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Analysis of Fatty Acid Steryl Esters in Tetraploid and Hexaploid Wheats: Identification and Comparison between Chromatographic Methods

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Fatty acid steryl esters (FASE) in whole meal of 14 genotypes of tetraploid wheats (*Triticum dicoccon* and *T. durum*) and 17 genotypes of hexaploid wheats (*T. spelta* and *T. aestivum*) were analyzed using different chromatographic strategies. By both GC-FID and HPLC-ELSD, tetraploid wheats are lacking two major peaks. The amounts of FASE, calculated on the basis of the GC-FID analysis, were double in hexaploid species as compared to tetraploids (40 and 20 mg/100 g db, respectively). HPLC with ESI-MS detection enabled the identification of FASE by the characteristic fragmentations and ion-adducts of each molecule. The distribution of steryl residues was not different between the wheat species: the main class of steryl derivatives found was the β-sitosteryl derivatives, followed by campesteryl derivatives with small amounts of stigmasteryl esters. The esterified fatty acids explain the difference between the hexaploid and tetraploid wheats. In particular, small amounts of campesteryl and β-sitosteryl, while no trace of stigmasteryl palmitates, were found in *T. durum* or its hulled ancestor *T. dicoccon*. Steryl oleates were not detectable in *T. aestivum* or its hulled ancestor *T. spelta*, which is consistent with the filogenesis of tetraploid and hexaploid species. Both chromatographic techniques (GC and HPLC) showed that FASE are useful to discriminate between hexaploid and tetraploid wheats from both qualitative and quantitative points of view.

KEYWORDS: Fatty acid steryl esters; GC-FID; HPLC-ELSD; HPLC-ESI-MS; tetraploid wheats; hexaploid wheats; *Triticum aestivum* L.; *T. durum* Desf.; *T. spelta* L.; *T. dicoccon* Schrank

INTRODUCTION

In humans, plant sterols play an important physiological role in serum cholesterol lowering and also have anticarcinogenic and immuno-modulating effects (*1*−*3*). Recent studies have shown that dietary plant sterols and their fatty acid esters can lower plasma levels of cholesterol by reducing LDL-cholesterol without affecting HDL-cholesterol (*4*). These compounds have recently gained much scientific and commercial interest in the production of bioactive ingredients and the development of functional foods (*5*−*10*).

Vegetable oils are the most important natural sources of plant sterols, although cereals and cereal byproducts are also significant sources, and in fact sterols represent more than 2% of the cereal lipid fraction (*11*−*14*). In cereals, plant sterols occur as free sterols, steryl esters with fatty acids, or phenolic acids, steryl glycosides, and acylated steryl glycosides (*10*, *11*, *15*). The fatty acid steryl ester (FASE) fraction in bread wheat flour generally has a high content of palmitate and a low amount of linoleate, whereas FASE in durum wheat semolina has low quantities of palmitate and high amounts of linoleate (*11*). When only steryl palmitate or saturated fatty acid steryl ester is measured, durum wheat semolina has 0.0−1.5 mg/100 g, whereas most hexaploids or soft wheat flours have 3.0−57.6 mg/100 g, which suggests that high saturated FASE could be used to detect contamination of *T. durum* with *T. aestivum* (*16*−*22*). However, information on the quantification and composition of FASE in wheat species and in particular in naked wheats is lacking.

Several chromatographic methods have been described for the analysis of FASE in complex mixtures isolated from plant and animal tissues with and without hydrolysis (*23*). FASE can be analyzed by thin-layer chromatography (TLC) (*16*, *17*), gas chromatography (GC) (*24*), high performance liquid chromatography (HPLC) (*25*, *26*), and on-line HPLC-GC (*27*). It is not possible to separate the different compounds using TLC, but the technique can be used successfully to purify the FASE fraction. On the other hand, HPLC is suitable for this purpose with the use of UV (*23*) or more appropriate detectors such as evaporative light scattering detectors (ELSD) (*24*).

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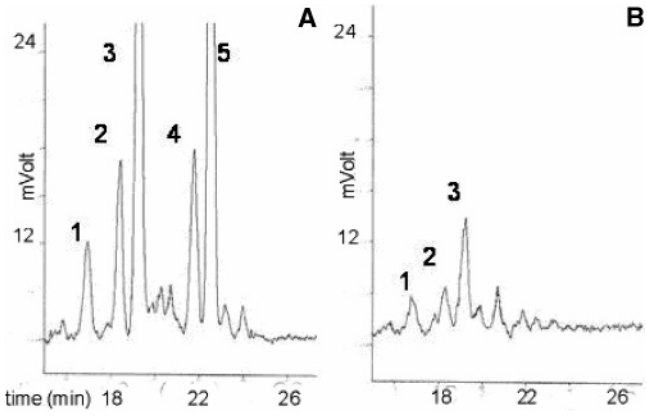

Figure 1. HPLC chromatograms of FASE of *TAe* (**A**) and *TDu* (**B**).

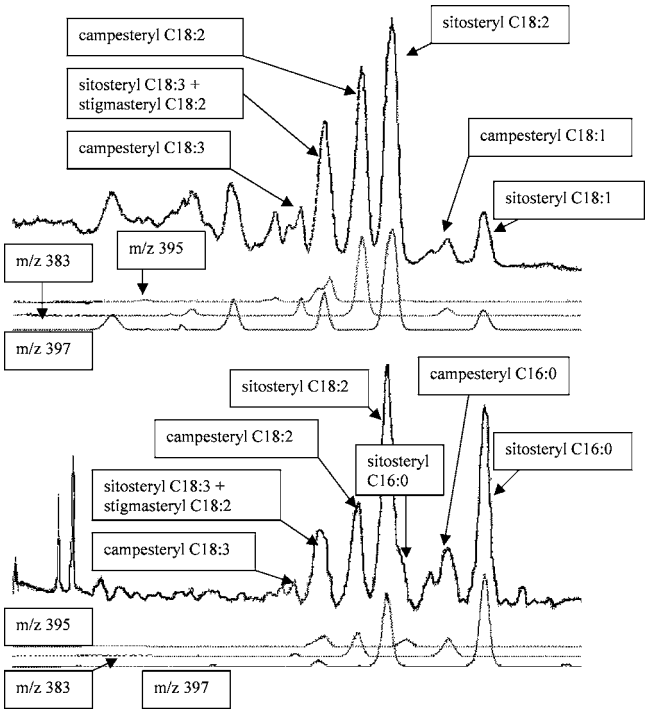

Figure 2. HPLC-ESI-MS chromatograms obtained by total ion current and by single ion extraction of FASE of *TAe* (upper chromatogram) and *TDu* (lower chromatogram).

Recent papers have documented the use of HPLC-APCI-MS (atmospheric pressure chemical ionization mass spectrometry) for plant sterol and FASE analysis in cholesterol-lowering spreads and in hexaploid wheats, which enables identification of single compounds even when chromatographic resolution is not complete (*27*, *29*). GC has rarely been adopted because of the high boiling point of FASE that requires very high temperatures or extreme analytical conditions for analysis (*29*−*32*). However, GC can easily be carried out on sterols derived from hydrolyzed FASE (*33*, *34*), although this would obviously provide only partial information; alkaline hydrolysis is preferred to acid hydrolysis because Δ7-sterols are labile under acid conditions (*35*, *36*). The analysis of FASE is problematic by both GC and HPLC because their molecular weight ranges from 650 and 700 Da and there are no strong chromophore groups.

This present report proposes some possible solutions using GC and HPLC to carry out determination of FASE through TLC purification, including GC-FID (flame ionization detector), HPLC-ELSD (evaporative light scattering detector), and HPLC-ESI-MS (electrospray ionization mass spectrometry) analysis. The main aim was to characterize FASE extracted from hexaploid and tetraploid free-threshing wheats and from their respective hulled ancestors. The cultivation of hulled wheats, which are ancient crops, is receiving increasing interest from farmers and the food industry because of greater consumer interest in organic and health foods (*29*, *37*−*39*). Identification and determination of FASE in hulled wheats could be very useful both to confirm the phylogenesis of tetraploid and hexaploid wheats and to characterize cereal-based ingredients enriched in these bioactive compounds for manufacturing functional foods.

MATERIALS AND METHODS

Samples. Grain samples of hexaploid (HW) and tetraploid (TW) free-threshing wheat species, *Triticum aestivum* L. (*TAe*) and *Triticum durum* Desf. (*TDu*), and their respective hulled ancestors, *Triticum spelta* L. (*TSp*) and *Triticum dicoccon* Schrank (*TDk*), were analyzed. In particular, the whole meal of five genotypes of *TAe* and *TDu*, 9 of *TDk* and 12 of *TSp*, were analyzed for FASE composition. All genotypes were grown in an experimental field in Salcito, southern Italy, under the same conditions.

The grain (100 g) of hulled wheats was de-hulled by passing it twice through rubber-coated rollers and removing the hulls by aspiration (OTAKE model FC2K, Irom Italy srl., Milan, Italy). The grain was milled in a laboratory mill (model IKA A10-IKAWERKE GmbH &CO. KG, Staufen, Germany). Pure standards of cholesteryl esters (decylate, C10:0; palmitate, C16:0; stearate, C18:0; oleate, C18:1; linoleate, C18:2; linolenate, C18:3) were purchased from Sigma Chemical Co. (St. Louis, MO).

FASE Determination. *Sample Preparation.* Lipids were extracted from 5 g of milled samples according to the Folch method (*40*) that was slightly modified as described (*41*). Twenty micrograms of cholesteryl decylate (as an internal standard) was added to 20 mg of extracted lipid and dissolved in a mixture of *n*-hexane/2-propanol 4:1 (v/v). The solution was loaded on a TLC silica plate (20 cm × 20 cm × 0.25 mm film thickness). FASE were collected after 15 cm TLC elution, to completely separate the FASE ($R_f = 1$) from the triglycerides ($R_f = 0.90-0.95$). The elution was obtained with *n*-hexane/ethyl ether 70/30 (v/v). The FASE band was visualized under UV light (254 nm), after spraying with a 0.2% ethanolic solution of 2,7-dichlorofluorescein. The FASE band was scraped off and extracted three times with chloroform; the solution was then dried under a nitrogen stream, redissolved on 50 μL of *n*-hexane, and analyzed by GC, HPLC-ELSD, and HPLC-ESI-MS.

FASE Analysis. *HPLC Analysis. HPLC-ELSD.* An Agilent (Palo Alto, CA) HPLC series 1100 system equipped with Chemstation software, a model G1379A degasser, a binary gradient pump, a model G1313A autosampler, a model G1315B diode array detector, was used. The HPLC system was connected in series with an evaporative light scattering detector (ELSD), model 45 S.E.D.E. R.E (Sedex, Vitry-sur-Seine, France). Chromatographic separation was obtained by RP-HPLC, using a C18-Luna column 25 cm × 4.6 mm ID, 5 μm (Phenomenex, Torrance, CA). The mobile phase composition was programmed from 35% to 75% of 2-propanol in acetonitrile during 35 min; the flow rate was 1.5 mL min^{-1}. The pressure of nebulization was 0.2 Mpa, and the evaporation temperature was 35 °C. A stainless steel three-way valve supplied from Lab Service Analytica (Anzola Emilia, Bologna, Italy) was assembled to split and collect the single fractions that were then injected in GC (under the conditions described below).

HPLC-ESI-MS. LC-MS analyses were carried out with the above-mentioned HPLC apparatus, equipped with a mass spectrometer detector, model G1946A (Agilent). Ionization source: atmospheric pressure electrospray (API-ES, or ESI), set on positive mode. The instrument settings were drying gas flow, 9 L min^{-1}; nebulizer pressure, 0.38 MPa; drying gas temperature, 350 °C; capillary voltage, 5000 V; fragmentor with a 90 V potential on the first skimmer; mass scanning range, 350−750 *m*/*z*. The HPLC method was adapted for MS detection; solvents were added to 1/10 (v/v) 20 mM aqueous ammonium acetate; gradient applied: from 50% to 75% of 2-propanol on acetonitrile within 25 min, standing at 75% for 75 min, then increased to 90% in an

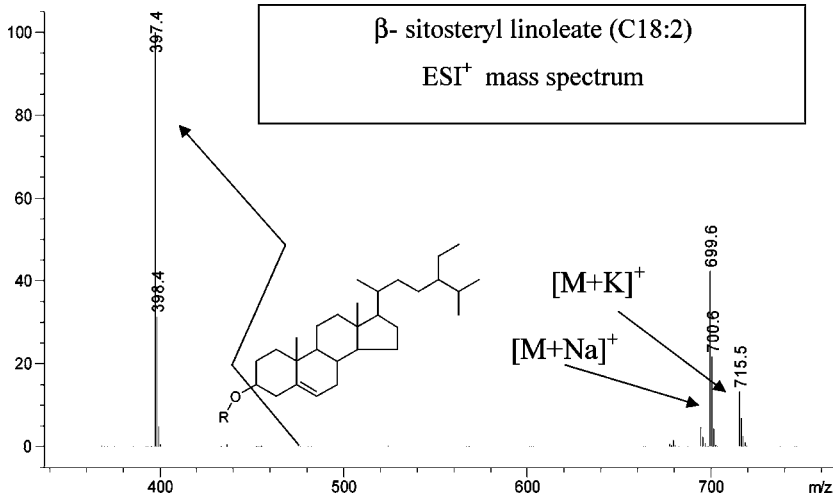

Figure 3. Mass spectrum of β-sitosteryl linoleate (C18:2) found in a sample of *TAe*.

Table 1. Expected Ions for Some FASE[a]

		palmitate (C16:0)		stearate (C18:0)		oleate (C18:1)		linoleate (C18:2)		linolenate (C18:3)	
steryl-residual	*m/z*	$[M+Na]^+$	$[M+K]^+$	$[M+Na]^+$	$[M+K]^+$	$[M+Na]^+$	$[M+K]^+$	$[M+Na]^+$	$[M+K]^+$	$[M+Na]^+$	$[M+K]^+$
campesterol, Δ7-campesterol	383	661	677	689	705	687	703	685	701	683	699
stigmasterol. avenasterol, (clerosterol, fucosterol, Δ7-avenasterol, Δ7-fucosterol, 24-methylene-lophenol)	395	673	689	701	717	699	715	697	713	695	711
β-sitosterol, stigmastanol avenastanol, Δ7-sitosterol	397	675	691	703	719	701	717	699	715	697	713
campestanol	385	663	679	691	707	689	705	687	703	685	701
sitostanol	399	677	693	705	721	703	719	701	717	699	715
24-methylene-Δ7-cholesterol	381	659	675	687	703	685	701	683	699	681	697
citrostadienol	409	767	703	715	731	713	729	711	727	709	725

[a] This table shows the *m/z* of sterolic residue and of the K^+ and Na^+ adducts.

additional 21 min and then isocratic for 10 min. Mobile phase flow rate was 1.5 mL min^{-1}. Mass response was then optimized by the systematic setting of the spray chamber parameters on the directly injected pure standard of cholesteryl esters. In particular, the applied voltage on the first skimmer was set to 90 V, as a compromise between high voltages, which enhance the total ion current, and low voltages, which aid to preserve the integrity of the molecular ions.

GC-FID Analysis. The FASE were analyzed using an HRGC 5300 Fisons (Rodano, Milano, Italy), equipped with a flame ionization detector (FID). Separation was carried out in a fused silica capillary column 8 m length, 0.25 mm i.d. coated with a stationary phase 5% phenyl−95% dimethyl-polysiloxane with a film thickness of 0.25 μm (Restek, Bellefonte, PA). Helium was used as the carrier gas at 0.8 mL min^{-1} flow; 1:20 split ratio. The injector and detector were kept at 340 °C. Oven temperature was programmed from 280 to 330 °C at a gradient of 3 °C min^{-1}. Data acquisition and processing were performed with Chrom-Card, version 1.21 (Fisons).

Statistical Analysis. The FASE results obtained by GC analysis are the averages of three repetitions ($n = 3$). Tukey's honest significant difference (HSD) multiple comparison one way (ANOVA) was used to identify differences at $P < 0.05$. A principal component analysis (PCA) was carried out to determine differences between the hexaploid and tetraploid wheats on the basis of FASE using Statistica software, version 6.0 (2001, Stat Soft, Tulsa, OK).

RESULTS AND DISCUSSION

HPLC Analysis of FASE. An HPLC method for separation of wheat FASE was carried out by using an HPLC-ELSD system, which was particularly useful when universal detection was required. Each analysis takes 30 min including the time to reset the chromatographic conditions. The HPLC profiles of *TAe* and *TDu* (**Figure 1**) were similar to those of *TDk* and *TSp*, respectively. Five major peaks appeared in the chromatograms of HW, whereas only three appear in TW, which lacked the last two peaks. The peaks were successively identified by using HPLC-ESI-MS.

Differences in the mobile phase composition between the ELSD and the ESI-MS detection were suggested by Kalo and Kuuranne (*42*), who added ammonium acetate to the mobile phase to enhance the electrospray ionizability. Chromatographic separation of FASE in aqueous ammonium acetate maintained the elution order observed in HPLC-ELSD, but gave retention times that were 3 times longer and elution peaks that had considerably broader profiles (**Figure 2**); nevertheless, this was unavoidable so as to obtain ionization of the compounds.

The chromatographic sequence of elution for a series of esters was deduced for the same sterol by using the retention times of the cholesteryl esters as standards. In particular, the order of elution was C10:0, C18:3, C18:2, C18:1, C16:0, C18:0, with oleate and palmitate being only slightly resolved; the complete series, between 3 and 0 unsaturations, was eluted within about 40 min. This behavior should extend to the other sterolic series, as the most representative β-sitosteryl, campesteryl, and stigmasteryl derivatives.

This led to two different conclusions: (a) esters of the same steryl series should be separated on a C18 HPLC column on the basis of the length of the fatty acid and the number of double bonds; and (b) the complete purified FASE fraction could have several peaks overlapping that belonging to different steryl series. The only possibility of distinguishing between them

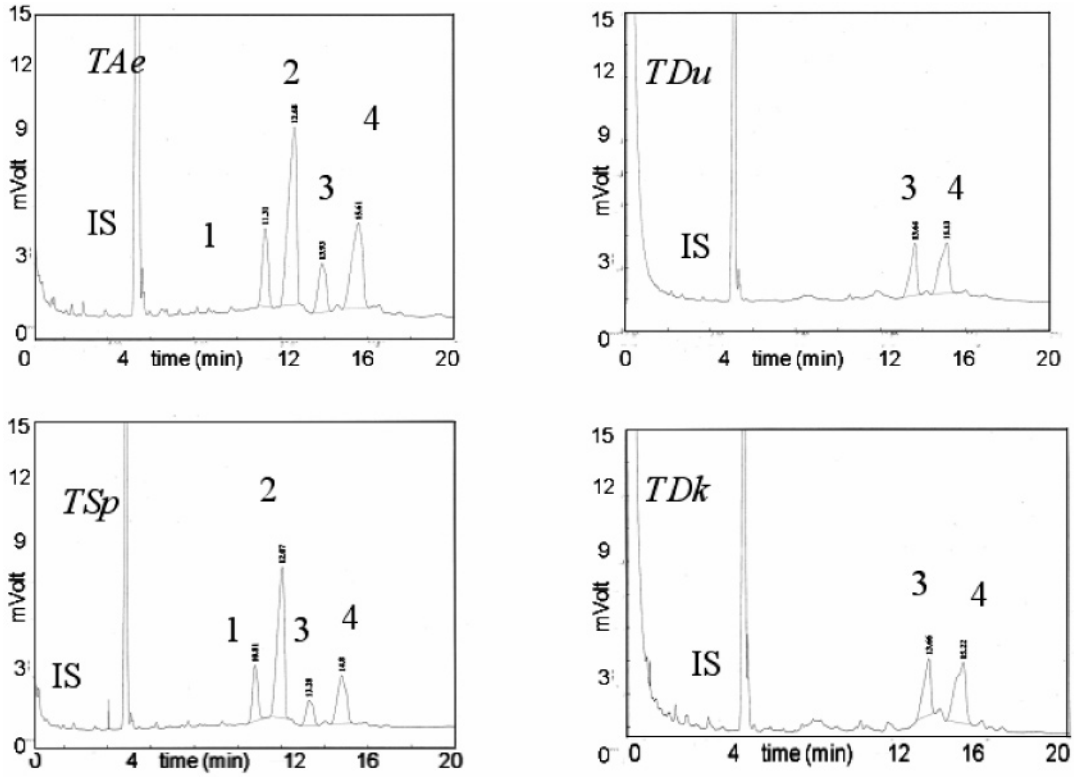

Figure 4. Gas chromatograms of FASE in *TAe*, *TDu*, *TSp*, and *TDk*.

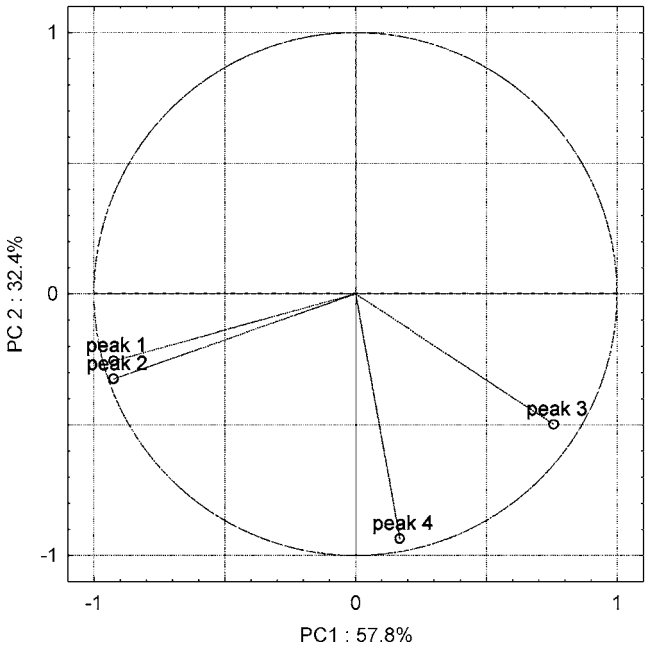

Figure 5. Principal component analysis (loading plot) of the single FASE on TW and HW.

would be the correct use of mass detection, extracting single ions. A typical mass spectrum of a FASE is presented in **Figure 3**, which shows the β-sitosteryl linoleate (C18:2) found in a sample of *TAe* and has three main peaks in two regions of the spectrum separated by about 300 units. The first region (around 400 *m*/*z*) represents sterol residuals, and only one ion, corresponding to $[\text{sterol} - \text{OH}]^+$, is given. In the second region of the spectrum, there are the ions corresponding to the molecular size, $[M + H]^+$, rarely found in abundance, whereas the alkaline adducts, $[M + Na]^+$ and $[M + K]^+$, typical of positive electrospray ionization are always latest and are diagnostic of the identity of molecular species. No ions were produced in the region below 350 *m*/*z*; for this reason, the identification of the fatty acids in the esters had to be deduced indirectly from the information on the entire molecule and the steryl species.

A list of some of the ions expected in the FASE found in wheats, which is based on the free sterols detected in the same samples (*14*), is proposed in **Table 1**. The fragmentation obtained by the single quadrupole used in this work does not allow isomer steryl derivatives such as stigmasteryl or avenasteryl esters to be distinguished. Campesteryl, sitosteryl, and lower amounts of stigmasteryl esters were easily found in the HPLC-ESI-MS chromatograms as revealed in **Figure 2**, which shows the total ion current (TIC) of FASE extracted from *TAe* and *TDu* and the extract ion of the main steryl derivatives; FASE, characterized by different fatty acids, can easily be recognized by extracting single ion chromatograms, as in the case of the partial overlapping of the C18:1 and C16:0 derivatives of the same sterol.

The chromatograms obtained by single ion extraction confirmed the main differences between *TDu* and *TAe* (**Figure 2**). The lack of β-sitosteryl palmitate in *TDu*, as observed by other authors (*18*, *19*, *21*), can be extended to other steryl palmitates. In particular, the ion extraction area of β-sitosteryl palmitate was about 1/20 of that of β-sitosteryl oleate in the tetraploid species, whereas it was completely different for HW, where oleate was nearly undetectable. The same results were observed in the case of campesteryl derivatives, where palmitate in HW and oleate in TW were both found in considerable quantities.

With regards to the stigmasteryl esters, no traces of palmitate were found in TW. The retention time for oleate is assumed to be the same as that for β-sitosteryl linoleate, which is the most representative FASE in all of the samples and is characterized by the same molecular ions ($M + Na = 699$ *m*/*z*; $M + K = 715$ *m*/*z*). Moreover, for this reason, under these analytical conditions it is not possible to confirm the absence of stigmasteryl oleate in HW. The chromatograms obtained from the single

Table 2. FASE Amount in HW and TW Species (mg/100 db)[a]

wheat species	fatty acid steryl ester				
	peak 1	peak 2	peak 3	peak 4	total
			Hexaploid Wheats		
Triticum aestivum					
Pandas	4.3	13.4	6.0	13.3	37.0
Centauro	5.6	17.7	6.6	16.6	46.6
Abbondanza	3.0	11.5	3.8	12.9	31.2
Mieti	3.1	13.8	7.3	19.7	43.9
Serio	4.3	13.6	3.6	9.3	30.9
mean	4.1b	14.0b	5.5b	14.4a	37.9a
CV%	25.3	15.3	29.4	26.1	19.6
Triticum spelta					
Hercule	2.8	12.8	5.9	14.5	36.0
Rouquin	5.4	19.7	9.7	14.2	48.9
Schwabenkorn	7.0	17.7	6.4	14.1	45.2
Oberkulmer	5.8	22.3	5.8	15.4	49.3
Triventina	3.4	18.4	4.4	11.7	37.9
Ebners Rotkorn	5.1	17.2	5.6	11.4	39.3
Frankenkorn	3.3	13.4	6.3	13.8	36.8
Redoutè	5.6	16.5	5.3	12.5	39.9
Huble	7.8	13.8	5.9	12.4	39.9
Ostar	5.9	16.5	4.9	10.5	37.7
Bertel	6.3	12.7	5.3	11.2	35.5
Balmegg	4.9	12.7	6.5	12.0	36.2
mean	5.3a	16.1a	6.0b	12.8a	40.2a
CV%	28.0	19.0	22.0	12.1	18.0
			Tetraploid Wheats		
Triticum durum					
Grazia 1	traces	traces	8.0	11.2	19.2
Grisian	traces	traces	8.2	15.4	23.6
Simeto	traces	traces	5.6	10.2	15.8
Creso	traces	traces	7.8	12.6	20.3
Grazia 2	traces	traces	9.4	13.5	22.9
mean			7.8a	12.6a	20.4b
CV%			17.1	15.7	19.9
Triticum dicoccon					
Molise	traces	traces	10.7	17.5	28.1
Lucanica	traces	traces	5.4	10.4	15.8
Farvento	traces	traces	7.2	10.8	18.0
Agnone	traces	traces	10.5	13.1	23.6
Fontesambuco	traces	traces	5.4	8.1	13.5
S. Angelo del Pesco	traces	traces	7.7	11.6	19.3
Davide	traces	traces	6.7	13.5	20.2
Garfagnana	traces	traces	10.8	13.6	24.5
Guardiaregia	traces	traces	11.2	14.0	25.3
mean			8.4a	12.5a	20.9b
CV%			27.9	20.9	22.3

[a] Within the same column, means with different letters are significantly different ($P < 0.05$). Peak 1, campesteryl C16:0; peak 2, β-sitosteryl C16:0; peak 3, campesteryl C18:1+C18:2; peak 4, β-sitosteryl C18:1+C18:2.

ion extraction of steryl residues suggest that a greater amount of β-sitosterol is present with respect to campesterol. On the basis of the HPLC-ESI-MS results, the presence of steryl palmitate and steryl oleate is characteristic of HW and TW, respectively. The HPLC-ESI-MS by using single ion monitoring demonstrated the lack of oleates in HW and confirmed a higher amount of steryl linoleate in wheats lacking a D genome. This difference was more pronounced when analyzing refined flour (endosperm), because the high content of palmitate provided by the D genome is expressed only in the endosperm. In fact, the fatty acids in FASE from other parts of the HW kernel always have a high linoleate content (*45*).

GC-FID Analysis of FASE. Quantitative analysis of wheat FASE was obtained by GC-FID because the high sensitivity of this technique allows a better baseline and integration as compared to HPLC-ELSD. The minimum amount detectable is 3×10^{-8} g, as measured by five injections of cholesteryl palmitate, corresponding to 0.02 ± 0.002 mg/100 g db of FASE. GC analysis carried out on a short nonpolar capillary column was rapid and permitted discrimination of HW from TW. The GC chromatograms of *TDu* and *TAe* were similar to those of the respective hulled ancestor wheats (*TDk* and *TSp*) (**Figure 4**). The analysis took 20 min, and a short capillary column coated with a nonpolar phase was used; similar chromatographic resolution (results not shown) can be obtained using a 30 m column coated with a polar thermostable phase (TAP, Chrompak, Middelburg, The Netherlands); in this case, the oven temperature reached 360 °C. Four major peaks appeared in the gas chromatograms; peaks 1 and 2 are sharp and characteristic of HW only, whereas peaks 3 and 4, common to both TW and HW, have a profile that suggested that more than one compound was coeluted in each peak.

The GC peaks were identified by re-injection of the main HPLC-ELSD collected fractions: peaks 1 and 2 contained mainly steryl palmitates, campesteryl palmitate (mw 672) in peak 1, and β-sitosteryl palmitate (mw 686) in peak 2 where stigmasteryl palmitate (mw 684) also elutes and is present in very small amounts; oleate, linoleate, and linolenate esters are distributed in peaks 3 and 4, where campesteryl derivatives are separated from β-sitosteryl derivatives on the basis of molecular

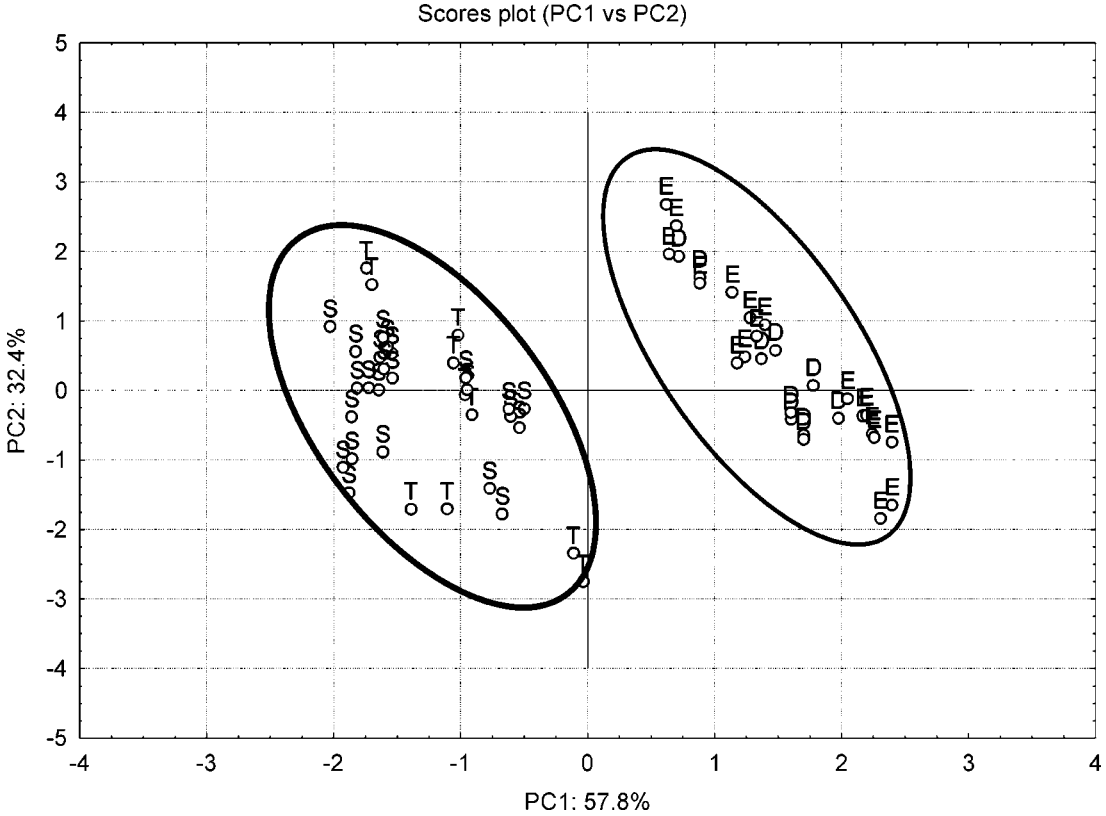

Figure 6. Principal component analysis (scores plot) of FASE of TW (*TDu* = *D*, *TDk* = *E*) and HW (*TAe* = *T*, *TSp* = *S*).

weight. Moreover, regular intervals in retention times of the homologue esters from the same sterol were observed by using cholesteryl esters in GC: the order of elution was C16:0, C18:3, C18:2, C18:1, C18:0. No stearates were found, but the difference in molecular weight between steryl oleate and linoleate, the main C18 esters, is not sufficient to resolve these esters of the same steryl residue. Campesteryl and β-sitosteryl linolenate elute before peaks 3 and 4, respectively, but these were not quantified because of their low amounts, ranging between 0.02 and 0.2 mg/100 g db. Peak 3 was identified as campesteryl oleate and linoleate (mw 684 and 682, respectively), and peak 4 was attributed to β-sitosteryl oleate and linoleate (mw 696 and 694, respectively). On the other hand, the HPLC separation in the C18-reversed phase gave the following elution order of the esters: stigmasteryl < campesteryl < sitosteryl; the saturated acidic C16:0 chain eluted after C18:3 and C18:2.

The amount of FASE in HW and TW, expressed as mg/100 g db on the basis of the four main peaks, is shown in **Table 2** and was calculated on the basis of the internal standard (cholesteryl decylate). The FASE quantification was significantly higher (min 30.9, max 49.3 mg/100 g db) in HW as compared to TW (min 13.5, max 28.1 mg/100 g db). *TSp* contained greater amounts of total FASE (average 40.2 mg/100 g db), which did not significantly differ from that of *TAe* (average 37.9 mg/100 g db). Similarly, *TDk* and *TDu* were not significantly different (mean 20.9 and 20.4 mg/100 g db, respectively). The single FASE identification could be performed by GC−MS, but only because the instruments giving high capacity vacuum system are suitable for this analysis.

Table 2 shows that campesteryl and β-sitosteryl palmitates (peaks 1 and 2) are present in HW only, confirming that the D genome adds the complete system for palmitate esterification (*43*, *44*). The total amount of steryl ester palmitates varied from 18.1 and 21.4 mg/100 g db, for *TAe* and *TSp*, respectively, and this difference was significant for both campesteryl palmitate and β-sitosteryl palmitate. Some authors have found that the content of sitosteryl palmitate ranges from 1.6 to 40 mg/100 g db in bread wheat and from 0.6 to 6 mg/100 g db in durum wheat (*20*). It should be noted that campesteryl oleate and linoleate (peak 3) were significantly higher in TW (mean 8.1 mg/100 g db) as compared to that in HW (mean 5.7 mg/100 g db). The amount of β-sitosteryl oleate and linoleate was 14.4 and 12.8 mg/100 g db for *TAe* and *TSp*, respectively, and did not significantly differ from *TDu* (13.4 mg/100 g db) and *TDk* (12.5 mg/100 g db).

Principal Components Analysis (PCA). The PCA results of the FASE composition of HW and TW are shown in **Figure 5**, which illustrates the score plot. In accordance with the findings of the analytical results, there was a significant difference between the HW and TW with regard to the composition of the FASE; more than 80% of variance is explained, and the loading plot (**Figure 6**) shows that the variance affects mainly principal component 1 (57.8%).

In addition, the score plot confirms that peak 3, containing mainly campesteryl C18 esters, behaves inversely to the other components considered and is more abundant in durum wheat and in emmer as compared to in bread wheat and spelt. Peak 1, peak 2, and consequently total FASE can discriminate HW. As the score plot shows, HW and TW can be discriminated, but *TAe* and *TDu* cannot be distinguished from their hulled ancestor *TSp* and *TDk*, respectively.

These findings confirm that the composition of FASE represents a unique fingerprint of botanical origin and underlines the genetic affinity of bread and durum wheat with their hulled ancestors *TSp* and *TDk*, respectively.

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