An optimized gossypol high-performance liquid chromatography assay and its application in evaluation of different gland genotypes of cotton

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A comparative study on gossypol content of various genetic types of pigment glands of cotton varieties was conducted through an optimized high-performance liquid chromatography (HPLC) on a C_{18} column (4·6 mm × 250 mm, 5 μm 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. Absolute recoveries were > 94%. The results revealed major differences among the different gland varieties or species of cotton, including the special and ordinary glandless and glanded *Gossypium hirsutum*, *G. barbadense*, and displayed the precious resources of different glands of extraordinary cotton.

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1. Introduction

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-microbial, anti-HIV, anti-oxidation and male contraceptive (Pharmacia Institute of China Medicine Academy 1995).

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 no or low-gossypol in seeds, roots, stems as well as in leaves, but its resistance to diseases, pests and even rats is reduced greatly (Zhang *et al* 1999, 2001). Therefore a cotton variety which is characterized by the presence of glanded roots, leaves and stems (to maintain the resistance trait) but glandless seeds (for safe utilization) is highly desired. Biologists and agronomists have long been studying the glanded characters and the glandless characters of cotton as well as their gossypol content (Wang *et al* 1985; Hron *et al* 1990, 1999; Xiang *et al* 1993; Yang *et al* 1995; Yuan *et al* 1999). There are many methods to determine gossypol, such as spectrophotometry, the non-aqueous titrimetric method, gas chromatography and high-performance liquid chromatography (HPLC) (Pharmacia Institute of China Medicine Academy 1995; Yang *et al* 1995). 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 (Nomeir 1982; Wang *et al* 1985; Yang *et al* 1995). Recently, Chinese scientists have bred some special varieties and genotypes of cotton

Keywords. Cotton (*Gossypium*); gossypol; high-performance liquid chromatography (HPLC); pigment gland

Abbreviations used: HPLC, High-performance liquid chromatography; ICIS, Industrial Crop Institute of Sichuan Academy of Sciences; RME, relative mean error; RSD, relative standard error.

(Zhang *et al* 2001, 2002). There is no report yet about their gossypol content measured by HPLC. In this experiment the comparative studies on gossypol content of various genetic types of pigment glands of cotton, including the new special and ordinary glandless and glanded *Gossypium hirsutum* and *G. barbadense*, were conducted through HPLC. The purpose 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.

2. Materials and methods

The cotton materials tested were divided according to their pigment gland and species, into 6 groups (numbered 1–16, figure 3): (a) three glanded varieties of *G. barbadense* – Mexico 8390 (No. 1), 5593Φ (No. 2) and 72-69 (No. 3); (b) four glandless varieties of *G. hirsutum* – Wufen383 (No. 4), Jijiaowufen (No. 5), SP21 (No. 6) and Xiangwu 93 (No. 7); (c) one special new cv. Xiangmian 18 (No. 8) with gland-less seed but glanded root, leaf and stem, obtained from National Center of Hybrid Cotton Development and Extension, Hunan (Zhang *et al* 2001); (d) two special glanded germplasms of *G. hirsutum* resistant to many races of Verticillium wilt dahliae, Chuan 737 (No. 9) and Chuan 2802 (No. 10) bred by Industrial Crop Institute of Sichuan Academy of Agricultural Sciences (ICIS) (Li *et al* 1996; Zhang and Cai 2002); (e) three glanded new resistant varieties of *G. hirsutum* Chuanmian 239 (No. 11), Chuanmian 243 (No. 12), Chuanmian 65 (No. 13) and 1 susceptible Chuanmian 45 (No. 14) to Verticillium wilt (Zhang and Cai 2002); and (f) two Bt transgenic lines or varieties, Zhongmiansuo 30 (No. 15), RP4-4 (No. 16) obtained from Cotton Institute of Academy of Agricultural Sciences of China. Materials in groups (a–e) were obtained from ICIS.

2.1 *Chemicals*

Acetonitrile and methanol (HPLC-grade) were purchased from Tedia (Fairfield, OH, USA). All other reagents (analytical grade) were purchased from Beijing Chemical Company (Beijing, People's Republic of China). Calibration gossypol was purchased from Sigma (USA).

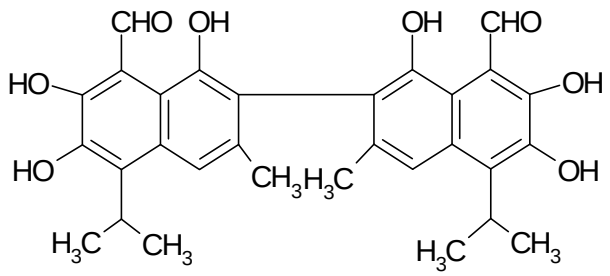

Figure 1. Structure of gossypol.

2.2 *Sample preparation*

Samples were prepared according to Wang *et al* (1985). The dried and powdered samples (about 0·1 g) 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-$CHCl_3$ solution to 25 ml.

2.3 *High-performance liquid chromatography*

HPLC was performed on a Waters system (Milford, MA, USA) 2487 dual-wavelength absorbance detector, a Waters 515 pump, and a Waters Rheodyne 7725i consisting of manual injector (Wang *et al* 1985). Compounds were separated on a Hewlett-Packard (Palo Alto, CA,USA) Zorbax Eclipse XDB-C18 column (4·6 mm × 250 mm, 5 μm particle) by a Supelco (Bellefonte, PA, USA) C_{18} precolumn (4·6 mm × 20 mm, 5 μm) (Wang *et al* 1985). The mobile phase was 90 : 10 (v/v) methanol-0·5% acetic acid aqueous solution at a flow rate of 0·8 ml/min. The wavelength for UV detection was 254 nm. A 5 μl sample was injected. The assays were performed at room temperature.

2.4 *Calibration procedure*

Calibration curves were produced by analysis of solutions containing 3, 6, 15, 30, 45, 60 μg/l standard gossypol (purity, > 99%) in chloroform containing 1% acetic acid.

2.5 *Precision, accuracy, and limit of detection*

The studies were performed with solutions containing gossypol at concentrations of 3 μg/ml (low), 15 μg/ml (medium), and 60 μg/ml (high). The solutions were stored in dark at room temperature, 4ºC, and at – 20ºC. The inter-day study was performed over a period of 15 days on days 0, 3, 6, 9, 12 and 15. 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% (Causon 1997).

2.6 *Recovery*

Recovery was studied by use of three sets of samples: (i) standard solutions containing 3 μg/ml, 15 μg/ml, and 60 μg/ml, (*a*); (ii) 0·2 g sample, (*b*); and (iii) standards added to

0·2 g sample, (*c*). All three sets of samples were subjected to the extraction procedure described above. The equations used to calculate recovery were: Absolute recovery (%) = [(*c*–*b*)/standard (unprocessed)] × 100; Relative recovery (%) = [(*c*-*b*)/*a*] × 100.

3. Results

3.1 *Optimization of mobile phase*

Several mobile phases were attempted using calibration grade gossypol 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% phosphate and methanol-0·5% acetic acid aqueous solution 90 : 10 (v/v), were 6·5 min and 7·1 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).

3.2 *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 7·1 min. Gossypol peak in figure 2b, c was separated to baseline from the most closely eluting component enabling accurate quantification (the minimum resolution was > 3·0).

3.3 *Linearity*

The analytical methodology established that the UV detector response to gossypol was highly linear through the concentrations range from 3 to 60 μg/ml. The equation of the calibration curve was $y = [2{\cdot}1026 \times 10^3)\ x - 3474{\cdot}8757$ ($r^2 = 0{\cdot}9991$, $n = 6$).

3.4 *Precision, accuracy, limit of detection and recovery*

The quantities used for assessment of precision and accuracy, RSD and RME, were always < 10% (table 1). According to Causon (1997), precision and accuracy are generally acceptable if RSD and RME are ≤ 15%, so the results in table 1 show that the reproducibility of the method was good. The limit of detection was 3 μg/ml (i.e.15 ng absolute), because RSD and RME were < 10%. All the recoveries were greater than 94%.

3.5 *Sample analysis*

The amounts of gossypol in the seeds depicted in figure 3 revealed great differences among the different genetic types of gland cotton. The contents of gossypol varied from 0 to 9·206 mg/g. The contents of gossypol of the special germplasms group (d) of glanded *G. hirsutum* and the glanded *G. barbadense* group (a) possessed high contents of gossypol in the seeds. The contents of gossypol of group (b) four glandless varieties of *G. hirsutum* were the lowest, from 0 to 0·287 mg/g, less or far less than the international food standard (0·4 mg/g). The special new cv., Xiangmian 18 with glandless seed but glanded plant possessed 0·382 mg/g of gossypol in its seeds, more than ordinary glandless cotton, but it is still lower than the international food standard. In addition, the contents of gossypol of 2 Bt transgenic lines or varieties

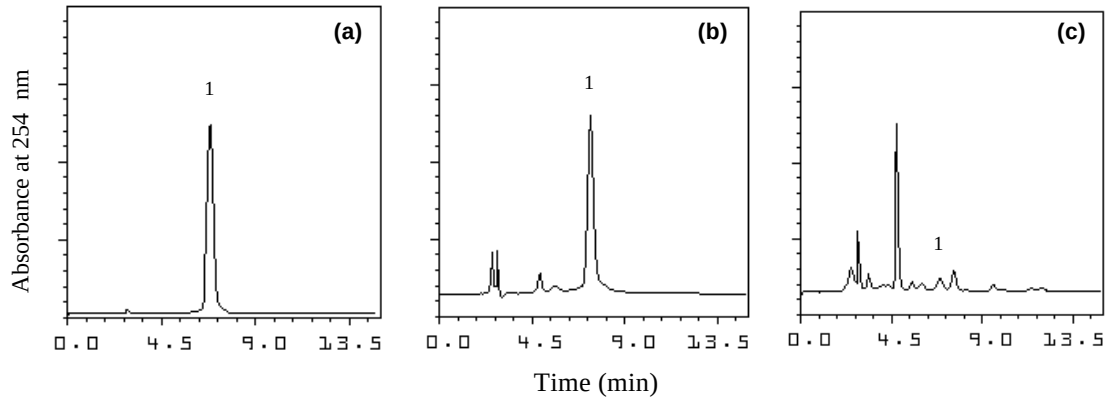

Figure 2. Chromatograms obtained from gossypol of standard (**a**), seeds sample (**b**) and leaf sample (**c**).

Table 1. Precision accuracy and recovery data.

	Storage	3 μg/ml	15 μg/ml	60 μg/ml
Intra-day precision	RT	7·8	4·6	1·2
Intra-day accuracy	RT	7·1	– 1·5	– 0·2
Inter-day precision	RT	8·6	3·4	1·6
	4°C	7·2	2·1	0·9
	– 20°C	6·9	1·7	0·2
Inter-day accuracy	RT	6·7	– 0·8	– 1·3
	4°C	5·9	– 1·9	0·5
	– 20°C	5·1	– 2·1	0·2
Recovery	Absolute	96·0 ± 2·8	97·3 ± 3·1	94·7 ± 2·9
	Relative	102·9 ± 6·2	103·2 ± 7·1	100·4 ± 5·8

RT, room temperature; mean ± SD, $n = 6$.

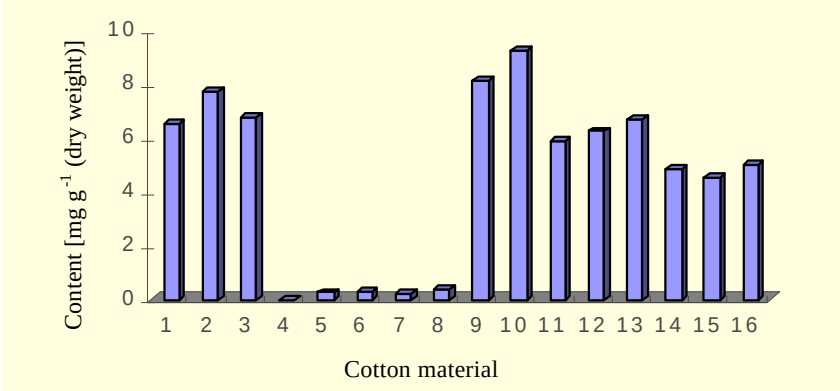

Figure 3. Comparison of gossypol content of the seed in different gland types of cotton materials 1–16 (mean ± SD, $n = 6$).

[group (f)] were very low compared with the three resistant glanded varieties of *G. hirsutum*.

The contents of gossypol of the top leaf in florescence of the material 72–69 (glanded), SP21 (glandless) and Xiangmian 18 were 0·384, 0·218, and 0·312 mg/g, indicating thereby that the contents of gossypol of the top leaf in florescence of Xiangmian 18 was between the glanded and glandless cotton.

4. Discussion

In this study different extracting methods of gossypol such as different solvents, treatments and extracting time etc. were assessed and the method of Wang *et al* (1985) proved to be the optimal. The different mobile phases were optimized. We established an accurate, rapid and highly reproducible method for determination of gossypol in cotton with precision (as RSD) and accuracy (as RME) < 10%, both intra-day and inter-day. Absolute recoveries were greater than 94%, and the correlation coefficient $r^2 = 0{\cdot}9991$ ($n = 6$).

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 (Yang and Xiang 1995), although, in some studies it has not been used as the extractant (Xiang and Yang 1993; Hron *et al* 1999). Wang *et al* (1985) 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 genetic types of gland among the special and ordinary glandless *G. hirsutum*, the special (germplasms) and ordinary glanded *G. hirsutum* and *G. barbadense* displayed the precious resources of different gland variety of a newly bred cotton. This is very helpful to have an accurate and informed learning of the valuable resources of new glandless or glanded cotton. The extraordinary kind Xiangmian 18 with glandless seed but glanded plant is of great significance, which may eventually lead to making cotton a crop simultaneously

producing lint (fiber), safe food (kernel protein) and oil. The two germplasms have been successfully used in practice as resources resistant to Verticillium wilt in China (Li *et al* 1996; Zhang and Cai 2002). So the special cotton with either high or low (zero) contents of gossypol has different extensive application.

In fact, the results also reflected the relationship between the resistance and the gossypol content of cotton. Generally *G. barbadense* among four cultivated species of *Gossypium* is most resistant to Verticillium wilt (Gu and Ma 1966) and the 2 glanded germplasms of *G. hirsutum* are two special resources resistant to many races of *Verticillium dahliae* including the leaf-fall races of the American and the Chinese Verticillium wilt (Yang and Xiang 1995). Both groups possessed high contents of gossypol in seeds. In contrast, most ordinary glandless cotton is susceptible to Verticillium wilt due to low or complete absence of gossypol (Zhang *et al* 1999). Some Bt transgenic cotton are not resistant to Verticillium wilt due to low content of gossypol (Sun *et al* 1998; Hron *et al* 1999). According to Xia *et al* (1994) the content of gossypol is remarkably correlated with the resistance of Verticillium wilt in cotton. The results of our experiments corroborate the above observations.

To our knowledge, this could be the first attempt to HPLC quantitate gossypol in these special new varieties and germplasms, and assess their agronomic importance.

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