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Gossypol Analysis in Bt and Non-Bt Cotton Seed Extracts by High-Performance Liquid Chromatography (HPLC)

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Present investigation on gossypol content of various seed extracts of Bt and non-Bt of cotton varieties was conducted through an optimized High-Performance Liquid Chromatography (HPLC) on a C_{18} column (4.6 mm × 250 mm, 5 mm particle) with methanol–0.5% acetic acid aqueous solution, 90: 10 (v/v), as mobile phase, at a flow rate of 0.8 ml/min and UV detection at 254 nm. The method was shown to be highly reproducible, with precision [as relative standard deviation (RSD)] and accuracy [as relative mean error (RME)] < 10%, both intra-day and inter-day. The results revealed slightly differences among the Bt and non-Bt varieties of cotton in gossypol content including the Gossypium hirusutum (non-Bt) and Bt varieties of cotton.

Keyword: Cotton seed, Gossypol, High-Performance Liquid Chromatography (HPLC).

1. Introduction

Cotton and related species have pigment glands located throughout the plant, and these glands contain a polyphenolic compound called gossypol[1]. Gossypol, and other related compounds, are an integral part of cotton's self-defense mechanism and protect the plants from pests and possibly some diseases[2]. This compound also has been reported to have antitumor activity[3] and possess contraceptive properties. Gossypol (figure 1), a polyphenolic binaphthyl dialdehyde stored in the pigment glands of cotton, is not only an important resistant substance for cotton but also an important phytochemical component of immense interest due to its several biological properties including anti-cancer, anti-HIV, anti-oxidation antimicrobial, and male contraceptive[4]. Gossypol content of cotton is mainly dependent on different genetic types of pigment glands. The glanded cotton normally contains gossypol in both seeds and plants that is toxic to human and non-ruminant animals. Ordinary glandless cotton contains low-gossypol in seeds, root bark, stems as well as in leaves, but its resistance to diseases, pests and even rats is reduced greatly[5,6]. There are many methods to determine gossypol, such as spectrophotometry, the non-aqueous titrimetric method, gas chromatography and high-performance liquid chromatography[7]. Each of these methods can reflect the relative levels of gossypol. However, the chemical methods are not very specific and gossypol analogs give positive values resulting into significant overestimation. In

contrast, the HPLC method is more accurate, effective and specialized[8,9,10]. In this experiment the comparative studies on gossypol content of various genetic types of Bt and non-BT cotton were conducted through HPLC.

2. Materials and Methods:

2.1 Extraction of Gossypol

For extraction of gossypol three grams of Bt (Vibha, Kaveri, JK, Rudra, Tulasi and Bhasker) and non-Bt Gossypium hirusutum varieties (Narasimha, Sivanandi, NDLH-1906, NDLH-1928, NDLH-1938, NDLH-1959) cotton seed kernels obtained manually were crushed and extracted with diethyl ether (5 x 20 ml)[11]. The solvent was evaporated at low temperature till an oily material containing gossypol was obtained. This was preserved for further use. Gossypol was extracted with aqueous acetone[12] with the same above method. The residual left after the extraction of free gossypol with aqueous acetone was soaked in 2M HCl solution (75 ml) for 10 min then refluxed for 30 min. After cooling, the solution was filtered. The residue was washed with absolute ethanol (15ml). Then chloroform was evaporated from the extract at low temperature till an oily material containing gossypol was obtained. Specific chemical tests were performed for detection of gossypol in samples of seed extracts. For this purpose 5 ml of seed extract crude was dissolved in small volume of ethanol in 25 ml conical flask and final volume was made up to the mark by adding more ethanol. Two ml of each sample solution was taken in the test tube separately and equal amount of solid antimony chloride was added in each test tube and mixed thoroughly. Similarly other types of tests were also performed with stannic chloride and lead acetate. Samples were prepared according to[13]. The dried and powdered samples were macerated with acetone for 16 h, then filtered through 0.45 µm micro-filter membrane and the residue washed. The extract was evaporated to dryness under vacuum. The residue was resuspended in 1% HOAc-CHCl3 solution to 25 ml.

2.2 Chemicals

Methanol (HPLC-grade) was purchased from Brampton (Brampton, Ontario L6t 3Y4 CANADA). All other reagents (analytical grade) were purchased from Beijing Chemical Company Calibration gossypol was purchased from Sigma (USA).

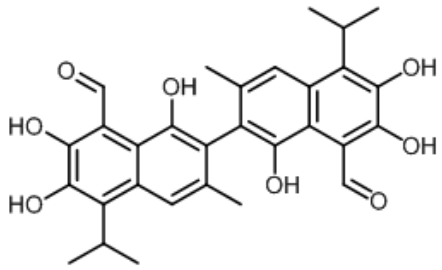

Fig 1: Structure of gossypol

2.3 High-Performance Liquid Chromatography

HPLC was performed on a Shimadzu's Prominence HPLC system (Japan), Prominence Pump model LC-20AD with High precision dual plunger design and Forced check valve design for excellent solvent delivery control .Dynamic Gradient Mixer suitable for high pressure, low pressure & semi-micro applications. Consisting of manual injector[9]. Compounds were separated on a Hewlett-Packard (Palo Alto, CA,USA) Zorbax Eclipse XDB-C18 column (4×6 mm × 250 mm, 5 mm particle) by a Supelco (Bellefonte, PA, USA) C18 precolumn (4×6 mm × 20 mm, 5 mm) (Wang et al 1985). The mobile phase was 90: 10 (v/v) methanol-0.5% acetic acid aqueous solutions at a flow rate of 0.8 ml/min. The wavelength for UV detection was 254 nm. A 5 ml sample was injected. The assays were performed at room

temperature. Calibration curves were produced by analysis of solutions containing 0, 1, 3, 5, 7, 10 mg/l standard gossypol in chloroform containing 1% acetic acid.

2.4 Precision, Accuracy and Limit of Detection

The studies were performed with solutions containing gossypol at concentrations of 1mg/ml (low), 5 mg/ml (medium), and 20 mg/ml (high). The solutions were stored in dark at room temperature, 4ºC, and at –20ºC. The interday study was performed over a period of 15 days on days 0, 1, 3, 6, 19 and 12. The equations used to calculate relative standard deviation of the mean (RSD) and relative mean error (RME) were: RSD (%) = [standard deviation/ mean] × 100; RME (%) = [(measured value – true value)/ true value] × 100. RSD and RME were used as measures of precision and accuracy, respectively. Limit of detection was calculated as the lowest concentration of standard for which both RSD and RME were less than 20%[14].

2.5 Optimization of Mobile Phase

Mobile phases were attempted using calibration grade gossypol (Sigma USA) at room temperature. The retention time with methanol-0×5% phosphate, in the ratios 80 : 20, 85 : 15 and 87 : 13 (v/v), were 31×7 min, 15×2 min and 9×5 min, respectively. The retention times with methanol-0×5% acetic acid aqueous solution, in the ratios 80 : 20, 85 : 15, 87 : 13 (v/v), were 28×2 min, 14×2 min and 10×5 min, respectively. The retention times of methanol-0×5% phosphates and methanol-0×5% acetic acid aqueous solution 90: 10 (v/v), were 3×4 min and 4×5 min, but the peak in the latter case was narrower and better than the former one. What is important that the separation effect of samples was best using mobile phase of methanol-0×5% acetic acid aqueous solution 90: 10 (v/v) (figure 2b, c). Therefore the optimal mobile phase was methanol-0×5% acetic acid aqueous solution, 90: 10 (v/v).

2.6 Chromatography

The chromatogram obtained from a solution of standard gossypol figure 2a showed that the standard was free from contaminants. The retention time of gossypol was 6.1 min. Gossypol peak in figure 2b, 2c was separated to baseline from the most closely eluting component enabling accurate quantification. The analytical methodology established that the UV detector response to gossypol was highly linear through the concentrations range from 1 to 10mg/ml. The equation of the calibration curve was y = [2×1026 ×103) x – 3474×8757 (r 2= 0×9991, n = 6). The quantities used for assessment of precision and accuracy, RSD and RME, were always <10%. According to Causon (1997), precision and accuracy are generally acceptable if RSD and RME are <15%, so the results show that the reproducibility of the method was good. The limit of detection was 1 mg/ml.

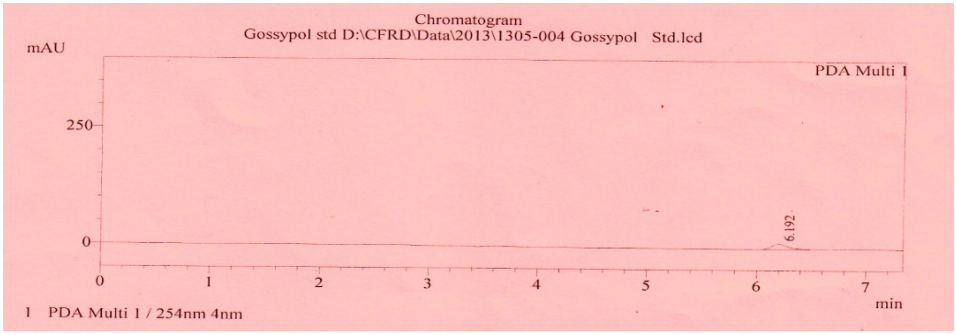

Fig 2: a. Chromatograms obtained from gossypol of standard

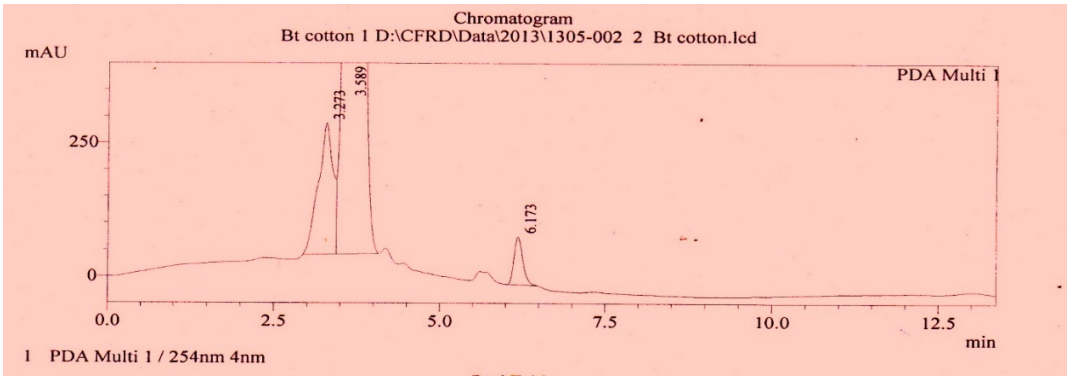

Fig 2: b. Chromatograms obtained from gossypol of Bt seeds sample

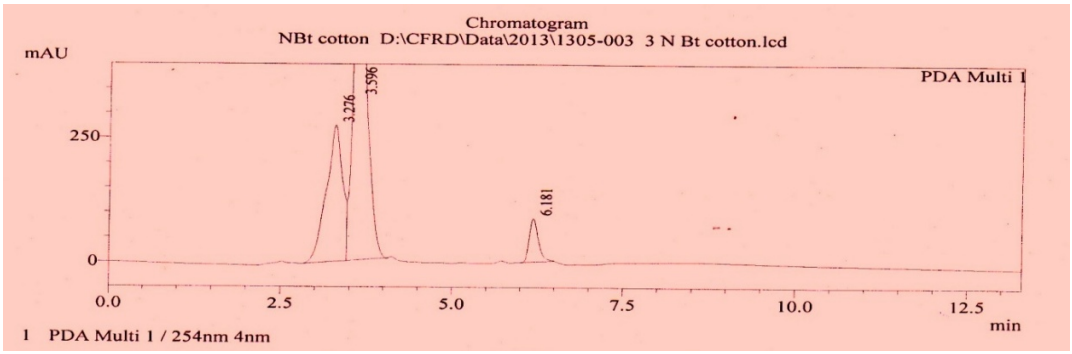

Fig 2: c. Chromatograms obtained from gossypol of non-Bt seeds sample

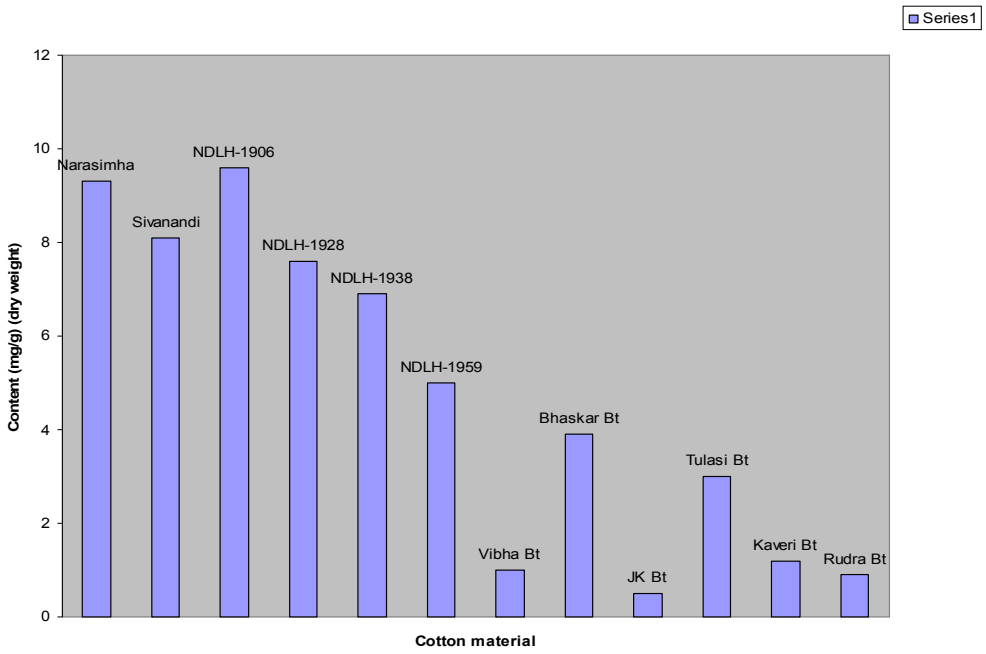

Fig 3: Comparison of gossypol content of the seed in different gland types of cotton materials.

3. Results and Discussion:

In the present study different extracting methods of gossypol such as different solvents, treatments and extracting time etc.

were assessed and the method of Wang et al[9] proved to be the optimal. The different mobile phases were optimized. We Followed an accurate, rapid and highly reproducible method for determination of gossypol in cotton with precision (as RSD) and accuracy (as RME).Generally the free gossypol other than the bound gossypol is toxic to human and non-ruminant animals. The American Oil Chemists' Society clearly defines that all of the gossypol extracted with 70% acetone aqueous solution is called free gossypol[7] although, in some studies it has not been used as the extract ant [14,15]. Wang[9] revealed that gossypol extractable with acetone and 70% acetone aqueous solution are the same, therefore we have used acetone as the extracting solvent for free gossypol. The studies revealed wide variation in the amount of gossypol in the seeds of various types of gland and glandless G. hirusutum and Bt cotton varieties. This is very helpful to have an accurate and informed learning of the valuable resources of new glandless or glanded cotton.

4. Conclusion

The amounts of gossypol in the seeds depicted in figure 3 revealed great differences among the different seed extracts of Bt and non-Bt cotton. The contents of gossypol varied from 0 to 9.206 mg/g. The contents of gossypol 2 varieties G. hirusutum possessed high contents of gossypol in the seeds. The contents of gossypol remaining four varieties of G. hirusutum were the lowest, from 0 to 0.287 mg/g. Contents of gossypol of 6 Bt transgenic varieties were very low compared with the 6 varieties of G. hirusutum.The purpose of this study is to stimulate the extensive exploitation of these resources, to provide a basis for isolating specific genes and to help understand the molecular mechanisms involved and agronomic importance of certain varieties of cotton.

5. Acknowledgement:

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