Evaluation of Selected Steroids as Chemical Markers of Past or Presently Occurring Fungal Infections on Tobacco*

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SUMMARY

Three fungal strains isolated from tobacco were cultured on tobacco water extracts. In these cultures, the mycelium weight was shown to be correlated with the concentration of a steroid, ergosta-5,7,22-trien-3 β -ol [ergosterol]. This steroid is not a tobacco constituent, but tobacco samples where mold or yeast infections had occurred exhibited significant amounts of it.

A method is proposed to quantify ergosterol in tobacco samples by reverse-phase high-performance liquid chromatography (HPLC) with UV detection at 282 nm. 7-Dehydrocholesterol can be used as internal standard.

When found in a tobacco sample, the ergosterol concentration exhibits a good correlation with that of another related steroid, ergosta-4,6,8(14),22-tetraen-3-one [ETO], for which an HPLC quantification method in tobacco is proposed. Because it is highly fluorescent, ETO is also amenable to a sensitive and quick determination by thin-layer chromatography (TLC).

Once produced on tobacco, ergosterol concentration remains stable through storage under normal conditions, and even primary processing does not alter it appreciably.

Possible applications of ergosterol analysis to the detection of fungal infections or the monitoring of fungal growth on tobacco are outlined. In addition, TLC estimation of the ETO concentration in a sample may constitute a convenient and fast screening method for fungal infections.

an Ergosta-5,7,22-trien-3 β -ol [Ergosterol] korreliert. Ergosterol ist kein tabakspezifisches Steroid; es wird jedoch in Tabakproben gefunden, die von Schimmelpilzen oder Hefen befallen sind.

Es wird eine Methode beschrieben, mit der sich Ergosterol in Tabak quantitativ erfassen läßt. Das Verfahren basiert auf einer HPLC-Analyse und der Bestimmung der Steroide im UV-Bereich bei 282 nm. 7-Dehydrocholesterol dient als interner Standard.

Die Konzentration von Ergosterol in Tabak korreliert sehr gut mit der Konzentration eines anderen Steroids, Ergosta-4,6,8(14),22-tetraen-3-on [ETO]. Dieses Steroid läßt sich ebenfalls mittels HPLC oder auch aufgrund seiner fluoreszierenden Eigenschaft dünnsschichtchromatographisch (TLC) schnell und genau bestimmen.

Unter normalen Lagerungsbedingungen wird Ergosterol in Tabak nicht abgebaut; seine Konzentration ändert sich auch während der Tabakaufbereitung nicht wesentlich.

Mögliche Anwendungen der Ergosterolbestimmung zum Zweck der Erfassung von Pilzbefall oder Pilzwachstum in Tabak werden aufgezeigt. Auch wird die dünnsschichtchromatographische Bestimmung (TLC) von ETO als einfache und zeitlich nicht aufwendige Screening-Methode zur Erfassung von Pilzbefall in Tabak vorgestellt.

RESUME

On a mis en culture dans des extraits aqueux de tabac trois souches de champignons isolés du tabac. Dans ces cultures, le poids du mycélium s'est avéré présenter une bonne corrélation avec la concentration d'un stéroïde, l'ergostatriène-5,7,22-ol-3 β [ergostérol]. Ce stéroïde n'est pas un constituant du tabac, mais on en trouve des quantités significatives dans des échantillons ayant été contaminés par des champignons ou des levures.

Pour l'analyse quantitative de l'ergostérol dans le tabac, les auteurs proposent une méthode de chromatographie

ZUSAMMENFASSUNG

In einem wäßrigen Tabakextrakt wurden drei aus Tabak isolierte Pilzstämme gezüchtet. Es zeigte sich, daß das Myzelgewicht mit dem Gehalt des Tabakextraktes

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en phase liquide à haute performance (HPLC) — phase inversée et détection UV à 282 nm — le déhydro-7-cholestérol pouvant être utilisé comme standard interne.

Lorsque l'ergostérol est présent dans le tabac, sa concentration est en bonne corrélation avec celle d'un stéroïde de structure voisine, l'ergostatétraène-4,6,8(14),22-one-3 [ETO] pour lequel un procédé d'analyse quantitative par HPLC est également proposé. Comme l'ETO est fortement fluorescent, sa concentration peut aussi être déterminée rapidement et avec précision par chromatographie en couche mince (TLC).

Une fois que l'ergostérol est apparu sur le tabac, sa concentration reste constante pendant le stockage dans des conditions normales, et même les opérations ultérieures de préparation du tabac ne la modifient pas de manière appréciable.

Les auteurs évoquent également les possibilités d'application d'une telle analyse quantitative de l'ergostérol au problème de la détection des infections fongiques ou à celui du suivi de la croissance de champignons sur le tabac. En outre, l'estimation par TLC de la concentration en ETO peut constituer une méthode rapide et pratique pour le triage des échantillons présentant des infections fongiques.

INTRODUCTION

Fungal infections are among the major causes of spoilage and quality losses that may occur during the storage of cured tobacco. It is therefore of importance to have means of assessing quantitatively the extent of a fungal contamination in a tobacco sample.

Microbiological counting methods suffer from serious drawbacks. It often takes a few days to complete the determination and one only gets an estimate of the viable spore population in the sample. This can lead to false positive results if spores have been deposited on the sample but no microbiological activity is actually taking place. Conversely, following an invasion, the microorganisms may have died or be in a vegetative form and a false negative result is obtained.

An alternative approach is provided by the identification and monitoring of chemical or biochemical markers. Several systems have been described (1), among which two are potentially applicable to screening of tobacco samples for fungal contaminations.

One involves glucosamine analysis after hydrolysis as a way to estimate the sample content in chitin, a major cell-wall component of most fungi (2, 3). This approach, however, has several shortcomings: amino sugars are present in significant amounts in tobacco, insect parts could cause false positive responses, and the procedure is rather tedious.

The second approach, which was found more sensitive and straightforward for the screening of cereals (4), involves the analysis of a specific steroid: ergosterol (5).

Figure 1.
Structure of the steroids used in the study.

Ergosterol [ergosta-5,7,22-trien-3 β -ol (I in Figure 1)] is a major steroid in practically all molds and yeasts (6), but does not occur in significant amounts in higher plants. Very few reports have been made of the presence of ergosterol in tobacco (7), and its occurrence was only linked to fungal contamination of the tobacco in a recent paper (8) which also mentioned the presence in the infected samples of a related steroid, ergosta-4,6,8(14),22-tetraen-3-one [ETO (II in Figure 1)].

In the present paper we propose a method for ergosterol analysis in tobacco and discuss the possibility of using it as a chemical marker of fungal contamination. The use of ETO detection as a quick screening technique to select samples with high ergosterol levels is also presented.

MATERIALS AND METHODS

Reagents

- Ergosterol (99%) was obtained from Sigma.
- 7-Dehydrocholesterol (purity > 98%) was obtained from Fluka.

- ETO was synthesized from ergosterol according to ELKS (9).
- All solvents used were of HPLC grade (Romil) and all other reagents of analytical grade (Fluka).
- Czapek agar / chloramphenicol was obtained from Difco.

Analysis of Ergosterol in Tobacco

Clean-up: The ground tobacco sample (~ 5 g) is re-fluxed for 60 minutes in 50 ml of a 0.5 M methanolic KOH solution, the mixture is cooled, and 10 ml of 15% aqueous NaCl and 50 ml of petroleum ether are added. The petroleum ether phase is collected after shaking and centrifugation, and the methanol is re-extracted with an additional 25 ml of petroleum ether. The pooled petroleum ether fractions are dried under reduced pressure (Büchi Rotavapor M) and the residues are washed with petroleum ether onto a silica pre-packed 500 mg cartridge (Millipore SEP-PAK) equilibrated with the same solvent. The cartridge is fitted to a vacuum manifold (Analytichem International Vac-elute), first washed with 10 ml of 4% diethyl ether in petroleum ether, and ergosterol is subsequently eluted with 10 ml of 60% diethyl ether in petroleum ether. The solvents are evaporated and the residues re-dissolved in methanol, adjusting the volume to 5 ml, for analysis by high-performance liquid chromatography (HPLC).

Chromatographic Conditions: The chromatographic equipment consisted of a HP 1084B liquid chromatograph with a HP 79841A autosampler, a HP 79875A variable wavelength detector with stopped-flow scanning, and a Pye-Unicam PU 4024 fluorimetric detector.

Conditions:

column: 250 mm × 4.6 mm column packed with 5 µ Lichrospher 100-CH-18/II (Merck),
 mobile phase: methanol (100%),
 flow rate: 1.7 ml/min,
 column temperature: 45 °C,
 injection volume: 10 µl,
 detection: UV at 282 nm.

A typical chromatogram obtained from a tobacco extract is shown in Figure 2.

For mass-spectrometric investigations, the chromatograph was connected to a HP 5988 mass spectrometer through a thermospray interface (ionization: filament on, 950 eV energy).

The same chromatographic conditions were used, with the following exceptions:

column: 200 mm × 2.1 mm column packed with 5 µ Hypersil MOS (Hewlett-Packard),

Figure 2.

HPLC chromatogram of a tobacco extract (conditions: see text).

flow rate: 0.4 ml/min,
 post-column addition: 0.1 M aqueous ammonium acetate (0.8 ml/min).

Precision and Accuracy of the Method: For repeated analyses of a tobacco sample containing about 10 ppm of ergosterol, the relative standard deviation at the 2-sigma level is 3%.

The lower quantification limit is about 400 ppb in tobacco.

Special care was devoted to checking the accuracy of the method, as some samples exhibited ergosterol contents considerably higher than what was expected from previous studies (8).

The peak purity was confirmed by the following experiments:

- Mass-spectrometric single-ion monitoring of the LC eluates confirmed the presence of ergosterol at the expected retention time. Ions monitored: m/e 379 ($MH^+ - H_2O$), 397 (MH^+), 414 ($M + NH_4^+$).
- The presence of interference by other UV-absorbing compounds co-eluting with ergosterol was ruled out by UV scanning of the eluates, and also by re-injecting the samples after destroying the ergosterol by UV irradiation (254 nm, 10 min).

Additionally, results were cross-checked by analyzing some samples by capillary gas chromatography after trimethyl silylation, operating as described by SEVERSON et al. (10). The results obtained by this method were in excellent agreement with those obtained by the proposed HPLC method.

Analysis of Ergosterol in Mycelium

Sample preparation for ergosterol analysis in mycelium is the same as the one followed for tobacco samples, except that the clean-up on silica cartridges is no longer needed: the pooled petroleum ether fractions are evaporated and the residues are directly re-dissolved in methanol, made up to volume (5 ml), and subjected to HPLC analysis.

Chromatographic conditions are identical to those used for analysis of tobacco samples.

Clean-up Recovery (use of an internal standard)

The yield of the clean-up procedure (including hydrolysis) used for ergosterol analysis in tobacco was determined by the standard addition technique. The recovery of the added ergosterol was found to be between 85% and 90%. Carrying out the hydrolysis under nitrogen gives slightly higher recoveries, which can reach 95%. While using the procedure detailed above is generally acceptable with regard to the tobacco heterogeneity, it may be desired to take directly into account all losses by the use of an internal standard. 7-Dehydrocholesterol (III in Figure 1) can be used for this purpose as it is structurally very close to ergosterol and exhibits a strong absorbance at 282 nm. It was verified

that its recovery through the analytical procedure is identical to that of ergosterol.

The method has to be modified as follows:

Sample Preparation: A constant amount of internal standard solution is added to the sample with a syringe before hydrolysis. The same amount is added to ergosterol standard solutions in order to obtain a relative response factor.

Chromatographic Conditions: The chromatographic conditions are the same as above, except for the mobile phase: water/methanol/acetonitrile = 5/47/48 and column temperature = 35 °C.

A typical chromatogram obtained under these conditions from a tobacco extract is shown in Figure 3.

Ergostetraenone (ETO) Analyses

HPLC Quantification: For quantitative determination of ETO concentration in samples subjected to ergosterol analysis, the procedure is as follows:

Sample Preparation: The sample is processed as for ergosterol analysis in mycelium.

Chromatographic Conditions: The conditions are the same as for ergosterol analysis, except that fluorimetric detection is used.

Filter bandwidth: excitation, 340–380 nm; emission, 460–650 nm.

Estimation by Thin-layer Chromatography (TLC): For TLC estimation of ETO concentration in tobacco, the ground tobacco is extracted with petroleum ether (1/10), and 1–2 μ l of the extract are applied directly onto a pre-coated silica plate (Merck 10 cm $\times$ 10 cm silica gel 60 F 254) and eluted with benzene/acetone (9/1). ETO appears under UV light (366 nm) as a blue spot with a R_f value of 0.65, and quantities as low as 20 pg per spot are visible.

By comparing the spot brightness to that of different standards of selected concentrations deposited on the same plate, the range of the ETO concentration in the sample can be roughly estimated.

Fungal Cultures in Tobacco Extracts

This study was carried out on three mold strains that had previously been isolated from tobacco and identified:

Aspergillus niger,
Penicillium brevicompactum,
Eurotium spiculosum.

A culture is started on agar plates and incubated at

25 °C. The spores are collected in distilled sterile water, and this solution is used to inoculate the tobacco extracts.

Cut flue-cured tobacco is extracted at 40 °C with water in a 1/5 ratio for 30 minutes. The tobacco is filtered off and the extract sterilized. A series of 50 ml samples is prepared, each of which is inoculated with the pure culture of the selected strain and incubated at 25 °C. After the selected incubation time (0 to 18 days) the sample is filtered on a glass microfiber filter (25 mm diameter Whatman GF/B), and the mycelium is rinsed with distilled water and dried at 70 °C for 4 hours to determine its dry weight. Both filtrate and mycelium are analyzed to obtain the total ergosterol content.

Fungal Population Counting

Ground tobacco (2.5 g) is extracted for 30 min with 50 ml of sterile isotonic solution at 4 °C. The solution is filtered through sterile gauze into a sterile flask. Sterile Petri dishes are inoculated with 4 ml of the tobacco extract — or a dilution thereof if needed — and filled with 20 ml of Czapek agar / chloramphenicol medium kept at about 55 °C. The dishes are incubated at 25 °C for 5 to 10 days, depending on the growth rate, until the number of colonies can be counted. This method will count viable spores of both yeasts and molds.

Tobacco Storage under Controlled Conditions

To promote the growth of the natural flora on a tobacco matrix, 5 kg of strips were stored in 300 l climate-controlled cabinets (Mazzali Umidotest U-830-K5) for up to 7 weeks. The temperature was regulated at 25 °C and the relative humidity kept at 80% (corresponding to equilibrium moisture contents of 22%–23%). Samples were taken at 1 week intervals, and their ergosterol concentrations were determined together with their fungal population (yeasts and molds).

RESULTS AND DISCUSSION

Bound and Free Ergosterol

Steroids may be present in plant material as fatty acid esters, as glycosides and/or in the free form. An alkaline saponification will hydrolyze the esters but not significantly the glycosides, and the converse is true under acidic conditions.

Samples from the same tobacco were subjected to acid and base hydrolyses as well as combinations of the two. This was done on a set of tobaccos of different type and origin, and the ergosterol yields obtained after each

Figure 3.

HPLC chromatogram of a tobacco extract with internal standard (conditions: see text).

of these treatments from a given tobacco were compared.

On average, about 60% of the total extractable ergos-

Figure 4.
Ergosterol and mycelium in liquid cultures (*Aspergillus niger*).

terol is present in tobacco in the free form, and the bound ergosterol (40%) is almost exclusively in the form of fatty acid esters. These proportions are practically independent of the type of tobacco considered. This means that an acid hydrolysis step can be omitted when estimating the total ergosterol concentration of a tobacco sample.

Cultures of Fungi on Aqueous Tobacco Extracts

Three fungal strains previously isolated from tobacco were cultured on water extracts of flue-cured tobacco. The results obtained with the strain *Aspergillus niger* are given in Figures 4 and 5. Qualitatively, very similar results were obtained with the other two strains, but the ergosterol/mycelium ratio was about twice as low for *Penicillium brevicompactum* as that of the other two strains. All three strains also grew and produced ergosterol when cultured on water extracts of Burley or Oriental tobaccos.

Figure 4 demonstrates the simultaneity and the close parallelism that prevails between the fungal mycelium growth and the ergosterol build-up in the culture. This is true from the very beginning of the infection and at

Figure 5.
Ergosterol vs. dry-weight mycelium (*Aspergillus niger* cultures).

least throughout the first part of the incubation, i.e. from day 0 to day 9, as long as growth is actually taking place.

After 9–10 days the mycelium weight stabilizes as growth essentially stops. The ergosterol production remains fairly constant however, until day 14.

The correlation between mycelium weight and ergosterol is shown in Figure 5. The experimental points can be very well fitted to a quadratic equation ($r^2 = 0.9996$).

Fungal Growths on Cured Tobaccos

Mold cultures in aqueous tobacco extracts allow a direct verification of the correlation that prevails between mycelium weight and ergosterol. However, these experiments are limited to a single strain growing in a liquid medium under axenic conditions.

Therefore two batches of Oriental and flue-cured strips were kept under conditions where fungal growth could be expected from their natural flora (yeasts and molds). Under these conditions, there is no practical way of estimating directly the mycelium weight of a sample.

Figure 6.
Ergosterol and fungal population in Oriental tobacco.

Figure 7.
Ergosterol and fungal population in flue-cured tobacco.

Fungal growth can then be evidenced indirectly, by monitoring the resulting increase in fungal population counts as determined by classical microbiological methods.

The results are given in Figures 6 and 7, which plot the ergosterol concentration of the tobacco together with the logarithm of the fungal population count as a function of time. The plots show clearly the simultaneity of the increases in both parameters.

Additionally, a sample of Burley tobacco which had received an excess amount of casing and in which fermentation had started, was incubated at 25 °C and 80% relative humidity and analyzed as above. The results are reported in Figure 8, and they show the same simultaneity between the increases in ergosterol concentration and fungal population as is observed for uncased raw tobaccos.

Stability of Ergosterol in Tobacco

In the free form in solution, ergosterol is fairly sensitive to UV irradiation and oxidizing agents. It displays, however, remarkable stability on a tobacco matrix:

- The ergosterol concentration of tobacco stored under normal conditions and where no fungal infections occur remains unchanged over a period of several months.
- The ergosterol concentration of cased Burley is not affected by high-temperature drying (after correcting for humidity changes).
- The average ergosterol concentration of a cut blend after tobacco processing is equal to the sum of the contributions of all the blend components measured before factory operation (after correcting for additional casing weight and moisture changes).

It appears from these observations that ergosterol in a tobacco matrix is stable through normal storage and is not appreciably degraded during primary processing.

Figure 8.
Ergosterol and fungal population in Burley tobacco.

Range of Ergosterol Levels in Tobacco

A large number of raw tobaccos of different grades and origins routinely used in the manufacture of tobacco products were analyzed and their average ergosterol concentrations determined.

The ergosterol level of Oriental tobaccos was always below 3 ppm. For flue-cured tobaccos it could however reach 20 ppm, and be as high as 35 ppm for Burley tobaccos, with no detrimental impact on their quality.

Quick Screening Method for Fungal Steroids

Any quality-control application of ergosterol monitoring suffers from a major drawback: the number of samples to analyze in order to obtain a representative overview of a large batch of tobacco would quickly become overwhelming if one had to use a procedure involving clean-up and HPLC determination.

A solution to this problem can be found in the analysis of ergostetraenone [ETO]. This steroid, structurally related to ergosterol, was isolated by TANCONE (8) from moldy tobacco. It has the advantage of being highly fluorescent, and thus low levels of it can be detected in a tobacco sample by a very fast method allowing a large sample throughput (TLC analysis, see Methods section).

To validate this approach, a series of tobacco samples of various types were analyzed by TLC while a precise determination of the ETO concentration was obtained by HPLC. Comparing both results showed that the TLC method does not suffer from any interference from other tobacco components.

ETO can be detected in a tobacco sample at a level of 100 ppb.

The possibility of using this quick, semi-quantitative TLC assay as a predictor of ergosterol concentration rests on the existence of a good correlation between the concentrations of both steroids in tobacco.

A second series of tobacco samples of various grades

Figure 9.
Ergosterol and ETO correlation in different types of tobacco.

and origins encompassing a wide range of ergosterol levels was analyzed by HPLC to determine their ETO concentrations as well. The results are plotted in Figure 9, which demonstrates the good correlation that exists between the levels of both steroids ($r^2 = 0.943$). From the slope of the regression line, the concentration ratio of ergosterol to ETO is about 25 to 1. The 100 ppb detection limit for ETO in tobacco therefore corresponds to about 2.5 ppm of ergosterol, which is quite sufficient for screening purposes.

CONCLUSION

A method was developed and assessed to quantify ergosterol in tobacco samples. It was shown that ergosterol production is directly linked to mycelium growth in mold cultures on aqueous tobacco extracts, and that it is also an indicator of mold or yeast growth on tobacco strips. Ergosterol appears therefore as an accurate and sensitive marker of fungal growth in tobacco products, even allowing detection of past infections due to its stability and non-volatility.

Ergosterol monitoring should find an application in confirming or replacing altogether microbiological determinations when estimating fungal growths (studies on storage or preservation of tobacco products). The fact that it remains on a sample after a microbial infection makes it also a valuable tool in assessing the origin of certain spoilages.

The correlation between the concentration of ergosterol and that of a related steroid (ETO) in tobacco samples was established. The fast screening of the presence and concentration range of ETO, therefore, can provide a means to quickly select samples with significant ergosterol levels when assessing larger tobacco batches.

REFERENCES

1. Matcham, S. E., B. R. Jordan and D. A. Wood: Methods for assessment of fungal growth on solid substrates; in *Microbiological methods for environmental biotechnology*, edited by J. M. Grainger and J. M. Lynch, Academic Press, London and New York, 1984, pp. 5–18.
2. Ride, J. P., and R. B. Drysdale: A rapid method for the chemical estimation of filamentous fungi in plant tissue; *Physiol. Plant Pathol.* 2 (1972) 7–25.
3. Lin, H. H., and M. A. Cousin: Detection of mold in processed foods by high-performance liquid chromatography; *J. Food Prot.* 48(18) (1985) 671–678.
4. Matcham, S. E., B. R. Jordan and D. A. Wood: Estimation of fungal biomass in a solid substrate by three independent methods; *Appl. Microbiol. Biotechnol.* 21 (1985) 108–112.
5. Seitz, L. M., D. B. Sauer, R. Burroughs, H. E. Mohr and J. D. Hubbard: Ergosterol as a measure of fungal growth; *Phytopathology* 69 (1979) 1202–1203.
6. Weete, J. D.: Sterols; in *Fungal lipid biochemistry* — Monographs in lipid research, Plenum Press, New York and London, 1974, Vol. 1, pp. 151–174.
7. Dymicky, M., and R. L. Stedman: Composition studies on tobacco, IV. Ergosterol, γ -sitosterol and a partially characterized steroidal glycoside from flue-cured leaves; *Tob. Sci.* 3 (1959) 4–8 and references therein.
8. Tancogne, J.: Phytosterols in French dark tobaccos — A study of their evolution in the plant and during smoking; *Ann. Tab. (Section 2)* 18 (1983–1984) 5–91.
9. Elks, J.: Studies in the synthesis of cortisone — Conjugated trienones derived from ergosterol; *J. Chem. Soc. (Lond.)* 1954, 468–469.
10. Severson, R. F., J. J. Ellison, R. F. Arrendale and M. E. Snook: Quantitative gas-chromatographic method for the analysis of aliphatic hydrocarbons, terpenes, fatty alcohols, fatty acids and sterols in tobacco; *J. Chromatogr.* 160 (1978) 155–168.

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