Rapid Measurement of Free Phytosterols in Tobacco by Short-Column GC/MS/MS

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Phytosterols occur in plants, including tobacco, in free and bound forms. A GC/MS/MS method to quantify free phytosterols in plant materials was investigated. Free phytosterols in tobacco were extracted and directly analyzed without any preinjection sample treatments. A short GC column was used to reduce the retention of sterols in the severe GC conditions, and the chances of dehydration or decomposition were reduced. A highly selective tandem mass spectrometric detection called multiple selected reaction monitoring not only eliminated all of the chemical background of the crude tobacco extract but also compensated for the loss of GC resolution due to the short column. In a single GC injection, sterols that had not been resolved by GC were detected individually with the multiple selected reaction monitoring scan. This direct GC/MS/MS method provides reliable and reproducible quantitative results for free phytosterols in tobacco.

Keywords: *Phytosterol; β-sitosterol; campesterol; stigmasterol; gas chromatography; multiple selected reaction monitoring; mass spectrometry*

INTRODUCTION

Phytosterols exist in plants as part of the lipid content. In tobacco, there are four major phytosterols: β-sitosterol, campesterol, stigmasterol, and cholesterol (Stedman, 1968). These sterols, except cholesterol, are the essential components of plant membranes (Goad, 1967). Quantification of phytosterols in plants and animal tissues has evolved from the precipitation method developed in late 1950s (Stedman and Rusaniswkyi, 1959) to gas−liquid chromatography/mass spectrometry (GC/MS) analysis (Dyer et al., 1995; Ballesteros et al., 1995; Pizzoferrato et al., 1993; Plank and Lorbeer, 1993). Phytosterols exist in plants as free sterols and glucoside ethers. The conventional GC/MS method to quantify free phytosterols in plant leaves usually involves extracting the dry plant material with organic solvent, isolating the lipid fraction of the extract, and derivatizing free sterols before GC injection. Prechromatography sample treatment procedures are lengthy and may generate contamination and other artifacts.

The one important step to analyze sterols with GC is to convert free sterols to their trimethylsilyl ethers (Schmarr et al., 1996; Dyers et al., 1995; Plank and Lorbeer, 1993) to increase the volatility. Without this derivatization step, free sterols with relatively low volatility are considered difficult for a regular capillary GC analysis. The high temperature and long retention time of the GC process may cause the free sterols to be dehydrated or even decomposed. To analyze free phytosterols without derivatization, dehydration or decomposition of sterols must be avoided. When a short capillary GC column is utilized instead of a regular length analytical column, retention times can be shortened for free sterols and the chances of dehydration can be reduced. Phytosterols may elute intact from a short capillary GC column and be analyzed subsequently. The biggest problem associated with the use of a short GC column is the poor GC resolution. Phytosterols cannot be resolved in short-column GC analysis. Another problem of using a short column is that the chemical interference is overwhelming when a complex mixture, such as a crude tobacco extract, is injected. Tandem mass spectrometry (MS/MS) is a powerful technique in natural material analysis (Biemann, 1993; Evershed, 1994; Gross, 1994). A highly selective scanning mode, selected reaction monitoring (SRM), detects a specific chemical while eliminating the interference of the chemical background (Buser and Mueller, 1994; Poletinni et al., 1993). A multiple SRM scanning can be set up to apply several SRMs to detect several compounds alternately in a single GC injection. Therefore, the interference of the chemical background is eliminated, and the unresolved chemicals can be detected individually. Thus, the poor resolution of using a short-column GC can be compensated.

In this paper, an analytical method is investigated to directly quantify free phytosterols in crude tobacco extracts without sample cleanup and derivatization. This method takes advantages of the short-column GC and the multiple SRM scanning of tandem mass spectrometry. Short-column GC shortens the retention time of sterols and reduces sterol dehydration or decomposition. The multiple SRM scanning compensates for the loss of resolving power of the GC due to the short capillary column and eliminates the interference of the chemical background of the crude tobacco extract. Three phytosterols (campesterol, β-sitosterol, and stigmasterol) in tobacco are quantified by directly injecting the crude tobacco extract into the GC/MS/MS system.

MATERIALS AND METHODS

Reagents and Tobacco Sample. β-Sitosterol (95% pure) was purchased from Sigma (St. Louis, MO). Stigmasterol (95% pure) was purchased from Aldrich Chemical (Milwaukee, WI). Both of them were used as standard materials without further purification. Campesterol with a relatively high purity was not available. Cholestane (97.8% pure), obtained from Sigma, was used as the internal standard. GC/MS grade dichloromethane was purchased from Fisher Chemicals (Fair Lawn, NJ). Two tobacco samples of 1994 crop (cultivar TR Madole) were obtained from the University of Tennessee Experimental Station in Springfield, TN. One of them was air-cured, and the other was fire-cured. Both tobacco samples were ground, and a 2 mm screen was used to collect the ground tobacco.

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A stock solution of β-sitosterol and stigmasterol was prepared with dichloromethane as the solvent. Five calibration solutions were prepared with the stock solution. Concentrations of β-sitosterol in the calibration solutions were 7.4, 14.8, 29.6, 59.2, and 118 μg/mL. Concentrations of stigmasterol were 10.0, 20.0, 40.0, 80.0, and 160 μg/mL. In each of the calibration solutions, cholestane was added as the internal standard at a concentration of 30 μg/mL. Calibration was carried out by injecting the calibration solutions serially, beginning with the solution of the lowest concentration and then reversing the calibration, continuing with the highest standard followed by decreasing concentrations. The average of relative integrated intensities was plotted against the injected amount as the calibration curve.

Tobacco extracts were prepared by adding cholestane (300 μg) to 1.00 g of ground tobacco in a 20 mL capped vial. Ten milliliters of dichloromethane was added to the vial. The vial was sealed and put in an ultrasonic bath (Model 2210 from Branson, Danbury, CT) and sonicated for 1 h at 35 °C. The dichloromethane extract was filtered through a 45 μm nylon filter and ready for GC injection. β-Sitosterol- and stigmasterol-spiked tobacco samples were prepared by adding the standard stock solution of β-sitosterol and stigmasterol to 1.00 g of ground fire-cured tobacco along with cholestane (300 μg). Dichloromethane (10.0 mL) was added to the spiked tobacco, and the vial was sealed and sonicated for 1 h at 35 °C in the ultrasonic bath. The dichloromethane extract was filtered with a nylon filter prior to GC injection. The spiked amounts were 26, 39, and 52 μg/mL for β-sitosterol, respectively, and 40, 60, and 80 μg/mL for stigmasterol, respectively.

Instruments and GC/MS/MS Analysis. A Hewlett-Packard (Palo Alto, CA) 5890 GC was interfaced with a Finnigan MAT (San Jose, CA) TSQ 700 triple-quadrupole mass spectrometer. The analytical column was a piece of 10 m long capillary column cut from a 30 m × 0.25 mm DB-5 column with 0.25 μm film from J&W Scientific (Folsom, CA).

GC injection port temperature was 250 °C. The helium carrier gas flow rate was 2.0 mL/min. The temperature of the transfer line was 250 °C. The initial oven temperature was 100 °C and was held for 1 min. The oven was then heated to 300 °C at a heating rate of 10 °C/min and was held at that temperature for 10 min. A 1 μL sample was injected in the splitless mode.

The SRM scan for phytosterols and cholestane was set as follows. The molecular ion was selected with the first quadrupole of the triple-quadrupole mass spectrometer and then collided with argon molecules in the second quadrupole. A fragment ion of the collision was selected with the third quadrupole and then detected. The fragment ion of collision was chosen to give a high detection sensitivity and a unique selectivity for the compound of interest. Cholestane had a shorter retention time than the three phytosterols. After a GC injection, the SRM scanning mode of the mass spectrometer was set to detect cholestane, the internal standard, first. The SRM scan for cholestane was set to use the first quadrupole to select m/z 372^+, the molecular ion of cholestane, and the third quadrupole to select m/z 217^+, a fragment ion of the molecular ion after its collision with argon gas. After the retention time passed 14.5 min, a multiple SRM scanning mode was applied to detect three phytosterols simultaneously. The multiple SRM was set to scan β-sitosterol (from m/z 414^+, the molecular ion, to m/z 329^+, a fragment ion of molecular ion after collision with argon gas), campesterol (from m/z 400^+ to m/z 315^+), and stigmasterol (from m/z 412^+ to m/z 379^+) alternately. The scanning time for each one of the three sterols was 0.5 s. Thus, the multiple SRM scanning resolution for each one of the sterols was 1.5 s. During the SRM scanning, the argon gas pressure in the second quadrupole was 0.3 mTorr and the collision energy was set at 25 eV. The MS total ion current was filtered into three separate traces corresponding to the individual SRM scanning for the three phytosterols. Intensities of the three separated traces were integrated for quantitative calculations.

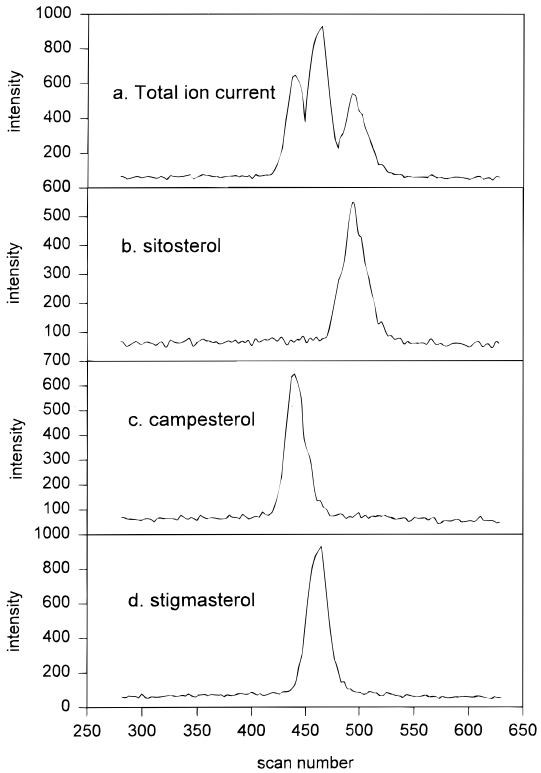

Figure 1. Short-column GC/MS/MS chromatogram of fire-cured tobacco extract: (a) total ion current, three phytosterols were not resolved; (b) trace filtered for β-sitosterol (from m/z 414^+ to m/z 329^+); (c) campesterol (from m/z 400^+ to m/z 315^+); and (d) stigmasterol (from m/z 412^+ to m/z 379^+).

RESULTS AND DISCUSSION

Underivatized phytosterols have relatively low volatility and are not suitable for direct capillary GC analysis. The chromatographic peaks of phytosterols are broad if a regular length GC column is used. Broad and tailing peaks indicate interactions of the analyte with the column materials. For sterols, dehydration or even decomposition may take place inside the GC column. It is difficult to determine the baseline of a broad tailing peak. Therefore, the integration for quantitative calculation is not applicable. As a short capillary GC column is used instead of a regular length one, the retention times for sterols are significantly reduced and chromatographic peaks are sharper. The resolution of the GC is tremendously reduced when the column is cut short. It is impossible to get baseline-resolved peaks for the three phytosterols (β-sitosterol, stigmasterol, and campesterol). The chromatogram of the three phytosterols in a tobacco extract when a 10 m capillary GC column was used and the multiple SRM scanning mode was applied for detection is presented in Figure 1a. It was obvious that the sterols were not well resolved. Since the highly selective SRM scan was used, the interference from background chemicals of crude tobacco extract was completely eliminated. Therefore, the baseline of the three overlapped peaks (three sterols) was flat, without significant tailings, and it was acceptable for quantitative purposes.

Since it was a multiple SRM scan, each one of the three phytosterols was scanned alternately every 1.5 s. Thus, the total ion current was filtered into three separate traces corresponding to the three sterols. Each of the three separate traces for β-sitosterol (Figure 1b), campesterol (Figure 1c), and stigmasterol (Figure 1d) showed good peak shapes with acceptable signal to noise

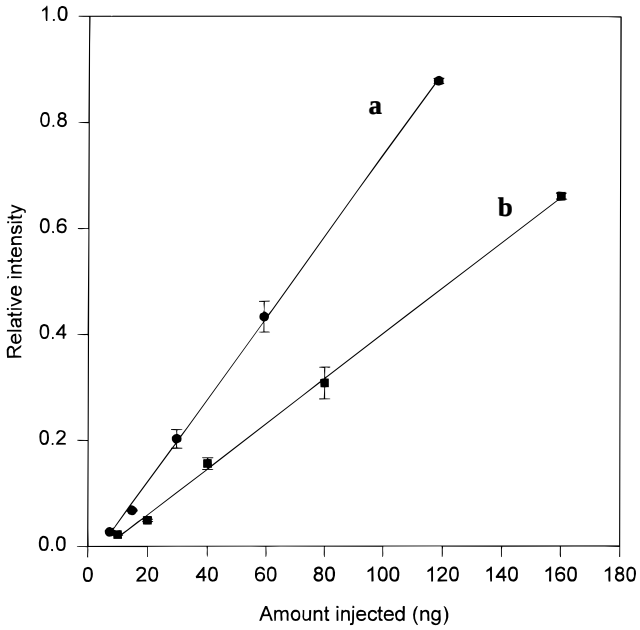

Figure 2. Linear response of the short-column GC/MS/MS method for (a) β-sitosterol and (b) stigmasterol. Cholesterol was used as the internal standard. The linear correlation coefficients were 0.9996 for β-sitosterol and 0.9994 for stigmasterol.

ratios (Figure 1b−d). The area of each peak could be easily integrated for each of the three sterols. Although the short-column GC did not resolve the three phytosterols, the multiple SRM scan of tandem mass spectrometry selectively detected each one of them in a single GC injection.

To test the combination of short-column GC with the multiple SRM mass spectrometric detection for the quantification of phytosterols, the linearity of the detected response toward the injection concentration was examined (Figure 2) as the calibration lines for β-sitosterol and stigmasterol. Cholestane was used as the internal standard during the calibration. Excellent linearity was observed for both sterols. The linear correlation coefficients were 0.9996 for β-sitosterol and 0.9994 for stigmasterol. The data plotted (Figure 2) were the average of two sets of measurements. One measurement started with the standard of lowest concentration and progressed to the highest standard by increasing concentrations. The other measurement was performed in a reverse order from the highest standard to the lowest one. The error bars are the ranges of the two measurements. Free phytosterols, without derivatization, had excellent linear responses at the injection range from 10 to about 200 ng in the GC/MS/MS analysis with a short GC column and the multiple SRM mass spectrometric detection.

To verify the reliability of the GC/MS/MS method, concentration differences of stigmasterol and β-sitosterol between the fire-cured tobacco spiked with these two sterols and unspiked tobacco were evaluated. The results and the recovery rates at three different spiking levels are listed in Table 1. In the table, the measured Δconcn was the difference of the sterol concentrations between the spiked tobacco and the unspiked tobacco samples. The spiked concentration was calculated from the amount of sterols added to the tobacco sample before extraction and the volume of the dichloromethane solvent used for extraction. Recovery rates for β-sitosterol were close to 100% for all three samples spiked with different levels of β-sitosterol. Also, for stigmasterol, recovery rates were close to 100% for tobacco samples spiked to 60 and 80 μg/mL stigmasterol. For the tobacco sample to which stigmasterol was spiked to 40 mg/mL, the recovery rate was 87%, which was low but still acceptable in quantitative analysis. The short-column GC/MS/MS method provides reliable results for the quantification of free sterols in plants without any sample treatments after organic solvent extraction.

Table 1. Recovery of β-Sitosterol and Stigmasterol Spiked in Fire-Cured TR Madole Tobacco

β-sitosterol	spiked concn (μg/mL)	0	26.0	39.0	52.0
	measured concn (μg/mL)	11.6	38.5	51.4	67.1
	Δconcn		26.9	39.8	55.5
	recovery rate (%)		103	102	107
stigmasterol	spiked concn (μg/mL)	0	40.0	60.0	80.0
	measured concn (μg/mL)	32.9	67.7	89.6	112
	Δconcn		34.8	56.7	79.1
	recovery rate (%)		87.0	94.5	98.9

Table 2. Free Phytosterols in TR Madole Tobacco Quantified with the Short-Column GC/MS/MS Method

	fire-cured TR Madole		air-cured TR Madole	
	concn (ppm)	%RSD ($n = 4$)	concn (ppm)	%RSD ($n = 4$)
β-sitosterol	140	7.4	246	8.0
stigmasterol	374	2.1	371	4.8
campesterol[a]	171	10	234	8.3
sum	685		851	

[a] Response factors for β-sitosterol were used for calculation.

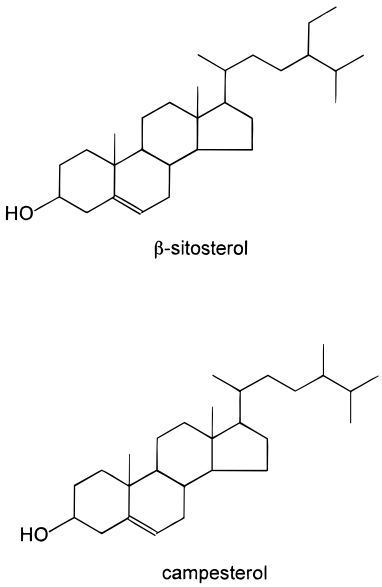

Figure 3. Structures of β-sitosterol and campesterol.

Table 2 lists the amount of β-sitosterol, stigmasterol, and campesterol in TR Madole tobacco quantified with the short-column GC/MS/MS method. Since campesterol with a relatively high purity was not available, the calibration for campesterol was not performed in this study. As is shown in Figure 3, the structure of campesterol is very close to that of β-sitosterol. The difference between them is that campesterol has a methyl group attached on the 24th carbon of the side chain of the cholesterol, while β-sitosterol has an ethyl group. The electron impact mass spectra of the two compounds are very close to each other except there is a difference of 14 amu for the corresponding fragment ions. The daughter ion spectra of molecular ions of these two sterols have the same fragmentation pattern. The most prominent fragment ion after collision with argon molecules is the $(M - 85)^+$ ion for both of them.

It is reasonable to assume that the mass spectrometric response factor of campesterol is similar to that of β-sitosterol. Therefore, campesterol can be quantified using the calibration line of β-sitosterol. The measured values of campesterol listed in Table 2 were calculated using the calibration for β-sitosterol.

The amount of phytosterols in tobacco varies with tobacco cultivar (Cheng et al., 1968; Davis et al., 1970) and cultural practices (Grunwald et al., 1971). The amount of phytosterols reported in the literature is usually the total sterols that contain the free sterols and the sterols hydrolyzed from their glucosides. There are several literature publications giving sterol contents as the percentage of the dry weight of the tobacco samples (Rao et al., 1988; Ellington et al., 1978; Keller et al., 1969). The total campesterol level and β-sitosterol level are both in the range from 200 to 600 ppm. The total stigmasterol level is in the range from 300 to 800 ppm. In this study, the tobacco sample (TR Madole) is a cultivar of dark tobacco. No report on the lipid composition of this cultivar was found during the literature search. The results listed in Table 2 are only free forms of sterols in tobacco. Since hydrolysis is not happening during the organic solvent extraction and there is no other sample treatment, sterols in their glucosides are not counted in the measured values listed in Table 2. The measured concentrations of free campesterol (234 ppm), β-sitosterol (246 ppm), and stigmasterol (371 ppm) in air-cured tobacco samples all fall in the ranges for different cultivars of tobacco reported in the literature. The composition pattern of the three free phytosterols is consistent with the literature results for total phytosterols in tobacco (Cheng and Sheen, 1972; Ellington et al., 1978).

Also in Table 2, the relative standard deviations (%RSD) of the measurements are listed. They are all within 10% for four measurements over two tobacco samples. This indicates that the short-column GC/MS/MS method detailed here provides reproducible results for free phytosterols in plant materials.

In conclusion, free phytosterols in tobacco can be quickly quantified after being extracted with organic solvent. A short GC column interfaced with a tandem mass spectrometer provides reliable and reproducible quantitative results for underivatized phytosterols in complex mixtures of crude tobacco extracts. All of the tedious preinjection sample treatments, such as cleanup and derivatization, are unnecessary. Analysis time is saved and the chances of contamination are reduced compared to conventional GC/MS quantification analysis.

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