

An Improved Ultrasound-Assisted Extraction Process of Gossypol Acetic Acid From Cottonseed Soapstock

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To investigate the extracted process of gossypol acetic acid (G-AA) from cottonseed soapstock and explore the improvement of its yield and purity, a novel ultrasound-assisted extraction and crystallization method was introduced to this process. Under the optimized conditions, preliminary G-AA with the yield of 1300 mg and the purity of 95.9% could be obtained from 100 g of fresh soapstock by ultrasound-assisted extraction. In addition, UV, IR, and NMR spectrum further confirmed the detailed chemical structure of G-AA. Assay of inhibiting human prostate tumor cell line PC-3 and human breast cancer cell line MDA-MB-231 revealed its biological activity, the values of IC_{50} are 9.096 $\mu\text{mol/L}$ and 14.37 $\mu\text{mol/L}$ respectively. In comparison with the conventional solvent extraction, this novel process increases the content of G-AA over 90%, reduces the time of crystallization by 75%, and retains the anticancer activity of gossypol. © 2009 American Institute of Chemical Engineers AICHE J, 55: 797–806, 2009
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Introduction

Gossypol is a yellow polyphenolic compound found in pigment glands distributed throughout the cotton plant (*Gossypium* sp.), which has been associated with a wide range of biological and medicinal activities, including antifertility,¹ antimalarial,² antitumor,³ and antiviral⁴ effects. Therefore, it is widely used in medical and pharmaceutical field. Recently, some bio-medical research^{5–11} suggested that gossypol is a

promising novel anticancer drug. Gossypol has two enantiomers. Benz et al.¹² found correlation of the antitumor and antimitochondrial properties of gossypol enantiomers, and believed that the antimitochondrial effect of (-)-gossypol correlated with its antiproliferative activity. It was also noted that (-)-gossypol modified the resistance of human carcinoma cells to other chemotherapeutic agents because of the increase in glutathione-S-transferase and protein kinase C content. Oliver et al.¹³ reported that (-)-gossypol acted directly on mitochondria to overcome the antiapoptotic function of Bcl-2 and Bcl-xL. Our previous report¹⁴ showed that (-)-gossypol inhibited the antiapoptotic proteins Bcl-2/Bcl-xL and radiosensitized prostate cancer cells in vitro and in vivo without augmenting toxicity. Currently, (-)-gossypol has

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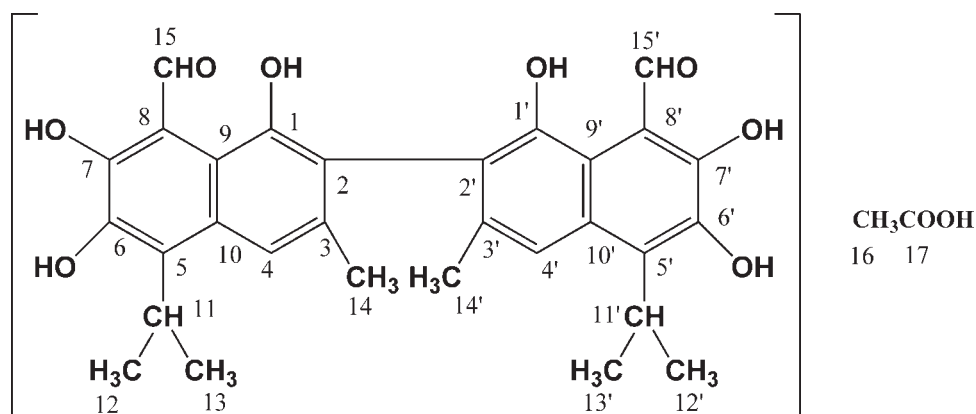


Figure 1. Chemical structure of G-AA.

shown promising results in Phase I clinical trial for cancer therapy and is now in Phase II clinical trial in the USA. With more and more biological and clinical investigation on gossypol, the demand for large amounts of pure and pharmacologically active gossypol will greatly increase. However, because gossypol was prone to be oxidized in air, it was usually turned into stable gossypol acetic acid (G-AA) (Figure 1) with glacial acetic acid during production.

To meet the growing demand for the medicinally active gossypol in pharmaceutical industry, several methods for extracting gossypol were developed. The initial preparations of gossypol employed cottonseed or defatted cottonseed,¹⁵ the root bark of the cotton plant,¹⁶ or isolated pigment glands.¹⁷ However, there were at least two severe shortcomings in these procedures. One is that the products are quite expensive and crude because of the low content of gossypol in raw materials and the high consumption of extracting solvent. The other is that these methods will lead to a potential risk in food and feedstuff safety due to some toxic organic extracting solvents remained in oil and protein. Therefore, it is important to develop a safer process for preparing large amounts of high purity gossypol with economical material.

Soapstock, the byproduct of alkali refining cottonseed oil, was developed as the substitute material for producing gossypol. It used to be thrown away as rubbish because of its ugly color and rotten odor, which results in severe atmosphere pollution. However, it was reported that gossypol in bound state is enriched at 2–8% in soapstock.¹⁸ It is clear that soapstock maybe a proper material for the recovery of gossypol, and this process will make for relieving environmental contamination in the refining oil factories. Dowd et al.¹⁸ proposed a conventional solvent extraction procedure to produce large amounts of research-grade gossypol by cottonseed soapstock. Despite their success in recovery of gossypol from cottonseed soapstock in lab, it is unclear whether their product had antitumor effect, and no tested data have been reported, and there are several puzzles in its industrialization, such as low purity, long producing cycle and complicated processing program.

Ultrasound-assisted extraction uses acoustic cavitations to cause molecular movement of solvent and samples, which offers advantages of improved efficiency, reduced extraction

time, low solvent consumption and higher level of automation compared with conventional extraction techniques.¹⁹ Consequently, the increasing use of ultrasound extraction has been investigated in the pharmaceutical, chemical and food industries. This is a well-established method in the processing of plant material, especially in extraction of low molecular weight substances,²⁰ and of bioactive substances from plants.²¹

Sonocrystallization is the use of power ultrasound to control the crystallization process. Power ultrasound was used principally to affect the initial nucleation stage of crystallization, that is, the width of the metastable zone is reduced and nucleation starts at a lower level of supersaturation.^{22–24} The utilization of ultrasound to crystallization in the pharmaceutical and fine chemicals industry has received further impetus in recent years. The corresponding equipments to prepare and purify complex chemical entities to different exacting standards²⁵ have been designed and applied. Therefore, ultrasonic crystallization has become a relative mature technique in the pharmaceutical and fine chemicals industry.

To improve the gossypol extraction procedure from soapstock, we have developed an improved process for recovering large amount, pure and active G-AA from cottonseed soapstock by the powerful ultrasound assisted extraction and crystallization technology. The yield of crude product reached at 1.3 g and the content of G-AA was 95.9% in the initial extracts. Our product has the same chemical structure with standard sample according to UV, IR, and H NMR spectrums. Primary biological test revealed that our product showed excellent antitumor activity. Hence, this novel process of ultrasound assisted extraction and crystallization may be adopted in preparing the large scale G-AA from cottonseed soapstock, and G-AA may have promising biomedical use in the future.

Materials and Methods

Materials preparation and handling

Soapstock from a local miscelle-refining cottonseed oil mill (Shanxi Shiyang, China) was collected immediately after centrifuge and was rapidly stored in the -20°C refrigerator. G-AA standard was offered by National Institute for the

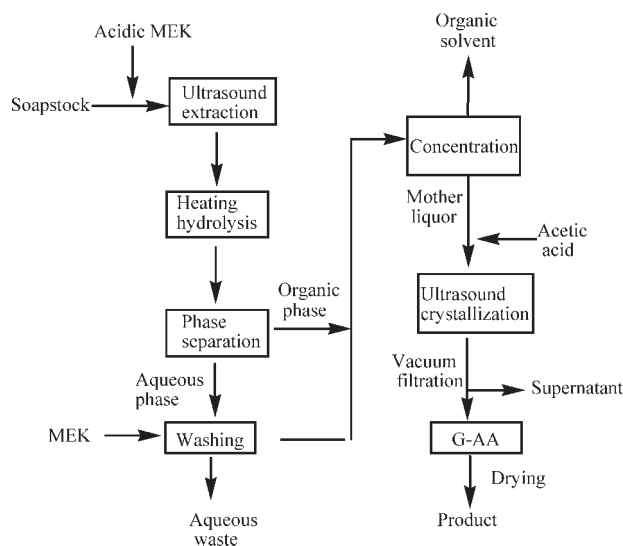


Figure 2. Process schematic of the recovery of G-AA from cottonseed soapstock by ultrasound.

Control of Pharmaceutical and Biological Products in China. All the chemical reagents were of analytical grade.

Soapstock gossypol concentration was determined by the American Oil Chemists' Society Official Method.²⁶ alkali concentration was estimated by dispersing 1.0 g of soapstock in 30 mL of deionized water and measuring pH. We used the soapstock containing 2% gossypol (calculated by G-AA) and pH 8.0 in this study.

Recovery process

On the basis of a method described by Down et al.,¹⁸ we proposed a modified procedure (Figure 2) which includes extracting gossypol from soapstock with ultrasound water bath, hydrolyzing bound gossypol in acid condition, partitioning the gossypol into an organic phase and gathering all extract, concentrating the combined gossypol solution by vacuum distillation, and crystallization by power ultrasound. Briefly, (100 ± 1) g of soapstock was dispersed in 100 mL of methyl ethyl ketone (MEK) containing phosphoric acid, and then bathed in the ultrasound producer (KQ3200DE, 40 kHz, 150 W, China). Subsequently, the system was processed by heat hydrolysis, centrifugal separation, and vacuum concentration. Acetic acid was added into concentrated extraction in the volume ratio of one to three. And the formation of G-AA was promoted by using ultrasound producer (KQ3200DE, 40 kHz, 150 W, China) at room temperature. It followed that the solution was stored for 3 hr in the dark to prevent photo-oxidation. Crude product was recovered by vacuum filter, washing with petroleum ether, and vacuum drying at room temperature for 12 hr. Finally, its weight was defined as the yield of sample.

To obtain of 99% pure G-AA, crude product was dissolved in acetone (1:10 w/v), filtered through filter paper, and acetic acid with the volume ratio of one to three was added to induce crystallization. And then the solutions were treated in the ultrasound bath at room temperature for 2 min. Pure G-

AA was recovered by the above-mentioned filtering, rinsing, drying technology.

Process optimization

Four important factors were optimized in the extraction and the crystallization steps (Figure 2). The optimization of the extraction conditions were restricted in the proper scope, such as phosphoric acid concentrations of 0.6, 1.0, 1.4, 1.8, or 2.0 mol/L, ultrasonic extracting times of 10, 20, 30, 40, or 50 min, and refluxing times of 30, 60, 90, 120, or 150 min, respectively. Ultrasound inducing time varying from 10 to 50 min was tested in the process of crystallization. To ensure the authenticity of the test, each experiment was repeated three times and relative deviation was controlled within 1.0%, and final value is the general average of above parallel trials.

Sample purity

Preparation of Standard Curve. Firstly, 50 mg of G-AA standard was weighed into a 100-mL volumetric flask and filled to the mark with new distilled chloroform (CDCl₃) to achieve the 0.5 mg/mL (m/v) standard solution for use. Secondly, a series volumes of above standard solutions, 50, 100, 150, 200, 250 μL, were transferred into 5-mL volumetric flasks, and respectively diluted to 5, 10, 15, 20, 25 μg/mL by the addition of the same chloroform. Thirdly, the diluted standard solutions were filled into quartz colorimetric cylinder and measured their absorbance at 365 nm in the UV equipment (Model Ultrospec 2100 pro, Biochrom, England) against the chloroform as the blank. According to the variation of absorbance (A) with the concentration (C) of standard solution, finally, the regression equation of standard curve was $A = -0.0201 + 0.03578C$ ($P < 0.0001$) ($R^2 = 0.9989$) by the software origin 7.0 (Microcal software).

Test of Sample Purity. The purity of crude gossypol products was determined by UV against a G-AA standard. The 15 μg/mL of crude product was prepared with the chloroform as described above. Its absorbance was determined at 365 nm against the blank solution. The ratio of its measured value to the predictive value base on standard curve was defined as the purity of sample.

Testing of the final G-AA product was conducted to ensure authenticity. The melting point was determined with a digital microscopical melting point apparatus (Model X-4, Beijing Tech Instrument, China). The UV/Vis spectrum was recorded in CDCl₃ with a spectrophotometer (Ultrospec 2100 pro). The IR spectrum was recorded by potassium bromide tableting with a spectrophotometer (Model EQUINX55, Bruker, Germany). To definite the accurate structure of product, the ¹H NMR was introduced by its solution in CDCl₃ solvent with a Super-conducting Fourier Digital NMR spectrometer (Model AVANCF 300 MHz, Bruker, Switzerland).

Antitumor activity assay

Cell Culture and Reagents. Prostate cancer and breast cancer cell lines used were obtained from the American

Type Culture Collection (Manassas, VA). Cells were routinely maintained in an improved Dulbecco's Modified Eagle's Medium (Biofluids, Rockville, MD) with 10% fetal bovine serum and 2 mmol/L L-glutamine. Cultures were maintained in a humidified incubator at 37°C and 5% CO₂. G-AA was dissolved in dimethyl sulphoxide (DMSO) at 20 mmol/L as the stock solution.

Growth Inhibition Assay. Cell growth inhibition by G-AA was determined by the methyl thiazolyl tetrazolium (MTT)-based assay using cell proliferation reagent water-soluble tetrazolium (WST-1) (Roche) according to the manufacturer's instruction. Briefly, cells (5000 cells/well) were plated in 96-well culture plates, and various concentrations of G-AA were added to the cells in triplicates. 4 days later, WST-1 was added to each well and incubated for 1.5 hr at 37°C. Absorbance was measured with a plate reader at 450 nm with correction at 650 nm. The results are expressed as the % of absorbance of treated wells vs. that of vehicle control. IC₅₀, the drug concentration giving 50% growth inhibition was calculated via sigmoid curve fitting using GraphPad Prism 5.0 (GraphPad).

Apoptosis Assay. PC-3 cells were incubated with G-AA sample or standard, and DMSO (control), for the 36 hr. At the end of treatment cells were washed in phosphate buffered solution (PBS) and analyzed for apoptosis. Morphology changes were observed by 4,6-diamino-2-phenylindole (DAPI) staining. After fixation with 70% ethanol for 15 min, cells were incubated with DAPI in PBS for 15 min. Cells were then washed with PBS for observation. Caspase-3 activities assay was performed by using Caspase-3 Assay Kit from BioVision following the manufacturer's instruction. Briefly, the cells were lysed in cell lysis buffer. The cell lysates were placed on ice for 10 min and centrifuged at 10,000 g for 5 min.

Subsequently, caspase-3 activity was determined by N-acetyl-Asp-Glu-Val-Asp-pNA (Ac-DEVD-pNA), which is a substrate of caspase-3. After reaction of 2 hr at 37°C, the free pentose nucleic acid (pNA) was then quantified using a spectrophotometer at 405 nm. Relative caspase-3 activity was evaluated by comparing the absolute absorbance from drugs treated cells with that of control.

Tumor cells were treated with G-AA at a series of concentrations, then trypsinized and washed with PBS, and fixed in 70% ethanol on ice. After centrifugation, cells were stained with 50 µg/mL propidium iodide and 0.1 µg/mL RNase A and analyzed by flow cytometry using a FACStar Plus cell sorter. Data were analyzed to calculate the percentage of sub-G1 population (apoptosis) using the CellQuest software (Becton Dickinson, Franklin).

Results and Discussion

Effect of process conditions on the yield and content of G-AA

Ultrasound Extracting Time. Currently, ultrasound assisted extraction has been widely utilized in chemical or medicine plants. Our ultrasound producer had invariable frequency of 40 kHz and power of 150 W; therefore, we mainly studied how ultrasonic extracting time affects on the mass and purity of the product. Different time was set varying

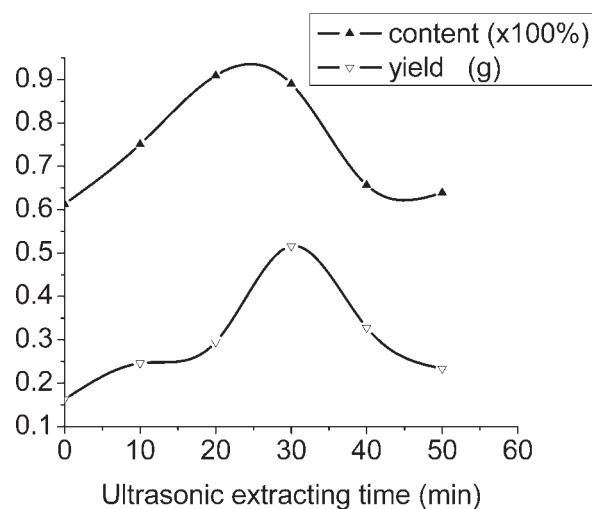


Figure 3. Effect of ultrasound extracting time on the content and yield of crude G-AA (acid concentration: 1.8 mol/L, refluxing time: 90 min, sonocrystallization time: 30 min).

from 0 to 50 min, and soapstock was treated as described in experimental section. It was shown in Figure 3 that time of ultrasonic treatment affected the quantity and quality of product significantly. The purity and yield of crude product was relatively low in the absence of ultrasound, and was increased distinctly in the presence of ultrasound. With the increase of ultrasonic extracting time, product yield and content appeared to reach a 'plateau' that represented the maximum. When the ultrasonic extracting time was beyond 30 min, the content decreased slowly; however, the yield was evidently declined from 30 min (0.5164 g) to 50 min (0.2337 g).

It is well known that powerful ultrasound may make especial chemical effect in an individual reaction system. And the degree of the chemical effect could be examined with some methods such as KI dosimetry and Fricke dosimetry.²⁷ The cavitation occurring in water yields hydroxyl radicals which lead to chemical decomposition,^{28,29} and the reduction in the yield could be attributed to the decomposition of G-AA in too long process time. Further study will be conducted to examine the mechanism of ultrasonic degradation of G-AA. The drop of sample purity might be attributed to substances mixed into G-AA during the continuous ultrasonic treatment.

Refluxing Time. To dissolve more gossypol into solvent, temperature is one of crucial factors in the hydrolysis of bound gossypol. It was obvious that the quality and quantity of crude G-AA changed with the hydrolysis time prolonging as shown in Figure 4. When the refluxing time was at 90 min, the content and yield of crude sample reached a peak. There was, however, a slight reduction in the yield of G-AA only when the refluxing time was beyond 90 min as the active aldehyde groups in the free gossypol tended to be turned into other inactive substance. Consequently, in view of the efficiency of hydrolytic reaction and the economy in energy, 90 min should be a reasonable refluxing time to ensure the stable quality of product.

Acid Concentration. It is well known that Schiff's complexes was formed between gossypol and phospholipids, or

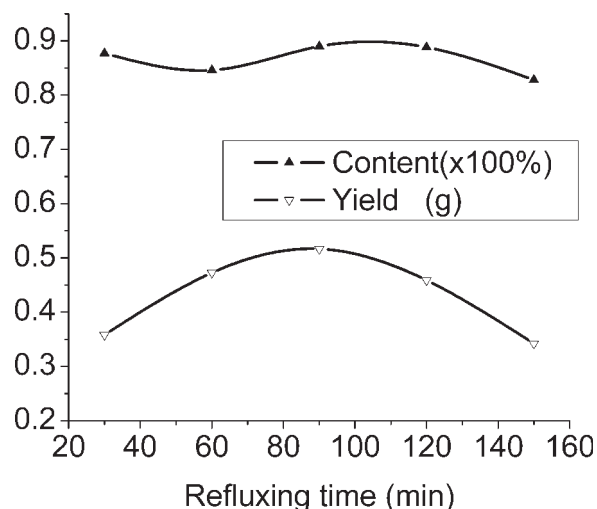


Figure 4. Effect of refluxing heating time on the content and yield of crude G-AA (ultrasonic extracting time: 30 min, acid concentration: 1.8 mol/L, sonocrystallization time: 30 min).

and peptide fragments, and any other amines in base conditions. Hence, gossypol with bound form is subsided in soapstock during alkali refining. However, gossypol could be released from its Schiff's complexes in acid conditions. In consideration of avoiding gossypol to be oxidized and retaining the intensity of acid hydrolysis, phosphoric acid was the better hydrolytic solvent than common acids including hydrochloric acid, sulfuric acid and nitric acid because of its high boiling point and low oxidization. As was it shown in Figure 5, the content of G-AA in the crude sample has gradually increased until the top value is 89.0% at 1.8 mol/L of acid concentration. Similarly, the yield of crude sample had a sharp increase and reached the peak at 1.6 mol/L. There was

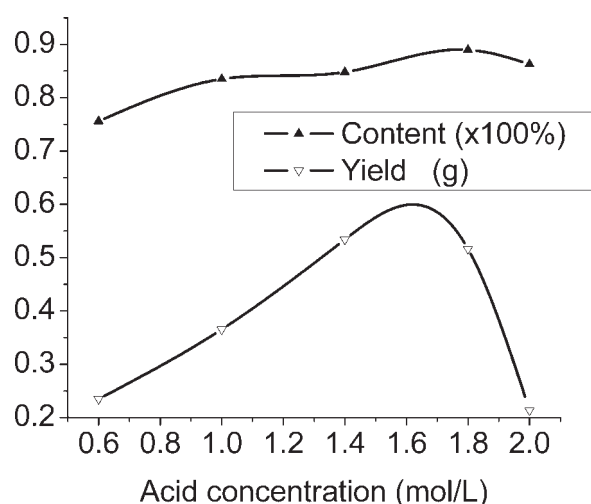


Figure 5. Effect of acid concentration on the content and yield of crude G-AA (ultrasonic extracting time: 30 min, refluxing time: 90 min, sonocrystallization time: 30 min).

a predominant decline in the quantity of G-AA, on the contrary, when acid concentration was beyond 1.8 mol/L. It was found that acid concentration had a notable influence on the content and yield of G-AA.

There are at least two main reasons for the changes of G-AA with different acid concentrations. One and important reason is that the partial hydrolysis would lead to less free gossypol released in the organic solvent at the low concentration of acid. Secondly, the small quantity of crude products was possibly due to low saturation of the crystallization mother solution. It is difficult to vaporize further MEK at the same concentrated condition because the relative superfluous acid increased the boiling point of solution at the high acid concentration. It is clear that the acid concentration should be within range of 1.4 mol/L and 1.8 mol/L.

Time of Ultrasonic Crystallization. Currently, rapid sonocrystallization, an undeveloped process in crystallization field, was investigated in the recent studies.^{30,31} With its intensive vibration, cavitation and transmission, ultrasound can uniformly blend the solutions on the microscopic scale, and hasten mass transfer of the insonated crystal products. Consequently, it was by changing the ultrasonic power, duration, and the volume of mixture that the mean size, size distribution and shape of crystal could be controlled efficiently.³⁰

To study the sonication effect on the recovery of G-AA, we adjusted sonocrystallization time in range of 0–50 min. The yield and content of G-AA had similar tend as ultrasonic inducing time prolonged as shown in Figure 6. The recovery of G-AA increased and reached peak when ultrasonic time was 30 min. After that, content of G-AA declined gradually because of the coprecipitation of some foreign materials. The decomposition of G-AA in long ultrasound crystallizing time also possibly led to the decline in yield of crude products. It was obvious that the recovery of G-AA was greatly different between ultrasonic process and nonultrasonic process. And we noticed that the crystal of G-AA began to form at least 24 hr later in the conventional standing crystallization

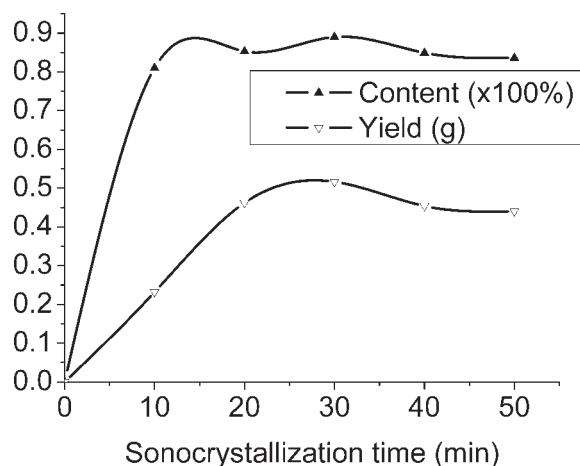


Figure 6. Effect of the induction time on the content and yield of crude G-AA (ultrasonic extracting time: 30 min, acid concentration: 1.8 mol/L, refluxing time: 90 min).

Table 1. Optimized Experiment Data of G-AA Extracted From Cottonseed Soapstock by Ultrasound $L_9(3^4)$

Essay No.	Factors			Recovery Value
	A (min)	B (mol/L)	C (min)	Mass of Pure G-AA (mg)
1	1 (20)	1 (1.4)	1 (20)	455
2	1	2 (1.6)	2 (25)	671
3	1	3 (1.8)	3 (30)	268
4	2 (25)	2	3	759
5	2	3	1	831
6	2	1	2	1243
7	3 (30)	3	2	672
8	3	1	3	453
9	3	2	1	358
K_1	1394	2151	1644	
K_2	2833	1788	2586	
K_3	1483	1771	1480	
k_1	465	717	548	
k_2	944	596	862	
k_3	494	590	493	
R	480	127	369	
Optimized combination	A_2	B_1	C_2	

A, ultrasound extracting time; B, acid concentration; C, sonocrystallization time.

K_1, K_2, K_3 , Sum of recovery values of same level in a factor.

k_1, k_2, k_3 , Mean of recovery values of same level in a factor.

R (range), Difference of maximum and minimum among k_i in a factor.

described by Dowd et al.¹⁸ So, powerful ultrasound is an advanced way to prompt crystallization during the preparation of G-AA up to the present.

Because of its inducing initial nucleation in nominally particle-free solutions at much lower supersaturation levels, ultrasound can speed up crystallization. The application of ultrasound to a liquid induces the phenomenon of cavitation in the process fluid. It is possible that cavitation plays an important role in the increase of nucleation rate which results in higher G-AA recovery in sonicated samples. The effect of cavitation to speed up the nucleation rate is a complex phenomenon and has not yet been fully explained.²⁵ Some believed that subsequent rapid local cooling rates, calculated at 10^7 – 10^{10} K/s, played a significant part in increasing supersaturation; others argued that the increases in localized pressure decreased the crystallization temperature; still others insisted that the cavitation events allow the excitation energy barriers related with nucleation to be surmounted.

It can also be speculated that the local solvent evaporation takes place within the cavitation nuclei due to hot spot for-

mation as a result of cavity collapse.³¹ On collapse of a cavity, these nuclei are introduced into the crystallization slurry and caused further cavitation opportunities due to the presence of these solids.³² In the absence of sonication samples, however, the rate of crystallization appears to be rather less, possibly due to comparatively slow mixing and nucleation.

As shown in Figure 6, the continuous ultrasonic seldom contributes to the crystallization of G-AA beyond 30 min. To shorten the cycle of crystallization and reduce the consumption of energy, consequently, the range of ultrasonic inducing time was chosen as 20–30 min.

Optimization of ultrasound extracting conditions of G-AA from cottonseed soapstock

The effects of ultrasonic extracting time, refluxing time, acid concentration, and ultrasound crystallizing time on the recovery of G-AA from cottonseed soapstock were analyzed. It was reasonable that refluxing time was 90 min for the stable quality of product. In view of the principle of homogeneous design, the extracting conditions were respectively limited in the proper scopes, that is, 20–30 min of ultrasonic extracting time (A), 1.4–1.8 mol/L of acid concentration (B), and 20–30 min of ultrasound crystallizing time (C). On the basis of the uniform combination among three factors and three levels, nine experimental points were shown in Table 1.

Nine experiments were carried out according to uniformly combinatorial conditions, and mass of pure G-AA is the multiplication result of the content of G-AA and the yield of every crude product as the final result. These results were then analyzed by DPS (Data Process System, made in Zhejiang University, China) and the analysis of their range and variance were shown in Table 1 and 2. It was found that ultrasound extracting time (A) and ultrasound crystallizing time (C) affected the recovery of G-AA significantly ($P < 0.01$). No.6 was chosen as the optimized experimental conditions ($A_2B_1C_2$) for its high recovery of G-AA. In other words, 1243 mg of pure G-AA coming from 1296 mg and 95.9% crude product was prepared when ultrasonic extracting time, acid concentration, and ultrasound crystallizing time were respectively settled at 25 min, 1.4 mol/L, and 25 min.

Characterization of the product

To definite clearly the purity of G-AA, melting point test and spectrum scan were used in this study. G-AA sample from the recrystallization in acetone solvent was tested with a digital microscopical melting point apparatus. The melting

Table 2. Analysis of Variance About the Data in the Optimization Experiment

Source of Variation	Sum of Squares	Degrees of Freedom	Mean Square	F-value	P
A (min)	433,460	2	216,730	313.45	0.00318 (**)
B (mol/L)	30,718	2	15,359	22.21	0.04308 (*)
C (min)	237,500	2	118,750	171.74	0.00579 (**)
Error	1383	2	691		
Total	703,060	8			

The result of analysis of variance was obtained by F-test method.

A, ultrasound extracting time; B, acid concentration; C, sonocrystallization time.

*Significant ($P < 0.05$); **Very significant ($P < 0.01$).

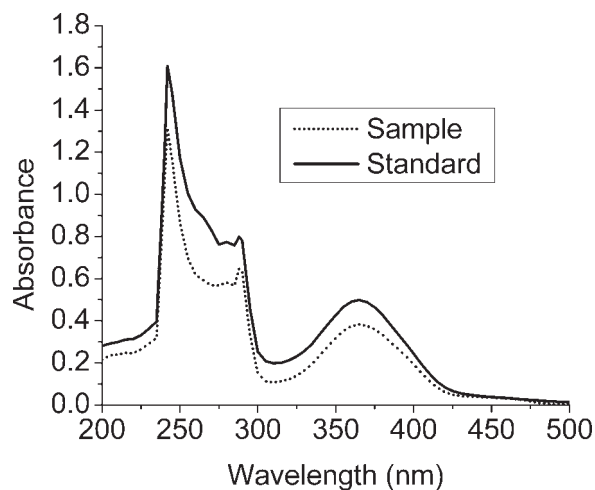


Figure 7. UV scan spectrum of G-AA in CDCl_3 (sample and standard concentrations are $12 \mu\text{g/mL}$ and $15 \mu\text{g/mL}$ respectively).

point of the material was $181\text{--}182^\circ\text{C}$ in reasonable agreement with values reported by Clark,³³ Campbell et al.,³⁴ and Murty et al.,³⁵ which ranged between 180 and 187°C . It was shown that there are a few crystal styles in our product.

UV-Vis spectrum analysis, as a fast method, is used to determine the structure of material according to the special absorbance of its functional group. The UV-Vis spectrum

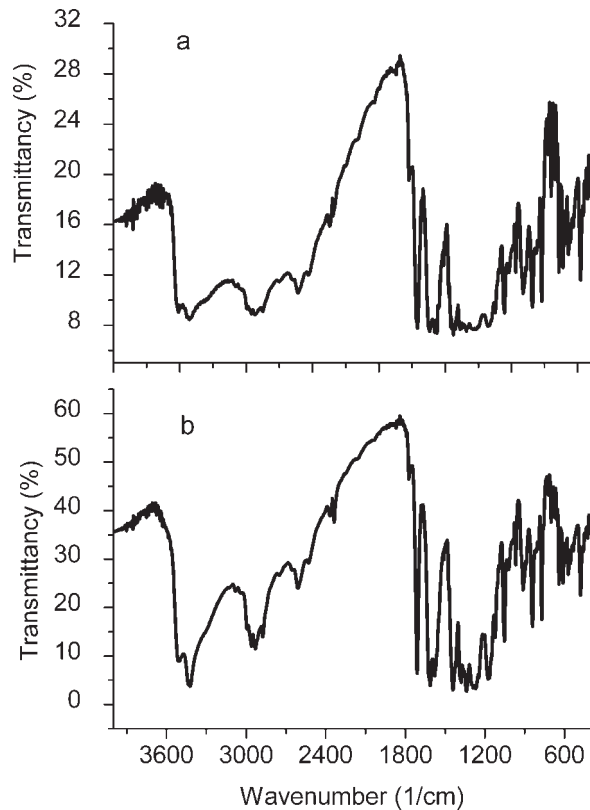


Figure 8. ^1H NMR of G-AA in CDCl_3 (a. Standard; b. Sample).

(Figure 7) of our product in chloroform (peaks: 243 , 278 , 289 , and 365 nm) was identical to the spectrum reported by Dowd et al.¹⁸ Therefore, we picked out the wavelength of maximum absorbent peak at 365 nm as the special absorbance of free aldehyde group, and the absorbance of all products were recorded for calculating the content of G-AA in crude materials.

IR spectrum is able to reveal directly the detail information of structure of chemical material. We got the IR scan result of G-AA shown in Figure 8 by the KBr pellet method. There are similar spectra between the sample (Figure 8a) and standard (Figure 8b) of G-AA and significant functional groups are markedly shown. The maximum absorption peaks around 3400 cm^{-1} illustrated the region of the $\nu(\text{O—H})$ vibrations of sample (3427 cm^{-1}) and standard preparation (3425 cm^{-1}). The peaks at 1710 cm^{-1} (a) and 1708 cm^{-1} (b) assigned to the $\nu(\text{C=O})$ vibration in aldehyde, ketone, or carboxylic acid. However, a series of peaks nearby 3000 cm^{-1} meant the $\nu(\text{O—H})$ vibration of carboxylic acid, and the bands appeared at 2610 cm^{-1} (a) and 2608 cm^{-1} (b) meant the vibration of bond between carbon and hydrogen, which demonstrated the existence of aldehyde group. This spectral feature at 1615 cm^{-1} and 1577 cm^{-1} revealed $\nu(\text{C=C})$ vibration in aromatic ring, and the $\nu(\text{C—O})$ vibration in naphthol was shown at 1269 cm^{-1} in Figure 8.

The ^1H NMR spectra of G-AA were recorded in CDCl_3 using a spectrometer (AVANCF, 300 MHz). As shown in Figure 9, there was the similar response signal between standard and sample of G-AA, which illustrated that they could be the same compound. The ^1H NMR data for standard and sample of G-AA were summarized in Tables 3. Therefore, the ^1H NMR spectrum further ensured the high purity of our product.

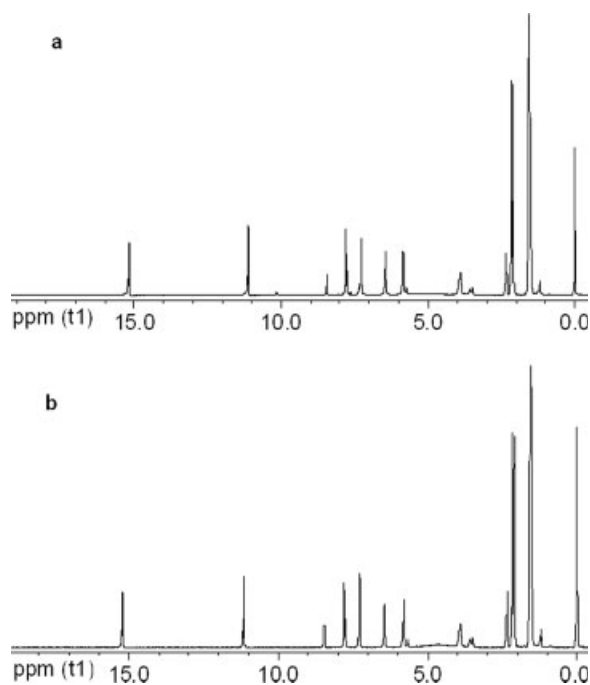


Figure 9. IR scan spectrum (a. Sample; b. Standard).

Table 3. ^1H NMR Chemical Shifts (ppm) for the G-AA in the CDCl_3

Compound	Chemical shift (ppm)									
	11 (11')- C(CH ₃) ₂	14 (14')- CH ₃	13 (13')- CH ₃	11 (11')- CH	1 (1')- C—OH	6 (6')- C—OH	4 (4')- C—H	17- COOH	15 (15')- CHO	7 (7')- C—OH
Standard	1.56 (d)	2.13 (s)	2.34 (s)	3.58 (m)	5.79 (s)	6.44 (s)	7.73 (s)	8.45 (s)	11.15 (s)	15.21 (s)
Sample	1.56 (d)	2.13 (s)	2.34 (s)	3.57 (m)	5.73 (s)	6.44 (s)	7.76 (s)	8.46 (s)	11.15 (s)	15.22 (s)

s, singlet; d, doublet; m, multiplet.

Antitumor activity

To test the tumor inhibition effect of G-AA prepared by the ultrasound extraction and crystallization, MTT-based cell growth assay was carried out as described method¹⁴ in experimental section. According to the results in Figure 10, the values of IC_{50} of G-AA sample were similar to G-AA standard in both human prostate cancer cell line PC-3 (Figure 10a) and human breast cancer cell line MDA-MB-231 (Figure 10b), which implied that our product had similar biological activity as standard. The ultrasound assisted extraction and crystallization technology sped up the process

of recovery for G-AA, and was able to avoid the degeneration of G-AA as result of little chance for touch with air.

Caspase-3 activation plays a pivotal role in apoptosis and also serves as a critical indicator for apoptosis. As shown in Figure 11, both G-AA sample and G-AA standard at the concentrations of 10 and 20 $\mu\text{mol/L}$ induced similar caspase-3 activation and in a dose-dependent manner, indicating that both of the compounds had similar apoptosis inducing effect.

Karyorrhexis is a typical morphologic change of apoptosis cells, which means the nuclei broken down into pieces. DAPI is a dye specifically stains the cell nuclei. As shown in Figure 12, after 36 hr incubation with 20 $\mu\text{mol/L}$ G-AA sample or standard, apoptotic PC-3 cells were observed in both treatments.

Low content (less 1%) materials including cottonseed, cottonseed oil, and the root bark of the cotton were utilized in the conventional process of recovering gossypol, which led to high cost of G-AA. In compared, soapstock with high content (2%) gossypol, an industrial waste in some oil refine plants, was developed in our process, which further decreased the cost of G-AA. Down et al. obtained the 87–92.2% pure G-AA product in 16 hr according to their conventional process and didn't show whether their product could inhibit the growth of tumor. After the ultrasound assisted extraction and crystallization technology was introduced in this novel process; we could achieve the initial product with 95.9% pure G-AA only in about 4 hr. Moreover, high content initial product would relieve the pressure from the recrystallization (standard grade G-AA was obtained by ultrasound recrystallizing in 2 min). It is notable that our product shown the similar biological activity to the standard in both the growth inhibition and apoptosis induction in prostate cancer cell line PC-3 and human breast cancer cell line

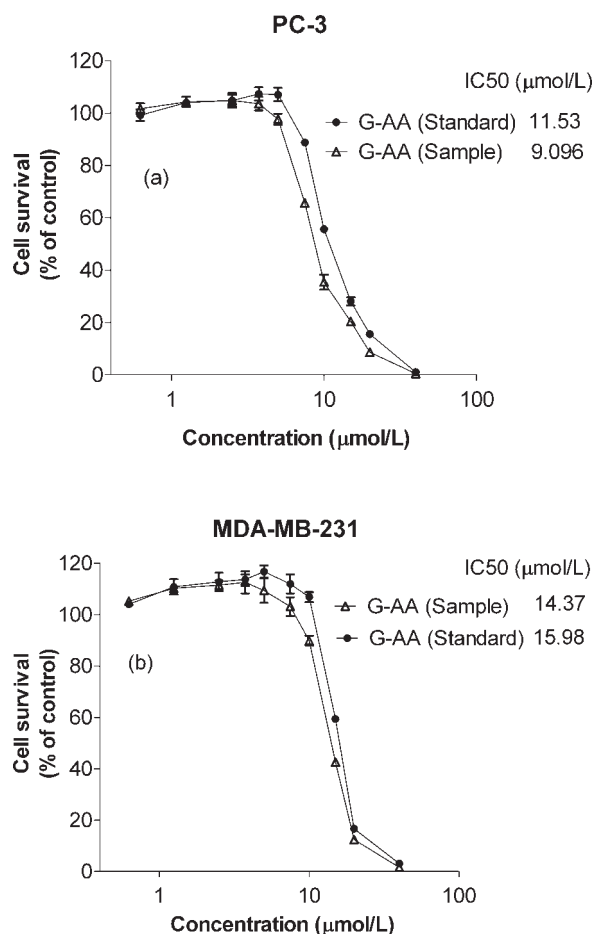


Figure 10. Inhibiting effect of G-AA on tumor cells (a. prostate cancer cell line PC-3 was inhibited by different concentration G-AA; b. human breast cancer cell line MDA-MB-231 by different concentration G-AA).

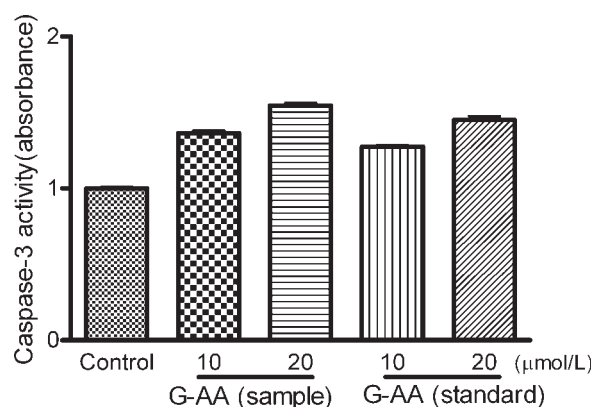


Figure 11. Caspase-3 activity assay.

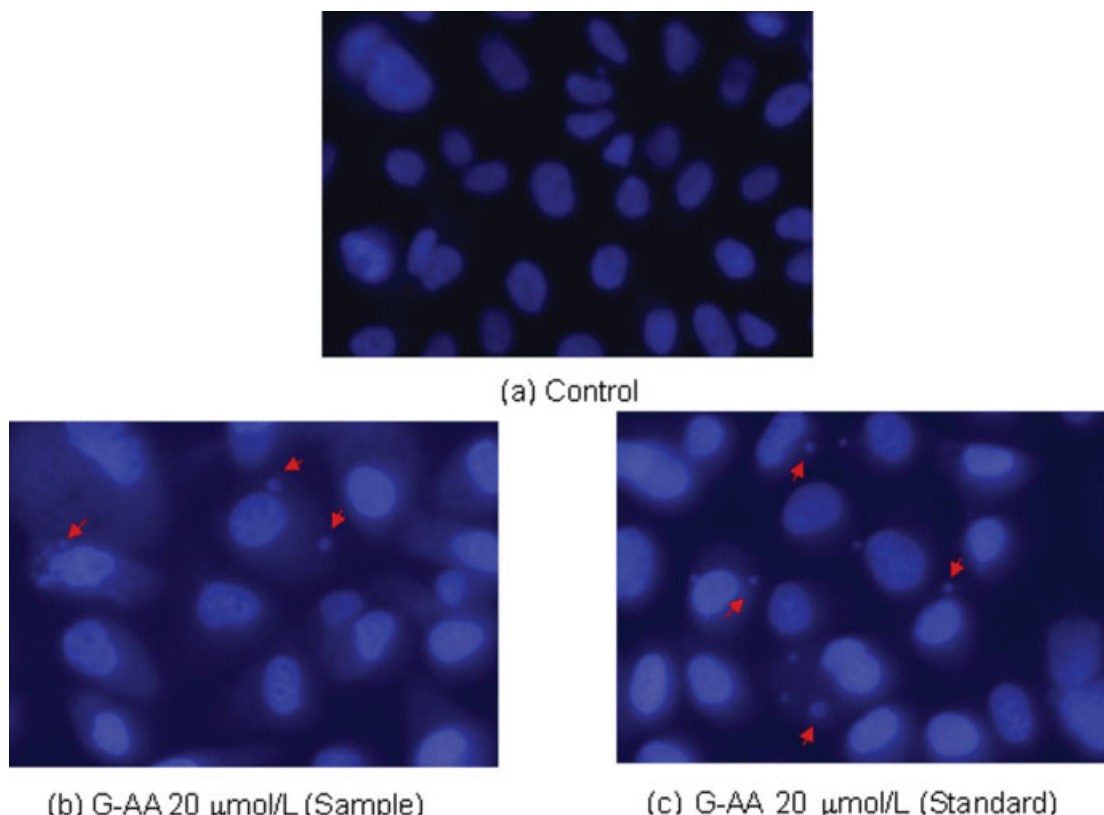


Figure 12. Apoptotic morphology changes caused by G-AA (400×).

[Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

MDA-MB-231. Therefore, with the further research about antitumor effect of gossypol and its derivatives which we succeed in synthesizing (Data was not shown), it is promising that this novel process offers abundant, low-cost, high pure and bioactive gossypol for treating cancer.

Conclusions

The novel process in our study can make good use of industrial waste and decrease the cost of G-AA. Ultrasound extraction and crystallization technology introduced in this novel process shortened product cycle, retained the antitumor activity of gossypol and boosted the quality of product. It is promising that large scale of G-AA with high antitumor activity is extracted from cottonseed soapstock by our novel ultrasound assisted method.

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