

Sterols in Pollen

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The acetone fraction (working name: Cernitin GBX) of a standardized pollen mixture from six plant species: *Zea mays*, *Pinus montana*, *Secale cereale*, *Phleum pratense*, *Alnus glutinosa*, and *Dactylis glomerata*, prepared for commercial use by AB Cernelle (Pharmaceutical Manufacturers), Ångelholm, Sweden, was used as crude material from which pollen sterols were purified. Approximately 15 g of sterols were crystallized from the non-saponifiable fraction of Cernitin-GBX. The chemical and physico-chemical properties of the sterols were investigated.

The discovery of an active animal hormone (folliculin, oestrone) in extracts of palm heart by Butenandt¹ led to an intensive search for sterols in different plant materials. The presence of sterols in pollen from various plants has been described. Active oestradiol was established by Skarzynsky² in *Salix* pollen, and an oestrogenic substance was found by Wafa *et al.*^{3,4} in pollen from the date palm. The existence of the same substance in pollen has also been reported by von Euler *et al.*⁵

The number of published reports on sterols in pollen is very small. The reason for this sparsity varies: difficulties associated with the collection of sufficient study material, as well as problems concerned with the separation of sterols from fats.

The main part of the studies concerning the chemical composition of the content of pollen was carried out by Sosa-Bourdouil,⁶⁻⁸ by Hügel *et al.*^{9,10} who employed mass-spectrography to demonstrate the presence of some C₂₇, C₂₈, and C₂₉ serols, and by Devys and Barbier¹¹ who showed large variations between pollens from five different species with regard to the composition of the sterol content. Extensive work has also been performed by Kwiatkowsky¹² who used thin-layer chromatography for separation and gas-chromatography for analysis, resulting in a rather crude separation of the ether fraction from pollen, and further by Standifer¹³ who employed column-chromatography for separation and thereby confirmed the presence of, *inter alia*, some 3 β -hydroxysterol in pollen.

The chemical composition of Cernitin-GBX has been investigated by Hallgren¹⁴ who found that it consisted chiefly of different lipids.

EXPERIMENTAL

A) Isolation of saponifiable and non-saponifiable fractions

Approx. 500 g Cernitin-GBX, acetone-free, was mixed with a 5 % solution of KOH in ethanol (100 g GBX in 200 ml KOH-ethanol soln.). The mixture was hydrolyzed for 2 h using a reflux-condenser, after which the ethanol was distilled off. Distilled water (approx. 500 ml to 100 g GBX) and concentrated HCl, to give pH 1–2, was added to the residue. The water mixture was extracted with ether. The greenish-brown ether fraction was washed with a 3 % solution of sodium carbonate (three times). The ether fraction was dried over a water bath. A thick-flowing, green-brown, oily fluid was obtained. (Fraction 1, non-saponifiable substances).

B) Acid hydrolysis of non-saponifiable substances

Fraction 1 was distilled under vacuum at a pressure of approx. 12 Hg mm (max. temp. approx. 80°C), as a result of which a very small quantity of distillate — containing mostly water and some higher alcohols — was separated. The residue (Fraction 2) was hydrolyzed in 3 M HCl in ethanol (approx. 50 ml of fraction in 100 ml HCl-ethanol soln.) for 2 h using a reflux-condenser, after which the ethanol was distilled off. 1000 ml distilled water was added to the residue (approx. 250 ml). The mixture was extracted with ether. The ether phase was washed with a 3 % solution of sodium carbonate (three times) and then dried with Dryerite (water-free calcium sulphate, no indicator). The solution was filtered and the solvent distilled off. The residue obtained consisted of approx. 200 ml of thick-flowing, oily, greenish-brown fluid. (Fraction 3. Residue=non-hydrolysable substances).

C) Isolation of a crystalline fraction from the non-saponifiable residue of Cernitin-GBX (Fraction 3): Preliminary identification of a sterol mixture

1. *Crystallization.* Fraction 3, approx. 200 ml, was mixed with 200 ml ethanol (99 %). The mixture was boiled under reflux for about 30 min, and then left to stand at a temperature of about 20°C for one week. After this time, the mixture contained crystals which were separated by means of vacuum filtration. The crystals were washed in ice-cold, water-free acetone and recrystallized five times from ethanol (99 %). The crystalline fraction was given the name GBX-S. The crystallization procedure was repeated with the filtrate (Fraction 3) three times. On the third occasion, Fraction 3 contained no crystals after 10 days had elapsed.

2. *Description of the crystalline fraction.* The crystals (14.2 g total weight) are white, soft, and have a mother-of-pearl sheen. The crystal form is somewhat irregularly rhomboid. The crystals are soluble in acetone, chloroform, ether, carbon tetrachloride; sparingly soluble in cold ethanol, methanol, and ethyl acetate; insoluble in water.

3. *Chemical properties.* a) Libermann-Burchard reaction. A chloroform solution of GBX-S (1 mg/ml) was investigated. The solution was blue-green in colour, $\lambda_{\max} \approx 5000$ Å, which showed the presence of sterols.

b) Precipitation of 3 β -sterols with digitonine. An acetone-ethanol (99 %) solution of GBX-S (1 mg/ml) was mixed with digitonine (3 mg/ml, solvent: ethanol 40 %). A white precipitate was formed which was filtered off. The precipitate and the filtrate were studied according to the Liebermann-Burchard method. Result:

| Sample | Colour |
|-------------------------------|------------|
| GBX-S digitonine precipitate | blue-green |
| GBX-S (control) | blue-green |
| Filtrate | colourless |
| Digitonine solution (control) | colourless |

It appears that the crystalline fraction consists entirely of substances which form complexes with digitonine (3 β -sterols).

c) Kober's test. Approx. 1 mg of GBX-S was dissolved in conc. sulphuric acid (1 mg/ml). The solution was stained orange and showed green fluorescence in UV light. The colour turned red when two drops of water were added and the solution heated. The reaction is semispecific for oestrogens.

d) Reaction with vanillin-perchloric acid according to Few:¹⁵ 1 mg GBX-S was dissolved in 1 ml glacial acetic acid. Approx. 1 mg vanillin and 0.5 ml conc. perchloric acid were added. The solution turned red ($\lambda_{\max} \approx 7000 \text{ \AA}$), indicating the presence of sterols.

4. *Thin-layer chromatography.* Sample solutions contained 3 mg GBX-S/ml of water-free acetone. The reference substances used were: oestrone, stigmasterol, β -sitosterol, and cholesterol. The concentration of all solutions used was 3 mg/ml. The investigations were carried out according to Stahl¹⁶ with the aid of a Desaga thin-layer chromatography apparatus. Stationary phase: Silica gel G.

Solvents. 1) chloroform, *p.a.* Merck, 2) chloroform + ethyl acetate 80 + 20, *p.a.* Merck, 3) chloroform + acetone, 90 + 10, *p.a.* Merck.

Development. (No. 123 and 120 c, according to Stahl): 1 vol. conc. phosphoric acid + 1 vol. water; 1.5 % sol. phosphoromolybdate (ethanol solution).

Procedure. The plates were sprayed until they were saturated and transparent. They were then heated to 120°C for approx. 7 min. The fluorescent spots were subsequently studied in UV light. Unsaturated sterols could be developed as follows: the plates were heated for 10 min, sprayed as described above with a freshly prepared solution of phosphoromolybdate, and then reheated for a further 5 min at 120°C.

Table 1. Thin layer chromatography of GBX-S and reference substances.

| | Sample | R_F | Colour | % |
|---|---------------------|-------|--------------|----|
| Solvent 1) (Development: No. 123 + 120c) | GBX-S, spot a) | 33 | blue | 50 |
| | spot b) | 26 | blue | 35 |
| | spot c) | 20 | blue | 15 |
| | oestrone | 19 | blue | |
| Solvent 2) (Development: No. 123) | GBX-S, spot a) | 53 | orange | 35 |
| | spot b) | 57 | (grey) lilac | 55 |
| | spot c) | 61 | pale yellow | 10 |
| | oestrone | 62 | pale yellow | |
| | β -sitosterol | 58 | purple | |
| | stigmasterol | 55 | (grey) lilac | |
| | cholesterol | 58 | purple | |
| Solvent 3) (Development: No. 123 + 120c) | GBX-S, spot a) | 37 | blue | 35 |
| | spot b) | 40 | blue | 50 |
| | spot c) | 44 | blue | 15 |
| | oestrone | 45 | blue | |

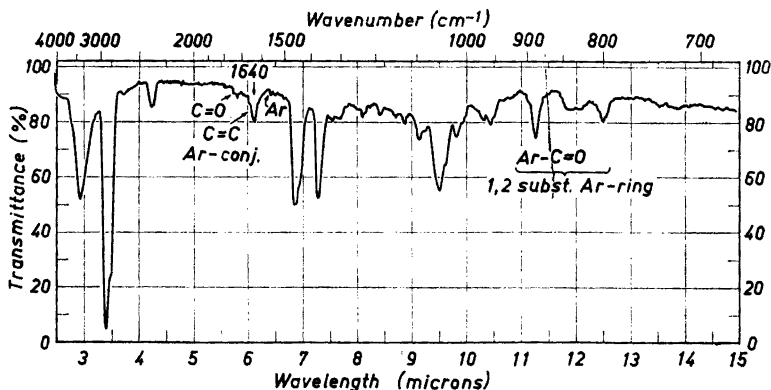


Fig. 1. IR diagram of crystalline sterols from pollen (in KBr medium).

Semi-quantitative optical methods of comparison were used to determine the approximate composition of GBX-S. Approx. 30 μg of all substances was applied to the chromatography plates. The reference and sample spots were then stained and compared (Table 1).

5. *Spectrophotometric investigations.* a) UV. This investigation was carried out with the aid of a Zeiss PMQII UV-Spectrophotometer. GBX-S was dissolved in ethanol (99 %) (approx. 0.1 mg/ml). The following absorption maxima were determined: 216, 240, and 281 μm .

b) IR. The investigation of IR-spectra was carried out with the aid of a Perkin-Elmer IR-Spectrophotometer, type 137. The KBr technique was used to prepare the samples (1.5 mg sample + 300 mg KBr). The reference substances used were: cholesterol, stigmaterol, β -sitosterol, and diosgenin.

Results.^{17-19, 21} Similarities between IR-spectra for GBX-S and the reference substance spectra in the "fingerprint" region show that GBX-S consists of sterols (Fig. 1). An absorption band at 1640 cm^{-1} showed complete deviation from the reference substances, indicating that at least one substance in the sample had a molecular structure comprising aromatic conjugated C=C, possibly C=C conjugated C=O. This latter group also appears at 1710 cm^{-1} , and similarly the aromatic ring shows at 1600 cm^{-1} . The 1,2-substituted aromatic ring is visible at 890, 840, and 800 cm^{-1} . The aforementioned C=O conjugated aromatic ring is also visible at 890 cm^{-1} . The intensity of these bands is medium and weak.

The IR spectra allow the tentative conclusion that GBX-S consists of at least two substances resembling stigmaterol and also a smaller quantity of an oestrogenic substance.

DISCUSSION

The results of the chemical investigations, used in the analysis of a crystalline sterol fraction of pollen, showed that this fraction — called GBX-S in the foregoing — consists entirely of 3β -sterols. These form quantitative complexes with digitonine. The Liebermann-Burchard reaction showed that at least some of these sterols have, as in the case of cholesterol, a side chain at C_{17} , and furthermore, that this side chain does not branch at C_{24} (type = stigmaterol, sitosterol) which would result in an extinction maximum at higher ($\sim 6500 \text{ \AA}$) wavelengths.²⁰ Kober's semispecific test showed the presence of an oestrogenic substance. The results obtained from thin layer chromato-

graphic investigations demonstrated that GBX-S is composed of at least three different sterols and that none of these is identical with either cholesterol or β -sitosterol. A similarity was found between a substance in GBX-S and stigmaterol, and between another substance and oestrone, with regard to R_F -values and colour reactions.

UV absorption spectra, with maxima at 2810 Å, are comparable with the UV absorption maxima of oestrone (2810 Å). The IR spectrum confirms that GBX-S comprises substances of oestrogenic nature and substances resembling stigmaterol.

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