# Effect of pH on a Gossypol Complex with Phospholipids: Gossypol–PE

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**ABSTRACT:** PE, one of the major phospholipids in oilseed soapstock, may react with gossypol to form Schiff bases. PE amounts to 20–30% of the phosphorus compounds in soapstock. In this report, the dependence on pH of the Schiff base products between PE and gossypol was investigated using a spectrophotometer, an HPLC equipped with an ELSD, and an LC–MS system. We observed that at pH 7 the Schiff reaction product and reactants were clearly detected by ELSD, absorption, and LC–MS spectra. The absorption spectra displayed the characteristic peak for the Schiff bases around 430–440 nm. The absorption spectra also indicated that the reaction was pH dependent. The reaction temperatures were 60 and 90°C. The LC–MS spectra supported the formation of Schiff bases as well as methyl ether derivatives of gossypol in alcohol at the elevated temperatures. The implications of these experimental findings are presented in this paper.

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**KEY WORDS:** Cottonseed meal, gossypol complex, pH effects, phospholipid, Schiff reaction.

Gossypol and phosphatides are extracted with TG when cottonseed meal and oil are produced by solvent extraction (1). By adding caustics to extracted oil, FFA, phosphatides, and gossypol are separated from oil, producing soapstock as a byproduct. Soapstock, separated from oil by centrifugation, is usually added back to animal feed (1). Cottonseed meal is one of the most popular feeds for ruminant animals.

Gossypol reacts with protein [ε-amino groups of lysine (2)], and with alkyl- and phenylamines (3,4) to form bound gossypol. Because of its toxicity problem, the amount of free gossypol allowed in animal feed is lower than that of bound gossypol (3). Although gossypol reactions with phosphatides are believed to form a class of bound gossypol (5,6), experimental investigations to confirm the reaction in detail are scarce. In particular, the reversibility of the reaction and the effect of pH on the reaction have not been reported thus far. It is extremely important to investigate the reappearance of free gossypol by reversion of the reaction from the Schiff product, gossypol-phosphatide, because of the animals' physiological sensitivity to free gossypol (7). In general, PE amounts to up to 20–30% of phosphatides in oilseed soapstock (1). The dependence on pH of the Schiff product between PE and gossypol was investigated in this paper using a spectrophotometer, a high-performance liquid chromatograph equipped with an ELSD, and an LC–MS system. The reaction temperatures were room temperature (RT), 60 and 90°C. The formation of the Schiff base product was confirmed by mass spectra obtained from an atmospheric pressure chemical ionization (APCI) source.

## MATERIALS and METHODS

*Materials.* A typical oilseed PE (1,2-di-9-octadecenoyl-*sn*-glycero-3-phosphatidylethanolamine; 99% pure with formula weight 716) extracted from soy (Avanti Lipids, Alabaster, AL), gossypol–acetic acid (GA) from cottonseed (89.61% gossypol by weight; SRRC, ARS, USDA, New Orleans, LA), and reagent-grade amines (isopropyl amine and aniline), HPLC solvents and reagents, and gossypol (G) (reagent grade; Sigma-Aldrich, St. Louis, MO) were used.

*Gossypol reactions with amines.* Ten micromoles of PE dissolved in 2 mL $CHCl_3$, and 5 µmol of gossypol acetic acid were placed in a 50-mL volumetric flask with 15 mL of isopropyl alcohol (IPA) at the predesignated temperatures [90, 60, and RT (25°C)] for 2 h, continuously and gently shaken. The final reaction products were analyzed using a spectrophotometer, a high-performance liquid chromatograph, and an LC–MS system. Analytical systems included an LC–MS system

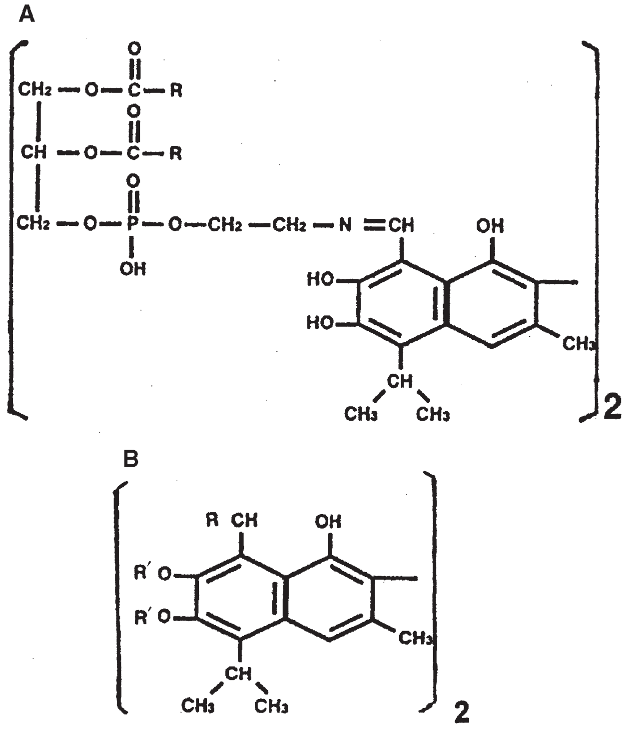

**FIG. 1.** (A) Predicted Schiff base product from the gossypol–PE reaction. (B) Gossypol alkyl ether derivatives.

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*To whom correspondence should be addressed at Southern Regional Research Center, ARS, USDA, P.O. Box 19687, New Orleans, LA 70170. E-mail: mskuk@srrc.ars.usda.gov with an APCI source (Model ZMD; Waters, Milford, MA), coupled with a liquid chromatograph (Model 2690; Waters), a spectrophotometer (Model 160A; Shimadzu, Columbia, MD), and an HPLC system (Model 10 VP; Shimadzu) equipped with an ELSD (Model IIA; Alltech, Burtonville, MD). The details of HPLC–ELSD were as follows: (i) column—normal phase, 25 × 4.6 cm (Supelco, Bellefonte, PA) packed with 5 µm silica; (ii) flow pattern—isocratic at 0.5 mL/min at RT; and (iii) mobile phase made of isopropanol/*n*-hexane/$H_2O$ in a volume ratio of 52:40:8. The absorption spectra of the PE–GA reaction product were compared with the same obtained from the reactions GA–isopropyl amine and GA–aniline. The final PE–GA Schiff base products were examined by using an LC–MS system with an APCI source. The APCI spectra of GA heated at 90°C in IPA for 2 h without the other reactant were also examined. The same was done for PE; spectral examination for PE was conducted at 90°C with and without the other reactants. The specifics of LC–MS were: (i) column—symmetry 3.5 µm C18, 2.1 × 50 mm (Waters); (ii) mobile phase and flow pattern—0.3 mL/min acetonitrile for 5 min followed by a 5-min gradient to 50:50% THF/acetonitrile, and MS loop injections at 0.2 mL/min isocratic with acetonitrile; and (iii) negative-ionization APCI with desolvation gas at 150 L/min, cone gas at 100 L/min, corona voltage at 2.91 kV, cone voltage at 50 V, source block temperature at 130°C, and APCI heater at 500°C. The reaction experiments were repeated with G in place of GA.

## RESULTS AND DISCUSSION

Equation 1 describes the possible gossypol reactions with PE:

$$\text{G (GA)} + \text{PE} \rightleftarrows \text{reaction products } (P_1, P_2, \ldots, P_n) + H_2O \qquad [1]$$

where $P_1$, $P_2$, and $P_n$ represent the reaction products in general.

Of the three tautomeric forms of gossypol (5,6) (aldehyde, ketone, and lactol) from this reaction equation, the Schiff base products with the aldehyde form (5,6) are most likely to be formed (Fig. 1A). The reactants at RT were detected well by the HPLC–ELSD system as shown in Figure 2. APCI spectra of the reaction product at 90°C (Fig. 3C) supported the formation of the reaction products. $(M-1)^-$ peaks of this Schiff base reaction at *m/z* 517, 499, and 481 represent the molecular ion of gossypol and anhydrogossypols, as shown by Matlin *et al.* (8,9) in their LC–MS for gossypol. The molecular ion for PE is shown at *m/z* 714 (Fig. 3A). The Schiff base products are shown at *m/z* 1256 and in this vicinity (Fig. 3C). When GA in IPA was heated, the spectra (Fig. 3B) indicated that gossypol methyl ether derivatives at *m/z* 541 (Figs. 3B and 3C) were formed, as predicted by Adams *et al.* (5). These authors suggested that ether derivatives are formed when GA or G is placed with acetic acid in alcohols. The spectra in Figure 3B provided a clear indication of the formation of degenerated compounds from GA or G in the presence of alcohols. The formation of these derivatives (Fig. 1B) did pose a problem in the early HPLC assays for gossypol (8–10).

The absorption spectra (Figs. 4A–C) indicated that the Schiff base products, which were predicted and classified as "gossyphosphatides" by early investigators (6), were clearly produced at both 90 and 60°C. A shoulder (Fig. 4B) shown around 430–440 nm is characteristic of a Schiff base (3,4). The same shoulder peak at 430–440 nm was observed from the spectra of Schiff bases produced from reactions with aniline–G (or GA) and isopropylamine–G (or GA) (Fig. 4D) even at RT. The Schiff base products were not formed within 2 h when either G or GA was placed with PE at RT. When a strong acid such as HCl was added to the reaction products of GA–PE until the pH value was lowered to 2, free gossypol was released from the reaction product. The conversion of this gossyphosphatide to free gossypol is clearly shown in the absorption spectra in Figures 4A–C. This indicated that binding of gossypol to phosphatide is a pH-dependent and reversible process.

In cottonseed processing to produce feed and edible oil, a number of steps for cooking use expanders. The temperature profile of expanders ranges from 110 to 140°C. Since free gossypol in cottonseed is in direct contact with phosphatides in these cooking, expanding, and extraction steps, a good portion of bound gossypol in the meal takes the form of a gossypol–phospholipid (gossyphosphatide) complex. Some of the gossyphosphatide, bound gossypol, can be converted back to the free form within the animals' digestive tract. Since pH values in the digestive tract of animals are in the vicinity of 2, there is a strong possibility that free gossypol may be formed from this gossypol–phosphatide complex as part of the diges-

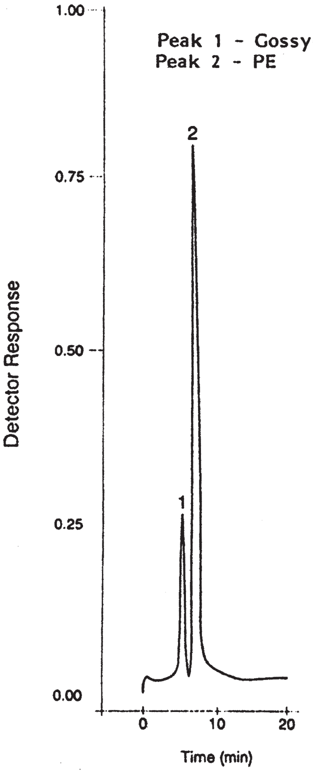

**FIG. 2.** HPLC–ELSD chromatogram for gossypol and PE.

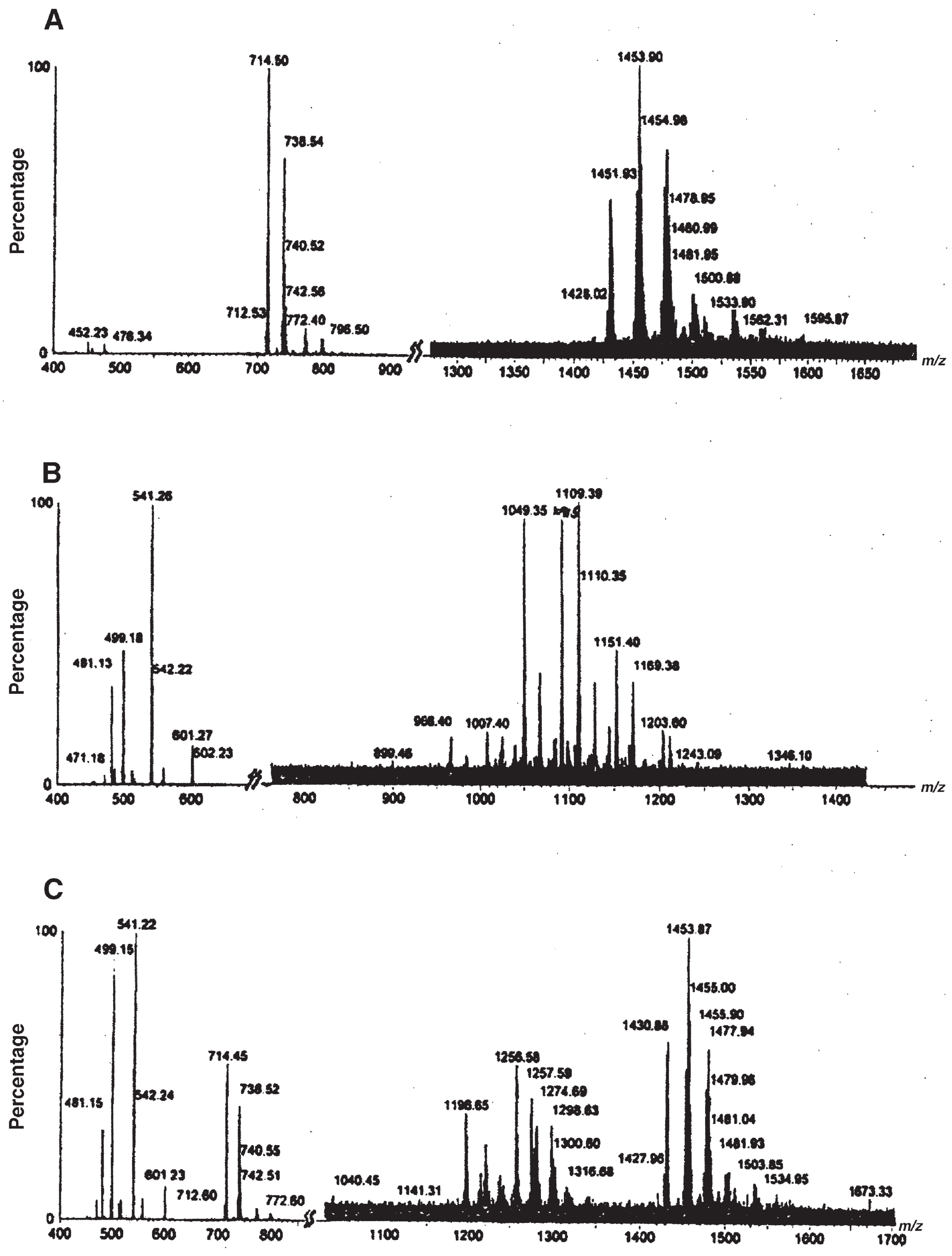

**FIG. 3.** (A) LC–MS atmospheric pressure chemical ionization (APCI) spectra for PE at 90°C. (B) LC–MS APCI spectra for gossypol–acetic acid (GA) and gossypol (G) at 90°C. (C) LC–MS APCI spectra for Schiff bases from GA (G)–PE reaction at 90°C.

tive process. Considering the similarity in the chemical structure between PS and PE, it is not difficult to project that PS forms a complex with gossypol similar to that with PE. The same reversibility and formation of free gossypol from the PS–gossypol complex is expected in the PE–gossypol complex. The conversion of bound gossypol from these gossypol–phospholipid complexes may not be solely dependent on pH value, but the conversion may well be determined by the physicochemical property of the Schiff product. As shown earlier (Fig. 4D), gossypol reacted readily with aniline or a small aliphatic amine at RT, whereas the G–PE reaction required higher temperatures. We can rationalize that the reaction between G and PE needs a certain amount of thermal energy from a source higher than ambient temperature, and that the amount of energy transferred to the G–PE reaction affects the reversibility of the Schiff base product. Further study is needed to examine how the energy input, transferred to cottonseed meal during the reaction and extraction steps of

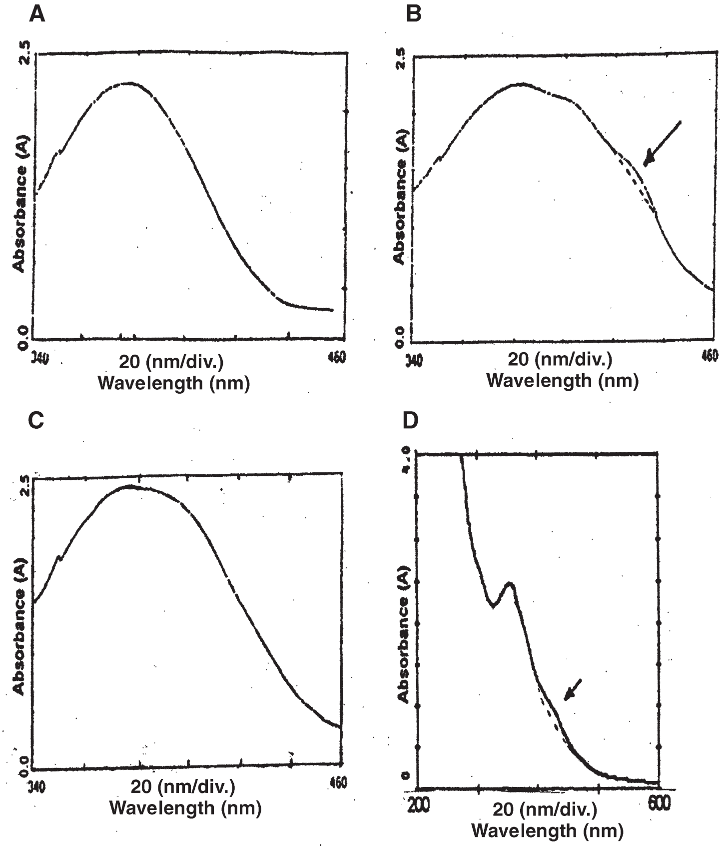

**FIG. 4.** GA (G) absorption spectra in isopropyl alcohol (IPA). (B) Schiff base absorption spectra from the GA (G)–PE reaction. (C) Absorption spectra of the reversed Schiff product at pH 2. (D) Absorption spectra of GA (G)–isopropylamine in IPA. For abbreviations see Figure 2.

cottonseed preparation, affects the reversibility of the G–PE reaction products.

Free gossypol substantially promotes erythrocyte fragility in animals and humans (11,12); it impedes the function of hemoglobin because gossypol binds with iron. Thus, the present understanding of gossypol–phospholipid reactions and the physicochemical properties of the gossypol–phosphatide complex occurring in the reaction and extraction steps of cottonseed must be expanded. Further investigation is necessary to elucidate the precise role of reaction variables such as the reacting components' (gossypol and phospholipids) residence time, temperature, and pH in the processing steps of this oilseed.

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