Biochemical Properties of Plant Polyphenols

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Abstract: Secondary metabolites are small organic molecules originated from primary metabolites during the embolism of plant. Polyphenols are secondary metabolites that are common in plant kingdom. Gossypol is a specific secondary metabolite in *Gossypium* species. The natural compound gossypol forms stable clathrates with vapor of diethyl ether solvent. Experiments carried out at room temperature, +40ºC and -5ºC. This work describes investigation of gossypol clathrates by X-Ray powder diffraction and TG-DSC analysis.

1 INTRODUCTION

Polyphenols are natural compounds that are part of daily consumed fruits, vegetables, cereals. They are one of the secondary metabolites in plants, which usually protect the plant from ultraviolet radiation and various pathogenic diseases, and are involved in increasing the plant's immunity (Claudine et al., 2004). In addition to fruits, vegetables, and grain products, secondary metabolites are formed in large quantities in the cotton plant, which is considered a technical crop. The cotton plant grown in Uzbekistan is an annual plant belonging to the *Malvaceae* family, *Gossypium* genus and *Gossypium hirsutum* species. During the ontogenesis of the cotton plant, secondary metabolites are formed in various organs, of which the substance belonging to the polyphenol class is gossypol. The cotton plant contains sesquiterpene-forming gene *GhTPS1* and monoterpene-forming gene *GhTPS2*, which play an important role in the biosynthesis of gossypol. The substance gossypol is synthesized in the glands of the root, stem, leaf and seed of the cotton plant, it exhibits the properties of a phytoalexin and protects against the attack of insects as well as external pathogenic effects (Tianlun et al., 2020).

Gossypol - $C_{30}H_{30}O_8$ (1,1'6,6',7,7'-hexahydroxy-5,5'-di-isopropyl-3,3'-dimethyl-(2,2'-binaphthalene)-8,8'- dicarboxyaldehyde) (Fig. 1), a yellow pigment, is an organic substance containing two naphthyl groups, six hydroxyl groups, two aldehyde groups, two methyl and two isopropyl groups.

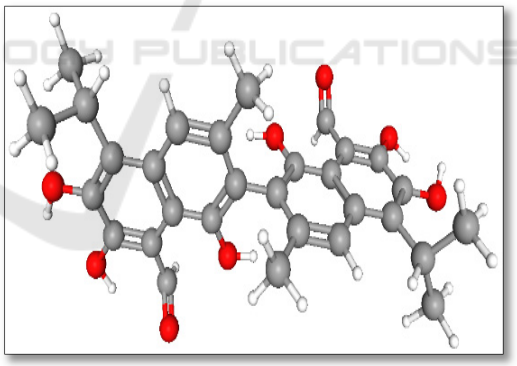

Figure 1: Structural formula of gossypol.

Aldehyde and hydroxyl groups in gossypol increase its biochemical activity. In the gossypol molecule, two "+" and "-" gossypol form mutual enantiomers, and this situation causes its biochemical properties to differ. According to the functional

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groups in the gossypol molecule, various chemical compounds are formed, in which the properties of gossypol are formed by bonds such as covalent bonds, ionic bonds, and hydrogen bonds. One of the unique properties of the gossypol molecule is that it creates a Van der Waals voltage due to the polarization of electron shells as a result of the interaction of dipole moments. Although these stresses are weak, they are important in the formation of various combinations of gossypol. In addition, there are several polymorphs of gossypol, and in the crystal structure of these polymorphs, gossypol molecules form an interlayer and a tubular channel. These polymorphs are well soluble in organic solvents with polar lower molecular weight and form the corresponding clathrates as a result of recrystallization (Honkeldieva et al., 2023), (Honkeldieva et al., 2015).

In recent years, the production of various medicinal preparations based on gossypol substance has been launched. It is important that the chemical composition of the gossypol substance does not change during the storage period of the medicinal product, and that additional intermediate products are not formed under the influence of external factors. The biochemical activity of gossypol polymorphs is determined by its content of many functional groups and the richness of hydrophilic and hydrophobic bonds. This article describes the study of the formation of appropriate clathrates by gossypol under the influence of vapors of organic polar solvents. Based on the results of the experiments, the stability of the properties of gossypol and its changes under the influence of heat were studied by the TG-DSC method. The transformation of gossypol polymorph substance in the initial crystal phases and formation of corresponding clathrates was proved by X-Ray Powder Diffraction X-ray phase method (Honkeldieva et al., 2015).

2 MATERIALS AND METHODS

X-ray Analysis. The X-ray powder diffractions were obtained using a Shimadzu X-ray diffractometer, model LabX XRD-6100, using CuKa radiation (λ=0.154 nm), current of 40 mA and operating voltage of 40 kV. The instrument features a vertical goniometer, and a linear scintillation detector with a graphite diffraction monochromator. Aluminium disks were used as sample supports, and the samples were swept with incidence angles from 4 to 35, at 0.02 increments, 2 s per increment.

Thermal analysis. The thermal analysis system was employed to acquire the simultaneous Thermogravimetry and Differential Scanning Calorimetry – TG-DSC curves (NETZSCH STA 409, Germany). Dry air and nitrogen were used individually as furnace atmosphere purge gases, with a flow rate of 50 mL min-1 in both cases. The temperature program consisted of heating the samples from 25 to 350°C, at a heating rate of 10°C min-1. Samples weighing 5 mg samples were placed in aluminum crucibles with a perforated cover. The application possibilities comprised the whole spectrum of TG and DSC analysis.

3 RESULTS AND DISCUSSION

A solid:gas phase was used to produce gossypol clathrates by absorption method. In the case of P3 polymorph of gossypol, which has polycrystalline properties in the solid phase (Zhao et al., 2020), organic polar solvent diethyl ether was chosen for the gas phase. Diethyl ether is an aliphatic ether with the chemical formula CH_3-CH_2-O-CH_2-CH_3. The boiling temperature of diethyl ether is T=34.15°C, it is a colorless liquid with a quick volatile and characteristic smell. In medicine, diethyl ether is used as a general pain reliever. A person who has worked a lot with diethyl ether may develop an asthetic tendency, as a result of which the disease "Ether zombie" is observed, and this disease leads to a decrease in memory and slowing down of physical activity.

The conducted experiments are based on the absorption of volatile diethyl ether vapors on the surface of polycrystalline gossypol polymorph, as a result of which the formation of the corresponding clathrate was studied. A chemical hermetic vessel was selected for the experiment, and a filter paper was cut into a box shape, placed in a four-layer case, and 2 ml of diethyl ether solvent was poured into it. As a result, a chamber saturated with diethyl ether vapor was created and placed in this chamber after weighing and determining the mass of two bulk scales. The first sample was considered as a control and was placed in a blank state without the gossypol polymorph 35 mg ($6{,}7 \cdot 10^{-5}$ mol) of the P3 polymorph of gossypol was taken into the second batch. Then, the hermetically sealed container was closed and left for 24 hours to allow absorption in the solid:gas phase. After one day, the first and second samples were repeatedly pulled, and it was observed that the mass of the empty sample did not change, while the mass of the sample containing the P3 polymorph of gossypol increased. The ongoing experiment was continued until the mass of the gossypol polymorph contained byuks reached

a constant. The corresponding experiments were carried out at room temperature, +40°C and -5°C. In carrying out these experiments, we used an electronic analytical balance of the Talent TE-64 Sartorius model, made in Germany, Weighing Paper, size: 3x3 inches, and Filter papers, size: 110 mm Dia, made in England for weighing substances.

A) *X-ray Analysis.* The formation of gossypol clathrates using the absorption method in the solid:gas phase was studied using X-ray phase analysis. According to the results of X-ray phase analysis, it was found that diethyl ether vapors were completely absorbed by P3 polymorph of gossypol at room temperature (Fig. 2).

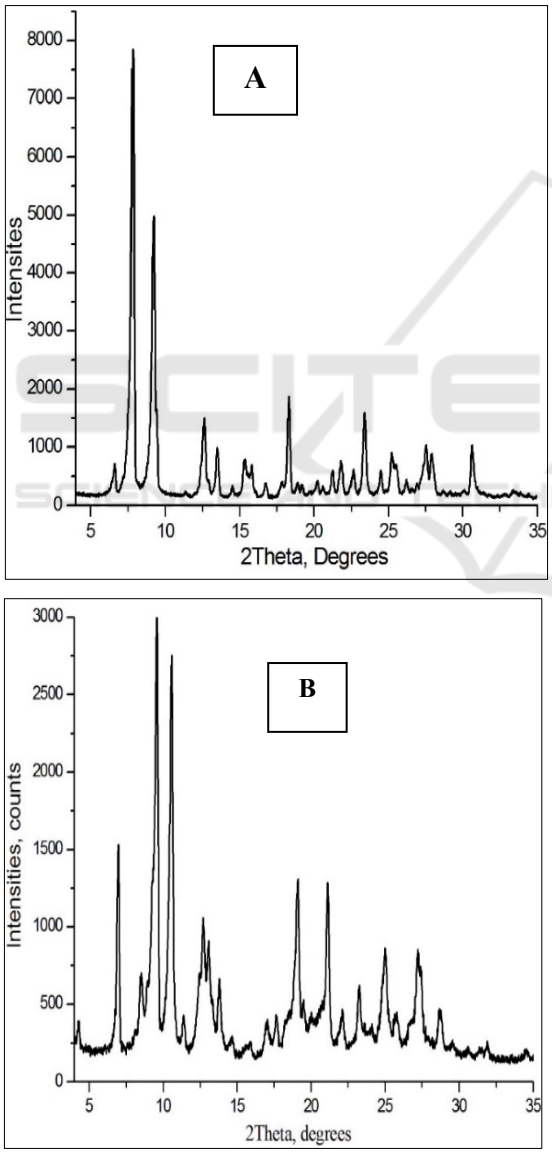

Figure 2: A) X-Ray Powder diffraction pattern of gossypol's polymorph P3 in room temperature; B) X-Ray Powder Diffraction pattern formation of clathrate gossypol:diethyl ether in room temperature.

It can be seen from the figure that A) X-Ray Powder Diffraction pattern is an analysis of the P3 polymorph of gossypol in pure form, where 2Theta=7.5º; 9.0º; 12.5º; 13.0º; 18.0º; 23.0º; 27.5º; X-rays falling at an angle of 31.0º respectively I_0=7997; 5000; 1500; 1040; 2001; 1700, 1100, 1200 showed intensity.

B) X-Ray Powder Diffraction pattern is the analysis of gossypol:diethyl ether clathrate at room temperature, where 2Theta=6.9º; 9.0º; 10.5º; 13.0º; 13.2º; 19.0º; 21.0º; 23.0º; 25.0º; 27.5º; X-rays falling at an angle of 28.0º respectively I_0=1500; 3000; 2750; 1000; 900; 1300; 1301; 600; 770; 800; 600 shows an intensity of 2Theta=13.0º; Formation of paired peaks was observed at 13.2º. This indicates the formation of new phases in the initial P3 polymorph, and as a result, it was determined that gossypol:diethyl ether clathrate was formed.

The pure P3 polymorph is a polycrystalline substance, the highest peak of its intensity peak was equal to I_0=8000. In the formation of gossypol:diethyl ether clathrate, the peak intensity is equal to I_0=3000, and it was observed that the polycrystalline nature of gossypol:diethyl ether clathrate decreased by 2.67 times compared to the polycrystalline nature of polymorph P3 (Table 1).

Table 1: X-ray analysis of pure gossypol P3 polymorph and gossypol:diethyl ether clathrate at room temperature.

Peaks	P3 polymorph		Gossypol:diethyl ether clathrate	
	2Theta	I/I_0	2Theta	I/I_0
1	7,5º	7997	6,9º	1500
2	9,0º	5000	9,0º	3000
3	12,5º	1500	10,5º	2750
4	13,0º	1040	13,0º	1000
5	18,0º	2001	13,2º	900
6	23,0º	1700	19,0º	1300
7	27,5º	1100	21,0º	1301
8	31,0º	1200	23,0º	600
9	-	-	25,0º	770
10	-	-	27,5º	800
11	-	-	28,0º	600

The results of the experiment conducted at a temperature of +40°C also showed that there was an absorption process between P3 polymorph of gossypol and diethyl ether vapors (Fig. 3).

Analysis of gossypol:diethyl ether clathrate at +40°C X-rays incident at an angle of 2Theta=6,9º; 8,0º; 9,0º; 11,0º; 11,5º; 12,5º; 13,0º; 14,0º; 17,5º; 21,0º; 22,3º; 27.0º respectively it was observed that

I_0=250; 600; 350; 810; 330; 370; 280; 210; 220; 400; 300; 350 exhibits intensity.

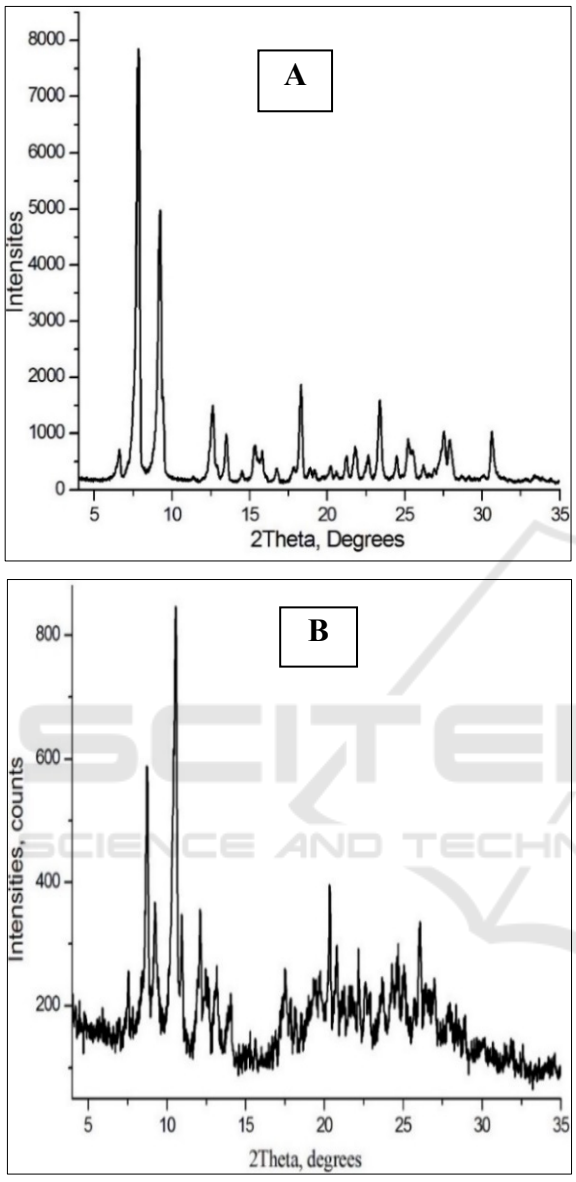

Figure 3: A) X-Ray Powder diffraction of pure gossypol's polymorph P3 in +40°C; B) X-Ray Powder Diffraction pattern formation of clathrate gossypol:diethyl ether in +40°C.

The pure P3 polymorph is a polycrystalline substance, the highest peak of its intensity peak was equal to I_0=8000. In the formation of gossypol:diethyl ether clathrate at a temperature of +40°C, the peak intensity is equal to I_0=3000, and it was observed that the polycrystalline nature of gossypol:diethyl ether clathrate decreased by 10 times compared to the polycrystalline nature of polymorph P3 (Table 2).

Table 2: X-ray analysis of pure gossypol P3 polymorph and gossypol:diethyl ether clathrate at +40°C.

Peaks	P3 polymorph		Gossypol:diethyl ether clathrate	
	2Theta	I_0	2Theta	I_0
1	7,5º	7997	6,9º	250
2	9,0º	5000	8,0º	600
3	12,5º	1500	9,0º	350
4	13,0º	1040	11,0º	810
5	18,0º	2001	11,5º	330
6	23,0º	1700	12,5º	370
7	27,5º	1100	13,0º	280
8	31,0º	1200	14,0º	210
9	-	-	17,5º	220
10	-	-	21,0º	400
11	-	-	22,3º	300
12	-	-	27,0º	350

This table 2 indicates that polymorph P3 lost its crystallinity under the influence of diethyl ether vapors and changed to an amorphous state.

Analysis of gossypol:diethyl ether clathrate at -5°C X-rays incident at an angle of 2Theta=5,0º; 13,0º; 14,2º; 17,5º; 21,0º; 21,7º; 22,0º; 22,5º; 24,0º; 25,3º; 28,0º respectively it was observed that I_0=1250; 1400; 1200; 410; 400; 500; 650; 410; 420; 490; 495 exhibits intensity (Fig. 4).

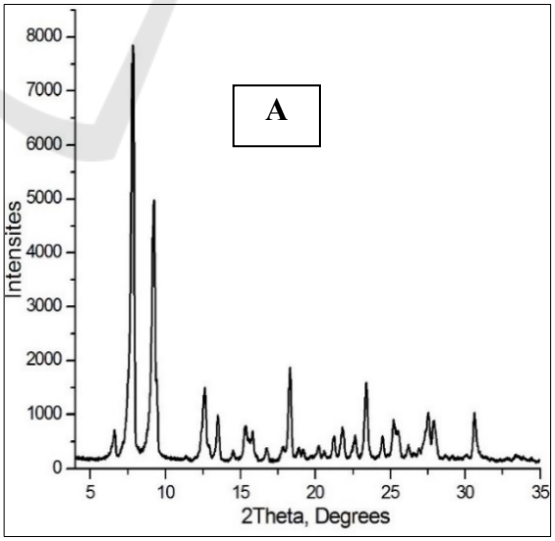

Figure 4: A) X-Ray Powder diffraction of pure gossypol's polymorph P3 in -5°C.

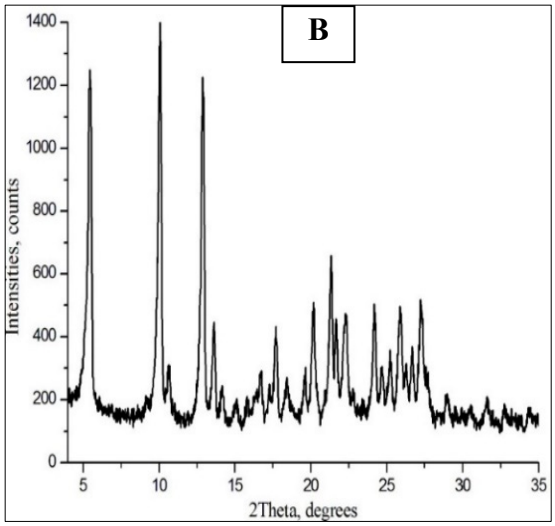

Figure 4: B) X-Ray Powder Diffraction pattern formation of clathrate gossypol:diethyl ether in -5°C.

The pure P3 polymorph is a polycrystalline substance, the highest peak of its intensity peak was equal to I_0=8000. In the formation of gossypol:diethyl ether clathrate, the peak intensity is equal to I_0=1400, and it was observed that the polycrystalline nature of gossypol:diethyl ether clathrate decreased by 5,72 times compared to the polycrystalline nature of polymorph P3 (Table 3).

Table 3: X-ray analysis of pure gossypol P3 polymorph and gossypol:diethyl ether clathrate at -5°C.

Peaks	P3 polymorph		Gossypol:diethyl ether clathrate	
	2Theta	I_0	2Theta	I_0
1	7,5º	7997	5,0º	1250
2	9,0º	5000	13,0º	1400
3	12,5º	1500	14,2º	1200
4	13,0º	1040	17,5º	410
5	18,0º	2001	21,0º	400
6	23,0º	1700	21,7º	500
7	27,5º	1100	22,0º	650
8	31,0º	1200	22,5º	410
9	-	-	24,0º	420
10	-	-	25,3º	490
11	-	-	28,0º	495

Thermal analysis. This study uses combined thermogravimetric analysis (TG)/differential thermal calorimetry (DSC). The use of thermal techniques such as thermogravimetry (TG) and differential scanning calorimetry (DSC) has been proposed as a reproducible, informative, rapid, low-cost and small-sample consuming method to characterize the complete quality continuum of organic materials.

TG is a thermogravimetric analysis, in which the phenomenon of degradation in the studied substance under the influence of temperature, the formation of polymorphic modifications as a result of phase changes, the processes of desolvation and decomposition, as well as the thermal stability of the substance, as well as the composition of the substance, are determined.

TG is a method of thermal analysis in which changes in physical and chemical properties of materials are measured as a function of increasing temperature (with constant heating rate), or as a function of time (with constant temperature and/or constant mass loss). TG can provide information about physical phenomena, such as:

- second-order phase transitions (including vaporization, sublimation, absorption, adsorption, and desorption);
- chemisorption;
- desolation (especially dehydration);
- decomposition;
- solid-gas reactions.

DSC is a thermo-analytical technique in which the difference in the amount of heat required to increase the temperature of a sample and reference is measured as a function of temperature. Both the sample and reference are maintained at nearly the same temperature throughout the experiment. Generally, the temperature program for a DSC analysis is designed such that the sample holder temperature increases linearly as a function of time.

DSC can provide information about physical phenomena, such as:

- Melting Point/Melting Range;
- Heat Capacity;
- Crystallization;
- Thermal Stability;
- Decomposition Temperature;
- Purity.

In the TG-DSC method, the studied substance is compared with the control substance. For this analysis, chemically pure gossypol P3 polymorph was selected as a control. The result of thermal analysis of Gossypol P3 polymorph is shown in Figure 5. TG-DSC curve was performed in the temperature range of 25-350°C. According to the TG-

thermogravimetric results, it was observed that the mass reduction of gossypol P3 polymorph proceeds in 2 stages. The 1st stage of mass reduction was observed in the temperature range of 100-140°C, and the mass difference of P3 polymorph was reduced from 100% to 94.94%, resulting in a mass difference of 5.06%. The 2nd stage of mass reduction started slowly at 175°C and rapidly decreased in the temperature range of 192.8-200°C. No mass change was observed starting at 230°C. In the 2nd stage, the mass difference was 6.15%.

According to DSC-differential scanning calorimetry analysis, a two-step "phase transition" process was observed when gossypol P3 polymorph was exposed to heat. The first "phase transition" process corresponds to 100-120°C, and in this temperature range, one molecule of water was released from the composition of the gossypol P3 polymorph. The second "phase transition" process corresponds to 175-192.8°C, and in this temperature range, another molecule of water is separated. The strongest lower peak of the endothermic peak corresponded to 192.8°C, where it was observed that the P3 polymorph of gossypol was converted to dianhydrogossypol, and the amount of heat required for the endothermic reaction was 227.6 J/g. In the DSC diagram, there was no peak at all from 230°C, which indicated the complete decomposition of the dianhydrogossypol molecule and the beginning of the carbonization process.

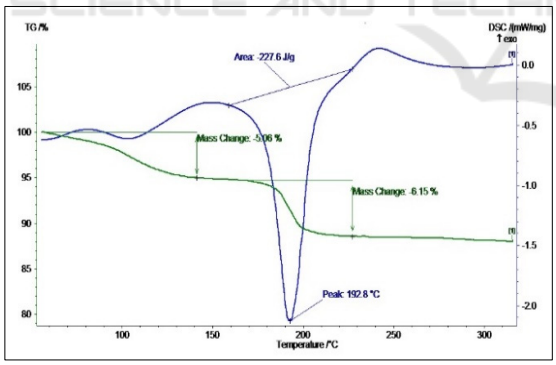

Figure 5: TG-DSC curve of gossypol P3 polymorph.

The result of thermal analysis of gossypol:diethyl ether clathrate produced at room temperature is shown in Figure 6. The mass reduction of gossypol:diethyl ether clathrate was observed to proceed in one step.

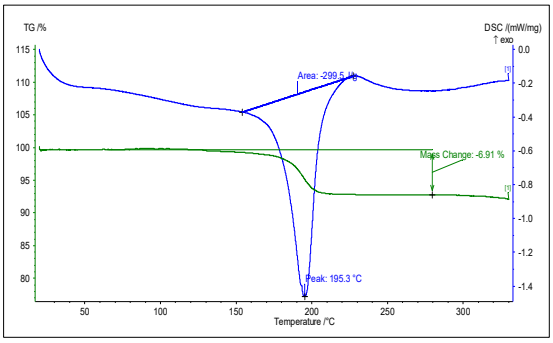

Figure 6: TG-DSC curve formation of gossypol:diethyl ether clathrate in room temperature.

Mass reduction was observed in the temperature range of 155-200°C, and the mass difference was 6.91%. In gossypol:diethyl ether clathrate, the "phase transition" process was not observed until 155°C, due to which the hydroxyl groups of gossypol P3 polymorph form mutual hydrogen bonds with diethyl ether. As a result, water and diethyl ether contained in gossypol:diethyl ether clathrate were simultaneously desolvated at 195.3°C, and dianhydrogossypol molecule was observed to be formed. The amount of heat required for the endothermic reaction was 299.5 J/g. The dianhydrogossypol molecule was completely decomposed and the beginning of the carbonization process coincided with the temperature of 280°C.

The result of thermal analysis of gossypol:diethyl ether clathrate produced at +40°C temperature is shown in Figure 7. The mass reduction of gossypol:diethyl ether clathrate was observed to proceed in one step.

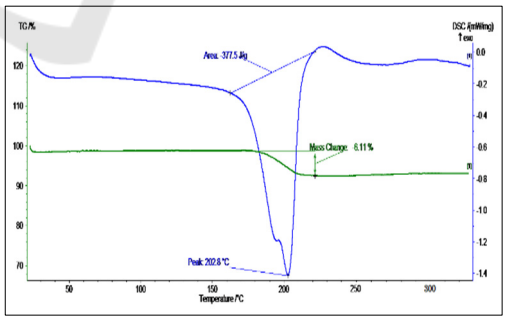

Figure 7: TG-DSC curve formation of gossypol:diethyl ether clathrate in +40°C.

Mass reduction was observed in the temperature range of 185-210°C, and the mass difference was 6.11%. In gossypol:diethyl ether clathrate, the "phase transition" process was not observed up to 165°C. This clathrate was desolvated with diethyl ether solvent at 195.3°C and water at 202.8°C, resulting in

the formation of dianhydrogossypol molecule, and the amount of heat required for the endothermic reaction was -377.5 J/g. The dianhydrogossypol molecule was completely decomposed and the carbonization process started at a temperature of 225°C.

The result of thermal analysis of gossypol:diethyl ether clathrate produced at -5°C temperature is shown in Figure 8. The mass reduction of gossypol:diethyl ether clathrate was observed to proceed in two steps. The 1st stage of mass reduction was observed in the temperature range of 80-145°C, and as a result of mass reduction of gossypol:diethyl ether clathrate from 100% to 88.82%, the mass difference was 11.18%. The 2nd stage of mass reduction started slowly at 160°C and rapidly decreased in the temperature range of 185.5-200°C. No mass change was observed starting at 230°C. In the 2nd stage, the mass difference was 5.42%.

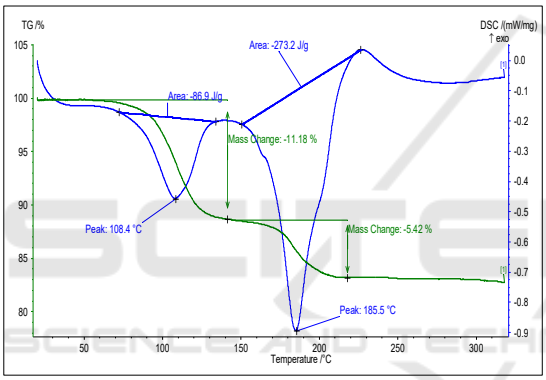

Figure 8: TG-DSC curve formation of gossypol:diethyl ether clathrate in -5°C.

Gossypol:diethyl ether clathrate formed at -5°C was observed to produce two endothermic peaks when affected by temperature. The first endothermic peak corresponded to the interval of 80-145°C, where diethyl ether was released, and the lowest endothermic peak corresponded to 108.4°C. The second endothermic peak was formed in the temperature range of 175.5-200°C, and the lowest peak of this endothermic peak corresponded to 185.5°C, where two molecules of water in the clathrate were separated and turned into dianhydrogossypol, and the amount of heat required for the endothermic reaction was respectively in the first stage it was -86.9 J/g, and in the second stage it was -273.2 J/g. The dianhydrogossypol molecule was completely decomposed and the beginning of the carbonization process coincided with the temperature of 220°C.

4 CONCLUSIONS

Based on the results of the analysis, it can be concluded that:

1. The P3 polymorph of gossypol was observed to form gossypol:diethyl ether clathrate in the solid:gas phase with diethyl ether at room temperature, +40°C and -5°C, respectively;
2. P3 polymorph was found to be stable up to 100°C, desolvation of water molecules was observed in the temperature range of 100-140°C;
3. The resulting gossypol:diethyl ether clathrate was stable up to 155°C at room temperature, and water and diethyl ether were found to desolvate simultaneously at 195.3°C;
4. Gossypol:diethyl ether clathrate formed at +40°C is stable up to 163°C, desolvation of diethyl ether at 195.3°C and water molecule at 202.8°C was observed;
5. Gossypol:diethyl ether clathrate formed at -5°C is stable up to 75°C, desolvation of diethyl ether solvent at 108.4°C and water molecule desolvation at 185.5°C was observed;
6. The stability of the formed clathrates was found to increase from left to right in the cross section: gossypol:diethyl ether clathrate formed at -5°C → gossypol:diethyl ether clathrate formed at room temperature → gossypol:diethyl ether clathrate formed at +40°C.

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