Review Article

Analysis of Phytosterols in Plants and Derived Products by Gas Chromatography – A Short Critical Review

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Abstract

Phytosterols are sterols present in plant cells and studies of these molecules suggested positive biological effects. Government agencies proposed to include free phytosterols in foods as well as its determination in many products to avoid adulterations by chromatography such as Gas Chromatography (GC). Limitations and suggested alternatives for Phytosterols analysis by GC are cited in this review article as information source for future studies.

Keywords: Phytosterol; Gas chromatography; Limitations; Alternatives

Introduction

Phytosterols are an important family of lipids present in plant cells and can be classified either as 4-desmethylsterols, 4-methylsterols, or 4,4'-dimethylsterols. Sitosterol, campesterol, and stigmasterol (4-desmethylsterols group) are found in abundance in most of the plants. However, there are over 200 different sterol structures that have been discovered in various plant species [1]. Phytosterols can exist in vegetables in their free form, as esters with fatty acids, ferulic acid, or p-coumaric acid, or as glycosides and acylatedsteryl glycosides [2-4].

Studies have been suggested that phytosterols have anti-inflammatory, antibacterial, antifungal, anticancerous and antiinflammatory activities [5,6]. Moreover, these molecules show ability to lower blood cholesterol [7,8]. Because of its positive biological effects, Food and Drug Administration (FDA) and European Union (EU) proposed to include free phytosterols in conventional foods and established labeling guidelines. Therefore, the determination of sterol profile in medicinal products and vegetable oils on the basis of its free form and extractive techniques based on principles of Liquid-Liquid Extraction (LLE) and Solid Phase Extraction (SPE) are performed to promote the extraction [17,18]. Moreover, SPE allows purification and higher concentrations of these molecules compared to LLE [18]. Regardless of the extraction technique, both procedures are required for phytosterols analysis by GC.

GC is a physical separation method which allows high efficiency analysis of different compounds based on volatilization of the analytes and interaction between analyte and stationary phase from capillary column, since mobile phase (carrier gas) is inert [19-21]. This equipment is an important tool in the process of detection, identification and quantification of Phytosterols [19,20] showing a wide range of columns for efficient and reliable analysis [21], with standard Gas Chromatographic-Flame Ionization Detection (GC-FID) and Gas Chromatographic-Mass Spectrometry (GC-MS).

Gas Chromatographic-Flame Ionization Detection (GC-FID) is widely used in the analysis of phytosterols because of easy handling, low cost and good sensitivity [15,22,23]. However, the difficulty of the analysis of phytosterols in foods and plants requires complex processes extractives, purifications and efficient derivatization (if necessary) [19,20,23,24]. Furthermore, chromatographic determination of these molecules shows some limitations such as co elution of some compounds. This behavior can happen when a fully nonpolar capillary column with methyl groups attached is used as stationary phase, such as dimethylpolysiloxane (100%) or phenyl-dimethylpolysiloxane (5%-95%). According to Laakso [23], delta-5-avenasterol/sitostanol, campesterol/campestrol and sitosterol/sitostanol demonstrated problematic separation with these columns.

A chromatogram developed in our laboratory used reference standards of cholesterol, brassicasterol, ergo sterol, campesterol, stigma sterol, beta-sitosterol and fucosterol (Sigma-Aldrich®, MO, USA) with intention to demonstrate the coelution in these analytes by

Phytosterols analysis by gas chromatography (GC)

Crude extract of plant and its derived products are considered complex matrices. Saponification is a sterol transformation method in its free form and extractive techniques based on principles of Liquid-Liquid Extraction (LLE) and Solid Phase Extraction (SPE) are performed to promote the extraction. Liquid-Liquid Extraction (LLE) and Solid Phase Extraction (SPE) are performed to promote the extraction.
GC-FID using capillary column Elite-5 (crossbond 5% diphenyl - 95% dimethyl polysiloxane) with 30 m x 0.25 mm x 0.25 μm film thickness. The result is demonstrated in the Figure 1, showing coelution between beta-sitosterol and fucosterol with this stationary phase. Different temperature programs were evaluated and the retention time of beta-sitosterol and fucosterol were quite close.

One alternative is the derivatization step to promote chromatographic separation, since most phytosterols having hydroxyl group (-OH group) can easily be derivatized by silylating agents as N,O-bis (trimethylsilyl) trifluoroacetamide with 1% trimethylchlorosilane (BSTFA) or N-methyl-N-(tert-butyl)methylchlorosilane with 1% tert-butylmethylchlorosilane (MTBSTFA) [25-28]. Indeed, an increase of sensitivity after this chemical reaction occurs due to better volatilization of these compounds because phytosterols requires high temperatures to perform the volatilization and consequently, its determination by GC. However, separation of the analytes cannot be modified because elution orders of the phytosterolsremains the same manner after derivatization. This particular behavior happened between beta-sitosterol and fucosterol.

To solve this problem, columns with different chemical characteristics are required. Columns with stationary phase with 14%cyanopropylphenil and 86% methyl polysiloxancan are used to promote the separation of these compounds [23]. Similarly, it is possible to use columns of medium polarity based in 50% phenyl and 50% methylpolysiloxane [20] or stationary phase with 95% dimethyl-5% diphenylpolysiloxane [29]. Nevertheless, chromatographic separation with a type of capillary column for determined phytosterols (ex.: beta-sitosterol and fucosterol) can be promote coelution among other sterols, mainly complex matrices with a mixture of these molecules.

Because of the phytosterols and matrices complexity, Multidimensional Gas Chromatography (MDGC) and comprehensive two-dimensional Gas Chromatography (GCxGC) would be interesting alternatives for chromatographic separations [30-33]. MDGC uses two columns of different polarities interconnected with a modulator transfer, where selected bands of overlapping compounds from conventional nonpolar column (first dimension) are passed to a short polar column (second dimension) [34]. For GCxGC, two columns (first dimension is a conventional column and second dimension is a short fast type) are connected sequentially with a modulator between them which is powered by liquid nitrogen. The modulator functions are continuously collect small fractions from first dimension, focus or refocuss of a narrow band and quickly transfer of fraction collected to second dimension [31]. Both techniques have been poorly explored for determination of phytosterols. MDGC was used to analyze of plant sterol profile from unsaponifiable lipid fraction [30] and GCxGC was applied to evaluate phytosterols in vegetable oil [32]. GCxGC, MDGC and traditional GC can be easily coupled to MS, ensuring other important parameter, mass spectrum, which allows the determination of these analytes.

In this regard, GC-MS is still considered the gold standard equipment for phytosterols determination [10,11,28,33]. Chromatographic separation problems remains if different types of columns are not used or the equipment is not a MDGC or GCxGC. However, mass fragmentagtion of each phytosterol allows selectivity of the compounds, including bands of overlapping compounds. By selection of the ions (m/z), chromatographic peak becomes evident and consequently, the identification of the compounds [24]. Figure 2 is a typical chromatogram produced by GC-MS in our laboratory showing coelution between beta-sitosterol and fucosterol and the identification of these analytes was performed based in ions of interest.

Nevertheless, determination of phytosterols is discouraged when these analytes shows very close retention time and similar mass fragmentagation. In this case, it is possible to identify these analytes using other types of advanced MS (triple quadrupole, time-offlight and hybrids MS), avoiding different strategies with respect to chromatographic separation [35-37].

For quantification of compounds, the most of the analysis requires Internal Standard (IS) [21,24]. Cholesterol has been used as Internal Standard (IS) in GC-FID for analysis of unsaponifiable compounds.
in vegetable distillate oils [38]. However, cholesterol is present in algae at significative levels [14] and small concentrations (less than 1%) of crude extra-virgin olive oil [39]. Cholesterol (5α-cholestan-3β-ol), dihydrocholesterol (5α-cholest-3β-ol), coprosterol (5β-cholestan-3β-ol) and epicoprosterol (5β-cholestan-3α-ol) have been used with success as IS for GC-FID and GC-MS [23,28,40-44]. Indeed, deuterated analytes are the best option as IS for phytosterols determination when GC-MS is available.

Conclusion

This critical review article showed limitations of GC for determination of phytosterols, demonstrating alternatives to solve chromatographic problems for future studies with these analytes in plants and derived products.

References


