Analysis of Free and Esterified Sterols in Wheat Flour and Semolina[1]

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ABSTRACT

Qualitative and quantitative determinations of free and esterified sterols in wheat are described. Determinations were carried out principally with column, thin-layer, and gas-liquid chromatography. Free sterols and sterol ester analyses were performed on U.S. and foreign wheats. The endosperm of HRS wheat contained small quantities of free sterols; conversely, a higher content of saturated sterol esters was observed. The endosperm of durum wheat contained an appreciable amount of free sterols, whereas the saturated sterol esters appeared only as a trace. The content of free sterols in the mill streams of several wheats increased as ash increased, whereas the saturated sterol ester content decreased. For HRS and durum wheat, the major portion of the free sterols is in the bran region. Sitosterol, campesterol, cholesterol, and brassicasterol were identified in the free sterols and total sterol esters by gas-liquid chromatography. Sitosterol and campesterol were most abundant.

A review by Bailey (1) covers most of the pertinent early work on wheat sterols. In 1939, Bernstein and Wallis isolated alpha$_3$-sitosterol (2) and proposed a structure for alpha$_1$-sitosterol (3). Campesterol was isolated from wheat germ oil and characterized by Fernholz and MacPhillamy (4). Delta^7-stigmasterol was isolated from wheat in 1953 by Idler and co-workers (5), who reported that delta^7-stigmasterol comprised 3% of the total sterols present and stated that it was a component of alpha$_3$-sitosterol.

In 1964 McKillican and Sims (6) reported the separation of unesterified sterols from wheat lipids on a silicic acid column. The column eluant was reported to contain additional material other than free sterols. Later McKillican (7) separated the free sterols by two-dimensional thin-layer chromatography. With gas-liquid chromatography, she reported over 70% of the sterols as beta-sitosterol and the remainder as gamma-sitosterol.

Considerable work has been reported on the isolation, characterization, and identification of the sterol esters, particularly sitosterol palmitate (1,8-13).

Garcia Faure et al. (14) reported that sitosterol palmitate was composed of sitostanol palmitate as well as sitosterol palmitate.

In addition to sitosterol palmitate, Gilles and Youngs (15) have reported the existence of sitosterol oleate, linoleate, and linolenate in durum and HRS wheats.

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MATERIALS AND METHODS

Ten varieties of HRS wheat, all grown at Fargo, N. Dak., in 1965, were milled on a Buhler experimental mill according to *Cereal Laboratory Methods* (16).

Justin and Thatcher (HRS) wheats, grown in 1966 at Warren, Minn., and Froid, Mont., respectively, were milled on a pilot mill according to a method of Shuey and Gilles (17). Two soft wheats (Gaines and Nugaines) and one club wheat (Omar), all grown in Washington in 1966, also were milled on a pilot mill. Five durum varieties (Leeds, Stewart 63, Wells, Mindum, and Lakota) were milled to semolina on an experimental Allis mill according to *Cereal Laboratory Methods* (16). All varieties were grown at Langdon, N. Dak., in 1965. Ten Italian soft wheat varieties and 58 HRW wheat samples from different locations in Oklahoma and Nebraska were milled on a Brabender quadruplex mill.

Lipid Extraction

A rapid extraction method (18) for flour, semolina, and other wheat kernel parts was employed. Ten grams of material was placed in a 250-ml. Erlenmeyer flask, and to this was added 30 ml. of chloroform. The flask and contents were placed on a Burrell shaker for 15 min. After this the solids were removed by vacuum filtration and washed twice with 10-ml. portions of chloroform. The filtrate was evaporated to dryness and weighed, or concentrated to a specific volume and spotted directly on a thin-layer plate.

Column Chromatography

Column chromatography, with 100-mesh silicic acid, was used for crude separation of free sterols and sterol esters from wheat lipids. Preparation of the column was followed as described by Hirsch and Ahrens (19). The lipids were fractionated by discontinuous elution as outlined below:

Eluting Solvent	*Eluted Fraction*
Petroleum ether (b.p. 30°–60°C.)	Hydrocarbons, sterol esters
Ethyl ether:petroleum ether, 1:11.5 (v./v.)	Triglycerides, free fatty acids
Ethyl ether:petroleum ether, 7:5 (v./v.)	Diglycerides, free sterols
Methanol (anhydrous)	Monoglycerides, polar lipids

The fractions were verified by TLC and those containing sterol esters and free sterols were retained for further analysis.

Saponification

The petroleum ether and ethyl ether:petroleum ether, 7:5 (v./v.) fractions containing the sterol esters and free sterols were evaporated to dryness under vacuum and stored under nitrogen. The samples to be analyzed by GLC were saponified with 6% alcoholic (methanol) potassium hydroxide for 15 hr. at room temperature.

Thin-Layer Chromatography

Thin-layer plates were coated with Adsorbosil-3, 0.25 mm. thick. The plates were air-dried for 15–20 min., then heated in an oven for approximately 1 hr. at 120°C., and stored in a closed container.

McKillican (7) reported the separation of free sterols from HRS, soft white, and durum wheat by two-dimensional TLC. However, no identification was made of the free fatty acids and the 1,3- and 1,2-diglycerides, which might interfere. In single-dimension chromatography, it has been shown that if the polarity of a petroleum ether-ethyl ether-acetic acid system is increased, the free sterols can be obscured by the 1,3-diglyceride spot (20).

The free sterols were separated on thin-layer plates with a solvent system of benzene:ethyl acetate, 95:24 (v./v.), a modification of a solvent system described by Avigan *et al.* (21). The sterol esters were separated with carbon tetrachloride as the developing solvent (15).

Visualization of the spots was accomplished most frequently with a 50% aqueous sulfuric acid spray, followed by 10–15 min. of heating in an oven at 200°–220°C. To identify the sterol and sterol ester spots, R_f values of the unknowns were compared with standards spotted on the same plate. Also, the colors of the unknowns and standards were compared after they were sprayed with aqueous sulfuric acid and heated for approximately 3 min. at 200°C. The sterols and sterol esters produced a pink color.

Quantitative data were obtained with a Photovolt densitometer, Model 530, equipped with a Varicord recorder, response setting 5. With this instrument, analysis of thin-layer plates was facilitated. Quantitative amounts of a standard were spotted on each plate, and for each plate a standard curve was drawn.

The Beckman Analytrol, Model RB, was used to analyze the area under the curves, recorded by the Varicord recorder from the densitometer.

Gas-Liquid Chromatography

All gas-chromatographic analyses were carried out on a Beckman GC-2A equipped with a Beckman flame ionization detector and a Brown recorder. Sterol separations were done on 6-ft. stainless-steel columns 0.125 in. in diameter and packed with either DC-560 or SE-30 on Gas Chrom Q, 60- to 80-mesh. The liquid phases were applied to the solid in 1% concentration by weight. The operating conditions for sterol analysis are shown below.

Carrier gas	helium
Rate of flow	42 ml./min.
Inlet pressure	30 lb./sq. in.
Column temperature	240°C.
Chart speed	0.5 in./min.

The peaks obtained through GLC were identified by comparing relative retention times of the unknowns with standards under identical operating conditions.

Trimethylsilyl ether derivatives were prepared as described in Technical Bulletin 11 from Applied Science Laboratories, Inc. (22). To remove as much pyridine as possible, it was necessary to evaporate the sample mixture to dryness. The sample then was redissolved in ethyl ether and injected into the gas chromatograph.

RESULTS AND DISCUSSION

Quantitative Analysis of Free Sterols and Saturated Sterol Esters

U.S. Wheats. Table I shows the results obtained in the analyses of free

TABLE I

FREE STEROL AND SATURATED STEROL ESTERS OF FLOUR MILLED FROM U. S. WHEATS

SAMPLE	FREE STEROL[a]	SAT'D STEROL ESTERS[b]	SAMPLE	FREE STEROL[a]	SAT'D STEROL ESTERS[b]	SAMPLE	FREE STEROL[a]	SAT'D STEROL ESTERS[b]
	mg. %	*mg. %*		*mg. %*	*mg. %*		*mg. %*	*mg. %*
HRW (NEBR.)[c]			HRW (OKLA.)[c]			HRS (N. DAK.)		
Lancer	7.0	67.3	Gage	7.9	53.5	Justin	7.7	34.2
Bison	7.1	72.0	Tmp × CI 12406	6.7	54.4	Fortuna	8.2	42.0
Omaha	5.2	71.5	Triumph	6.4	51.6	DURUM (N. DAK.)		
Ottawa	6.3	68.5	Tmp × T-ac	5.5	43.0	Leeds	24.5	.....[e]
Turkey	9.1	67.1	Wichita	4.5	58.4	Stewart 63	38.4	.....
Gage	7.3	63.9	Caddo	5.0	40.1	Wells	27.0	.....
Scout	5.6	62.2	HRS (N. DAK.)			Mindum	37.4	.....
Cheyenne	5.6	72.5	Selkirk	7.7	34.2	Lakota	32.7	.....
HRW (OKLA.)[c]			II-54-30	5.9	39.9	WHITE (WASH.)		
Kaw	5.6	53.3	ND 264	12.5	40.4	Nugaines	15.0	45.3
Parker	8.9	51.8	Nordman	8.4	37.7	Gaines	15.1	50.4
F_8 cross[d]	5.5	44.2	Manitou	9.1	36.6	CLUB (WASH.)		
Scout	6.8	45.0	Chris	5.9	34.4	Omar	30.8	25.5
Concho	10.5	41.2						

[a] Crude lipids extracted in chloroform.
[b] Crude lipids extracted in petroleum ether.
[c] Represents composite from different locations within the state.
[d] Composite.
[e] Too small to measure.

sterol and saturated sterol esters (principally sitosterol palmitate) in U.S. wheats. A noticeable difference in the free sterol content is seen between *Triticum aestivum* L. (common wheat) and *T. durum* Desf. Likewise, a difference in saturated sterol ester content is observed. *T. aestivum* contains a considerable amount of the saturated sterol esters, but very little free sterols. *T. durum* contains only trace amounts of saturated sterol esters, but a considerable amount of free sterols. Reasons for these differences between the two wheat species were not investigated. However, the results could indicate a difference in metabolic pathways initiated by enzyme activity. These data suggest that the enzyme could hydrolyze the saturated sterol esters into free sterols and palmitic acid. Youngs (18) has reported slightly more palmitic acid present in four durum wheats than in Selkirk, a HRS wheat. Also, the free fatty acids of durum contained more palmitic acid than the HRS wheat.

Omar, a club wheat (*T. compactum* Host), shows durum characteristics with reference to free sterol content (relatively high value), but shows characteristics between those of *T. aestivum* and *T. durum* (Table I) with respect to saturated sterol ester value. The two soft wheats examined show characteristics similar to those of the HRS wheats. Perhaps the sterol content of wheat is genetically controlled.

Typical thin-layer separations of free sterols and sterol esters are shown in Figs. 1, 2, and 3.

Foreign Wheats. It has been reported that some Italian soft wheats contain very little sitosterol palmitate as measured by the Matveef method (23,24). Through the co-operation of Prof. Fabriani at the University of

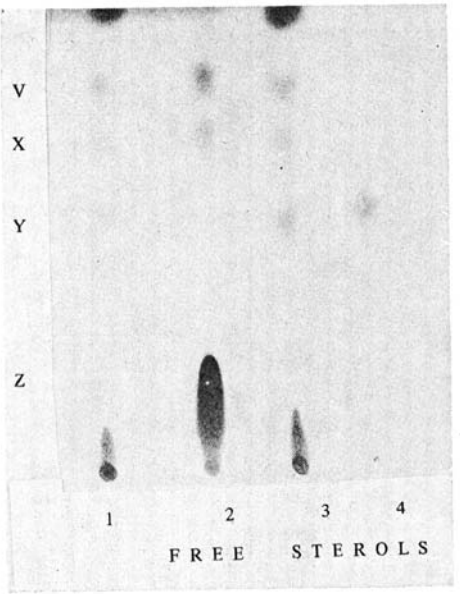

Fig. 1. Thin-layer chromatoplate of separation of free sterols. Left to right: 1, HRS wheat; 2, monoglyceride; 3, durum wheat; 4, standard sitosterol. Top to bottom: V, 1,3-diglyceride; X, 1,2-diglyceride; Y, free sterol; Z, monoglyceride.

TABLE II

ANALYSIS OF FREE STEROLS AND SATURATED STEROL ESTERS IN FLOUR MILLED FROM FOREIGN WHEATS

SAMPLE	FREE STEROLS	SAT'D STEROL ESTERS	SAMPLE	FREE STEROLS	SAT'D STEROL ESTERS
	mg. %	*mg. %*		*mg. %*	*mg. %*
Italian soft wheats			Italian soft wheats		
Aquilla	9.1	57.6	Impeto	53.8	7.3
Leone	13.8	56.1	Funone	43.3	7.0
Produttore	14.9	53.2	Damiano	63.5	6.0
Campodoro	62.7	8.4	San Pastore	40.3	5.1
Leonardo	33.1	7.9	Argentina		
Generoso 7	52.0	7.6	Tagenrog (durum)	28.8	....

Rome, ten selected Italian soft wheats were obtained and analyzed for saturated sterol ester content (principally sitosterol palmitate) by Gilles and Youngs (25). The results were in general agreement with Fabriani's (Table II). The free sterols of these wheats were analyzed (Table II).

Of the ten Italian soft wheat varieties tested, seven showed durum characteristics with reference to saturated sterol esters (low values). They also showed durum characteristics with reference to the free sterols (high values). The other three samples (Aquilla, Leone, and Produttore) gave the expected high saturated sterol ester and low free sterol values. With the exception of the one club wheat, the seven Italian soft wheats were the only

nondurum wheats tested that gave durum characteristics with reference to saturated sterol ester and free sterol content.

Mill Stream Analysis. Samples were collected from various mill streams of an Allis mill (durum), and a pilot mill (HRS). All the samples were extracted with chloroform. The free sterols and saturated sterol esters present in each mill stream were analyzed by TLC and densitometry; see table below (in which no values for saturated sterol esters are given because the amounts present were very small) and Tables III, IV, and V.

Allis Mill Streams

Streams	*Free Sterols mg. %*	*Streams*	*Free Sterols mg. %*
1st Break	48.2	4th Break (bran)	89.1
2nd Break	60.4	Semolina from 2nd and 3rd break	36.5
3rd Break	79.1	Semolina	36.5

The data given here and in Table III show that durum wheat is similar to HRS wheat concerning free sterol distribution in the kernel. The free sterol content increases as the amount of bran increases. The saturated

TABLE III

ANALYSIS OF FREE STEROLS AND SATURATED STEROL ESTERS IN HRS WHEAT (PILOT MILL STREAMS)

SAMPLE	THATCHER			JUSTIN		
	Free Sterols	Sat'd Sterol Esters	Ash	Free Sterols	Sat'd Sterol Esters	Ash
	mg. %	*mg. %*	*%*	*mg. %*	*mg. %*	*%*
Patent flour	9.3	51.1	0.367	6.2	52.3	0.436
Tailings	18.4	42.4	0.527	15.4	46.1	0.649
Low-grade	26.1	35.9	0.663	23.5	36.8	0.884
Red dog	52.0	24.8	1.043	68.1	28.4	1.880
Head shorts	132.5	13.2	3.575	102.0	19.0	4.325
Tail shorts	151.5	12.8	3.850	124.3	18.0	4.845
Bran	142.5	11.6	5.585	126.3	14.7	7.365

sterol esters, however, do not occur in a measurable amount in the durum kernels.

An interesting relationship is shown in Table III. When the free sterol

TABLE IV

PERCENT FREE STEROLS AND SATURATED STEROL ESTERS IN LIPIDS EXTRACTED FROM DIFFERENT MILL STREAMS OF JUSTIN[a]

MILL STREAM	TOTAL LIPID		MILL STREAM	TOTAL LIPID	
	Free Sterols	Sat'd Sterol Esters		Free Sterols	Sat'd Sterol Esters
	%	%		%	%
Patent flour	0.45	3.76	Head shorts	2.24	1.66
Tailings	0.65	3.00	Tail shorts	2.32	1.42
Low-grade	0.75	2.57	Bran	3.45	1.42
Red dog	1.68	2.14			

[a] All samples were adjusted to equal lipid concentration before they were spotted on thin-layer plates.

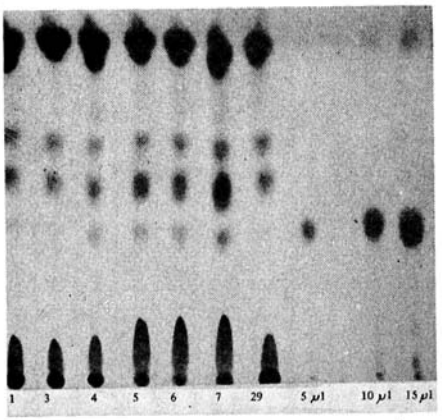

Fig. 2 (left). Thin-layer separation on a chromatoplate of free sterols obtained from different (Miag) mill streams. Left to right: 1, low-grade flour; 3, tailings; 4, red dog; 5, tail shorts; 6, head shorts; 7, bran; 29, patent flour; and standards.

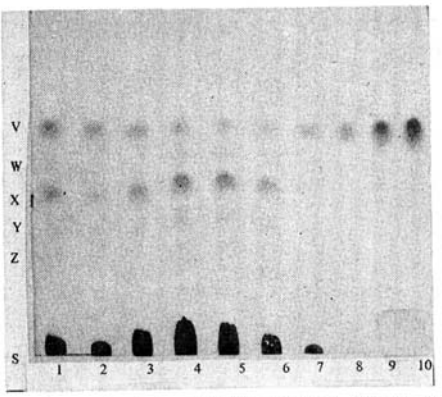

Fig. 3 (right). Thin-layer separation on a chromatoplate of sterol esters obtained from different (Miag) mill streams. Left to right: 1, low-grade; 2, tailings; 3, red dog; 4, tail shorts; 5, head shorts; 6, bran; 7, patent flour; 8-10, standards. Top to bottom: V, sitosterol palmitate; W, sitosterol oleate; X, sitosterol linoleate; Y, sitosterol linolenate; Z, unidentified; S, sample (origin).

content increased, the ash content of the corresponding stream also increased; conversely, the saturated sterol ester content decreased as the ash increased. Typical thin-layer separations are shown in Figs. 2 and 3.

Lipids were extracted from the mill streams and were adjusted to equal concentration. Equal amounts of the lipids from each mill stream were spotted on thin-layer plates and the free sterols and saturated sterol esters were measured. The results (Table IV) confirm the free sterol-saturated sterol ester relation shown in Table III.

The two soft white wheats, Nugaines and Gaines, and the club wheat, Omar (Table V), showed different free sterol distributions in the head shorts and the bran compared to HRS wheats.

TABLE V

ANALYSIS OF FREE STEROLS AND SATURATED STEROL ESTERS IN SOFT WHEATS (Pilot Mill Streams)

Sample	Omar		Nugaines		Gaines	
	Free Sterols	Sat'd Sterol Esters	Free Sterols	Sat'd Sterol Esters	Free Sterols	Sat'd Sterol Esters
	mg. %	*mg. %*	*mg. %*	*mg. %*	*mg. %*	*mg. %*
Patent flour	27.0	30.8	15.5	45.4	15.5	49.8
Head shorts	114.4	18.4	95.6	20.4	101.3	19.4
Bran	78.0	11.2	53.5	14.2	57.4	13.7

IDENTIFICATION OF INDIVIDUAL STEROLS BY GAS-LIQUID CHROMATOGRAPHY

Chloroform was used to extract lipids from the wheat samples. The crude

lipids were fractionated on a silicic acid column, and the sterol ester and free sterol fractions were saved.

The free sterol fraction, which included other lipids such as diglycerides, was saponified with potassium hydroxide; also, the sterol esters. In some instances the free sterols were recrystallized from ethanol after saponification. Trimethylsilyl ether derivatives were prepared from the crystals. In other cases, the trimethylsilyl derivatives of the free sterol fraction were prepared directly from the chloroform extracts of the unsaponified material. When the derivatives were injected into the gas chromatograph, similar sterol distributions resulted from each method.

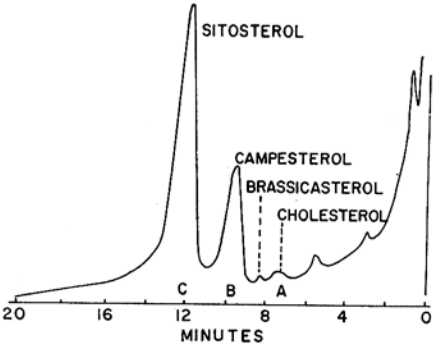

Fig. 4. Gas chromatograph of trimethylsilyl ether derivatives of wheat sterols.

Sitosterol, campesterol, brassicasterol, and cholesterol were identified (Fig. 4). Brassicasterol was present only in trace amounts when fairly large samples were applied to the column; therefore, no values are cited in the tables. No separation was obtained between sitosterol and sitostanol, or between cholesterol and cholestanol. Other peaks in Fig. 4 were not identified.

Although cholesterol has been reported in other plant lipids (26,27,28), its presence as a constituent in wheat lipids seemed highly unusual. While identification was based solely on retention time, several wheat lipid samples were tested. In each case, the retention time consistently checked with a known standard. The addition of cholesterol to a sample of wheat lipids invariably enlarged the area of the peak labeled as cholesterol.

The relative distribution of the individual sterols was determined by measuring the peak areas obtained from gas chromatographic analysis (Tables VI and VII). Results represent an average of at least three analyses for each value given. The letters in the column heading refer to the peaks in Fig. 4.

Table VI shows that the majority of the sterol composition is campesterol and sitosterol for both free sterols and total sterol esters in durum wheat.

Table VII gives results of the free sterol and total sterol ester composition from the various mill streams of the pilot mill. Again campesterol and sitosterol are the two major sterols. Of special interest is the large amount of un-

TABLE VI

GAS-LIQUID CHROMATOGRAPHY OF FREE STEROL AND STEROL ESTER FRACTIONS OF DURUM[a]

	FREE STEROL FRACTION				STEROL ESTER FRACTION			
	Unknown Peaks	A Cholesterol	B Campesterol	C Sitosterol	Unknown Peaks	A Cholesterol	B Campesterol	C Sitosterol
	%	%	%	%	%	%	%	%
Lakota semolina		trace	30.2	69.8				
Wells semolina		trace	26.4	73.6				
Composite semolina		trace	26.4	73.6	0.4	1.7	25.9	72.4
Composite bran	8.5	9.7	14.0	67.8	1.0	....	29.9	69.1
Discard flour		1.1	17.1	81.8	4.3	5.4	14.2	76.1

[a]All analyses done on a 6-ft. stainless-steel column containing 1% SE-30 on 100- to 200-mesh Gas-Chrom Q.

TABLE VII

GAS-LIQUID CHROMATOGRAPHY OF FREE STEROLS AND STEROL ESTERS IN PILOT MILL STREAMS

	UNKNOWN PEAKS		A (CHOLESTEROL)		B (CAMPESTEROL)		C (SITOSTEROL)	
STREAM	SE-30	DC-560	SE-30	DC-560	SE-30	DC-560	SE-30	DC-560
	%	%	%	%	%	%	%	%
Free sterols								
Low-grade	5.6	7.4	6.4	2.0	19.0	17.5	68.9	73.0
Tailings	6.5	2.5	1.7	1.6	18.8	22.2	73.0	73.7
Red dog			6.6	6.2	19.9	19.8	73.5	74.0
Tail shorts		0.6	1.5	2.4	26.8	30.0	71.7	67.0
Head shorts		1.5	4.3	3.6	28.3	27.0	67.4	67.9
Bran		1.6	0.8	0.1	23.1	25.4	76.0	72.9
Patent flour	6.4		6.9	3.8	15.9	17.1	70.8	79.1
Sterol esters								
Low-grade			6.5	6.6	16.3	16.2	77.2	76.8
Tailings	2.4	3.8	2.5		16.3	24.2	78.8	72.0
Red dog		3.2	4.9	0.8	19.0	19.5	76.1	76.5
Tail shorts		1.5	1.8	1.2	23.8	22.0	74.4	75.4
Head shorts	1.3	1.3	0.4		24.9	25.0	73.8	73.7
Bran	3.1	3.0	0.8	0.6	22.9	20.6	73.2	73.8
Patent flour	21.9[a]	21.3[a]		3.1	15.2	16.1	62.9	59.5

[a]All but 2% occurred as a single peak just preceding that of cholesterol.

known present in the sterol esters of patent flour. All but 2% of this occurred in a single peak just preceding that of cholesterol. This peak did not occur in such large amounts in any of the other fractions.

SUMMARY AND CONCLUSIONS

The separation of free sterols, as a class, from other wheat lipids was done by TLC. Separation of the individual components of the free sterols by this method was not successful.

Free sterol and saturated sterol ester analyses were performed on U.S. and foreign wheats. The endosperm of HRS wheat contained small quantities of free sterols; conversely, a higher content of saturated sterol esters

was observed. The endosperm of durum wheat contained an appreciable amount of free sterols, whereas the saturated sterol esters appeared only as a trace. The white wheats were similar to the HRS wheats. The endosperm of one club wheat had a free sterol content similar to that of durum. The saturated sterol ester content was intermediate between that of HRS and durum wheat. Of ten Italian hard wheats analyzed, only three were similar to HRS wheat varieties. With respect to free sterols and saturated sterol esters, the others showed durum characteristics.

The mill streams of several wheats were analyzed. The content of free sterols increased as ash increased, and concomitantly the saturated sterol ester content decreased. For HRS and durum wheat, the major portion of the free sterols exists in the bran region. The soft white and club wheats contained more free sterols in the shorts than in the bran.

Sitosterol, campesterol, cholesterol, and brassicasterol were identified in the free sterols and total sterol esters by GLC. Sitosterol and campesterol were most abundant.

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