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Analytical Methods

Direct saponification preparation and analysis of free and conjugated phytosterols in sugarcane (*Saccharum officinarum* L.) by reversed-phase high-performance liquid chromatography

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ABSTRACT

A simple method based on direct saponification followed by RP-HPLC analysis was developed for quantification of free and conjugated sterols in sugarcane. Acid hydrolysis prior to alkaline saponification was used to determined acylated steryl glycoside and steryl glycoside in sugarcane. The applicability and generality of this method were improved with intensive investigation. Compared to traditional solvent extraction method, this method was more time saving and appropriate for characterization of sterol fractions in sugarcane. This method was successfully applied for determination of free and conjugated sterols in different sugarcane samples. The results exhibited that stigmasterol (varied from 883.3 ± 23.5 to 1823.9 ± 24.5 μg/g dry weigh) and β-sitosterol (varied from 117.6 ± 19.9 to 801.4 ± 33.5 μg/g dry weight) were major phytosterols in the sugarcane sample, and their glycosylated forms accounted for almost 87.0% of stigmasterol and 87.5% of β-sitosterol in sugarcane, respectively. In addition, among other parts of sugarcane, tips contained the greatest amount of phytosterols.

1. Introduction

Phytosterols (plant sterols) recently have received increased attention due to their capability to inhibit cholesterol intestinal absorption, resulting in lowering serum total plasma cholesterol and low-density lipoprotein levels (Klingberg et al., 2012; Plat & Mensink, 2001). In all plant tissues, phytosterols exist in four common forms: the free sterol (FSs) and conjugated sterols including steryl esters (SEs), steryl glycosides (SGs) and acylated steryl glycosides (ASGs) (Breinholder, Mosca, & Lindner, 2002; Moreau, Whitaker, & Hicks, 2002). Many of the commonly applied methods for the analysis of phytosterols in plants are based on procedures for cholesterol analysis (Mattila, Lampi, Ronkainen, Toivo, & Piironen, 2002; Orozco-Solano, Ruiz-Jimenez, & de Castro, 2010), which hydrolyzes the conjugated sterols to free sterols before analysis. However, as cholesterol is commonly not found as a glycoside, these procedures do not include acid hydrolysis, therefore the glycosylated sterols are not included in the analysis of the total sterol content. Therefore, it is problematic to quantitate the total plant sterols content of all four common forms (FSs, SEs, SGs and ASGs) in plant tissues.

Currently, the most common methods for analysis of phytosterols usually follow the pattern of extraction of the lipid fraction, alkaline saponification, derivatization of sterols, chromatographic separation and quantification (Abidi, 2001). SGs and ASGs are always overlooked, because polar conjugated sterols are not soluble in non-polar lipid phase and may not be included in the direct lipid extracts (Moreau et al., 2002). On the other hand, acetal bond between the sterol hydroxyl group and the sugar cannot be hydrolyzed in alkaline conditions (Toivo, Lampi, Aalto, & Piironen, 2000). Acid hydrolysis has been used to determine the composition of ASGs and SGs, as acid hydrolysis can cleave the glycosidic bond (Aguirre, Ruiz-Mendez, Velasco, & Dobarganes, 2012; Piironen, Toivo, & Lampi, 2002). As solvent extraction method fails to quantify SGs and ASGs, direct acid and alkaline hydrolysis is used to analyze the sterol content in oil and food matrices because of its distinct advantages to traditional method (Jonker et al., 1985; Lagarda, Garcia-Llatas, & Farre, 2006). Gas chromatography (GC) (Derewiaka, Szwed, & Wolosiak, 2014; Garcia-Llatas, Vidal, Cilla, Barbera, & Lagarda, 2012; Zhao, Shen, Chang, & Kim, 2013) and high performance liquid chromatography (HPLC) (Baila-Rueda et al., 2013; Liu & Ruan, 2013; Zhang et al., 2014) in combination

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with different detection systems has been used to identify and quantify the major phytosterols in foods. GC is the most frequently used technique for the analysis of phytosterols. However, it has several drawbacks, such as complex sample preparation procedures, a high temperature of operation and destructive detection techniques (Lagarda et al., 2006; Slavin & Yu, 2012). HPLC has also been used in both analytical and preparative scale for the analysis of sterols (Abidi, 2001). To our knowledge, valid reversed-phase-HPLC method for analyses phytosterols in sugarcane is limited in the literature.

Sugarcane (*Saccharum officinarum* L.), one of the most important economic plants, is considered as a key commodity in many regions and countries. Previous studies on sugarcane and sugarcane industry by-products demonstrate that sugarcane is a promising potential source of phytosterols, such as β-sitosterol, stigmasterol and other minor steroids (Georges, Sylvestre, Ruegger, & Bourgeois, 2006; Nuissier, Bourgeois, Fahrasmane, & Grignon-Dubois, 2008). Glycosylated sterols are also potential health benefiting dietary components that can reduce cholesterol absorption as efficiently as SE (Lin, Ma, Moreau, & Ostlund, 2011; Lin, Ma, Racette, Spearie, & Ostlund, 2009). Literatures on the occurrence, contents, and sterol composition of ASGs and SGs in plant foods are still limited. SGs and ASGs indeed are an important group of sterols in plants. For example, they contributed up to 60% of total sterols in some potato cultivars (Nystrom, Schar, & Lampi, 2012). Exclusion of ASGs and SGs can cause an underestimation of 37% in the content of total sterol content in nuts and seeds (Phillips, Ruggio, & Ashraf-Khorassani, 2005). It is of great importance to investigate the precise sterol content of sugarcane, especially the SGs and ASGs content. However, to the best of our knowledge, little information is available regarding the phytosterol content and composition of FSs, SEs, SGs and ASGs in sugarcane.

The aim of the present study is to develop a reliable sample preparation and RP-HPLC measurement method of total sterol concentrations including FSs, SEs, SGs and ASGs for individual sterols in sugarcane. The sterol content and composition of different parts (rind, pith, node, tip) of two sugarcane cultivars are also determined according to the method developed.

2. Materials and methods

2.1. Chemicals

Phytosterol standards 6-ketocholestanol, brassicasterol, campesterol, stigmasterol, β-sitosterol, cholesterol and ergosterol were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All the other chemicals used were of HPLC grade or analytical grade.

2.2. Sugarcane samples

Two sugarcane cultivars of Badila (red-rind) and Yuetang 54–474 (green-rind) were collected at commercial maturity from Zhejiang, PR China. Four parts of two sugarcane cultivars namely rind (RR), pith (PR), node (NR), tip (TR) of red-rind sugarcane and rind (RG), pith (PG), node (NG), tip (TG) of green-rind sugarcane were air dried at 30 °C for about 18 h until constant weight was achieved, smashed into powder with a pulverizer (Huangchen HC-280T, Zhejiang, China), passed through a 60-mesh sieve and kept dry at −18 °C until use. All results were expressed by dry weight (DW).

2.3. Preparation of standard solution

Stocks solutions containing 1 mg/ml of sterols were prepared in HPLC-grade methanol and stored in the dark at −5 °C. Assessed by spectrophotometric assays, the stock solutions were stable for at least 1 month when stored in the dark at −5 °C. Working standard solutions of phytosterol were prepared by diluting the standard stock solution to 5, 10, 20, 40, 80 μg/ml. 6-ketocholestanol was used as the internal standard (IS) (Lu, Ren, Zhang, & Gong, 2009) and was added into each calibration solution at the concentration of 10 μg/ml.

2.4. High-performance liquid chromatography conditions

Chromatography was carried out using LC-2010 (Japan AT) system with an ultraviolet–visible (UV) detector. Separation was performed on a Luna 5 μm C-18 column (250 mm × 4.6 mm i.d., Phenomenex Ltd.) with mobile phases: methanol and acetonitrile in a ratio 80:20 (by volume). The chromatographic conditions were isocratic and the duration of the HPLC run was 30 min. Injection volume was 20 μl, flow rate 1.2 ml/min and detection wavelength 210 nm. Column temperature was maintained at 30 °C. Quantification was based on internal calibration and the final concentrations were expressed as μg/g DW.

2.5. Analytical procedures

2.5.1. Determination of FSs

For the free phytosterols' determination (Fig. 1), the ground sugarcane sample (1.0 g by adding 120 μg 6-ketocholestanol) was placed into conical flask, soaked with 10 ml anhydrous ethanol, placed on a vibrator and extracted for 4 h at 130 rpm at 30 °C. Then, the ethanol extract was filtered, evaporated to dryness using rotatory evaporation, redissolved in 10 ml of absolute methanol and filtered with 0.45 μm membrane filters before chromatography analysis.

2.5.2. Determination of FSs and SEs

The ground sugarcane sample (0.5 g by adding 120 μg 6-ketocholestanol) was accurately weighed into a 50-ml round-bottom flask and was mixed with 5 ml 4 mol/l ethanolic KOH solution. The sample was refluxed at constant temperature 70 °C for 1 h.

The unsaponifiable matter was extracted twice with 10 ml of diethyl ether and the diethyl ether phase was collected. The diethyl ether extracts were washed with 10 ml water twice and dried with

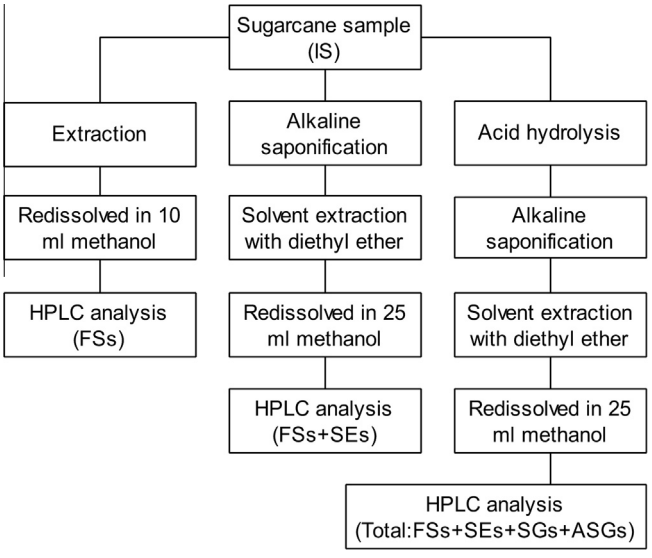

Fig. 1. Main steps of the analytical procedures. IS, internal standard (6-ketocholestanol); FSs, free phytosterols; SEs, steryl esters; SGs, teryl glycosides; ASGs, acylated steryl glycosides.