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Specific Method for Spectrophotometric Determination of Gossypol

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Abstract: Simultaneous reactions of aniline on the aldehyde groups and ammonium molybdate on the ortho-diphenolic groups of gossypol were studied in tartaric acid buffer solution. The results indicated in the range 300-520 nm that the spectrum of dianilino-dimolybdate of gossypol complex obtained presents two peaks of absorption at 340 and 450 um whereas the dianilino-gossypol and the dimolybdate of gossypol present one peak at 444 and 420 um, respectively. The pH effect on the intensity of the absorption indicated that the optimum pH was 6.5. Gossypol is the only compound in the cottonseed extract having two peaks of absorption in the range 300-520 nm. The calibration curve was linear over the range 0.634-4.953 ppm (r^2 = 0. 9949) with the detection limit of 0.474 ppm. The stability of the complex was 90 min. The method was sensitive, specific and accurate for the determination of total gossypol in glanded cottonseed, crude, neutral and refined glanded cottonseed oil.

Key words: Gossypol, specificity, spectrophotometry, aniline, molybdenum, glanded cottonseed oil

INTRODUCTION

In many African tropical regions, glanded cottonseed oil is the main source of food lipids. The quality of this refined glanded cottonseed oil is very uncertain because crude glanded cottonseed oil contains gossypol (1) (Fig. 1), a toxic constituent for men and animal's species (Yuan and Shi, 2000; Yildirim-Aksoy *et al*., 2004). The presence of gossypol in cottonseed is also a major economic problem because it is a factor reducing the value of glanded cottonseed oil on the market place due to dark colour (Osman *et al*., 1976; El-Nockrashy *et al*., 1976; Wan, 1996). Gossypol is a pigment not only found in cotton plant material, but also in many Malvaceae species used in traditioual medicine (Angela *et al*., 2005). Its derivatives exhibit many benefits biological properties (Blackstaffe *et al*., 1997; Bushunow *et al*., 1999; Dao *et al*., 2003; Abe *et al*., 2004). In both the nutritional and pharmacological point of view, the determination of gossypol is important. The quantitative HPLC developed is the sensitive and specific method for the analysis of gossypol but is an expensive method (Hron *et al*., 1999; Gamboa *et al*., 2001; Benbouza *et al*., 2002; Cai *et al*., 2004) in African cottonseed oil industries. The spectrophotometric method (AOCS, 1997) for determining gossypol involved reaction of gossypol with aniline to form a yellow dianilino-gossypol (2) (Fig. 1). The AOCS method is relatively inexpensive and a fast method for determining gossypol but lacks sensitivity and specificity (Mahoney *et al*., 1985). The selective reaction of ammonium molybdate with o-diphenolic group was used for determining gossypol as gossypol dimolybdate (3) (Fig. 1) in glanded cottonseed

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(Tchatchueng *et al*., 1992). In the present study an improved spectrophotometric method was developed for quantitative and specific analysis of gossypol. The developed method is based on the simultaneous reaction of ammonium molybdate and aniline on the gossypol to form a yellow complex called dianilino-dimolybdate of gossypol (4) (Fig. 1).

MATERIALS AND METHODS

Apparatus

A spectronic 20D+ of Milton Roy and Co (USA) was used for the measurement of absorbance. For the pH measurements, PH-meter consort p207 of bioblock equipped with calomel glass electrode was used. Eppendorf micropipette of 100-1000 µL was used for appropriate volume of a sample solution and reagent additions. A A200s Sartorius analytic balance, accurate to 0,0001 g was used for weighing all the compounds required. Officially calibrated Pyrex glassware was used throughout this study and a prolabo water bath accurate to 2°C for heating the solution.

Materials and Reagents

Glandless cottonseeds, crude, neutral and refined glanded cottonseed oils used were kindly supplied by the Cotton Development Society Sodecoton of Garoua and Maroua in Cameroon. All chemicals from Sigma France S.A: Aniline A9880, Ammonium molybdate A7302, Caffeic acid C0625, DMSO D8779, Gossypol G4382, Kaempferol K0125, Myricetin M6760, Quercetin Q0125, Tartaric acid T8277, Glacial acetic acid A0808, Zinc dust 19834-0010 (Acros), acetonitrile, ethyl alcohol and isopropyl alcohol were of analytical reagent-grade and were used as received. Doubly distilled water was used throughout.

A 30.2 ppm ethyl alcohol Stock solution of gossypol acetic (24.92 ppm pure gossypol) was prepared and stored in borosilicate glass vessel in refrigerator at 4°C. Every day working solutions were obtained by appropriate dilution of stock solution. Reagent 1 was an 800 ppm solution of ammonium molybdate in a mixture of isopropyl alcohol and dimethylsulfoxyde (1:1). The binary solvent was a good solvent for ammonium molybdate, aniline and gossypol. Reagent 2 was 1% freshly redistilled aniline under zinc dust in a mixture of isopropyl alcohol and dimethylsulfoxyde (1:1). This reagent can be stored in a brown bottle in a refrigerator and used for three or four days. The acid buffer media of a given pH was prepared by mixing various volumes of 0.2 mol L^{-1} of tartaric acid in DMSO/isopropyl alcohol (1:1) and 0.2 mol L^{-1} sodium hydroxide solutions to the required pH value (2-8) under a pH-meter control.

Procedure

To construct the calibration curve and measure the absorbance at the maximum wavelength, place aliquots of gossypol acetic standard solution in 10 mL calibrated flasks in order to obtain a final concentration range of 0.298-4.985 ppm (pure gossypol). Add 2 mL of buffer solution of a given pH, 1 mL of reagent 1 and 1 mL of reagent 2. Incubate the resulting solution for 15 min in a water- bath at 70°C accurate to 2°C. After cooling, dilute to volume with appropriate buffer solution. Obtain he spectrum of the complex by recording the absorbance from 320 to 520 um. Measure the absorbance of the complex at 450 um against a blank. For the analysis of the sample, the procedure described above was followed except that the standard solution of gossypol was replaced by the sample solution of gossypol firstly extracted according to the method developed by Marquié and Bourrély (1991).

Application and Comparison with the Reference AOCS Method

For the analysis of the samples and the comparison purpose, the proposed method and standard AOCS method (1997) were simultaneously tested for glanded cottonseeds, crude, neutral and refined glanded cottonseed oils. The contents of gossypol in the samples were obtained with a calibration graph.

RESULTS AND DISCUSSION

Basis of the Proposed Method

The shame of the reactions that are on the basis of the proposed method is presented in Fig. 1. Gossypol (1) reacts with aromatic amines such as aniline to form dianilino-gossypol (2). This reaction has been the basis of the colorimetric analysis of gossypol (Smith, 1958).

Tchatchueng *et al*. (1992) using the reaction of molybdenum ions on o-diphenolic group demonstrated that gossypol could be determined by means of the molybdate gossypol complex (3). Combining the reactions of aniline and molybdenum ions on gossypol at pH 6.5, dianilino-dimolybdate of gossypol (4) was obtained (Fig. 1).

Figure 2 shows two possible structures of o-diphenolic compounds. One o-diphenolic function can react with one molybdenum ion giving a compound of structure (6) or two o-diphenolic functions can react with one molybdenum ion giving a compound of structure (7) depending on the pH and the o-diphenolic compound structure. The structure of the gossypol molybdate complex was established by the mole-ratio method using spectrophotometric analysis (Fig. 3). The experiments were performed at pH 6.5. The result obtained was 2.1:1. This result confirms the proposed structure of dimolybdate of gossypol (3).

Interference Studies

To evaluate the influence of other cottonseed phenolic compounds such as flavonoids, ortho-diphenolic acids on the determination of gossypol, known amounts of the desired compound were added in the standard solution of gossypol (2 ppm) under the optimal conditions of the proposed

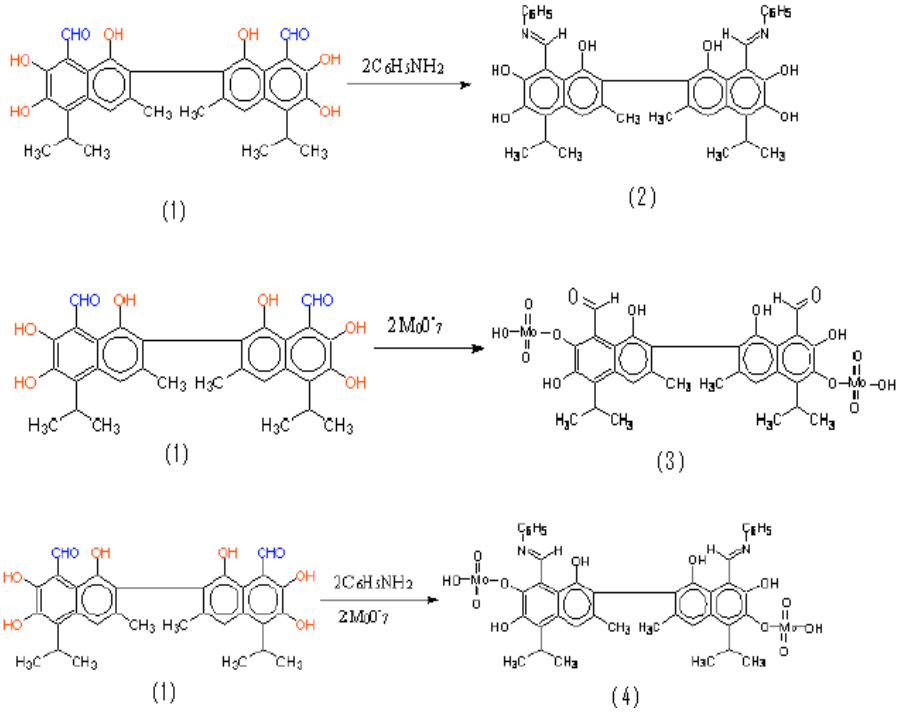

Fig. 1: Structure of gossypol complexes, (1) gossypol, (2) dianilinogossypol, (3) dimolybdate of gossypol, (4) dianilino-dimolybdate de gossypol

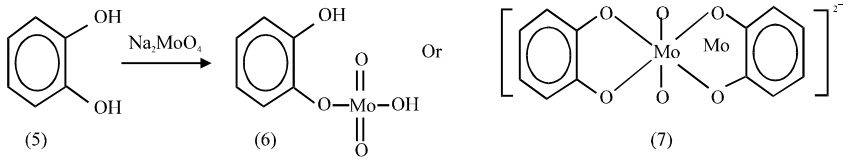

Fig. 2: Metallic ortho-diphenolic complexes

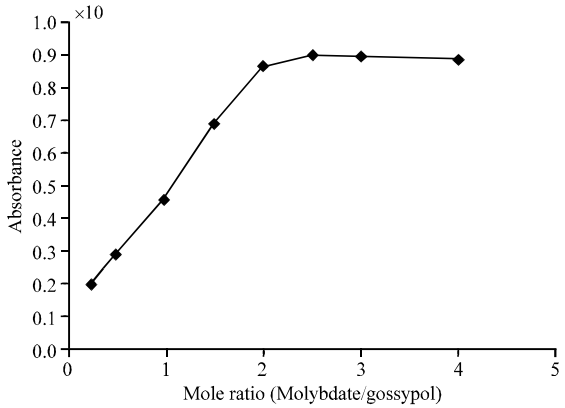

Fig. 3: Determination of mole-ratio of molybdenum and gossypol

Table 1: Effect of other phenolic compounds on the determination of 2 mg L^{-1} of gossypol

Tolerance limit Interferants/gossypol	Phenolic compound
0.3	Myricetin
0.5	Quercetin
2.0	Kaempferol
2.0	Caffeic acid

procedure. The tolerance limits of other phenolic compounds were taken as the quantity that caused an error not more than ±5% in the absorbance (Table 1). These tolerance limits expressed as the ratio of interferants and gossypol was: Myricetin (0.3), Quercetin (0.5), Kaempferol (2), Caffeic acid (2). According to Stipanovic *et al*. (1977, 1988), the sum of the content of all these interferants could not practically found in the cottonseed extract. These compounds could not interfere with the method in the studied experimental conditions.

Fingerprint of Gossypol and Specificity of the Method

The spectrum of dianilino-gossypol (2) has one peak at 444 um and dimolybdate of gossypol (3) has also one peak at 420 nm. The spectrum of dianilino-dimolybdate of gossypol (4) has two maxima at 450 and 340 um (Fig. 4).

In the cottonseed extract, other terpenoid aldehydes identified besides gossypol are divided into two groups: the first group that contained methoxylated gossypol and some of its derivatives: 6-methoxygossypol (9), 6,6'-dimethoxygossypol (10), methoxyhemigossypol (11), methoxyhemigossypolone (12), heliocides of B series (13) cannot undergo simultaneous reaction with aniline and molybdenum ions. The second group of compounds such as hemigossypol (14), hemigossypolone (15) and heliocides of H series (16) (Fig. 5) in spite of getting simultaneous reaction of aniline and molybdenum ions like gossypol have the molar absorption coefficient very lower (ε = 7000-8300) than the molar absorption coefficient of aniline derivative of gossypol (ε = 41450).

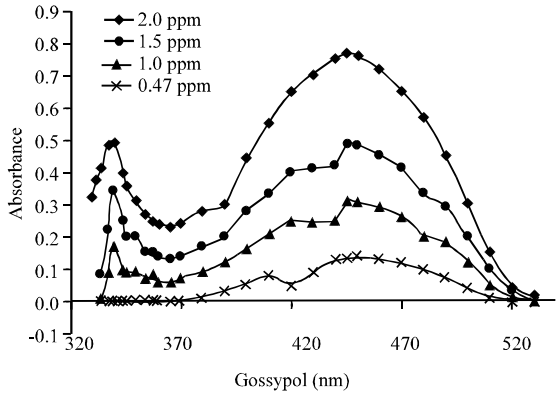

Fig. 4: Spectra of dianilino-dimolybdate of gossypol

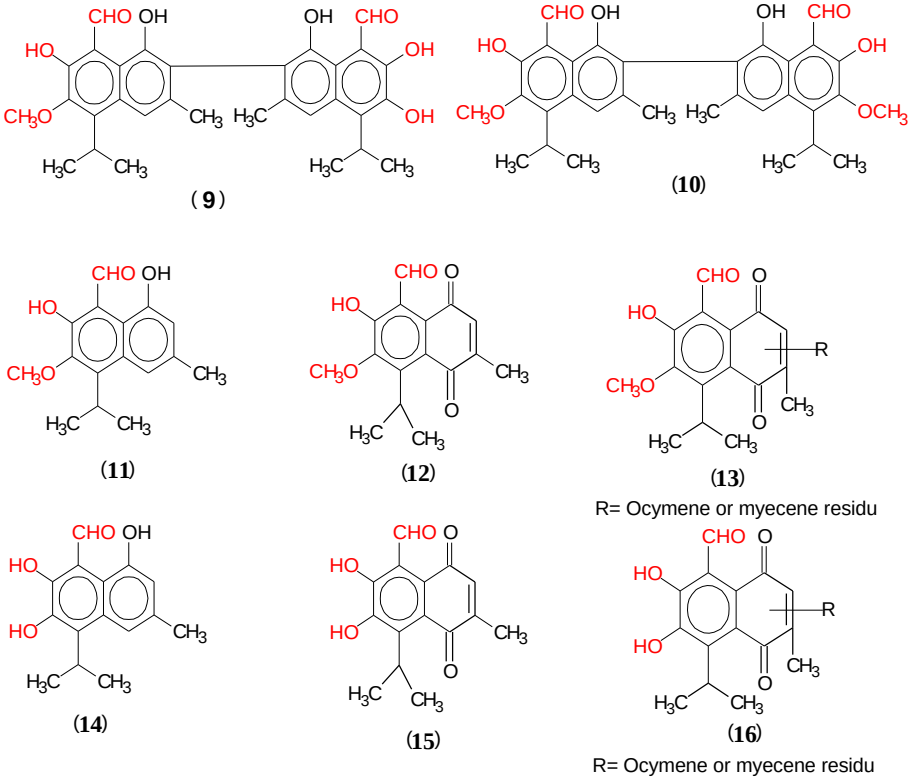

Fig. 5: Other terpenoid aldehydes in cottonseed: (9) 6-methoxygossypol, (10) 6,6'dimethoxygossypol, (11) 6-methoxyhemigossypol, (12) 6-methoxy gossypolone, (13) helioside B_1 and B_2, (14) hemigossypol, (15) hemigossypolone, (16) helioside H_1 and H_2

In addition to this according to Stipanovic *et al*. (1977), the content of other terpenoid aldehydes besides gossypol in the cottonseed was less than 10%. This result associated with the interference study show that gossypol is the only terpenoid aldehyde in the cottonseed products presenting in

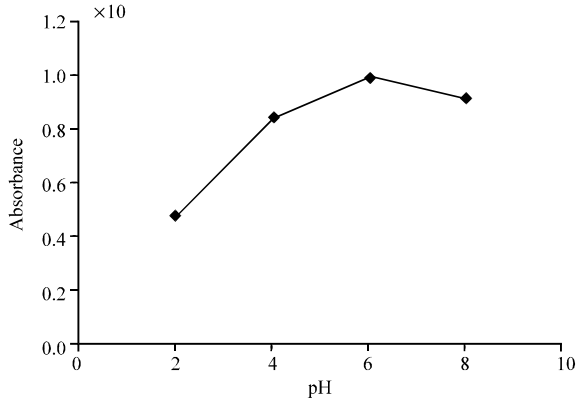

Fig. 6: Effect of pH on the absorption of gossypol complex, Concentration of gossypol (2 mg L^{-1})

Table 2: Recovery and reproducibility of the determination of 0.298 to 4.985 mg L^{-1} of gossypol

Gossypol added (mgL^{-1})	Mean±SD (n = 5)	RSD	Recovery (%)
0.298	0.226±0.027	11.94	75.83
0.498	0.474±0.016	3.37	95.27
0.796	0.773±0.021	2.71	97.11
0.997	0.954±0.023	2.41	95.70
1.994	1.932±0.023	1.12	96.80
2.991	2.924±0.035	1.12	97.78
3.988	3.927±0.027	0.68	98.47
4.985	4.953±0.034	0.68	99.36

the studied experimental conditions a spectrum having two maxima at 450 and 340 nm in the range 300-520 um. The peak at 340 nm is the fingerprint of gossypol. The proposed method is specific for the determination of gossypol in cottonseed and cottonseed products.

Effect of the pH on the Absorption of Dianilino-Dimolybdate of Gossypol

The effect of pH on the intensity of the absorption of the dianilino-dimolybdate of gossypol was studied over the pH range 2-8. As shown in Fig. 6, the pH of the medinm had a great effect on the absorbance of the dianilino-dimolybdate of gossypol. The experimental results obtained indicate that the maximum absorbance was at pH 6.5 that was used in subsequent experiments.

Precision and Accuracy

The accuracy and precision of the proposed method were evaluated by performing replicate analyses (n = 5) at each of six different concentrations of gossypol in the range of the calibration graph (0.634-4.953 ppm). The relative standard deviation and the recovery of gossypol were calculated. The standard errors for the five replicate determinations were less than 3% showing good reproducibility of the method. The recovery percentage was found to be in the range 95.7-99.4% giving certitude that the proposed method is accurate (Table 2).

Calibration Graph, Detection and Determination Limits

The calibration graph obtained using the proposed method under the optimized conditions was constructed (Fig. 7). There is a linear relationship between the absorbance and the gossypol concentration from 0.634 to 4.985 ppm. The detection limit was obtained by analysing series standard solutions of gossypol (0.298-4.985 ppm) under optimized conditions. The specificity of the method is the appearance of two peaks of absorption. Then the detection limit was defined as concentration

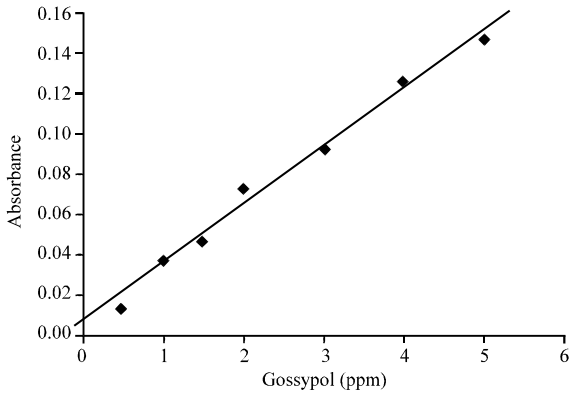

Fig. 7: Calibration graph with optimized conditions using proposed method

Table 3: Gossypol content in cottonseed and cottonseed oil

	AOCS method		Proposed method	
Samples	Mean±SD (N =5)	RSD	Mean±SD (N = 5)	RSD
Refined oil	LOQ		LOQ	
Neutralized oil (Garoua)	LOQ		LOQ	
Neutralized oil (Maroua)	LOQ		LOQ	
Crude oil (Garoua)	761.4 0±45.60	5.98	712.60±3.92	0.55
Crude oil (Maroua)	773.30±43.70	5.65	702.80±3.96	0.56
Cottonseeds (Garoua)	1201.50±51.60	4.29	1160.60±3.45	0.29
Cottonseeds (Maroua)	1217.10±62.50	5.13	1101.10±3.49	0.31

of gossypol y_a (0.474 ppm) under which only one peak at 450 nm was observed (Fig. 4). The determination limit was then y_a+10Sa (Miller and Miller, 2000) and equal at 0.634 ppm.

Stability of Dianilino-Dimolybdate of Gossypol

The stability of the dianilino-dimolybdate of gossypol was determined by analyzing a standard concentration of the compound (2 ppm) immediately after preparation (reference value) and after storage times. Stability was defined as being less than 2% loss of the initial absorbance in the stated time. The absorbance of the dianilino-dimolybdate of gossypol (4) remains constant for 1 h and decreases by 2% after 3 h and 5% after 5 h. The stability of the dianilino dimolybdate of gossypol was then 3 h. The system gives ample time to measure the absorbance of large nnmber of samples during cottonseed industrial processes. The relatively short reaction time of 30 min, the simplicity of the method are remarkable advantages over the spectrophotometric method reported by others Fisher *et al*. (1987), which requires more than 60 min and 10 steps to complete the reaction.

Application of the Method

The applicability of the proposed method for the quantification of gossypol in a glanded cottonseed, crude, neutral and refined glanded cottonseed oils was examined by analysing these products obtained from Cameroon cottonseed industries. The results of the proposed method were statistically compared with reference AOCS method (1997) and summarized in Table 3. The contents of the total gossypol obtained from the Maroua cottonseed samples were slightly lower than those obtained from the Garoua cottonseed samples. The AOCS methods produced higher estimation of total gossypol in all cottonseed samples with lower reproducibility compared to the proposed method.

CONCLUSION

The specificity of the proposed method was characterised by two peaks of absorption at 340 and 450 nm whereas standard AOCS methods and the method developed earlier (Tchatchueng *et al*., 1992) give spectra of the complex of gossypol showing a peak at 444 and 420 nm, respectively. The fingerprint of the gossypol is the appearance of the peak at 340 nm. The simplicity of the proposed method and the results obtained in the application to real samples demonstrate that this new method can be conveniently used for the determination of free or total gossypol during glanded cottonseed oil process. The method cannot be used to determine gossypol in which ortho-hydroxyl and aldehyde groups are not in the free form without pre-treatment such as hydrolysis with sulphuric acid as reported by Carruth (1918).

The perspectives of the present study are the systematic study of bifunctional molecule to search a probable specific spectrophotometric signature of the molecule and practically, for the general project, we intend to selectively extract using selective ion exchange resin the toxic gossypol from crude glanded cottonseed oil before refining process.

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