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Gas Chromatographic Determination of Purines and Pyrimidines from DNA Using Ethyl Chloroformate as Derivatizing Reagent

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Abstract

An analytical method has been proposed for the separation and determination of guanine, adenine, cytosine, thymine and uracil by gas chromatography (GC) following precolumn derivatization using ethyl chloroformate. The GC separation was achieved from HP-5 (30 m × 0.32 mm id) column with layer thickness 0.25 µm. The linear calibrations were observed within 0.5-50.0 µmol/L for each of the compound and limits of detection were within 0.1-0.17 µmol/L. The derivatization, separation and quantitation was repeatable with intra (n=5) and inter (n=5) variation in terms of peak height/peak area and retention time with relative standard deviation (RSD) within 4.70-6.43%. The method was applied for the analysis of isolated DNA from human blood and plant leaves after acid hydrolysis. The concentration of thymine, adenine, cytosine and guanine in blood samples were observed within 0.602-2.135 µmol/L of each compounds with RSD 2.60-6.00%. The recovery of the nucleobases by standard addition was calculated within 98-108% with RSD 2.5-7.8%.

Keywords: Gas chromatography; Guanine; Adenine; Cytosine; Thymine; Ethyl chloroformate.

Introduction

Deoxyribonucleic acid (DNA) is a bio micro molecule and plays an important role in genetic information storage and biological synthesis of proteins [1]. It is a linear polymer synthesized by nucleotide units. The nitrogenous bases Purines [adenine (A) and guanine (G)] and Pyrimidines [Cytosine (C), Uracil (U) and Thymine (T)] are essential building blocks of RNA and DNA. They are considered as key to DNA's stability, replication and capacity for information storage. They also participate in various processes in living organism such as energy transduction, metabolic cofactors and cell signaling [2]. A variation in the concentration of pyrimidines and purines in the organism may be caused by certain diseases such as Parkinson's, epilepsy, prostatitis, childhood and breast cancers [3]. Therefore it is significant to analyze the concentration of T, A, C and G in DNA for clinical pathology [4]. This has attracted the attention of analytical chemists to develop methods to detect and determine these bases of DNA for understanding the mechanism of replication and damage process in the DNA structure.

Several analytical methods have been developed for the determination of DNA bases including electro-analytical [1-5], spectrophotometry [6], mass spectrometry [7], high performance liquid chromatography [8, 9],

capillary electrophoresis [10], gas chromatography [11-14] and gas chromatography-Mass spectrometry. For the simultaneous determination of pyrimidines and purines, HPLC is more frequently used, but capillary GC is free from use of expensive solvents and their disposal.

GC analysis of purines and pyrimidines is completed, however they are not volatile enough and stable thermally to elute out from the column of GC. The derivataization with suitable reagent is carried out prior to GC analysis. The reagents such as bis(trimethylsilyl) trifluoro-acetamide [12-15], pentafluorobenzoyl chloride, pentafluorophenylsulfonyl chloride or heptafluorobutyric anhydride [16], *N,N*-tert-butyl-dimethylsilyl-trifluoroacetamide [13] and *N*-(tert-butyldimethylsilyl) *N*-methyl trifluoroacetamide [14] are used. The derivatization with different silyl reagents though effective, but requires non-aqueous medium for derivatization. Simple and inexpensive reagent which could be used in aqueous phase may be valuable in GC determination of purines and pyrimidines. Ethyl chloroformate has been used as derivatizing reagent in aqueous-organic phase for the GC determinations of amines and amino acids [17]. Husek has reported the applications of chloroformates as reagents for general purpose in GC [18], Simek and Husek have reported the applications of alkyl chloroformates as esterifing reagents [19]. GC analysis of a number of amino compounds has been carried out using chloroformates [20].

The present work suggests the applications of ethyl chloroformate as reagent for derivatizing the GC analysis of G, A, C, T and U, after acid hydrolysis of isolated DNA. The optimization of the derivatization conditions, limits of quantitation and detection, reproducibility of separation and quantitation are reported.

Materials and Methods

The compounds thymine (T), adenine (A), uracil(U), cytosine (C)guanine (G) 99% purity were purchased from sigma Chemicals Co. St. Louis, USA, ethyl chloroformate (ECF) (Fluka, Buchs, Switzerland), methanol (> 99% purity) (Rdh Chemicals Co. Spring Valley, CA) chloroform, acetonitrile (Fluka, Buchs, Switzerland) and pyridine (E-Merck, Darmstadt, Germany) were used.

Guaranteed reagents grade hydrochloric acid (37%), perchloric acid (70%), acetic acid, potassium chloride, ammonium acetate, sodium acetate, sodium tetraborate, boric acid, sodium carbonate, sodium bicarbonate, ammonia solution and ammonium chloride were from E-Merck, Dramstadt, Germany.

Stock solution of G, A, C, T and U containing 1 mmol/L were prepared in water-methanol. Further solutions were prepared by appropriate dilution. Buffer solution (0.1 M) between pH 1-12 at unit interval were prepared from the following: potassium chloride adjusted pH with hydrochloric acid (pH 1-2), sodium acetate-acetic acid (pH 3-6), ammonium acetate (pH 7), sodium tetraborate-boric acid (pH 7.5-8.5), sodium carbonate-sodium bicarbonate (pH 9), ammonia-ammonium chloride (pH 10) and potassium hydroxide-potassium chloride (pH 11-12).

Collection of samples

The samples of blood were collected by vein puncture in EDTA tubes with verbal permission at Laboratory of Molecular Biology, Liaquat University of Medicine and Health Sciences (LUMHS), Jamshoro for PCR analysis. A part from extracted DNA at Molecular laboratory LUMHS was obtained with permission and was used after acid hydrolysis for the determination of G, A, C and T contents. Similarly rice, cotton and jasmine leaves were collected and extraction of DNA was carried out at Nuclear Institute of Agriculture (NIA) Laboratory, Tando jam, Atomic Energy Commission of Pakistan. The extracted DNA was analyzed immediately as received.

Instrumentation

The measurements of pH were performed by an Orion 420A pH meter (Orion Research Inc., Boston, USA) with glass electrode combined along with internal reference electrode. GC analysis was

carried out on an Agilent model 6890 network GC system, connected with split injector and flame ionization detector (FID), pure nitrogen (British oxygen company (BOC), Karachi, Pakistan) and hydrogen generator (Parker Balston, Analytical Gas System, H_2-90, Parker Hannifin Haverhill, USA) and computer with software of Chemstation controlled the GC. Capillary column HP-5 (30 m × 0.32 mm id) with film thickness 0.25 µm (J&W Scientific GC column, Wilmington NC (USA) was used during entire study.

GC-FID procedure

Acetonitrile-water-methanol-pyridine (40: 40:10:10 v/v/v/v) (0.5 mL), sodium carbonate buffer solution (pH 9) (0.5 mL) and ethyl chloroformate (ECF) (0.5 mL) were mixed with the solution (0.2-1.0 mL) containing G, A, C, T and U (0.5-50 µmol/L). The solution was kept in an ultrasonic bath for 15 min at room temperature (30°C) after which chloroform (0.5 mL) was mixed in the solution mixture. The contents were shacked well and the two layers were allowed to separate from one another. A part from extracted mixture (0.5 mL from 1 mL) was pipette and then transferred to screw capped vial and 1 µL was injected in to GC. The split ratio was set at 10:1 and carrier gas nitrogen was used having flow rate of 1.5 mL/min. The column oven temperature was programmed from 90°C (held for 1 min) to 250°C at 25°C/min, then held for 1 min, the temperatures of detector and injector were 280°C and 270°C respectively. The flow rates for FID were fixed for nitrogen as make up gas 45 mL/min, hydrogen 40 ml/min and air 450 mL/min.

Isolation of DNA from blood samples

The DNA isolation from human blood samples was performed as reported [21], following inorganic method [22]. Cooled 5 mL of blood at -20°C was thawed and added tris EDTA buffer (15 mL), mixed and centrifuged. Supernatant was discarded and the buffer was again added and process repeated till pellets became light pink. The pellets were re-suspended in a buffer and treated with SDS and proteinase K overnight. Completely digested pellets were cooled and added sodium chloride solution. The mixture was cooled and centrifuged at 3000 g to pellet down the salts and protein. The supernatant was collected and centrifuged again at 3000 g. The Supernatant was added equal volume of isopropanol to precipitate DNA. The contents were centrifuged and supernatant was discarded. The DNA pellet was washed with 70% ethanol and air dried at 37°C. The precipitate was dissolved in Tris HCl-EDTA buffer at 37°C by placing in shaking incubator overnight. The DNA was inactivated by placing in shaking water bath at 70°C. The final volume 1.0 mL was placed in screw capped vial at -20°C.

Extraction of DNA from plant tissue

The slightly modified method was applied for DNA extraction from tissues of plant samples as reported [23]. The 5g of cotton, rice and jasmine leaves were collected, cleaned, washed and were ground by adding 5 mL of cetyltrimethyl ammonium bromide (CTAB) buffer in ice machine for 20-30 min and then left to attain room temperature. The 5 mL of isoamyl alcohol-chloroform (1:24) was added in mixture and shacked well. The mixture contents were centrifuged at 4000 g for 30 min. The supernatant was collected and treated again with 5 mL of isoamyl alcohol-chloroform (1:24). The 5 mL of 2-propanol was added in to collected aqueous layer and finally at the bottom of the tube off white threads were appeared which were centrifuged 1000 g for 5 min. The residue was dissolved in 1 mL Tris-EDTA (T.E) buffer.

Acid hydrolysis of DNA

The sample of DNA was prepared (0.1 mL) by adding perchloric acid (1 mL) in screw capped tube and heated at 95-100°C in water bath for 1 hr. Sodium hydroxide (2 M) was used to adjust the pH of the solution to 7 and solution was filtered. The solution (0.2-0.5 mL) was treated as GC-FID procedure. The quantitation of G, C, A and T was made from external calibration curve prepared from linear regression equation $y = mx + b$.

Analysis of G, A, C and T from acid hydrolyzed DNA using linear calibration curve with spiked samples

The acid hydrolyzed DNA (0.5 mL) was taken in duplicate. A portion was processed as GC-FID procedure and the other was added to the solution of G, C, A, T (0.5 mL) containing 1 µmol/L each and GC-FID procedure was again followed. The quantitation was carried out from external calibration curves and from the increase in response with added standards.

Results and Discussion

The reaction of ECF towards purines and pyrimidines; G, A, C, T and U was examined to form their derivatives and their elution from GC column was investigated reaction is shown in Fig. 1.

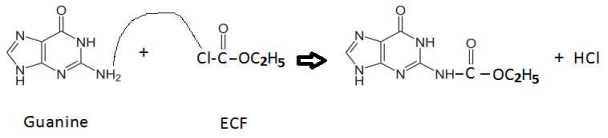

Figure 1. **Represents the reaction of ECF with one of the Purine base (Guanine).**

Effects of pH, reaction medium and time and solvent extraction of the derivatives were optimized. Each time GC-FID response (peak area/peak height) for each compound was checked. The experimental results that showed maximum response (peak height/peak area) were considered optimum. The effect of pH was examined between 1-12 at unit interval and quantitative and reproducible reaction was observed with maximum peak area/peak height in alkaline medium with bicarbonate buffer having pH 9. Husek et al also reported favourable reaction of amino acids with ECF at pH 9 [17]. Husek proposed acetonitrile-water-methanol-pyridine (40:40:10:10 v/v/v/v) as reaction media for the derivatization of amines and amino alcohols. In the present work the solvent system suggested by Husek [17] and methanol, actronitrile, aqueous acetronitrile, aqueous methanol and an aqueous pyridine solution were examined as reaction media. The solvent system proposed by Husek proved optimum for the derivatization reaction for reproducible and quantitative response, and was used. The reaction mixture was sonicated at room temperature (30°C) for 5-20 min at an interval of 5 min. A maximum response was obtained for the sonication time of 15 min and above and 15 min was selected. The addition of ECF was varied from 0.1-0.6 mL at an interval of 0.1 mL and it was noted that the addition of ECF was not critical as long excess was available in reaction medium. The addition of 0.5 mL was selected for derivatization and synergetic extraction. The effect of addition of variable volumes of the carbonate buffer pH 9 was varied from 0.2-1.0 mL at an interval of 0.2 mL. The effect of addition of variable volumes of buffer solution was not significant and the addition of 0.5 mL was selected. The solvents such as tertiary butanol, chloroform, ethyl acetate and 1,2-dichloroethane were studied for the derivatives extraction. The solvents 1,2-dichloroethane and Chloroform showed better extraction efficiency, but chloroform was chosen as extraction solvent as suggested earlier [17]. A solution of each derivative was separately analyzed, and each of the derivative eluted as a single peak and separated from the derivatizing reagent. GC response after derivatization was examined after different time intervals and no change in response (average peak height/peak area, (n=4) was observed for the purines and pyrimidines investigated up to 18 h.

GC separation of G, A, C, T and U was examined from GC column HP-5 (30 m × 0.32 mm id) using different temperature programs and nitrogen flow rates. Total run time was 10 min as shown in Fig. 2a and b.

The order of elution of the compounds was T, U, C, A and G with capacity factor (k') 1.37, 1.66, 2.04, 2.45 and 2.87 respectively. Repeatability of the separation was studied with regard to peak height and retention time (n=5) and RSDs were obtained in the range of 4.70% and 6.43% respectively. Gelifkens et al. reported the separation of U, T, C, A and G as trimethylsilyl derivatives from 12 m SE 30 fused silica column within 20 min [24]. Glavin et al separated 7 nucleobase standards; U, T, C, hypoxanthine (HX), A, xanthine (X) and G as N,N-tert-butyl-dimethylsilyl-trifluoroacetamide from 15 m × 0.25

mm id, 5% diphenyl, 95% dimethylpolysiloxane column with the Helium flow rate 1.3 ml/min within 30 min [13]. The present work report separation of 5 nucleobases within 8.5 min.

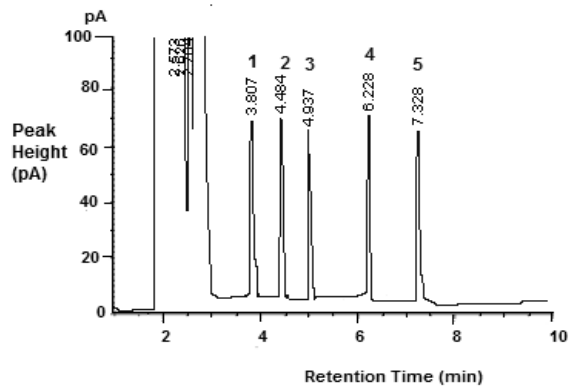

Figure 2a. **Separation of (*1*) T, (*2*) U, (*3*) C, (*4*) A, (*5*) G as ECF derivatives**

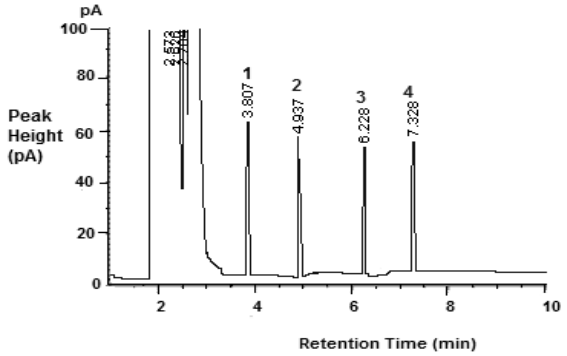

Figure 2b. **GC analysis of acid hydrolyzed DNA from human blood sample (*1*) T, (*2*) C, (*3*) A, (*4*) G as ECF derivatives.**

Linearty of calibration curves for the five DNA nucleobases checked by recording mean peak area/peak height against concentration was obtained in the range of 0.5-50 µmol/L with coefficient of determination (r^2) within 0.990-0.993. LODs were calculated as signal to noise ratio (3:1) within 0.10-0.17 µmol/L. Limits of quantitation (LOQs) measured as signal to noise ratio (10:1) were within 0.30-0.51 µmol/L. Co-efficient of determination and Calibration ranges are given in Table 1.

Repeatability of separation, derivatization and quantitation was studied in intra (n=5) and inter (n=5) day variation by the same operator in terms of retention time and peak height/peak area. RSDs were obtained within 4.70% and 6.43% respectively. Five test solutions of the nucleobase G, A, T, C and U were determined within the range of calibration and relative error was obtained within 2.6-5.2%.

The existence of glucose, ascorbic acid, methyl parabin, gum acacia, Na, K, Ca, Mg, Cl, CO_3, SO_4, NO_3 did not affect the determination atleast twice the concentration of nucleobases G, A, C, T and U.

Table 1. **Results of quantitaion of purine & pyrimidine bases using ethyl chloroformate as derivatizing reagent.**

Purine & Pyrimidine Bases	Limit of Detection (LOD) µmol/L	Limit of Quantitation (LOQ) µmol/L	Calibration Range µmol/L	Co-efficient of determination (r^2)
Thymine T)	0.10	0.30	0.50-50	0.990
Uracil (U)	0.10	0.30	0.50-50	0.990
Adenine (A)	0.15	0.45	0.50-50	0.993
Cytosine C)	0.15	0.45	0.50-50	0.991
Guanine (G)	0.17	0.51	0.50-50	0.993

Analysis of samples

In order to access the possible analytical applications of the developed procedure, the simultaneous analysis of A, G, T, C and U in the acid hydrolyzed DNA of human blood and plants were performed. The fresh calibration curves were prepared for simultaneous determination from the standards and the values of nucleobases in samples were determined by direct interpolation in the linear regression equation. The obtained results are briefed in Table 2 and typical chromatogram is shown in Fig 2b. T, C, A and G were determined from both human blood and plants leaves DNA samples, but U was detected below LOD.

The identification of the peaks was based on comparison of retention times to that of the standards of nucleobases. The amount of T, A, C and G from human blood samples were detected 0.625-2.061 µmol/L, 0.615-2.132 µmol/L, 1.019-1.520 µmol/L and 1.031-1.505 µmol/L respectively with RSDs within 2.60-6.00%. The ratio between A: T and C: G for each sample of blood was calculated and observed within 0.96-1.01 and 0.97-1.04 respectively. The values of (A+ T)/ (C+ G) for the each DNA sample were calculated 0.98-1.02.

Table 2. **Analysis of acid hydrolyzed DNA of human blood and plants (cotton, rice and jasmine) for T, C, A and G concentration.**

Purine & Pyrimidine Bases	Blood Sample 1 µmol/L	Blood Sample 2 µmol/L	Blood Sample 3 µmol/L	Blood Sample 4 µmol/L	Blood Sample 5 µmol/L	Cotton Sample 1 nmol/g	Cotton Sample 2 nmol/g	Rice Sample 1 nmol/g	Rice Sample 2 nmol/g	Jasmine Sample 1 nmol/g	Jasmine Sample 2 nmol/g
T	1.383 (2.80) * 1.383 (2.5)	0.625 (2.79)	1.903 (2.96)	1.823 (2.75)	2.061 (5.11)	0.946 (5.0) *0.948 (2.8)	1.039 (3.0)	0.886 (3.8) *0.878 (3.9)	1.106 (4.9)	0.558 (8.3) *0.554 (2.7)	0.540 (5.0)
C	1.520 (4.15) * 1.512 (3.3)	1.019 (3.64)	1.387 (3.52)	1.479 (4.38)	1.413 (6.00)	0.802 (3.4) *0.798 (3.8)	0.799 (4.3)	1.021 (4.8) *1.022 (5.7)	0.910 (3.3)	0.849 (2.9) *0.845 (3.0)	0.725 (2.68)
A	1.370 (2.76) * 1.365 (3.2)	0.615 (2.90)	1.890 (3.08)	1.836 (3.85)	2.132 (2.84)	0.955 (4.3) *0.954 (6.1)	1.043 (4.8)	0.900 (2.7) *0.904 (4.9)	1.060 (5.0)	0.563 (4.7) *0.562 (7.8)	0.555 (3.0)
G	1.485 (3.52) * 1.603 (4.9)	1.031 (3.42)	1.397 (2.60)	1.505 (2.94)	1.473 (2.64)	0.857 (3.4) * 0.846 (5.0)	0.780 (3.6)	1.016 (2.8) *1.015 (6.9)	0.912 (4.7)	0.860 (4.8) *0.858 (4.7)	0.699 (5.2)

Note: The values given in parenthesis represent % RSD.
T= thymine, C= cytosine, A= adenine and G= guanine.
* = Spiked sample.

Similarly six DNA samples of plants were determined and the detected amounts of T, A, C, G found were 0.540-1.106 nmol/g, 0.555-1.060 nmol/g, 0.725-1.021 nmol/g and 0.699-1.016 nmol/g respectively with RDS within 2.68-8.3.0%. Similarly the results for A: T, C: G and (A+ T)/ (C+ G) for each plant sample were detected within 0.94-1.10, 0.95-1.06, and 0.98-1.03 respectively. The samples from each acid hydrolyzed human blood and plant DNA were spiked with 1.0 µmol/L of T, A, C and G each and determination was carried out using GC-FID method. The results obtained agreed with external calibration and recovery was obtained within 98-108% with RDS 2.5-7.8%.

Comparison of the obtained results with reported values

The results for Cytosine (C) agreed with that of Xia et al. [25] and also for Cytosine (C), Thymine (T) and Adenine (A) with Xia et al. [26] for samples of human plasma. Our results for plants DNA nucleobases also satisfied with the reported values by Zhao et al. [27]. The developed GC procedure was also compared with reported procedures in terms of analysis time, calibration range, applications and LOD Table 3.

Our proposed procedure compares favorable with the reported methods [27-31] in terms of broader calibration range and shorter analysis time for all the compounds detected. The present method is less sensitive than GC-MS-MS [28] and is ESI-MS [30], but GC-MS-MS and ESI-MS are not available at many of the working laboratories. However, the present method is based on GC-FID, for which the equipment is ordinary available in most of the laboratories.

Table 3. **Comparative study of the reported methods used for the determination of purine & pyrimidine bases with present method.**

Method	Compounds Determined	Derivatizing Reagent	Calibration Range	Limit of Detection	Analysis Time	Application	Reference
C.E	Urea, Creatinine, T, A, U, G, Adenosine, Histidine	-	5-500 µmol/L	0.84-4.25 µmol/L	20 min	Urine	[27]
GC-MS-MS	U, T, Dihydrouracil, Dihydrothymine	*tert*-Butyl dimethyl silyl trifluoroacetamide	25-2500 pmol/L	0.80-8.0 pmol/L	12 min	Urine Samples	[28]
HPLC-Amperometric Detector	C, G, T, A, 5-Methylcytosine	-	0.1-10 µmol/mL	26.3- 162.1 nmol/mL	15 min	Biological Samples (Calf Thymus and Fish Sperm)	[29]
Hydrophilic Interaction Chromatography (HILIC) and Electrospray Ionization Mass Spectrometry (ESI-MS)	T, U, Thymidine, Uridine, A, C, G, Xanthine, Hypoxanthine	-	0.001-5.00 µmol/mL	0.15-10.0 nmol/mL	60 min	Natural *Cordyceps (Chinese Medicinal Plant)*	[30]
HILIC	C, U, G, Cytidine, Xanthine	-	0.0025-1.816 µmol/mL	0.07-30.49 nmol/mL	110 min	Geosaurus and Leech	[31]
GC-FID	A, C, T, G and U	Ethyl Chloroformate (ECF).	0.5-50 µmol/L	0.1-0.17 µmol/L	10 min	Human Blood & Plant DNA (Cotton, Rice & Jasmine)	Present method

Conclusion

A GC-FID procedure has been proposed for the detection of T, A, C, G and U from aqueous media using ECF as derivatizing reagent with short GC separation time (10 min). The proposed analytical procedure is applicable for the determination of DNA nucleobases after acid hydrolysis of DNA of human blood and plants.

References

1. L. Svorc and K. Kalcher, *Sens. Actuat. B: Chem.,* 194 (2014) 332.
2. A.L. Burueho, R. Cabrerizo, N. G. Mamsilla, S. Sookoian and C. J. Pirola, *J. Nutr, Biochem.,* 24 (2013) 6.
3. P. Cekan and S. T. Singurdnon, *J. Am. Chem. Soc.,* 131 (2009) 18054.
4. A. J. Li, P. McAllister and B. Y. Karlan, *Gynecol Oncol*, 116 (2010) 105.
5. Q. Xu, X. Liu, H-bo Li, Li-na and X. Hu, *Biosens. Bioelect.*, 42 (2013) 355.
6. I. Heisler, J. Ketler, R. Tauber, M. Sutherland and H. Fuchs, *Anal. Biochem.*, 302 (2002) 114.
7. Y. F. Huang and H. T. Chang. *Anal. Chem*, 79 (2007) 4852.
8. K. Kanek, I. T. Yamanobe and S. Fujimori, *Biomed. Chromatogr.*, 23 (2009) 858.
9. C. Carrilo-Carrion, S. Armenta, B. M. Simonet, M. Valearcel and B. Lendi, *Anal. Chem*, 83 (2011) 9391.
10. M. Haunschmidt, W. Buchberger and C. W. Klampfl, *J. Chromatogr. A*, 1213 (2008) 88.
11. F-Q. Yang, L. Geb, J. W. H. Yong, S. N. Tan and S-P. Li, *J. Pharm. Biomed. Anal.,* 50 (2009) 307.
12. T. Kuhara, C. Ohdoi and M. Ohse, *J. Chromatogr. B.*, 758 (2001), 61.

13. D. P. Glavin, H. J. Cleaves, A. Buch, M. Schubert, A. Aubrey, J. L. Bada and P. R. Mahaffy, *Planet. Space Sci.*, 54 (2006) 1584.
14. S. M. Horst., R. V. Yelle., A. Buch., N. Carrasco., G. Cerno-gora., O. Dutuit, E. Quirico., E. Sciamma-o Brien., M. A. Smith., A. Somogyi., C. Szopa., R. Thissen and V. Vuitton, *Astrobiology,* 12 (2012) 1.
15. D. B. Lakings and C. W. Gehrke, *Clin. Chem.*, 18 (1972) 810.
16. A. Nazareth, M. Joppich, S. Abdul-Baky, K O'Connell, A. Setissh and R. W. Giese, *J. Chromatogr.*, 314 (1984) 201.
17. P. Husek, *J. Chromatogr.*, 552 (1991) 289.
18. P. Husek, *J. Chromatogr. B*, 717 (1998) 57.
19. P. Husek and P. Simek, *Current, Pharm. Anal.,* 2 (2006) 23.
20. M. Y. Khuhawar, L. A. Zardari and A. J. Laghari, *Chromatorgaphia,* 67 (2008) 847.
21. S. Ali, B. Ahmed, S. Bashir, N. Mahmood and J. Ali, *Life Sci. J.,* 10 (2013) 310.
22. J. Grimderg, S. Nawosehik, L. Belluscis, R. Mckee, A. Truck and A. Eisenberg, *Nucleic Acid Res.,* 17 (1989) 8390.
23. K. Choudhary, N. Mathur, O. P. Choudhary and U. Pillaiio, *Adv. Bio. Res.*, 2 (2008) 83.
24. C. F. Gelijiken, D. L. Smith and J. A. McCloskey, *J. Chromatogr.,* 225 (1981) 291.
25. J-F. Xia, Q-L. Liang, P. Hu, Y-M. Wang, P. Li and G-A. Luo, *Clinica Chimica Acta*, 412 (2011) 940.
26. J-F. Xia, Q-L. Liang, P. Hu, Y-M. Wang, P.Li and G-A. Luo, *Clinica Biochem,* Vol. 42 (2009) 215.
27. H-Q. Zhao, X-Wang, H-M. Li, B. Yang, H-J. Yang and L. Huang, *Molecules,* 18 (2013) 9755.
28. T. Adam, P. Lochman and D. Friedeck, *J. Chromatogr. B,* 767 (2002) 333.
29. U. Hofmann, M. Schwab, S. Seefried, C. Marx, U. M. Zanger, M. Eichelbaum and T. E. Murdter, *J. Chromatogr: B*, 791 (2003) 371.
30. T. A. Ivandini, K. Honda, T. N. Rao, A. Fujishima and Y. Einaga, *Talanta,* 71 (2007) 648.
31. P. Chen, W. Li, Q. Li, Y. Wang, Z. Li, Y. Ni and K. Koike, *Talanta,* 85 (2011) 1634.