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Development and validation of methodologies for the quantification of phytosterols and phytosterol oxidation products in cooked and baked food products

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ABSTRACT

Chromatography–mass spectrometry (GC–MS) methodologies for the analysis of the main phytosterols (PS) and phytosterol oxidation products (POPs) present in 19 different foodstuffs cooked or baked using margarines with or without added plant sterols are presented. Various methods for fat extraction were evaluated to allow the GC–MS analysis of large numbers of prepared vegetable, fish and meat products, egg and bakery items in a practically feasible manner. The optimized methods resulted in a good sensitivity and allowed the analysis of both PS and POPs in the broad selection of foods at a wide range of concentrations. Calibration curves for both PS and POPs showed correlation coefficients (R^2) better than 0.99. Detection limits were below 0.24 mg kg^{-1} for PS and 0.02 mg kg^{-1} for POPs, respectively. Average recovery data were between 81% and 105.1% for PS and between 65.5 and 121.8% for POPs. Good results were obtained for within- and between-day repeatability, with most values being below 10%. Entire sample servings were analyzed, avoiding problems with inhomogeneity and making the method an exact representation of the typical use of the food by the consumer.

1. Introduction

Plant sterols and stanols, generally referred to as phytosterols (PS), have been widely shown to exert low-density lipoprotein (LDL)-cholesterol-lowering effects. A daily intake of 1.5–3 g of PS has been documented to reduce LDL cholesterol by 7–12% [1–3]. Despite the availability of a reasonably diverse range of foods added with plant sterols, for many consumers it is not easy to obtain this level of intake. Clearly the use of margarines with added plant sterols (PS-margarines) in hot cooking methods, i.e. frying, roasting, or baking, would provide consumers with an attractive and easy route for meeting their intake goal. To generate data on the actual PS intakes and to study the fate of the sterols and the possible formation of phytosterol oxidation products (POPs) during the (hot) cooking operation, new analytical methods for monitoring the retention of the sterols in a wide range of cooked foods, as well as methods for monitoring POPs in these foods are needed.

Hot applications of PS-margarines may cause significant losses of sterols, depending on the actual foodstuff and the heating process applied [4,5]. PS are susceptible to oxidation by reactive oxygen species leading to the formation of POPs [6,7]. Although studies on the potential biological effects and safety of POPs are still scarce and contradictory [8–10], there is some concern regarding their safety [11]. Monitoring POP levels is hence relevant and their presence has actually been reported in a variety of food products [12]. Unfortunately, the formation of POPs in heated PS-margarines has only been established under standardized laboratory conditions and may therefore not fully reflect POP formation 'in the consumer's kitchen'.

For the analysis of PS and POPs in prepared foods, two routes can be envisaged: (i) fat extraction followed by analysis of the target analytes in the fat, or (ii) direct (strong) saponification of the sample [4,13,14]. To obtain the most realistic estimate of the intake of plant sterols when eating foodstuffs prepared with PS margarines we opted for the analysis of the entire sample. Direct saponification is less suited for this as it would require very large volumes of hazardous reagents. Exhaustive fat extraction followed by saponification of the fat phase only is a more practical route. Clearly this route requires method optimization for the different sample matrixes.

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The Folch [15] and Bligh and Dyer [16] methods, based on the use of mixtures of chloroform and methanol, are considered the classical and most reliable methods for extracting lipids from various types of foods [17]. But several alternative methods are available that can be easier while still providing similar extraction yields. Shin et al. [18] for example pointed out that almost no difference was observed between the fat content determined by the Folch method and that determined by automated Soxhlet (Soxtec) in bakery products. However, for some bakery samples an acid hydrolysis step prior to extraction was required to 'open' the sample matrix [13]. Modifications of the Folch or Bligh and Dyer methods to make them more environmentally friendly, i.e. using *n*-hexane:isopropanol (IPA) instead of chlorinated solvents [19], were successfully applied for lipid extraction from French fries and potato crisps [20,21]. Despite all the positive results however, careful selection of the extraction conditions remains needed and methods that perform well for one matrix might show poor recoveries for other samples.

Once the fat extract is obtained, the analysis of the PS is rather straightforward and well documented [13]. After hot saponification of the extracted fat the unsaponifiable matter is extracted with an organic solvent and, if gas chromatography (GC) is used for analysis, the PS are converted into their trimethylsilylether (TMS) derivatives [22]. Both GC with flame ionization detection (GC-FID) as well as GC-mass spectrometry (GC–MS) have been widely used [13,23,24]. Several studies have been conducted on the analysis of the sterol content by GC in foods with added PS such as spread, milk, yogurt drink, milk-based fruit beverage and fruit beverage [24–27].

The determination of POPs on the other hand is highly challenging due to the very large number of compounds, the low levels and the instable nature of the oxides [28]. Enrichment and purification by means of solid-phase extraction (SPE) or liquid chromatography (LC), for example, are needed prior to further separation and quantification by GC–MS. However, there is a clear lack of uniformity in analytical methodologies for sterol oxides studies resulting in a large variation of results in food products of very similar nature [12,14]. Most of the studies are focused on one food matrix or a few similar matrixes [26,29,30]. When studying the full range of food products that can be prepared and different cooking techniques and conditions, however, matrix interactions, chemical reactions and oxidation routes can be very different. Analytical methodologies improved in terms of selectivity and sensitivity are needed to investigate the effect of the different matrixes on oxysterol formation under specific cooking conditions.

The aim of the present study was to develop and validate GC–MS methodologies including sample preparation for the analysis of the main PS present in a large quantity and variety of prepared foods cooked and baked using PS-margarines. Additionally, methods for assessing POP formation were developed to investigate the stability of the sterols during the culinary treatments. The application of the analytical methods to a wide range of food products is discussed and practical issues seen in the analysis of large series of samples are mentioned. A summary of the quantitative results is given. Detailed quantitative results on the sterols and sterol oxides present, including the consequences for blood cholesterol levels, are presented elsewhere [31].

2. Materials and methods

2.1. Chemical and materials

2.1.1. Solvents and reagents

Chloroform, citric acid, dichloromethane, diethyl ether, ethanol, heptane, *n*-hexane, hydrochloric acid (HCl) 37%, methanol, petroleum ether, isopropanol and anhydrous sodium sulfate (purity ≥ 99%) were obtained from Merck & Co. Inc. (Whitehouse Station, NJ, USA). Brassicasterol (purity ≥ 95%), 5α-cholestane (purity ≥ 98.4%), cholestanetriol (purity ≥ 98%), cholesterol (purity ≥ 98%), 5,6β-epoxycholesterol (purity ≥ 80%), 7β-hydroxycholesterol (purity ≥ 95%), 7-ketocholesterol (purity ≥ 90%), β-sitosterol (purity ≥ 98%), stigmasterol (purity ≥ 95%) and potassium chloride (KCl) (purity ≥ 99%) were obtained from Sigma–Aldrich (Saint Louis, MO, USA). Campesterol (purity ≥ 94%) was obtained from Larodan (Soldan, Sweden). Potassium hydroxide (KOH) was from BDH Prolabo® Chemicals (VWR, Radnor, PA, USA). 19-Hydroxycholesterol (purity ≥ 99%) was from Steraloids (Wilton, NH, USA). 7α-Hydroxycholesterol (purity ≥ 99%) and 5,6α-epoxycholesterol (purity ≥ 99%) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). Tri-Sil Reagent was obtained from Thermo Fisher Scientific (Rockford, IL, USA). Demineralised water was prepared using a Merck Millipore Q-Pod Element (Merck Millipore, Billerica, MA, USA).

2.1.2. Samples

A wide variety of food samples (19 in total) prepared using standard kitchen techniques (shallow- and stir-frying and baking) were studied: Vegetable and potato samples: onion, green beans, cabbage and potato; Meat: steak, roast beef, stew, chicken, pork and minced meat; Fish samples: salmon, shallow-fried cod, microwaved cod and fish sticks; Egg; and Bakery products: sponge cake, banana bread, muffins and cookies. All ingredients used in their preparation were purchased at local supermarkets and foods were prepared in five repetitions with PS-margarine (7.5% PS equivalents) or a control margarine (control). The amounts prepared were the portion size for an individual or 2 person household. To minimize the previously discussed inhomogeneity issues, unless stated otherwise after each cooking procedure the entire food sample was collected trying to mimic as much as possible the common kitchen and eating practices. Samples were put into 500 mL prelabeled dark glass pots which were flushed with N_2 and stored at −20 °C awaiting analysis.

2.2. Lipid extraction methods

2.2.1. Folch extraction

The entire meat serving was finely minced and mixed with 250 mL chloroform:methanol (2:1, v/v). The mixture was homogenized with an Ultra-Turrax® T25 homogenizer (Jankel & Kunkel GmbH, Staufen, Germany). 50 mL of chloroform:methanol (2:1, v/v) were used to rinse all the material used and were added to the mixture. Then, the mixture was centrifuged for 10 min at 3000 rpm using a Sigma 6–15 centrifuge (Sartorius Stedim Biotech, Goettingen, Germany). Subsequently, the mixture was filtered through Whatman® filter paper, Grade 1 (GE Healthcare Life Sciences, United Kingdom) using a Büchner flask. The sample residue was re-extracted with 100 mL of chloroform and the extracts were combined. 300 mL of a KCl solution (0.88% in demineralized water) was added and the mixture was mixed gently. The biphasic system was allowed to separate at 4 °C and the upper aqueous phase was discarded. Solvent was evaporated with a BÜCHI Rotavapor® Model R-124 (Büchi, Flawil, Switzerland) with a BÜCHI B-480 waterbath and coupled to a VARIO® chemistry pumping unit PC 3001 VARIO^{pro} (Vacuubrand GMBH + CO KG, Wertheim, Germany), and further evaporated under a nitrogen stream using a Thermo Scientific™ Reacti-Vap™ Evaporator (Thermo Fisher Scientific, Inc, MA, USA) at 30 °C. The lipid content was then determined gravimetrically. The fat phase was stored under nitrogen in the dark at −20 °C untill further use.