ELSEVIER

Food Chemistry 68 (2000) 239–245

Food Chemistry

www.elsevier.com/locate/foodchem

Analytical, Nutritional and Clinical Methods Section

Factors affecting sample preparation in the gas chromatographic determination of plant sterols in whole wheat flour

Jari Toivo*, Anna-Maija Lampi, Satu Aalto, Vieno Piironen

Department of Applied Chemistry and Microbiology, University of Helsinki, PO Box 27, Viikki-D, FIN-00014 University of Helsinki, Helsinki, Finland

Received 18 November 1998; received in revised form 5 July 1999; accepted 5 July 1999

Abstract

A fractional factorial experimental design was applied to study factors affecting sample preparation prior to a gas chromatographic determination of total plant sterols in cereal samples. Whole wheat flour was chosen for the representative matrix. Altogether six factors were studied at two levels. The most affecting factors were a type of hydrolysis (combined acid hydrolysis and alkaline hydrolysis over alkaline hydrolysis alone), extraction solvent after saponification (chloroform over hexane–ether), and sample amount (1.0 g over 2.0 g). Also, it was shown that there is no need to perform total lipid extraction prior to hydrolysis. The use of acid hydrolysis prior to alkaline saponification was found to be essential in a plant sterol analysis of flour matrix. In a further method development, that procedure is to be optimized with various foods of plant origin in order to establish a general method to determine plant sterols. Toivo et al., Factors in the determination of plant sterols © 1999 Elsevier Science Ltd. All rights reserved.

1. Introduction

Dietary plant sterols have received increasing attention in recent years because of their positive effects on health. Experimental studies have shown that plant sterols reduce serum total cholesterol and LDL cholesterol levels (Ling and Jones, 1995). Also, animal studies have shown plant sterols to inhibit a development of certain types of cancer (Rao & Koratkar, 1997).

Howard and Kritchevsky (1997) suggested that nutritional databases must be further developed to include better information on consumption of plant sterols in our diets. To achieve this goal, appropriate and well validated methodology must exist and must be used. At the time being, many methods used in plant sterol analysis are derived from cholesterol analysis. Those methods may, however, lead to serious errors in plant sterol values, e.g. sterol glycosides are overlooked, although they are widely present in foods of plant origin and they have positive health effect (i.e. reduce serum cholesterol) (Tateo, Yoshikawa, Takeuchi, Fujii, Mizobuchi & Takeuchi, 1994).

In foods, plant sterols occur as free sterols, sterol esters, sterol glycosides, and acylated sterol glycosides (Fig. 1). Currently, the most common methods for determination of plant sterols involve extraction of the lipid fraction from sample material followed by alkaline hydrolysis (saponification), extraction of the nonsaponifiables, derivatization of sterols, and separation and quantification of the sterol derivatives by gas chromatography using a capillary column. This approach has been applied, for example, by Thompson and Merola (1993) who developed a simplified alternative method to the AOAC Official Method, sections 43.283–43.291 (AOAC, 1984) to determine cholesterol in multicomponent foods. The saponification step was shortened, for instance. This method was reported also to be capable of determining major plant sterols.

Alternatively, samples are directly saponified and then the nonsaponifiables are extracted as presented, for example, by Klatt, Mitchell and Smith (1995) who proposed a direct saponification method for determination of cholesterol in various foods. Their method was a modification of AOAC Official Method, sections 976.26 and 983.23, and it was adopted first action, as section 954.03, by AOAC (1995). Also, it has been recommended that direct saponification can be used to determine plant sterols in pasta products (Kovacs, 1990).

* Corresponding author. Tel.: +358-9-7085-8407; fax: +358-9-7085-8475.

E-mail address: jari.toivo@helsinki.fi (J. Toivo).

0308-8146/99/$ - see front matter © 1999 Elsevier Science Ltd. All rights reserved.
PII: S0308-8146(99)00201-0

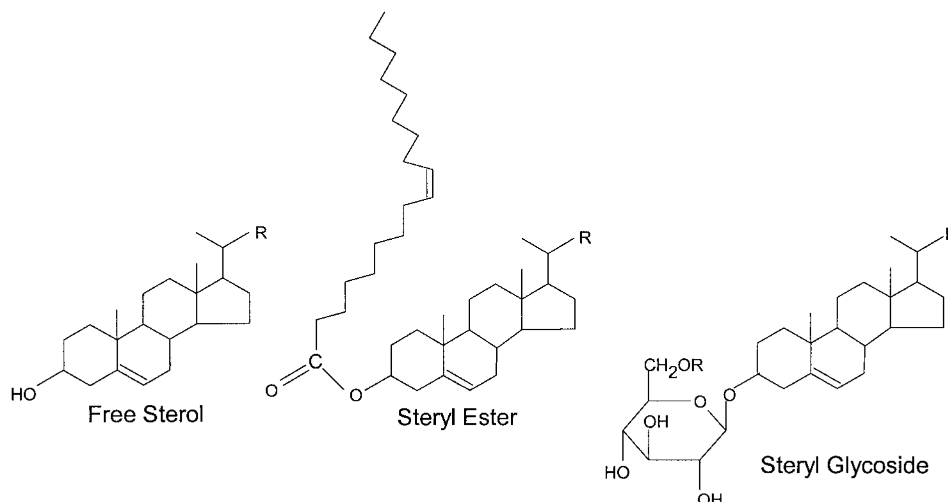

Fig. 1. Basic structures of free alcoholic sterol and two forms of bound sterols: esterified and glycosylated, respectively. *R* (side chain) varies between plant sterols, *R'* corresponds to hydrogen or acyl residue.

It must be pointed out that there is not available any official method to determine especially plant sterols generally in foods. In determination of plant sterols, free sterols as well as esterified sterols are included in total sterol yield when methods involving alkaline hydrolysis are used. However, sterol glycosides are totally overlooked, because the acetal bond between the sterol hydroxyl group and the sugar (Fig. 1) cannot be hydrolysed in alkaline conditions. This leads to underestimation of total plant sterol concentration in sample material. Inclusion of an acid hydrolysis step has been suggested as one alternative to release sterols from their glycosides (Jonker, van der Hoek, Glatz, Homan, Posthumus & Katan, 1985). This problem does not occur when only cholesterol is to be determined, because it is not present as glycosides.

The aim of this study was to examine which factors are affecting sample preparation in the gas chromatographic determination of plant sterols in cereals. Whole wheat flour was chosen to act as a sample matrix, because it was known to contain appreciable amounts of sterols in their all main classes (i.e. free sterols and bound sterol esters, sterol glycosides, and acylated sterol glycosides). In this study, a fractional factorial experimental design was applied to study six different factors. Fractional factorial design experiments are useful when significant factors as well as interactions between the given factors are to be identified in processes (Haaland, 1989). Here, our approach was to apply a method developed in our laboratory to determine sterols in edible oils and fats (Toivo, Piironen, Kalo & Varo, 1998) with modifications to study affecting factors in the determination of plant sterols in cereal matrix. Further, findings of this study will be used in a focused method optimization in order to establish a general, well-validated, method to determine plant sterols in various food matrixes of plant origin.

2. Materials and methods

2.1. Sample matrix, standards and reagents

Whole wheat flour (Melia, Raisio, Finland) used as a sample matrix was bought in a local grocery store. The flour matrix was stored in small portions in polyethylene plastic bags in the darkness at -18°C until analyzed.

Betulin (Lup-20[29]-ene-3β,28-diol) used for an internal standard was purity of $>98\%$ and obtained from Sigma Chemical Co. (St. Louis, MO, USA).

All organic solvents used were HPLC-grade and purchased from Rathburn Chemicals Ltd. (Walkerburn, UK), except diethyl ether was p.a. grade and obtained from E. Merck (Darmstadt, Germany). Potassium hydroxide and hydrochloric acid were p.a. grade and purchased from E. Merck. Milli-Q Plus water (Millipore, Bedford, MA, USA) was used in the study.

2.2. Gas chromatography

GC analysis of derivatized sterols was performed with a Hewlett-Packard 5890 Series II gas chromatograph equipped with a flame ionization detector (300°C), an on-column injector (70°C), and a 25 m ×0.32 mm i.d. NB-17 (50% phenyl–50% methylpolysiloxane, film thickness 0.25 μm) capillary column (Nordion, Finland). Column temperature program used was: 70°C (1 min); 60°C min^{-1} ramp to 245°C (1 min hold step); 3°C min^{-1} ramp to 275°C and 10 min hold. Helium was used as a carrier gas at 1.4 ml min^{-1}.

2.3. Mass spectrometry

Sterols were analyzed on a Finnigan MAT Incos 50 (San Jose, CA, USA) mass spectrometer connected to a Varian 3400 (Walnut Creek, CA, USA) gas chromatograph. An

NB-17 (25 m×0.32 mm i.d., film thickness 0.25 μm) column was used with oven temperature 255°C (isothermal) and helium as carrier gas at 10 psi. Splitless injection of 1.0 μl was performed at 270°C. The MS spectra of sterols was obtained at an ionizing voltage of 70 eV when ion-source and transfer-line temperatures were 160 and 260°C, respectively.

2.4. Experimental design

When the experimental design was set up, a method developed and used in our laboratory (Toivo et al., 1998) was chosen to act as a basic method. This method was originally developed to determine major sterols in edible oils and fats. Now, we wanted to examine its application to determine sterols in cereal matrix. Briefly, in that method sample was saponified in KOH by refluxing 20 min at 80°C. Then, internal standard, betulin, was added and nonsaponifiables were extracted with chloroform. Sterol extract was further purified using C18 solid-phase extraction (SPE). Finally, sterols were derivatized to their trirnethylsilyl ethers (TMS) and analyzed by capillary gas chromatography (GC).

A Taguchi (1986) fractional factorial design was chosen to be applied, because it allows to examine many factors and possible factor interactions at the same time. Earlier, same kind of an experimental design system has been successfully used by Oles et al. (1990) for determination of cholesterol in food matrixes. A 16-trial orthogonal array was chosen as the experimental design, when six factors at two levels were to be examined. Factors and their levels are shown in Table 1 and the analytical conditions in 16 trials are shown in Table 2. In the experimental design, Level 1 always corresponds to the analytical condition in a basic method (briefly described above), while Level 2 is an alternative condition.

Total lipid extraction (Factor A) was found to be essential to examine, because its existence may play an important role on sterol recovery. In cholesterol analyses it has been shown that direct saponification may lead to a better recovery (Fenton, 1992). On the other hand, it can be assumed that total lipid extraction procedures do not totally extract polar sterol conjugates (i.e. sterol glycosides) which leads to underestimation of a total sterol value in a sample.

In hydrolysis procedure (Factor B) we wanted to study if acid hydrolysis step prior to alkaline hydrolysis is producing a greater sterol yield, i.e. to include sterol glycosides as reported by Jonker et al. (1985).

Solvent to extract nonsaponiflables (Factor C) was chosen to be examined, because it was shown in our preliminary studies that the extraction of nonsaponifiables is a critical step to maintain a good sterol recovery. Different approaches were chosen to be studied: chloroform forms a lower phase while hexane–diethyl ether forms an upper phase.

Also, two different approaches were studied in SPE cleanup step (Factor D). C18 SPE was a reversed-phase system while silica SPE represented a normal phase mode. An elution solvent used for silica SPE was optimized in our preliminary studies.

Introduction point of an internal standard (Factor E) may be very critical to the sterol value obtained. We wanted to study if there is a significant effect on sterol value when an internal standard is added after saponification or in the beginning of the assay. Betulin (1.00 mg/sample) was used as an internal standard.

Sample amount (Factor F) was varied from 1.0 to 2.0 g. Because the volumes of all solutions were kept constant all the time, different ratios of sample amount to hydrolysis and extraction solutions were produced.

2.5. Description of the methods in sample preparation

2.5.1. Total lipid extraction

A method used for a total lipid extraction (Level 2) was a modification of widely used Folch (1957) procedure as described by Christie (1987). Extractions were carried out in screw-capped test tubes of 50 ml. Extraction was repeated three times. Each time 30 ml of extraction solvent was used and the tubes were shaken for 20 min. All three extracts were filtered and combined and then evaporated to dryness in a rotavapor at 40°C.

2.5.2. Hydrolysis

In a saponification step (Level 1), 7 ml of 0.5 M ethanolic KOH was added and the sample refluxed for 20 min at 80°C. After saponification the sample was let to cool to room temperature before adding any extraction solvent (i.e.

Table 1
Factors and their two levels examined

Factor	Level 1	Level 2
A. Total lipid extraction	No	Yes
B. Hydrolysis	Alkaline	Acid + alkaline
C. Extraction solvent after saponification	Chloroform	Hexane–diethyl ether (85 + 15)
D. SPE-cleanup	C18	Silica
E. Introduction point of an internal standard	After saponification	In the beginning of the assay
F. Sample amount	1.0 g	2.0 g

Table 2
The analytical conditions and the total plant sterol content for each trial

Trial	A. Total lipid extraction	B. Hydrolysis	C. Extraction solvent	D. SPE-cleanup	E. Internal standard introduction	F. Sample amount (g)	Total plant sterols,[a] mean ± SD (mg/100 g)
1	No	Alkaline	$CHCl_3$	C18	After hydrolysis	1.0	58.2 ± 1.5
2	No	Alkaline	$CHCl_3$	Silica	Beginnning	2.0	33.7 ± 0.9
3	No	Alkaline	Hexane–ether	Silica	After hydrolysis	2.0	50.3 ± 3.1
4	No	Alkaline	Hexane–ether	C18	Beginnning	1.0	35.0 ± 0.5
5	No	Acid + alkaline	$CHCl_3$	C18	After hydrolysis	1.0	66.0 ± 2.1
6	No	Acid + alkaline	$CHCl_3$	Silica	Beginnning	2.0	47.4 ± 8.8
7	No	Acid + alkaline	Hexane–ether	Silica	After hydrolysis	2.0	44.5 ± 2.5
8	No	Acid + alkaline	Hexane–ether	C18	Beginnning	1.0	54.4 ± 2.2
9	Yes	Alkaline	$CHCl_3$	C18	After hydrolysis	2.0	27.7 ± 2.2
10	Yes	Alkaline	$CHCl_3$	Silica	Beginnning	1.0	63.2 ± 2.9
11	Yes	Alkaline	Hexane–ether	Silica	After hydrolysis	1.0	47.7 ± 9.1
12	Yes	Alkaline	Hexane–ether	C18	Beginnning	2.0	24.5 ± 5.3
13	Yes	Acid + alkaline	$CHCl_3$	C18	After hydrolysis	2.0	64.1 ± 6.3
14	Yes	Acid + alkaline	$CHCl_3$	Silica	Beginnning	1.0	57.0 ± 0.6
15	Yes	Acid + alkaline	Hexane–ether	Silica	After hydrolysis	1.0	36.0 ± 2.4
16	Yes	Acid + alkaline	Hexane–ether	C18	Beginnning	2.0	48.1 ± 0.4

[a] A sum of β-sitosterol, campesterol, stigmasterol, and Δ5-avenasterol, mean ± SD for triplicate determinations.

Level 1: chloroform, and Level 2: hexane–diethyl ether, 85:15, v/v) to extract nonsaponifiables.

In an acid hydrolysis step (Level 2), 7 ml of 1.0 M ethanolic HCl was added and then refluxed for 20 min at 80°C. After the sample was let to cool to room temperature it was neutralized using 10 M NaOH solution. The pH checked (pH 6–7) sample was then saponified, and further extraction solvent was added as above.

2.5.3. *SPE-cleanup*

A 1 ml aliquot from the organic layer was taken into a cleanup step. Sample solution was pH adjusted (pH about 5) with 6 M HCl and passed through a 0.45 mm nylon membrane fitter (Whatman Inc., Clifton, NJ, USA).

In an SPE procedure a vacuum manifold (Bond-Elut, Varian, Harbor City, CA, USA) was used. In a case of C18 SPE (Mega Bond-Elut, 1.0 g, Varian) cartridge (Level 1) was activated with 5 ml methanol followed by 5 ml deionized water, while in case of silica SPE (Extra-Sep, 1.0 g, Lida Corp., Kenosha, WI, USA) cartridge (Level 2) was activated with 5 ml hexane.

In C18 SPE sterols were eluted with 15 ml 5% methanol in chloroform. When silica SPE was used sterols were eluted with 10 ml 1% isopropanol in hexane. Solutions were eluted dropwise until the cartridges were dry. The eluates were evaporated to dryness under vacuum in a rotary evaporator and then redissolved to 0.5 ml dichloromethane.

2.6. *Identification and quantitation*

Sterols were analyzed on GC as their trimethyisilyl (TMS) ether derivatives. Throughout the study, samples were silylated at the same way. In a presilanized sample vial, solvent was evaporated under nitrogen (50°C) and then 100 µl of anhydrous pyridine (E. Merck, Darmstadt, Germany) and 100 µl of derivatization reagent containing 99% of BSTFA (E. Merck, Darmstadt, Germany) and 1% of TMCS (Fluka Chemie AG, Switzerland) were added. To complete the silylation, solutions were allowed to stand overnight at room temperature before GC-analysis.

The GC–MS analysis was used to identify the major sterols in sample matrix. MS analysis was also used to evaluate peak purities of sterols. Later, however, routine analyses were carried out by using a GC equipped with flame ionization detector. Then, sterols were identified according to their relative retention times.

The sterols quantitated were sitosterol, campesterol, stigmasterol, and Δ5-avenasterol. Quantitation was based on an internal standard method (betulin). GC calibration procedure and GC performance has been reported earlier (Toivo et al., 1998). In brief, all the sterols quantified were well separated under GC conditions used and, also, according to MS analysis all these sterols eluted as pure compounds. The limit of determination for individual sterol was 2 mg/100 g.

2.7. *Analysis of results*

In the experiment, the output variable for the given trial was a total plant sterol value (a sum of sitosterol, campesterol, stigmasterol, and Δ5-avenasterol) obtained in sample matrix in the corresponding analytical conditions. Each trial was performed in triplicate, and for each trial average value and standard deviation were calculated. Further, the relative significance (ρ, %) of each factor

and interaction of certain factors (A×B, C×D, B×E, B×F, and A×E) was determined after insignificant factors were pooled. Data analysis was conducted using ANOVA procedure (Taguchi, 1986).

3. Results and discussion

The goal of this experiment was to identify analytical conditions that yielded the greatest total plant sterol value in whole wheat flour sample matrix. Means of total plant sterol values and their standard deviations from triplicated determinations of each trial are shown in Table 2. In all, it can be seen that there was a wide range in the 16 trials: total plant sterol value varied from 24.5 to 66.0 mg/100 g. Relative standard deviations (RSD) were acceptable (<10%) in most cases, an average RSD being 7.2%. However, in three trials RSD was about 20%. A wide range in sterol values between the trials suggested that there will be some factors that have more effect on sterol yield than the others. Trial 1 totally corresponded our basic method developed and validated for determination of sterols in edible oils and fats. That method yielded a total sterol value of 58.2 mg/100 g and its repeatability was very good (RSD 2.6%, $n=3$). Individual sterol values were as follows: sitosterol 40.0, campesterol 13.4, stigmasterol 2.7, and Δ5-avenasterol 2.1 mg/100 g. The relative proportions of individual sterols appeared to remain constant in different analytical variations studied. This finding suggests that the chemical behaviour between these individual sterols was the same under the given conditions. Later, only total sterol values are commented upon.

The ANOVA table obtained based on a statistical data analysis is shown in Table 3. Significant main effects and interactions are illustrated in Fig. 2. The significant factors affecting the total plant sterol value were a hydrolysis procedure used (Factor B), an extraction solvent after saponification (Factor C) and a sample amount (Factor F). Each of them corresponded to about 20% relative significance. Also, significant were interaction of hydrolysis procedure (Factor B) and sample amount (Factor F) as well as interaction of total lipid extraction step (Factor A) and introduction point of an internal standard (Factor E).

Table 3
ANOVA table for the affecting factors and their interactions

Source[a]	D_f[b]	S[c]	F[d]	S'[e]	ρ(%)[f]
A	1	27.98			
B	1	372.49	12.0	366.26	20.5
C	1	369.90	11.9	363.37	20.3
D	1	0.17			
E	1	60.30			
F	1	372.10	11.9	365.87	20.5
A×B	1	3.03			
C×D	1	60.29			
B×E	1	37.10			
B×F	1	215.50	6.9	209.27	11.7
A×E	1	269.12	8.6	262.89	14.7
Error	5	31.15		218.02	12.2

[a] See Materials and methods for details.
[b] D_f = degrees of freedom.
[c] S = sum of squares.
[d] $F = S$/error.
[e] S' = sum of squares after pooling the insignificant factors with error.
[f] ρ = relative significance of main effects.

3.1. Hydrolysis

A combination of acid hydrolysis and alkaline saponification yielded about 20% greater sterol value than alkaline saponification alone (Fig. 2). This is in agreement with findings of Jonker et al. (1985). It can be assumed that the increase in sterol values was due to the fact that acid hydrolysis liberated glycosidic sterols from their sugar conjugates. However, it must be pointed out here that no optimization for acid hydrolysis conditions was carried out during this study. Therefore, it cannot be strictly concluded that there is a 20% proportion of sterol glycosides in total sterols in a whole wheat flour studied. Acid hydrolysis step will be a very important subject to focus on in the further method development. Jonker et al. (1985) reached an increase of tens of percentages in sterol values in various plants when acid hydrolysis (6 M HCl, 30 min in a boiling water bath) combined to saponification was compared to the values obtained from saponification only.

3.2. Extraction solvent

Chloroform was shown to be more effective in the extraction of the nonsaponifiables than a solvent combination of hexane–diethyl ether (85+15, vol/vol). In preliminary tests, it was found that the both are very effective in dissolving sterols. An important remark here, however, is that after saponification chloroform phase was separated better than hexane–diethyl ether phase. In some cases with hexane–diethyl ether, a clear organic layer was not formed. This may have had an effect on sterol yields.

3.3. Sample amount

A remarkably greater sterol value was achieved when a sample amount of 1.0 g was used rather than 2.0 g. It can be hypothesized that, in these conditions, the sample amount of 2.0 g was too large for the quantitative liberation of the bound sterols in the matrix. This hypothesis leads to a question if 1.0 g also was too large sample size. Therefore, this issue was later studied with a sample size of 0.5 g and it was shown that the total sterol yield did not significantly differ from the one with

a sample size of 1.0 g. This subject, however, would need to be studied in a further method optimization with different sample matrixes.

3.4. Interaction of hydrolysis and sample amount

When alkaline saponification alone was used as a hydrolysis procedure there was a remarkable difference in sterol values with different sample amounts (Fig. 2). When sample amount was doubled from 1.0 to 2.0 g, sterol value was decreased nearly by 20 mg/100 g. There was also a slight decrease in a case of combined acid hydrolysis and alkaline hydrolysis. That difference was, however, only 2–3 mg/100 g. It was clearly shown that the conditions used in alkaline saponification were not powerful enough to quantitatively liberate and extract sterols from the sample matrix. Assumably, acid hydrolysis liberated glycosylated sterols very effectively. Later, in the saponification step, these sterols already were in free alcoholic form. Also this interaction of hydrolysis and sample amount suggests that a sample amount must always be optimized to certain hydrolysis conditions.

3.5. Interaction of total lipid extraction and introduction point of an internal standard

Data clearly shows (Fig. 2) that when there was no total lipid extraction and betulin was used as an internal standard, it is to be introduced after saponification. This was in agreement with the finding observed when the basic method (Toivo et al., 1998), same as Trial 1, was developed and validated. Basically, an internal standard should be added to the sample at the earliest possible step to compensate any possible losses which can take place during different steps. When a total lipid extraction step was included, it was found that sterol values were higher when an internal standard was introduced in the beginning of the assay. It can be assumed that a sterol loss occurred during the total lipid extraction and to compensate this it was necessary to add internal standard before the initial lipid extraction step. It is well worth noting that an introduction point of an internal standard alone, however, was not a significant factor.

3.6. Factors that were not affecting sterol value

In this experiment setup, existence of a total lipid extraction step, SPE-cleanup, and introduction point of an internal standard were not significantly affecting sterol values obtained in whole wheat flour sample matrix. In recent years, many methods published have omitted a total lipid extraction step. It has been shown that in determination of cholesterol in samples of animal origin direct hydrolysis can even produce a better recovery than methods including total lipid extraction (Fenton, 1992). However, sterols are known to be differently bound in sample matrixes of plant origin. Our study shows that a total lipid extraction step can be included, but, on the other hand, is not essential.

When total lipid extraction step is included, analysis time needed is notably longer. Also, worth noting is that the need of organic solvents in assay is much greater when a total lipid extraction step is used. However, total lipid extraction will be useful, if there is also an interest in other lipid fractions, i.e. fatty acid composition, and also gravimetric total fat content can be determined (Phillips et al., 1997).

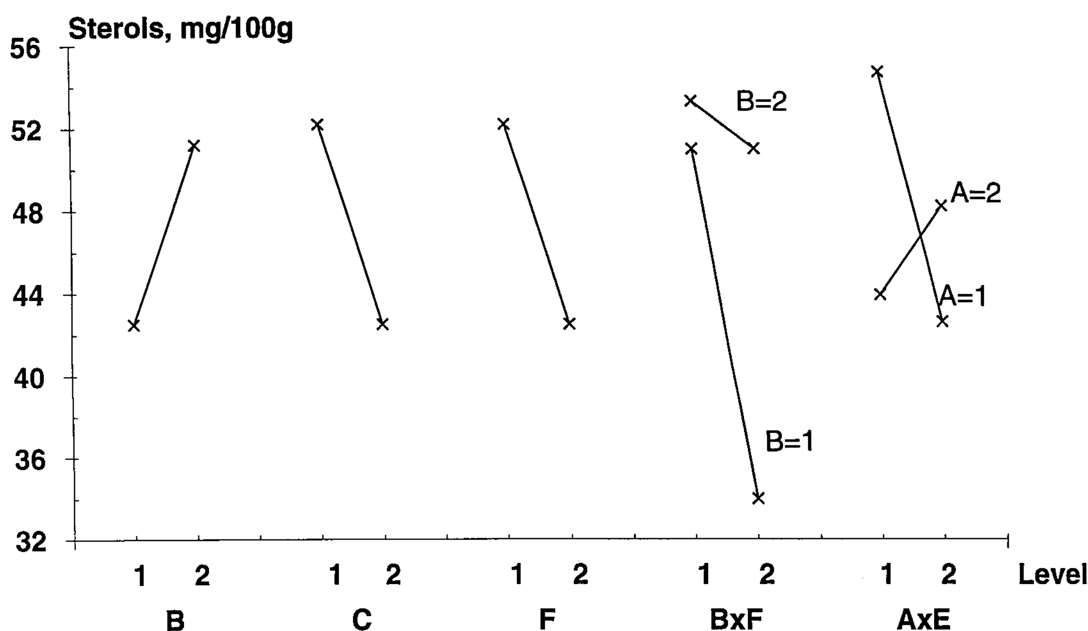

Fig. 2. Significant main effects and interactions. See Materials and methods for details.

Both SPE-cleanup methods used in this study were well validated. It was shown that the both methods were able to extract and purify sterols from the fraction of nonsaponifiables. This experiment confirmed that there was no difference in those cleanup methods that affected sterol values. Later, it would be interesting to examine if it is possible to analyze sterols from the solvent-extracted nonsaponifiables without further purification.

3.7. *Analytical points to focus on in the further method optimization*

In order to establish a general method to determine plant sterols — including free and different classes of bound sterols — in foods, the optimization of certain analysis steps is needed. Also, a wider range of sample matrixes must be studied. Based on the findings of this study, the most important point to focus on was the optimization of the acid hydrolysis step. In addition, the sample amount of a given sample matrix must be optimized and an importance of a further cleanup of nonsaponifiables is to be examined.

Acknowledgements

The authors would like to thank Leena Laukkanen for technical assistance and the Academy of Finland and Fund of Jenny and Antti Wihuri for support.

References

AOAC (1984). *Official methods of analysis of the association of official analytical chemists* (14th Ed.). Arlington, VA: AOAC.

AOAC (1995). *Official methods of analysis of the association of official analytical chemists* (16th Ed.). Arlington, VA: AOAC International.

Christie, W. W. (1987). *High-performance liquid chromatography and lipids*. Oxford, UK: Pergamon Press.

Fenton, M. (1992). Chromatographic separation of cholesterol in foods. *Journal of Chromatography, 624*, 369–388.

Folch, J. (1957). A simple method for the isolation and purification of total lipids from animal tissues. *Journal of Biological Chemistry, 226*, 497–509.

Haaland, P. D. (1989). *Experimental design in biotechnology*. New York : Marcel Dekker Inc.

Howard, B. V., & Kritchevsky, D. (1997). Phytochemicals and cardiovascular disease. A statement for healthcare professionals from the American Heart Society. *Circulation, 95*, 2591–2593.

Jonker, D., van der Hoek, G., Glatz, J. F. C., Homan, C., Posthumus, M. A., & Katan, M. B. (1985). Combined determination of free, esterified and glycosilated plant sterols in foods. *Nutrition Reports International, 32*, 943–949.

Klatt, L. V., Mitchell, B. A., & Smith, R. L. (1995). Cholesterol analysis in foods by direct saponification–gas chromatographic method: collaborative study. *Journal of the Association of Official Analytical Chemists International, 78*, 75–79.

Kovacs, M. I. P. (1990). Determination of cholesterol in pasta products using gas–liquid chromatography. *Journal of Cereal Science, 11*, 291–297.

Ling, W. H., & Jones, P. J. H. (1995). Dietary phytosterols: a review of metabolism, benefits and side effects. *Life Sciences, 57*, 195–206.

Oles, P., Gates, G., Kensinger, S., Patchell, J., Schumacher, D., Showers, T., & Silcox, A. (1990). Optimization of the determination of cholesterol in various food matrixes. *Journal of the Association of Official Analytical Chemists, 73*, 724–728.

Phillips, K. M., Tarrago-Trani, M. T., Grove, T. M., Grün, I., Lugogo, R., Harris, R. F., & Stewart, K. K. (1997). Simplified gravimetric determination of total fat in food composites after chloroform-methanol extraction. *Journal of the American Oil Chemists Society, 74*, 137–142.

Rao, A. V., & Koratkar, R. (1997). Anticarcinogenic effects of saponin and phytosterols. In F. Shadidi, *Antinutrients and phytochemicals in food* (pp. 313–324). Washington: (ACS Symposium Series). American Chemical Society.

Taguchi, G. (1986). *Introduction to quality engineering*. Dearborn, MI: American Supplier Institute.

Tateo, M., Yoshikawa, M., Takeuchi, H., Fujii, S., Mizobuchi, H., & Takeuchi, H. (1994). Effects of sterylglycosides from soybean on lipid indices in the plasma, liver, and faeces of rats. *Bioscience, Biotechnology, and Biochemistry, 58*, 494–497.

Thompson, R. H., & Merola, G. V. (1993). A simplified alternative to the AOAC official method for cholesterol in multicomponent foods. *Journal of the Association of Official Analytical Chemists International, 76*, 1057–1068.

Toivo, J., Piironen, V., Kalo, P., & Varo, P. (1998). Gas chromatographic determination of major sterols in edible oils and fats using solid-phase extraction in sample preparation. *Chromatographia, 48*, 745–750.