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Original Research Article

Quantification of plant sterols/stanols in foods and dietary supplements containing added phytosterols

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ABSTRACT

Plant sterols and plant stanols, collectively referred to as phytosterols, are currently added to conventional foods and dietary supplements for the purpose of reducing the risk of coronary heart disease (CHD). The objective of the present study was to validate a method for the determination of the content and composition of plant sterols/stanols in foods and dietary supplements containing added phytosterols. Chromatographic conditions with this method permitted the near-baseline resolution of the five major phytosterols (campesterol, campestanol, stigmasterol, β-sitosterol, sitostanol) that are the subject of the United States Food and Drug Administration's (FDA) health claim on the relationship between phytosterols and reduced risk of CHD. Analyzed samples ($n = 25$) showed total phytosterol contents that varied from 0.2 to 55.2 g/100 g, or 0.02 to 2.3 g/serving. The mean total phytosterol content was 105 ± 14% of label declarations (range: 83–137%). Thirteen (13) products (52%) carried the FDA's health claim. This work is the first to evaluate the content and composition of phytosterols from the wide variety of products containing added phytosterols currently available in the United States and their use of the FDA's health claim for phytosterols and reduced risk of CHD.

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1. Introduction

Q3 Plant sterols and plant stanols, collectively referred to as phytosterols, are natural constituents of plant-based foods which have been shown to lower serum total and low density lipoprotein cholesterol when added to the diets of humans (Demonty et al., 2009; Laitinen and Gylling, 2012; Gylling et al., 2014). Over 100 different phytosterols have been identified, of which the most common include campesterol, β-sitosterol, stigmasterol, brassicasterol, and Δ5-avenasterol (Moreau et al., 2002). Plant stanols (e.g., campestanol, sitostanol) are found in some unhydrogenated vegetable oils and cereal grain lipids (Ostlund, 2002) or they may be synthesized from plant sterols by desaturation of the double bond at the Δ5 position (Carr et al., 2010). Foods naturally rich in phytosterols include vegetable oils, legumes, nuts, seeds, and whole grains (Moreau et al., 2002; Phillips et al., 2002, 2005; Robbins et al., 2011). The phytosterol contents of these and many other foods are available in the United States Department of

Abbreviations: BSTFA, *N,O*-bis(trimethylsilyl)trifluoroacetamide; CHD, coronary heart disease; FDA, United States Food and Drug Administration; FID, flame ionization detector; IS, internal standard; NIST, National Institute of Standards and Technology; PTFE, polytetrafluoroethylene; SRM, Standard Reference Material; TCF, theoretical correction factor; TMCS, trimethylchlorosilane; TMSE, trimethylsilyl ether.

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Agriculture's (USDA) National Nutrient Database for Standard Reference (USDA, 2011), as well as other national and international databases (Jimenez-Escrig et al., 2006; Normen et al., 2007; Plumb et al., 2011). Because of the well-documented cholesterol-lowering effect of phytosterols, these compounds are added to a wide variety of conventional foods and dietary supplements for the purpose of reducing risk of CHD (Devaraj and Jialal, 2006; Carr et al., 2010; Choudhary and Tran, 2011).

In 2000, in response to two health claim petitions, FDA issued an interim final rule authorizing health claims on the relationship between plant sterol/stanol esters and reduced risk of CHD (65 FR 54686). This action, which was codified in 21 CFR 101.83, required that products carrying the health claim declare, on their labels, contents of total phytosterols in a single serving. In addition, the plant sterol and stanol ester mixtures that are the subject of the health claim were required to contain specified amounts of the five major phytosterols (i.e., campesterol, campestanol, stigmasterol, β-sitosterol, and sitostanol; Fig. 1) for which beneficial health effects have been reported (Weststrate and Meijer, 1998; Hallikainen et al., 2000; Jones et al., 2000; Vanstone et al., 2002). According to the interim final rule (65 FR 54686), plant sterol ester mixtures were to be composed of a minimum 80% combined weight of campesterol, stigmasterol, and/or β-sitosterol, whereas for plant stanol ester mixtures, 80% of the combined weight was to be composed of campestanol and/or sitostanol. In 2010 in response to a health claim petition submitted by Unilever United States, Inc., FDA proposed to amend its health claim regulation based on evidence previously considered and on data that were published since the agency first authorized the health claim (75 FR 76526). The proposed rule would amend, among other provisions, the authorized use of the claim by modifying the nature of the substances to include nonesterified (i.e., free) phytosterols for conventional foods, would modify the daily dietary intake of the substance specified in the claim as necessary for the claimed benefit by adjusting the minimum amount of total phytosterols required for a food to bear the claim, and would expand the types of products that may carry the claim to include a broader range of foods. The proposed rule would also remove the distinction between sterols and stanols to require that the content of total phytosterols be comprised of at least 80% of the combined weight of the five major phytosterols (75 FR 76526).

Analytical methods used to measure phytosterols most commonly involve a saponification procedure followed by gas chromatographic (GC) separation of phytosterol trimethylsilyl ether (TMSE) derivatives (Winkler-Moser, 2011). Currently there is no AOAC Official Method for phytosterols in foods. FDA considered several methods before determining in its proposed rule (75 FR 76526) that the most appropriate method to date for quantification of total phytosterols was that of Sorenson and Sullivan (2006), which meets performance criteria for an AOAC Single Laboratory Validation study and is a modification of AOAC Official Method 994.10 (AOAC, 2000). However, the method of Sorenson and Sullivan (2006) was not validated for quantification of plant stanols, nor was it applied to the range of total phytosterol contents observed in foods and dietary supplements containing added phytosterols (i.e., >1% of total weight). The methods of Laakso (2005) and Clement et al. (2010) have been validated for the analysis of a wide variety of foods containing added phytosterols. However, neither of these methods meets performance criteria suitable for accurate quantification of the five major phytosterols that are the subject of the FDA's health claim, nor have they been applied to the analysis of dietary supplements.

Therefore, the objective of the present study was to validate a method for the determination of the content and composition of plant sterols/stanols, including the five major phytosterols that are the subject of the FDA's health claim, in foods and dietary supplements containing added phytosterols. This method, which uses epicoprostanol (cholestan-3-ol, (3α,5β)) as an internal standard, involves the saponification of homogenized samples followed by liquid–liquid extraction of unsaponifiable material and GC separation of phytosterol TMSE derivatives. In addition, several minor method modifications may be applied (e.g., acid hydrolysis treatment, modifications for dietary supplements) to expand the range of product matrices and applicable range of total phytosterol contents that may be analyzed. This method meets requirements for a Level Two single laboratory validation according to the FDA's Office of Foods' *Guidelines for the Validation of Chemical Methods for the FDA Foods Program* and is appropriate for the determination of the content and composition of phytosterols in the wide variety of foods (e.g., vegetable spreads/margarines, liquid and powdered beverages, baked goods) and dietary supplements containing added phytosterols that are currently available in the US.

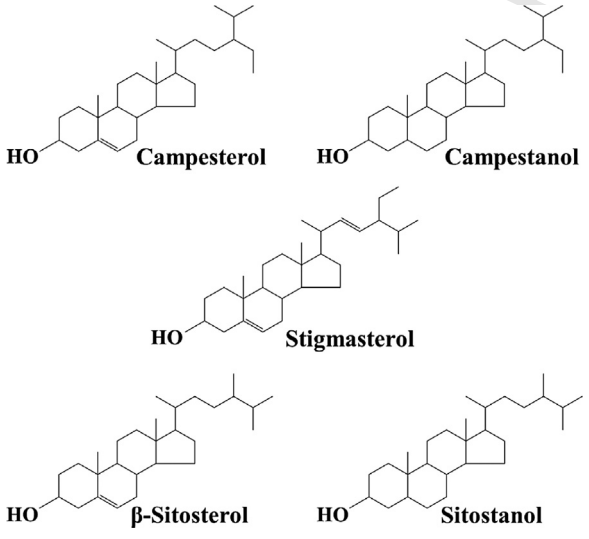

Fig. 1. Chemical structures for the five phytosterols that are the subject of the FDA's health claim on the relationship between phytosterols and reduced risk of CHD.

2. Materials and methods

2.1. Reagents and standards

ACS reagent grade chemicals (*N*,*O*-bis(trimethylsilyl)trifluoroacetamide, BSTFA, containing 1% trimethylchlorosilane, TMCS; chloroform; ethyl ether, anhydrous, stabilized with butylated hydroxytoluene, ≥99%; hydrochloric acid, HCl, 37%; petroleum ether, 35–60 °C boiling point; potassium hydroxide, KOH, pellets, ≥85%; sodium sulfate, Na_2SO_4, anhydrous, granular, ≥99%), HPLC grade solvents (acetone, 99.7%; heptane, 99.5%), and pyridine (anhydrous, 99.8%) were purchased from Fisher Scientific (Pittsburgh, PA, USA) or Sigma–Aldrich (St. Louis, MO, USA). Ethanol (200 proof) was purchased from VWR International (Radnor, PA, USA).

The internal standard, epicoprostanol (cholestan-3-ol, (3α,5β)), was purchased from Steraloids (Newport, RI, USA; ≥98%) and Sigma–Aldrich (≥95%). GC calibration standards were purchased from ChromaDex (Irvine, CA, USA; campesterol, 91.4–96.6%), Sigma–Aldrich (stigmasterol, ~95%), Avanti Polar Lipids (Alabaster, AL, USA; β-sitosterol, ≥99%), and Matreya (Pleasant Gap, PA, USA; sitostanol, 97–98%). Cholesteryl stearate (≥98%) was purchased from Steraloids. The phytosterol reference standard, phytosterols (mixture of soya sterols; β-sitosterol, 95–100%; P/N P18680), was purchased from Pfaltz & Bauer (Waterbury, CT, USA).

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