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A method for simultaneous analysis of phytosterols and phytosterol esters in tobacco leaves using non aqueous reversed phase chromatography and atmospheric pressure chemical ionization mass spectrometry detector

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ABSTRACT

While numerous analytical methods for phytosterols have been reported, the similar polarity and large molecules of phytosterol esters have made the methods lengthy and complicated. For this reason, an analytical method that could completely separate phytosterol esters including the higher fatty acids such as palmitic acid, stearic acid, oleic acid, linoleic acid and linolenic acid in addition to phytosterols without preliminary separation was developed. The separation was accomplished by non-aqueous reversed phase chromatography technique using only acetone and acetonitrile. An atmospheric pressure chemical ionization/mass spectrometry detector configured at selected ion monitoring mode was hyphenated with the separation system to detect phytosterols and phytosterol esters. Twenty-four types of these were consequently separated and then identified with their authentic components. The calibration curve was drawn in the range of about 5 to 25,000 ng/mL with a regression coefficient over 0.999. The limit of detection and limit of quantification, respectively, ranged from 0.9 to 3.0 ng/mL and from 3.0 to 11.0 ng/mL. Recovery rates ranged from 80 to 120%. The quantification results were subjected to statistical analysis and hierarchical clustering analysis, and were used to determine the differences in the amounts of phytosterols and phytosterol esters across tobacco leaves. The newly developed method succeeded in clarifying the whole composition of phytosterols and phytosterol esters in tobacco leaves and in explaining compositional differences across the variety of tobacco leaves.

1. Introduction

Phytosterols (Fig. 1) are major components observed in plants, particularly in the cell membrane and in seed oil, and are known to provide various physiological functions [1]. They consist of a tetracyclic structure generated from 2,3-epoxy squalene and including "28 or 29 carbons and one or two carbon–carbon double bonds, typically one in the sterol nucleus and sometimes a second in the alkyl side chain" [2]. While these structural varieties enable phytosterols to play numerous roles in plants, each structure is rigidly recognized inside the organism and utilized for each physiological function [3]. A representative example of structural recognition of phytosterols is seen on the recently prevalent phytosterol enriched food such as oil, mayonnaise and margarine. They are recommended to inhibit intestinal cholesterol absorptions and to reduce plasma cholesterol concentrations due to the structural difference from cholesterol [4]. In addition to structural diversities, the composition of phytosterols varies in plants, and therefore becomes the crucial index and the analytical target for qualitative discrimination [1,5]. These analytical studies have been extended not only to phytosterols but also to phytosterol derivatives such as phytosterol esters (Fig. 1) [6–8], phytosterol glycosides [9] and phytosterol oxides [10].

Tobacco leaf, *Nicotiana tabacum*, is known to be a source of numerous types of components such as cembranoids, labdanoids, terpenoids, carotenoids, hydrocarbons, fatty acids, glycosides, alkaloids, polyphenols, amino acids, sugars and organic acids [11]. Many analytical studies concerning these components have been published in order to elucidate the differences across growing districts, leaf positions and ripening and curing processes that highly affect the quality of tobacco leaves [12]. In particular, the resin components in tobacco leaf such as cembraonoids and terpenoids have become more focused on due to their relation with the taste and aroma of cigarettes [13–15]. However, little attention has been

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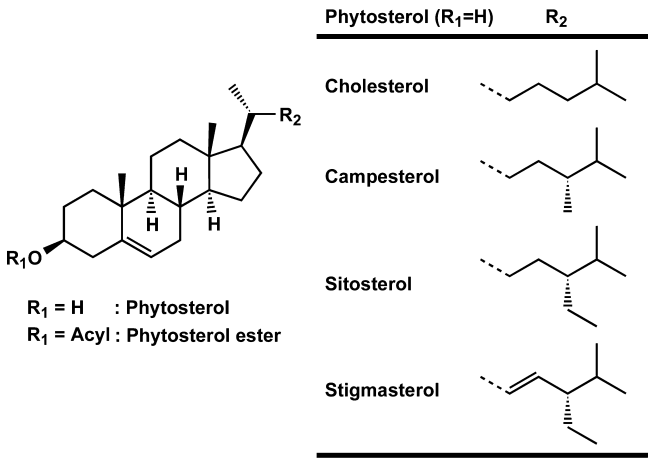

Fig. 1. Structure of phytosterols consisting of a tetracyclic structure and an alkyl group. Phytosterol esters are the esterified phytosterol at the hydroxyl group (R_1). Phytosterol includes an alkyl group with the various numbers of carbon atoms and double bonds (R_2).

paid to the whole composition of phytosterols and phytosterol esters contained in the resin of tobacco leaves. Although a few previous reports refer to how significant simple analysis of phytosterols and phytosterol esters was [16,17], the details remain unclear mainly due to the lack of a feasible analytical method. Considering these backgrounds, the objective of this research is to develop a simultaneous yet simple analytical method for phytosterols and phytosterol esters in the resin of tobacco leaves, to clarify the compositional differences across various tobacco leaves and to explain how different these components are across tobacco leaves.

Numerous analytical methods for phytosterols have been reported, using gas chromatography (GC) coupled with flame ionization detector (FID) or mass spectrometry detector (MSD) and, as other combinations, liquid chromatography (LC) coupled with photodiode array detector (PDAD), refractive index detector (RID), electrospray ionization mass spectrometry detector (ESI-MSD) and atmospheric pressure chemical ionization mass spectrometry detector (APCI–MSD). These combinations were summarized in the previous review [18]. On the other hand, the analytical method for phytosterol esters has not advanced as much as for phytosterols due to their structural similarities causing insufficient separation and the large molecular weight limiting usable instruments. The previous methods therefore incorporated the following techniques into the whole analytical system for phytosterol esters or remained partial separation of them: preliminary separation such as thin layer chromatography to isolate steryl ester fraction from total lipid followed by GC-based analysis [19], flash chromatography to separate free phytosterol and phytosterol esters followed by GC-based analysis for phytosterol esters [20] or solid phase extraction used to separate phytosterols, phytosteryl conjugates and matrix constituents, followed by a GC-based separation of the SPE-fractions which gave complete separation of phytosteryl as well as of phytostanyl fatty acid esters except for coelutions of the esters of saturated and monounsaturated fatty acids [21,22], separation with mass filter to characterize phytosterol esters using GG by GC-based separation [23], or partial separation of phytosterol esters giving coelutions of the esters of saturated and monounsaturated fatty acids by LC [24]or GC–LC [25] without preliminary separation. The main separation parts for phytosterol esters have been classified into GC-based and LC based chromatography. GC-based technique has provided the great reproducibility and, thus has been frequently used for the quantification of hydrolyzed phytosterols. However, neither could apolar column of GC separate the esters of saturated fatty acids from monounsaturated ones, nor could polar column separate them as previously mentioned [4]. LC-based technique on normal phase (NP) chromatography has been used a lot for the separation of the whole phytosterol esters fraction from lipid matrix largely due to its effectiveness in differentiating the component polarity [4] and has not been used as a main separation part. Similarly, reversed phase (RP) chromatography has still remained the insufficient separation of phytosterol esters, even though it has the capability of differentiating the components consisting of different carbon numbers [20]. The approach enabling the detection of large molecules including the hydrolysis of ester counted for the following techniques: hydrolysis of phytosterol esters for detection on GC/FID or GC/MSD [6–8,16], use of high temperature GC (HTGC)/FID [23], or use of LC/APCI–MSD or capillary electrophoresis chromatography (CEC) hyphenated with DAD caring thermally liable components [26]. As a whole, to the author's knowledge, complete separation of phytosterol esters including the higher fatty acids such as palmitic acid (C16), stearic acid (C18), oleic acid (C18:1), linoleic acid (C18:2) and linolenic acid (C18:3) in addition to free phytosterols without preliminary separation has not been achieved.

In this research, non-aqueous reversed-phase chromatography (NARPC) [27] was selected to separate phytosterols and phytosterol esters, because this technique has accomplished clear separation of solanesol and solanesyl esters [28], chlorophyll metabolites [29] and triacylglycerols [30], which are also known as low polar non-volatile components. The detection was achieved through APCI–MSD and its selected ion monitoring mode (SIM) improving peak purity and signal-to-noise ratio. The previous research using reversed phase chromatography hyphenated with APCI–MSD [24] has already taken the similar approach to analyze phytosterol esters, yet it provided the coelutions of esters due to the use of reversed phase chromatography including water as eluent. The application of NARPC was therefore expected to improve the coelution. In the first part of this study, the separation and identification of phytosterols and phytosterol esters in tobacco leaves are investigated. The newly developed method for quantification is then validated by linearity of the calibration curve, limit of detection (LOD), limit of quantification (LOQ) and recovery rates for all phytosterols and phytosterol esters. The last part provides the quantification results for each phytosterol and phytosterol ester in various tobacco leaves and the overview of compositional difference across tobacco leaves based on the result of statistical analysis (principal component analysis (PCA)) and hierarchical clustering analysis.

2. Experimental

2.1. Materials

Tobacco leaves, *N. tabacum* (Flue-cured Virginia (FCV) in Indonesia, Spain, China, America, Thailand, Zimbabwe, Argentine, Malawi, Tanzania, Brazil and Japan harvested in 2005 to 2012, Burley (BLY) in Philippine, Brazil, Malawi, Zimbabwe, Italy, Serbia, Thailand, India, America and Japan harvested in 2004 to 2012, Oriental (ORI) in Greek, Turkey, Macedonia, Albania, Thailand, China and Bulgaria harvested in 2005 to 2012, Dark-air cured (DAC) in Philippine, Brazil and India harvested in 2004 to 2006, Dark-fire cured (DFC) in Tanzania harvested in 2007, Sun-air cured (SAC) in India harvested in 2004 to 2006 and stem in Brazil, Philippine and Japan harvested in 2006 to 2010) and *Nicotiana rustica* (Rustica) in India harvested in 2004 to 2006 were stored in a warehouse of Japan Tobacco Inc. and used for the analysis of phytosterols and phytosterol esters. Solvents for extraction and liquid chromatography, all of HPLC grade, were purchased from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan).