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Effect of water cooking on free phytosterol levels in beans and vegetables

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Abstract

Plant sterols (phytosterols) are known to decrease plasma cholesterol, mainly the atherogenic LDL cholesterol. In an earlier study, the thermal stability of phytosterols in vegetable oils was reported. The aim of this present work was to investigate the potential effect of cooking (30 min in boiling water), for eight plant products (broad bean, celery, cabbage, courgette, carrot, cauliflower, onion, pepper), on the free phytosterol level. Sitosterol was the most abundant sterol, followed by campesterol. After cooking, the level of total sterols was higher in all vegetables than that before cooking, if dry matter is considered. Acid hydrolysis (active for glycosylated phytosterols) yielded a higher sterol value than alkaline hydrolysis alone (active for esterified phytosterols). This indicated that studied vegetables contained appreciable amounts of steryl glycosides. Their cooking induced higher values of free phytosterols. Cooked vegetables could give better protection against cardiovascular diseases thanks to higher phytosterol levels.

Keywords: Cardiovascular diseases; Cooking; Free phytosterol determination; GC; Plant products; Vegetables

1. Introduction

Plant sterols (phytosterols) are a group of naturally occurring compounds derived from the 1,2-cyclopentanophenanthrene structure. The most abundant are β-sitosterol (24-α-ethylcholesterol), campesterol (24-α-methylcholesterol) and stigmasterol (Δ-22, 24-α-ethylcholesterol). β-sitosterol usually makes up more than 50% of the desmethyl sterols. In unprocessed foodstuffs, sterols can be found as free sterols (alcohol form) or as several types of conjugates in which the 3β-hydroxyl group is esterified by a fatty-acid, ferulic acid or a hydroxycinnamic acid or glycosylated with a hexose (generally glucose) or a 6-fatty acyl hexose. Glycosides seem to be the most common form found in vegetables (Piironen & Lampi, 2004).

Phytosterols are known to have hypocholesterolemic properties. Their esters and the saturated (phytostanols) analogues have been suggested as effective-lowering agents offering cardiologic health benefits. The higher the dietary intake of phytosterols, the lower the cholesterol absorption and the lower the serum cholesterol level. Phytosterol potency in decreasing serum atherogenic low-density lipoprotein (LDL) cholesterol levels and thus, in protecting against cardiovascular diseases, mainly coronary heart disease, has led to the development of functional foods enriched with plant sterols. The level of protective high-density lipoprotein (HDL) cholesterol was unaffected

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(Piironen & Lampi, 2004; Piironen, Lindsay, Miettinen, Toivo, & Lampi, 2000).

So, in Western countries, commercial spreadable fats, margarines, milks and yogurts formulated with esterified phytosterols are available. The usual human diet currently contains around 200–300 mg day^{-1} of phytosterols (Gylling & Miettinen, 1999). The dose of phytosterols seems to be important, such that about 2 g day^{-1} of sterols seems to offer an ideal dose for significant cholesterol lowering. Higher doses may not improve efficacy and may produce adverse effects (Awad, Downie, Fink, & Kim, 2000). Several studies have indicated that phytosterols may have health-promoting effects such as anticancer activity (breast cells, prostate cells, colon etc.) (Awad et al., 2000). Phytosterols were also precursors in the synthetic synthesis of several steroid hormones (Moreau, Whitaker, & Hicks, 2002).

An earlier paper showed the thermal stability of phytosterols in vegetable oils at 100 °C during 1 h. Degradation was observed above 140 °C (Thanh et al., 2006). Several studies on phytosterol oxidation (at high temperature and/or under UV or day light) were done with GC/MS characterisation of oxidized compounds (Dutta, Przybylski, Appelqvist, & Eskin, 1996; Lutjohann, 2004; Porter, Caldwell, & Mills, 1995; Savage, Dutta, & Rodriguez-Estrada, 2002; Smith, 1996; Yoon & Kim, 1994).

Typically, the analysis of individual sterols includes the acid hydrolysis of glycosylated sterols, then extraction of free and esterified sterols by the Soxhlet method, basic hydrolysis of esterified sterols. Both column chromatography (CC) and thin-layer chromatography (TLC) are adequate for sample clean up and purification. Finally, free phytosterols are determined by capillary gas chromatography (GC), after derivatisation to their trimethylsilyl (TMS) ethers, which offer higher thermostability, lower polarity and improved peak shape (Abidi, 2001; Lagarda, Garcia-Llatas, & Farre, 2006; Päivi, 2005). The ISO 12228 norm method was used, but with small modifications adapted for vegetable analysis (ISO (International Organization for Standardization), 1999).

To our knowledge the influence of cooking vegetables and fruits at 100 °C in boiling water on phytosterol level was not yet studied. The aim of this present work is to show the potential effect of cooking different foods (beans and vegetables) on the free phytosterol levels.

2. Materials and methods

2.1. Standards, reagents, samples

Analytical grade standard stigmasterol was obtained from the ICN Company (91400 Orsay, France). A mixture of standards (β-sitosterol, campesterol and stigmasterol) was obtained from ICN. Δ5-Avenasterol and Δ7-Avenasterol were characterised from rapeseed oil.

All reagents were of high purity. Ethanol, *n*-hexane, hydrochloric acid, potassium hydroxide and sodium hydroxide were obtained from Carlo-Erba (27106 Val de Reuil, France). The derivatisation reagents *n*-methyl-*N*(trimethylsilyl)-heptafluorobutyramide (MSHFBA) and 1-methyl imidazole were obtained from Sigma Compagny (38297 Saint Quentin Fallavier, France).

Celite®545 (CAS 68855-54-9) and aluminium oxide (alumina CAS 1344-28-1) were provided by Acros Organics (93166 Noisy-le-Grand, France and SDS (13124 Peypin, France) companies, respectively. TLC silica plates (20 × 20 cm, 0.25 mm Kieselgel 60 film thickness) were obtained from Merck Company (94126 Fontenay-sous-Bois, France).

Milky drink Pro-Activ® (Fruit d'Or Company - France), enriched with phytosterol esters, was analysed in repeatability tests.

Eight plant products (broad beans; green vegetables such as stick of celery, cabbage, courgette; not green vegetables such as red carrot, white cauliflower, yellow onion, red pepper), cultivated in France, were studied. They were bought from grocery stores. Each of them was obtained from equal mixture of 3–6 different varieties. Lyophilised cabbage, obtained by us, was analysed in repeatability tests.

2.2. Sample preparation

The moisture contents of the fresh or cooked products were determined by azeotropic distillation with toluene using the Dean Stark apparatus as described in European Pharmacopoeia current edition. All samples were homogenised in a grinder or a commercially available grain mill. Cooked vegetables were obtained after 30 min in boiling water. After spinning, they were homogenised like uncooked vegetables.

2.3. Analytical methods

2.3.1. Acid hydrolysis

Each sample was weighed to approximately 2.5 g. Sample was put in a 250 ml flask with 100 ml of 3 M HCl. Mixture was heated for 1 h and shaken vigorously. Then, the flask was cooled to ambient temperature. About 2.5 g Celite were added. After shaking, the mixture was filtered on a double humid filter paper. The solid residue was washed by bi distilled water until neutrality of the washing water. We drained the mixture for 2 h and then dried at 105 °C over 90 min.

2.3.2. Solvent extraction

The double filter with the solid residue was placed in a Soxhlet apparatus. A 250 ml flask with 200 ml of hexane was used. Reflux heating, with shaking was carried out for 6 h. Then the content of the flask was evaporated under reduced pressure thanks to a rotary evaporator.

2.3.3. Basic hydrolysis and purification

The residue obtained after solvent volatilisation was transferred to a 50 ml flask. Five ml 0.5 M alcoholic KOH and 1.00 ml betulin solution as internal standard (1.00 mg/ ml of acetone) were added. Saponification and

double purification (column chromatography and thin-layer chromatography) were done according to ISO 12228 norm (ISO (International Organization for Standardization), 1999).

2.3.4. Silylation

A 100 µl mixture of MSHFBA and 1-methyl imidazole (20/1; v/v) was added to this purified residue of phytosterols. The flask was heated at 105 °C for 15 min. After cooling, the excess was volatilised under nitrogen. Finally, the residue was dissolved in 1000 µl of hexane before GC injection.

2.4. GC conditions

2.4.1. Qualitative analysis

GC-MS was used to identify phytosterols in the vegetable samples. GC analysis of the derivatised sterols was performed with a Hewlett-Packard 5890 instrument (Agilent Technologies, Massy, France) equipped with a quadrupole MS Hewlett-Packard 5972. A capillary column (30 m × 0.25 mm id × 0.25 µm film thickness HP5-MS) was used. Splitless injection of 2 µl was performed at 280 °C. The initial column temperature was held at 100 °C for 2 min and then ramped at 20 °C min^{-1} to 280 °C and 1 °C.min^{-1} until 290 °C. TMS-phytosterols were identified by SCAN technique according to their retention times (RT) and mass spectra. The results of standards (RT and specific *m/z* peaks of MS) are shown in Table 1.

2.4.2. Quantitative analysis

GC-FID is most commonly used to quantify sterols in various sample matrixes by virtue of a large linear mass range, of response of the FID (flame ionisation detector) system. TMS-phytosterols were analysed by a Perkin-Elmer Autosystem XL, equipped with a FID and a Turbochrom Perkin-Elmer software. The same column as GC-MS was used. 1 µl of solutions was injected automatically. Injector and detector temperatures were 280 °C. Initial column temperature was 80 °C (hold for 1 min), then heated at 10 °C min^{-1} until 280 °C (hold for 15 min) and then 5 °C min^{-1} until 300 °C (hold for 10 min). Hydrogen was used as the carrier gas with a pressure of 8.0 psig in the head column.

The main phytosterols, present in the studied vegetables and observed by GC-FID, were: cholesterol, campesterol, stigmasterol, β-sitosterol, Δ-5 avenasterol and Δ-7 avenasterol, with retention times of about 32.1, 35.4, 36.3, 38.3, 38.6 and 40.1 min, respectively. Betulin (internal standard) was present at 44.3 min.

Table 1
GC-MS data of the trimethylsilyl standard ethers

Standards	RT (min)	Molecular ion, most abundant and characteristic peaks *(m/z)*
TMS-Campesterol	18.7	472, 457(a), 382(b), 367(c), 343(d), 129(d)
TMS-β-Stigmasterol	19.1	484, 469(a),394(b), 379(c), 355(d), 129(d)
TMS-Sitosterol	20.0	486, 471(a), 396(b), 381(c), 357(d), 129(d)
TMS-Δ5-Avenasterol	20.2	484, 469(a), 394(b), 379(c), 355(d), 129(d)
TMS-Δ7-Avenasterol	20.6	484, 469(a), 379(c)

(Indicated TRs (retention times) are mean values of 10 tests; RSD was lower than 0.5% for all standards; identification was done according to the MS library DATABASE/NBS 75 K.L.
MM = molecular mass; a = MM – 15; b = MM – 90; c = MM – 15–90; d = MM – 129; 15, 90, 105 and 129 correspond to the loss of methyl group, trimethylsilanol, methyl group and trimethylsilanol and to the fragmentation of 1,2-cyclopentanophenanthrene structure, respectively.

2.4.3. Quantitation of phytosterols by the use of GC peak areas

(1) The method of internal normalisation (ratio of area of each sterol *vs* total area of all sterols) gave the relative percentage of each sterol: $RL = (A_i * 100)/A_t$ RL = relative level of each sterol (%); A_i = area of each sterol; A_t = total area of sterols (except betulin).
(2) The method of internal standard (betulin) was used in the determination of total sterol level in each sample: $S_t = (A_t * m_{Bet} * 100)/(A_{bet} * m_s)$ S_t = total sterols (mg kg^{-1}); m_{Bet} = mass of added betulin (mg); A_{Bet} = Area of betulin; m_s = mass of sample (g).

2.5. Refining of the method (acid hydrolysis)

Acid hydrolysis time was further studied at levels of 0.5 h, 1.0 h and 2.5 h. However, sterol values remained the same or were lower than in the 1 h acid hydrolysis. Therefore, 1 h was chosen for acid hydrolysis in the method proposed.

Hydrochloric acid concentration was also studied at levels of 3 M and 6 M. 3 M was chosen because the sterol level was much higher than at 6 M. A part of the phytosterols was degraded.

Tests were done at several acids hydrolyse temperatures: 55, 80 and 100 °C. At 100 °C, the phytosterol level was higher than that at 80 °C and itself higher than that at 55 °C. So, 100 °C was chosen as the acid hydrolysis temperature.

2.6. Statistics

Sampling results were analysed using Excel® 10, WinStat® v2003.1 add-on and were expressed as mean ± standard deviation ($m \pm SD$) and compared by Student's t-test. A p-value (comparison between raw and cooked plant) < 0.05 was considered as significant.

3. Results

3.1. Repeatability and reproducibility

To evaluate the repeatability of the analytical method, three sets of determination were conducted.

In the first study (Table 2) on the milky drink Pro-Activ® (1st batch), the repeatability of the automatic injection, showed a very weak RSD (about 1%) for a high level of phytosterol (β-sitosterol at 71%) and for total sterols. In contrast the RSD is higher (5.8%–9.8%) for low levels of phytosterols (lower than 3%).

The second set of determination (Table 3) was done by preparing 10 different tests on the same liquid sample (Pro-Activ® 2nd batch) and then injecting each of them once. The RSD was at 1.05% for high levels of phytosterol (β-sitosterol at 71%); in contrast, the RSD was higher for phytosterol levels lower than 1% (cholesterol and stigmasterol).

The third set of determinations (Table 4) was done by preparing six different tests on a powder (lyophilised cabbage) sample and then injecting each of them once. Here also, the RSD was very low (lower than 0.8%) for β-sitosterol and very high for very low levels of phytosterols.

The test of reproducibility (Table 5) was done on a mixture of four phytosterol standards and betulin as the internal standard. It shows a RSD lower than 5% for phytosterol levels higher than 16%.

In conclusion, the RSD for the determination of phytosterols is lower than 5% for abundant sterols.

3.2. Determination of phytosterols in raw and cooked vegetables

Only the edible part (fresh matter FM) of vegetables was analysed. In Tables 6 and 7 are presented: moisture level in each sample, abundant phytosterol contents, total sterols,

Table 2
Repeatability of automatic Injection of the same sample of Pro-Activ®

	% of each phytosterol *vs* total sterols						Total sterols (g kg^{-1})
	Chol	Brassi	Camp	Stigm	β-sito	Sito	
m	1.25	2.41	15.39	0.77	71.0	7.15	13.6
SD	0.11	0.14	0.31	0.07	0.89	0.23	0.148
RSD (%)	9.20	5.82	1.99	9.78	1.25	3.08	1.09

Ten successive automatic injections done on the 1st batch; Chol = cholesterol; Brassi = brassicasterol; Camp = campesterol; Stigm = stigmasterol; β-sito = β-sitosterol; Sito = sitostanol; *m* = average; SD = standard deviation; RSD = relative standard deviation.

Table 3
Repeatability of analysis of the same sample of Pro-Activ® (intra-day precision)

	% of each phytosterol *vs* total sterols						Total sterols (g kg^{-1})
	Chol	Brassi	Camp	Stigm	β-sito	Sito	
m	0.93	2.67	15.5	0.56	71.5	7.77	13.0
SD	0.25	0.04	0.44	0.39	0.75	0.52	1.26
RSD (%)	27.4	1.54	2.82	68.6	1.05	6.69	9.77

Ten different tests as described in analytical methods for the 2nd batch; Chol = cholesterol; Brassi = brassicasterol; Camp = campesterol; Stigm = stigmasterol; β-sito = β-sitosterol; Sito = sitostanol; *m* = average; SD = standard deviation; RSD = relative standard deviation.

Table 4
Repeatability of analysis of lyophilized cabbage (intra-day precision)

	% of each phytosterol *vs* total sterols						Total sterols (g kg^{-1})
	Chol	Camp	Stigm	β-sito	Sito	Identified sterols (%)	
m	0.91	17.3	0.36	80.0	0.18	99.2	1.66
SD	0.27	0.49	0.03	0.61	0.17	0.42	0.113
RSD (%)	29.9	2.79	7.30	0.77	96.0	0.42	6.78

Six different tests as described in analytical methods; Chol = cholesterol; Camp = campesterol; Stigm = stigmasterol; β-sito = β-sitosterol; Sito = sitostanol; *m* = average; SD = standard deviation; RSD = relative standard deviation.

Table 5
Reproducibility of analysis a phytosterol standards mixture (intra-month precision)

	% of each phytosterol *vs* total sterols				
	Cholesterol	Campesterol	Stigmasterol	β-Sitosterol	Betulin
m	16.4	10.4	5.54	21.05	46.7
SD	0.81	0.59	0.32	0.83	1.66
RSD (%)	4.96	5.71	5.83	3.96	3.57

Ten tests were done in the same conditions (1 test month^{-1} from May 2005 to April 2006) on a standard phytosterol mixture, maintained at +4 °C; TMS derivatization was done before each test. *m* = average; SD = standard deviation; RSD = relative standard deviation.

Table 6
Analytical data for raw and cooked green vegetables and beans

	Broad bean		Cabbage		Courgette		Celery	
	Uncooked	Cooked	Uncooked	Cooked	Uncooked	Cooked	Uncooked	Cooked
Moisture (%)	75.8	80.8	88.0	93.4	96.6	96.6	90.2	92.4
Cholesterol (%)	2.96 ± 2.41	4.87 ± 0.82 (*)	8.05 ± 6.43	1.48 ± 0.32(*)	1.32 ± 0.78	1.37 ± 0.29	6.66 ± 3.76	2.28 ± 1.44 (**)
Campesterol (%)	11.0 ± 0.55	11.0 ± 0.21	7.50 ± 6.24	11.9 ± 0.13	ND	0.18 ± 0.21	14.4 ± 0.81	13.0 ± 0.76 (**)
Stigmasterol (%)	5.43 ± 0.33	5.44 ± 0.26	0.24 ± 0.57	ND	4.06 ± 0.28	3.09 ± 0.32(*)	38.0 ± 2.20	35.2 ± 1.07
β-Sitosterol (%)	77.1 ± 1.31	75.3 ± 1.60	78.7 ± 6.75	82.7 ± 0.60	51.6 ± 0.83	47.0 ± 2.05(*)	40.3 ± 2.73	49.2 ± 1.80(**)
Δ-5 Avena (%)	2.55 ± 0.90	2.03 ± 0.98	ND	ND	ND	ND	ND	ND
Δ-7 Avena (%)	ND	ND	ND	ND	39.8 ± 0.62	41.0 ± 1.34	ND	ND
Compounds NI (%)	0.89 ± 1.06	1.34 ± 0.68	5.50 ± 3.10	3.77 ± 0.67	3.06 ± 1.37	7.40 ± 3.40	0.71 ± 1.09	0.25 ± 0.50
Total sterols (mg kg^{-1} FM)	282 ± 5.95	236 ± 11.65(***)	87.0 ± 18.20	199 ± 8.70(***)	154 ± 4.08	259 ± 15.29(***)	5.69 ± 1.71	8.62 ± 0.67(**)
Total lipids (g kg^{-1} FM)	7.18 ± 0.74	6.79 ± 0.49	2.90 ± 0.44	3.37 ± 0.32	1.60 ± 0.47	2.61 ± 0.64	1.27 ± 0.57	1.83 ± 0.75
Stérols/lipids (‰)	39.3	34.8	30.0	59.0	96.3	99.0	4.48	4.71
Total sterols (mg kg^{-1} DM)	1166 ± 120.1	1230 ± 60.7(*)	725 ± 151.7	3012 ± 13.2(***)	4529 ± 120.0	7603 ± 449.7(**)	58.0 ± 17.43	113 ± 8.81(***)

p-values of student *t*-test (comparison between raw and cooked plant) <0.05 (*), <0.01 (**), <0.001 (***).
Avena = avenasterol; NI = not identified; ND = not detected; FM = edible fresh matter; DM = dry matter; data are mean ± SD of three measures done on three different samples and each one was analysed in duplicate in GC.

Table 7
Analytical data for raw and cooked not green vegetables

	Red carrot		White cauliflower		Yellow onion		Red pepper	
	Uncooked	Cooked	Uncooked	Cooked	Uncooked	Cooked	Uncooked	Cooked
Moisture (%)	93.0	96.2	91.4	96.4	94.5	96.6	96.0	94.6
Cholesterol (%)	2.20 ± 1.44	1.92 ± 0.73	0.91 ± 0.54	1.30 ± 0.28	32.8 ± 6.12	18.1 ± 0.63 (**)	4.03 ± 1.40	2.64 ± 1.00 (***)
Campesterol (%)	9.90 ± 0.49	11.5 ± 0.24 (***)	23.1 ± 1.04	22.2 ± 0.42	2.21 ± 0.30	3.21 ± 0.09 (***)	24.2 ± 1.08	23.7 ± 0.46
Stigmasterol (%)	20.1 ± 0.90	23.2 ± 0.44 (***)	5.61 ± 2.41	4.68 ± 1.17	0.28 ± 0.37	0.25 ± 0.15	3.04 ± 1.30	4.11 ± 0.16
β-Sitosterol (%)	61.4 ± 2.09	66.8 ± 0.80 (**)	69.4 ± 0.83	70.5 ± 0.70 (*)	36.6 ± 4.32	40.5 ± 4.40	67.9 ± 1.41	68.4 ± 0.46
Δ-5 Avena (%)	ND	ND	ND	ND	ND	ND	ND	ND
Δ-7 Avena (%)	ND	ND	ND	ND	ND	ND	ND	ND
Compounds NI (%)	0	0	0.92 ± 0.73	1.45 ± 0.27	31.9 ± 2.29	36.3 ± 1.89 (*)	1.69 ± 2.36	1.18 ± 0.61
Total stérols (mg kg^{-1} FM)	93.0 ± 19.48	103 ± 4.53	274 ± 5.78	225 ± 11.12 (***)	13.8 ± 1.64	16.1 ± 0.36 (**)	7.33 ± 1.13	12.2 ± 1.21 (***)
Total lipids (g kg^{-1} FM)	2.90 ± 0.36	1.50 ± 0.18	3.50 ± 0.70	5.90 ± 0.59	3.07 ± 0.38	1.17 ± 0.12	3.03 ± 0.60	4.17 ± 1.85
Stérols/lipids (‰)	32.1	68.9	78.3	38.2	4.50	13.8	2.42	2.92
Total sterols (mg kg^{-1} DM)	1336 ± 165.8	2721 ± 326.5 (***)	3186 ± 637.2	6250 ± 625.0 (***)	251 ± 31.1	474 ± 48.6 (**)	142 ± 28.1	160 ± 71.0 (**)

p-values of student *t*-test (comparison between raw and cooked plant) <0.05 (*), <0.01 (**), <0.001 (***).
Avena = avenasterol; NI = not identified; ND = not detected; FM = edible fresh matter; DM = dry matter; data are mean ± SD of three measures done on three different samples and each one was analysed in duplicate in GC.

total lipids and the ratio total sterols/total lipids. Total lipids level was determined, by weighing the residue, after acid hydrolysis, Soxhlet extraction and solvent volatilisation. The total phytosterol content was always higher in cooked samples than in uncooked samples, when results are expressed according to dry matter (DM):

- broad bean 1166–1230; green vegetables: cabbage 725–3012; courgette 4529–7603; celery 58–113 mg kg^{-1} DM.
- not green vegetables: red carrot 1336–2721; white cauliflower 3186–6250; yellow onion 251–474; red pepper 142–160 mg kg^{-1} DM.

3.3. Influence of the hydrolysis conditions on total phytosterol level – Examples of raw and cooked carrot and cauliflower

Cooked and uncooked carrot and cauliflower samples were studied using the method recommended (see Experimental–Analytical method) and the method without the acid hydrolysis step. Also, both materials were analysed using these two approaches and with total lipid determination after Soxhlet extraction.

The tests on carrot were done according to three methods of hydrolysis (Table 8):

Table 8
Results of phytosterols obtained for raw and cooked carrot according to hydrolysis conditions

	Uncooked carrot			Cooked carrot		
	Acid + basic hydrolysis	Only acid hydrolysis	Only basic hydrolysis	Acid + basic hydrolysis	Only acid hydrolysis	Only basic hydrolysis
Moisture (%)	93.0	93.0	93.0	96.2	96.2	96.2
Cholesterol (%)	2.35 ± 1.44	1.70 ± 0.32	9.49 ± 1.90	1.92 ± 0.73	1.50 ± 0.28	22.3 ± 4.23 (**)
Campesterol (%)	10.6 ± 0.49	11.8 ± 0.35	24.7 ± 4.44	11.5 ± 0.24 (***)	10.8 ± 0.22 (**)	14.7 ± 3.23 (**)
Stigmasterol (%)	21.4 ± 0.90	23.3 ± 4.09	10.8 ± 2.36	23.2 ± 0.44 (***)	23.4 ± 3.51	10.9 ± 2.40
β-sitosterol (%)	65.7 ± 2.09	63.3 ± 1.90	39.9 ± 2.16	66.8 ± 0.80 (**)	64.1 ± 1.79	48.9 ± 2.44 (**)
Compounds NI (%)	ND	ND	15.2 ± 4.52	ND	0.30 ± 0.14	3.30 ± 1.32
Total sterol (mg kg^{-1} FM)	93.0 ± 19.48	76.4 ± 11.50	15.8 ± 4.74	103 ± 4.53	61.2 ± 9.19	18.4 ± 5.52
Total lipids (g kg^{-1} FM)	2.90 ± 0.36	2.10 ± 0.52	3.70 ± 0.66	1.50 ± 0.18	1.40 ± 0.56	2.10 ± 0.32
Sterols/lipids (‰)	32.1	36.4	4.27	68.9	43.7	8.76
Total sterols (mg kg^{-1} DM)	1336 ± 165.8	1091 ± 164.2	226 ± 67.8	2721 ± 119.2 (***)	1590 ± 238.9 (**)	478 ± 143.2

p-values of student *t*-test (comparison between raw and cooked plant) <0.05 (*), <0.01 (**), <0.001 (***).
NI = not identified, ND = not detected, FM = edible fresh matter, DM = dry matter; data are mean ± SD of three measures done on three different samples and each one was analysed in duplicate in GC.

(1) Acid hydrolysis, then extraction and finally basic hydrolysis,
(2) acid hydrolysis and then extraction,
(3) extraction and then basic hydrolysis.

Results obtained on cauliflower are given in Table 9 (acid hydrolysis alone was not done).

In the two examples given (raw and cooked carrot), we observed higher values for acid hydrolysis than basic hydrolysis. This confirms that phytosterols were mainly in a glycosylated form rather than in an esterified form.

4. Discussion and conclusion

Cholesterol is the predominant sterols in animals, wherein free cholesterol serves to stabilise cell membranes and cholesteryl fatty-acid esters are a storage/transport form, usually found associated with triacylglycerols (Gylling & Miettinen, 1999). Plant membranes contain little or no cholesterol but include a methyl or ethyl group at C-24. In general, phytosterols are also thought to stabilise plant membranes with an increase in the sterols/phospholipids ratio leading to membrane rigidification (Heftmann, 1971). Individual phytosterols differ in their effect on membrane stability. For example, stigmasterol has a disordering effect on membranes and the molar ratio of stigmasterol to other phytosterols in the plasma membrane increases during senescence (Marsan, Muller & Milon, 1996; Stalleart & Geuns, 1994).

After cooking, broad bean and five vegetables (carrot, cauliflower, cabbage, celery and onion) had moisture content higher than that of the uncooked form: 80.8 and 75.8, 96.2 and 93.0, 96.4 and 91.4, 93.4 and 88.0, 92.4 and 90.2, 96.6 and 94.5, respectively (Table 6). Courgette had the same level of moisture before and after cooking (96.6%).

Cholesterol percentage was practically unchanged during cooking for carrot, cauliflower and courgette, whereas it decreased for cabbage, onion, celery red pepper and increased for broad bean. Campesterol was practically constant, except for in cabbage. Stigmasterol was practically unchanged or slightly increased for red pepper. β-sitosterol was always the most abundant phytosterol and with a slight increase during cooking, except for broad bean and courgette. Δ5 and Δ7 avenasterols were present in broad bean and in courgette, respectively. Their levels were similar before and after cooking. In spite of this, avenasterols (mainly Δ5 avenasterol) may undergo acid-catalysed isomerisation, while no other major sterols were known to isomerise (Kamal-Eldin, Maatta, Toivo, Lampi, & Piironen, 1998). However, Δ5 avenasterol is not an abundant sterol in food samples or typical diet. It can be hypothesised that some sterols undergo reactions (e.g. dehydration) in acidic conditions. There were few unidentified compounds except for yellow onions. In courgette the level increased. This could be attributed to partial degradation, transformation or isomerisation of identified sterols. The obtained values of total phytosterols were 5.7, 7.3, 13.8, 87.0, 93.0, 154, 274 and 282 mg kg^{-1} fresh weight (raw edible matter) for celery, red pepper, yellow onion, green cabbage, red carrot, courgette, white cauliflower and broad bean, respectively. According to Piironen, Toivo, Puupponen-Pimiä, and Lampi (2003) total phytosterols were 130, 153 and 310 mg kg^{-1} for carrot, Chinese cabbage and cauliflower, respectively. Three varieties of yellow onion were observed in the interval 59–137 mg kg^{-1} edible portion; only one of them contained brassicasterol (Piironen et al., 2003). In our study, one of the unknown sterols (10.2%) could be brassicasterol.

We observed that the level of total phytosterols was increased significantly for cabbage, courgette, celery, yellow onion and red pepper, after cooking. In contrast, it decreased for cauliflower and broad bean.

Lipids were always present in low levels:

- <10‰ for celery, onion, pepper.
- 10–70‰ for broad bean, cabbage, courgette, carrot, cauliflower.

Table 9
Results of phytosterols obtained for raw and cooked cauliflower according to hydrolysis conditions

	Uncooked cauliflower		Cooked cauliflower	
	Acid + basic hydrolysis	Only basic hydrolysis	Acid + basic hydrolysis	Only basic hydrolysis
Moisture (%)	91.4	91.4	96.4	96.4
Cholesterol (%)	0.91 ± 0.54	2.61 ± 0.44	1.30 ± 0.77	30.0 ± 5.52 (***)
Campesterol (%)	23.1 ± 1.04	30.2 ± 0.57	22.2 ± 1.00	26.9 ± 0.51 (***)
Stigmasterol (%)	5.61 ± 2.41	4.78 ± 0.19	4.61 ± 1.98	ND
β-Sitosterol (%)	69.4 ± 0.83	60.0 ± 1.14	70.5 ± 2.40 (*)	40.3 ± 0.78 (***)
Compounds NI (%)	1.02 ± 0.73	2.39 ± 0.42	1.33 ± 0.53	3.10 ± 0.55
Total sterols (mg kg^{-1} FM)	274 ± 5.78	46.0 ± 9.63	225 ± 4.75 (***)	29.0 ± 6.07 (**)
Total lipids (g kg^{-1} FM)	3.50 ± 0.70	2.40 ± 0.48	5.90 ± 1.18	0.70 ± 0.14
Sterols/lipids (‰)	78.3	19.2	38.2	41.4
Total sterols (mg kg^{-1} DM)	3186 ± 637.2	534 ± 111.8	6250 ± 625.0 (***)	806 ± 166.7 (*)

p-values of student *t*-test (comparison between raw and cooked plant) <0.05 (*), <0.01 (**), <0.001 (***).
NI = not identified; ND = not detected; FM = edible fresh matter; DM = dry matter; data are mean ± SD of three measures done on three different samples and each one was analysed in duplicate in GC.

The ratio of total sterols/total lipids increased for carrot, cabbage, onion and courgette. It decreased for broad bean and cauliflower.

After cooking, the seven vegetables and beans showed an increase of phytosterol contents according to the dry matter. Very few sets of data are available on the stability of plant sterols in different food preparation procedures used at home. It seems that there was not a significant difference between raw and cooked samples at a group level but significant sterol losses occur at high temperature for the samples used for deep-frying (Normén, Johnsson, Andersson, van Gameren, & Dutta, 1999).

Stigmasterol and β-sitosterol levels (Table 8) were lower from acid and basic hydrolysis than for basic hydrolysis only. This decrease was also observed in raw and cooked carrot. In contrast, cholesterol and campesterol levels increased.

Regarding cauliflower (Table 9), we observed the same effect (decrease of stigmasterol and β-sitosterol, and increase of cholesterol and campesterol). It has been observed in a number of species that the proportion of stigmasterol in the esters was lower than in the free sterol fraction (Dyas & Goad, 1993).

Total phytosterols, obtained after acid and basic hydrolysis, were 6 times higher than those obtained after basic hydrolysis alone, respectively, for raw (5.9) and cooked (5.7) carrot (Table 8). These values were 6.0 and 7.75, respectively, for cauliflower (Table 9). A method including acid and basic hydrolysis yielded a notably higher sterol value than alkaline saponification alone, indicating that those matrices contain an appreciable amount of steryl glycosides.

In conclusion, in all of the food sample matrixes, sitosterol was retrieved the most abundant sterol, followed by campesterol. After cooking, the studied level of total phytosterols was always higher than that before cooking on a dry matter basis. Acid hydrolysis yielded a notably higher sterol value than alkaline saponification alone, indicating that those matrixes contain an appreciable amount of steryl glycosides.

GC-FID (or MS, when peak identity confirmation is needed) can be considered the method of choice for the determination of phytosterols in foods. It should also be noted that for each food matrix the conditions have to be checked, to optimise the accuracy/yield of all forms of sterols present.

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