, analytes that are ionized will interact with the chiral stationary phase differently than their neutral conjugates.

In reversed phase chromatography, the most frequently used organic modifiers are methanol and acetonitrile. Changing a solvent that can accept and donate hydrogen bonds (methanol) to one that cannot provide hydrogen bonding (acetonitrile) will often have an affect on the observed separation. In this study, acetonitrile has been used as organic modifier, and acetonitrile tends to be a stronger eluent than methanol. The use of TEAA buffer (pH 8.3) was not useful for several of the chiral analytes. They were in general eluted at very short retention times. Nearly half of the chiral analytes did not indicate enantio separation with decreasing content of TEAA buffer in mobile phases. The analytes with a free carboxyl group are negatively charged at pH 8.3. Several enantioseparation of amino acids were achieved at various mobile phase compositions. Suitable mobile phase was used for ACN and TEAA buffer (pH 4.1) in different ratios of volumes. Under these condition (pH 4.1) the enantiomeric elution order was determined. Partial enantioseparation of chiral analytes was observed in mobile phase ACN/TEAA (pH4.1) 30/70 (v/v, as shown in Fig.4.

Higher resolution values were observed if TEAA (pH 4.1) was used instead of TEAA (pH 8.3). Under this condition (pH 4.1) amino acids were uncharged. These analytes were baseline enantio separated in mobile phase of ACN/TEAA buffer (pH 4.1) with higher contents of TEAA buffer (30/70 v/v). The analysis time was around 10 min. The enantio selectivity of amino acids having two chiral centers was studied and the enantiomer elution order (EEO) determined by injection of the authentic enantiomer dansyl-L-amino acids. Enantiomer L eluted before D, indicating a stronger interaction of D-enantiomer with the chiral selector.

The use of acetonitrile/AMACbuffer (pH 4.7) was useful for several analytes. It was found that the separation result changed significanly as compared to the chiral analytes with mobile phases of ACN/100 mM TEAA buffer (pH 4.1) 30/70 (v/v. In general, they could be eluted at long retention times due to the strong interaction between the chiral selector on CSP and compunds. The analyte that is more tightly bound to the CSP has

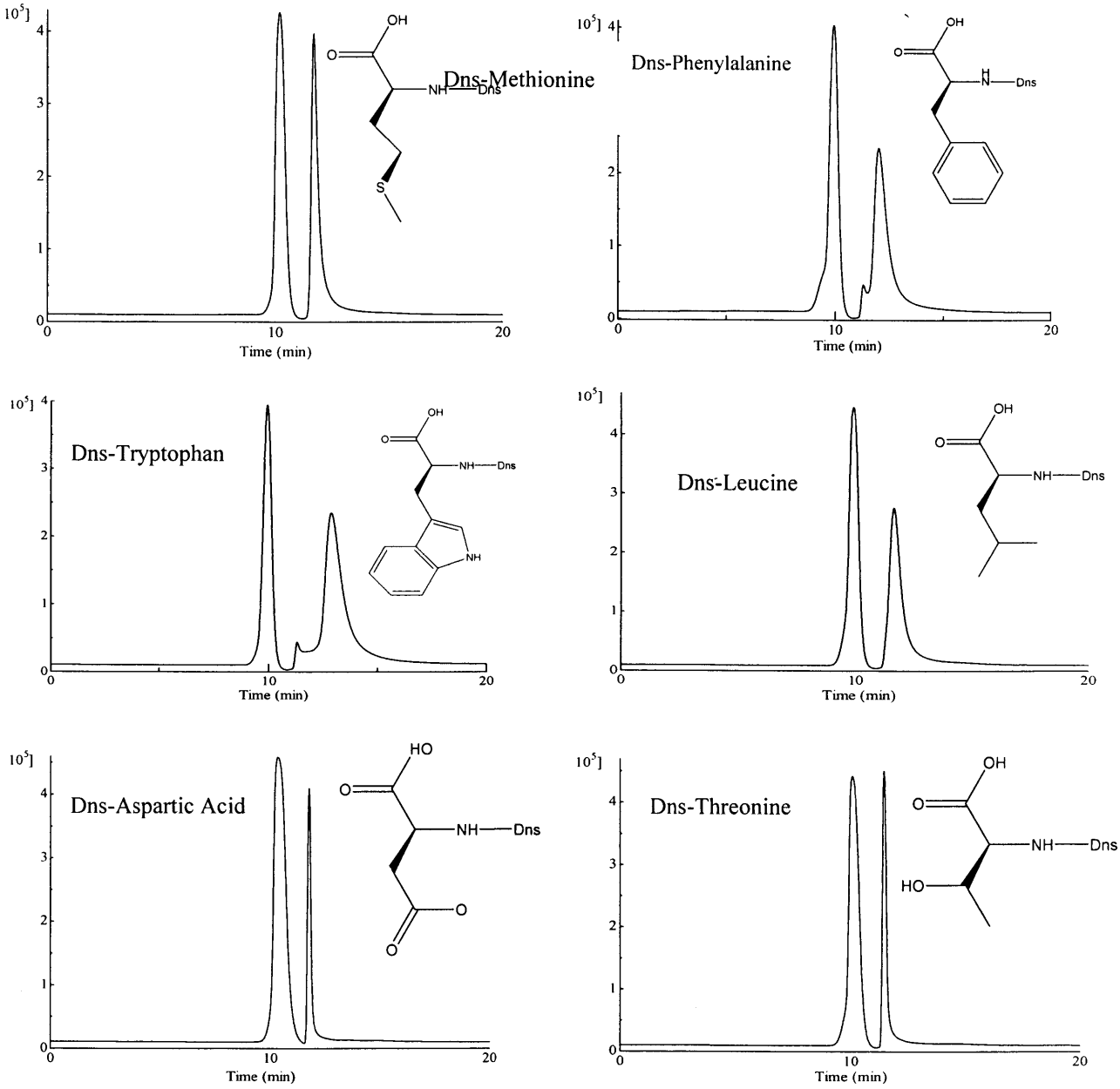

Fig. 4 Chromatograms of the separation of chiral analytes with mixture of ACN/100 mM TEAA buffer (pH 4.1 30/70 (v/v. Conditions:column, 100x0.32 mm I.D.; flow rate, 1µL/min; wavelength of UV detection, 254 nm.

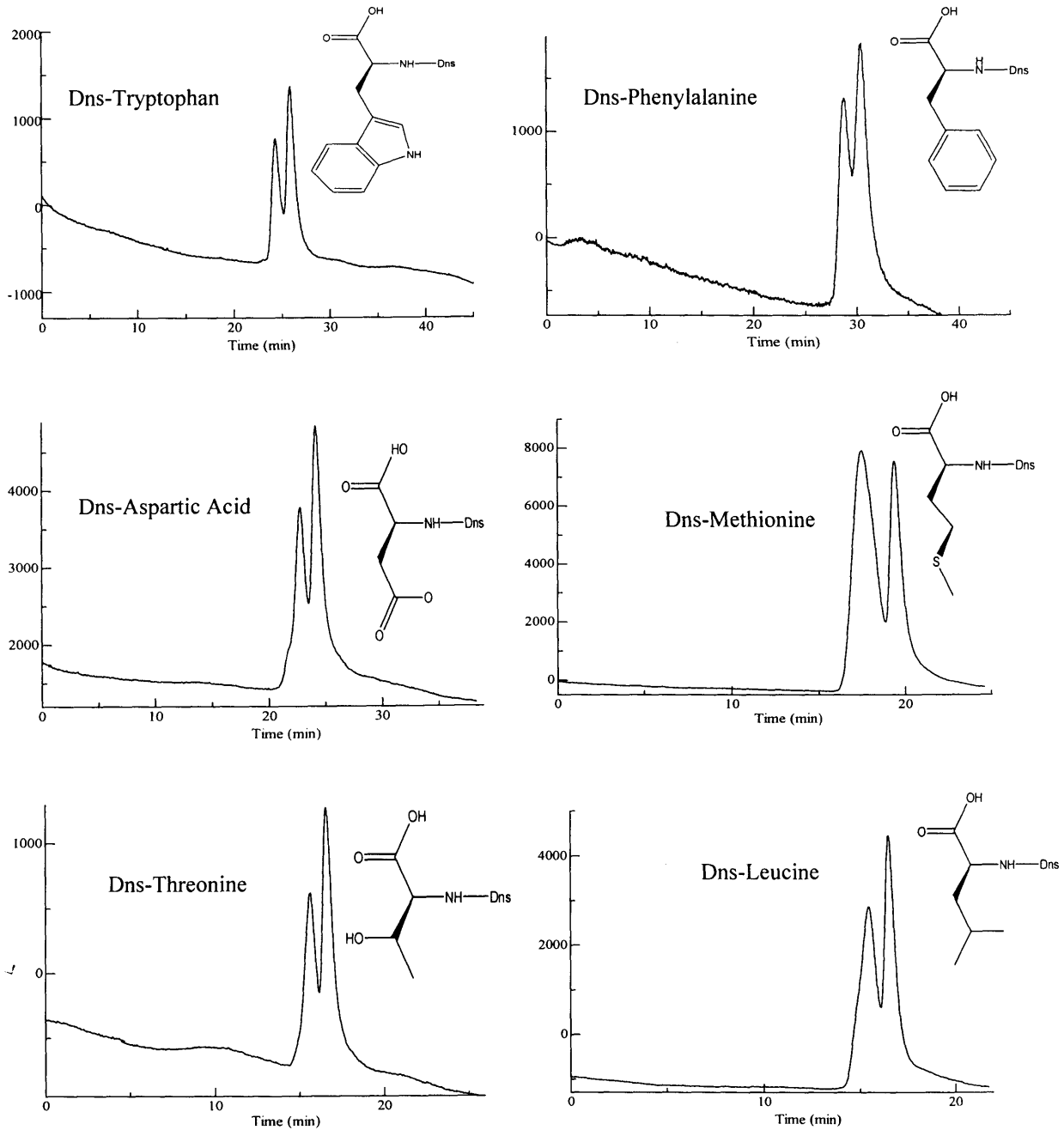

Fig. 5 Chromatograms of the separation of chiral analytes with mixtureof ACN/60 mM AMAC buffer (pH 4.7 30/70 (v/v.Conditions: column, 100x0.32 mm I.D.; flow rate, 1µL/min; wavelength of UV detection,254 nm.

greater negative free energy and hence is retained longer. Under these conditions, the enantiomeric elution order was determined by injection of dansyl-L-amino acids, indicating a weaker interaction of L-enantiomer with the chiral selector, while the peak of the compound obtained still overlapped. Partial enantio separations of chiral analytes are shown in Fig. 5.

Effect of pH

The effect of pH on retention and enantioselectivity is shown in Fig.6 and Table 2. The retention was obtained for Dansyl-DL-threonine on the poly(GMA-GUG-β–CD-co-EDMA) monolithic column. The effect of pH on enantioselectivity (α) for Dansyl-DL-amino acids was favored at lower pH. It is well known that chiral recognition mechanism is based on the specific short-range interaction and hydrogen bonding following the non-specific long-range interactions (hydrophobic and ionic forces which allow the approach of solute to the chiral selector [17].

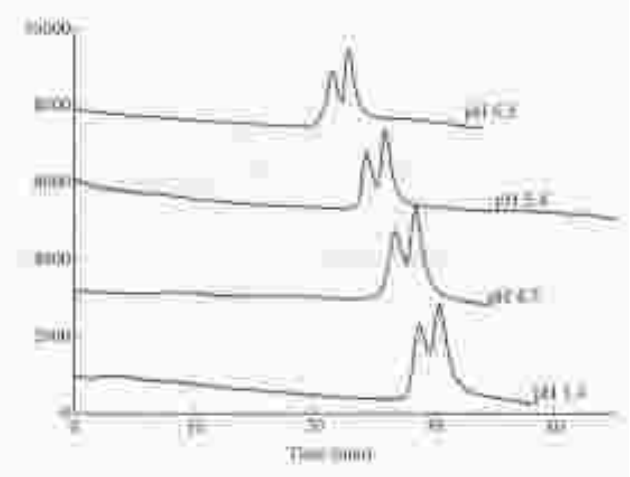

Fig. 6 The influence of pH on retention of Dansyl-DL-threonine. Conditions: column, 100x0.32 mm I.D.; mobile phase, ACN/60 mM AMAC buffer (pH 4.7 30/70 (v/v; flow rate, 1μL/min; wavelength of UV detection, 254 nm; column temperature, 20°C.

Table 2 Enantioseparation data of Dansyl-amino acids on cyclodextrin

Amino Acids	Retention Factor ($k^{(a}$	$\alpha^{(a}$	Retention Factor ($k^{(b}$	$\alpha^{(b}$
Methionine	$k_1 = 4.752$; $k_2 = 6.182$	1.30	$k_1 = 8.386$; $k_2 = 10.296$	1.23
Phenylalanine	$k_1 = 4.612$; $k_2 = 7.052$	1.53	$k_1 = 19.686$; $k_2 = 21.406$	1.09
Tryptophan	$k_1 = 4.742$; $k_2 = 7.942$	1.67	$k_1 = 15.086$; $k_2 = 16.895$	1.12
Leucine	$k_1 = 4.712$; $k_2 = 6.612$	1.40	$k_1 = 6.085$; $k_2 = 7.435$	1.22
Asp. Acid	$k_1 = 5.152$; $k_2 = 6.322$	1.23	$k_1 = 13.667$; $k_2 = 15.314$	1.12
Threonine	$k_1 = 4.642$; $k_2 = 6.172$	1.33	$k_1 = 6.306$; $k_2 = 7.256$	1.15

[a]Separation of chiral analytes with MP mixtureof ACN/100 mM TEAA buffer (pH 4.1) 30/70 (v/v)
[b]Separation of chiral analytes with MP mixtureof ACN/60 mM AMAC buffer (pH 4.7) 30/70 (v/v)

With a constant organic modifier and buffer composition, the chromatograms at various buffer pH are shown in Fig. 6, *i.e.*,the effects of pH on retention times of Dansyl-DL-threonine. The retention time of the solutes decreased with the buffer pH in the range of operational pH (3.4 – 6.5.) Since at higher pH, the carboxylic acid of chiral solute is mainly ionized, the interaction between the dansyl group and β-CD decreases leading to the decrease in the separation factor (α).

Conclusions

Poly(GMA-GUG-β–CD-co-EDMA monolithic columns were prepared *via* the copolymerization of β–CDin one

pot approach. The prepared column was investigated for enantio selective separation of derivatized amino acids. The experiment with polymer monolith is less time consuming and reproducible and with further option of variation of monomer ratio composition. The enantioselective separation was conducted under reversed phase conditions.

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RNI No. MAHENG/2017/74063
ISSN No. (Print) 2581-5911

Determination of some toxic and essential elements in some food stuffs by Induced Coupled Plasma Optical Emission Spectrophotometry

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Abstract
The goal of the present investigation is to establish a suitable method for the extraction of heavy metals and essential elements from some food stuffs. Potato chips samples were collected from local markets in Kalar- Sulaimaniya, Iraq. The samples were converted into solution with the aid of two digestion methods, viz. wet ashing digestion and the closed vial domestic microwave digestion. The analysis was carried out by means of inductively coupled plasma optical emission spectrophotometry (ICP-OES). The assessment of the accuracy of the sample preparation procedures was performed by evaluating the percentage spike recovery. The Pearson's method was employed to study the correlation between element contents. Consequently, the matrix effect could be evaluated. Percentage spike recovery in the range of 85-100% could be achieved for wet ashing digestion. Meanwhile, the recovery was in the range of 89-110% for the closed vial domestic microwave digestion.

Keywords: *potato chips, acid digestion, ICP-OES, heavy metals, essential elements.*

Introduction

The consumption of packed foods is one of the features of modern life reflecting the shortage of time and the need for ready-made foods instead of cooked foods. In addition to their low nutritional values, these foods could be a source of toxic material like organic compounds and heavy metals to human body. Two groups of elements may be distinguished; micro-elements that are essential for the living organisms and those with neutral effects for biota. Micro-elements in living organisms usually occur in trace amounts defined for specific species. Both the deficiency and excess of these metals has a harmful influence on living organisms, causing, among other things, disorders of the nervous system due to the tendency to accumulate in the brain or liver[1, 2].

The determination of various elements in foods is important because of their significant roles in the stabilization of healthy life. Elements in foodstuffs are grouped as essential and heavy toxic elements. The essential elements are represented by sodium, potassium, silicon,

calcium, magnesium, and sulfur as major constituents; boron, copper, iron, manganese, molybdenum, zinc, nickel, cobalt, chromium, selenium, and vanadium as micronutrients. Micronutrients are at critical concentrations[3]. The foremost sources of toxic metals can be registered as industrial processes, medical processes, glass and ceramic manufactures, dyeing and agriculture dyeing. The examples for toxic elements are silver, aluminium, arsenic, barium, beryllium, cadmium, mercury, lead, and lithium.

Potato chips are among the commonly consumed snack foods. They are packed as thin slices of potato with a variety of spices to suit the apetite of children and occasionally adults. Potato chips may be polluted due to the raw materials used, processing, packing, transportation, storage, marketing and consumption. The presence of heavy metals in vegetable oils may contribute to the overall content of the metal ions in potato chips[4]. Chemically, the pollutants are organic as well as inorganic in nature. Organic pollutants may be carcinogenic while metal ion can support the generation of ultra-oxide and hydroxyl radical which, consequently may lead to deterioration of lipids, nucleic acids and proteins [5]. Heavy metal contamination of food is linked with some diseases [6].

Among food sample preparation methods, the dry mineralization is popular. However, the method has some disadvantages including the loss of volatile ingredients, mechanical loss, and risk of secondary contamination. In addition, they are time consuming procedures and require a second stage process dissolution in acid after the incineration[4].

The European Commission published a report on the determination of selected poisonous metal ions in canned food by laboratories from 36 countries. The majority of laboratories (more than 74%) reported satisfactory results for the selected metals. The best performances were obtained for total As, Cd and Pb[7]. The significance of food pollutants is related to their levels and their chemical nature[8]. Thus, the concentration levels of metals in food items at the point of consumption are necessary for the estimation of human exposure to metals[9]. Farhan et al.,[10] employed arc spectrography for the determination of trace metals in canned vegetables. They added pure sulfur to the samples before calcination to stabilize the trace metals as sulfides and prevent volatilization. Inductively coupled plasma optical emission spectrophotometry, ICP OES, is one of the most reliable and powerful analytical methods for the determination of trace elements in a myriad of samples due to its accuracy and precision. The technique is based upon the spontaneous emission of photons from atoms and ions that have been excited in a radio frequency discharge. Liquid and gas samples can be injected directly into the instrument by a nebulizer, while solid samples must be extracted by one of the extraction methods or acid digestion so that the analytes will be done in solution[11].

The purpose of the present investigation is to establish suitable and reliable methods for the quantitative extraction of some toxic and essential elemental elements from potato chips for their determination using ICP OES.

Material and Methods

Apparatus

The sample solutions were analysed by aspiration into radial view ICP-OES, (Spectro Arcos, Germany). The instrumental parameters are given in **Table 1**. The sample homogenization was performed on an ultrasound bath sonicator (740, Germany).

Materials and solutions

Samples Ten different samples of potato chips were obtained from local supermarkets in Kalar, Sulaimaniyah, Iraq, and their names and manufactures are given in Table 2. The samples were dried in an oven at 105^{o}C overnight. The dried samples were crushed by means of a mortar and pestle separately and stored in closed plastic containers to be used for acid digestions.

Chemicals The standard solutions were prepared by serial dilution of the 1000 mg mL^{-1} stock solutions

supplied from Bernd Kraft GmbH, Germany. Distilled deionized water was used for the dilution and the washing of the glass ware.

Sample preparation for analysis

Two digestion methods were used to convert potato chips samples into solutions to be analysed for metal ion estimation wet acid digestion, WD, and closed vessel domestic microwave digestion, MWD.

Wet Acid Digestion method

Approximately about 1 g of the dried sample was placed in a beaker, and concentrated HNO_3 4 mL and 30% H_2O_2 2 mL were added. Similar digestion mixture was employed by Jaradat and Tarawneh[12] for the dissolution of potato and corn chips to estimate heavy metal content. The mixture was homogenized by placement for 10 minutes on an ultrasound sonicator. The homogenous solution was heated on a hotplate at 100°C for about 1 hour and left in a fume hood for another 1 hour to ensure complete reaction. Finally, the cold solution was filtered and diluted into a 25 mL volumetric flask using deionised water as diluent and kept at 4°C until measurement, usually within a few hours before running by ICP-OES.

Domestic Microwave Digestion Method

Accurately about one gram of the potato chips samples were placed in a beaker together with concentrated HNO_3 4 mL and 30% H_2O_2 2 mL. The mixture was sonicated for about 10 minutes and the homogenous material was then heated in a closed vial in a domestic microwave at 300 W, for 30 second. The vial content was allowed to stand for one hour at room temperature prior to their dilution to 25 mL in calibrated flasks with deionised water. The solutions were kept at 4°C until measurement with induced coupled plasma, ICP, emission.

Quality Control

To estimate the precision and accuracy of the analytical digestion methods, applicable quality assurance techniques and precautions were accomplished. Throughout the experiments, all glassware and apparatus were soaked in 5% HNO_3 overnight followed by rinsing three times with deionised water. Blank and standard solutions at different concentrations were used for instrumental calibration. The value of method limit detection LOD was measured. The method LOD was determined by measuring blank solution 10 times, sample standard deviation (S) was found and multiplied by 3 (3S).

In order to assess the quality control of the digestion methods for specific elements, the percentage recovery of spike was carried out. Known amounts of selected elements were added as volumes of the standard solutions to pre-determined weight of the samples and intimately ground while wet, followed by drying. The dried material was subjected to the same digestion procedures as above. The spiked recovery was calculated by the following equation:

$$\%S_r = \frac{measured\ conc.}{added\ conc.} x100\%$$

where $\%S_r$ is the percentage spike recovery. The calculation was done for most elements with appreciable concentration in analysed samples.

Results and Discussion

The limits of detection, LOD, measured by ICP-OES for the analysed elements using both methods of digestion were evaluated. The elements measured in this investigation were classified into three dissimilar categories in accordance with the level and role in the body: macro-essential elements, Ca, K, Mg, Na, and P; micro-essential elements: Al, Co, Cr, Cu, Fe, Mn, Mo, Ni, Sr, Tl; non-essential toxic elements: Ag, As, Ba, Be, Cd, Hg, Li, Pb, Sb, Se, Sn, V, and Zn. The results of the quality control section expressed in mg kg^{-1} are presented in Table 3-5**.**

Table 6 demonstrates the range of macro-essential elements in potato chips. By method A calcium concentrations ranged from 28.09 mg kg^{-1} in D to 92.45 mg kg^{-1} in sample E, which are very close to values obtained by method B of digestion: 30.64 for sample D

and 93.97 μg g^{-1} for sample E, respectively. For potassium, the concentrations were in the range 4963.8 to 4953.7 mg kg^{-1} in sample E to 8139.0, and 8057.7 mg kg^{-1} in sample H for methods A and B respectively. The levels of phosphorus concentrations ranged between 46.67 $mgkg^{-1}$ in sample A and 7967 $mgkg^{-1}$ in sample J for method A, compared with 46.30 to 78.61 $mgkg^{-1}$ for A and J in turn. A comparison of the Ca, Mg and P contents with the two digestion procedures can be seen in Fig. 1. Further, a correlation was established between the results of potassium by the two methods as shown in Fig. 2.

The concentrations of microessential elements Ba, Se, Zn in the analysed potato chips using two different methods are given in Table 7. Selenium occurs in all the samples with a maximum value of 0.72 $mgkg^{-1}$ and lowest of 0.45 $mgkg^{-1}$. Zinc was detected in all the samples with a maximum value of 0.48 $mgkg^{-1}$ and lowest of 0.27 $mgkg^{-1}$.

The concentrations of nonessential toxic elements in the studied potato chips using two different methods are given in Table 8. Copper existed in all the samples in the range of 0.02-0.32 mgkg-1. Barium could be detected in all of the samples with maximum content of 1.3 $mgkg^{-1}$ in sample B and lowest value of 0.5 mg kg^{-1} in sample J. Aluminium could be detected in the potato chips and the maximum content of 1.0 ug g^{-1} was in sample A, whereas the lowest value of 0.35 mg kg^{-1} was in sample C.

Discussion

The three elements, Ca, P and Mg, are present at 30-90 $mgkg^{-1}$ dry wt. Fig. 1 shows the results using the two methods. The relatively high K content of the chips may suggest the high nutrient value of the chips.

Magnesium concentrations fluctuated from 38.57 and 38.20 mg kg^{-1} for sample D to 62.36 to 62.34 $mgkg^{-1}$ for sample G for methods A and B respectively. The lowest level of sodium Na was found to be 1709.3, and 1714.8 $mgkg^{-1}$ in sample B, in comparison with the highest level of 5548.2, and 5503.9 $mgkg^{-1}$ in sample C as analysed by methods A and B, respectively.

The results revealed that cobalt, chromium, molybdenum and thallium contents are less than the detection limits of the method. Meanwhile, nickel could be detected only in Sample B at a low level of 0.08 mg kg^{-1}. Also, all the potato chips samples are free of elements like Ag, As, Be, Cd, Hg, Li, Pb, Sb, Sn and vanadium. However, the Zn content in the present study was lower by ten folds than that reported for samples from Turkey[13].

However, potato chips from Turkey showed a much higher copper content [14]. Iron content, on the other hand was in the range of 0.2 -0.7 $mgkg^{-1}$. Again the Turkish potato chips showed iron content of 1.7 to 13 mg kg^{-1} [14]. However, the Zn content of our potato chips samples was lower by ten folds that reported for samples from Turkey[15]. It seems that all the potato chips samples are free of elements like Ag, As, Be, Cd, Hg, Li, Pb, Sb, Sn and vanadium.

Pearson's Correlation

The results were correlated in accordance with Pearson's rule and variety of correlations could be obtained for the results obtained by the two digestion methods as can be seen in Table 9. Transition metals like Cu, Zn, and Mn and Fe are very well correlated with each other as a result of the almost similar chemistry and the ability to be exchanged depending on the geochemistry of the soils in which the potatoes were cultivated[16]. It is interesting that selenium, a toxic element, showed very high correlation coefficients with transition metals like Cu, Zn, and Mn (R^2= 0.850, 0.899, 0.716 respectively.) This results is indicative that selenium occurs as selenides of such elements and that its toxicity is highly controlled. This is because the chemistry of selenium reveals the very low solubility of the metal selenides and consequently selenium is not available in the fluids of the human body. Very weak correlation can be shown between P and Mg and/or Ca, and relatively strong

correlation with K, which indicates that the phosphate is available for the humans as soluble compounds.

Conclusions

Essential and toxic elements can be determined using both methods, WD and MWD but method one (WD) has better recovery as compared to method two (MWD). By using method MWD higher recovery can be achieved for Al, Ba, Co, Cr, Cu, Fe, Li, Mn, Ni, and Zn. There was a good correlation for some elements. Furthermore, method WD is easier for filtration as compared to MWB.

Table 1: The instrumental parameters of ICP-OES measurements.

Parameters	Radial view
Plasma power	1400 W
Argon coolant flow	14.000 L/min
Argon auxiliary flow	0.900 L/min
Nebuliser flow	0.800 L/min
Strategy	Standard "28 sec per run"
Pump speed	30 pm

Table 2: Samples details

Sample name	**symbol**	**Taste**	**Source**
Duna duna	A DT	Tomato	Iran
Duna duna	B DV	Vinegar	Iran
Bato	C BS	Salt	Iran
Bato	D BL	Lemon	Iran
Kish	E KL	Lemon	Iran
Kish	F KC	Chilli pepper	Iran
Lezz	G LT	Tomato	Saudi Arabia
Lezz	H LC	Cheese	Saudi Arabia
Jacker	I JC	Chicken	Malaysia
Jacker	J JS	Spice	Malaysia

Table 3: LOD and wavelength of the macro-elements and strontium using both methods of digestion.

Elements/ λ (nm)	**LOD, mgkg^{-1}**	
	Method A*	**Method B***
Ca/315.887	0.004	0.005
K/766.491	0.032	0.057
Na/330.237	0.057	0.065
P/177.495	0.002	0.002
Sr/407.771	0.001	0.002
Mg/279.074	0.006	0.007

Table 4: LOD and wavelength of the micro-essential and trace elements using both methods of digestion.

Elements/ λ (nm)	LOD, $mgkg^{-1}$	
	Method A*	Method B*
Co/228.616	0.001	0.002
Cr/267.716	0.001	0.001
Cu/324.754	0.0012	0.001
Fe/259.941	0.00145	0.002
Li/670.780	0.001	0.001
Mn/257.611	0.001	0.001
Mo/202.095	0.001	0.001
Ni/231.604	0.001	0.001
Tl/190.864	0.004	0.003
Al/396.152	0.004	0.003

Table 5: LOD and wavelength ë of the Non-essential Toxic elements using both methods of digestion, $mg.kg^{-1}$.

Elements/ λ (nm)	LOD, $mgkg^{-1}$	
	Method A*	Method B*
As/189.042	0.00264	0.003
Ag/328.068	0.00121	0.001
Ba/455.404	0.00441	0.004
Be/313.042	0.001	0.001
Cd/214.438	0.001	0.001
Hg/184.940	0.001	0.001
Li/670.780	0.001	0.001
Pb/220.353	0.00345	0.004
Sb/206.833	0.00676	0.007
Se/196.090	0.002	0.003
Sn/189.991	0.001	0.001
V/292.402	0.00245	0.004
Zn/213.856	0.001	0.001

*Method A is wet ashing digestion on hotplate and *Method B is domestic microwave ashing digestion.

Table 6: Concentration of macro-essential elements in potato chips samples, mgkg^{-1} dry wt.

Samples	Methods	Ca	K	Mg	Na	P
A	Method A*	64.50	4964.6	54.89	3695.5	46.67
	Method B**	63.57	4990.2	54.31	3703.2	46.30
B	Method A	42.53	5132.9	63.86	1709.3	53.35
	Method B	40.73	5149.7	64.55	1714.8	52.30
C	Method A	50.81	7080.3	59.38	5548.2	71.38
	Method B	50.03	7072.9	59.71	5503.9	70.64
D	Method A	28.09	6340.1	38.57	3428.7	53.13
	Method B	30.64	6351.0	38.20	3597.3	52.16
E	Method A	92.45	4963.8	47.00	4519.6	77.68
	Method B	93.07	4953.7	47.50	4658.8	77.46
F	Method A	66.78	6470.9	45.29	4549.9	68.93
	Method B	68.23	6504.3	46.28	4556.8	68.96
G	Method A	33.78	8057.7	62.36	4653.2	76.65
	Method B	35.24	8113.9	62.34	4668.4	76.35
H	Method A	33.45	8130.0	49.36	2334.6	75.84
	Method B	32.98	8070.1	50.22	2370.0	74.96
I	Method A	32.57	6424.0	41.57	3809.3	62.50
	Method B	31.81	6403.3	40.79	3989.3	62.78
J	Method A	31.61	7263.8	50.38	2578.2	79.67
	Method B	31.43	7248.6	50.78	2548.8	78.61

Table 7: Concentration of microessential elements µg g^{-1} dry weight in potato chips samples in two different digestion methods:

Samples	Ba		Se		Zn	
	Methods of digestion					
	A	B	A	B	A	B
A	0.59	0.58	0.59	0.61	0.44	0.47
B	1.30	1.29	0.61	0.60	0.42	0.45
C	1.05	1.09	0.70	0.72	0.27	0.27
D	0.56	0.60	0.68	0.62	0.46	0.43
E	0.55	0.55	0.45	0.47	0.31	0.39
F	0.56	0.57	0.49	0.47	0.27	0.29
G	0.64	0.61	0.45	0.41	0.48	0.41
H	0.89	0.87	0.57	0.51	0.51	0.45
I	0.79	0.75	0.48	0.55	0.36	0.38
J	0.50	0.52	0.45	0.47	0.38	0.35

* Hotplate digestion method; ** microwave digestion method

Table 8: Concentration of nonessential toxic elements in mg/kg of potato chips using two digestion methods.

Sample	Method	Al	Cu	Fe	Mn	Sr
A	Method A*	1.04	0.20	0.51	0.39	12.76
	Method B**	0.99	0.24	0.54	0.37	14.00
B	Method A	0.52	0.20	0.46	0.25	11.40
	Method B	0.57	0.21	0.47	0.27	12.09
C	Method A	0.44	0.18	0.43	0.24	9.44
	Method B	0.35	0.12	0.15	0.20	3.95
E	Method A	0.79	0.11	0.22	0.15	16.55
	Method B	0.76	0.10	0.24	0.17	17.26
F	Method A	0.66	0.24	0.45	0.23	11.55
	Method B	0.75	0.21	0.50	0.25	13.22
G	Method A	0.53	0.32	0.18	0.23	3.78
	Method B	0.52	0.29	0.17	0.22	4.51
H	Method A	0.76	0.25	0.71	0.26	5.62
	Method B	0.75	0.28	0.68	0.22	4.17
I	Method A	0.71	0.04	0.40	0.22	3.21
	Method B	0.73	0.02	0.42	0.27	3.68
J	Method A	0.90	0.11	0.52	0.23	4.42
	Method B	0.88	0.12	0.50	0.22	4.47

Table 9: Spiked recovery determined for some represented elements for two methods used

Elements	Method A	Method B
	Recovery, %	
Al	90.9	104
Ba	94	96.9
Ca	96.9	120
Cd	100.6	93
Co	86.6	91.6
Cr	89.2	95.8
Cu	90.1	101.6
Fe	68.1	89.1
Li	88.9	93.9
Mg	99.6	143
Mn	89.2	97.8
Na	85.9	145
Ni	86.2	92.9
Sr	90	109
Zn	86.8	96.3

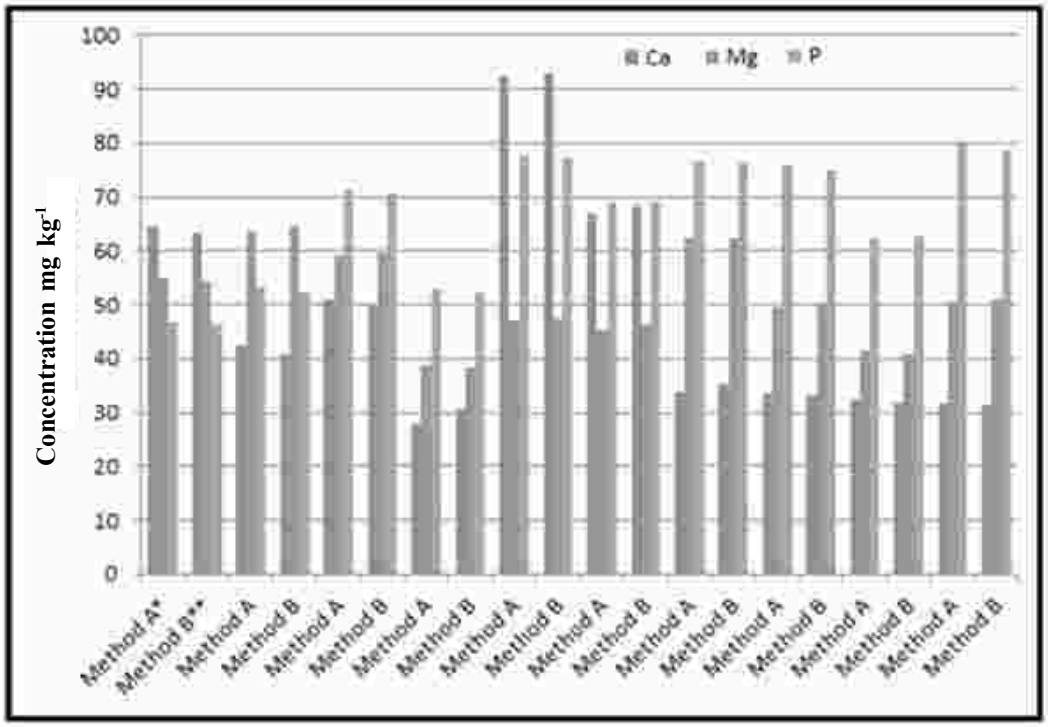

Fig. 1: Direct comparison of Ca, Mg, and P concentrations in the analysed samples by the two methods of digestion.

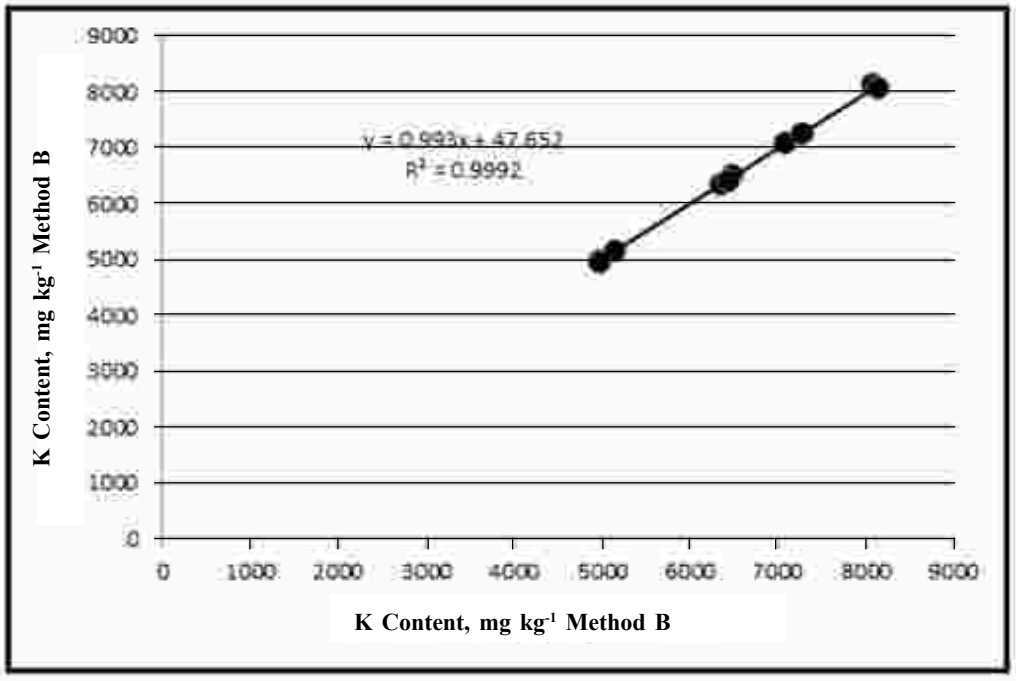

Fig. 2: Correlation of the results of potassium by the two methods.

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