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Studies on the Nature of the Chloroplast Lamella. I. Preparation and Some Properties of Two Chlorophyll–Protein Complexes*

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ABSTRACT: Chloroplasts have been treated with the anionic detergent sodium dodecylbenzene sulfate (SDBS) and two chlorophyll–protein complexes were obtained by electrophoresis in polyacrylamide gels. These components (complexes I and II) account for approximately 25 and 50%, respectively, of the lamellar protein. Complex I has a chlorophyll a/b ratio of 12:1 and complex II has a ratio of 1.2:1. Complex I is more readily extracted by successive SDBS treatments. Sublamellar particles, prepared using digitonin, depleted in photochemical system I, give higher proportions of complex II, and those deficient in system II yield more complex I. We conclude that complexes I and II are derived from systems I and II, respectively, and that complex I represents a more superficial particle in the lamellar matrix.

The lipids, pigments, and cations associated with the gross lamellar lipoprotein material of the chloroplast are well documented (Lichtenthaler and Park, 1963), but little is known of the number and composition of the macromolecules in this complex. This paucity of knowledge is due mainly to the difficulty encountered in rendering the lamellae soluble at physiological pH. Bailey *et al.* (1966) have reviewed recently the methods used to solubilize the lipoprotein complex and concluded that in their hands anionic detergents were the most useful reagents for the complete dissociation of the lamella into its components.

Solutions of the anionic detergents, SDS[1] and SDBS, have been used frequently to dissolve the insoluble material of chloroplasts (Smith and Pickels, 1941; Wolken, 1956; Chiba, 1960; Itoh *et al.*, 1963) and a single sedimenting boundary (2–5.5 S) has been observed in the ultracentrifuge with these extracts (a similar sedimentation coefficient has also been observed for the lipid-free total protein of lamellae dissolved in SDS solution (Biggins and Park, 1965)). However, the most recent work on the solubilization of the lamellae with anionic detergents (Bailey *et al.*, 1966; Ogawa *et al.*, 1966; Sironval *et al.*, 1966) has shown that several protein components are present, some of which contain chlorophyll and all of which presumably contain detergent. Characterization of the macromolecules in such extracts is essential to our understanding of the structure and function of the lamella.

In this communication a method is described for the isolation of two major chlorophyll-containing proteins and evidence adduced for their possible relation to photochemical systems I and II (Witt *et al.*, 1961; Duysens *et al.*, 1961). A subsequent communication will describe the composition of the two complexes. A preliminary note on the isolation and characterization of the two major chlorophyll–protein components has been reported (Thornber *et al.*, 1966).

Methods

Preparation of Chloroplasts. Chloroplasts were

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[1] Abbreviations used in this work: SDS, sodium dodecyl sulfate; SDBS, sodium dodecylbenzene sulfate; Chl, chlorophyll; NADP, nicotinamide–adenine dinucleotide phosphate.

isolated from spinach beet (*Beta vulgaris*) leaves (160 g) by differential centrifugation of an homogenate in 400 ml of 0.5 M sucrose–0.05 M $KHCO_3$ adjusted to pH 7.5 with CO_2 (Bailey *et al.*, 1966). Preparations were also made from spinach (*Spinacia oleracea*), tobacco (*Nicotiana tabacum*), and oat (*Avena sativa* var. condor) leaves.

Solubilization of Lamellae. The isolated chloroplasts were suspended in water (total volume about 50 ml) using a Potter-Elvehjem homogenizer and centrifuged at 100,000*g* for 20 min. The washed chloroplasts were dispersed in 0.5% SDBS–0.05 M sodium borate, pH 8.0 (SDBS:Chl, 2.5:1, w/w), by grinding in an homogenizer, and the suspension was centrifuged at 100,000*g* for 50 min. The plug was reextracted with detergent solution using the same volume as for the first extraction, and recentrifuged. Further extractions were carried out to obtain five supernatants and a final residue. The volume of detergent solution used for obtaining the fourth and fifth extracts was one-half that used for the first extraction. All operations were carried out at 4°.

Zone Electrophoresis of SDBS Extracts. Electrophoresis was carried out in 0.2% SDBS–0.05 M sodium borate, pH 8.0, in the apparatus described by Ornstein and Davis (1962). The polyacrylamide gels (concentration, 9%; cross-linking, 1.67%) were equilibrated by electrophoresis (110 v, 6 ma/tube) for 15 min before applying the SDBS extract which was mixed with propylene glycol (10%, v/v) and layered under the buffer onto the top of the gel columns. After further electrophoresis for 10 min the gels were stained with Amido Black 10B solution, and destained by washing in methanol–acetic acid–water (5:1:5, v/v).

Analysis of Stained Polyacrylamide Gels. After electrophoresis and staining the gels were placed in EEL colorimeter tubes and a Chromoscan, Model J297 (Joyce, Loebl & Co., Ltd., Gateshead, England) was used to scan the gels at right angles to the column. The transmission of white light was recorded and the concentration of stain in each zone was determined by integration of the areas under the corresponding peak.

Isolation of Chlorophyll–Protein Complexes. The complexes were separated from the second or third SDBS extracts using polyacrylamide gel electrophoresis in 0.2% SDBS–0.05 M sodium borate, pH 8.0. Gels of the same composition as described above, but of greater size (the gel column was 1.7 cm in diameter, 7.0 cm high), were equilibrated by electrophoresis (140 v, 20 ma/tube) for 45 min, and then electrophoresced for a further 40 min after the sample (containing about 0.5 mg of chlorophyll, 0.3 mg of N) had been loaded onto the column. After electrophoresis, disks containing the separated components were dissected from the gel column and cut into small pieces. The pieces were disintegrated further by extrusion from a syringe through 100-μ nylon bolting cloth (Henry Simon, Ltd., Stockport, Cheshire, England). The gel particles were then transferred to the top of a G-200 Sephadex column (3.2 × 12 cm) equilibrated in 0.02% SDBS–0.05 M sodium borate, pH 8.0, and the isolated components were eluted through the column.

Chlorophyll Determination. The concentrations of chlorophylls a and b were estimated by the method of Mackinney (1941).

Nitrogen Content. Nitrogen was determined by micro-Kjeldahl digestion followed by distillation and titration (Chibnall *et al.*, 1943).

Measurement of Photochemical Activities. A. HILL REACTION. The evolution of oxygen by a suspension of chloroplast material in a Chappell–Rank polarographic oxygen electrode (Rank Brothers, Bottisham, Cambridgeshire) was recorded electronically. In the 5-ml reaction volume there were 250 μmoles of phosphate buffer, pH 7.1, 5 μmoles of KCl, 2.5 μmoles of $(NH_4)_2SO_4$, 5 μmoles of $K_3Fe(CN)_6$, and chloroplasts containing from 0.03 to 0.2 mg of chlorophyll. The cell, jacketed with water at 20°, was illuminated from the side by a 40-w tungsten lamp. A red filter (cutoff below 608 mμ) and a heat filter were used and the incident illumination was 8–16 × 10^3 lux. Under these conditions the chloroplasts were light saturated.

B. REDUCTION OF NADP. Chloroplast material containing from 0.02 to 0.1 mg of chlorophyll was suspended in each of two 1-cm spectrophotometer cuvets in volumes of 3 ml. Each cuvet contained 150 μmoles of phosphate buffer, pH 7.1, 3 μmoles of KCl, 1.5 μmoles of $(NH_4)_2SO_4$, 0.42 μmole of NADP, 0.15 mg of ferredoxin, 0.03 mg of ferredoxin–NADP reductase, 0.5 μmole of 2,6-dichlorophenolindophenol, and 30 μmoles of sodium ascorbate. Ferredoxin was prepared by the method of San Pietro and Lang (1958), and ferredoxin–NADP reductase was prepared by the method of Tagawa and Arnon (1962). One cuvet was used as a reference and as an unilluminated control when the other cuvet was illuminated for periods of 15–60 sec by a 100-w lamp, using a condenser. The cuvet received 5–10 × 10^4 lux, *i.e.*, saturating intensity. The increase in extinction at 340 mμ of the illuminated cuvet relative to the control was measured; allowance was made for any observed rate of reoxidation of the NADPH after illumination.

Results

Solubilization of the Lamellae with Detergent. Earlier findings (Bailey *et al.*, 1966) had indicated that SDBS solutions dissociate the lamellae so that all the solubilized material entered polyacrylamide gel on electrophoresis. SDS solutions were similarly successful but in this case the gels proved difficult to stain with Amido Black 10B solution.

The results which will be presented and discussed here were obtained from experiments with *B. vulgaris*. To establish the more general validity of our conclusions other species (*S. oleracea*, *N. tabacum*, and *A. sativa*) were examined; no significant differences were observed.

In order to solubilize the lamellae as completely as possible, five successive extractions of the chloroplast pellet were necessary. At higher ratios (5:1) of SDBS:Chl only three successive extractions were required to obtain the same degree of solubilization,

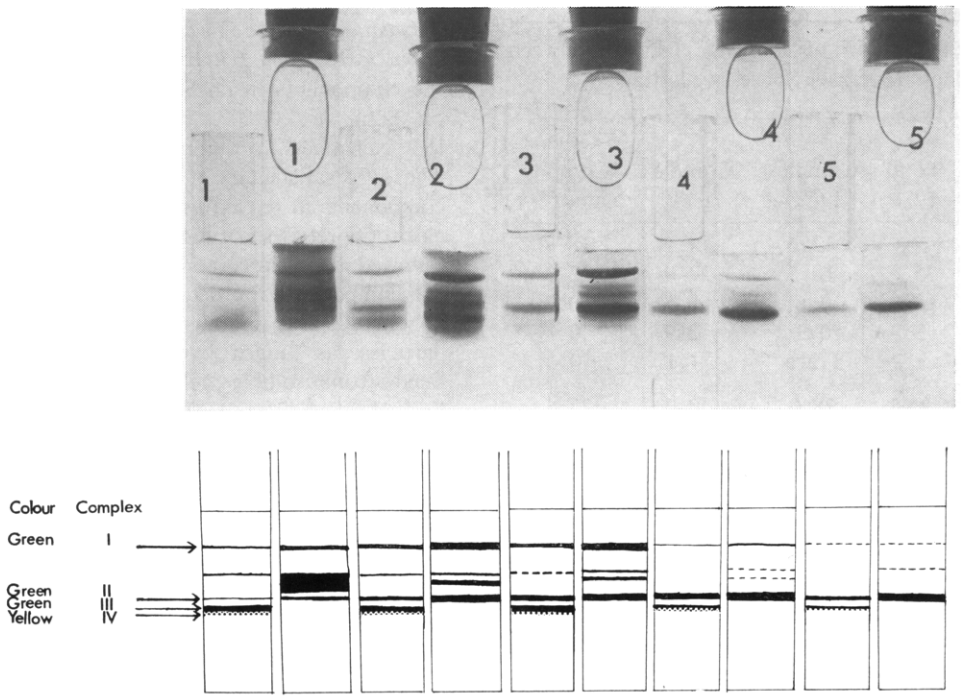

FIGURE 1: Photograph of the electrophoretic patterns of SDBS extracts 1–5 (see Methods) on polyacrylamide gels (concentration 9%, height 5.5 cm, diameter 0.7 cm). The right-hand gel of each pair has been stained with Amido Black 10B. Below the photograph of the gels a diagrammatic representation is presented of each gel perfectly aligned. Only those bands which are blue are given in the drawings of the stained gels. The band corresponding to complex III in the photograph of the stained gels is brown.

and 75% of the lamellae protein was then found in the first extract. Under both conditions a small green residue remained after the final extraction.

Analysis of SDBS Extracts. A. CHLOROPHYLL AND PROTEIN NITROGEN CONTENT. The majority of nitrogen in the extracts was assumed to be derived from protein. Corrections have been made for nitrogen derived from chlorophyll, but not for that originating from other nitrogenous sources which might be present. A typical analysis of the chlorophyll and protein nitrogen content and the chlorophyll a:b ratio of each of the five extracts are shown in Table I. The results show that the nitrogen-containing substances are more readily released than the chlorophyll. Compared with whole lamellae the first two extracts are characterized by higher chlorophyll a:b ratios, whereas subsequent extracts give lower values. The residue, containing some 4% of the total chlorophyll and 1.3% of the total nitrogen in the starting material, has a chlorophyll a:b ratio greater than the final extract.

B. ELECTROPHORESIS. The electrophoretic patterns obtained for each extract before and after staining the gels are shown in Figure 1. It was observed that the prominent green zones (complexes I and II) (seen more clearly in gel 3, Figure 1) were stained, whereas the faster green and yellow zones (complexes III and IV) were not. Complex II is obtained predominantly from extracts 2–5, whereas complexes I, III, and IV are almost exclusively confined to the first three extracts. Extract 1 contains many protein bands, but rarely gives a very satisfactory electrophoretic pattern. The presence of some of the additional proteins in the first extract is probably due to incomplete removal of the stroma from the chloroplast pellet; therefore it is not possible from the present results to conclude which of these minor proteins actually occurs in the

TABLE I: The Chlorophyll and Protein Nitrogen Content of Successive Extracts of *B. vulgaris* Chloroplast Pellet with 0.5% SDBS Solution.[a]

Extract	N (%)	Chlorophyll %	Chlorophyll a:b Ratio	Chl:N (w/w)
Chloroplast pellet	100	100	3.0	1.02
1	46.6	20.0	5.0	0.44
2	34.7	42.5	3.2	1.25
3	12.9	22.5	2.0	1.80
4	3.3	7.8	1.4	2.37
5	1.2	3.2	1.5	2.56
Residue	1.3	4.0	2.0	3.22

[a] There was 73.1 mg of chlorophyll and 71.6 mg of protein nitrogen in the chloroplast pellet.

TABLE II. Distribution of Lamellar Protein between Complexes I and II of Each Successive Extract.

Extract	Total (%)	Complex I (%)	Complex II (%)	Other Components (%)
1	47	13.6	14.5	18.8
2	35	10.5	22.0	2.5
3	13	3.6	8.5	0.9
4	3.3	0.3	2.9	0.1
5	1.2	Trace	1.1	0.1
Total	99.5	28.0	49.0	22.4

lamellae. The presence of these minor nonpigmented proteins in the first extract accounts for its low chlorophyll:nitrogen ratio (Table I). Other protein components which are present in most of extracts 1–5, and therefore probably originate from the lamellae, are a minor chlorophyll–protein complex and a colorless protein, both of which migrate between complexes I and II.

The amounts of each protein in each extract were estimated by scanning the stained gels in a densitometer; from a knowledge of the percentage of the protein nitrogen in the original chloroplast pellet obtained in each extract (Table I), it was then possible to calculate the approximate percentage of each component in whole lamellae. The results obtained (Table II) are dependent on the same quantity of dye being adsorbed by the same weight of each protein, but since this has not been verified then the figures can only be used as a guide to the composition of the lamellar complex. Preparations of the equivalent five extracts from leaves of *Spinacia*, tobacco, and oats gave identical electrophoretic patterns.

Isolation of the Two Major Chlorophyll–Proteins (Complexes I and II). It proved impossible to resolve the components in an SDBS extract by gel filtration on Sephadex G-200, chromatography on cellulose ion exchangers, or ammonium sulfate precipitation. The only satisfactory method for the purification of components in the extracts has so far proved to be an electrophoretic separation on polyacrylamide gels, followed by disintegration of discs of gel containing the component (for details see Methods). Each component was then readily eluted from the slurry of gel particles, as judged by removal of the color, and freed from soluble gel contaminants by filtration through a Sephadex G-200 column.

Analytical scale gel electrophoresis was used to determine which extract was the most suitable starting material for the isolation of the complexes. The second or third SDBS extract (SDBS:Chl = 2.5:1) was always the more suitable, since it contained large quantities of complexes I and II and very few other proteins. In the instance shown in Figure 1 extract 3 would be used. Other extracts obtained using SDBS:Chl = 2.5:1 or 5:1 were not used since they either gave poor electrophoretic separations, contained many different proteins, or had low concentrations of complex I.

Chlorophyll a:b Ratio of Complexes I and II. The ratios obtained for 15 different preparations of complex I were between 8:1 and 17:1 with a mean value of 12:1. The ratios for complex II lay between 1.1:1 and 1.6:1 with a mean of 1.2:1. The values for the equivalent complexes of other plant species were similar to those of spinach beet.

Relation of Complexes I and II to Photochemical Systems I and II. SDBS when added to chloroplasts at a final concentration of 0.012% caused the Hill reaction to cease within a few seconds, and at this concentration no NADP reduction could be observed. Hence the possible relation between complexes I and II and any photochemical activities cannot be determined by direct measurement. The detergent

TABLE III. Analysis of Particles Prepared from Digitonin-Treated Chloroplasts.[a]

Fraction	Total Chl (mg)	Chl a:b	Hill Act.[b]	NADP Reduction[c]	Hill:NADP Act.	Complex II:I Unstained[d]	Complex II:I Stained[d]
Chloroplasts in phosphate	–	2.7	92	103	0.9	–	–
Chloroplasts in digitonin	29.2	2.7	34	12.3	2.8	–	–
1000*g* precipitate	22.0	2.0	14	7.8	1.8	3.7	3.6
10,000*g* precipitate	5.4	1.9	25	6.4	3.8	6.7	3.9
50,000*g* precipitate	8.5	2.1	20	10.6	1.9	4.6	2.5
144,000*g* precipitate	3.2	3.4	0	10.0	0	0.5	0.8
144,000*g* supernatant	0.3	4.1	0	>10.0	0	–	0.6

[a] According to the schedule of Anderson and Boardman (1966). [b] Activity expressed as micromoles of O_2 evolved per milligram of chlorophyll per hour. [c] Activity expressed as micromoles of NADP reduced per milligram of chlorophyll per hour. [d] These ratios were determined as described under Methods. Unstained gels were scanned using a red filter (cutoff below 610 mμ) and stained gels using white light.

had no effect on the spectral bands of the cytochromes when it was added to an acetone-dried powder of chloroplasts to a final concentration of 0.3%.

Anderson and Boardman (1966) have, however, partially separated systems I and II in an active state using digitonin. We analyzed fractions prepared by their schedule for the presence of complexes I and II using SDBS to dissociate the particles further so that they enter the gels; a similar approach has been set out by Sironval *et al.* (1966). Chloroplasts were prepared and washed once in a medium containing 0.5 M sucrose, 0.05 M phosphate buffer, pH 7.1, 10^{-3} M KCl. A small portion was suspended in 0.05 M phosphate buffer, 0.01 M KCl, and the bulk in the same medium containing 0.5% digitonin. The digitonin-treated chloroplasts were fractionally centrifuged according to Anderson and Boardman's (1966) schedule but at pH 7.1, and the fractions were analyzed for chlorophylls a and b, Hill reaction, and NADP reduction activity. A pH of 7.1 was used since under our conditions this pH gave optimum Hill reaction rates for *B. vulgaris* chloroplasts; above this value the rate falls and approaches zero at pH 7.4. SDBS solution was added to each fraction to 0.2% final concentration and the solutions were dialyzed for 18 hr at 4° against 0.2% SDBS in 0.05 M sodium borate buffer, pH 8.0, to equilibrate them for analysis on polyacrylamide gels. After electrophoresis, the relative proportion of the two chlorophyll–protein bands (complexes I and II, Figure 1) in each fraction were estimated using a Chromoscan densitometer. The complexes were then stained and the estimation was repeated. Table III presents the results of these analyses. When the ratio of the activities in each fraction is compared with the ratio of complexes II and I there is a strong indication that complex I is concerned with NADP reduction and complex II with the Hill activity.

Discussion

The present study has shown that the anionic detergent, SDBS, dissociates over 96% of the chlorophyll and protein in the lamellae of chloroplasts (Table I) so that all the solubilized substances enter polyacrylamide gel on electrophoresis as detergent complexes. Exhaustive extraction of the chloroplast pellet with detergent solution always yielded a small insoluble residue, but whether this contained components different from those observed in the soluble portion has not been ascertained. The residue, containing less than 1.3% of the lamellar protein, we regarded as negligible.

The bulk of the lamellar material is composed of the two major chlorophyll–proteins (complexes I and II) and two proteins which migrate between them, one of which is pigmented. The approximate composition of the total lamellae protein has been estimated (Table II) as complex I, one-quarter; complex II, one-half; other proteins, one-quarter. A green complex III and a yellow complex IV are also present in the extracts; these complexes do not contain protein and are free pigment probably released from the pigment–proteins (Ogawa *et al.*, 1966).

Analysis of the successive extracts (Figure 1; Tables I and II) has shown that complex I is the more readily released pigment–protein. This is in agreement with previous reports (Boardman and Anderson, 1964; Brown and Duranton, 1964; Vernon *et al.*, 1966) that a chlorophyll a enriched fraction of lamellae is the more readily extracted by detergent solutions. We therefore suggest that complex I is the more superficial particle in the lamellar matrix.

Certain differences were noticed between the components isolated by us using polyacrylamide electrophoresis and those prepared by Ogawa *et al.* (1966) using a similar method. Our chlorophyll a:b ratio was higher for complex I (12:1 compared to 7:1) and lower for complex II (1.2:1 compared to 1.9:1). Differences in sedimentation characteristics and the carotenoid and quinone contents will be reported in a later paper.

Strong evidence for the hypothesis that complex I is derived from system I and complex II from system II has been adduced. Firstly, chlorophyll b is believed (Losada *et al.*, 1961) to be specifically involved in system II, and we find it almost entirely in complex II. Secondly, the particles of Anderson and Boardman (1966) which specifically carry out photochemical reaction I give rise to a greater proportion of complex I, and the larger particles which have greater Hill activity give a correspondingly greater proportion of complex II. The distribution of xanthophylls between complex I and II, which gives further corroboration of the hypothesis, will be reported in a later paper.

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Wax Esters of Mullet (*Mugil cephalus*) Roe Oil

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ABSTRACT: Lipids of mullet roe were separated and analyzed by liquid and gas chromatographic methods. Nearly 70% of the oil consists of wax esters in which saturated and monounsaturated fatty alcohols are bound to fatty acids with up to six double bonds. Alcohols and acids from wax esters were identified and quantified. Both contain appreciable amounts of odd-numbered straight-chain compounds. The structures of monoenoic alcohols are the same as those of monoenoic acids, *e.g.*, 9-heptadecenoic, 9- and 11-octadecenoic, and nonadecenoic. The total content of odd-numbered alcohols was between 10 and 25% while that of odd-numbered acids was consistently somewhat lower. Several polyunsaturated acids such as 4,7,10,13,16-heneicosapentaenoic or 4,7,10,13,16,19-docosahexaenoic and related acids were newly identified from mullet. They occur more in wax esters than in triglycerides of roe or of the body oil.

The fatty acids in wax esters of mullet are more typical for marine oils than those in any other wax esters of marine source so far reported. The extensive combination of polyunsaturated acids with saturated or monounsaturated alcohols emphasizes strongly the limitation of unsaturation in the latter. With acids being the most likely precursor for these alcohols, the biological reduction apparently cannot take place with polyenoic acids. Gas–liquid partition chromatography of hydrogenated wax esters showed that the alcohols and acids are combined to form C_{30} to C_{40} esters, with maximum amounts for C_{32} and C_{34}. The combination of chain lengths was random with a sample which contained about 10% each of C_{14} and C_{15} alcohol, but it was not random when each of these alcohols occurred at the level of 20%.

In a recent study of the fatty acids of mullet (*Mugil cephalus*) oil in this laboratory it was found that they contain 15–30% straight-chain odd-numbered components (Sen and Schlenk, 1964). The oil samples investigated had been obtained from the whole fish or from the fillets, and they consisted mainly of triglycerides. It appeared of interest to check the distribution of odd-numbered fatty acids in specific parts of the body and in lipid classes other than triglycerides. In the course of such investigation it was found that wax esters represent the major portion of the lipids in roe. The structures of fatty alcohol and acid components of the waxes are reported here and their possible biological correlation is discussed.

Procedures and Results

Mullet was caught on the coast of the Gulf of Mexico near Pascagoula, Miss., in the fall of 1964 and 1965. Fish containing roe were about 28–30 cm long, weighed 250–500 g, and had between 15 and 55 g of roe.

Lipids were extracted from the roe with $CHCl_3$–CH_3OH (2:1) in an Omni-Mixer. About 20% of the wet weight was obtained as a yellow oil which partly solidified in the refrigerator. Thin layer chromatography on silicic acid with hexane–diethyl ether–acetic acid (85:15:1) indicated the presence of wax esters, triglycerides, acids, alcohols, free cholesterol, and cholesterol esters, listed in order of decreasing amounts. A small

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