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Free Phytosterol Analysis and Characterization in Tobacco by Ultra Performance Liquid Chromatography-Atmospheric Pressure Chemical Ionization-Mass Spectrometry

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This paper presents that the ultra-performance liquid chromatographic atmospheric pressure chemical ionization mass spectrometer as an efficient method for the identification and quantification of phytosterols in tobacco. The sample preparation consisted of extraction by methanol solution, separated on an Acquity UPLC BEH C18 column with a gradient of methanol/water at a flow of 0.4 mL/min. The determination was performed in selective ion monitoring mode. The quality parameter of the developed method was established using 6-ketocholestanol as internal standard. The limits of quantification of phytosterols varied from 0.28 to 2.21 μg/g. The intra-day and inter-day determination precision for 5 free phytosterols were less than 10 % in relative standard deviations and their recoveries were located in the range of 90.5-110.8 %. The generally and applicability of this improved method for analyzing phytosterols in tobacco were validated after a series of comparison were done. Compared with traditional methods, this method not only simplified procedures, but also save time and solvent.

Keywords: Tobacco, Phytosterols, Ultra performance liquid chromatography.

INTRODUCTION

Phytosterols are bioactive components of natural subtances which are made up of a tetracyclic cyclopenta[α]-phenanthrene ring and a long flexible side chain at the C-17 carbon atom[1]. Generally, four major sterols could be found in tobacco, they are cholesterol, campesterol, stigmasterol and β-sitosterol. Besides, ergosterol could also be found in mildew tobacco[2,3]. Phytosterols existed in tobacco as free sterol (FSs) and as conjugates including sterylesters (SEs), steryl glycosides (SGs) and acylated steryl glycosides (ASGs)[3]. Some of phytosterols' structures are shown in Fig. 1. Sterols are important precursor of carcinogenic polycyclic aromatic hydrocarbons in tobacco. Some studies have suggested that approximately 61 % of benzene[α]pyrene is produced by pyrolysis of sterols in tobacco[4-6]. Measurement of tobacco phytosterols'levels in tobacco is mandatory because phytosterols are converted to polycyclic aromatic hydrocarbons, which are thought to be harmful to human health[7].

Sterols are typically measured by gas chromatography[8], but this require derivatization procedure, not only time-consuming and formation of artifacts, but also derivatization may lead to reduced recovery and imprecise and can not distinguish between cholesterol and α-tocoperol. HPLC methods offer the advantage of being able to separate and detect sterols. Moreover, HPLC methods followed by UV detection with a diode array detector (DAD) are highly reproducible, but they are quite tedious as they need a high degree of purification and the sample throughput is low, which makes the cost of the analysis high. Analysis of sterols in complex matrixes such as plant tissue can be especially challenging. However, the problem of the incomplete resolution of compounds in complex samples can be overcome by coupling chromatographic separation with mass spectrometry (MS). The use of LC-MS or LC-MS/MS makes it feasible to investigate sterol, in much more detail and with less sample preparation even in complex biological samples. However, the analysis of sterols by electro-

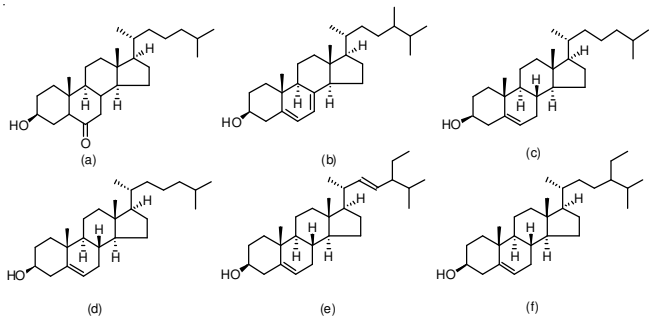

Fig. 1. Structures of phytosterols. (a) 6-ketocholestanol.; (b) ergosterol; (c) cholesterol; (d) campesterol; (e) stigmasterol; (f) β-sitosterol

spray ionization (ESI) is technically challenging, because these compounds lack ionizable groups. Atmospheric pressure chemical ionization (APCI) has generally been found to be more effective. In the present study, we developed and validated a sensitive method for the analysis of sterols in tobacco.

EXPERIMENTAL

HPLC grade of *n*-hexane, cyclohexane, ethyl acetate, dichloromethane, methanol were purchased from Tedia Company (USA). Deionized water from a Millipore system (Millipore, Bedford, USA) was used throughout the work. Ammonium aceate and acetic acid were also purchased from Sigma. Ergosterol (99 %); cholesterol (95 %); stigmasterol (95 %); β-sitosterol (98 %); campesterol (98 %); 6-ketocholestanol (98 %) were purchased from Chromadex Corporation.

Preparation of standard solutions: Stock solutions containing 0.5 mg/mL of various sterols were prepared in HPLC-grade methanol and stored in the dark at 4 °C for not more than one month. The final stock solution concentration was calculated taking into account the purity of commercial standards. Work standard solutions were prepared from these stock solutions and diluted with methanol prior to analysis .

Detection and quantification were performed with a tandem quadrupole mass spectrometry (API 4000, Applied Biosystems, Foster City, CA, USA) equipped with a atmospheric pressure chemical ionization-mass (APCI) source. The ultra performance liquid chromatography system with binary pump and autosampler (Waters, Milford, MA, USA). The separation were performed with An Acquity BEH C18 column (2.1 mm × 100 mm, 1.7 µm) in gradient mode, the column temperature was at 40 °C. Methanol and water were used as mobile phases. The flow rate was 0.4 mL/min and the injection volume was 5 µL. The methanol concentration was linearly increased from 95 to 100 % in 6 min and held for 1.5 min, finally brought back to 95 % in 0.5 min and held for 4 min to the next injection. Analyts were detected with an APCI probe in the positive electrospray ionization mode. Interface parameters were set as follows: source temperature 500 °C, curtain gas 20 psi, ion source gas 1 (nebulizer gas) 60 psi, ion source gas 2 (auxiliary gas) of 70 psi. Other MS parameters were shown in Table-1. Each phytosterol was quantified by MRM mode.

Sample preparation: Tobacco samples were supplied by Hongta cigarette factory, China. All tobacco samples were dried for 1 h at 40 °C and then ground to 40 mesh. Sample of 0.2 g was weighed into a 100 mL conical flask with cover and 40 mL of methanol was added. Samples were shaking for 1 h with oscillator and then filtered through a 0.22 µm membrane and transferred to an auto-sampler vial. Finally, 5 µL aliquot was injected into the UPLC/MS/MS system.

RESULTS AND DISCUSSION

Selection of extraction solvent: In order to improve the extraction efficiency, compare of methanol, *n*-hexane, cyclohexane, ethyl acetate, methylene chloride extracted samples respectively. To prevent *n*-hexane, cyclohexane, ethyl acetate, methylene chloride damage to the instrument, the extract was evaporated to dryness and with a constant volume of methanol and analyzed under the same conditions. The results show that the chromatogram peak and separation is preferably obtained by methanol extraction system, but *n*-hexane, cyclohexane, ethyl acetate, methylene chloride solvent extraction in the process of phase inversion sterol content will be lost. The methanol extraction residue was washed with methanol and extracted twice, the filtrate was analyzed and almost do not see sterol peaks. So methanol can be extracted completely.

Characterization of sterols in mass spectrometry

Mass spectrometry for each phytosterol uder APCI mode were acquired direct infusion of 10 µg/mL sample at the flow rate of 5 µL/min by syringe pump. The results showed that the molecular ion of sterols could not be seen, although add ammonium acetate and acidification of sample with formic acid, $[M + H\text{-}H_2O]^+$ is the most intense ion fragments (*m/z* 379.5, 369.3, 383.3, 397.5, 395.5 for ergosterol, cholesterol, campesterol, β-sitosterol and stigmasterol, respectively), indicating the loss of water $[M + H\text{-}H_2O]^+$ seems to be the characteristic of unsaturated sterols. However, the most intense ion is the fragment ion $[M + H]^+$ for 6-ketocholestanol.

Internal standard: For minimize analytical errors due to fluctuations in human operation and other experimental variables, peaks of sterols are described by their retention times. On the other hand, losses of analytes during isolation and separation must be corrected for the final results of analyte quantification by using radioisotopes or internal standards not present in samples[9]. According to reported paper[10,11], 6-ketocholestanol was used as the internal standard in the present work, because it is usually absent in tobacco and had a suitable retention time against other sterol variants in the present UPLC system (Fig. 2).

Linear range and limits of detection: To determine sterols in tobacco, an internal standard calibration curve was constructed with 5 standard solutions containing ergosterol, cholesterol, campesterol, stigmasterol, β-sitosterol respectively. Cholesterol, campesterol, stigmasterol, β-sitosterol from 0.07 to 8 µg/mL, ergosterol ranging from 0.05 to 5 and 0.16 µg/mL

TABLE-1
OPTIMIZED MS PARAMETERS OF PHYTOSTEROLS

Compound	Quantificational ion pair *m/z*	DPV	CEV	CXPV	Qualitative ion pair *m/z*	DPV	CEV	CXPV
Ergosterol	379.5/69.3	28	39	4	379.5/159.2	28	39	15
Cholesterol	369.3/147.2	37	33	9	369.3/161.3	37	32	9
Campesterol	383.3/147.3	50	37	12	383.3/161.0	50	37	12
Stigmasterol	395.5/83.1	41	36	4	395.5/147.3	38	37	15
β-sitosterol	397.5/161.3	24	29	9	397.5/147.2	24	29	9
6-Ketocholestanol	403.4/385.3	44	16	10	403.4/367.4	50	20	10

TABLE-2
CARLIBRATION CURVE PARAMETERS FOR 5 STEROLS

Compound	Standard cure	Correlation coefficient (r)	LOD (μg/g)	LOQ (μg/g)
Ergosterol	Y = 0.205X-0.0111	0.9994	0.29	0.97
Cholesterol	Y = 0.516X-0.454	0.9993	0.08	0.28
Campesterol	Y = 0.763X-0.314	0.9998	0.14	0.47
Stigmasterol	Y = 0.0687X-0.0122	0.9996	0.58	1.95
β-Sitosterol	Y = 0.244-0.0874	0.9998	0.66	2.21

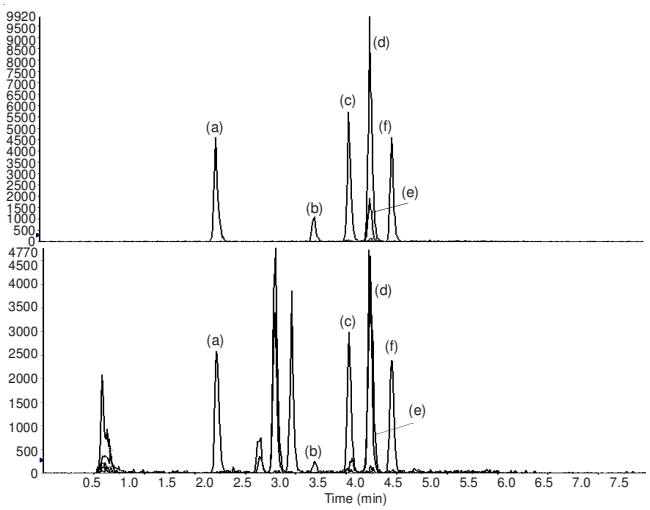

Fig. 2. Typical extracted ion chromatogram (XIC) of 5 phytosterols standard and sample. (a) 6-ketocholestanol.; (b) ergosterol; (c) cholesterol; (d) campesterol; (e) stigmasterol; (f) β-sitosterol

6-ketocholestanol (the internal standard). Linear calibration curves were obtained in the tested concentration ranges for tobacco. Table-2 lists the linear regression equations and the corresponding correlation coefficients (r). The limits of detection (LOD) and the limits of quantification (LOQ) achieved were 0.29 and 0.97 μg/g for ergosterol, 0.08 and 0.28 μg/g for cholesterol, 0.14 and 0.47 μg/g for campesterol, 0.58 and 1.95 μg/g for stigmasterol, 0.66 and 2.21 μg/g for β-sitosterol. So, besides the dramatic reduction in analysis time when using the UPLC-APCI-MS methodology, the narrower peaks produced a significant increase in sensitivity.

Precision and accuracy: The precision of the developed method was evaluated with representative sample. The sample was pretreated and subjected to UPLC-MS/MS analysis. For evaluation of the intra-day precision, five parallel test solutions were prepared and analyzed in 1 day and the relative standard for the 5 measurements were examined. For evaluation of inter-day precision, test solution were prepared and analyzed in consecutive 5 days and the RSDs for measurements performed in the 5 days were examined. The observed results of intra-day and inter-day for the 5 sterols are less than 10 %.

The accuracy of the developed method was validated with spiking-recovery tests. Different types of tobacco samples, contain Oriental tobacco, Burly tobacco, Flue-cured tobacco, Virginia and blended type cigarette were used as the control sample and the 5 sterols were spiked into the sample at three concentration levels. Satisfactory recoveries ranging between 90.5 and 110.8 % were obtained by the developed method for the tested sterols.

The developed method was applied to test different spices tobacco samples, There were 20 collected samples were pre-treated and analyzed with the method. In accordance with previous UPLC-APCI-MS/MS methods, ergosterol detected in three kinds of tobacco samples ,that illustrate the three samples of tobacco occurred mildew. It was found that stigmasterol was the predominant phytosterol in tobacco.

Conclusion

The presented method of UPLC-APCI-MS-MS has been proved to be a rapid, efficient and simple determination of phytosterols in tobacco. The sample pre-treatment avoided the difficult and time-consuming procedure, such as cleanup and derivatization. Samples were immersed in organic solvent and extracted in an oscillator. Sterols were separated on a C18 column. and determined by UPLC-MS/MS, Detection was performed by APCI-MS-MS and the total analysis time was 8 min. This UPLC-MS/MS assay was fully validated. The sample pre-treatment method was greatly simplified, analysis time was sharply shortened and the LOQ was lowered to conventional GC/MS quantification analysis.

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