Quantitative analysis of phytosterols in *Aristotelia chilensis* (Maqui) leaves using GC/MS

1*Muñoz, O. and Ramos, F.

1Faculty of Sciences, Chemistry Department. University of Chile. Las Palmas 3425, Ñuñoa, Santiago, Chile.
2CNC - Center for Neuroscience and Cell Biology, Health Sciences Campus, Faculty of Pharmacy, University of Coimbra, Azinhaga de Santa Comba, 3000-548 Coimbra, Portugal

Abstract

The prevalence of metabolic syndrome, hypertension, heart disease and diabetes has been increasing rapidly in Chile in recent years. The rate of increase has paralleled the replacement of traditional Mediterranean diets, which emphasize vegetables and fruits, with the fast food that now prevails, which has patterns rich in saturated fat. It is well-established that high phytosterol intakes can lower total and LDL cholesterol concentrations in human serum. *Aristotelia chilensis* (Mol.) Stuntz (Elaeocarpaceae), a 4–6 m tall evergreen tree with yellow flowers and edible black-coloured fruit, grows in central and southern Chile and in southwest Argentina and is typically consumed fresh or used to make jam, tea, wine, and juice. *A.chilensis* can also be used as a source of phytosteroles, which led us to begin our investigation into these compounds. To do so, a method based on GC-MS for separating, quantifying and identifying the phytosterols present in *Aristotelia chilensis* extracts was described. Our study shows that the main phytosterol found was β-sitosterol. Furthermore, the use of *Aristotelia chilensis* as a food supplement is also discussed.

Introduction

Nowadays, cardiovascular diseases (CVD) are the main cause of death in developed countries, a trend that is spreading to developing countries (Leif et al., 2006). It has recently been suggested that the ingestion of phytosterols could reduce cholesteroolemia, and thereby contribute to reducing the development of CVD (Ramos and Saraiva, 2009). Phytosterols are the collective term for plant-derived sterols and stanols. They are 28- or 29-carbon alcohols that resemble cholesterol in vertebrates both in terms of function (stabilization of phospholipid bilayers in plant cell membranes) and structure (steroid nucleus, 3β-hydroxyl group, 5,6 double bond) (Lagarda et al., 2006).

Clinical trials find that daily consumption of phytosterol-enriched foods can significantly lower LDL cholesterol concentrations (Ostlund, 2004). However, relatively few studies have considered the effects of normal dietary phytosterol intakes on serum LDL cholesterol concentrations. Dietary phytosterol intakes have been estimated to range from about 150-500 mg/day in a range of populations (Ostlund,. 2002; Jones, 2008; AbuMweis et al., 2014). Limited evidence suggests that dietary phytosterols may play an important role in decreasing cholesterol absorption. A cross-sectional UK study found that dietary phytosterol intakes were inversely related to serum total and LDL cholesterol concentrations even after adjusting for saturated fat and fiber intake (Andersson et al., 2004). Similarly, an analysis in a Swedish population found that dietary intake of phytosterols was inversely associated with total cholesterol in both men and women and LDL cholesterol in women (Klingberg et al., 2008). In single meal tests, removing 150 mg of phytosterols from corn oil increased cholesterol absorption by 38% (Ostlund et al., 2002), and removing 328 mg of phytosterols from wheat germ increased cholesterol absorption by 43% (although more research is needed, these findings suggest that normal dietary intakes of phytosterols from plant foods could have an important impact on cardiovascular health (Ostlund et al., 2003).

*Aristotelia chilensis* is a fruit-bearing shrub that thrives in the forests of central to southern Chile and western Argentina. It belongs to the Elaeocarpaceae family and is commonly known as “maqui.” *A. chilensis* yields a small edible purple/black berry (Schreckinger et al., 2010). The leaves and fruit of *A. chilensis* have been used in folk medicine to treat a...
variety of ailments including sore throat, pains, ulcers, fever, inflammation, and diarrhea (Minsal, 2009). In addition, it is commonly used as a natural dye due to its high content of anthocyanin pigments (Escribano-Bailon et al., 2006; Muñoz et al., 2011). Studies on the phytochemical composition of the *A. chilensis* berry have indicated the presence of phenolic acids, proanthocyanidins, and anthocyanins and other flavonoids (Gironés-Vilaplana et al., 2012) while examination of the leaves showed the presence of quillizidine alkaloids (Muñoz et al., 2011).

Recently, fruit from this plant has been used to manufacture a range of flavored drinks, which seek to take advantage of the antioxidant properties of the antocyanins and polyphenols found therein (Gironés-Vilaplana et al., 2012). Meanwhile, other research groups have focused on preparing maqui concentrates to be used in preparing functional foods (Alonso, 2012). For the above reasons, and in order to complement the application of these phytochemicals in the food industry, the purpose of the present study, which utilizes a GC-MS method (Mo et al., 2013), was to evaluate and quantify the composition of phytosterols in *A. chilensis*. To the best of our knowledge, this is the first report of the phytosterols composition reported from Aristotelia chilensis to the present.

**Materials and methods**

**Materials**

A Mettler Toledo PC2000 balance (Greifensee, Switzerland), Gilson micropipettes, a vortex agitator (UnimagZX), a Sonorex RK100 ultrasound bath (Bandelin, Berlin, Germany) and heating blocks with a nitrogen evaporation system (Reagente 5, Oporto, Portugal) were used for the steps of the extraction procedure. The vials and crimp caps used in the derivatization procedure were supplied by Chromacol (Dias de Sousa, Lisbon, Portugal), and determination of phytosterols was achieved using an Agilent Technologies (AT) (Soquimica, Lisbon, Portugal) GC–MS system, controlled by an HP Compaq computer and containing an automatic AT 7683B injector and AT 6890N gas chromatograph equipped with an HP-5MS column (30 cm × 250 µm i.d. × 0.25 µm film thickness) connected to an AT 5975 mass detector.

**Standards and reagents**

β-sitosterol, campesterol, β-sitostanol, campestanol, brassicasterol, stigmasterol and cholestane, used as an internal standard (IS), were obtained from Sigma (Madrid, Spain). The reagents used were absolute ethanol for standard solution preparations, and cyclohexane for sample extraction (Merck, Darmstadt, Germany). N-methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA; Sigma, Madrid, Spain), 1,4-dithioerythritol (DTE; Merck, Lisbon, Portugal), and trimethylsiodsilane (TMIS; Sigma) at a proportion of 5 mL:10 mg:10 µL (MSTFA:DTE:TMIS) were used as derivatization reagents (Saraiva et al., 2011).

**Standard preparation**

All standard stock solutions, including the IS, were prepared quarterly in ethanol at a 1 mg/mL concentration. Working solutions prepared weekly in ethanol were made at the required concentrations by properly diluting stock solutions. The IS working solution, cholestane, was prepared at a 50 µ/mL concentration. The temperature of all solutions was kept at −20±2°C in a freezer.

**Samples**

Leaves from *Aristotelia chilensis* (Mol.) Stuntz (Elaeocarpaceae) were collected in October 2011 by Dr. Orlando Muñoz, at the Juan Gómez Milla Campus, University of Chile, Metropolitan Region, Santiago, Chile, and identified by Prof. Dr Carla Delporte. A voucher specimen was kept at the School of Chemistry and Pharmacy Herbarium (SQF 22257) at the University of Chile.

Air-dried and powdered vegetal material (3.2 g) was extracted three times using hot methanol (50°C; 0.3l each) and then filtered. A rotary evaporator was used to remove methanol and obtain dry extract. An aqueous extract was prepared from dried and ground vegetal material to which water was added, after which the resulting mixture was extracted three times using CH$_2$Cl$_2$ (0.2l each). This extract was suspended in 90% hexane-MeOH. The methanol extract was concentrated under reduced pressure followed by lyophilization to produce the sterol extract. The sterol extract obtained was put into a round flask and lyophilized in vacuo for 2 days, (ILSHIN TFD 5505 apparatus; -53°C, 5 mTorr) and then ground into a fine powder for further use.

**Sample preparation**

10.0 ± 1.0 mg of lyophilized sample was dissolved in 2 mL of cyclohexane by vortexing (1 min.) and sonication (15 min.) in order to obtain a homogenous
solution. Then 500 µL of cyclohexane solution was transferred to a derivatization vial and evaporated to dryness using a nitrogen stream at 60ºC. The dry residue was derivatized with 50 µL of N-methyl-N-trimethylsilyl-trifluoracetamide (MSTFA), 1,4-dithioerythritol (DTE), and trimethylsiodisilane (TMIS) at a proportion of 5 mL:10 mg:10 µL (MSTFA:DTE:TMIS) at 60ºC for 30 min (Saraiva et al., 2011).

**Chromatography**

Phytosterol was determined by injecting 1 µL of the derivatized sample at 250ºC in splitless mode for 1 minute. The constant helium mobile phase flow was 1.0 mL/min. The initial column temperature of 200ºC was held for 1 min., after which it was increased by 20ºC/min until reaching a temperature of 300ºC, at which it was held for 10 min. The temperature of the detector was 280ºC. The mass spectrometer was operated in the electron ionization mode (EI) at 70 eV. Data was obtained in scan mode using electron impact ionization. Scan range: m/z 50 to m/z 650 Scan speed: 500 ms.

Beta-sitosterol and campesterol in the samples were identified by comparing the respective standard chromatographic retention times and mass spectra. The range of each standard curve was beta-sitosterol: 0.1 – 10 µg/mL and campesterol: 0.1 – 10 µg/mL. Figure 1 shows a typical chromatogram and demonstrates that only β-sitosterol can be accurately determined in A.chilensis samples.

**Results**

GC-MS analyses (Figure 1) showed that the typical plant sterol, β-sitosterol was the main sterol component in A.chilensis; it was found at 4.3 ± 1.0 micrograms per gram of dehydrated sample while campesterol, sitostanol and campestanol were present in negligible concentrations. In this study, retention times and the mass spectra obtained from the referred peaks were compared with the most common phytosterols and no match was made. β-sitosterol is probably the most abundant and widely distributed plant sterol. (Beta-sitosterol: 0.1 ug/mL Campesterol: 0.1 ug/mL); campesterol peaks between 10.80 and 10.90 min. However, as the peak is not very distinguishable from background noise, it is not possible to quantify campesterol levels in A. chilensis.

**Discussion**

A number of studies have demonstrated clinical effectiveness as a cholesterol-lowering agent and in treating benign prostatic hyperplasia (Bergues et al. 2000; Wilt et al., 2011). Dietary vegetables are a daily source of β-sitosterol, which is mainly known and used for its cholesterol-lowering properties. However, other studies have shown that this phytochemical may have other health benefits, such as easing the symptoms of benign prostate enlargement, reducing the risk of cancer and preventing oxidative damage as a result of its antioxidant activity. β-sitosterol has promising antidiabetic and antioxidant effects and may be included in clinical studies as part of the development of drugs (Gupta et al., 2011). β-sitosterol also has antihyperlipoproteinaemic, antibacterial and antimicotic properties and has been shown to inhibit in vivo tumor promotion (Yasukawa et al., 1991) and carcinogenesis (Woyengo et al., 2009).

Phytosterol supplements marketed as beta-sitosterol are available in the U.S. free of prescription. 60-130 mg/d doses of beta-sitosterol have been found to alleviate symptoms of benign prostatic hyperplasia (BPH) in some clinical trials (gel chews providing 0.5 g of plant stanols are marketed as being able to lower cholesterol at a recommended dose of 2 g/d. Phytosterol supplements should be taken with meals containing fat (Drake, 2008).

Relatively few studies have considered the effects of dietary phytosterol intakes on serum LDL cholesterol concentrations (Woyengo et al., 2009) The intakes reported of dietary phytosterol tend to be variable: thus, some research groups have informed values estimated between 150-450 mg/day (Ostlund et al., 2002), while others have indicated values of between 250-500 mg/day (AbuMweis et al., 2014). Although more research is needed, these findings suggest that dietary intakes of phytosterols from plant foods could have an important impact
on cardiovascular health. Present-day dietary phytosterol intakes have been estimated to vary from 150-450 mg/day in different populations (Ostlund et al., 2002). Vegetarians, particularly vegans, generally have the highest intakes of dietary phytosterols (Ramos and Saraiva, 2009).

The prevalence of metabolic syndrome, hypertension, obesity, vascular disease and diabetes has grown rapidly in Chile in recent years, reflecting the replacement of traditional diets based on vegetables, fruits, and legumes with fast food dietary patterns rich in foods of animal origin and high in saturated fat (Solis et al., 2012). With the above in mind, A. chilensis, a plant native to southern Chile well known for the benefits of its fruit and leaves, could be used in food. Enriching products such as margarine, milk, bread, yogurt and butter – among others – with Aristotelia sterols could deliver significant economic and social benefits to a population besieged by diabetes, obesity and hypertension, and which prefers fast food to healthier, more natural.

The most important impact of phytosterols is in blocking cholesterol absorption in the intestines. However, the levels naturally present in food are insufficient to have an impact on high cholesterol levels. A. chilensis is widely used in popular medicine, and as occurs with other plants, the quantity of phytosterols it contains is rather low compared with some other foods (A. chilensis 4.3±1.0’10-3mg/gr vs 0.65mg/gr peanuts). However, these quantities can be compensated by adding phytosterols imported from other plants, currently a common practice in the industry (Sernac, 2004; Valenzuela and Ronco, 2004; ISP, 2013).

Conclusions

β-sitosterol was the main sterol component in A. chilensis; and was found at 4.3 ± 1.0 micrograms per gram of dehydrated sample. We propose that these concentrations be increased by adding supplementary phytosterols for subsequent use as a dietary supplement.

References


ISP. 2013. Instituto de Salud Pública de Chile. Comision Regimen de Control Aplicado .Fitosteroles puros de Pino que ayudan a la reduccion de la absorcion de colesterol Importacion de fitosteroles USA. Internet: Downloaded from http://www.ispch.cl/sites/default/files/documento/2013/07/acta_5_comision_regimen_de_control_aplicable_2013.pdf


