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Assessment of the Potency of Ceftiofur Sodium Powder through Validated Microbiological Method

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Abstract

Ceftiofur Sodium is a semi-synthetic beta-lactamase broad spectrum third generation of cephalosporin antibiotic. The development and validation of a two-level agar diffusion (2+2) bioassay to quantify ceftiofur in powder for injection is described in this paper. Bacterial strain *Bacillus subtilis* ATCC 6633 (MTCC 441) was selected and used as the most significant strain against ceftiofur sodium. The mean potency recovery value of ceftiofur sodium in marketed sample XCEFT powder for injection was estimated to be 101.79%. All potency results were statistically analyzed and found to be linear ($r^2 = 0.9916$) in the range of 1.0-10 μgmL^{-1}, with the intermediate precision RSD between days was 0.81%; intermediate precision RSD between analyst was 0.43% and accuracy 101.08%, RSD = 0.28%. The findings backed up the proposed microbiological technique, which allowed for accurate ceftiofur sodium quantification in pharmaceutical samples. Furthermore, bioassay is a useful, easy and cost-effective method for controlling the quality of ceftiofur sodium in raw material as well as in pharmaceutical preparations.

Keywords: Ceftiofur sodium, *Bacillus subtilis*, Bioassay, Quality Control, Potency.

Introduction

Ceftiofur sodium is a semi synthetic, beta-lactamase broad spectrum third generation cephalosporin group antibiotic, approved for use in veterinary medicine by Food and Drug Administration (FDA). Ceftiofur sodium has been developed as dry powder injection which is reconstituted in sterile water prior to intramuscular administration. Ceftiofur is used to treat respiratory disease in swine, ruminants and horses associated with *Actinobacillus (Haemophilus) Pleuropneumoniae, Pasteurella multocida, Pasteurella himolytica, Salmonella choleraesuis* and *Streptococcus suis*[1-4]. Ceftiofur is resistant to beta lactamase producing organisms and has an antibacterial activity against both Gram-positive and Gram-negative bacteria.[4,5] Escherichia coli strains resistant to ceftiofur have been reported.[6]

Ceftiofur sodium is sodium 7-[(Z)-2-(2-amino-1,3-thiazol-4-yl)-2-methoxyimino)acetamido]-3-(2-furoylthiomethyl)-3-cephem-4-carboxylate (Fig. 1) with the chemical formulae $C_{19}H_{16}N_5NaO_7S_3$ and a molecular weight of 545.5. It is an off-white crystalline powder.[7]

The pharmacopoeias recommend using high performance liquid chromatography (HPLC) with UV detection and a mobile phase composed of water, phosphate buffer, and acetonitrile for official quality control of ceftiofur sodium as a raw

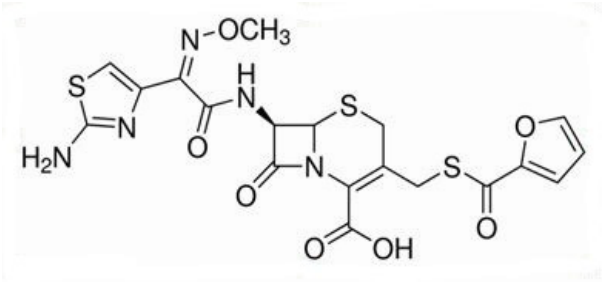

Fig. 1: Chemical Structure of Ceftiofur sodium

ingredient or in pharmaceutical preparations.[7]

There is only one method reported in literature which employs a 3×3 agar diffusion assay using *Micrococcus luteus* (ATCC 10240) as the test organism for their microbiological assay for the quantitative determination of ceftiofur sodium powder.[8] However, two level (2×2) factorial agar diffusion microbiological assay method, which was validated in this present study for quantitative determination of ceftiofur sodium in dry powder injection, is mentioned only in the Indian Pharmacopoeia.[7] Several methods for determining ceftiofur in cow milk and serum using high performance liquid chromatography have been reported in the literature.[9-10]

Microbiological assay for other antibiotics such as streptomycin, spiramycin, tylosin and oxytetracycline used in veterinary practice have been described in Indian Pharmacopoeia.[7] Because ceftiofur sodium is used to treat bacterial infections in animals, alternative methods for quantification in pharmaceutical dosage forms must be developed. To calculate the antimicrobial activity of antibiotics using bioassay method, their inhibitory effect on the growth of the test microorganisms is evaluated.[7,11-13] Moreover, the bioassay can monitor subtle adjustments which can no longer be detected by means of traditional chemical approach. The bioactivity as well as the potency of an antibiotic can be estimated by microbiological assay. The cylinder plate method extensively used in assay of antibiotics, correlates the area of inhibition with the dose of the antibiotic being tested. This study has taken in to account the theoretical relationship between the area of inhibition zone and the concentration of antibiotic in a solution carried out in perforated plate. Practically the area of inhibition zone is directly proportional to the antibiotic concentration.

The reproducibility and precision of data from the cylinder plate method have led to the development of advanced statistical techniques.[14] The agar diffusion method can also be used to create calibration zones that isolate the range of susceptible, resistance, or confer with more sophisticated responses of microorganisms to antibiotic drugs.[15] Microbiological assay strategies provide an appropriate amount of antibiotic potency while posing only a minor risk of interference from degradation products or biologically inactive components.[16]

This article presents a study to develop and validate an easy and precise microbiological assay by agar diffusion technique for quantitation of ceftiofur sodium as a raw material and injectable formulation, as an alternative to the physicochemical methods prescribed in pharmacopoeias.

Material and Methods

Chemicals

A working standard of ceftiofur sodium (purity assigned 98.0%) was used as a reference substance, and a commercially sample XCEFT dry powder for injection containing ceftiofur sodium 250 mg per vial was obtained from the market. Reference and test solution were prepared in sterile distilled water.

Equipment

Calibrated and validated equipment was used for bioassay study. Sterilized glassware of Class B such as pipettes, volumetric flasks, petri plates and sterile borer were used during the test. Microbiological media was sterilized at 121 ^{o}C and 15 psi for 15 min in an autoclave. For the microbiological assay, glycerol stocks of microbial cultures stored in deep freezer were used. Petri plates of microbiological assay were incubated at 37 ^{o}C in BOD Incubator. Antibiotic zone reader was used for measuring the area of circular inhibition zones.

Microbial strains

Bacterial strains were purchased from national culture collection centre such as Microbial Type Culture Collection (MTCC) and National Collection of Industrial Microorganisms (NCIM) which was equivalent (eq.) to international culture collection centre as American Type Culture Collection (ATCC) and National Collection of Type Culture (NCTC). Total 9 bacterial strains were used in bioassay among them 4 are Gram-negative and 5 are Gram-positive bacteria. The Gram-negative bacteria are *Escherichia coli* (MTCC 1687 eq. to ATCC-8739), *Salmonellae enterica serotype abony* (MTCC 3858 eq.

to NCTC-6017), *Bordetella bronchiseptica* (NCIM 5389 eq. to ATCC-4617), *Pseudomonas aeruginosa* (MTCC 1688 eq. to ATCC-9027) and Gram-positive bacteria are *Bacillus subtilis* (MTCC 441 eq. to ATCC-6633), *Staphylococcus aureus* (MTCC 737 eq. to ATCC-6538P), *Staphylococcus aureus* (MTCC 96 eq. to ATCC-9144), *Staphylococcus epidermidis* (MTCC 3615 eq. to ATCC-12228), *Kocuria rhizophila* (MTCC 1541 eq. to ATCC-9341).

Microbiological media

The purpose of the media is to promote the quick development of the tested organism being used in the bioassay. Media used for bioassay were procured from Mumbai. Base layer and seed layer was prepared using an Antibiotic Assay Medium No. B. The composition of the media contained 6.0 gL^{-1} peptone, 3.0 gL^{-1} yeast extract, 1.5 gL^{-1} HM peptone B and 15.0 gL^{-1} agar powder; final pH was adjusted at 6.55 ± 0.05. For bacterial growth, fresh slants of tryptone soya agar were used. Distilled water was used for media preparation and sterilized in an autoclave at 121°C and 15 psi for 15 min. The final pH was adjusted in accordance with the instructions on the media container.

Solution of reference substance

A sufficient quantity of reference substance of ceftiofur sodium was accurately weighed and dissolved in 25 mL sterile distilled water to obtain 1000 μgmL^{-1}. The stock solution was kept in a refrigerator. On the day of the experiment, different dilutions viz. 10, 5.0, 4.0, 2.0 and 1.0 μgmL^{-1} were prepared in sterile distilled water from the stock solution. The concentrations of reference solution 4.0 μgmL^{-1} and 1.0 μgmL^{-1}, both with a 4:1 dilution ratio were chosen as reference standard high (S_H) and reference standard low (S_L), respectively.

Sample solution

To make a 1000 μgmL^{-1} stock solution of sample, 250 mg of XCEFT powder for injection sample was diluted in 250 mL of sterile distilled water. Aliquots of this stock test solution were diluted in ratio of 4:1 in distilled water to get the sample high concentrations (T_H = 4.0 μgmL^{-1}) and the sample low concentration (T_L= 1.0 μgmL^{-1}), which were used in bioassay.

Standardization of inocula preparation

Glycerol stocks of bacterial cultures were revived and cultured on the slants of Tryptone soya agar to maintain the growth of bacteria. Tryptone soya agar slants were incubated for 24-48 h at 37 °C. After incubation, washed out with 3 mL sterile saline (0.9%) to harvest the growth of organism from the surface of agar slants and diluted appropriate amount of harvest suspension to determine the target value which gave approximately 25% transmission at 530 nm using UV spectrophotometer. This diluted inoculum was stored under refrigeration and used for further experiments of microbiological assay.

Bioassay method using agar diffusion

The cup plate method for two-level factorial microbiological assay was carried out in quadruplicate. The cup plate method relies on antibiotic diffusion through a solidified agar layer in a Petri plate through a vertical cup or cavities. The growth of the specific microorganism inoculated in the agar is prevented in a circular area around the cup or cavities containing the solution of the antibiotic.[7] In a 100 mm × 20 mm Petri dish, pour 21 mL un-inoculated base layer of assay medium and allow it to harden in to a smooth base layer of uniform depth. After solidification, determined target value of suspension of microorganisms was added to seed layer agar medium to prepare double-layer plates of assay by pouring 4 mL to spread the inoculums uniformly over a solidified base layer surface and allow to solidified.[11] These plates were left to solidify for at least 30 min. Four circular holes were bored having a diameter of 8 mm in to the solidified agar plate with the help of sterile borer. These holes were marked as low and high concentration with respect to reference and sample solutions. Through micropipette, these labelled holes were filled with 100 µL reference substance and sample solutions of low and high concentration respectively. To minimize the impact of time differences between the applications of the different solutions, agar petri plates loaded with solutions were left at room temperature for 1-4 h. Then agar petri plates were incubated for 18-24 h at 37 °C in BOD incubator. After completion of the incubation period, diameters (mm) of zone of inhibition were accurately measured through antibiotic zone reader and calculate the results accordingly (Fig. 2). All of the experiments were carried out in a Biosafety Cabinet.

The percentage potency of the XCEFT injection was calculated using the Indian Pharmacopoeia's model equation.

Percentage potency = Antilog (2.0 ± a log I)

In which, $$a = \frac{(T_H+T_L) - (S_H+S_L)}{(T_H-T_L) + (S_H-S_L)}$$

T_H and T_L are the sum of the zone diameters with

Fig. 2: Two level agar diffusion assay method using a test microorganism Bacillus subtilis ATCC-6633 (MTCC 441) at concentration of Reference solution SH = 4 μgmL^{-1}, S_L = 1 μgmL^{-1} and Sample solution S_H = 4 μgmL^{-1}, S_L = μgmL^{-1}).

high and low level sample solutions, S_H and S_L are the sum of the zone diameters with high and low level reference standard solutions, and I = dilution ratio.

Results

Selection of most suitable microorganism

Most suitable microorganism was selected on the basis of sharp & clear edges and large measurable zone diameter under antibiotic behaviour. Microbiological assay of ceftiofur sodium was performed on 09 strains of bacteria for their response and susceptibility.

Result shows that microbial strains B. *bronchiseptica* (NCIM-5389), *P. aeruginosa* (MTCC-1688), *S. aureus* (MTCC-737), *S. epidermidis* (MTCC-3615) *S. enterica serotype abony* (MTCC-3858) growth were not inhibited by ceftiofur sodium and do not show any inhibitory effect. *S. aureus* (MTCC-96) and *E. coli* (MTCC-1687) were susceptible against ceftiofur sodium and shows intermediate zone of inhibition. However, *K. rhizophila* (MTCC-1541) showed large and light zone where as *B. subtilis* (MTCC-441) exhibit a highest and considerable inhibition zones against ceftiofur sodium (Table I). Therefore, B. *subtilis* (MTCC-441) was selected as the most appropriate organism and used for further bioassay study.

Determination of optimal inoculum concentration

In the current study, a test was conducted to determine how to choose the inoculum concentration. On the basis of sharp and clear zones, optimal inoculum concentration was selected. Low inoculum concentration implies poor growth and an unusually large zone diameter, whereas excessive inoculum concentration indicates an overlapping growth pattern and a lower zone diameter. Optimal inoculum concentration should lie between these two limits.

Five different inoculums concentrations, i.e., 0.5%, 1.0%, 1.5%, 2.0% and 3.0% were used in this study to test their influence on area of inhibition zones, which was previously optimised at 25% transmittance (Table II). For the microbial bioassay, the optimal inoculum concentration of *B. subtilis* (MTCC-441) was selected as 2.0%.

Table I: Selection of most suitable microbes for microbiological assay of ceftiofur sodium.

Name of microbes	Zones of inhibition (mm) of growth at 4 μgmL^{-1}	Interpretation
Staphylococcus aureus (MTCC-96)	10.1	Intermediate zone
Escherichia coli (MTCC-1687)	13.2	Intermediate zone
Kocuria rhizophila (MTCC-1541)	24.1	Large and light zone
Bacillus subtilis (MTCC-441)	19.1	Sharp and Clear zone
Staphylococcus epidermidis (MTCC-3615)	-	No inhibition zone
Bordetella bronchiseptica (NCIM-5389)	-	No inhibition zone
Pseudomonas aeruginosa (MTCC-1688)	-	No inhibition zone
Salmonellae enterica serotype abony (MTCC-3858)	-	No inhibition zone
Staphylococcus aureus (MTCC-737)	-	No inhibition zone

Table 2: Action of different inoculum concentration on the area of growth inhibition zones.

Inoculum concentration (%)	Antibiotic Conc. (μgmL^{-1})	Area of growth inhibition zones (mm)	Interpretation
0.5	4	22.5	Light and overlapped zone
1.0	4	21.2	Light zone
1.5	4	20.1	Zone without Sharp edge
2.0	4	19.5	Very clear and sharp edge zone
3.0	4	18.1	Sharp Zone

Determination of optimal antibiotic concentration

The concentration of antibiotic is a critical factor that prevents the growth of microbes. On the basis of clear, sharp edge and measurable zone size, the concentration of reference solution was estimated. From the 1000 μgmL^{-1} standard stock solution of ceftiofur, different concentration of 10, 5.0, 4.0, 2.0 and 1.0 μgmL^{-1} were made. It was determined that a concentration of 4 μgmL^{-1} of reference solution gave a clear, sharp edge and measurable zone. Table III shows the effect of various concentrations of the ceftiofur reference substance on zone inhibition.

Estimation of percentage potency calculation

For all experiments, triplicate plates were used. The area of the circular inhibition zones of reference and sample solutions at high and low concentration levels was measured using an antibiotic zone reader. The Pharmacopoeia's standard model equation estimated the mean percentage potency for XCEFT powder for injection as 101.79%.

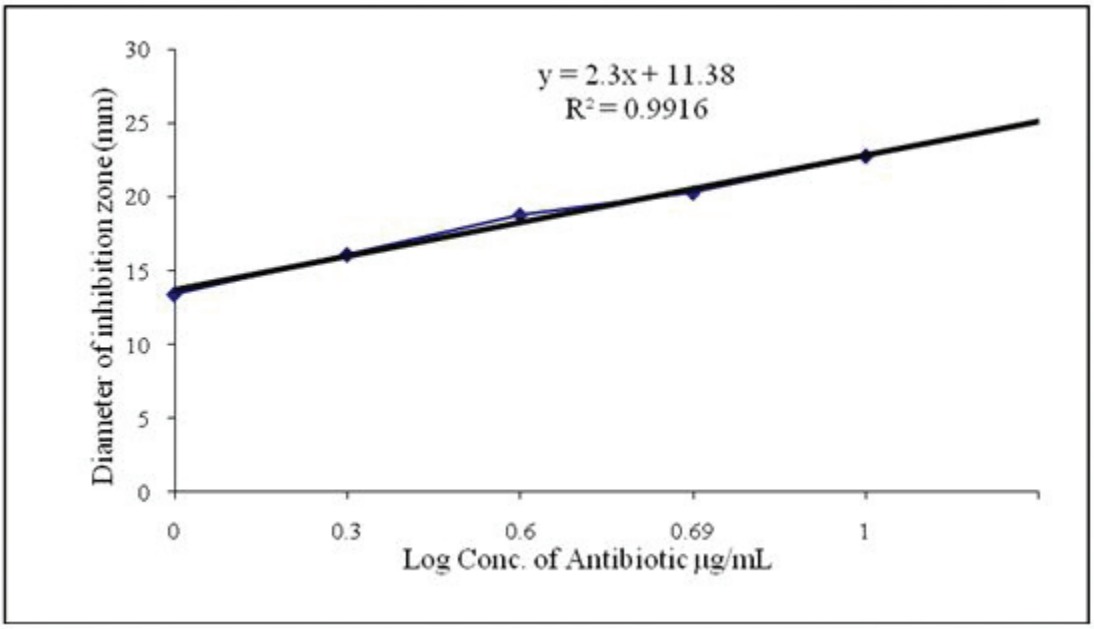

Fig. 3: Bioassay derived calibration curve for ceftiofur sodium reference substance

Table 3: Effect of ceftiofur reference substance concentrations on inhibition zone.

Reference substance concentration (μgmL^{-1})	Area of growth inhibition zones (mm)
10	22.8
5	20.3
4	18.8
2	16.1
1	13.4

Table 4: Repeatability evaluation for XCEFT powder for injection by bioassay.

Stated amount (mg/vial)	Experimental amount (mg/vial)	% potency	Mean % potency	RSD (%)
250 mg	251.15	100.46	101.79	1.07
	254.88	101.95		
	252.15	100.86		
	258.60	103.44		
	255.88	102.35		
	254.13	101.65		

Method validation

Prior to validation, all microbiological assay parameters were optimised in order to properly evaluate the performance of the proposed microbiological assay method. The microbiological assay method was validated using the International Conference on Harmonization's criteria for linearity, precision, accuracy, and robustness.[17,18]

Linearity

The linearity of the bioassay was measured using concentrations of 1.0, 2.0, 4.0, 5.0 and 10 µgmL-1 of ceftiofur sodium reference substance solution. The data were subjected to least squares regression analysis after plotting a calibration curve for log10 of ceftiofur concentrations (μgmL^{-1}) vs area of growth inhibitory zone (mm) was plotted. In regression analysis, determined linear equation was Y = 2.3 x + 11.38 and regression coefficient was ($r^2 = 0.9916$) (Fig. 3).

Range

Range was measured by the selected reference substance concentrations of the calibration curve and confirmed by linearity, precision, and accuracy of the method.

Precision

The relative standard deviation (RSD) for different precision parameters i.e. repeatability and intermediate precision were calculated. The repeatability of XCEFT powder for injection was determined in six replicates on the same day by the same analyst (Table IV). The intermediate precision was calculated by repeating the analysis on two distinct days (inter day) and between different analysts (inter analyst) (Table V).

Accuracy

Accuracy was measured at 80%, 100%, and 120% of the nominal analytical concentration for the microbiological assay method. The calculated mean accuracy was 101.08%, with an RSD of 0.28% indicating that the approach can accurately determine ceftiofur sodium concentrations in the 80–120% range (Table VI).

Robustness

Table 5: Intermediate precision evaluation for XCEFT powder for injection by bioassay.

Precision	Experimental % potency	Mean % potency	RSD (%)
Inter-day Precision			
Day 1	98.81	99.57	0.81
	99.59		
Day 2	100.69		
	99.20		
Inter-analyst Precision			
Analyst 1	101.06	101.05	0.43
	100.44		
Analyst 2	101.39		
	101.32		

Table 6: Accuracy for XCEFT powder for injection by bioassay

Hypothetical % potency	Experimental % potency	Mean % potency	Accuracy (%)	RSD (%)
	81.75			
80	81.26	81.10		
	80.28			
	100.69			
100	102.07	101.05	101.08	0.28
	100.39			
	120.78			
120	120.36	120.97		
	121.76			

It was determined after examining the same material under various settings. The buffer used for standard dilution, inoculum concentration and various microbial strains were all taken into account. To investigate the resilience, some test settings were altered, including the solvent used for the standard and sample dilution (phosphate buffer pH 7.0), inoculum concentration (3.0%), and incubation temperature (30°C). When the experimental settings were changed to the required specifications, no significant variations in potencies were observed, as shown in Table VII.

Discussion

The development and validation of analytical method for the potency estimation has received considerable attention from regulatory bodies because of their importance in pharmaceutical analysis. The selection of an appropriate analytical approach is critical for effective drug control and is influenced by a variety of parameters such as the source of the drug, its complexity, sample quantity, qualitative or quantitative purpose of the method, and the availability of equipment and literature.

In various pharmacopoeias and literatures, HPLC methods and other chemical techniques have been developed and used for potency estimation of ceftiofur sodium in pharmaceutical products. In this case a bioassay method was proposed as a suitable method for estimation of potency of ceftiofur in powder for injection. Antibiotic potency can be determined by comparing the inhibition of growth of a susceptible microbe induced by known quantities of the antibiotic under investigation and their related reference substances. For assessing the ceftiofur sodium content in dry powder injectable pharmaceutical dosage forms, a two-level (2×2) factorial microbiological assay was proposed7.

In the literature studies, there is an established microbiological assay technique by three level (3×3) cylindrical plate method for evaluating ceftiofur sodium activity against *Micrococcus luteus* ATCC 10240.[8] Souza et al. 2007, confirmed the antibacterial activity of ceftiofur sodium against *Micrococcus luteus* ATCC 10240 on Grove-Randall's culture medium (Diffco).[8] In proposed study, microbiological assay of ceftiofur sodium was performed on 09 bacterial strains for their reaction and susceptibility. Few of them show positive response i.e. susceptibility against ceftiofur sodium but due to its ability to form sharp edge zone of

Table 7: Factors studied in the robustness.

Factors	Parameters	Mean % potency	RSD (%)
		101.62	
Solvent	Phosphate buffer pH 7.0	100.42	0.60
		101.20	
		101.39	
Inoculum concentration	3.0%	100.79	0.50
		100.39	
		100.69	
Incubation temperature	30 °C	99.17	0.78
		99.61	

inhibition on Antibiotic assay medium B, *B. subtilis* MTCC-441 was chosen as the most appropriate test organism.

On the basis of clear and sharp edge zone of inhibition, concentration of inoculum should be validated for quantification of an antibiotic throughout a microbial bioassay.[14] Experiments were carried out to determine the importance of inoculum concentration while all other conditions remains constant, as it is well known that inoculum concentration affects the size of the resulting zone.[14,19] Petri plate containing media with high concentration of test organism produce cloudy growth and no antimicrobial action of antibiotic while low inoculum concentration of test organism produce a light or immeasurable zone. Therefore it is necessary to optimize inoculum concentration for microbiological assay. The inoculum concentration of *M. luteus* ATCC 10240 was chosen as 1.0% for the development of ceftiofur sodium bio assay.[8] In proposed study different inoculums concentrations of *B. subtilis* MTCC-441 i.e. 0.5%, 1.0%, 1.5%, 2.0%, and 3.0% were tested and selected optimize inoculum concentration was 2.0% for microbial bioassay.

For this experiment, the area of growth inhibition zones was measured using a selected range of concentrations of the reference substance ceftiofur sodium. The chosen concentration was determined by the microbes susceptibility to low concentrations, the size of inhibitory zones at high concentrations, and the linear relationship between the logarithm of concentration and the mean area of the inhibition zone, which was limited by the size of the petri dish. Other authors used a concentration of ceftiofur sodium of 2-8 μgmL^{-1} for method development. In our study, different concentrations i.e., 1.0, 2.0, 4.0, 5.0 and 10 μgmL^{-1} of reference substance of ceftiofur sodium were tested against selected test microorganism and the high and low concentration of ceftiofur sodium reference substance were chosen as 1.0 µgmL-1 and 4.0 μgmL^{-1} for potency estimation. A good linearity was established in the range of specified concentrations of ceftiofur sodium reference substance by plotting the logarithm of antibiotic concentration (µgmL-1) vs mean diameter of inhibition zone (in mm) (Fig. 3). The representative linear equation for ceftiofur sodium was Y = 2.3 x + 11.38 and the regression coefficient ($r^2 = 0.9916$) obtained was very significant for this approach.

Relative Standard Deviation was used to express precision. The repeatability of the sample was determined by analysing it several times on the same day; the mean ceftiofur sodium content in XCEFT powder for injection was 101.79%, with an RSD value of 1.07%. The mean ceftiofur sodium content for interday precision assay was 99.57% with an RSD of 0.81% and the mean content between analysts was 101.05% with an RSD of 0.43%. Accuracy was measured at 80%, 100%, and 120% of the nominal analytical concentration for the microbiological assay method with an RSD of 0.28% and the calculated mean accuracy was 101.08%.

The performed validation and result obtained in this study were satisfactory and proven that bioassay is a good option for pharmaceutical analysis of ceftiofur sodium in powder for injection. It is a useful analytical tool when used in addition to or instead of the physicochemical approach.

Conclusion

For ascertaining the quality of pharmaceutical products, it is mandatory to utilize validated and authenticated analytical methods. HPLC methods for estimating the potency of ceftiofur sodium have been developed in the various pharmacopoeias. However, there is a genuine microbiological method for estimating ceftiofur sodium potency that has not been described in any pharmacopoeias. According to literature survey, microbiological assay method have been developed for ceftiofur sodium against test organisms *M. luteus* ATCC 10240. The purpose of the proposed experimental investigation was to design and validate a bioassay method for estimating ceftiofur sodium potency. The choice and optimization of the microbial assay was performed through the use of various conditions and *B. subtilis* MTCC-441 was found to be the most susceptible organism against ceftiofur. Several parameters including buffer pH, inoculum concentration and standard solution concentration were studied using the test organism *B. subtilis* MTCC-441 against ceftiofur sodium. The potency of ceftiofur sodium in XCEFT powder for injection sample was estimated as 101.79% through bioassay. The results demonstrated that the proposed microbiological assay method for estimating the potency of ceftiofur sodium in pharmaceutical products is accurate, with the obtained results confirming its good accuracy, robustness, precision and significant linearity of response. Therefore, the proposed bioassay method can be useful for the quality control of ceftiofur sodium in the studied formulation.

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References

1. Salmon S.A., Watts J.L., Case C.A. , Hoffman L.J., Wegener H.C. and Yancey R.J.: Comparison of MICs of ceftiofur and other antimicrobial agents against bacterial pathogens of swine from the United States, Canada, and Denmark, J. Clin. Microbio., 1995, 33(9): 2435–2444.
2. Stanek C. and Kofler J.: Use of sodium ceftiofur in the combined therapy of complicated septic diseases in cattle, Tierarztl Prax Ausg G: Grosstiere Nutztiere., 1998, 26(6): 314–317.
3. Wisselink H.J., Veldman K.T., Eede C.V.D., Salmon S.A. and Mevius D.J.: Quantitative susceptibility of Streptococcus suis strains isolated from diseased pigs in seven European countries to antimicrobial agents licenced in veterinary medicine, Vet. Microbiol., 2006, 113(1-2): 73–82.
4. Erskine R.J., Wilson R.C., Tyler J.W., McClure K.A., Nelson R.S. and Spears H.J.: Ceftiofur distribution in serum and milk from clinically normal cows and cows with experimental Escherichia coli-induced mastitis, Am. J. Vet. Res., 1995, 56(4): 481–485.
5. Deshande L., Pfaller M.A. and Jones R.N.: In vitro activity of ceftiofur tested against clinical isolates of Escherichia coli and Klebsiella pneumoniae including extended spectrum β-lactamase producing strains, Int. J. Antimicrob. Agents., 2000, 15(4): 271-275.
6. Donaldson S.C., Straley B.A., Hegde N.V., Sawant A.A., DebRoy C. and Javarao B.M.: Molecular epidemiology of ceftiofur-resistant Escherichia coli isolates from dairy calves, Appl. Environ. Microbiol., 2006, 72(6): 3940–3948.
7. Indian Pharmacopoeia, Indian Pharmacopoeia Commission, Govt. of India, Ministry of Health and Family Welfare, Ghaziabad, 2018, 3 (50-56), 1539-1540.
8. Souza M.J.E., Rolim C.M.B., Melo J., Philo P.S.S. and Bergold A.M.: Development of a microbiological assay to determine the potency of ceftiofur sodium powder, J. AOAC Int., 2007, 90(6): 1724-1728.
9. Jacobson G.A., Martinod S. and Cunningham C.P.: Determination of ceftiofur in bovine plasma by HPLC-DAD, J. Pharm. Biomed. Anal., 2006, 40(5): 1249-1252.
10. Keever J., Voyksner R.D. and Tyczkowska K.L.: Quantitative determination of ceftiofur in milk by liquid chromatography-electrospray mass spectrometry, J. Chromatogr. A., 1998, 794(1-2): 57-62.
11. United States Pharmacopoeia, United States Pharmacopoeial convention, Rockville, MD, USA, 2021, 3, 6488-6508.
12. British Pharmacopoeia, The stationary office, London, 2021, 5, VA439- VA446.
13. Cazedey E.C.L. and Salgado H.R.N.: Development and validation of a microbiological agar assay for determination of orbifloxacin pharmaceutical preparations, Pharmaceutics., 2011, 3(3): 572-581.
14. Dafale N.A., Agarwal P.K., Semwal U.P. and Singh G.N.: Development and validation of microbial bioassay for the quantification of potency of the antibiotic cefuroxime axetil, Anal. Methods., 2013, 5: 690–698.
15. Bauer A.W., Kirby W.M., Sherris J.C. and Turck M.: Antibiotic susceptibility testing by a standardized single disk method, Am. J. Clin. Pathol., 1966, 45(4): 493-496.
16. Simon H.J. and Yin E.J.: Microbioassay of antimicrobial agents, J. Appl. Microbiol., 1970, 19(4): 573-579.
17. ICH Harmonized Tripartite Guideline, Validation of Analytical Procedures: Text and Methodology Q2 (R1) Current Step 4 Version, November, 2005.
18. Queiroz K.M., Silva M.L.M., Prado N.D., Lima P.M.A., Diniz R.D.L., Cesar I.C., Pianetti G.A. and Santos D.A.: Comparison of microbiological assay and HPLC-UV assay for determination of fluconazole in capsules, Braz. J. Pharm. Sci., 2009, 45(4): 693-700.
19. Hewitt W.: Microbiological Assay for Pharmaceutical Analysis: A Rational Approach (1st Indian Reprint), Interpharm/CRC Press LLC, Boca Raton, FL, 2009.

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FTIR Characterization and Physico-chemical Studies of Honey Samples in Kanpur Nagar

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Abstract

Honey is a valuable food entity that gains a lot of attention due to its potential benefits for health and its uses to sweeten foods. It contains about 60% invert sugar and appreciable amounts of maltose and sucrose. In this study, the Physico-chemical characteristics of pure and adulterated honey samples were investigated. The grade and limitation of adulteration of the honey samples also were checked. The moisture, ash and pH were carried out. The Fourier Transform Infrared Spectroscopy (FTIR) of different samples were analyzed. The result of FTIR spectroscopy provides reliable results.

Keywords: Honey, Moisture, Total ash, pH.

Introduction

Honey is a natural sweet substance obtained from honeycombs. It is a unique sweetening substance that can be used by humans without processing.[1] Honey is used in health described in traditional medicine and as an alternative treatment for clinical conditions and is also used in brewing honey wine.[2] The major component of honey is fructose and glucose with a lesser amount of water. The composition of honey varies with the floral and honeydew sources utilized by honey bees as well as climate conditions.[3] The chief sources of microorganisms in honey are the nectar of flowers and the honeybee yeasts have been shown to come from the nectar and from the intestinal content of the bee, bacteria also come from the later source. Honey has been found to contain lysozyme, an enzyme with a bacteriostatic as well as a lytic effect on most gram-positive bacteria. The use of antibiotics, such as neomycin[4] and streptomycin[5] is widespread in beekeeping, and these antibiotics have been found in the honey obtained from treated larvae and bees. The gluconobacter and lactobacillus are the two main groups of bacteria present during the maturation of nectar to honey.[6] Honey is extracted from the comb, strained, and marked. Honey contains larger quantities of fructose[7] (about 38%) than glucose (31%) and Sucrose[8] constitutes about 2% of the total sugar[9] content. Honey has the capacity to retain water, and hence, cakes, candies, etc., made with honey remain moist for a longer period than those made with other sweetening agents[10] determined by the Physico-chemical parameter i.e., refractive index,

specific gravity, water-insoluble solids, and total soluble solid, etc. The physical properties such as conductance, surface tension, and pH determine by using digital instruments.[11] Honey is essentially a concentrated aqueous solution of invert sugars, but it also contains a very complex mixture of other saccharides, proteins, enzymes, amino acids, organic acids, polyphenols, and carotenoid-like substances, vitamins, and minerals.[12] Dietary frauds in particular adulteration are practices in constant progress.[13] If any coloring matter other than prescribed in respect of the amounts is not within the prescribed limits of variability present in substances.[14,15] The colored inverted sugar syrups are used to adulterate honey.[16,17,20] One of the methods that provide information on the total chemical composition of any sample is infrared spectroscopy.[18,19] So, the aim of this study was to confirm the pure and adulterated honey samples to be collected from the local market of Kanpur Nagar by physicochemical and IR spectroscopy methods.

Materials and Methods

Sample Collection

Three samples of honeys produced in Kanpur Nagar were collected from beekeepers. The samples were stored in refrigerator in airtight plastic containers until analysis.

Physico-Chemical Analysis

Determination of moisture content

The sample material was taken in a flat bottom dish and kept for 12 hours in an oven at 100 -110 °C and weighed. The loss in weight was a measure of moisture content.

Moisture content = M_1- M_2 / M_1-M_0

Where

M_0 - Weight of the dish

M_1 - Weight of the fresh sample +dish

M_2 - Weight of the dried sample +dish

Determination of ash content

Ashes were obtained in a muffle furnace at 550 °C for 5-6 hours to obtain constant weight.

The percentage of Ash was calculated:

Ash (%) = (Weight of crucible + Ash) – (Weight of empty crucible) X 100 / sample weight

Determination of pH

The pH of honey is determined by using a digital

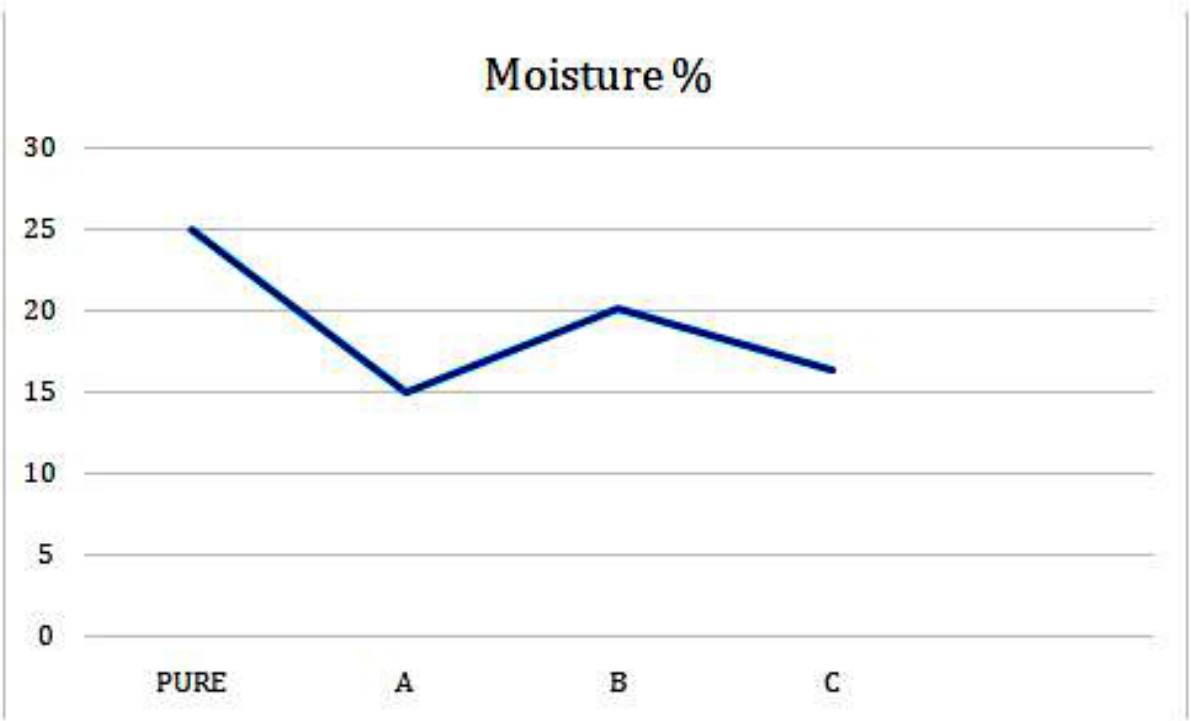

Fig. 1: Moisture in various honey samples

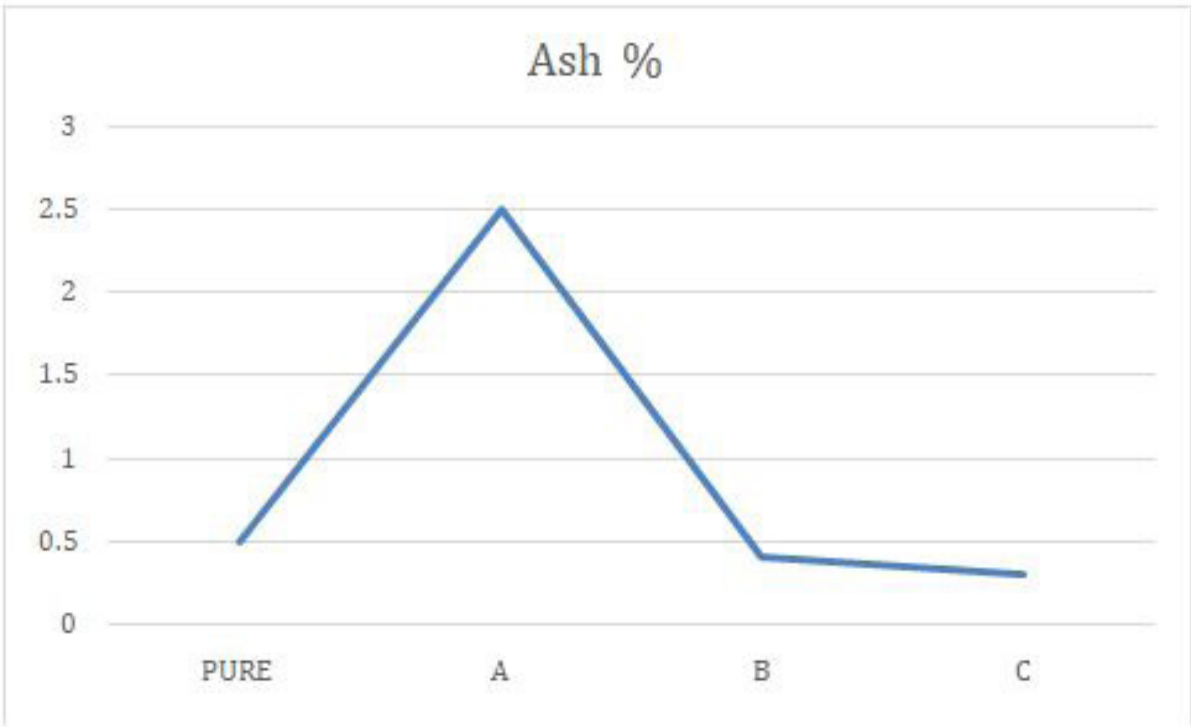

Fig. 2: Ash content in various honey samples

pH meter. The pH meter was calibrated with buffers at pH 4,7 and 10. The sample was taken in a beaker dissolved in distilled water by using a Magnetic stirrer. Electrode inserted in the beaker containing the sample and recorded the pH.

Determination of Adulteration

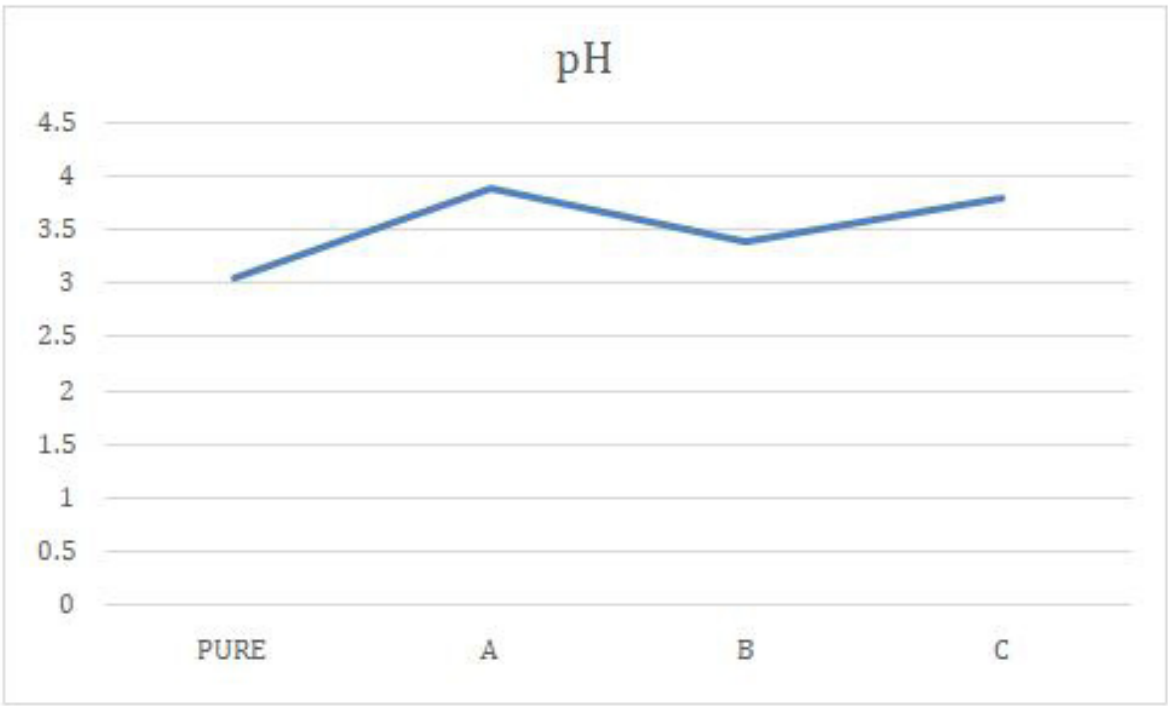

Fig. 3: Data of the pH in various honey samples

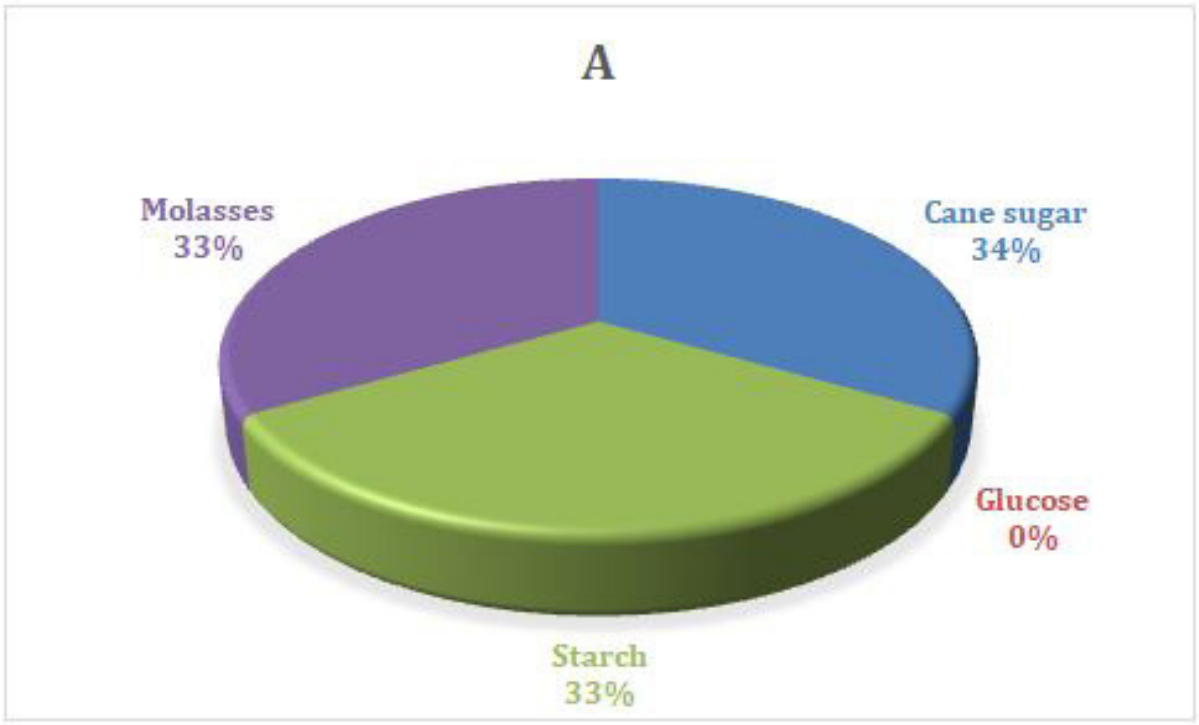

Fig 4: Adulteration in Honey samples

FTIR Spectra

IR spectra of honey samples were recorded using the Fourier transform infrared spectrometer (FTIR Spectrometer). The spectral range from 1200 to 700 cm-1 (Maximum absorbance established at 1026 cm-1) was subjected to further statistical analysis since the adulteration in honey has been identified in this particular spectral region.

Results and Discussion

The moisture content of honey samples had a value of 15%, 20.11%, and 16.42%. The moisture levels are one of the most crucial parameters of honey which affects the optical density, refractive index, surface tension, and viscosity. Honey is an excellent hygroscopic product and tends to absorb atmospheric moisture and thus readily increase its moisture levels. In BIS the maximum content of moisture is 25%.

The Ash content in Fig 2, sample A has a high amount of Ash value, Sample B has a little high

Table 1: Infrared Band Assignments for pure honey and compare with other honey samples collected from local market of Kanpur Nagar

Functional Group	Pure Honey Frequency (cm-1)	Sample A Frequency (cm-1)	Sample B Frequency (cm-1)	Sample C Frequency (cm-1)
O-H (H-bonded)	3276.79	3521.42	3518.92	3521.81
C-H stretching	2948.35	2929.28	2929.16	2928.32
O-H (Water) bonding	1638.19	-	-	-
-CH2 bending (Strong)	1426.05	1419.86	1426.12	1421.02
-CH2 bending (Medium)	1362.05	1346.20	1362.68	1361.66
C-C, O, -CH2, C-OH bending	1254.15	1255.66	1259.19	1260.14
C-O Stretching	1032.05	1034.24	1057.47	1057.64
Extra peak	-	3095.92	3099.01	-
Extra peak	-	2343.51	-	2343.72

but Sample C has a low according to the Bureau of Indian standard (BIS). Bureau of Indian standard (BIS) maximum percentage of ash is reported as 0.5%.

Data of pH of samples shown in Fig. 3. pH content of honey varied a 3.9, 3.4, and 3.8. Bureau of Indian standard (BIS) reported pH in the range of 3.05 – 4.50. According to data pH range is not more than the reported value.

FTIR Spectrum Analysis

In this study, FTIR was used to compare honey samples based on their spectral difference in the range 4000- 650 m-1.[11] The spectrum of honey samples is shown in Fig. 5, 6, and 7. Table-1 represents the bond assignment along with the corresponding vibration and compares it with pure honey.

The O-H stretching vibration band in carboxylic acids is very broad and occurs in the field of 3300-2500 cm-1.[12] The pure honey samples obtained the O-H peak at 3276.79 cm-1 but adulterated honey samples 1, 2 and 3 show a peak at 3521.42, 3518.92, and 3521.81, respectively. Fig. 5,6,7 and Table:1 show a band at 2929 cm-1 was observed which according to Anions et.al.[13] also corresponds to C-H stretching of carboxylic acids and NH3 stretching band of free amino acids. However, the band under 1440-1395 cm-1 cannot be distinguished from C-H bending bends which also occur in the same frequency region. The O-H water bonding peak is approx. 1638 cm-1 did not appear in sample1, 2, and 3. The vibration with the maximum bandwidth of about 1255 cm-1 1259 cm-1 and 1260 cm-1 are characteristic and bending vibrations of C-C, C-CH2, and C-OH grouping. The band range from 1250 to 1140 cm-1 is in turn vibration characteristic for the stretching vibration of the C-H in carbohydrates or stretching vibration of CO in carbohydrates. The vibration with the maximum at approximately 1100 and 1080 cm-1 constitutes a band that can originate in the vibration of C-O stretching but samples A, B, and C showed these peaks at 1034 cm-1, 1057 cm-1, and 1057 cm-1. Some extra peaks i.e., 3095 cm-1 and 3099 cm-1 showed in samples A and B but not shown in sample C. Similarly, the peak 2343 cm-1 appeared in samples A and C but not appeared in samples B. The extra peaks which showed in Table-1 were due to adulteration.

Conclusion

Honey is a low-cost natural product that can be used for different purposes. Now, commercially honey is used in various industries for product formation and this trend is increasing day by day as industrialists are finding honey to be a cheap

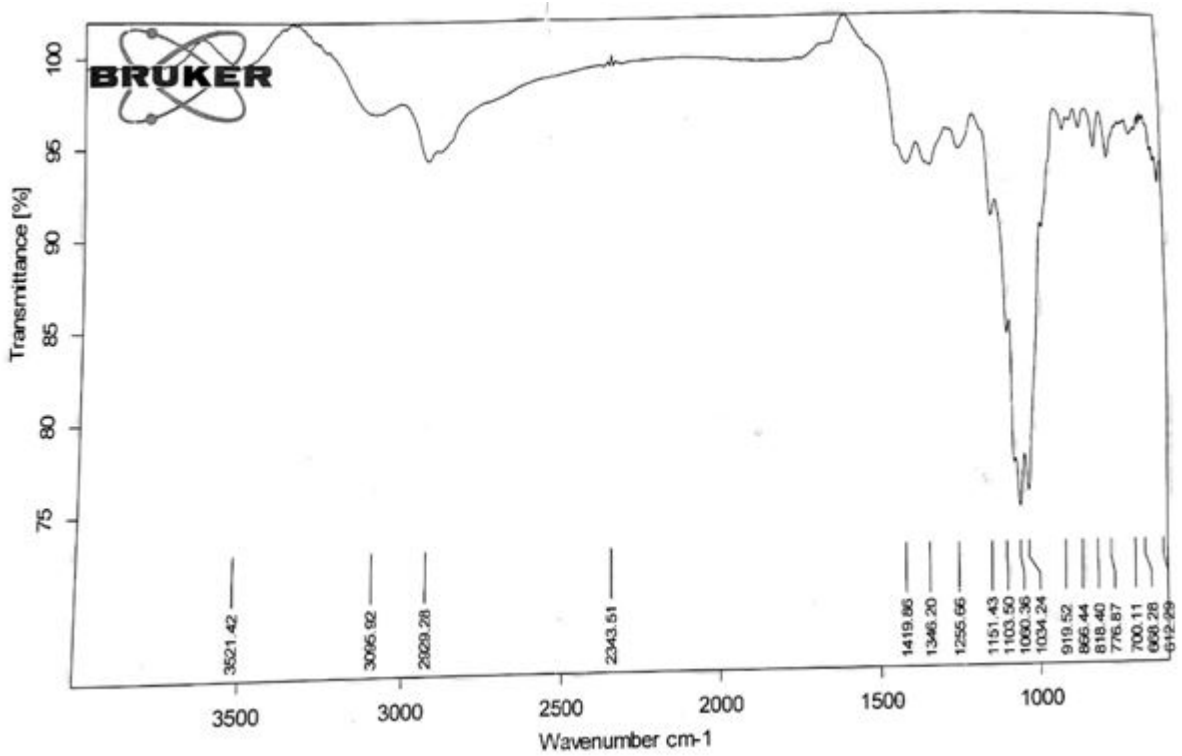

Fig. 5: FTIR Spectra of sample A. **Source:** https://www.bruker.com/en.html

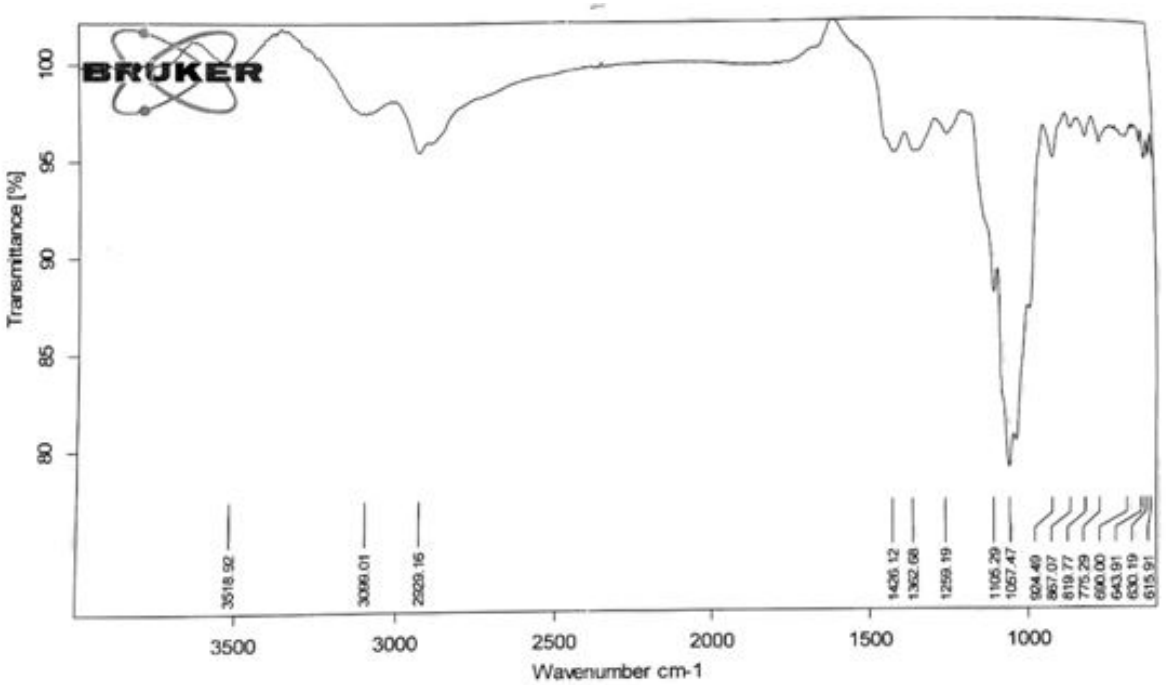

Fig. 6: FTIR Spectra of sample B. **Source:** https://www.bruker.com/en.html

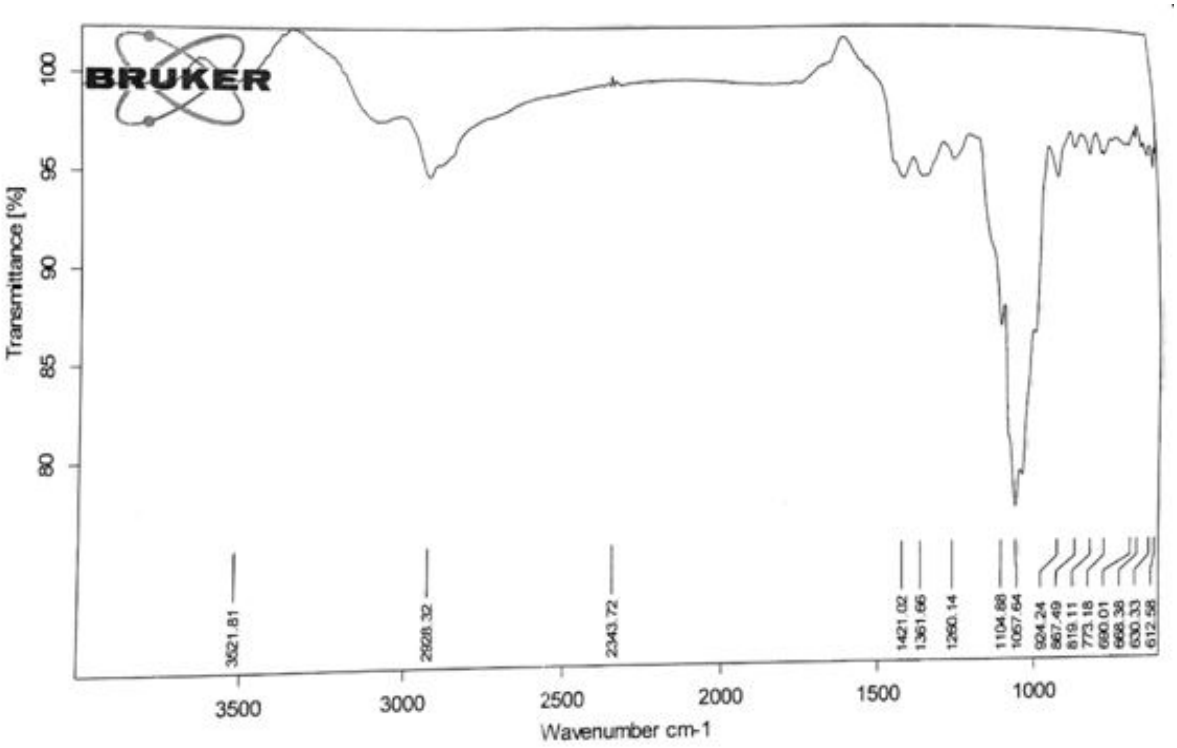

Fig. 7: FTIR Spectra of sample C. **Source:** https://www.bruker.com/en.html

source of sweetening agent without any side effects as in the case of synthetic sweeteners. Due to variation in botanical origin honey differs in appearance, sensory perception, and composition. According to obtained data from studied literature, it is to some extent obvious that nearly the majority of physiochemical properties of honey depend on floral sources. The honey samples collected from Kanpur Nagar were not found to have good quality physicochemical characteristics and adulterants

also found in some samples also might have been due to unhygienic handling during processing and storage. The results of this study showed that IR spectroscopy provides equally reliable results, but also represents a rapid and cheap analytical tool in comparison to commonly used standard analytical methods. Therefore, it is expected that IR spectroscopy will contribute to the authentication and quality control of honey samples.

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References

1. Sharbt, M.N.A. and S.A. Abdel- Fattah. (1994),"Honeybees production" Bulletin from technology transfer components (In Arabic) Ministry of Agric and land Reclamation.
2. Saeed Samarghandian, Tahereh Farkhondeh, and Fariborz Samini Honey and Health: A Review of Recent Clinical Research Pharmacognosy Res. 2017 Apr-Jun; 9(2): 121–127.
3. Stefan Bogdonov, Brenda Massel, Bruce Robert D Arcy (1999), International regulatory standards: review of the work of the International Honey Commission. Mitteilungen aus dem Gebiete der Lebensmitteluntersuchung und Hygiene 90(1):108–125.
4. USDA International regulatory standards: review of the work of the International Honey Commission. Mitteilungen aus dem Gebiete der Lebensmitteluntersuchung und Hygiene 90(1): USDA Washington DC.
5. Definition of Honey and Honey Products, Approved by the National Honey Board June 15, 1996, Updated September 27,2003.
6. Ahmed, Jasim, S.T. Prabhu, G.S.V. Raghavan, M.Ngadi (2007), " Physico- chemical , rheological, calorimetric and dielectric behavior of selected Indian honey" Journal of food engineering, vol 79, 1207-1213.
7. White J W, Composition of Honey, A comprehensive survey, Heinemann Edition: London 1975, pp 157-206
8. Siddoqui I R, The sugar of honey advances in carbohydrate chemistry and biochemistry, 1970, pp 285-309
9. OféliaAnjos, Maria Graça Campos, Pablo Contreras Ruiz, Paulo Antunes Application of FTIR-ATR spectroscopy to the quantification of sugar in honey Food Chemistry, Volume 169, 15 February 2015, Pages 218-223
10. S.M. Abdel – Aal, H.M.Ziena, M.M.Youssef, Adultration of honey with high fructose corn syrup:detection by different method.48(2),1993,209-212.
11. Codex Alimentarius. 2001 Revised codex standard for honey.(no. CODEX STAN 12-1981, Rev.1 (1987),Rev.2 (2001).
12. SHC, Yeow, Tee Suan Chin, Jian Ai Yeow, KS Tan, Consumer purchase intentions and honey related products. Enterpreneurship vision 2020: Innovation development stainability and economic growth.
13. AOAC. *Association of Official Analytical Chemists,* 16th ed., (1999). Official Methods of Analysis, Washington, DC
14. Bagdanov, S. (1989). Determination of pinocembrin in honey using HPLC. Journal of Apicultural Research, 28(1), 55-58.
15. Steeg, E., & Montag, A.(1988). Quantitative Bes timmungAromatischerCarbonsauren in Honig. Zeitschrift fur LebensmittelUntersuchung and Forschung, 187, 115-120.
16. Mateo, R., & Bosch Reig. F. (1997). Sugar profile of Spanish unifloral honeys. Food Chemistry, 60(1),33-41.
17. Terrab, A., Recamales, A.F., Hernandez, D., Heredia, F.J.,Characterisation of Moroccan Food Chem. 2004, 88, 537.
18. K.Hemalatha, P.Satyanarayana Elementary analysis of different floral honey of east Godavari dist. Andhra Predesh, IOSR Journal of environmental science and toxicology and food technology 9(12),2015,56-60.
19. M.V. Balasubramanyam, Chemical characteristics of multifloral wild and apiary honey from western ghat of Karnataka. The bioscan 6(3),2011,467-469.
20. Lidija SVEČNJAK, Nikola BILIŠKOV, Dragan BUBALO, Domagoj BARIŠIĆ, Application of Infrared Spectroscopy in Honey Analysis Agriculturae Conspectus Scientific, Vol. 76 (2011) No. 3 (191-195

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A Meticulous Review on Arson Inquest Using Gas Chromatography

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Abstract

Arson investigation is hands down the most tedious and the robust form of investigation to go with. It is by far the multifarious and endearing type of investigation as investigating at the arson scene demands high endeavour in order to withstand from all the challenges that stood up at the scene of crime. Stringent norms have been framed by the government that defines it under section 435, 436 and 438 of IPC. In such cases, the chain of custody plays a dynamic and a crucial role as from the procurement of the most fragile samples to transporting it in the most desired form to follow the best sample preparation method before subjecting to GC analysis in order to avail the quality result. Keeping in mind the susceptibility of fire evidences American Society for Testingand Materials International has established standard guidelines for subjecting sample before GC-MS introducing solid phase micro-extraction technique linking to various GC detectors. New minds and innovation has bumped into better and more approachable forms of GC by making hybrids of it such as E-nose i.e. headspace-mass spectrometry, a strategic mass spectrometry based instrument with both qualitative and quantitative applications surpassing the bygone separation process. Revolution in the arson and analytical world is making way for non destructible techniques to serve as a better alternative to any of the GC detectors.

Keywords: Arson, Indian Penal Code, Gas Chromatography -Mass Spectrometry, Headspace-Mass Spectrometry, Electronic nose.

Introduction

With the emergence of forensic science, advancements or entailment of scientific applications in the field is the need of the hour as the scientific examination allows a forensic scientist to put forth the opinions over the evidences admissible in the court with full certainty. Over the years, due to the diversification in respect of crime and the ways of doing it has made a forensic expert to question the existence of even those exhibits that were once neglected but now serve as a corroborative one that helps in carve out a way to further proceedings.[1] There was a time when ignition of fire was considered natural or accidental, but with the time many theories have come up that doubts fire being natural, accidental or malicious i.e. Arson.[2] Deliberate fire cases were novelly grouped under violent offences but were later classed under crimes against property and public safety. New transitions and amendments with the time have now referred arson as miscellaneous IPC crime. In India, these arson cases get registered under the Section 435, 436 and 438 of Indian Penal Code (IPC).[3] Although many factors are responsible for enhancing the destruction caused by arson but

the essential and the most widely used ingredient that counts as the major cause and also serves as the major step in fire investigation is the use of accelerant.[4,5] Accelerants are basically the chemical substances knowing to initiate as well as spread the fire to a wide stretch. It can be available in all the three states i.e. either in the form of solid, liquid or gaseous.[6] Amongst the three available states, liquid accelerants especially the petroleum products like petrol, kerosene and diesel are widely abused. Other inflammable solvent accelerants that came into light are alcohol, ether, thinner and industrial solvents.[7] Identification and determination of the type of accelerant involved in arson is the key step that helps in establishing the origin as well as cause of fire. Arson investigation has always come up as a challenging one to deal with as more of the evidence usually gets destroyed due to the combustion and thermal degradation, with lot of interferences with the petroleum products that gets sum up due to abiotic factors and also sometimes many other similar accelerants in fire debris creates hurdles at the time of examination and in result of which the only existence of accelerants in fire debris cannot be considered as a definitive indicator for an arson suspected.

This review has listed the uncompensated methods showing the way to proceed in the arson inquest describing sample preparation as the key and utmost step to consider and thoroughly go with before proceeding with any analysis along with the advancements in the most indispensible tool employed for its analysis i.e. GC with its hybrid version. This review has also focused on the current needs allowing researchers to shift from destructive type of analysis to indestructive type of examination.

Arson

Fire – That can be unintentional i.e. either natural or accidental, basically a chemical reaction that came into existence when heat, oxygen, fuel came up and act simultaneously in an uninhibited chain reaction.[8] **Whilst**, the willful and malicious setting of fire in one's own property or of other's property because of fraudulent or criminal intent is universally termed as **Arson.**[8] **Forensically**, its investigation is considered as the most tedious and troublesome task to accomplish because of the destructive nature it possess. It not only damages the evidences and exhibits but this has the potential to destroy the scene of crime as well and left the investigator with empty handed, as some gets destroyed during burning process and rest in the extinguishing process.[9] Also many arson cases have shown a set range and a set pattern of ignition indicating towards the serial arsonist referred as pyromaniacs with major psychological issues.[10] In the American Psychiatric Association's DSM5 classification system this condition has been considered as a part of diagnosis as many case reports have revealed the fact that a true pyromaniac usually hold one thing in common in all of his criminal acts and their possible way of conviction is to identify that very possible existing evidence that they leave behind.[11] Not just pyromaniac, but they have been accompanied by people who suffer from serious schizophrenia.[10] Bradish in 1999 in one of his studies putforth the statistical data of FBI crime index that showed higher percentage of children and adolescents were held responsible for the cases of arson.[11]

Chemistry of Fire

According to Dawson Powell, six basic elements are responsible for ignition, input heat, fuel, oxygen, proportioning, mixing, ignition continuity. Fire has been categorized under different classes[9]-

- **Class 'A' Fire**- Involves setting up of fire in solid materials as in woods, paper or textiles. The commonly used fire extinguisher for this class are said to be AFF Foam, water, wet chemicals as well as dry powder.
- **Class 'B' Fire**- Involves flammable liquids such as petrol, diesel or lubricating oils. The preferred extinguishers are AFF Foam as well as carbon dioxide.
- **Class 'C' Fire**- Involves gases with the preferred extinguisher be dry powder.
- **Class 'D' Fire-** Involves metals with the preferred extinguisher be L2 and M28 powder.
- **Class 'E' Fire-** Involves live electrical apparatus with the preferred extinguisher is carbon dioxide and dry powder.

❖ **Class 'F' Fire-** Involves cooking oils such as in deep fat fryers with the preferred extinguisher be wet chemicals.

Sequential events followed by fire - Mostly in a closed apartment fire follows certain sequential levels from the beginning to the end.

❖ *Incipient stage or the growth phase* - Is the first and foremost level of fire development that begins from the moment of ignition with the flames properly localized and the fire is regulated not only by the oxygen available but also be the mass and properties of fuel as

well.[12]

- *Flash over stage* - Incipient stage is followed by flash over which is a transition stage between the growth and fully developed stage and possess collection of gases due to constant burning that will accumulate all over the room and sums up to more spread of fire. Also presence of any state of fuel will lead to form a specific characteristic "V" pattern at the fire scene.[12]
- *Fully developed or post flash over stage* – Flash over stage is followed by *fully developed or post flash over stage* that involves intense burning and also has the potential to get in contact to every inch of the apartment.
- *Smoldering or decay* - With time when the supply of oxygen become less as well as fuel and heat to keep the fire ignited falls is the stage when fire reaches to its end called as the stage of *smoldering or decay*.[12]

Various aspects of fire can be easily turned into arson and thus it became crucial to know each and every aspect that the chemistry of fire holds before actually carrying out the investigation.

Investigation begins with the understanding and finding of the origin of fire, causes of fire as well as finding the type of accelerants used to ignite the fire using an indispensible technique and approach.[14]

Methods

Progressive Patterns of Firsetting

Identifying the patterns formed by the fire is the first and the original physical evidence that is encountered and dealt with. These patterns are formed when number of processes such as oxidation, distortion, melting, charring takes place during and after the fire ignited and can be visually analyzed as well as measured.[16] After the fire gets extinguished, clear demarcations appear as formation of broad lines takes place that indicates the levels of heat and smoke. Formation of such demarcations are a result of a number of factors involving the surface, length of heat exposure, heat source, fire enhancing materials, fire suppression ways and ventilation as well. Studies have revealed that amongst smooth and rough surfaces, the tendency to sustain more damage rests with rough surface and also increase and decrease of damage has been attributed by materials such as paint, bricks, tiles, wallpapers and any other article. Usually, the spread of fire is upward in direction thus downward penetration is the matter of concern and needs to be examined thoroughly.[16] Penetration of fire in the downward direction is quiet unusual and can be encountered in cases like burning of furniture involving chairs, couches and mattresses. Examination of areas around a hole provides us information regarding

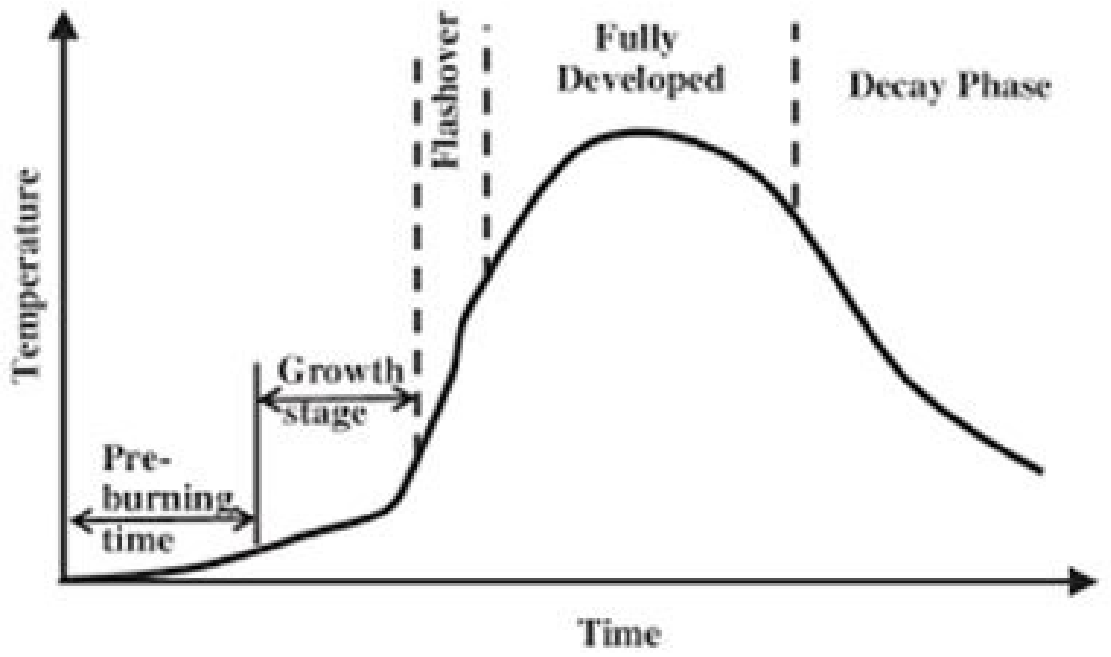

Fig. 1: Depicting Stages of fire.[13]

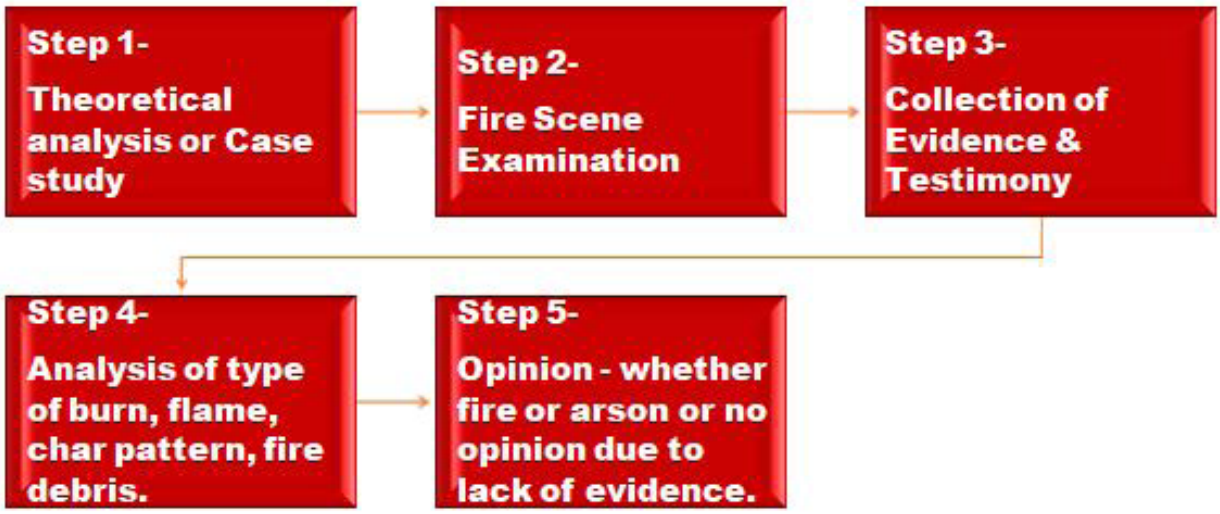

Fig. 2: Flow chart depicting series of Fire/Arson investigation.[15]

the direction of burn and its penetration. Around the hole if the sides are wider from the top along with inward slope then it indicates that the arrival of fire is from above whilst if the sides are wider from the bottom and slope upward towards the centre of hole shows that the arrival of fire is from below. The fire debris also adds up in knowing the direction that fire chooses to travel. Pattern "V" is the most common pattern encountered at the fire scene and is also referred as plume generated patterns.[16] It was claimed and believed for a while that fast burning fire usually forms narrow "V" pattern whereas a slow burning fire forms wide "V" pattern. There are other patterns as well-

1. **Confinement patterns** – When hot gas layer gets trapped beneath the ceiling and interacts with the walls give rise to pattern named as confinement patterns. Presence of these patterns at the fire scene provides investigator with a sequential data of smoke horizon and heat horizon that indicates that failure of ceiling takes place after the patterns has been formed. Such patterns are the source of information that tells about the origin in multilevel buildings and also sometimes this is the only type of pattern left at the fire scene.[17]
2. **Movement patterns** – In an apartment where the fire travels from a room to another it generally documents movement patterns at or near the doorways. These are the diagonally formed patterns tracing of which can lead to its origin.[17]
3. **Irregular patterns** – Gets formed on places particularly floors where fires have gone to flashover and remain exposed to burning for some time. Such pattern can lead to the cause of fire as it indicates the presence of ignitable liquids at the fire scene.[17]

Persistent Accelerants At The Arson Scene

The existence of liquid fuels or solvents at the fire scene is commendably a potential evidence to prove the fire scene as suspected arson.[18] Type of substrate plays a vital role in the detection and recovery of the ignitable liquid used, as from porous surface it

Table 1: Depicting comparison between widely used accelerants (7) (19).

Accelerants	Petrol	Kerosene	Diesel
n-alkane hydrocarbon range	n-C4 to C12	n-C6 to C 16	n-C8 to C 21
Composition	Paraffin, isoparaffin, olefins, naphthenes and aromatics.	Saturated aliphatic and aromatic chain of alkane and cycloalkanes.	Saturated and aromatic hydrocarbons.
Boiling range	Low	Moderate	High
Application	As fuel in automotive spark ignition engine	As fuel for jets and rockets and for cooking purposes	In high speed engines, domestic burners

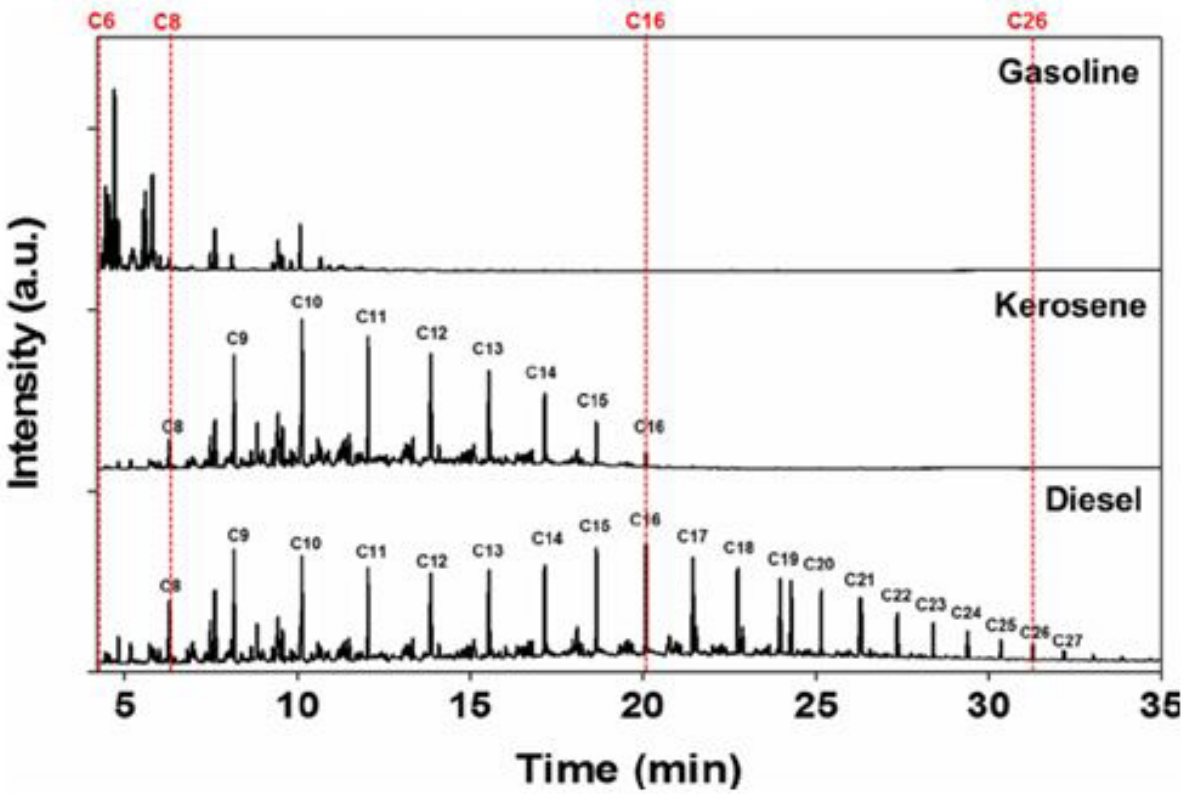

Fig. 3: Depicting difference in petrol, kerosene and diesel using chromatogram peaks.[21]

has been stated that recovery is way easier instead of procuring it from semi-porous as well as non-porous surfaces.

Petrol

The widely found accelerant in most of the arson cases is petrol with lesser and lighter hydrocarbon components than other petroleum products. On ignition, petrol being more volatile in nature has the tendency to form an explosive air vapor mix that led to huge destruction.[7] In order to establish a link between suspect and an arson crime scene where petrol has been detected, research have been carried out to determine whether any unique fingerprint can be obtained from petrol that belongs to different sources and analyzed that by examining more volatile components of it this can be successfully achieved.[20] But with time, this was considered to be less feasible approach to go with. As petrol exhibits a quite distinctive pattern as shown in figure 3 from other respective accelerants, many attempts were made to distinguish it from the rest of the accelerants by interpreting the chromatogram peaks present.[21] In 2003, a more promising study was carried out by Sandercock and Pasquier considering 35 random unevaporated petrol of different grades to focus on the desired poly aromatic hydrocarbons which were extracted by much advanced solid phase micro extraction process and finally analyzed under GC-MS.[7] In one study carried out by Mach, poly aromatic hydrocarbon were detected in burned exhibits of petrol and concluded that certain components are exceptional and particularly belongs to petrol.[22]

Kerosene

It is considered to be the second most widely encountered petroleum product after gasoline. Due to its lower volatility, it finds difficulty at the time of ignition but will hold longer burning duration.[5,7] Like diesel, it holds a larger proportion of lighter hydrocarbon compounds and because of its high boiling point it is more likely to leave behind traces that can be detected after a fire extinguish.[7] Similar to petrol, it also shows significant chromatogram peaks that makes it distinctive from the rest. Its chromatogram projects eight uniformly spaced peaks with its characteristic retention time. Despite of it being used for criminal act, it also has its legitimate use as it is used for cooking purposes so encountering it from places cannot directly link it with a criminal conduct and demands other factors to be analyzed before concluding.[23]

Diesel

After petrol and kerosene, the popular accelerant encountered is diesel. It is the petroleum product with heavier hydrocarbon components possess high boiling point along with low volatility and thus finds difficulty at the time of ignition i.e. consumes a long time to ignite.[7,24] Like kerosene, it also exhibit significant chromatogram pattern as it shows first eight peaks in same time interval as that of kerosene but later extends to eight more making it unique from the commonly used petrol and other accelerants.[2]

Hybrid of Gas Chromatography – for efficient analysis

From past 30 years, for the identification, characterization and analysis of accelerants from suspected arson cases the preferred instrument has been the Gas Chromatography.[25] With the gas chromatography the most apt detector employed is Mass Spectrometry known for its better separation and identification of specific type of accelerants from the fire debris.[26] For the identification and classification of ignitable liquid residues the American Society for Testing and Materials International (ASTM International) has provided standard guidelines to consider at the time of analyzing the residues under GC-MS.[27] Procured samples from fire debris cannot be directly subjected to chromatographic analysis until and unless it should be in volatile liquid form. Thus, sample preparation of fire residues is the utmost step and hence multiple ways have been stated for it and has been accepted by ASTM.

Passive Headspace Concentration with Activated Charcoal (ASTM E1412) the standard and most widely employed isolation method for ignitable liquid residues from fire debris.[28] This type of isolation has been adopted by U.S. majorly whilst isolating using solid phase micro extraction using Tenax as sorbent has been known in European countries. ASTM E1412, despite being the most sensitive, non-destructive and easier method to process with it holds a limitation of working only with the availability of solvent to extract volatile compounds from the activated charcoal strips followed by the injection of the solvent into the GC system.[6,29] Apart from the adsorbent employed earlier, St. Pierre et al in his recent study shown the use of zeolites as the most reliable adsorbent for the extraction of oxygenated ignitable liquids from fire debris samples as it demands only 4 hours of heating at 120 degree celsius which is quite rapid than the ASTM E1412.[30] Similar to ASTM E1412 it also requires a solvent (MeOH) only for oxygenated ignitable liquids.

Solid Phase Micro-Extraction (SPME) has been considered as the best alternative to the activated charcoal strip method as it is more reliable, rapid in nature, more sensitive and above all does not require any of the solvent for extraction.[6,31] American Society for Testing and Materials International has endorsed SPME as the screening test for fire residue analysis. SPME despite of the alternative developed stated unfavorable because of the low robustness of the fibers, known to be useful for shorter period and also difficult to automate when the extraction time is minimal.[6,32]

Nevertheless, of the preconcentration method described above, the major analysis of the accelerants involved remains associated with the most indispensible and versatile gas chromatography that provides visual pattern recognition of the total ion chromatogram (TIC), extracted ion chromatograms (EIC), and target compound analysis.[6] Inspite of the fact that the following stated method works well, it is laborious and also interpretation of results are totally dependent on analyst knowledge and experience as it does not holds any automation. So, this drawback seeks the employment of chemometric tools in order to help the analyst to identify and classify accelerants easily in a shorter span. With respect to chemometric tools, such as hierarchical cluster analysis (HCA), linear discriminant analysis (LDA) or soft independent modeling of class analogy (SIMCA) the combination with total ion spectrum (TIS) enables set procedures to differentiate and classify the fire debris sample and prepared an automated database search tool for fire analyst.[6, 33]

Hs-Ms As E-Nose

Studies have incorporated many forms of GC-MS making it varied number of hybrids by associating it with multiple techniques in order to acquire better efficiency and surpassing the limitations that the previously established instruments holds. Recently, researchers have brought headspace-mass spectrometry (HS-MS) also known as E-nose into existence.[6,34] In the E-nose based MS detection system, the pattern obtained is basically the summed ion spectrum quite similar to that obtained from TIS but with quick response as it does not demands chromatographic separation. Also, the E-Nose technique was optimized for the analysis of fire debris samples not even including any kind of

adsorbent to isolate the ILRs and using as the pre-concentration method where the gas syringe takes the headspace from the vial and directly injecting into the mass spectrometer without any prior chromatographic separation.[34]

Gonzalez et al. were the first to proposed direct analysis of ILRs without performing extraction and adsorption processes.[6] For his study, six different samples i.e. pine wood, cork, paper, newspaper, cardboard, and cotton sheet were initially burned without any ignitable liquid and later with the commonly available petrol, kerosene, diesel, citronella, paraffin, and ethanol. For the analysis of sample, E-Nose system composed of an HS 100 static headspace auto sampler along with Kronos quadrupole mass spectrometer (MS) was used performing different sets of burning experiments with each liquid and the burned pine wood sticks from the different sets but same ignitable liquid were used with the objective of ensuring that all of the burned samples contained the same ILRs.[6]

Results

The study carried out by Gonzalez et al. showed E-Nose as the preferred alternative because it does not require any solvent unlike the ASTM E1412 and turned to be safer for the users, eco-friendly and cheaper as well as it does not even require any absorbent.[6] Its sensitivity and selectivity is also higher as it provides different and specific fingerprints that would be beneficial for the discrimination of different Ignitable Liquid Residues.[6]

Future Perspectives

Recent studies have made arson analyst to form a different perspective for the arson inquest by evolving from the conventional, destructive gas chromatography and shifting to the non-destructive and better alternatives techniques for fire debris analysis.[26] To deal with every limitation a fire debris analysis holds, the spectroscopic techniques has came into light keeping in mind the nature of the burnt sample.

Infra-red spectroscopic techniques

It is based on the absorption of radiations by the molecules of a substance. The absorption takes place when the frequency of the radiations equals the energy difference between the two vibrational energy states of that molecule. As the frequency that get absorbed is specific for different vibrational energy states will thus helps to elucidate the structure of the molecule.[35]

Raman spectroscopic techniques

It is based on the scattering of the radiation. Around the nucleus an electron cloud exists that gets polarized by the radiation of single frequency that in turn creates an unstable excited virtual state that immediately radiates photons. Due to nuclear motion energy transfer takes place because of which the photons consist of more or less energy than the incident radiation. This difference of energy in the scattered radiations is known as Raman scattering.[35]

Discussion

Due to the destructive and less availability of the desired sample from the arson scene has made ASTM to stretch its limits and reframe the standard protocol by making sample preparation an important asset of investigation. Amongst activated charcoal strip method and solid phase micro-extraction, SPME has been preferred and used with varied GC- detectors.[31]

Almirall, *et al*, (1996) performed the recovery of accelerants in aqueous samples from fire debris using solid phase micro extraction with GC-FID and inferred that high sensitivity of solid phase micro extraction(SPME) in comparison to solvent extraction procedure for light petroleum distillate and petrol while solvent extraction of diesel did not produce identifiable chromatograms.[31]

Bodle, *et al*, 2007 performed the multivariate pattern recognition of petroleum based accelerants by solid phase micro extraction with GC-FID and inferred that soft independent modeling of class analogy (SIMCA) to be effective class predictor of accelerants.[36]

Further studies are making way to carry out the identification and classification using non-destructive techniques-

Rodriguez, *et al*, 2011 performed fire debris analysis on carpet, a DVD case, nylon, foam packaging and CD cases using Raman spectroscopy by burning them with petrol, kerosene, diesel and ethanol. The better results were obtained for the samples burnt with petrol and kerosene.[35]

Gonzalez, *et al*, 2015 on the basis of difference in research octane number discriminated two different petrol samples. Near Infra-red radiation spectrophotometry along with hierarchical cluster analysis (HCA) and linear discriminant analysis (LDA) were used to classify the gasoline samples

and found LDA as the better analysis tool than HCA.[35]

Kerr, *et al*, 2017 conducted a study using exhibits involving high density polyethylene i.e. bottle, low density polyethylene i.e. a bag, polyvinyl chloride i.e. a panel, polymethyl methacrylate i.e. flooring, cotton i.e. towel. And tried to investigate the presence of accelerants from fire debris and the results were concluded using Principle Component Analysis (PCA).[35]

Conclusion

Despite of having the best of knowledge as well as the equipments, there exist a number of other factors that unintentionally left unconsidered and sometimes leave the expert with empty handed. The Crimes like arson and its investigation itself holds a baggage of limitations and surpassing of which is a way difficult task to deal with. Despite of all the odds, a forensic expert always tries to stretch its potential by carving the most novice ways to investigate such crimes in which even traces of fire debris can tell us what the fire scene actually reflects. From stating whether the fire is natural, accidental or deliberate to stating the every specification like the origin, cause as well as the rationale behind setting up of fire by considering the psychological aspects as well, in order to exonerate the innocent. Its investigation is the most strenuous task that can be successfully executed by using gas chromatography which is the most versatile instrument that can act as an asset to the inquest. For the identification, characterization and analysis of accelerants from suspected arson cases the preferred instrument has been the Gas Chromatography. Gas chromatography has been used with varied detectors mostly FID and MS amongst which MS has always topped the list with more susceptible methods in terms of sample collection to sample preparation in order to provide the detailed study of arson debris. To maintain the standard of testing American Society for Testingand Materials International (ASTM International) has contributed by establishing set guidelines to operate and process before subjecting to GC instrument. New minds and innovation has bumped into better and more approachable forms of GC by making hybrids of it and the most talked in the recent years is E-nose i.e. headspace-mass spectrometry a strategic mass spectrometry based instrument with both qualitative and quantitative applications surpassing the bygone separation process. This technique emerges out as the most promising tool and an apt alternative hybrid GC technique to carry out the identification of ignitable liquids from the fire debris opening varied ways to conclude the arson investigation. From the beginning to till now GC has ruled in arson inquest but revolutionary ideas and implementations are making way for spectroscopic techniques such as infrared and Raman spectroscopy with an aspect of them being non-destructible in nature. Yet, more studies are required and also in queue to make it reliable and susceptible among the analytical world.

References

1. Brannigan FL. Fire investigation handbook. National Bureau of Standards Handbook 134; August 1980;: P. 197.
2. Mulimani CF. A review of trends and crime patterns of arson offences in India. Research Square; 2009-2018.
3. Sandercock P Mark. Fire investigation and ignitable liquid residue analysis a review: 2001-2007. Forensic Sci Int. 2008;176(2-3):93110. doi: 10.1016/j.forsciint.2007.09.004, PMID 17949931.
4. McCurdy RJ, Atwell T, Cole MD. The use of vapour phase ultra-violet spectroscopy for the analysis of arson accelerants in fire scene debris. Forensic Sci Int. 2001;123(2-3):191-201. doi: 10.1016/s0379-0738(01)00549-7, PMID 11728747.
5. Bumbrah Gurvinder Singh. Derivative ultravioletspectrophotometry: A rapid, screening tool for the detection of petroleum products residues in fire debris samples.Malays JForensic Sci. 2016;17-26::7(1).
6. Ferreiro-González Marta.Determination of Ignitable liquids in fire debris- direct analysis by electronic nose. Sens. 2016:16, 695.
7. Borusiewicz R. Fire debrisanalysis -A Survey of techniques used for accelerants isolationand concentration. Z Zagadnieñ Nauk S1dowych, z. L. 2002;p. 44-63.
8. Pert Alastair D, Baron MG, Birkett JW. Review of analytical techniques for arson residues. JForensic Sci. 2006;: p;515:1033-49. doi: 10.1111/j.1556-4029.2006.00229.x.
9. Bumbrah Gurvinder Singh. Developments in analysis of fire debrisresidues.JForensic ChemToxicol. January–June 2017;: p;3(1).
10. Bob Green. Firesetting patterns, symptoms and motivations of insanity Acquittees charged with arsonoffences. PsychiatryPsycholLaw. 2014 July:(1-10).
11. Shane McCardle. Adolescent firesettingA New

Zealand case-controlled study of risk factors for adolescent firesetting. Fire research report; 2004.

12. Bwalya AC, Bénichou N, Sultan MA. Literature review on design fires.InstResConstr. June 25, 2003:40.
13. Muhammad Masood Rafi TA. A suggested model for mass fire spread. SustainResil Infrastruct. 2018 October.
14. Kinateder Max T, Kuligowski Erica D, Reneke Paul A, Peacock Richard D. Gorbett et al. Risk perception in fire evacuation behavior revisited: definitions, related concepts, and empirical evidence. Fire Sci Rev. 2015;4(1):1. doi: 10.1186/s40038-014-0005-z, PMID 27656350.
15. Sharma DMSaDA. Forensic Investigation in Fire and Arson cases; 2020 June. Available from: https://read.nxtbook.com/wordsmith/evidence_technology/june_2020/fire_and_arson.html
16. DeHaan JD. Kirk's fire investigation New Jersey. 3rd ed. Brady Publishing Co.; 1991.
17. J.J. L. Fire Patterns and Their Interpretation. Encyclopedia of forensic sciences. 2013;: p. vol. 3, pp. 396-405.
18. Dove JD. Oil dries used in arson scenes: new absorbent material. Honors Theses. 2016;354:1-22.
19. Emiel Rorije EMJV, JAdK. Service Request on a critical review of the environmental and physicochemical methodologies commonly employed in the environmental risk assessment of petroleum substances in the context of Reach registrations. European Chemical Agency (ECHA); 2012. p. 4.
20. Li Y, Liang D, Shen H. An analysis of background interference on fire debris. Procedia Eng. 2013;52:(664-70). doi: 10.1016/j.proeng.2013.02.203.
21. Kwon Dongwook, Ko M, Yang J, Kwon MJ, Lee S, Lee S, Identification ofrefined petroleum products in contaminated soils using an identification index for GC chromatograms. Environ SciPollut Res. April 2015;22(16):12029-34. doi: 10.1007/s11356-015-4465-z.
22. Bland HH. Petrol, paraffin and arson. JForensic Sci Soc. 1979;19(2):(81-6). doi: 10.1016/s0015-7368(79)71257-6, PMID 536722.
23. Lentini JJ. The mythology of arson investigation. Scientific Protocols for Fire Investigation. CRC Press. 2006.
24. Ugena L, Moncayo S, Manzoor S, Rosales D, Cáceres JOIdentification and discrimination of brands of fuels by gas chromatography and neural networks algorithm in forensicresearch. JAnal MethodsChem. 2016;2016:(6758281). doi: 10.1155/2016/6758281, PMID 27375919.
25. Aparicio-Ruiz R, García-González DL, Morales MT, Lobo-Prieto A, Romero IComparison of two analytical methods validated for the determination of volatile compounds in virgin olive oil: GC-FID vs GC-MS. Talanta. 2018 May;187:(133-41). doi: 10.1016/j.talanta.2018.05.008, PMID 29853026.
26. Chauhan Ashish,MKGaPC. GC-MS technique and its analytical applications in science and technology. AnalBioanal Tech. 2014;5(6). doi: 10.4172/2155-9872.1000222.
27. Stauffer EricLentini John JASTM standards for fire debris analysis: a review. Forensic SciInt. 2003;132(1):(63-7). doi: 10.1016/s0379-0738(02)00459-0, PMID 12689753.
28. Megan E.Harries SSWJLBKMJ. Characterization of a headspace sampling method with a five component diesel fuel surrogate. Applied chemicals and materials.
29. Rankin JG. Interpretation of ignitable liquid residues in fire debris analysis: effects of competitive adsorption, development of an expert system and assessment of the false positive/ incorrect assignment rate-final report. United. States Department of Justice; 2014 September.
30. St Pierre Kathryne A, Desiderio Vincent J, Hall Adam B. Recovery of oxygenated ignitable liquids by zeolites, Part I: Novel extraction methodology in fire debris analysis. Forensic SciInt. 2014 February;240:137-43. doi: 10.1016/j.forsciint.2014.02.017, PMID 24780556.
31. Almirall Jr, Bruna J, Furton KG KF. The recovery of accelerants in aqueous samples from fire debris using solid-phase microextraction (SPME). SciJustice. 1996 February;36(4):(283-7). doi: 10.1016/S1355-0306(96)72615-1.
32. Cacho JI, Campillo N, Aliste M, Viñas P, Hernández-Córdoba M. Headspace sorptive extraction for the detection of combustion accelerants in fire debris. Forensic Sci Int. 2014;238:(26-32). doi: 10.1016/j.forsciint.2014.02.006, PMID 24631666.
33. Frisch-Daiello Jessica L, Williams Mary R, Waddell Erin E, Sigman Michael E. Application of self-organizing feature maps to analyze the relationships between ignitable liquids and selected mass spectral ions. Forensic Sci Int. 2014;236:(84-9). doi: 10.1016/j.forsciint.2013.12.026, PMID 24529778.
34. Conner L, Chin S, Furton KG. Evaluation of field sampling techniques including electronic noses and a dynamic headspace sampler for use in fire investigations. SensActuators B. 2006 May;116(1-2):(121-9). doi: 10.1016/j.snb.2005.12.069.
35. Yadav Vijay Kumar, Nigam Kriti, Srivastava Ankit. Forensic investigation of arson residue by infrared and Raman spectroscopy: From conventional to non-destructive techniques. MedSciLaw. 2020;60(3)(206-15). doi: 10.1177/0025802420914807, PMID 32279580.

36. Bodle Eric S, Hardy James KMultivariate pattern recognition of petroleum-based accelerants by solid-phase microextraction gas chromatography with flame ionization detection. AnalChim Acta. 2007 March;589(2):(247-254). doi: 10.1016/j.aca.2007.03.006, PMID 17418188.

Review Article

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DNA Methylation: An Approach to Forensic Age Prediction by Molecular Mechanism

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Abstract

Epigenetic processes have an important role in gene expression which is affected by living environments conditions. Occurrence of epigenetics is conciliated by two major molecular mechanisms which includes histone modification and DNA methylation. DNA methylation is an epigenetic channel action, this is a natural process where transmission of a methyl group on the C5 position of the cytosine to form 5-methylcytosine at genome. So, epigenetic change is normal and continual fact that may be influenced by many factors including age, the environment / lifestyle, and disease state. The normal process of aging causes a span of transformation of tissues and organs which gathered over life time. Now this could be possible to be examined through molecular based method. In view of this methylation levels of age associated marker have been used for highly accurate age prediction in this area and depicted that an age associated methylation marker on specific gene location found to be useful with marginal lapse. In Indian scenario there are no such study published on epigenetic markers for age estimation. In future for forensic cases work Molecular biomarkers may be significant to estimate age.

Keywords: DNA methylation, Epigenetics, Age prediction, Age associate CpGs

Introduction

DNA methylation is an epigenetic action that regulate gene expression and involves adding methyl group to a cytosine base. The study of inheritable modification in gene activity or its function due to alteration of point mutation, deletion, insertion and translocation of base on DNA sequence is called genetics. In contrast of this epigenetic is also a constant heritable change in gene activity or function that is not associated with any modification of DNA sequence itself. In this phenomenon all cells in an organism contains the same genetic information that means not all genes are expressed simultaneously by all cell types. Unlike genetic, epigenetics is the study of inheritable alteration in gene expression which do not modify DNA sequence rather change in phenotype without a change in genotype. Epigenetic alteration are those changes which affects directly gene expression by means of DNA methylation, histone modifications, acetylation and chromatic remodelling. An epigenetically re-programming of the gene is express by removal or addition of methyl group, the process of a removal of methyl group from DNA is known as demethylation where agene can be activated by removal of methyl group and leads to gene expression by promoting transcription. In non-coding region of DNA, GC (Guanine-Cytosinebases) rich DNA is highly

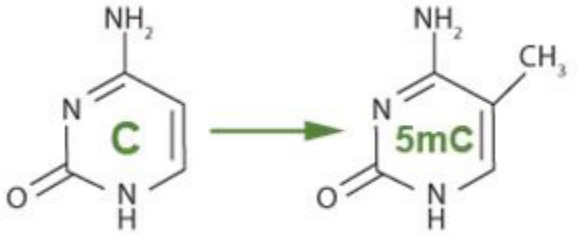

Fig 1. Conversion of Cytosine to 5-methylcytosine

methylated where 5th carbon of cytosine base is more prone to methylation which convert into the 5-methylcytosine (5mC). Basically, where these 5mC commonly occurs in CG rich region called CpG island in somatic cells and in embryonic cells these 5mC is occurred at non-CpG region.At post replication modification in which addition of methyl group happened to the CpG island then this process deactivates the gene activity. In this phenomenon an active gene can be deactivated by additional methyl group on its promoter region, by doing this it inhibits the binding of proteins and enzymes involved in regulate gene expression. Promoter region of gene are non-coding CG rich region of DNA which are more prone to DNA methylation.

In multicellular organism epigenetic action are determined in the diversified gene expression profiles in different types of cells and tissues.[1] So, epigenetic change is normal and continual fact that may be affected by many factors including age, environmental conditions, heredity, lifestyle, treatment history, autoimmune diseases and certain disorders. So, DNA methylation is the genetic process by which methylation of DNA molecule can differ the activity of DNA segment without changing in the sequence. DNA methylation is necessary for normal development and is related with many crucial processes including genomic imprinting, X-chromosome inactivation, repression of transposable element, aging and carcinoma. When this present on gene's promoter region it supresses the gene transcription. An abnormal methylation pattern has been affiliated with many conditions and disease.[2,3] After the development of whole genome bisulfide sequencing, this technique has been applied to epigenetic studies.In this article, age associated DNA methylation marker are reviewed for future aspect of investigation in forensic field.

Role of DNA Methylation

DNA methylation involves the addition of a methyl group (CH3) at 5'position of cytosine residue, which occurs mainly in CpG dinucleotide.The CpG is the region of genomic DNA where a cytosine nucleotide is coming after guanine nucleotide in the linear form of bases at 5′ → 3′ direction and that CpG is shorthand for this nucleotide sequence order, when CpG present in higher frequencies in a genomic region than they know as CpG island. The whole human genome contains 30 million dinucleotides, which exist in methylated and unmethylated state. It is estimated that 30-90% of CpGs are methylated in mammals and these methylated CpGs found in CpG Island in human genome.[4,5] Advancement in research is consistently finds the role of epigenetics in a various fatal disease and genetic disorders of humans. In this regard various research studies have shown that the age-associated CpGs are specific in tissue, and DNA methylation patterns present widely divergent among tissues. Therefore, over the last decade it has become clear that epigenetic markers can be significant for age estimation in forensic investigation.[6,7]

Human tissues change as grow older and this age process is regulated by DNA, many researchers have been successfully used this ageing process to predicts individual's age. The determination of potential change in DNA methylation forms could help in various forensic investigations, such as differentiating monozygotic twins, identifying the tissue source or determining the age of tissue donors.[8] Age prediction is an important part in forensic field. Traditionally age determination is used by morphological method based on bone ossification, chemical analysis of tooth dentine and radiocarbon analysis. Age estimation based on such method has very less accuracy i.e estimation error margin >10 years, these techniques require expert medical experienced anthropologist.The normal process of aging causes a range of alteration of tissues and organs which accumulate over life time.[9] Now this is possible to be examined at molecular level from small remains of biological samples.

Methodology for Methylation

It is an essential task to developing an accurate, sensitive and robust method which could be analyses the age associated CpG sites in forensic samples. Though predicting age across a broad spectrum of human tissue and cell types might be very challenging task but it would be very advantageous to translate and observed age associated DNA methylation differences in the chronological age of an individual. In view of this, for analysis of DNA methylation, currently

researcher have used methodologies of gene specific number of CpG sites on cell types.[10]

Most of the researcher have calculated age, based on targeted methylation detection by the molecular technology such as Bisulfited conversion pyrosequencing, qPCR, EpiTYPER system and massive parallel sequencing. Among all Pyrosequencing found to be gold standard for DNA methylation. Apart from this base on genome-wide profiling has lad more extensive followed for gene regulation epigenetic mechanism. Illumina's Human methylation bead Chip technology is one of the most commonly used method for methylation which allows for simultaneous measurements of the methylation status of 27,578(27K chip) or 482421(450K Chip) CpG site in the genome at single resolution. To minimise prediction errors and increased accuracy, other statistical software has also been used together for regression analysis.[11]

Age associated – methylation marker on specific gene location have been reported useful with marginal lapse of 3.75 years in blood samples and 4.86 years from teeth.[12] Such techniques may be significant in police investigations to determine the age of criminal and unidentified bodies that can leads to identification. In Indian scenario there are no such study published on epigenetic markers for age estimation. Extensive research has been done on the effects of age upon DNA methylation by various working groups in order to address forensic investigation and its importance.[13]

Analysis of DNA methylation from Age-associated CpGsin various tissues

Bocklandt et. al 2011 reported first age predictive model base on DNA methylation data of saliva samples using Illumina 27K from 34 pairs of twin subject aged ranged 21 to 55 years. The model was composed of three age associated CpGs of the EDARADD, NPTX2, and TOM1L1 genes and showed an average precision of 5.2 years.[14]

Koch and Wagner 2011 identified epigenetic age signature which is applicable for various tissue to predict age of donor. The Illumina's Human Methylation 27 Bead Chip array (Illumina 27K) were used which represents 27,578 CpG sites. They used 19 CpG hyper-methylated sites with aging from five public data sets, which were generated from 20-30 samples dermis, epidermis, uterine cervical smear, T-lymphocyte, and monocyte. Among 19 CpG sits four CpGs sites in the NPTX2, TRIM58, GRIA2, and KCNQ1DN genes, and another hypo-methylation site in the BIRC4BP gene were chosen to carried out an age prediction. The developed predictive model that showed mean absolute deviation from chronological age of 12.7 years in eight validation sets with an accuracy. In that study total of 766 samples were collected for validation which includes mainly peripheral blood (PB) leukocytes, saliva, breast tissue, CD34 + hematopoietic progenitor cell, PB monocytes, PB lymphocytes, buccal epithelial cells and cord blood monocytes. DNA methylation were found to be lower in breast tissue in comparison to other cell types used at the CpG site of the BIRC4BP gene and the prediction accuracy increased by11.4 years. The workers of this study were performed and focused on most significant CpG sites in the KCNQ1DN, NPTX2, and GRIA2 for age prediction.[15]

DNA methylation were investigated at the specific locus (IGF2/H19) by C. pirazzini etal. in 2012 for age of homogeneous individuals (males of restricted age range between 30-50 years) who belonging to four Italian districts as well as representative of the major genetic clans. Similarly in a companion of monozygotic (MZ) and dizygotic (DZ) twins of different ages (22-97 years). The analysis of twins' personal life histories suggests that the all-twin pairs difference is likely the result of the aging process, because they sharing a same environment for long periods had no effect on DNA methylation divergence. He reported aging more than population genetics is responsible for the inter-individual variability in DNA methylation patterns in humans; DNA methylation variability appears to be highly region-specific.[16]

The study of Johansson A 2013 reported that age alters DNA methylation at around one third (29%) of the sites (Bonferroni adjusted P-value <0.05), of which 60.5% turns into hypomethylated and 39.5% hypermethylated with growing age. They examined DNA methylation in genome of white blood cells from a community group (N=421) age ranging from 14 to 94 years at 476,366 sites throughout the genome.Their study suggested that the methylation sites on DNA are more often convert into hyper-methylated within CpG islands in compared to sites outside an island. It appears that the change in DNA methylation partially overlap with segment that change histone modifications with age. Naturally happened epigenetic correction and change in gene expression throughout the time generally reflects normal process of aging and variation between individuals. It indicating an interaction between two major epigenetic mechanisms which aid to the elaborate of age-related phenotypes and diseases such as cardiovascular, type II diabetes and autoimmune disease.[17]

Bekaert B 2015. have studied the age-associated genes (ASPA, ELOVL2, EDARADD and PDE4C) from the published literature and demarcated CpG methylation levels from 206 blood samples of both living and deceased personal's (age upto 91 years) and to understand chronological age prediction they used both linear and non-linear regression models with an accuracy. Their report suggested that methylation levels of ELOVL2 showed highest accuracy with quadratic regression model with a Mean Absolute Deviation (MAD) between chronological age and predicted age of 3.75 years. There was no difference in accuracy among both living and deceased personals including among two genders. They also analysed 29 teeth samples from other personals whose age ranged 19-70 years, using same set of age associated markers resulting MAD between age 4.86 years. On validation of both types of samples, results from blood demonstrated the more powerful and reproducible assay. They suggested that set of these 4 CpG DNA methylation markers is capable of producing highly accurate age predictions for blood samples in both living and deceased individuals.[18]

Giuliani. C et. al. 2016 have conducted a study to analyze DNA methylation status at specific CpGs location in FHL2, ELOVL2 and PENK gene and corelates with age in modern teeth (cementum, dentine and pulp) by using Mass spectrometry. They considered 21 modern teeth samples from 17-77 years old subjects to get methylation data and develop a mathematical model that able to utilize DNA methylation values to predict age of an individuals. They reported that the methylation status of the examined regions in the FHL2, ELOVL2 and PENK genes could be used to predict age from modern teeth. Given the accuracy of such approach, these biomarkers promise to be highly informative also when applied to paleo-epigenetics investigations, where teeth are commonly available. This study has suggested DNA methylation is a robust method to predict age for anthropological applications. Based on their work cementum and pulp has 1.25 ± 2.5 years, cementum is 2.45±3.3 years and dentine age 7.07 ± 7 years.[19]

A study was carried oud out by Naue J et.al 2017 using massive parallel sequencing (MPS) and random forest regression on chronological age prediction based on DNA Methylation. This has reported that MPS allowed accurate DNA methylation determination of pre-selected markers and neighbouring CpG sites are best age predictive markers for the age prediction tool. From 208 individuals blood samples were used for training of the algorithm and another 104 individual's samples were used for model evaluation with known age ranged 18-69 years. For this prediction fifteen age-dependent markers of different loci were chosen form publicly available 450K microarray data, and then thirteen were finally selected for the age tool based on MPS which includes DDO, ELOVL2, F5, GRM2, HOXC4, KLF14, LDB2, MEIS1-AS3, NKIRAS2, RPA2, SAMD10, TRIM59, and ZYG11A. This study reported that the validation of the training set leads to a mean absolute deviation (MAD) of 3.21 years and a root-mean square error (RMSE) of 3.97 years. On further evaluation of model were conducted using the test set showed a MAD 3.16 years and RMSE 3.93 years respectively. On considering a reduced model based on only 4 markers (ELOVL2, F5, KLF14, and TRIM59) the result was found RMSE of 4.19 years and MAD of 3.24 years for the test set whereas for cross validation training set: RMSE were 4.63 years and MAD 3.64 years were observed. In this model of study,no statistical difference was found for accuracy of age prediction.[20] Similarly in another study M. Spólnicka et al have analysed DNA methylation profile,for calculation of forensic age estimation in three groups of individuals who diagnosed with three types of medical conditions by observing five markers from five different genes (ELOVL2, C1orf132, KLF14, FHL2, and TRIM59). A converted DNA methylation profile and adjusted age prediction accuracy were reported in all medical conditions. The results of their work demonstrated that the preferred age related CpG sites have found unaffected age prediction capacity in Alzheimer's disease subjects at late onset. Abnormal hyper-methylation and weakened prediction accuracy were found for TRIM59 and KLF14 markers in the group of early onset Alzheimer's disease suggesting increased aging of patients. In the Graves' disease subjects, aberrant hypermethylation observed on TRIM59 and FHL2 for the former and aberrant hypomethylation for the latter. Their study has emphasized high efficacy of the ELOVL2 and C1 or f132 markers for estimation of chronological age in forensics by demonstrating unchanged prediction accuracy in affected individuals by three systematic diseases. Their study also demonstrated that artificial neural system could be a suitable alternative for the forensic predictive DNA analyses.[21] In 2019 a study was conducted by Jung SE considering that the age associated DNA Methylation marker are tissue specific, in view of this a total of 448 samples of various tissue types (blood, buccal swab, saliva) were examined for age prediction at 5CpG sites from ELOVL2, C1 or f132/ MIR29B2C, FHL2, KLF14 and TRIM59 gene. DNA

Table1: Age -Related DNA methylation based other studies for age prediction

Author	Age-related CpG marker	Sample types	Age rang	Technique	Findings
Zbieć-Piekarska R et.al.2015 (27)	ELOVL2	Blood	2-75	pyrosequencing	Accuracy are ±5.75 years, high correlation with increasing age.
Park JL et.al 2016 (28)	ELOVL2,ZNF 423, CCDC102B	Blood	11-90	pyrosequencing	Accuracy ±3.156 years & markers ae useful for analysis in Asian population
Eipel M et.al. 2016 (29)	PDE4C, ASPA, ITGA28, CD6, SERPINB5	Complete blood, Buccal epithelium	1-85	Bisulfite-converted pyrosequencing	Accuracy are ±4.3 years in training set and ±7.03 years in validation set and ±5.09-5.12 years in specific cell combination
Freire-Aradas A et.al. 2016 (30)	ELOVL2 ASPA PDE4C.FHL2, CCDC102B C1 or f132, chr16:85395429	Blood, Peripheral blood, cell lines	Control:19-101, Peripheral blood: 18-104, Twin blood: 42-69	Flanking Sequencing Epi TYPER	Accuracy with ±3.07 years
Freire-Aradas A et.al.2018 (31)	SDS, PGLYRP2, HKR1, TOM1L1, KCNAB3, PRKG2, EDARADD, FL146365, ITGA2B, ELOVL2 ASPA, PDE4C, FHL2, CCDC102B MIR29B2CG, chr16:85395429	Blood	2-18	Flanking Sequencing Epi TYPER® DNA methylation analysis system	Accuracy are ±0.94 years. KCNAB3 has strongest correlation with age of childhood & adolescences
Shi L et.al 2018 (32)	DDO, PRPH 2, DHX8, ITGA2B, Unknown gene (Illumina ID 22398226)	Blood	6-15	Bisulfite Sequencing	Accuracy are ±0.47 years. in boys & ±0.33 years in girls.
Aliferi A et.al 2018 (33)	VGF, TRIP10, KLF14, CSNKID, FZD9, C21ORF63, SSRP1, NHLRC1, ERG, FXN, P2RXL1, SCGN	Blood, Saliva Semen	Blood:11-93 Saliva:16-90.5 Semen:23-50	Bisulfite conversion, Massive parallel sequencing (Illumina MiSeq)	Accuracy between 4.13 - 4.9 years in blood,7.3 -11.1 in saliva & no DNA methylation in semen samples.
Feng L et.al 2018 (34)	TRIM59, RASSF5 Clorf132, CSNK1D, ELOVL2, PDE4C, chr 17 21452808	Blood	15-75	pyrosequencing	Accuracy with ±2.89 years
Peng F 2019 (35)	TRIM59, RASSF5 Clorf132, PDE4C, chr 10:22334463/65, CCDC102B, ELOVL2	Blood stain	18-66	Bisulfite Sequencing EpiTYPER	Accuracy with ±2.94-3.55 years
XuY et.al.2019 (36)	SALL4, MBP, C17OR76, B3GALT6, NOC2L, SNN, NPTX2, SLC22A18, TMEM106, ALEP, SCAP, C16ORF30, FLJ25410	Non blood tissue (Taking form platform Human Methylation 27 Beadchip DNA & Human Methylation 450 Beadchip DNA	0-90	Pyrosequencing	Accuracy are ±4.66 years by MAD. and ±6.08 years by Gradient Boosting Regressor MAD
Fleckhaus J & Schneider PM 2020 (37)	ELOVL2, CDC102B, KLF14, FHL2, C1 or f132, SST, PDE4C, EDARADD	Blood DNA	-	pyrosequencing	ShowedSignificant correlation with age by all marker except FHL2
Correia Dias H et.al 2020 (38,39)	ELOVL2, FHL2, EDARADD PDE4C, C1 or f132,	Blood (deceased) Blood (living)	24-86 1-95	Bisulfite Sequencing	Accuracy are ±6.08 years by MAD in deceased Accuracy are ±5.35 years by MAD in living

methylation were measured by SNaPshot assay and age prediction models were built separately for each samples type. Their results showed high accuracy for age prediction with Mean absolute deviation from chronological age of 3.478 in blood, 4.293 years in buccal swab and 3.552 years in saliva. This study supports their model is useful for forensic analysis.[22]

Correia Dias H etal in 2020 have reported DNA methylation in blood samples of living and deceased subjects for age estimation by SNaPshot assay. They used 5CpG sites located on 5genes (ELOVL2, FHL2, KLF14, C1 or f132 and TRIM59) to compare DNA methylation status between two populations(Portuguese and Korean individuals) previously investigated apart from living and deceased subjects. For that 59 blood samples includes male & females with age ranged 1-94 years, and 62 deceased subjects age ranged 28-86 years from both male & females were examined. They used linear regression model for relationship between methylation level and chronological age. The results showed that for living subject 3CpG sites at ELOVL2, FHL2 and C1 or f132 genes were 4.25 years with mean absolute deviation from chronological age (96.3% of age variation). In deceased subjects 4CpG site at ELOVL2, FHL2 and C1or f132 and TRIM59genes were 4.97 years with MAD (92.5% of age variation). In comparison of Korean and Portuguese population there were some differences found in the extent of age association at targeted locations. Their study suggested usefulness of multiplex methylation by SNaPshot assay for forensic analysis in blood samples. In case of population there is possibility that markers can be population specific.[23]

In 2021 a study was conducted by Zapico SC and co-workers to identify methylation pattern from the erupted third molar dental pulp samples of healthy individuals and correlated this pattern with age. They wanted to understand the current age-at-death estimation for Forensic anthropological point of view as age estimation is less accurate in adult individuals based on degenerative changes in bones and teeth. They collected samples from individuals whose age were 22-70 years. The ELOVL2, FHL2, NPTX2, KLF14, and SCGN marker at CpG sites of genes were used to predict chronological age by different multivariate regression models and reported that mean absolute error (MEA) of 1.5-2.13 years for age-at-death estimation in adult personals. This study suggested excellent accuracy and potential maker for age estimation.[24]

DNA methylation is the most usable and described part of the chromatin marks that formed epigenome. It is a specific chemical conversion of highly stable biological macromolecule. The DNA methylation is an attractive detection and diagnostic biomarker on chosen gene that can serving as an ideal target for epigenetic studies in human populations.[25] The age associated CpGs have been located in a specific tissue as well as across tissues, as DNA methylation profiles are highly dissimilar in different tissues.Among all possible age dependent marker, ELOVL2 is found to be most promising age predictive marker at CpG sites of genes in blood samples, which showed high association of age with DNA methylation by using the 450K Bead Chip array.[26]

Conclusion

Determination or prediction of age of individuals in forensic investigation is an important and difficult aspect. DNA analyses have facilitated scientists to accomplish particular genetic profiles of individuals from DNAs isolated from biological specimens, apart from identify, predictions of age and gender also important part in the investigation. Current approach for age prediction relies on examining the structure and composition of teeth and bone,resulting imprecise estimation. DNA methylation presents another sensitive molecular approach to age prediction.Scientist has identified genomic regions whose DNA methylation patterns is age sensitive. Molecular biomarkers may be significant to estimate age in future for forensic cases solving. Epigenetic approach provides new perspective for using biomarkers. Using such loci will attribute more precise biological age prediction.

References

1. Lisa D Moore, Thuc Le, and Guoping Fan. DNA Methylation and Its Basic Function. Neuropsychopharmacology. 2013; 38: 23-38.
2. Kader F, Ghai M. DNA methylation and application in forensic sciences. Forensic Science International. 2015;249: 255-265.
3. Vidaki A, Ballard D, Aliferi Aet.al. DNA methylation-based forensic age prediction using artificial neural networks and next generation sequencing. Forensic Science International: Genetics. 2017;28: 225-236.
4. Suchita R,kushawaha KPS.Application of DNA Methylation in Forensic Science: A review. Indian Journal of Forensic Medicine and Toxicology. 2016;10 :129-131.
5. Lee HY, Lee S D and Shin K-J. Forensic DNA methylation profiling from evidence material for investigative leads.BMB Rep. 2016; 49: 359-369.

6. Weber-Lehmann J, Schilling E, Gradl G et.al. Finding the needle in the haystack: Differentiating ''identical'' twins in paternity testing and forensics by ultra-deep next generation sequencing. Forensic Science International: Genetics.2014; 9: 42-46.
7. Horvath S, Zhang Y, Langfelder P, Kahn RS, Boks MP, van Eijk K, van den Berg LH, Ophoff RA. Aging effects on DNA methylation modules in human brain and blood tissue, Genome Biol. 2012;13: R97.
8. Forat S, Huettel B, Reinhardt R et.al. Methylation Markers for the Identification of Body Fluids and Tissues from Forensic Trace Evidence. PLoS ONE. 2016;11: 1-19.
9. Xu, C. et al. A novel strategy for forensic age prediction by DNA methylation and support vector regression model. Sci. Rep. 2015;5:17788.
10. Kurdyukov S and Bullock M. DNA Methylation Analysis: Choosing the Right Method. Biology. 2016; 5, 3; 2-21.
11. Tiffany J. Morris□ and Stephan Beck. Analysis pipelines and packages for Infinium HumanMethylation450 BeadChip (450k) data. Methods. 2015;15; 72: 3-8.
12. ChoS, Jung S.-E, HongS.R, LeeE.H, LeeJ.H, Lee S.D, Lee H.Y. Independent validation of DNA-based approaches for age prediction in blood, Forensic Sci. Int. Genet. 2017; 29: 250-256.
13. Salehi J, Abdelaal L, Gomaa R. Use of mRNA marker for age prediction in healthy and unhealthy individuals of Indian subcontinent. International Journal of Sciences: Basic and Applied Research (IJSBAR).2018; 37:175-184.
14. Bocklandt S, Lin W, Sehl ME, et al. Epigenetic predictor of age. PLoS One. 2011;6:e14821.
15. Koch C.M, Wagner W. Epigenetic-ageing-signature to determine age in different tissues. Ageing (Albany NY). 2011; 3:1018-27.
16. Pirazzini C, Giulana C, Bacalini M.G et.al. Space/ population and time/age in DNA methylation variability in humans: a study on IGF2/H19 locus in different Italian populations and in mono- and dizygotic twins of different age, Ageing(Albany NY). 2012; 4:509-520.
17. Johansson A, Enroth S, Gyllensten U. Continuous Aging of the Human DNA Methylome Throughout the Human Lifespan PLOS ONE.2013;8; e67378.
18. Bekaert B, Kamalandua A,Zapico S C, de Voorde WV, Decorte R. Improved age determination of blood and teeth samples using a selected set of DNA methylation markers. Epigenetics.2015;10: 922-930.
19. Giuliani C, Cilli E, Bacalini M G, et.al.Inferring Chronological Age from DNA Methylation Patterns of Human Teeth. Am J Phys Anthropol.2016; 159:585-95.
20. Naue J, Hoefsloot HCJ, Mook ORF, et al. Chronological age prediction based on DNA methylation: massive parallel sequencing and random forest regression. Forensic Sci Int Genet. 2017; 31:19-28.
21. M. Spólnicka1, E. Pośpiech, B. Pepłońska.et.al DNA methylation in ELOVL2 and C1orf132 correctly predicted chronological age of individuals from three disease groups. Int J Legal Med.2018; 132:1-11.
22. JungSE , LimS M , HongS R , LeeE H , ShinKJ , LeeH Y. DNA methylation of the ELOVL2, FHL2, KLF14, C1orf132/MIR29B2C, and TRIM59 genes for age prediction from blood, saliva, and buccal swab samples. Forensic Sci Int Genet. 2019 ;38:1-8.
23. Correia Dias H, Cordeiro C, Corte Real F, Cunha E, Manco L. Age estimation based on DNA methylation using blood samples from deceased individuals. J Forensic Sci. 2020; 65:465-70.
24. ZapicoS C, GauthierQ, Antevska A, . McCordB R. Identifying Methylation Patterns in Dental Pulp Aging: Application to Age-at-Death Estimation in Forensic Anthropology.Int J Mol Sci. 2021;22: 3717.
25. Hannum G, Guinney J, Zhao L, Zhang L, Hughes G, Sadda S, Klotzle B, Bibikova M, Fan J B, Gao Y. et al. Genome-wide methylation profiles reveal quantitative views of human aging rates. Molecular cell. 2013;49: 359-367.
26. Weidner C I, Lin Q, Koch C M, Eisele L, Beier F, Ziegler P, Bauerschlag D O, Jockel K H, Erbel R, Muhleisen T W. et al. Aging of blood can be tracked by DNA methylation changes at just three CpG sites. Genome biology. 2014;15: R24.
27. Zbieć-Piekarska R, Spólnicka M, Kupiec T, Makowska Ż, Spas A, Parys-Proszek A, et al. Examination of DNA methylation status of the ELOVL2 marker may be useful for humanage prediction in forensic science. Forensic Sci Int Genet. 2015;14:161-7.
28. Park JL, Kim JH, Seo E, Bae DH, Kim SY, Lee HC, et al. Identification and evaluation of age-correlated DNA methylation markers for forensic use. Forensic Sci Int Genet.2016; 23:64-70.
29. Eipel M, Mayer F, Arent T, Ferreira MR, Birkhofer C, Gerstenmaier U, et al. Epigenetic age predictions based on buccal swabs are more precise in combination with cell typespecific DNA methylation signatures. Aging (Albany NY).2016; 8:1034-48.
30. Freire-Aradas A, Phillips C, Mosquera-Miguel A, Girón-Santamaría L, Gómez-Tato A, Casares de Cal M, et al. Development of a methylation marker set for forensic age estimation using analysis of public methylation data and the Agena Bioscience Epi TYPER system. Forensic Sci Int Genet.2016; 24:65-74.
31. Freire-Aradas A, Phillips C, Girón-Santamaría L,Mosquera-Miguel A, Gómez-Tato A, Casares de Cal MÁ, et al. Tracking age-correlated DNA methylation markers in the young. Forensic Sci Int Genet. 2018; 36:50-9.

32. Aliferi A, Ballard D, Gallidabino MD, Thurtle H, Barron L, Syndercombe Court D. DNA methylation-based age prediction using massively parallel sequencing data and multiple machinelearning models. Forensic Sci Int Genet. 2018; 37:215-26.

33. Shi L, Jiang F, Ouyang F, Zhang J, Wang Z, Shen X. DNA methylation markers in combination with skeletal and dental ages to improve age estimation in children. Forensic Sci Int Genet. 2018; 33:1-9.

34. Feng L, Peng F, Li S, Jiang L, Sun H, Ji A, et al. Systematic feature selection improves accuracy of methylation-based forensic age estimation in Han Chinese males. Forensic Sci Int Genet. 2018; 35:38-45.

35. Peng F, Feng L, Chen J, Wang L, Li P, Ji A, et al. Validation of methylation-based forensic age estimation in time-series bloodstains on FTA cards and gauze at room temperature conditions. Forensic Sci Int Genet. 2019; 40:168-74.

36. Xu Y, Li X, Yang Y, Li C, Shao X. Human age prediction based on DNA methylation of non-blood tissues. Comput Methods Programs Biomed. 2019; 171:11-8.

37. Fleckhaus J, Schneider PM. Novel multiplex strategy for DNA methylation-based age prediction from small amounts of DNA via pyrosequencing. Forensic Sci Int Genet. 2020; 44:102189.

38. Correia DiasH, CunhaE, Corte RealF, MancoL. Age prediction in living: Forensic epigenetic age estimation based on blood samples.Legal Medicine. 2020;47:101763.

39. Correia Dias H, Cordeiro C, Corte Real F, Cunha E, Manco L.Age estimation based on DNA methylation using blood samples from deceased individuals. J Forensic Sci. 2020;65:465-70.

Review Article

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Forensic Wildlife: A Review

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Abstract

Wildlife crime, defined as the illegal capture, disturbance, ownership, exchange, or movement of animals and/or their derivatives, is a rising international problem that poses a threat to many species' survival. The 'crime scene' in such investigations can range from an animal carcass to terrain with topography as diverse as woodland or desert, as well as a variety of natural and man-made structures. The location of a wildlife crime scene is frequently remote, with insufficient facilities for thorough investigation and evidence collecting. These characteristics may pose particular issues in poorer sections of the world and countries suffering societal turmoil. Working at a wildlife crime scene necessitates the use of tools, investigation procedures, and scientific technologies that are all suited for the situation and the best available. A combination of portable and easy-to-use laboratory equipment, as well as current data gathering and information transmission systems, is likely to be required for effective inquiry in the field. It is critical to use an interdisciplinary approach. These tactics can be used to combat wildlife crimes and punish those involved in the illegal trafficking of animals, flora, and fauna, as well as their products. Footprint analysis, morphological and anatomical studies, microscopic inspections of bodily components, and molecular biology techniques such as serological, mitochondrial DNA, rRNA, and protein analysis are among these techniques. The well-documented methodology and techniques, as well as their disadvantages and advantages, have been thoroughly examined in this review, and will undoubtedly aid the court of law and scientists working in this field to reduce wildlife crime rates through scientific investigations.

Keywords: Wildlife crime, Illegal trade, Species determination, Sex identification, Molecular marker

Introduction

Wildlife crime encompasses illicit wildlife trafficking, ownership of wildlife animals, plants, and their products, as well as other violations of national and international law. It can also be defined as the unauthorized capture, ownership, trading, or movement of animals, plants, or their derivatives in violation of international, regional, or national law. Some also include cruelty to wild animals, both free-living and in captive, as well as persecution. Illegal wildlife flora and fauna trading is currently taking place all throughout the country. International trade poses a serious threat to the world's population, with at least a 50 percent reduction anticipated in the last 47 years. India's legal and policy framework for regulating and restricting wildlife trading is strong. In the current Indian environment, however, strong policies and implementation are critical.[1] After narcotics and guns, wildlife items are the third most illegally traded item. Wildlife crime is on the rise, and the

scope of the problem is unknown. Illegal trade in elephant ivory, wildlife animal skin, sport, food, and clothing are the leading cause of exploitation of wildlife natural resources. More than 60 to 70 percent of the world's population relies on herbal medication for their health needs.[2] The Tibetan Antelope (Pantholops hodgsonii) has been reclassified as Near Threatened from Endangered. Commercial poaching for the shahtoosh, which is used to produce shawls, is the main cause. The Bornean Yellow Muntjac (Muntiacusa therodes) is on the verge of extinction. Hunting for meat, skins, and traditional treatments are the main dangers. In 2016, the Grey Parrot (Psittacus erithacus) was upgraded from Vulnerable to Endangered on the IUCN Red List.[3,4]

Poachers are increasingly sophisticated tactics financed in many cases by covert or corrupt organisations, and countries facing what could be seen as a war on their environments often lack the manpower in terms of conservation officers, rangers, or law enforcement personnel to act as a deterrent or to handle the often overwhelming need.[5] Furthermore, poor countries face a severe lack of funding for military operations. There is frequently a lack of understanding, if not outright ignorance, of what is required in terms of investigative procedures. Even in countries with sufficient financial, personnel, and regulatory assistance, there is frequently a lack of experience and understanding of how to properly evaluate, investigate, document, and present these crimes in court.[6,7]

The illegal wildlife trade in mammals, birds, insects, and reptiles is well-connected all over the world, and thousands of wild animals, birds, insects, and fish are sold every year. In India, projects to protect wild animals such as sea turtles, crocodiles, hangul (red deer), tigers, elephants, and one-horned rhinos have been launched. The horns of one-horned rhinos are poached. The Ministry of Environment and Forests recently developed the Indian (One Horn) Rhino Vision 2020.[8] The goal of this initiative is to increase the number of participants by 2020. Seahorses and pipefish are illegally traded in Tamil Nadu, Kerala, Maharashtra, and Karnataka, India. The usage of it in anti-aging drugs, asthma, high cholesterol, goitre, kidney problems, skin ailments, and infertility is the main reason for exploitation.[9] Footprints analysis at crime scenes, morphological studies of the species, microscopic investigations of body parts, anatomical studies, and serological procedures are all diagnostic markers for species identification in wildlife forensics around the world. Hair, lengthy bones, species-specific tooth morphology, and other traits are used to make this determination. Cooked and dried meats, dried shark fins, egg shells, animal hairs, bone, ivory, rhinoceros' horns, turtle shell, feathers, and fish scales are among the evidences used to identify species in wildlife forensics.[10,11]

Crime Scene Investigation, Collection, And Preservation of The Sample

The primary goal of a wildlife crime scene inspection is to identify the species and cause of death, i.e., to connect the suspected wildlife criminals, victim, and crime scene. The essential precautions are to demarcate the search area, avoid congestion, put on sterile gloves before collecting samples, create a crime map, preserve, package, and forward the evidence. This section focuses on the measures that should be followed during the collecting of samples before they are sent to a forensic laboratory.[12]

With the advancement of DNA analysis technology, there is a significant benefit for wildlife inquiry. The identification of biological evidences for determining the species, gender, individual identity of samples, population, molecular taxonomy, and animal relationship or relatedness is a major challenge for wildlife DNA forensics. Because DNA evidences are often contaminated and degraded in the environment, it is critical to be cautious while sampling biological evidences.[13] As a result, it's best to gather samples from a crime scene as quickly as feasible. During sampling, careful work is required so that things can be properly documented, sized, packed, and kept for inspection at wildlife laboratories. Samples should be appropriately labelled with digits or alphabets, and their descriptions should be included in the forwarding notes, along with a crime scene sketch that shows where the samples were obtained. Samples must be appropriately packed and sealed. In addition, a sample of the seal should be included in the sending message. To verify that no tampering has occurred throughout the transit of items, maintaining chain of custody is a vital aspect of crime investigation.[14,15]

Contamination Precaution

Contamination is the most critical concern associated with the handling of biological DNA samples. During the collecting and transfer of evidence, there is always a substantial potential of DNA contamination. As a result, precautions must be taken to avoid contamination during the collection and preservation of DNA evidence.

The most important Pollution in DNA evidence occurs when other biological sources are mixed in with relevant DNA evidence, or when surface contamination comes into contact with the sample, or when faulty collection and preservation result in a significant danger of biological activity destroying the DNA authenticity.[16] To avoid and limit DNA contamination, the following principles should be followed.

Secure the area and restrict access to only the most important people. Always use sterilized, high-quality gloves and replace them frequently. As much as feasible, handle the evidence with care. Avoid touching any areas where there is a chance of finding DNA evidence. Before or after sampling, use disposable or single-use objects, as well as clean sterilized non-disposable things. It's best not to tread on or over evidence. As much as possible, avoid speaking, sneezing, or coughing near the crime scene or over evidence. Individual scientific evidence should always be recorded and stored in sealed envelopes or containers with suitable documentation. Before packaging, thoroughly air dry the evidence. For any forensic biological evidence, it is recommended that you avoid using plastic packaging for the inner or outer covering and instead use paper bags, envelopes, cartons, or other comparable materials. To avoid cross contamination, each piece of evidence was packed separately. If you are hurt, get out of there. A sufficient care is made to avoid direct bodily and mouth contact with investigation tools that may have come into contact with contaminated surfaces. Avoid eating, drinking, chewing gum, smoking, or using tobacco around a crime scene as much as possible.[17,18]

Sampling

When sampling biological material for genetic analysis in a wildlife crime investigation, the same chain of custody, labelling, transport, and storage processes and protocols must be followed as with other samples. All collection material and tools must be free of "external" DNA from the collector or other potential sources of contamination during sampling. It is suggested that you use sealed and disposable tools.

Sampling should be done by qualified and trained persons in general. To avoid cross-contamination, personal protective equipment (PPE) must be used, and gloves should be replaced between sampling of various materials and/or samples.[53]

To avoid material degradation and contamination, storage and transportation are critical, and various materials require different circumstances. If you have any doubts, you should always contact the laboratory that performed the genetic analysis for more information.

A large range of commercial kits for collecting genetic material is now accessible.[54]

In wildlife crime situations, using these kits is highly advised because it decreases the danger of inappropriate sample collection and processing. All directions from the manufacturer regarding usage, storage, and shipment mode must be followed.

- ***Soft Bodily Tissue:*** Any soft bodily tissue (organs, muscles, etc.) from a carcass can be retrieved for genetic study. Except for samples of very degraded remains, a minimal amount (5 gram) is usually sufficient. Tissues should be stored at -20°C in an airtight, shatter-proof container. Samples should be sent in a cooler with ice packs by express postal service.[55]
- ***Bone, Teeth, Eggshell:*** Bones, teeth, antlers, and eggshells should not be cleaned or bleached before being processed. DNA extraction works best with large molar teeth or large bones (humerus, femur). These items can be stored and sent at room temperature.[56]
- ***Hair/ Feather:*** Hair/Feathers: Gloved hands remove a quantity of hairs and feathers from the body. Do not cut the hairs with scissors or other tools because the best effects are obtained by cutting the hair roots, adhering to the sample. Hairs and feathers must both be removed. Kept and sent at room temperature in a paper envelope or bag. Putting these together, Degradation is increased by placing samples in a tight-sealing plastic container. Mold has infected the samples, rendering them unusable for genetic study.[57]
- ***Blood:*** Blood can be obtained from very fresh carcasses using sterile veterinary equipment and preserved in an anticoagulant tube. Blood tubes should be kept at a temperature of +4°C or less. Avoid thawing and refreezing multiple times since DNA deterioration will occur. Samples should be sent in a cooler with ice packs by express mail. However, in the vast majority of wildlife crime situations, taking blood samples on the spot will be impossible. Blood may be taken during necropsy in such circumstances. Wet blood can be transferred to swabs or specific filter paper and stored at room temperature in a swab tube or a paper envelope. Dried blood on the carcass or any other

object of interest can be collected with a slightly dampened swab and dried in a swab tube or paper envelope. Blood stains on ice or snow can be collected in a tightly sealed container with the original material. The samples should be kept frozen at all times. Avoid thawing and refreezing multiple times since DNA deterioration will occur. Samples should be sent in a cooler with ice packs by express mail.[58,59]

- ***Saliva:*** Swabs can be used to sample saliva on any object or a wound of an animal allegedly killed by a predator. The swab should be inserted in the swab tube after air drying at room temperature and stored and delivered at room temperature.[60]
- ***Urine:*** Fresh urine should be collected in a tight-sealing container (minimum 50ml) and frozen as soon as feasible at -20°C. Samples should be sent in a cooler with ice packs by express mail. Dried urine on any object can be collected with a slightly moistened swab and dried at room temperature in a swab tube or paper envelope. Urine on snow or ice should be collected in the same way as blood stains are collected on these surfaces.[61]
- ***Feaces:*** Wet faeces should be sampled as soon as possible and frozen at -20°C in a tight-sealing, shatter-proof container. Dry faeces should be stored and sent at room temperature in a paper bag or a breathable container.[62]

Wildlife Crime Analysis And Technique Used

The identification of confiscated materials and protected species must be sent to forensic laboratories that deal with wildlife crimes or research institutions that deal with wildlife. In forensic laboratories, several unique traits of species or confiscated materials are used to identify wildlife species. Footprint analysis at crime scenes, morphological research of the species, serological approach, and molecular biology techniques are currently used for animal species identification.[19]

Analysis by Footprints

Footprints of wildlife species are crucial evidence in species forensic investigation. Footprints are the imprints of an animal's foot on surfaces where it walks or in captivity. The pattern of the footprints and their size are used to determine the species type and age. The main issue with using footprint imprints to identify species is that they are typically unnoticeable on hard surfaces, and these footprint locations are frequently contaminated by the presence of other animals.[20]

Morphological Analysis

The easiest form of wildlife forensic procedure in the identification process of evidences and the least expensive forensic analysis is morphological or physical characteristics. Important hints based on external appearance are provided in the identification of species based on morphological characteristics of wildlife flora and fauna. Different species have different physical characteristics such as skin coat colour, pattern of coloration, eyes, pinna, tails, ivory, and so on. Ivory is a type of dentine that has a distinct pattern known as schreger that is illegally trafficked from Asian or African elephants.[21] It also features an angled pattern that serves as a means of distinguishing it from other species. When a whole skin or skeleton is given as evidence, morphological, anatomical, and microscopic study can aid in species identification. In morphology-based identification, experience and a database of known or reference samples become extremely important. When animal skin evidence is seized, for example, hair analysis and comparison with a reference sample are critical in determining the species of origin.[22] This type of evidence is examined and compared to existing reference materials such as taxonomic keys and species monographs. The lack of availability of the whole animal or its intact components preserved as such is a fundamental challenge with morphological identifications, and morphological analysis is again limited to the level of genus or its higher taxonomic level.[23]

Examination by Microscope

The morphology, elemental analysis, and cuticular scale pattern of the hair are all included. Hair is an important feature that can be utilised to identify a species. The identification of animals based on hair evidence can be done using a scanning electron microscope. Scanning electron microscopy gives a larger magnification range and linked Energy Dispersive Spectra, allowing elemental analyses such as sodium, potassium, calcium, and sulphur to be used to identify geographical regions. For mammalian species, hair scale patterns of various species have been documented in Australia and Europe. The fundamental drawback of microscopic techniques is that they require well-preserved samples for microscopic inspection.[24]

Serological Techniques

Serological approaches, like the lock and key paradigm, are based on the interaction between antibodies and their corresponding antigen. Species-specific antibodies are employed to identify the biological evidence's species of origin. The lack of species-specific antibodies in our laboratory is one of the most worrying issues with serological techniques. Antibody cross-reactions in non-target species have also been shown to give favorable outcomes. As a result, in forensic analysis, it is only used as a presumptive test.[25]

Infra Red Techniques

Spectroscopies such as mid-infrared, near-infrared, and Raman, in combination with chemometric approaches, have been shown to be particularly effective in distinguishing species and in identifying the geographical origins of herbal remedies. Because of its great dependability, low cost, and ease of examination of biological evidence such as soil, food, and beverages, NIR spectroscopy is widely used. Wildlife can be identified using near-infrared spectroscopy. Nuclear DNA, mtDNA, and DNA fingerprinting are among the molecular technologies used to determine the origin of species, which aids in the enforcement of wildlife protection laws. Geographic origin has also been determined using genetic approaches. The provenance has also been determined using assignment tests and microsatellites.[26,27]

Radio Isotrope Tracer Technique

In wildlife forensics, this approach is used to discover trace components in the evidential remnants. Radioisotopes are more plentiful in different places, and the presence of such radioisotopes indicates their most likely origin based on isotope abundance maps. Essential and trace elements are the two categories of elements assimilated into the body. Aluminum, arsenic, beryllium, cadmium, chromium, molybdenum, nickel, lead, and tin are examples of non-essential elements. Increased levels of trace metals in the body can be caused by intake and inhalation of trace metals from the diet and environment. As a result, radioisotope tracer techniques are being used in the discovery of species origin. Mt-DNA markers have also been used to determine a person's geographic origin. In such instances, identification based on DNA analysis becomes crucial in wildlife forensics when physical traits of species are lost. Forensic mt-DNA markers like as 16S rRNA, 12S rRNA, Cytochromeb, and Cytochrome Oxidase are employed in wildlife for identification, as well as phylogenetic study. Interspecific variation can be seen in these mtDNA-based markers. Interspecific variation can be seen in these mtDNA-based markers. The genetic marker that is available for many species and subspecies from various geographical ranges is useful in wildlife forensics, but much work has to be done in the present and near future to build a marker of DNA sequence repository of wildlife flora and fauna.[28,29,30]

Laboratory Investigation in Wildlife Crime

General Pathalogical Investigation

In any situation involving visible dead wildlife, veterinary pathology tests are essential. The pathologist can determine not only the cause of death, including the manner and reason for death, but also any underlying pathology and any cause of illness. In addition, forensic pathologists are professionals who may analyse and evaluate the need for additional investigations based on pathological results. As a result, in suspected illegal homicides, a detailed and thorough pathologic investigation at a recognised laboratory is critical! Remember that corpses are more than just dead bodies; they're also bundles containing valuable knowledge and prospects for the future.[31,32]

Species Determination

While determining unique species in mammals such as the wolf, bear, and lynx is simple, correctly identifying birds of prey by physical traits can be difficult. In some species, such as harriers, this is especially true for young individuals aged three years or less. Although various field guides are available to help with bird and other animal identification, the examiner may consult biologists, particularly ornithologists, if in doubt. If you don't have a working relationship with these experts yet, any natural history museum or a university's biology department can help you find one. If morphological criteria, such as the corpse being badly decomposed, do not allow for a correct species classification, a genetic examination of any tissue from the carcass will provide the necessary information.[33]

Age Determination

In a rare circumstance, determining the age of an individual victim may be simple if the person is "known." This could be the situation if the animal was collected and marked during scientific research and then released as part of a reintroduction campaign. The age of an individual is usually determined

over the course of these projects/studies, therefore contacting the project directors is beneficial. In all other circumstances, determining one's age might be difficult, and experts may be consulted. Various physical traits are utilized depending on the species, just as they are for species identification:[34]

Individual size and weight; pelage/plumage colour; ossification of various bones and bone sutures; ossification of various chondral tissues; dimension of bony structures; dentition, eruption, and tooth wear (in mammals). The dental characteristics of large carnivores, particularly the incisors, show regular changes with age and so allow assigning an individual to at least different age categories, such as 1 year, 1–2 years, 3–6 years, 7–9 years, 10–13 years, and 14 years in lynx. Microscopic analysis of the so-called cementum annuli rings of the incisors, canines, or premolar teeth can yield very precise age determination findings in wolves, bears, and lynx, but this approach is only used by a few specialised laboratories. With the exception of some species of birds of prey, whose plumage displays distinct coloration until they are about three years old, age determination in birds is often limited to simply classifying them as juvenile or adult due to the lack of distinct morphological characteristics that allow for a more precise age classification. It's important to remember that genetic testing won't help you figure out your age.[35,36]

Determination of Post Morteum Interval

Many chemical and physical processes begin in a carcass shortly after death, leading to decomposition and, in most cases, eventual skeletisation if left undisturbed. The autolysis and, in rare cases, the putrification processes are both involved in decomposition. Mummification, or the drying of the corpse, may occur in specific circumstances. During necropsy, the pathologist may be able to ascertain at least a reasonable estimate of the time since the individual's death based on the sequence of events. All of these processes, however, are highly varied and are influenced by a variety of intrinsic and extrinsic factors, such as the animal's species and size, ante-mortem activity, cause of death, and environmental conditions, among others. Enormous creatures, such as a bear with thick winter fur and large fat depots, cool more slowly than starving birds.[37] Postmortem cooling models have been established in human forensic sciences during the first few hours after death, however there is very limited data on different animal species, and human data cannot be easily generalised to each animal species. Scavenger activity and mummification after death might also be mistaken as stages of decomposition. As a result, determining the post-mortem interval simply based on the parameters listed above is not well established scientifically, and no practical field application exists. When analysing the findings, use caution! The pathologist must, however, carefully analyse and document the post-mortem alterations detected in a carcass. The intraocular pressure has recently been discovered to be a good predictor for brief (less than 12 hours) post-mortem intervals, and this method could have future promise if verified in a variety of animal species. Other strategies, such as differences in various body fluids properties, have failed to work in humans, let alone animals. Carcasses are frequently discovered later in the post-mortem period in animal forensic cases, rendering the methods indicated above worthless. In such circumstances, entomological examinations of the insects and various stages of larvae that occupy a carcass after death may yield useful information on the time since death.[38,39]

Pathalogical Finding Due to Electrocution

Birds that cut the electricity when perching on and, especially, lifting off from electrical lines are nearly exclusively electrocuted. Due to the low strength of current, electrical fences used to fence off a pasture, for example, will not harm ground-dwelling animals. From the contact point to the point of exit, electrical current normally takes the quickest path through the body. The current runs through the body's least resistant tissues, which include neurons, blood vessels, and moist tissues. Acute cardiac fibrillation, cardiopulmonary arrest, or brain injury are the most common causes of death.[40]

Pathological results can range from severe thermal burns to hardly visible local markings, especially when the tips of feathers were the only areas of contact.

Due to the current disturbing the neurological system, traumatic amputation of wings, legs, or digits may occur as a result of extreme muscular contractions. During necropsy, look for burnt feathers on the wings and wrists. However, because these might be difficult to tell apart from dirt, a dissecting microscope is suggested. Using an additional light source at 530 to 570 nm through a red filter, burnt feathers and skin can also be identified. Look for any discolorations on the feet. Additionally, inspect the whole skin of the carcass for current entry and exit holes. Only minor charring may be visible in these holes.[41,42]

Internal damage might include serious thermal burns if the current is present in the body for an extended length of time. Muscles may show signs of fatigue.

Cooked appearance, discoloration, and visceral ruptures are possible. Internal damage, on the other hand, is essentially non-existent in several situations when the electrocution was quite brief.

In the skin, histopathology may reveal coagulation necrosis and intra-epidermal separation.[43]

Pathalogical Finding Due to Collision With Vechicles

Vehicles and trains may crash with a variety of mammals and birds. A frontal collision in a small species will result in various and severe traumatic lesions, largely blunt force, such as bone fractures, luxations, laceration, and ruptures of interior organs and tissues. There will be a lot of bleeding in different tissues. Due to projection of the animal, coup-contrecoupe effects caused by the quick acceleration/deceleration process in accidents, and other factors, injuries to body parts outside of the immediate impact location may occur. Collision victims frequently have several abrasions on their skin and dirt embedded in their fur.[44]

When large crash victims with vast muscles and/or thick layers of fat, such as bears, are hit in the peripheral region, only minor pathological alterations such as bleeding in the skin and musculature may occur. Although there may be no visible wounds on the outside, look for vehicle paint chips. However, due to the pressures involved in crashes, death can result from the rupture of internal organs and subsequent internal haemorrhage. Larger or faster-moving vehicles can also kill flying birds by creating a downdraft, so there is no direct hit.[45,46]

Lung lacerations are a common occurrence in windmill victims.

Genetic Investigation

Human forensics has used DNA analysis, the molecules that carry genetic information, since the late 1980s. The linear shaped nuclear DNA found in the nucleus of a cell and the circular shaped mitochondrial DNA found in the cell's mitochondria are found in practically every cell of an animal. Mature red blood cells are a significant exception to DNA-carrying cells. Because linear shaped nuclear DNA is very sensitive to deterioration in disintegrating tissue, mitochondrial DNA is frequently better suited for forensic reasons. Furthermore, mitochondrial DNA can be found in hundreds of copies in a single cell, compared to only two copies in nuclear DNA. While nuclear DNA is transmitted down by both parents, making it more susceptible to recombination, mitochondrial DNA is only carried down through the maternal line, making it more stable. However, in each forensic case, the genetic lab will determine which DNA to utilize, as this will be determined by the question that the forensic genetic inquiry is addressing.[47,48]

Species Identification

Blood stains, traces of saliva, faces, feathers, and hairs (all materials that, with the exception of hairs and feathers, cannot be conclusively identified to a single species) are frequently used in wildlife crime investigations. Furthermore, because organic tissues deteriorate to the point where it is hard to identify the species from which they originate based on physical traits, genetic analyses may aid in the identification of various species. Most species and subspecies now have publicly accessible genetic reference databases, allowing examiners to compare the DNA profile of a sample in issue to the unique profiles of species in the databases.[49]

Population Origin

Animal or animal parts may be traced back to their source using genetic analysis. To do so, a reference database is required to match a sample's DNA profile to the known diverse DNA profiles of animals from different areas and/or populations. Because the various DNA profiles of the nine main populations in Europe are known, wolves can be assigned to the population and location from where they originated.[50]

Individual Identification

Reference DNA profiles of the unique individual are required for allocating, for example, a hair sample to a single individual or identifying an animal found dead individually. For this reason, it is advised that all individuals treated during conservation initiatives, such as those bred in captivity for release or animals taken for marking, be sampled for genetic analysis.[51]

Sex Determination and Sample Matching

Male and female mammals have different DNA sequences (for example, the XX chromosomes in female mammals vs the XY chromosomes in male mammals), genetic study can easily determine the individual's gender. Genetic studies can be performed to identify if two or more samples are

from the same person, such as whether blood spots on a car were from a found carcass, whether antlers match a certain individual, and other concerns.[52]

Discussion

The lack of species-specific antibodies in serological analysis, undetectable footprints and erosions by other animals in footprint analysis, the need for samples in well-preserved form in microscopic analysis, and the lack of taxonomic keys and wild animal monographs are all major limitations in wildlife forensics. In the current situation, wildlife conservation is critical in order to limit or prevent the illegal trade in wildlife flora and fauna. Poaching, encroachment into forest areas, and wildlife-related crimes must all be avoided. Wildlife forensics aids law enforcement agencies in prosecuting wildlife criminals and smugglers. Wildlife forensics in India is still underdeveloped, and animal officers are undertrained in applying forensic scientific approaches to wildlife crimes. Having a basic understanding of wildlife forensics will greatly improve their ability to handle wildlife-related offences. A single feature of a species, such as hair morphology, may be shared by closely related species. It is advisable to combine the results of more than one technique in the case of any doubt in species identification. Although molecular approaches can identify a species by themselves and are quite exact and specific, species-specific characteristic markers are not accessible for all species.

References

1. Lawton MPC, Cooper JE (2009) Wildlife crime scene visits. ApplHerpetol 6: 29-45.
2. Mayer WV (1952) The hair of Californian mammals with keys to dorsal guard hairs of Californian mammals Am Midland Nat 48: 480-512.
3. Sreepada RA, Desai UM, Naik S (2002) The plight of Indian sea horses: need for conservation and management. Indian Academy Sci 82: 377-8.
4. Farnsworth NR, Soejarto DD (1991) Global importance of medicinal plants. Cons med plants 25-51.
5. Vincent AC, Foster SJ, Koldewey HJ (2011) Conservation and management of seahorses and other Syngnathidae. J Fish Biol 78 1681-724.
6. Bell B, Machin S (2011) The impact of migration on crime and victimisation. London: UK.
7. Wong KL, Wang J, But PPH, Shaw PC (2004) Application of cytochrome b DNA sequences for the authentication of endangered snake species. Forensic sci Int 139: 49-55.
8. Chapman DD, Abercrombie DL, Douady CJ, Pikitch EK, Stanhopen MJ, et al. (2003) A streamlined, bi-organelle, multiplex PCR approach to species identifica¬tion: application to global conservation and trade monitoring of the great white shark, Carcharodon carcharias. Cons Gen 4: 415-25.
9. Moore SE, Grebmeier JM, Davies JR (2003) Gray whale distribution relative to forage habitat in the northern Bering Sea: current conditions and retrospective summary. Canadian J Zoology 81: 734-42.
10. Branicki W, Kupiec T, Pawlowski R (2003) Validation of cytochrome b sequence analysis as a method of species identification. J Forensic Sci 48: 83-7.
11. Del-Prado R, Cubas P, Lumbsch HT, Divakar PK, Blanco O, et al. (2010) Genetic distances within and among species in monophyletic lineages of Parmeliaceae (Ascomycota) as a tool for taxon delimitation. Mol PhylsEvol 56: 125-33.
12. Wasser SP (2007) Molecular identification of species of the genus Agaricus. Why should we look at morphology? Int J Med Mushrooms 9: 85-8.
13. Hsieh HM, Huang LH, Tsai LC, Kuo YC, Meng HH, et al. (2003) Species identification of rhinoceros horns using the cytochrome b gene. Forensic Sci Int 136: 1-11.
14. Lo TW, Pickle CS, Lin S, Ralston EJ, Gurling M, et al. (2013) Precise and heritable genome editing in evolutionarily diverse nematodes using TALENs and CRISPR/Cas9 to engineer insertions and deletions. Genetics 195: 331-48.
15. Thakur M, Singh SK, Shukla M, Sharma LK, Mohan N (2013) Identification of Galliformes through Forensically Informative Nucleotide Sequencing (FINS) and its Implication in Wildlife Forensics. J Forensic Res 4: 195.
16. Espinosa EO, Mann MJ (1993) The History and Significance of the Schreger Pattern in Proboscidean Ivory Characterization; The American Institute for Con¬servation of Historic & Artistic Works 32: 241.
17. Singh RR, Goyal SP, Khanna PP, Mukherjee PK, Sukumar R (2006) Using morphometric and analytical techniques to characterize elephant ivory. Forensic Sci Int 162: 144-51.
18. Linacre A, Tobe SS (2011) An overview of the investigative approach to species testing in wildlife forensic science. Investig Genet 2: 2.
19. Dahiya MS, Yadav SK (2013) Scanning electron microscopic characterization and elemental analysis of hair: a tool in identification of felidae animals. J Forensic Res 4: 1.
20. Hausman LA (1930) Recent studies of hair structure relationships. Science Monthly 30: 258-77.
21. Cole HI (1924) Taxonomic value of hair in Chiroptera. Philippine J Sci 14: 117-21.
22. Williams CS (1938) Aids to the Identification of

Mole and Shrew Hairs with General Comments on Hair Structure and Hair Determination. J Wildlife Man 2: 239-50.

23. Brown FM (1942) The Microscopy of Mammalian Hair for Anthropologists. Proc American Philos Soc 85: 250-74.
24. Martiniaková M, Grosskopf B, Omelka R, Vondráková M, Bauerová M (2006) Differences among species in compact bone tissue microstructure of mammalian skeleton: use of a discriminant function analysis for species identification. J Forensic Sci 51: 1235-9.
25. Hollmann T, Byard RW, Tsokos M (2008) The processing of skeletonized human remains found in Berlin, Germany. J Forensic Leg Med 15: 420-5.
26. Hillier ML, Bell LS (2007) Differentiating human bone from animal bone: a review of histological methods. J Forensic Sci 52: 249-63.
27. Ubelaker DH (2009) The forensic evaluation of burned skeletal remains: a synthesis. Forensic Sci Int 183: 1-5.
28. Cuijpers AGFM (2006) Histological identification of bone fragments in archaeology: telling humans apart from horses and cattle. Int J Osteoarchaeol 16: 465- 80.
29. Kuo HW, Kuo SM, Chou CH, Lee TC (2000) Determination of 14 elements in Taiwanese bones. Sci Total Environ 255: 45-54.
30. Kakuschke A, Griesel S, Fonfara S, Rosenberger T, Prange A (2008) Concentrations of Selected Essential and Non-Essential Elements in Blood of Harbor Seal (PhocaVitulina) Pups of the German North Sea. Biol Trace Elem Res 127: 28-36.
31. Graham EAM (2007) DNA reviews: ancient DNA. Forensic Sci Med Pathol 3:221–225
32. Hart M, Budgen P (2008) Forensic record-keeping and documentation of samples. ApplHerpetol 5:386–401
33. King DP, Dukes JP, Reid SM, Ebert K and others (2008) Prospects for rapid diagnosis of foot-and-mouth disease in the field using reverse transcriptase-PCR. Vet Rec 162: 315–316
34. Kuiken T, Simpson VR, Allchin CR, Bennett PM and others (1994) Mass mortality of common dolphins (Delphinus delphis) in southwest England due to incidental capture in fishing gear. Vet Rec 134:81–89
35. Lawton MPC, Cooper JE (2009) Wildlife crime scene visits. ApplHerpetol 6:29–45
36. Lawton ME, Sutton JG (1982) Species identification of deer blood by isoelectric focusing. J Forensic Sci 22:361–366
37. McDowall IL (2008) DNA technology and its applications in herpetological research and forensic investigations involving reptiles and amphibians. ApplHerpetol 5: 371–385
38. Merck MD (2007) (ed) Veterinary forensics. Animal cruelty investigations. Blackwell, Ames
39. Morgan RM, Wiltshire P, Parker A, Bull PA (2006) The role of forensic geoscience in wildlife crime detection. Forensic Sci Int 162:152–162
40. Wolfes R, Mathe J, Seitz A (1991) Forensics of birds of prey by DNA fingerprinting with P-labelled oligonucleotide probes. Electrophoresis 12:175–180 Yates B (1999)
41. The morphology of secondary guard hairs. Proc Internatl Assoc Forensic Sci 75th Triennial Meeting, August 22–28, 1999
42. Los Angeles, CA Zhang Y, Wang X, Ryder O, Li H, Zhang H, Yong Y, Wang P (2002) Genetic diversity and conservation of endangered animal species. Pure Appl Chem 74:575–584
43. Ross HM, Wilson B (1996) Violent interactions between bottlenose dolphins and harbour porpoises. Proc R Soc Lond B Biol Sci 263:283–286 Townley L, Ede R (2004) Forensic practice in criminal cases. The Law Society, London White P (2004) (ed)
44. Allentoft ME, Sikora M, Sjögren KG, Rasmussen S, Rasmussen M, et al. (2015) Population genomics of Bronze Age Eurasia. Nature 522: 167-72.
45. Wadsworth C, Buckley M (2014) Proteome degradation in fossils: investigating the longevity of protein survival in ancient bone. Rapid Commun Mass Specom 28: 605-15.
46. Rohman A, Nugroho A, Lukitaningsih E, Sudjadi (2014) Application of Vibrational Spectroscopy in Combination with Chemometrics Techniques for Authen¬tication of Herbal Medicine. ApplSpectrosc Rev 49: 603-13.
47. Laasonen M, Harmia-Pulkkinen T, Simard CL, Michiels E, Räsänen M, et al. (2002) Fast identification of Echinacea purpurea dried roots using near-infrared spectroscopy. Anal Chem 74: 2493-9.
48. Chang CW, Laird DA, Mausbach MJ, Hurburgh CR (2001) Near-Infrared Reflectance Spectroscopy–Principal Components Regression Analyses of Soil Proper-ties. Soil Sci Soc Am J 65: 480–90.
49. Cen H, He Y (2007) Theory and application of near infrared reflectance spectroscopy in determination of food quality. Trends Food Sci Tech 18: 72-83.
50. Huang H, Yu H, Xu H, Ying Y (2008) Near infrared spectroscopy for on/in-line monitoring of quality in foods and beverages: A review. J Food Eng 87: 303-13.
51. Palumbi SR, Cipriano F (1998) Species identification using genetic tools: the value of nuclear and mitochondrial gene sequences in whale conservation. J Hered 89: 459-64.
52. Roman J, Bowen BW (2000) The mock turtle syndrome: genetic identification of turtle meat purchased in the south-eastern United States of America. Anim Conservation 3: 61-5.

53. Waits L, Taberlet P, Swenson JE, Sandegren F, Franzén R (2000) Nuclear DNA microsatellite analysis of genetic diversity and gene flow in the Scandinavian brown bear (Ursus arctos). Mol Ecol 9: 421-31.

54. Seeb JE, Kruse GH, Seeb LW, Weck RG (1990) Genetic structure of red king crab populations in Alaska facilitates enforcement of fishing regulations. Proc Int symposium on King and Tanner crabs. Alaska Sea Grant, Fairbank 491-502.

55. Martiniaková M, Grosskopf B, Omelka R, Vondráková M, Bauerová M (2006) Differences among species in compact bone tissue microstructure of mammalian skeleton: use of a discriminant function analysis for species identification. J Forensic Sci 51: 1235-9.

56. Hollmann T, Byard RW, Tsokos M (2008) The processing of skeletonized human remains found in Berlin, Germany. J Forensic Leg Med 15: 420-5.

57. Hillier ML, Bell LS (2007) Differentiating human bone from animal bone: a review of histological methods. J Forensic Sci 52: 249-63.

58. Kuo HW, Kuo SM, Chou CH, Lee TC (2000) Determination of 14 elements in Taiwanese bones. Sci Total Environ 255: 45-54.

59. Kakuschke A, Griesel S, Fonfara S, Rosenberger T, Prange A (2008) Concentrations of Selected Essential and Non-Essential Elements in Blood of Harbor Seal (PhocaVitulina) Pups of the German North Sea. Biol Trace Elem Res 127: 28-36.

60. Ogden R, Dawnay N, McEwing R (2009) Wildlife DNA forensics—bridging the gap between conservation genetics and law enforcement. Endang Species Res 9: 179-95.

61. Wilson-Wilde L, Norman J, Robertson J, Sarre S, Georges A (2010) Current issues in species identification for forensic science and the validity of using the cytochrome oxidase I (COI) gene. Forensic Sci Med Pathol 6: 233-41.

62. An J, Lee MY, Min MS, Lee MH, Lee H (2007) A molecular genetic approach for species identification of mammals and sex determination of birds in a forensic case of poaching from South Korea. Forensic Sci Int 167: 59-61.

Case Article

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Dyadic Death: Roles Unveiled

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Abstract

Dyadic death is a scenario of two persons dying in a series in a different manner, usually Homicide followed by suicide. Shooting, poisoning, and hanging are the methods used for committing dyadic death. Dyadic deaths by cut-throat methods are rarely reported in the literature due to the extremely painful and violent nature of the injury. The interpretations of cut-throat wounds and other incised wounds could fit into both homicide and suicide. Therefore, a thorough analysis ofthe crime scene is required for corroboration with post-mortem findings. A crime scene is a cluster of evidence like fingerprints, footprints, bloodstains, patterns, etc. The author's report a case of Dyadic death involving cut-throat, stab and incised wounds. The authors intent to highlight the importance of correlating the crime scene findings, which unveiled the sequence of events thereby helping the investigating agency.

Keywords: Murder-suicide, Dyadic death, Homicidal cut-throat, Crime scene, Defense wound.

Introduction

Dyadic death is the term used when a person kills another and then commitssuicide.[1-4] These deaths happen in series, with variants, like Homicide-suicide or Suicide-Suicide (suicide pact).[5] The frequency of dyadic deaths is less in the world.[6] It is commonly seen among relations like husband-wife, husband-wife-children, brothers etc. The common reasons being infidelity in case of couples, while other reasons are property disputes, poverty, and unemployment.[7] "Honour Killing"- a term used in India is similar to dyadic death generally involving homicides when son/daughter goes into a relationship or desires to marry a person against their family wishes out of their caste, statusor religion. The perpetrator in the dyadic death is a person who is in a moral or emotional relationship with the victim like a father, brother, spouse, lover,

co-worker, etc while the perpetrator in an honour killing are family members.[8-11]

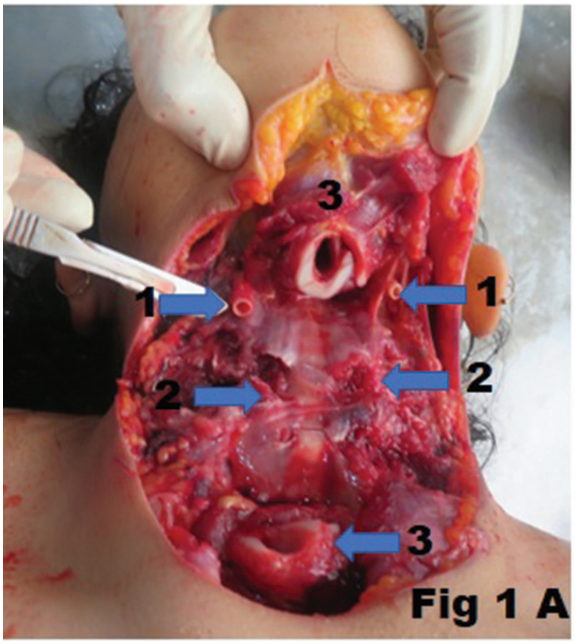
Shooting, poisoning, and hanging are the methods in the decreasing order used for committing dyadic death.[2-4,10,12] The perpetrator generally uses the same weapon or method to end their life.[2,3,10] Cut-throat methods in dyadic death sareless frequently reported in the literature due to painful and violent nature of the injury.[13-15] In case of dyadic death due to cut-throat wounds, the autopsy surgeon significantly contributes in establishing the sequence of events. The findings of the homicidal and suicidal cut-throat wounds overlap each other, as highlighted by Knight and Saukko.[16,17] Hence differentiating between them is an important part of Medicolegal investigation, which would be difficult without proper crime scene examination.The crime scene is a cluster with shreds of evidence like fingerprints, footprints, bloodstains, patterns, etc. Previous case studies also highlight the consideration of crime scenes, to arrive at a better conclusion regarding the sequence of events.[18] The authors, performed a thorough autopsy examination and correlated the findings observed with the crime scene, and unveiled the sequence thereby helping the investigating agency.

Case Report

A university student living in a rented accommodation was not responding to multiple phone calls. His room was broke open by his friends. The boy along with a girl was found dead. The entire scene of occurrence was having different patterns of bloodstains. The girl's body was found lying on a king-size cot and the boy's body was found lying on the floor nearby. During the preliminary assessment by police, both of them were found to have deep cut throat injuries. Two sharp, blood-stained kitchen knives were discovered at the scene of the crime. Fingerprints, footprints, and samples of bloodstains were collected by the forensic crime team experts. Then both bodies were transferred to Department of Forensic Medicine & Toxicology, All India Institute of Medical Sciences (AIIMS), New Delhi for conduction of autopsies.

Autopsy findings of the deceased girl

There was a single deep cut-throat injury on the front aspect of the neck, with dried bloodstains on the face depicting an arterial spurt. Vital structures including bilateral common carotid arteries, jugular veins, vagal nerves, esophagus, and trachea were severed showing clean-cut edges (Fig. 1A). The anterior aspect of corresponding vertebral bodies showed imprinted shallow transverse sharp cuts



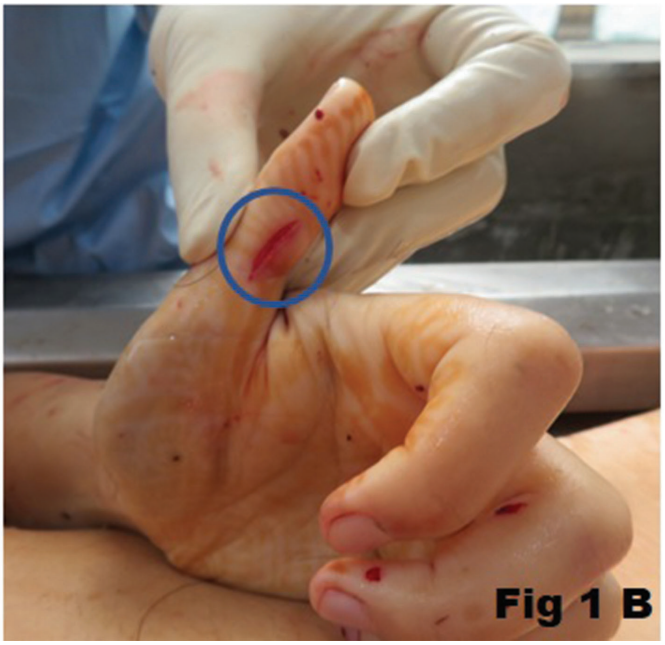


Fig 1A: 1 – Severed upper ends of left and right common carotid artery.
2 – Horizontal cuts are also noted over the underlying vertebra.
3 – Severed upper and lower ends of Trachea.

Fig 1B: An incised wound was present at the inner aspect of the left thumb

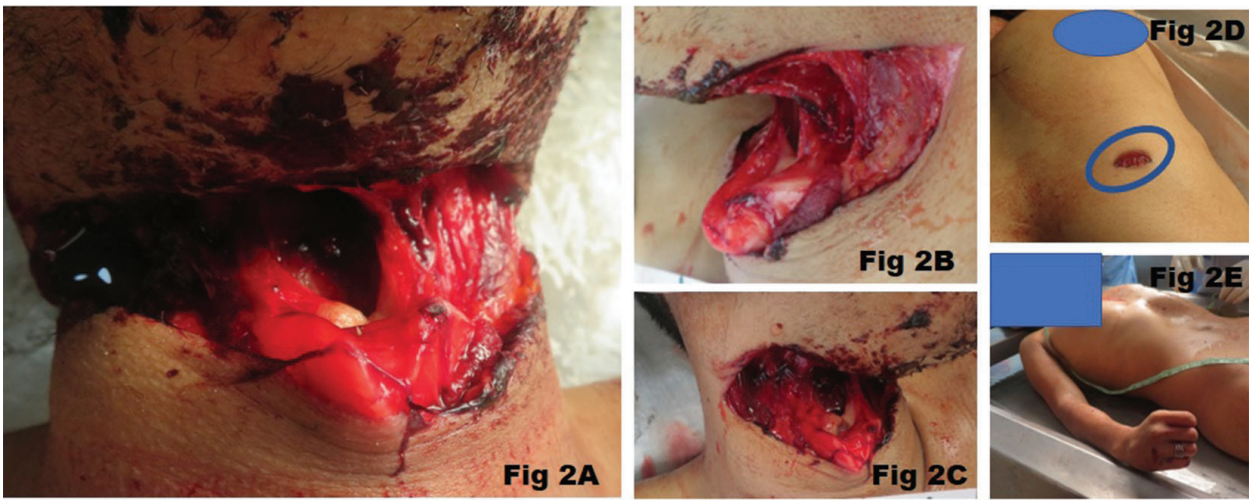


Fig 2A: A shallow cut-throat wound was present at the anterior aspect of the neck.

Fig 2B & C: The wound is shallow on the left side of the neck and became deeper towards the right

Fig 2D: A stab wound over the left hypochondriac region.

Fig 2E: Cadaveric spasm depicting the stab towards the left chest.

(Fig. 1A). There was air embolism to the right side of the heart, sub-endocardial hemorrhages over the anterior surface of the left ventricle, severe pallor, and aspirated blood in lower airways. An incised wound suggestive of self-protection / defense injury was present on the inner aspect of the left thumb (Fig 1B). She was wearing a pair of pink socks devoid of bloodstains.

Autopsy Examination of deceased boy

A cut-throat wound was present at the anterior aspect of the neck of the boy (Fig 2A). The wound was shallow on the left side of the neck and became deeper towards the right with a clean cut edge (Fig 2B). The external jugular vein on the right side, the airway at the level of the thyrohyoid membrane was severed (Fig 2C). A single deep transverse incised wound on the front of the left forearm transecting all flexor muscle bellies and tendons was present. Multiple linear superficial incised wounds suggestive of hesitation cuts were present in front of the left lower forearms. A stab wound just piercing the diaphragm was noted in the left hypochondriac region (Fig 2D). Both the layers of cloth worn by the boy showed fresh cuts corresponding to the stab injury. Air embolism and aspiration of blood into the lower airway, and generalized pallor were observed.

Discussion

A cut-throat injury could be a homicide, suicide, or an accident on rare occasions.[17] Thus, an autopsy surgeon needs to analyze every single factor while framing an opinion. In our case both the girl and boy were known to each other, studying in the same college and allegedly in a relationship, indicating a possibility of a suicide pact. The door was locked from the inside ruling out outside interference. The literature review suggested that the perpetrators in extra familial homicides suicides could either be adults or adolescents. Adolescents when compared with adults were less likely to complete suicide following the homicide.[9]

There were no hesitant cuts on the body of the girl. The cut-throat wound was a single sharp clean cut, severing both the carotids. Two horizontal cuts were also noted over the underlying vertebra. Homicide wounds are deeper, extending to the vertebra due to the excessive use of force by the assailant.[19] The same was also observed in the present case. An incised wound suggesting defensive action of the deceased was also present onthe inner aspect of the left thumb. Défense wounds were more commonly present in homicide cases contradictory to hesitation cuts which are characteristic of suicidal cases.[19-21] According to Taylor, defense injuries arose when the victim tried to defend themselves and the palmar surface of the hand was the most commonest site to occur.[22] Knight and Saukko pointed out that web space between the base of the thumb and the index finger to bea most common site for defense wounds due

to the pincer action.[23] Kumar[24] had reported the presence of hesitation cuts in a homicidal cut-throat to the neck. They justified that on the repeated cutting of the neck by the assailant, hesitant cuts can be produced. However, our case finding corresponds to Yadav et al. with the presence of a combination of a cut-throat wound and a défense wound.[17] The presence of defense wounds on the girl's hand and horizontal cuts at the vertebra ruled out the possibility of a suicide pact.

The cut-throat wound found on the boy's neck was muscle deep severing the thyrohyoid membrane and external jugular vein with less severity when compared to the girl's wound. The findings observed followed the mechanism as depicted by Knight and Saukko as well as Taylor.[22,23] There was no injury found to the carotid arteries due to the hyper extension by the boy at the time of severing the neck which protected the carotids in the carotid sheath, as it lied posterior to the sternocleidomastoid, even though, a cut was noted on both sternocleidomastoid muscles.

A single stab injury over the left chest pierced the diaphragm without any damage to the heart and lung. The manner was concluded as a suicidal stab wound considering the number and site which was congruent with literature.[16] The deceased had stabbed the chest through his clothing while the literature highlighted that the deceased removes the clothing before stabbing. Also, there were multiple horizontal fresh tentative cuts noted over the inner aspect of the left wrist joint and forearm. The incised wound at the wrist joint was deeper to transect the tendons. The cadaveric spasm of the right hand depicted the posture of the deceased stabbing his left chest. The posture was flexion at the wrist with all fingers incompletely flexed enough to hold the knife recovered from the crime scene (Fig 2E). All the above-mentioned points favoured that the boy had completed the homicide suicide act. Further, the secured crime scene was visited post autopsy by autopsy surgeons. The room was locked from the inside during the incident as per the investigating officer and evidence of a broken lock was present. The arterial spurt pattern was observed on the wall corroborating with the girl's head position,who was lying on the bed. Blood stained footprints of the boy were identified due to the evident disparity in dimensions of the foot of both, which was noted during the autopsy. Later, police also recovered a vague suicide note written in the boy's personal diary.

Conclusion

The detailed workup of the case helped in unfolding the sequence of events that occurred during the death circumstance. In such cases, there is a thin line of demarcation in interpreting the incised wounds and cut throat wounds into homicide or suicide and needs cautious approach. Hence, proper correlation of findings at autopsy along with crime scene findings should be done like in the present case which helped in unveiling the role of the assailant.

References

1. Lew EO. Homicidal hanging in a dyadic death. Am J Forensic Med Pathol. 1988;9(4):283-6.
2. Byard RW, Knight D, James RA, et al. Murder-suicides involving children. A 29-year study. Am J Forensic Med Pathol. 1999;20(4):323-7.
3. Jena S, Mountany L, Muller A. A demographic study of homicide-suicide in the Pretoria region over a 5 year period. J Forensic Legal Med. 2009;16:261-5.
4. Shiferaw K, Burkhardt S, Lardi C, Mangin P, La Harpe R. A half century retrospective study of homicide–suicide in Geneva–Switzerland: 1956–2005. J Forensic Leg Med. 2010;17(2):62-6.
5. Prat S, Rérolle C, Saint-Martin P. Suicide pacts: six cases and literature review. J Forensic Sci.2013;58(4):1092-8.
6. Viero A, Giraudo C, Cecchetto G, Muscovich C, Favretto D, Puglisi M, Fais P, Viel G. An unusual case of "dyadic-death" with a single gunshot. Forensic Sci Int. 2014:244,e1–e5.
7. Aggrawal A. Injuries: Classification and Medicolegal Aspects. In: Textbook of Forensic Medicine and Toxicology. Avichal publishing company: New Delhi. 2014; p 209.
8. Avis SP, Hutton CJ. Dyadic suicide. A case study. Am J Forensic Med Pathol. 1994;15(1):18-20.
9. Liem M. Homicide followed by suicide: a review. Aggress Violent Beh. 2010;15:153-161.
10. Santoro JP, Dawood AW, Ayral G. The murder-suicide. A study of post-agressional suicide. Am J Forensic Med Pathol. 1985;6(3):222-5.
11. Chan CY, Beh SL, Broadhurst RG. Homicide-suicide in Hong Kong, 1989-1998. Forensic Sci Int. 2004;140:261-7.
12. Ateriya N, Saraf A, Kanchan T, Shekhawat RS. Dyadic death-an unusual case of post-mortem mutilation. J Forensic Sci. 2018;8(1):1-6.
13. Karger B, Niemeyer J, Brinkmann B. Suicides by sharp force: typical and atypical features. Int J Legal Med. 2000;113:259–62.
14. Chadly A, Marc B, Paraire F, Durigon M. Suicidal stab wounds of the throat. Med Sci Law.

1991;31(4):355–6.

15. Driever F, Schmidt P, Madea B. Differentiation between self-inflicted and homicidal stab wounds to the neck. Arch Kriminol. 2000;205:92–101.
16. Knight B and Saukko P.Self Inflicted injury. In: Knight's forensic pathology, 4thed. London: CRC, 2016; p230–33.
17. Yadav A, Raheel MS, Kumar R L, Sharma SK, Kanwar H. Cut-throat wounds: Suicidal and homicidal—two case reports and review of literature. Med Sci Law. 2016;56(1):53-7.
18. Waghmare PB, Bhise SS, Nanandkar SD. Cut Throat Injury: Homicidal or Suicidal? Crime Scene Visit Solved the Mystery. IJHRLMP. 2016:2:138-40.
19. Reddy KSN. The essentials of forensic medicine and toxicology, 31sted. Hyderabad: K Suguna Devi, 2012; p179.
20. Rao NG. Textbook of forensic medicine and toxicology, 2nd ed. New Delhi: Jaypee Brother's Medical Publishers Pvt Ltd, 2010; p.254.
21. Vij K. Textbook of forensic medicine and toxicology: Principles and practice, 5th ed. New Delhi: Elsevier, 2011; p226–227.
22. Mant K A. Taylor's Principle and practice of medical jurisprudence, 13th ed. New Delhi: B I Churchill Livingstone,2000; p238.
23. Knight B and Saukko P. Incised wounds. In: Knight's forensic pathology, 4th ed. London: CRC, 2016; p159.
24. Kumar SA, Kumar MS V, Babu YR, Prasad M. A case of "atypical homicidal" cut-throat injury. Med Leg J. 2016;84(3):156-8.

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Results

Present your results in logical sequence in the text, tables, and illustrations, giving the main or most important findings first. Do not repeat in the text all the data in the tables or illustrations; emphasize or summarize only important observations. Extra or supplementary materials and technical details can be placed in an appendix where it will be accessible but will not interrupt the flow of the text; alternatively, it can be published only in the electronic version of the journal.

Discussion

Include summary of key findings (primary outcome measures, secondary outcome measures, results as they relate to a prior hypothesis); Strengths and limitations of the study (study question, study design, data collection, analysis and interpretation); Interpretation and implications in the context of the totality of evidence (is there a systematic review to refer to, if not, could one be reasonably done here and now?, What this study adds to the available evidence, effects on patient care and health policy, possible mechanisms)? Controversies raised by this study; and Future research directions (for this particular research collaboration, underlying mechanisms, clinical research). Do not repeat in detail data or other material given in the Introduction or the Results section.

References

List references in alphabetical order. Each listed reference should be cited in text (not in alphabetic order), and each text citation should be listed in the References section. Identify references in text, tables, and legends by Arabic numerals in square bracket (e.g. [10]). Please refer to ICMJE Guidelines (http://www.nlm.nih.gov/bsd/uniform_ requirements.html) for more examples.

Standard journal article

[1] Flink H, Tegelberg Å, Thörn M, Lagerlöf F. Effect of oral iron supplementation on unstimulated salivary flow rate: A randomized, double-blind, placebo-controlled trial. J Oral Pathol Med 2006; 35: 540-7.

[2] Twetman S, Axelsson S, Dahlgren H, Holm AK, Källestål C, Lagerlöf F, et al. Caries-preventive effect of fluoride toothpaste: A systematic review. Acta Odontol Scand 2003; 61: 347-55.

Article in supplement or special issue

[3] Fleischer W, Reimer K. Povidone iodine antisepsis. State of the art. Dermatology 1997; 195 Suppl 2: 3-9.

Corporate (collective) author

[4] American Academy of Periodontology. Sonic and ultrasonic scalers in periodontics. J Periodontol 2000; 71: 1792-801.

Unpublished article

[5] Garoushi S, Lassila LV, Tezvergil A, Vallittu PK. Static and fatigue compression test for particulate filler composite resin with fiber-reinforced composite substructure. Dent Mater 2006.

Personal author(s)

[6] Hosmer D, Lemeshow S. Applied logistic regression, 2nd edn. New York: Wiley-Interscience; 2000.

Chapter in book

[7] Nauntofte B, Tenovuo J, Lagerlöf F. Secretion and composition of saliva. In: Fejerskov O,

Kidd EAM, editors. Dental caries: The disease and its clinical management. Oxford: Blackwell Munksgaard; 2003. p. 7-27.

No author given

[8] World Health Organization. Oral health surveys - basic methods, 4th edn. Geneva: World Health Organization; 1997.

Reference from electronic media

[9] National Statistics Online—Trends in suicide by method in England and Wales, 1979-2001. www.statistics.gov.uk/downloads/theme_health/HSQ20.pdf (accessed Jan 24, 2005): 7-18. Only verified references against the original documents should be cited. Authors are responsible for the accuracy and completeness of their references and for correct text citation. The number of reference should be kept limited to 20 in case of major communications and 10 for short communications.

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