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# On-line liquid chromatography–gas chromatography: A novel approach for the analysis of phytosterol oxidation products in enriched foods

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ABSTRACT

A novel methodology for the automated qualitative and quantitative determination of phytosterol oxidation products in enriched foods via on-line liquid chromatography–gas chromatography (LC–GC) was established. The approach is based on the LC pre-separation of acetylated phytosterols and their corresponding oxides using silica as stationary phase and a mixture of *n*-hexane/methyl *tert*-butyl ether/isopropanol as eluent. Two LC-fractions containing (i) 5,6-epoxy- and 7-hydroxyphytosterols, and (ii) 7-ketophytosterols are transferred on-line to the GC for the analysis of their individual compositions on a medium polar trifluoropropylmethyl polysiloxane capillary column. Thus, conventionally employed laborious off-line purification and enrichment steps can be avoided. Validation data, including recovery, repeatability, and reproducibility of the method, were elaborated using an enriched margarine as example. The margarine was subjected to a heating procedure in order to exemplarily monitor the formation of phytosterol oxidation products. Quantification was performed using on-line LC–GC-FID, identification of the analytes was based on on-line LC–GC-MS. The developed approach offers a new possibility for the reliable and fast analysis of phytosterol oxidation products in enriched foods.



## 1. Introduction

Margarines enriched with phytosterols and their fatty acid esters were among the first products belonging to the so-called "functional foods" that were authorized in the European Union [1]. As phytosterols were shown to lower blood cholesterol levels, they serve as the functional ingredients of foods with claims regarding the reduction of the risk of cardiovascular diseases [2,3]. Meanwhile, phytosterol-enriched foods have increased considerably in number and variety [4]. This is not only drawing attention to the safety of the food ingredients as such, but also to the formation, occurrence and potential intake of oxides thereof, that may be formed in the presence of heat, oxygen, water, light and transition metals [5]. For cholesterol, oxidation reactions are known to occur in foods; several adverse health effects have been described for dietary cholesterol oxidation products; there is particularly evidence for an impaired hepatic function and lipid metabolism, as well as for pro-atherosclerotic effects [6–9]. As phytosterols share the principal structural features with cholesterol, they are also susceptible to oxidation reactions [5]; similarly, phytosterol oxidation products may be expected to be detrimental to human health. Thus far, a cytotoxic and pro-inflammatory potential of phytosterol oxidation products has been demonstrated in several studies, albeit they were mainly performed in vitro [5,10–12]. They revealed 7β-hydroxy and 7-keto compounds to be the derivatives showing the strongest cytotoxic capacity among those oxides that are quantitatively dominating in foods. As far as a pro-atherogenic potential of phytosterol oxidation products is concerned, data deriving from an in vitro study are indicative of an impairing effect [13], while the results of two available in vivo studies are inconclusive [14,15]. Though, the more recent study compared the effects of dietary cholesterol oxidation products and dietary phytosterol oxidation products in $LDLR^{+/-}$ mice; it was shown that phytosterol oxidation products increased the proportion of severe atherosclerotic lesions in a manner similar to cholesterol oxidation products [15]. Knowledge regarding the absorption of phytosterol oxidation products is scarce. One study showed low lymphatic absorption rates for phytosterols (2.2% sitosterol and 5.5% campesterol) in contrast to cholesterol (37.3%); however, the lymphatic absorption rates of sitosterol oxidation products (9.1%) and campesterol oxidation products (15.9%) were significantly higher than those of the non-oxidized phytosterols [14]. Further studies investigating differences between individual phytosterol oxidation products revealed their absorption to be impacted by the structure of the side

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chain and the type of oxidation [16–18]. The data on the potential implications of phytosterol oxidation products on human health underline the relevance of an extensive qualitative and quantitative assessment, both in (patho-) physiological and analytical regards. In this context, foods enriched with phytosterols and/or their fatty acid esters are of particular interest due to their increasing abundance and thus the availability of substrates for oxidation reactions when compared to non-enriched foods.

For a quantitative analysis of phytosterol oxidation products in foods, saponified or transesterified lipid extracts are usually subjected to off-line procedures such as column chromatography, liquid chromatography, thin layer chromatography (TLC) or solid phase extraction (SPE) for clean-up and pre-concentration by removal of matrix components and of the abundant phytosterols [19,20]. Subsequently, the phytosterol oxidation products are analyzed via gas chromatography (GC) or liquid chromatography (LC). However, *de facto*, this sequence is encountered with several difficulties. Phytosterol oxidation products comprise a variety of compounds with high structural similarity, exhibiting challenges regarding chromatographic separation as well as mass spectrometric identification. These issues are aggravated by the lack of commercially available reference compounds. In addition, in foods, phytosterol oxidation products occur only in trace amounts and analyses may be complicated by matrix constituents, making at times several purification steps necessary. The commonly applied off-line techniques for the isolation and purification of phytosterol oxidation products are time-consuming and carry the risks of sample losses, degradation of the labile oxides or artifact formation. These analytical obstacles result not only in a limited quantitative dataset on the occurrence and formation of phytosterol oxidation products in selected enriched foods; also the comparability of the existing data is often difficult as the employed analytical methodologies are not standardized [11].

An efficient and elegant alternative to the laborious off-line approaches is the on-line coupling of LC and GC [21]. This two-dimensional chromatographic technique has been used for several analytical purposes; recent work focused in particular on the analysis of phytosterols, phytosteryl/phytostanyl fatty acid and ferulic acid esters in oils, fats, cereals, and nuts, as well as on the analysis of phytosterols and phytosteryl/phytostanyl fatty acid esters in enriched foods [22–28]. Via LC, a highly selective clean-up, preconcentration and fractionation of the sample can be achieved; the fraction(s) of interest can then be transferred on-line to the GC [29]. This allows analyses being performed in a closed and fully automated system, minimizing the risks of sample losses, contaminations, and undesired reactions [30,31]. Due to these distinct and advantageous features, on-line LC–GC was considered to be a suitable novel analytical platform for the analysis of phytosterol oxidation products, circumventing the drawbacks of the conventional sample preparation techniques. Therefore, the aim of the present study was the development of an on-line LC–GC based analytical technique in order to enable a sensitive, automated and fast analysis of phytosterol oxidation products in enriched foods. The crucial step of enrichment and purification of the phytosterol oxidation products should be accomplished by LC separation, followed by an on-line transfer of the oxides to the GC for the analysis of individual compounds. The established approach should be extensively validated using as example an enriched margarine, which was subjected to a heating procedure in order to monitor the formation phytosterol oxidation products.

## 2. Experimental

### 2.1. Sample

Margarine enriched with phytosteryl esters ("Becel pro.activ" produced by Unilever, Hamburg, Germany) was purchased in a local supermarket (Freising, Germany). In the list of ingredients, the following information was included: 7.5 g plant sterols, 40 g fat per 100 g. The phytosteryl ester composition of the margarine was analyzed according to a previously described methodology [21]; the following profile was determined: 41.9% sitosteryl-18:2, 22.1% sitosteryl-18:0/18:1, 9.7% campesteryl-18:2, 6.6% campesteryl-18:0/18:1, 5.3% sitostanyl-18:2, 5.0% sitosteryl-16:0/16:1, 4.9% sitostanyl-18:0/18:1, 1.5% brassicasteryl-18:2, 1.1% campesteryl-16:0/16:1, 0.7% sitostanyl-16:0/16:1, 0.7% campestanyl-18:0/18:1, and 0.5% campestanyl-18:2.

### 2.2. Chemicals and reagents

Acetic anhydride (≥99%), cholesterol (95%), 5,6α-epoxycholesterol (90%), 5,6β-epoxycholesterol (≥95%), 7β-hydroxycholesterol (≥95%), 7-ketocholesterol (≥90%), pyridine (99.8%), sodium methoxide (reagent grade, 95%), stigmasterol (~95%), were purchased from Sigma-Aldrich (Steinheim, Germany). A mixture containing sitosterol and campesterol (75% sitosterol, 12% sitostanol, 10% campesterol, 2% campestanol) was purchased from Acros Organics (Morris Plains, NJ, USA). 7-Ketostigmasterol (92%) was purchased from Steraloids (Newport, RI, USA). Chloroform (AnalaR Normapur), *n*-hexane (HiPerSolv Chromanorm), isopropanol (HiPerSolv Chromanorm) were purchased from VWR International (Darmstadt, Germany). Citric acid anhydrous (for synthesis) was purchased from Merck (Darmstadt, Germany). Methyl *tert*-butyl ether (MTBE) was supplied by Evonik Industries AG (Essen, Germany) and was distilled prior to use.

### 2.3. Preparation of reference compounds of sterol oxidation products

Reference compounds of 5,6α-epoxy-, 7-keto-, 7α-hydroxy-, and 7β-hydroxysterols were obtained via a semi-preparative HPLC-separation of thermo-oxidized sterol standard compounds, based on the principles of a method described by Kemmo et al. [32]. Briefly, 15 mg of sterol standard were heated at 180 °C for 180 min in an open 11 mL glass vial in a ventilated oven. After cooling in a desiccator, the oxidized standard was dissolved in 1200 μL *n*-hexane/isopropanol (96:4, v/v) using a sonicator. The separation was carried out using a Dionex HPLC system (UltiMate 3000 series, Dionex Softron GmbH, Germering, Germany) equipped with a wavelength detector-3100 adjusted to 206 nm. The sample (1 mL) was injected onto a Nucleosil 50-5 column (250 mm × 8 mm, 5 μm; CS-Chromatographie, Germany) tempered at 30 °C. A linear gradient was used with *n*-hexane and isopropanol at 3.5 mL/min; the amount of isopropanol was raised from 4% to 12% within 30 min. This resulted in the separation of 5,6α-epoxy-, 7-keto-, 7α-hydroxy-, and 7β-hydroxysterols. The purities and identities of the substances were confirmed via GC/FID and GC/MS of TMS derivatives according to mass spectra from the literature [33–36]. 5,6β-Epoxysitosteryl acetate and 5,6β-epoxycampesteryl acetate were synthesized according to a procedure previously described by Kenny et al. [37] for the synthesis of 5,6β-epoxydihydrobrassicasteryl acetate. The reference compounds were stored under nitrogen at −10 °C.

### 2.4. Sample preparation

#### 2.4.1. Thermo-oxidation of stigmasterol

Stigmasterol standard (9 mg) was weighed into an 11 mL glass vial and heated in an oven at 180 °C for 60 min. The sample was cooled in a desiccator and dissolved in 1 mL of *n*-hexane/isopropanol (96:4, v/v). An aliquot of 500 μL was evaporated to dryness and acetylated using 1 mL pyridine and 100 μL acetic anhydride (room temperature, 12 h), based on a protocol of