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New method to determine free sterols/oxysterols in food matrices using gas chromatography and ion trap mass spectrometry (GC–IT-MS)

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ABSTRACT

Sterols/oxysterols in food may be free or bound i.e. esterified with fatty acids. Methods commonly applied to determine those compounds in such matrices (based on various analytical techniques) usually start with hydrolysis of the food lipid fraction, which means that the results are no good indication of concentration of free sterols/oxysterols only. But only free oxysterols are proatherogenic factors, bound ones are not. There are some published methods selectively sensitive to free oxysterols only, but they are capable to determine only a few compounds and feature very low recovery rates.

The aim of this work was to develop a method to determine various free (non-esterified) sterols/oxysterols in various food matrices. The developed method is based on the GC–IT-MS technique used in the chemical ionization mode. It was applied to determine 16 different free sterols/oxysterols in egg powder, cheese, butter, milk and salami. Fat extracted from the given matrix is purified on a specially prepared silica-gel bed to separate the sterol fraction from the oxysterol one. Sterols are silylated using N, O-bis(trimethylsilyl)trifluoroacetamide and trimethylchlorosilane BSTFA:TMCS, then GC–IT-MS analysed. The method features high recovery rates (75–95%), high reproducibility (RSD < 20%), and sensitivity within the 0.01–0.3 mg 100 g^{-1} range, depending on the analysed compound.

The method is ideally suited for determination of free sterols/oxysterols. Besides, should total concentration of both free and bound forms be of interest, food lipids may be transesterificated before the silica-gel bed purification step.

1. Introduction

Atherosclerosis is a major death cause among inhabitants of industrialized countries. Almost 60% of the Western population, including Poles, dies of vascular diseases, of which the most important are heart attacks and strokes. Pathogenesis and development of these diseases are not fully understood. Treatment and prevention are based on observations related to disease development. The first research on atherosclerosis consisted in epidemiological observations. Dependency of atherosclerosis morbidity and mortality rate on fat-high diet (mainly consisting of saturated fatty acids and rich in cholesterol > 50 mg 100 g^{-1}, (unit meaning: 50 mg of cholesterol per 100 g of product) was demonstrated [1–3].

It has long been known that improper diet results in an elevated level of cholesterol in blood plasma. As knowledge was progressing it became clear that concentration of cholesterol could not fully explain the diet-atherosclerosis relationship. Concentration of lipoproteins (primarily low-density lipoproteins, low density lipoproteins – LDL) that directly affect blood cholesterol levels and are directly involved in formation of atherosclerotic plaques [4–6] is perhaps a more important factor. Such plaques are formed of lipid-filled cells (foam cells) derived from LDL damaged by reactive oxygen species ROS like H_2O_2, O^{2-}, OH. However, it is not clear what initiates formation of the plaques. Damage to blood vessel endothelium is known as a very important factor. Monocytes accumulate in the damaged place, penetrate the vessel wall, and differentiate into macrophages. Monocytes and macrophages produce reactive oxygen species that oxidize lipoproteins (mainly LDL), next trapped by “scavenger” – type receptors for modified LDL. Foam cells formed immediately afterwards may accumulate into an atherosclerotic plaque. There is an increasing evidence that

Abbreviations: GC-IT-MS, gas chromatography connected to ion trap mass spectrometry; SiOH, silica-gel sorbent; LDL, low density lipoproteins; ROS, reactive oxygen species; BHT, butylhydroxytoluene; 3,5DIEN-7ONE, cholesta-3,5-dien-7-one; CHOL, cholesterol; 7βHC, 7β-hydroxycholesterol; 22(S)HC, 22(S)-hydroxycholesterol; 22(R)HC, 22(R)-hydroxycholesterol; S-EROL, stigmasterol; 5β,6βEC, cholesterol 5β,6β-epoxide; 7α,25DIHC, 7α,25-dihydroxycholesterol; 5α6αEC, holesterol 5α,6α-epoxide; SITO, β-sitosterol; 5α,3ONE, 5-cholesten-3-one (3-keto-5-cholestene); S-ANOL, stigmastanol; 20αCH, 20α-hydroxycholesterol; 5α,3,5,6TRIOL, 5α-cholestane-3,5,6-triol; 25HC, 25-hydroxycholesterol; 7KC, 5-cholesten-3β-ol-7-one (7-ketocholesterol); BET, betulin (syringe standard)

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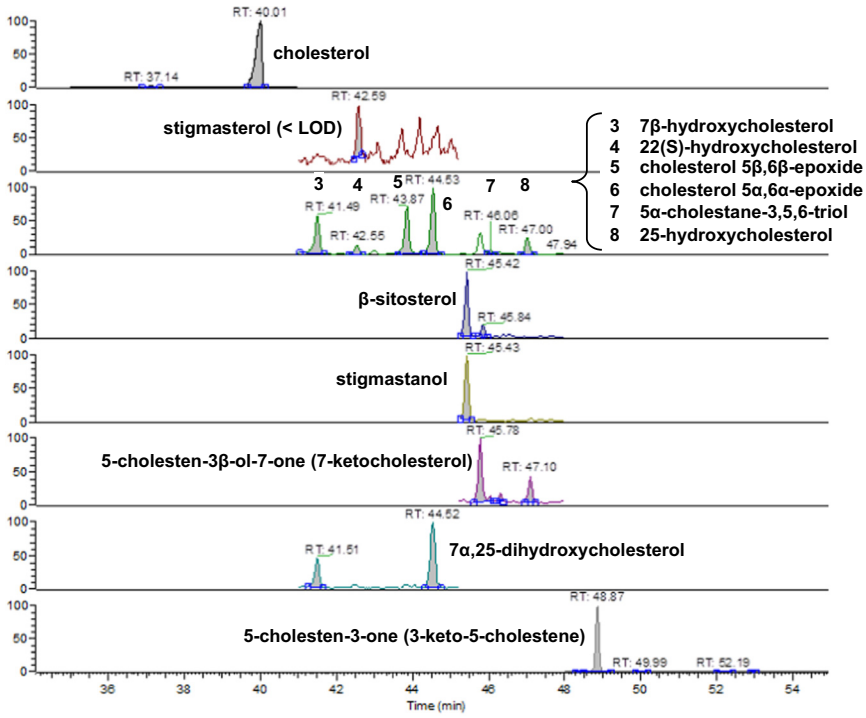

Fig. 1. MS/MS chromatogram of butter (free sterols/oxysterols only).

endothelial dysfunction may be caused by residues of oxidized fatty acids and oxidized forms of zoosterols/phytosterols (oxysterols) absorbed from food [7–10]. Recent studies have shown that oxidized sterols (both animal- and vegetable-origin) contribute to the development of atherosclerosis to a greater extent than oxidized fatty acids [11]. Studies conducted worldwide have shown that cholesterol oxidation products (both primary and secondary) are not only cytotoxic and atherogenic, but also mutagenic and carcinogenic [3,11–13].

Since concentration of oxysterols may vary widely from foodstuff to foodstuff, it is important to analyse their content in all kinds of food: fresh [14–17], processed [16,18–20], and long-term-stored [21]. That variability results from the fact that oxysterols may be formed either by enzymatic reactions [22–25] or by non-enzymatic (mainly auto-oxidation) reactions [26,27]. Temperature [20,28] and low water activity [29] catalyze oxysterol auto-oxidation reactions. To estimate daily intake of sterols/oxysterols with food it is necessary to test a large number of different foodstuffs. Therefore, from the point of view of human health it is important to study content of sterols/oxysterols in various food articles marketed in various countries.

Sterols/oxysterols appear in food in two forms: free and bound [31]. The latter are most commonly esterified with various fatty acids [32]. Most probably they are not as hazardous as free (non-esterified) forms [30] that exhibit proatherogenic activity [2,30]. To be absorbed from human digestive tract into the bloodstream they avoid hydrolyzed. However, effectiveness of enzymes that hydrolyze sterols/oxysterols within the tract is probably not high, although scientific evidence for that presumption is insufficient [30]. Free forms of sterols/oxysterols in food are more important also from the nutritional point of view as they are absorbed from human digestive tract along with fatty acids [33].

Analysing foreign literature it can be concluded that determination of sterols/oxysterols in food is always carried out after a prior acidic, alkaline, or enzymatic hydrolysis of the fat fraction. Either transesterification or saponification is applied before GC–MS or LC–MS analysis with various methods of molecule ionization [31,34]. However, basically none of the published method is capable to determine just free sterols/oxysterols, key compounds from the diet-related atherosclerosis point of view. In a method described by Guardiola et al. [35] egg powder samples were purified on a silica-gel bed and the method was indeed capable to determine free oxysterols in the samples. Unfortunately, 90% recovery rate was obtained only for 7-ketocholesterol (7KC), rates obtained for other analysed oxysterols were low or very low (about 48% for 25-hydroxycholesterol (25HC), about 6% for 7β-hydroxycholesterol (7βHC), less than 1% for cholesterol-5α,6α-epoxide (5α,6αEC), cholesterol-5β,6β-epoxide (5β,6βEC) and 5α-cholestane-3,5,6-triol (5α,3,5,6TRIOL). Methods described by Linseisen and Wolfram [33] and Helmschrodt et al. [36] were successfully used to determine free sterols/oxysterols in blood plasma. However, they were not as successful in food, a much more complicated matrix. Many authors have pointed out that successful analysis of sterols/oxysterols in food (e.g. using the GC–MS technique) is difficult due to fat content [17,37]. In his review article, Griffiths et al. [31] concluded that state-of-the art approach to analysis of sterols/oxysterols in food was based on prior hydrolysis of samples. It means that following that approach one really determines total content of sterols/ oxysterols in lipid fraction. However, as shown already in 1998 by Linseisen and Wolfram [33] and later confirmed by several authors (e.g. Garenc et al. [30]), esterified sterols/oxysterols probably do not play any significant role in formation of atherosclerotic plaques, only free forms facilitate formation of such plaques. An efficient method to determine free sterols/oxysterols in food would greatly facilitate research on enzymatic digestion of sterols/oxysterols in human gastrointestinal tract and on impact of human diet on atherosclerosis.

The aim of this study was to develop a method to determine free sterols/oxysterols in various foods. Content of free sterols/oxysterols will be compared with total content after alkaline hydrolysis using the transesterification of the lipid fraction.