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A UHPLC method for the rapid separation and quantification of phytosterols using tandem UV/Charged aerosol detection – A comparison of both detection techniques

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ABSTRACT

The presented work describes the development and validation of a rapid UHPLC-UV/CAD method using a core–shell particle column for the separation and quantitative analysis of seven plant sterols and stanols. The phytosterols (ergosterol, brassicasterol, campesterol, fucosterol, stigmasterol, and β-sitosterol) and the phytostanol stigmastanol were separated and analyzed in 8.5 min. The sample pre-treatment procedure was optimized to be less time-consuming than any other published method, especially due to no need of derivatization, evaporation and even reconstitution step. The chromatographic separation was performed on the Kinetex 1.7 μ Phenyl-hexyl column (100 × 2.1 mm) with a mobile phase acetonitrile/water according to the gradient program at a flow rate of 0.9 mL min^{-1} and a temperature of 60 °C. A tandem connection of PDA and CAD (Corona Charged Aerosol Detector) was used and both detection techniques were compared. The method was validated using saponification as a first step in sample pre-treatment and an universal CAD as the detector. Recoveries for all analyzed compounds were between 95.4% and 103.4% and relative standard deviation ranged from 1.0% to 5.8% for within-day and from 1.4% to 6.7% for between-day repeatability. The limits of detection were in the range of 0.4–0.6 μg mL^{-1} for standard solutions and 0.3–1.2 μg mL^{-1} for phytosterols in real samples. Although several gradient programs and different stationary phases were tested, two compounds, campesterol and campestanol, were not separated. Their peak was quantified as a sum of both analytes.

1. Introduction

Phytosterols, phytostanols (plant sterols and stanols) and their esters are natural steroids that are important structural components of plant membranes, and contribute to the regulation of the permeability of cell membranes. Most phytosterols contain 28 or 29 carbons and one or two carbon–carbon double bonds. Phytostanols are a fully saturated subgroup of phytosterols (contain no double bonds). In addition to the free form, both phytosterols and phytostanols occur as four types of conjugates in which the 3-OH group is esterified to a fatty acid or hydroxycinnamic acid, or glycosylated with a hexose (usually glucose) or a 6-fatty-acyl hexose [1,2]. Food sources naturally rich in plant sterols and stanols include a wide range of cereals, fruits, nuts, legumes and vegetable oils. Also, some processed foods are fortified with phytosterols, such as fat spreads, chocolates, snack bars and salad dressings. Phytosterols found in these fortified food matrices include β-sitosterol, campesterol, stigmasterol, brassicasterol and stigmastanol [2,3].

They have the ability to decrease low density lipoprotein cholesterol (LDL-cholesterol) in humans. Phytosterols have also been found to help protect against the development of various types of cancer, to encourage the production of skin collagen and have anti-inflammatory and anti-oxidative effects. Phytosterols from the plant *Serenoa repens* have been used as food supplements and drug products from saw palmetto extract (campesterol, β-sitosterol and stigmasterol) [2,4,5] for auxiliary treatment of prostatic hyperplasia. Since 1995, phytosterols and phytostanols enriched products have been launched in several countries and the market for phytosterols, as food supplements, has led to a rapidly growing worldwide market for functional foods in the EU and in the USA [2,6].

High performance liquid chromatography (HPLC) is nowadays one of the most widely used techniques in modern pharmaceutical analysis and quality control. Due to its wide field of application, the most common detection technique for HPLC is UV/vis spectrophotometry. However, both UV/vis and fluorescence

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detection reach their limits when the analyte molecules lack a suitable chromophor or fluorophor. In these cases, mass spectrometry (MS) and electrochemical (EC) detection or postcolumn derivatization with UV detection are often employed. However, all these alternatives have their own limitations. Derivatization is difficult to validate as different compounds in a mixture may interact differently with the derivatization agent. Moreover, degraded products from the derivatization reagent can interfere with the chromatogram. The MS-based quantitative multi-component analysis in plant materials is associated with unpredictable matrix effects and the use of expensive isotope-labeled internal standards [7,8].

Progress in chromatography and detector technology now allows higher separation power by ultra-high performance LC (UHPLC) and universal detection beyond the diode array detector (DAD), notably by the corona-charged aerosol detector (CAD), which was introduced in 2002 by Dixon and Peterson [9]. An optimized version for UHPLC applications, Corona Ultra, was introduced in 2009 [8]. The mobile phase from the chromatographic system is nebulized using a flow of nitrogen and the resulting aerosol is transported through a drift tube, where the volatile components and solvents are evaporated. A secondary stream of nitrogen becomes positively charged as it passes through a high-voltage corona wire. The charged nitrogen is mixed with the stream of analyte particles, where their charge migrates to the analyte. The charged analyte particles proceed to a collector region, where a highly sensitive electrometer produces a signal that is proportional to the weight of the sample present, independent of its chemical structure [10]. As with all other evaporation based detectors, a prerequisite for detection by CAD is that the solute be considerably less volatile than the mobile phase. This means that the choice of mobile phase additives is limited and that volatile mobile phases must be used [7].

The measurement of phytosterols in the different forms containing plant extracts, such as food supplements, fortified foods, or vegetable oil requires significant sample preparation including saponification and/or acid hydrolysis to convert all of the conjugated phytosterols into their free forms. The sample preparation steps are then followed by liquid-liquid solvent extraction, sample pre-concentration, and possible solvent exchange prior to the chromatographic analysis [5]. The most commonly used methods for the analysis of phytosterols are based on gas chromatography with mass spectrometry detection (GC–MS) [11–14], flame ionization detection (GC-FID) [3,4,15,16], and liquid chromatography with MS detection (LC–MS) [5,17–20]. Few papers deal with the analysis of phytosterols using the HPLC-UV method, e.g. [21,22]. This is due to the lack of chromophores in molecules of phytosterols. LC methods typically offer only a limited resolution of the relevant phytosterols due to similarities in sterol and stanol structures and polarity [5].

While GC methods are more common, they are not ideal because the phytosterols usually must be derivatized before performing the analysis except for MS detection. For GC analysis, sterol extracts are frequently derivatized using silylating agents to render the target analytes volatile and thermally stable [3].

This paper presents a new UHPLC method for the fast determination and quantification of seven plant sterols and stanols employing a Corona-charged aerosol detector (CAD). In liquid chromatography methods, columns with C8/C18 are mostly used for the analysis of phytosterols [18–21]. In our work, the separation of all analytes was achieved on an alternative narrow-bore core–shell column with a phenyl-hexyl stationary phase. The method was validated and successfully applied for the assay of all analyzed compounds in the presence of other substances in the different food supplement preparations available on the local market.

2. Materials and methods

2.1. Chemicals and reagents

The HPLC-grade solvents used were 2-propanol and *n*-hexane, and LC–MS grade was acetonitrile (ACN), all of which were supplied by Sigma–Aldrich (St. Louis, USA). All reagents used were of analytical grade. Ultra-pure water was obtained using a Milli-Q system (Millipore, USA). Nitrogen 5.0 (99.999%) for the Corona detector was obtained from Linde gas (CZ). Standards of brassicasterol (95%), ergosterol (95%), fucosterol (93%), campesterol (65%), β-sitosterol (95%), stigmasterol (95%), and 1-nonadecanol internal standard (99%) were purchased from Sigma Aldrich. Standards of stigmastanol (95%), and campestanol (98%) were purchased from ChemCruz and ChemFaces, respectively.

2.2. Instrumentation and the chromatographic conditions

The UHPLC chromatographic system by Thermo Scientific, the Dionex UltiMate™ 3000 RSLC system (California, USA) equipped with a binary pump, an autosampler, a column oven, a Diode Array detector and a Corona Ultra CAD (ESA Biosciences, part of Thermo Fisher Scientific, CA, USA) was used. The system control, data acquisition and data evaluation were performed using the Dionex - Chromeleon™ 7.2 Chromatography Data System (Thermo Fisher Scientific, CA, USA). Acetonitrile used as mobile phase B and Milli-Q water, filtrated through a 0.22 μm filter, as mobile phase A were delivered at a flow rate of 0.9 mL min^{-1} according to the following elution gradient program: 0.0–4.0 min 40–65% mobile phase B, 4.0–7.0 min 65–70% mobile phase B, 7.0–7.2 min 70–40% mobile phase B, and 7.2–8.5 min 40% mobile phase B. Separation was performed on the Kinetex Phenyl-hexyl (100 × 2.1 mm) core–shell column with a particle size of 1.7 μm at a temperature of 60 °C. A tandem connection of the PDA detector and CAD with a sampling rate of 30 points per sec for both detectors was used. Nitrogen with 99.999% purity was delivered at 40.0 psi to the Corona detector. The temperature of the autosampler cooling system was set at 20 °C to avoid excessive evaporation of the diluent and precipitation of the analytes at the same time, which was observed under lower temperatures. An injection volume of 2 μL was used.

2.3. Preparation of the standard solutions

A standard stock solution of 400 μg mL^{-1} was individually prepared of each phystosterol in *n*-hexane. An internal standard (1-Nonadecanol) stock solution of 2000 μg mL^{-1} was prepared in 2-propanol. All the stock solutions were stored at 4 °C in a dark place until used. The mixed stock standard solution with a concentration of 50 μg mL^{-1} was prepared by mixing aliquots of each of the standard solutions, including the internal standard stock solution, and was used for the system suitability test.

2.4. Sample preparation

A mass of 0.1–1.2 g of a homogenized content of soft capsules and 1.0 g of 20 homogenized tablets was accurately weighed into a 25 mL volumetric flask. The sample was saponified with 15 mL of ethanolic solution of 2 M KOH with the addition of 2.5 mL of the internal standard solution. The optimal time of saponification was studied and selected depending on the yields of free phytosterols from the samples (Section 3.3). The mixture was incubated at 80 °C for 30 min in a water bath and was shaken regularly. The mixture was cooled down to the laboratory temperature and filled up to the mark of the volumetric flask with ethanol. Then 1 mL of the mixture was pipetted into a 20 mL volumetric flask with 8 mL of diluted aqueous HCl and 10 mL of *n*-hexane. It was then vortex mixed for