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Characterization of Total, Free and Esterified Phytosterols in Tetraploid and Hexaploid Wheats

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Dietary plant sterols have received increasing attention in recent years due to their favorable health benefits. The present research focused on quantification of phytosterols as free, esterified and total forms in different tetraploid (5 cultivars of *Triticum durum* Desf., 9 cultivars of *Triticum dicoccon* Schrank) and hexaploid (5 cultivars of *T. aestivum* L., 12 cultivars of *Triticum spelta* L.) wheats. Tetraploid wheats showed the highest content of total sterol (79.4 and 79.5 mg of sterols /100 g dry weight for *T. durum* and *T. dicoccon*, respectively). Hexaploid cultivars were the best source of esterified sterols (40.7% and 37.3% of total sterols for *Triticum aestivum* and *T. spelta*, respectively). Significant amounts of free sterols (65.5% and 60.7% of total sterols for *T. durum* and *T. dicoccon*, respectively) were found in the tetraploid cultivars. The most abundant phytosterol in all wheat samples was sitosterol accounting for 45.1−59.1, 46.6−57.4 and 38.6−59.5% of total, free and esterified sterol fraction, respectively. These results demonstrate that although the sterol profile present in tetraploid and hexaploid wheat species is the same, differences in their relative amounts and distribution allow statistical differentiation between hexaploids and tetraploids, and between soft and durum wheats.

KEYWORDS: Phytosterols; tetraploid wheats; hexaploid wheats; whole grain; gas chromatography

INTRODUCTION

In recent years, both nutritionists and consumers have begun to regard foods as more than just sources of energy and essential nutrients. Indeed, certain minor components of food are now recognized for their health-promoting benefits, and in particular for their roles in preventing certain chronic diseases such as cardiovascular disease and some cancers. These minor components, called phytochemicals, are naturally occurring in raw foods and have biological activity in humans.

Plant sterols or phytosterols, especially 4-desmethyl sterols, have recently gained much scientific and commercial interest as food or dietary supplements to lower serum cholesterol level. Due to the structural similarity between cholesterol and 4-desmethyl plant sterols, the intake of optimal amounts of plant sterols and their saturated forms (stanols) decreases intestinal cholesterol absorption and leads to 6−15% reduction in serum LDL cholesterol (*1*). The recommended dosage of plant sterols in sterol-enriched foods is 2 g/day (*2*−*5*), whereas the average daily intake of natural plant sterols is estimated to be from 200−300 mg (*6*). Tapiero et al. (*7*) summarized several reports, and concluded that the intake of phytosterols (1.5−2 g/day) can lower circulating cholesterol levels by 5−15% (total and LDL). Thus, plant sterols may lead to physiological benefits as part of a well selected diet or through intake of sterol-enriched foods.

The positive biological effects of phytosterols have led to an intense interest in elucidating the phytosterol content of many foods, which is also useful for the creation of a reliable food composition database that provides information about the presence of these compounds in the diet and their potential effects on health (*8*). Furthermore, industry has dedicated substantial efforts to research and development of phytosterol-enriched foods. According to Directives 2006/59, 2004/4289, 2004/334, 2004/336 and 2000/500 of the European Community, the following products can be enriched with phytosterols, phytostanols or phytosterol esters: yellow fat spreads, salad dressings, milk products, fermented milk products, yogurt-type products, spicy sauces, milk-based fruit drinks, soybean drinks, cheese products and rye bread.

Cereals are considered to be a good source of dietary phytosterols. Although their levels in whole grains are moderate (0.4−1.2 mg/g) (*6*, *9*, *10*), the total amount of phytosterols is significant because of the large amounts of cereals consumed. Cereal based intake of phytosterols is about 30%. Sterols can be found in four forms in cereal grains, namely, free, ester of fatty acids, hydroxycinnamic acids (usually ferulate), and

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Table 1. Total Sterol Content (mg/100 g dry weight) of Hexaploid and Tetraploid Wheats[a]

	campesterol	campestanol	stigmasterol	sitosterol	sitostanol	other sterol	total
Hexaploid Wheats							
Triticum aestivum							
Pandas	13.8 b,c	3.8 m,n	1.0 f−l	40.2 e,f	5.7 m	3.3 a−d	67.7 n−p
Centauro	13.2 c−e	3.0 n	0.9 h−l	38.4 g−i	6.4 m	2.3 c,d	64.5 p−r
Abbondanza	12.2 e−l	3.8 m,n	0.7 l	37.3 i,l	6.4 m	2.5 b−d	63.2 q−s
Serio	9.0 o,p	4.7 i−m	0.8 i,l	34.8 m,n	7.6 l	2.8 b−d	60.0 s
Mieti	8.6 o,p	4.4 l,m	0.7 l	37.0 i,l	8.8 i,l	2.7 b−d	62.6 r,s
mean	11.4	3.9	0.9	37.5	7.0	2.7	63.4
range	8.6−13.8	3.0−4.7	0.7−1.0	37.0−34.8	5.7−8.8	2.3−3.3	60.0−67.7
coefficient of variation	21	16	13	5	17	14	5
Triticum spelta							
Hercule	12.0 e−l	5.6 h−l	1.1 c−l	40.6 e,f	9.4 h,i	2.5 b−d	71.3 m,n
Rouquin	10.9 l−n	6.8 h	1.1 b−l	40.5 e,f	11.1 e−g	2.7 b−d	72.9 l,m
Schwabenkorn	9.9 n,o	6.1 h	1.2 a−l	39.9 e−g	12.8 c,d	3.1 b−d	73.0 l,m
Oberkulmer	11.2 g−n	6.5 h	1.2 a−l	41.2 d,e	8.5 i,l	2.4 b−d	71.0 m,n
Triventina	7.8 p	6.0 h	1.1 e−l	32.5 p,q	12.5 c,d	2.8 b−d	62.8 s,r
Ebners Rotkorn	11.0 i−n	5.7 h,i	1.2 a−l	40.0 e−g	10.0 g,h	3.2 a−d	71.1 m,n
Frankenkorn	11.5 f−m	6.4 h	1.4 a−f	41.2 d,e	10.8 f,g	2.7 b−d	74.0 i−m
Redoutè	11.0 h−n	5.8 h,i	1.7 a	35.9 l,m	9.5 h,i	2.6 b−d	66.5 o−q
Hubel	11.0 i−n	4.7 i−m	0.9 g−l	37.9 h,i	7.7 l	2.2 d	64.3 o−r
Ostar	13.7 b−d	5.9 h,i	1.3 a−i	47.6 a	9.6 h,i	3.7 a−d	81.9 b−e
Sertel	12.3 e−l	5.8 h,i	1.1 d−l	42.9 c,d	9.5 h,i	3.9 a,b	75.7 h−l
Balmegg	12.2 e−l	4.7 i−m	0.9 g−l	45.5 b	8.7 i,l	3.8 a−c	75.8 g−l
mean	11.2	5.8	1.2	40.5	10.0	3.0	71.7
range	7.8−13.7	4.7−6.8	0.9−1.7	32.5−47.6	7.7−12.8	2.2−3.9	62.8−81.9
coefficient of variation	13	11	20	10	15	19	7
Tetraploid Wheats							
Triticum durum							
Grazia 1	14.9 b	11.3 c	1.5 a−f	40.8 e,f	12.2 d,e	2.7 b−d	83.3 b−d
Grisian	13.9 b,c	10.9 c,d	1.6 a−d	41.1 e	11.9 d−f	2.3 c,d	81.7 b−e
Simeto	12.5 c−h	9.6 e,f	1.5 a−f	35.7 l,m	11.8 d,e,f	2.7 b,c,d	73.7 l,m
Creso	12.5 c−g	11.7 b,c	1.4 a−h	32.6 p,q	13.5 c	4.8 a	74.6 g−l
Grazia 2	14.9 b	11.4 c	1.6 a−d	41.0 e	12.3 d,e	2.9 b−d	84.0 b,c
mean	13.7	11.0	1.5	38.2	12.3	3.1	79.4
range	12.5−14.9	9.6−11.7	1.4−1.6	32.6−41.1	11.8−13.5	2.3−4.8	73.7−84.0
coefficient of variation	9	8	5	10	12	31	6
Triticum dicoccon							
Molise	13.5 b−e	10.0 d,e	1.4 a−g	34.6 m−o	16.5 a	3.6 a−d	79.8 d−g
Lucanica	10.5 m,n	8.1 g	1.4 c−l	33.2 n−p	12.2 d,e	2.7 b−d	68.3 n,o
Farvento	13.0 c−e	11.7 b,c	1.6 a−c	35.7 l,m	15.8 a,b	2.9 b−d	80.6 c−f
Agnone	12.4 d−i	9.9 d,e	1.3 a−i	31.4 q	15.5 a,b	3.7 a−d	74.3 i−m
Fontesambuco	12.9 c−f	10.6 c−e	1.5a−e	33.1 o−q	15.6 a,b	2.6 b−d	76.4 g−l
S. Angelo del Pesco	12.8 c−f	9.7 d−f	1.7 a	35.1 m	15.3 b	3.7 a−c	78.3 e−h
Davide	11.5 f−m	8.5 f,g	1.7 a,b	39.3 f−h	13.5 c	3.1 b−d	77.6 f−i
Guardiaregia	13.8 b−d	12.7 b	1.6 a−f	37.3 i,l	16.2 a,b	3.3 a−d	84.9 b
Garfagnana	16.5 a	14.4[a]	1.7 a−d	43.5 c	16.5 a	3.1 b−d	95.7 a
mean	13.0	10.6	1.5	35.9	15.2	3.2	79.5
range	10.5−16.5	8.1−14.4	1.3−1.7	31.4−43.5	12.2−16.5	2.6−3.7	68.3−95.7
coefficient of variation	13	19	12	10	9	13	10

[a] Other sterols included Δ^7-campesterol, clerosterol, fucosterol, Δ^7-sitosterol, Δ^7-avenasterol. Mean values in the same column with different letters show statistically significant differences ($p \leq 0.05$).

conjugated with sugars (mostly with glucose, called sterol glycosides and acylated sterol glycosides). In wheat, the free phytosterols identified include sitosterol, campesterol and stigmasterol. Esterified forms with a fatty acid chain of $C_{16}-C_{18}$ (*11*) as well as saturated forms (stanols) (*12*−*15*) have also been identified in wheat. These compounds are reported to have similar health benefits (*16*).

Hulled wheats (*Triticum dicoccon* Schrank and *Triticum spelta* L.) are ancestral wheats that are related to durum and soft wheats. In reality, the renewed nutritional interest in these cereals is related to the presence of phytochemicals in whole meal or unrefined flour, which are widely used for preparation of cereal products (*17, 18*). Moreover, the quantitative and qualitative evaluation of different sterol fractions is a useful parameter to distinguish tetraploid from hexaploid wheats, and also permits detection of adulteration of semolina by flour from soft wheat (*11, 19, 20*). A number of investigations have described the quantity of phytosterols in specific cereals and their distribution in the aleurone layer, endosperm, pericarp, testa and germ. In contrast, there is little quantitative information on the concentration of different phytosterol forms.

The aim of this study was to examine the different sterol composition of four species of *Tritucum*. For this purpose, 31 genotypes (17 hexaploid and 14 tetraploid cultivars) were analyzed to verify the possible genetic variation on the basis of phytosterol composition. The present study is thus the first of its kind to determine differences in sterol classes (e.g., total, free, esterified sterols) and to examine if the various forms of sterols can allow the differentiation of wheat species.

Table 2. Free Sterol Content (mg/100 g dry weight) of Hexaploid and Tetraploid Wheats[a]

	campesterol	campestanol	stigmasterol	sitosterol	sitostanol	other sterol	total
Hexaploid Wheats							
Triticum aestivum							
Pandas	6.4 e,f	0.9 f	0.9 g−m	18.5 h,i	2.6 q	1.5 a,b	30.9 g,h
Centauro	5.2 f−h	1.0 f	0.9 g−m	16.3 l,m	4.0 l−o	1.3 a,b	28.8 h−l
Abbondanza	6.4 e,f	1.5 e,f	1.0 f−m	18.4 h,i	4.2 l−n	1.2 a,b	32.8 g
Serio	4.7 g−l	1.3 e,f	0.8 h−m	17.4 i,l	4.6 h−l	1.7 a,b	30.5 g,h
Mieti	6.3 e,f	2.0 e,f	1.0 f−m	22.4 e,f	5.7 h	1.2 a,b	38.7 f
mean	5.8	1.3	0.9	18.6	4.2	1.5	32.4
range	4.7−6.4	0.9−2.0	0.8−1.0	16.3−22.4	2.6−5.7	1.2−1.7	28.8−38.7
coefficient of variation	14	34	11	12	26	12	12
Triticum spelta							
Hercule	4.2 h−l	1.2 f	1.0 g−m	14.7 m−o	3.8 l−p	1.2 a,b	26.3 i−m
Rouquin	5.1 f−i	1.9 e,f	1.0 f−m	15.6 ln	4.5 i−m	1.1 a,b	29.4 g−i
Schwabenkorn	3.5 l	1.1 f	0.6 l,m	10.2 r	2.5 q	1.0 a,b	19.1 n
Oberkulmer	4.2 h−l	1.6 e,f	1.3 b−h	13.7 n−q	3.3 n−q	1.1 a,b	25.5 l,m
Triventina	5.6 f,g	2.3 e	1.1 d−l	17.0 i,l	5.4 h,i	1.4 a,b	33.1 g
Ebners Rotkorn	3.7 i,l	1.1 f	1.1 e−m	12.4 q	3.6 l−q	0.7 b	22.8 m,n
Frankenkorn	4.8 g−l	1.7 e,f	1.0 g−m	15.6 l−n	4.2 l−n	0.9 b	28.5 h−l
Redoutè	4.2 h−l	1.1 f	0.8 h−m	12.7 p,q	3.4 m−q	0.7 b	23.0 m
Hubel	4.3 h−l	1.3 e,f	0.7 i−m	12.8 o−q	2.9 p,q	0.7 b	22.8 m,n
Ostar	4.4 g−l	1.2 e,f	1.0 g−m	12.8 o−q	3.2 n−q	0.7 b	23.5 m
Sertel	4.3 g−l	1.2 e,f	0.9 g−m	13.3 o−q	3.2 n−q	0.9 b	23.9 m
Balmegg	4.1 h−l	1.0 f	0.6 m	14.3 m−p	3.0 o−q	0.8 b	24.0 m
mean	4.4	1.4	0.9	13.8	3.6	1.1	25.2
range	3.5−5.6	1.0−2.3	0.6−1.3	10.2−17.0	2.5−4.5	0.7−1.4	19.1−29.4
Coefficient of variation	13	29	21	13	22	25	15
Tetraploid Wheats							
Triticum durum							
Grazia 1	8.2 c,d	5.0 b,c	1.5 b−e	24.0 c−e	7.8 f,g	1.5 a,b	48.4 b−d
Grisian	10.6 a	6.5 a	1.6 a	27.9 a	7.4 g	1.4 a,b	55.7 a
Simeto	8.1 c,d	4.7 c,d	1.5 b−f	25.7 b	8.3 e−g	1.6 a,b	50.2 b,c
Creso	7.0 d,e	6.3 a	1.4 b−g	23.0 d−f	9.0 c−e	2.2 a	49.1 b−d
Grazia 2	10.3 a,b	5.9 a,b	1.6 b,c	28.1 a	9.0 c−e	1.4 a,b	56.7 a
mean	8.8	5.7	1.5	25.8	8.3	1.9	52.0
range	7.0−10.6	4.7−6.5	1.4−1.6	23.0−28.1	7.4−9.0	1.4−2.2	48.4−56.7
coefficient of variation	17	14	7	9	9	16	7
Triticum dicoccon							
Molise	9.2 b,c	3.7 d	1.4 b−g	24.6 b−d	10.8 a	1.5 a,b	51.4 b
Lucanica	7.0 d,e	3.7 d	1.4 b−g	23.5 c−e	8.9 c−f	1.4 a,b	46.1 d,e
Farvento	8.4 c,d	4.4 c,d	1.2 c−i	23.5 c−e	9.5 b−d	1.0 b	48.2 b−d
Agnone	8.0 c,d	4.4 c,d	1.3 b−h	21.4 f,g	10.3 a,b	1.4 a,b	47.0 c−e
Fontesambuco	7.1 d,e	4.1 c,d	1.2 c−i	20.3 g,h	8.9 c−f	1.3 a,b	43.1 e
S. Angelo del Pesco	8.0 c,d	3.9 c,d	1.7 b	23.1 d−f	10.7 a	1.3 a,b	49.0 b−d
Davide	8.7 c	3.9 c,d	1.6 b,c	23.7 c−e	10.0 a−c	1.1 a,b	49.2 b−d
Guardiaregia	8.2 c,d	4.3 c,d	1.6 b−d	23.6 c−e	10.2 a,b	1.5 a,b	49.7 b−d
Garfagnana	9.1 b,c	4.4 c,d	1.7 b,c	25.1 b,c	8.7 d−f	1.0 a,b	50.0 b,c
mean	8.2	4.1	1.4	23.2	9.8	1.5	48.2
range	7.0−9.2	3.7−4.4	1.2−1.7	20.3−25.1	8.7−10.7	1.0−1.5	43.1−51.4
coefficient of variation	9	7	15	6	8	15	5

[a] Other sterols included Δ^7-campesterol, clerosterol, fucosterol, Δ^7-sitosterol, Δ^7-avenasterol. Mean values in the same column with different letters show statistically significant differences ($p \leq 0.05$).

MATERIALS AND METHODS

Sampling. Seventeen hexaploid wheats were selected including 5 Italian cultivars of *Triticum aestivum* L. (Pandas, Centauro, Abbondanza, Serio, Mieti) and 12 cultivars of *Triticum spelta* L.: Hercule, Rouquin, Schwabenkorn, Oberkulmer, Ebners Rotkorn, Frankenkorn, Redoutè, Hubel, Ostar, Sertel and Balmegg (European origin) and Triventina (Italian origin). In addition, fourteen Italian tetraploid wheats were selected including 5 cultivars of *Triticum durum* Desf. (Grazia 1, Grisian, Simeto, Creso, Grazia 2) and 9 cultivars of *Triticum dicoccon* Schrank (Molise, Lucanica, Farvento, Agnone, Fontesambuco, S. Angelo del Pesco, Davide, Guardiaregia, Garfagnana). All genotypes of each wheat species were grown in an experimental field located in Salcito-Campobasso (southern Italy) under the same agronomic conditions and harvested in 2002. A split plot design with three replications for each cultivar was adopted. After harvest, the grain (0.5 kg) from each plot was mixed (1.5 kg). Hulled wheats were dehulled by passing them twice through rubber-coated rollers, and the hulls were removed by aspiration (OTAKE FC2K, Irom Italy s.r.l., Italy). The mixed samples were milled to pass 0.5 mm sieve (IKA A10-IKAWERKE GmbH & Co. KG, Staufen, Germany) and stored at −20 °C before analysis. The moisture content in whole meal was determined using AACC method 44.15A.

Extraction of Sterol Fraction. *Total Sterol Extraction and Purification.* The total sterol (TS) fraction was obtained by acid hydrolysis performed using AACC method 30.10 (*22*) with some modifications. As an internal standard (IS), 1 mg of dihydrocholesterol (Sigma-Aldrich Co., St. Louis, MO) was added to the sample before extraction. About 6 g of whole meal was weighed in a glass tube, and 30 mL of 30% HCl was added. The tube was shaken vigorously and placed into an 80 °C water bath for 30 min. After heating, the tube was cooled and 25 mL diethyl ether and 25 mL petroleum ether were added to the digested sample. The organic phase was transferred to a glass flask, and the lower phase was washed twice with 25 mL of a diethyl ether:petroleum ether (1:1, v/v) mixture. The organic fractions were pooled and then evaporated at 35 °C under vacuum using a rotary evaporator. The lipidic fraction obtained was saponified at room

Table 3. Esterified Sterol Content (mg/100 g dry weight) of Hexaploid and Tetraploid Wheats[a]

	campesterol	campestanol	sitosterol	sitostanol	total
Hexaploid Wheats					
Triticum aestivum					
Pandas	4.9 a,b	1.6 m	16.6 a,b	2.9 m−o	26.0 c,d
Centauro	4.7 a−c	1.8 i−m	14.0 c−e	5.7 a,b	26.4 b−d
Abbondanza	4.2 b−g	2.0 h−m	12.5 e,f	3.2 l−o	21.9 e
Serio	5.2 a	3.9 a−c	17.6 a	6.2 a	33.0 a
Mieti	4.3 a−f	2.3 g−m	11.7 f	3.5 h−o	21.9 e,f
mean	4.7	2.4	14.5	4.3	25.8
range	4.2−5.2	1.6−3.9	11.7−16.6	2.9−6.2	21.9−33.0
coefficient of variation	8	39	18	36	17
Triticum spelta					
Hercule	4.6 a−d	2.3 g−m	15.1 b−d	3.6 g−o	25.6 c,d
Rouquin	4.5 a−e	1.6 l,m	16.4 a,b	4.4 e−i	26.9 b−d
Schwabenkorn	4.0 b−h	2.3 g−m	15.3 b−d	4.8 c−g	26.4 b−d
Oberkulmer	3.9 b−i	2.3 g−m	15.3 b−d	4.8 c−f	26.3 b−d
Triventina	3.7 d−m	3.6 a−f	13.5 d−f	4.6 c−g	25.4 c,d
Ebners Rotkorn	4.0 b−h	2.9 d−l	15.3 b−d	2.8 n,o	25.1 d
Frankenkorn	3.7 c−m	2.7 d−m	17.9 a	3.9 f−n	28.2 b−d
Redoutè	3.8 c−l	2.5 g−m	14.8 b−d	4.1 e−l	25.2 d
Hubel	4.6 a−d	2.6 e−m	17.9 a	4.0 e−m	29.1 b
Ostar	4.5 a−e	2.3 g−m	16.5 a,b	3.9 f−n	27.2 b−d
Sertel	4.3 a−f	1.8 i−m	15.8 a−c	4.6 c−g	26.5 b−d
Balmegg	4.5 a−e	2.4	16.5 a,b	4.5 d−h	27.8 b−d
mean	4.2	2.5	15.9	4.1	26.7
range	3.7−4.6	1.6−2.9	13.5−17.9	2.8−4.8	25.1−29.1
coefficient of variation	8	20	8	14	5
Tetraploid Wheats					
Triticum durum					
Grazia 1	3.0 i−m	3.3 c−g	5.9 i,l	3.0 m−o	15.2 l,m
Grisian	3.1 h−m	2.6 f−m	7.4 g−i	2.5 o	15.6 i−m
Simeto	3.1 h−m	3.2 c−h	6.6 h−l	4.4 e−i	17.3 h−l
Creso	3.6 e−m	3.2 c−h	8.7 g,h	3.8 f−n	19.2 e−h
Grazia 2	3.4 f−m	3.4 c−g	6.7 h−l	3.0 m−o	16.4 h−m
mean	3.3	3.2	7.1	3.3	16.9
range	3.1−3.6	2.6−3.4	5.9−8.7	2.5−4.4	15.2−19.2
coefficient of variation	7	10	15	22	10
Triticum dicoccon					
Molise	3.9 b−i	3.9 a−c	7.0 h−l	3.6 f−o	18.6 g,h
Lucanica	3.7 d−m	3.9 a−d	4.9 l	5.6 a−c	18.1 g−l
Farvento	2.9 l,m	2.7 d−m	5.0 l	3.3 i−o	14.0 m
Agnone	2.8 m	3.2 c−h	6.4 i,l	4.6 c−g	17.1 h−l
Fontesambuco	3.3 f−m	3.0 c−i	9.5 g	4.5 d−h	20.3 e−g
S. Angelo del Pesco	3.3 g−m	4.7 a	8.7 g,h	5.1 a−d	21.8 e,f
Davide	3.5 f−m	3.3 c−g	7.0 h−l	4.9 c−e	18.8 g,h
Guardiaregia	2.8 l,m	3.7 a−e	7.0 h−l	4.7 c−g	18.3 g−i
Garfagnana	3.0 i−m	4.4 a,b	6.9 h−l	4.8 c−g	19.0 f−h
mean	3.3	3.4	7.1	4.6	18.4
range	2.8−3.9	2.7−4.7	4.9−9.5	3.3−5.1	14.0−21.8
coefficient of variation	12	12	21	16	11

[a] Mean values in the same column with different letters show statistically significant differences ($p \leq 0.05$).

temperature using 40 mL of methanolic 0.5 M KOH for 18 h in the dark under constant stirring (*23*).

After saponification the organic fraction was washed with deionized water and the unsaponifiable matter was extracted three times with diethyl ether. The solvent was removed under vacuum. Finally, about 20 mg of unsaponifiable fraction was recovered and purified by TLC silica (20 cm × 20 cm × 0.25 mm film thickness). Elution was performed using a mixture of *n*-hexane:diethyl ether 70:30 (v/v). The band corresponding to sterol was visualized under UV light (254 nm), after spraying with a 0.2% ethanolic solution of 2,7-dichlorofluorescein sodium salt, scraped off separately, extracted three times with chloroform, dried under a nitrogen stream and stored in *n*-hexane:2-propanol (4:1 v/v) at −18 °C until GC analysis.

Free Sterol and Esterified Sterol Extraction and Purification. Free (FS) and esterified sterols (ES) were obtained after the extraction of lipids according to Folch with slight modification (*24*, *25*). Dihydrocholesterol and cholesteryl decylate (Sigma-Aldrich Co., St. Louis, MO) were added to 15 g of whole meal in order to quantify the FS and ES fractions, respectively. FS and ES were obtained by TLC separation under the same conditions used for total sterol at an R_f of 0.2 and 0.9, respectively. The FS band was scraped off and recovered with chloroform, and then dried and stored in *n*-hexane:2-propanol (4:1 v/v). ES, after collection by TLC under the conditions previously described, were obtained after basic hydrolysis (2 N methanolic KOH, 40 min at 40 °C) and stored in *n*-hexane:2-propanol (4:1 v/v) until GC analysis. Bound sterol (BS) fraction was calculated as TS − (FS + ES).

Gas Chromatography (GC) Analysis. The three sterol fractions (TS, FS and ES) were analyzed by GC after sylilation (*26*). The trimethylsilyl derivatives (TMS) of sterols were analyzed by GC using a Clarus 500 instrument (Perkin-Elmer, Norwalk, CT) equipped with a flame ionization detector (FID) using the following conditions: the column was coated with diphenyl dimethyl polysiloxane (Restek, Bellefonte, PA, 30 m × 0.32 mm i.d., film thickness = 0.10 μm); helium carrier gas at 1.0 mL/min; split ratio 1:20; injector temperature, 330 °C; oven temperature, 270 to 330 °C at 3 °C/min. Analyses were performed in duplicate for each cultivar. Identification of sterol compounds was based on relative retention times compared with a standard and mass spectral data obtained by GC−MS (Agilent Hewlett-

Packard 6890GC gas chromatograph equipped with a MS detector Hewlett-Packard 5970 MSD, Scientific Instrument Service, NJ). Chromatographic conditions were the same for the GC−FID. The quadrupole was used in the electronic impact mode (70 eV) and a mass range of 40−650 *m*/*z* was monitored.

The method was validated with the recovery and repeatability studies. Dihydrocholesterol and cholesteryl decylate at two concentration levels (80 and 40 mg/100 g dry weight) were added to wholegrain wheat meal to evaluate the recovery of free and esterified sterols, respectively. The performance of the method was evaluated by analyzing 10 replicate analyses, giving a coefficient of variation of 2.5%. The recoveries of standards added were 97.6 (3.7% (*n*) 8) and 87.0 (4.2% (*n*) 8), respectively. All results are given as means of replicate samples on a dry weight basis.

Statistical Analyses. One-way analysis of variance, ANOVA (Tukey's honest significant difference multiple comparison) and PCA analysis were evaluated using Statistica 6.0 software (2001, StatSoft, Tulsa, OK). *P* values less than 0.05 were considered statistically significant. All chemical analyses were carried out in duplicate, and the analytical data were used for statistical comparisons.

RESULTS AND DISCUSSION

TS Evaluation. Different sterols were identified by MS, and the results were in overall agreement with the data reported by Pelillo and co-workers (*14*). Mass spectra data showed three major peaks with *m*/*z* of 472, 484 and 486, which corresponded to Δ^5-campesterol, Δ^5-stigmasterol and Δ^5-sitosterol, respectively, and the corresponding stanols at *m*/*z* 474, 486 and 488. Several minor peaks with *m*/*z* of 472, 484, 484, 486 and 484 were identified, in agreement with Pelillo et al. (*14*), as Δ^7-campesterol, Δ^5-clerosterol, Δ^5-fucosterol, Δ^7-sitosterol and Δ^7-avenasterol, respectively.

For quantitative purposes and statistical analyses, only the more abundant compounds were considered: campesterol, stigmasterol, and sitosterol included the sum of minor sterols (such as Δ^7-campesterol, clerosterol, fucosterol, Δ^7-sitosterol and Δ^7-avenasterol). **Table 1** reports the total sterol contents (TS) of tetraploid and hexaploid wheats. This fraction comprises both FS and ES. In this study, the determination of total sterols was obtained by acid hydrolysis of samples and subsequent saponification of lipid extracts. Direct saponification allows the determination of plant sterols in cereal and cereal products. However, the acetal bond between sterol and carbohydrate moiety cannot be hydrolyzed in alkaline conditions, and therefore direct saponification methods fail to quantify steryl glycosides. As discussed elsewhere, previous studies on phytosterols in cereals have underestimated the content by 20−35% since the extraction methods used did not extract the sterol glycosides. As reported by Toivo et al. (*27*) and Piironeen et al. (*28*), in order to release bound sterols, acid hydrolysis must be utilized in combination with saponification. 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 cannot be hydrolyzed in alkaline conditions. This leads to underestimation of total sterol concentration in sample analyzed. Inclusion of an acid hydrolysis step has been suggested as one alternative to release sterols from their glycosides (*27*).

Statistical differences in TS between tetrapoid and hexaploid wheats were observed. The highest phytosterols content was in tetraploid wheats, which contained on average 79.4 mg/100 g dry weight. Among hexaploid wheats, the dehulled cultivars contained the lowest amounts (63.4 mg/100 g dry weight).

The sum of sitosterol, campesterol and stigmasterol was 96−97% TS, whereas minor sterols (clerosterol, Δ^7-campesterol, fucosterol, Δ^7-sitosterol and Δ^7-avenasterol) represented about 3−4% TS. For the major sterols, the results are in general agreement with those previously reported by Dutta and Appelqvist (*29*), Piironen et al. (*13*), Normen et al. (*6*), Toivo et al. (*26*), Ruibal-Mendieta et al. (*15*) and Nyström et al. (*30*) for *T. aestivum*. The higher content of TS in tetraploid species was also reported in a recent work by Nurmi and co-workers (*31*). The presence of ergosterol and cholesterol in wheat samples has been reported by Ruibal-Mendieta et al. (*15*) and Seitz et al. (*32*), but it has not been confirmed by other studies. Naewbanij et al. (*33*) studied the ergosterol content of wheat, maize and sorghum grains as a means of estimating the extent of fungal contamination. Other researchers have described that at very low levels of fungal contamination ergosterol levels positively correlated with increasing levels of preharvest fungal contamination (*34*).

Based on these results, the difference between tetraploid and hexaploid wheats is related to differences in stanol content. In fact, as reported in several studies (*13*, *15*, *35*), the unsaponifiable fraction is characterized by the presence of significant quantities of sterols. In tetraploid wheats, up to 30% of total sterols consisted of saturated phytosterols, such as sitostanol and campestanol, whereas in hexaploid species the saturated sterols represented about 18% of total sterols. Therefore, the sterol/stanol ratio can distinguish tetraploid wheats from hexaploid wheats. In fact, this ratio is 4.7 and 3.5 in *T. aestivum* and *T. spelta*, whereas in tetraploid wheats the sterol/stanol ratio is lower, with values of 2.0−2.4. Therefore, the sterol/stanol ratio can be used as an index to distinguish *T. spelta* from *T. dicoccon*, and *T. durum* from *T. aestivum*.

FS and ES Evaluation. The FS and ES sterol contents are detailed in **Tables 2** and **3**, respectively. The use of two standards (free and esterified forms of sterols) that follow the fate and had the same recovery of the studied sterols ensured correct quantification. FS and ES are easily extracted with a strong lipophilic solvent (extraction by the Folch method). As reported by Määttä et al. (*35*) using only extraction with solvent, the same sterols remain intact in the sample (due to glycosylation and/or entrapment within the matrix of starch carbohydrate), and consequently, sterols are underestimated. These results show that there was no difference in the FS content of cultivars belonging to the same species, but there were significant differences in FS between tetraploid and hexaploid wheats. As reported in **Table 2**, the FS were distributed differently: in tetraploid wheats, the majority of phytosterols were found as free forms, approximately 60−66%, whereas in hexaploid wheats and in particular in *T. spelta*, only 35% existed in the free form. In FS, as for TS sitosterol is also the main sterol (approximately equal to 13.8−18.6 mg/100 g dry weight for hexaploid wheats and 23.2−25.8 mg/100 g for tetraploid wheats); the other sterols, in a decreasing order, are campesterol (4.4−5.8 g/100 g for hexaploid wheats and 8.2−8.8 mg/100 g in tetraploid wheats), stigmasterol and saturated sterol such as sitostanol and campestanol.

The results relative to ES are reported in **Table 3**. As can be seen, the sterols characteristic of this fraction are campesterol, sitosterol and the respective saturated sterols. Minor sterols are present only in trace amounts. Analysis of ES revealed differences in the species analyzed. In particular, in hexaploid wheats the total ES was 25.8−26.7 mg/100 g compared to 16.9−18.4 mg/100 g in tetraploid wheats.

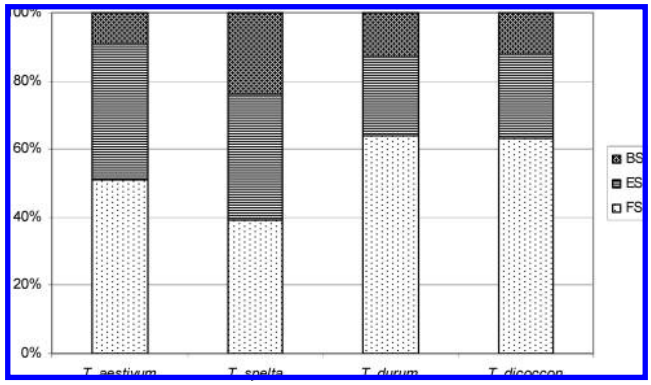

Figure 1. Distribution (%) of free sterols (FS), esterified sterols (ES) and bound sterols (BS) as a percentage of total sterols in tetraploid (*Triticum durum*, *Triticum dicoccon*) and hexaploid wheats (*Triticum aestivum*, *Triticum spelta*).

Evaluation of combined FS and ES showed that the two fractions of phytosterol are similar for varieties belonging to the same species; in hexaploid wheats they represented 47.7−55.7 mg/100 g, whereas in tetraploid wheats the combined FS and ES were 63.6−65.4 mg/100 g. The sum of different FS and ES in wheats is in agreement with the data presented by Ruibal-Mendieta et al. (*15*), who reported that the combined FS and ES content of spelt and soft wheat were 52.7−52.8 mg/100 g, in accordance with another study of unhydrolyzed sterol esters (*11*).

When the mean values for TS, FS and ES presented in **Tables 1**, **2**, and **3** were compared, it is evident that the sterol content largely increased after acid hydrolysis, indicating that about 13−16% in tetraploid wheats and 8−28% in hexaploid wheats of the total sterols were present in a bound form (BS). **Figure 1** shows the percentage distribution of FS, ES and BS. As can be observed in tetraploid wheats, the FS fraction is predominant with TS values of 61−66%, whereas in hexaploid wheats the FS fraction is 51% for *T. aestivum* and only 35% for *T. spelta*. These results clearly indicate that the evaluation of single fractions of phytosterol, and in particular quantitative analysis of ES, can be used to distinguish tetraploid from hexaploid wheats.

Principal Component Analysis (PCA). Figure 1 shows the distribution of the different forms of sterols. PCA carried out on the 31 samples allows discrimination between the tetraploid and hexaploid classes. **Figure 2** shows the loading plot of the first two principal components of PCA applied to the different classes of sterols in wheats. The first two principal components accounted for 52.1% and 34.2% of the variance, and the plot thus describes more than 95% of the total variance.

Figure 3 shows the score plot of the principal components applied to the TS in wheat samples. Therefore, this figure provides a visual interpretation of the results previously discussed. The first and fourth principal components accounted for 52.1% and 3.2% of the variance, and thus the plot describes more than 95% of the total variance.

As is evident in **Figure 3**, these variables can account for the differences found in the two wheat species (tetraploid and hexaploid wheats). In particular, acid hydrolyzed extracts formed two well-defined clusters in relation to the different levels of TS found.

In agreement with the findings of the analytical results reported in **Figure 1** and PCA analysis, tetraploid wheats showed the highest content of FS. In contrast, hexaploid cultivars showed the highest content of ES.

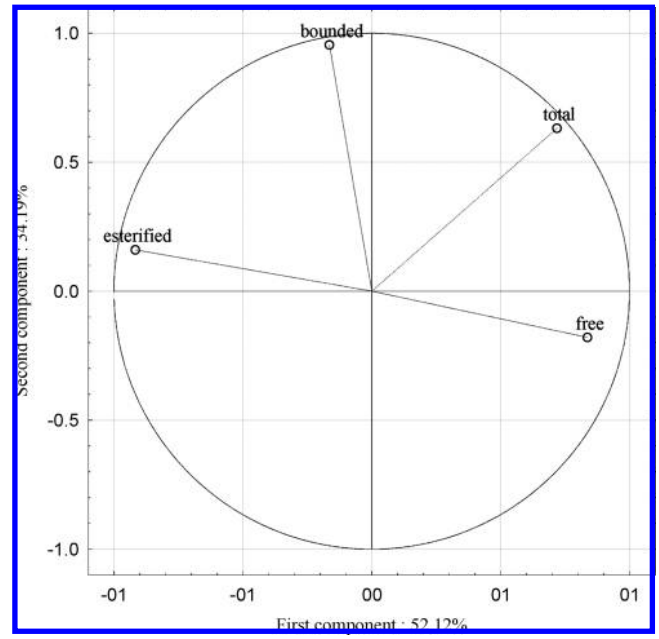

Figure 2. Principal component analysis (loading plot) of the sterolic fraction in tetraploid (*Triticum durum*, *Triticum dicoccon*) and hexaploid wheats (*Triticum aestivum*, *Triticum spelta*).

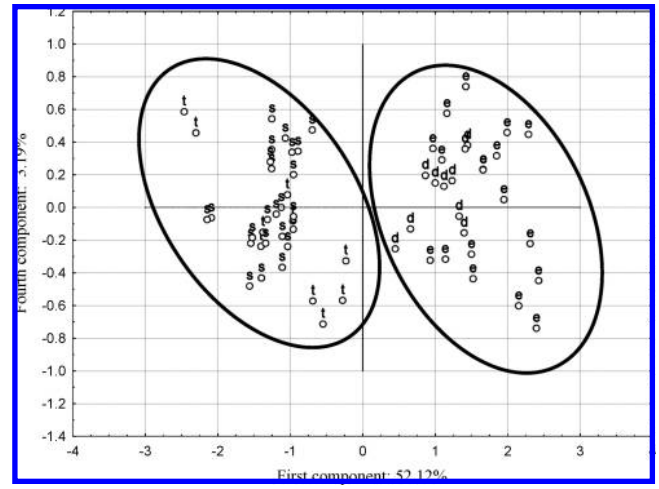

Figure 3. Principal component analysis (scores plot) of total sterols in tetraploid (*Triticum durum* (d), *Triticum dicoccon* (e)) and hexaploid wheats (*Triticum aestivum* (t), *Triticum spelta* (s)).

In conclusion, the tetraploid and hexaploid wheats exhibit the same qualitative pattern but not the similar sterol amount. The results presented in this paper show that tetraploid wheats contain significantly higher amounts of sterol than hexaploid wheats, with the free fraction accounting for most of this difference (about the 60% of total sterol). In contrast, hexaploid species show higher content of esterified sterols than tetraploid wheats.

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