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Fatty Acids, Phytosterols and Chlorophylls in Leaves of Apple Cultivars (*Malus Domestica* Borkh)

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Abstract. The aim of the present study was to investigate the fatty acids, sterols and chlorophylls content and profile in leaves of apple cultivars *(Malus domestica* Borkh) which are intended to be cryopreserved. Quantitative GC-FID was used for phytosterols and fatty acids analysis, while chlorophylls were determined by a spectrophotometric method.

Total phytosterols ranged from 189.6 to 463 mg/total lipid extract. The highest phytosterols content was found in Romus 3 and Romus 4 cultivars. The most important phytosterols in all leaves samples were: β-sitosterol, sitostanol, Δ5-avenasterol, 24-methylenecycloartenol and Δ7-stigmastenol. Some other minor sterols were also identified: campesterol, stigmasterol, citrostadienol.

Fatty acids analysis revealed important quantitative differences, with a total content ranging from 77.4 mg/g to 245 mg/g. The major fatty acid in all samples was α-linolenic acid with 40 - 55 %, followed by palmitic acid with 17.5 - 25 %, linoleic acid 7 - 14 % and oleic acid 4.7 - 9.6 % of total fatty acids.

Polyunsaturated fatty acids represent the major fraction, comprising 54-65 %. The ration between unsaturated and saturated fatty acids ranged between 2.1 - 3.2. The high degree of unsaturation is characteristic to green tissues. The chlorophyll *a* content ranged between 1.39 – 2.3 mg/g fresh weight, while chlorophyll *b* was found at 0.6 - 0.98 mg/g. The ratio chl a/chl b was close to 2.3 for all apple cultivars.

Keywords: apple leaves, fatty acids, phytosterols, chlorophylls.

INTRODUCTION

Cryopreservation is an essential technique for long-term storage of germplasm, in order to conserve genetic diversity (Engelmann, 2000). Hence, it is very important to understand the mechanism of plants cold tolerance and cold acclimation.

Cold acclimation involves significant changes in membrane lipid composition having as result an increase of membrane fluidity. Among these changes on can mention the increase of fatty acids unsaturation, the increase of membranes phospholipid content and modification of sterol composition (Mazliak, 1989; Quinn, 1989; Dufourc, 2008a).

The objective of the present study was to investigate the fatty acids, sterols and chlorophylls content and profile in leaves of apple cultivars *(Malus domestica* Borkh) which are intended to be cryopreserved.

This is a first part of a study which aims to preserve apple biodiversity by cryopreservation.

MATERIALS AND METHODS

The apple leaves were collected from the experimental field of Research Institute for Fruit Growing Pitesti–Mărăcineni, Romania.

Sterols analysis. Total lipids were extracted with a solvent system of chloroform: methanol (2:1) and stored at -20°C until use. A part of total lipid extract was used for total sterol analysis. After the addition of 5α-cholestane-3β-ol as an internal standard, the samples were saponified by refluxing with 1M KOH ethanol/water (8:2, v/v) solution for 1 h. The unsaponifiables (the total sterols) were extracted successively with petroleum ether and diethyl ether. The ether phases were combined, washed with 5% NaCl solution, and dried over sodium sulfate. The ether phase was evaporated to dryness, transferred in a vial with petroleum ether and stored until derivatisation process. The sterols were derivatized with N,O-bis-(trimethylsilyl)-trifluoroacetamide (BSTFA) containing 1% of trimethylchlorosilane (TMCS) in pyridine. Trimethyl silyl ether (TMS) derivatives of phytosterols were separated on a fused silica capillary column coated with 5% phenyl / 95% dimethylpolysiloxane (30 m x 0.25 mm i.d., film thickness 0.25 μm; Rtx-5; Restek Corporation, Bellefonte, PA, USA) and using a Shimadzu GC 17A GC system.

The temperature program was: 5 min at 200 ^{o}C, 10 ^{o}C / min to 300 ^{o}C (hold 20 min). An aliquot of 1.0 μl of sample solution was injected into the column via split injection (split ratio 1:40). The carrier gas was helium. Peaks were identified by comparison of their retention times with those of commercially available standards (Sigma). All extractions and GC-FID runs were performed in triplicate and mean values were calculated.

Fatty acids analysis. An aliquot of the total lipid extracts were transesterified with BF_3/methanol in benzene, respectively for 30 min, at 80°C. The methyl esters of fatty acids were dissolved again in hexane and injected for GC analysis. A Shimadzu GC 17A with FID detector and a Crompack Silica 25 MXO capillary column (25m x 0.25 mm i.d., film thickness 0.25 μm) was used.

The temperature program was: 5 min at 150 ^{o}C, 4 ^{o}C / min to 235 ^{o}C (hold 5 min). The injector temperature was 260˚ C and the detector temperature - 260˚ C. The carrier gas was helium. The fatty acids were identified by comparison with the retention times (t_R) of standards (Sigma) and quantified by area integration.

Determination of chlorophylls content. Fresh samples (0,2 g) were homogenized and extracted with 90 % acetone in water, using a magnetic stirrer until the residue was colorless. The absorbance was read at 645 and 663 nm.

The following formulas were used in order to quantify the chlorophylls a and b:

Chl a (mg/g) = (11.75 x A663 – 2.35 x A645) x V/g

Chl b (mg/g) = (18.61 x A645 – 3.96 x A663) x V/g

were A645 – absorbance at 645 nm
A663 – absorbance at 663 nm
V – volume of the extract (ml)
g - sample s weight (mg)

RESULTS AND DISCUSSIONS

Nine apple cultivars were subjected to the analysis of fatty acids, sterols and chlorophylls. GC analysis (Fig. 1) allowed us to establish the sterols profile and individual content in apple leaves, by using available sterols standards and relative retention time of peaks compared to sitosterol (Abidi, 2001). The most important phytosterols in all samples were: β-sitosterol (64-82 %), sitostanol (8-21 %), Δ5-avenasterol (5-12 %), 24-methylenecycloartenol and Δ7-stigmastenol. Some other minor sterols were also identified: campesterol, stigmasterol, citrostadienol (Fig. 2).

Over 200 sterols were identified in plants, in quantities and proportions that differ from one species to another. Generally vegetal oils contain sitosterol as the major compound (70%), followed by stigmasterol (20%) and campesterol (5%) (Gunstone *et al.*, 1994). Sitosterol and stigmasterol (24-ethyl sterols) are involved in embryonic growth in plants (Schaller, 2003) and increase membrane cohesion in order to maintain plant membranes in a state of dynamics less sensitive to temperature shocks (Beck et al, 2007; Dufourc, 2008b). Dufourc also suggested that ethyl groups of sitosterol and stigmasterol may reinforce the attractive van der Waals interactions leading to more membrane cohesion and therefore to less temperature sensitivity. Thus, the sterol composition of plants is a response of plant adaptation to temperature variation (Dufourc, 2008a).

Total phytosterols ranged from 189.6 to 463 mg/total lipid extract. The highest phytosterols content was found in Romus 3 and Romus 4 cultivars. The high variability of total sterols is reflected also in the total lipids content of leaves (Tab. 1).

It should be noted that there are not literature data on the sterol composition of apple leaves. Further studies are needed in order to confirm the structure of minor compounds by gas chromatography coupled with mass spectrometry.

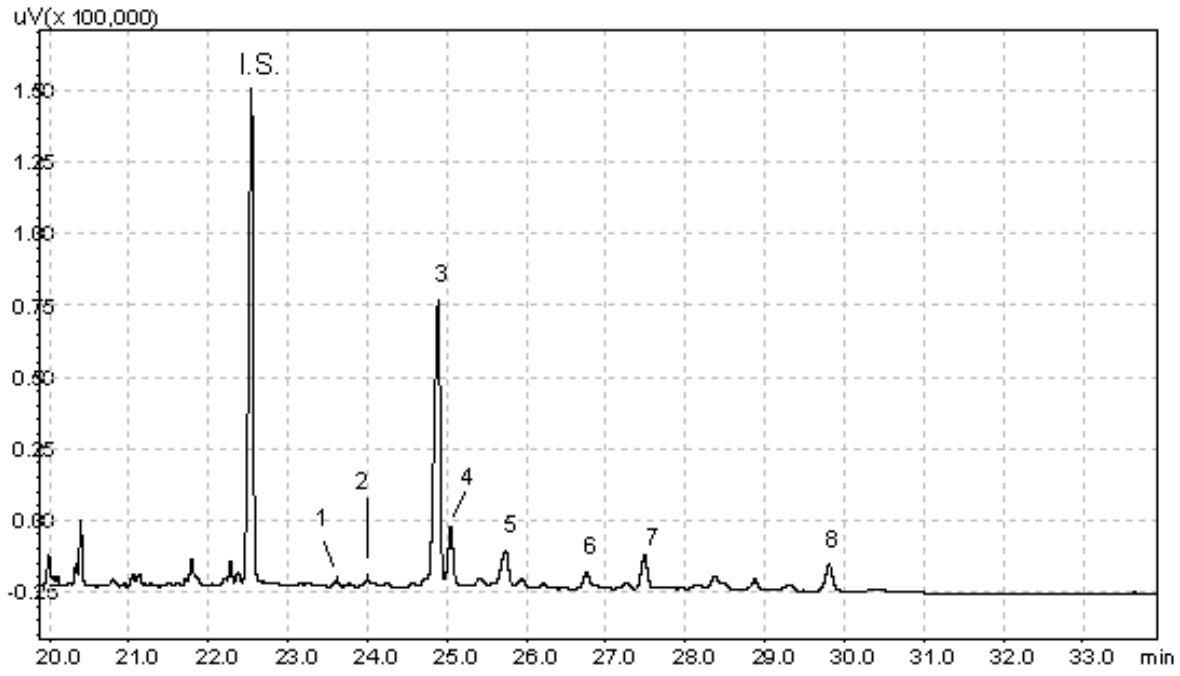

Fig. 1. GC separation of apple leaves phytosterols

Peak identification: I.S.: 5α-Cholestan-3β-ol; 1: Campesterol; 2: Stigmasterol; 3: β-sitosterol; 4: Sitostanol; 5: Δ5-avenasterol; 6: Δ7-Stigmastenol; 7: 24- Methylencycloartanol; 8: Citrostadienol.

Tab. 1

Total lipids and total sterols content in apple leaves
(mean ± S.D)

Nr.	Sample	Total lipids (mg/g fresh weight)	Total sterols (mg/100 g total lipids)
1.	Romus 4	20,5 ± 1.0	340.18 ± 20.4
2.	M9T337	38.4 ± 1.4	285.95 ± 16.5
3.	Rebra	39.2 ± 1.4	229.33 ± 14.2
4.	M 26	34.7 ± 1.4	317.07 ± 19.8
5.	Golden Rush	45.0 ± 0.9	199.93 ± 13.9
6.	Colmar	50.6 ± 1.1	197.64 ± 14.0
7.	Romus 3	30.2 ± 1.0	463.42 ± 23.1
8.	Idared	61.8 ± 1.3	323.43 ± 15.8
9.	Florina	52.7 ± 1.1	189.63 ± 14.5

Total fatty acids, as fatty acids methyl esters, were analyzed by GC-FID technique and identified using commercial standards (Fig. 3).

The major fatty acids in apple leaves were α-linolenic acid with 40-55 %, followed by palmitic acid with 17.5-25 %, linoleic acid 7-14 % and oleic acid 4.7-9.6 % of total fatty acids (Tab. 2).

Polyunsaturated fatty acids represent the major fraction, comprising 54-65 %. The highest unsaturation degree was recorded in Colmar and Idared cultivars, with 76 and, respectively, 74 % unsaturated fatty acids. The ratio between unsaturated and saturated fatty acids ranged between 2.1 - 3.2 in all samples, and the high degree of unsaturation is characteristic to green tissues.

Membrane lipid composition undergoes significant changes when plants are exposed to environmental factors, including low temperature. The degree of fatty acid unsaturation and the content of phospholipids increase during cold acclimation, in order to enhance membrane fluidity, which in turn enhance membrane integrity and cellular functions (Uemura *et al.*, 2006, Wang *et al.*, 2006).

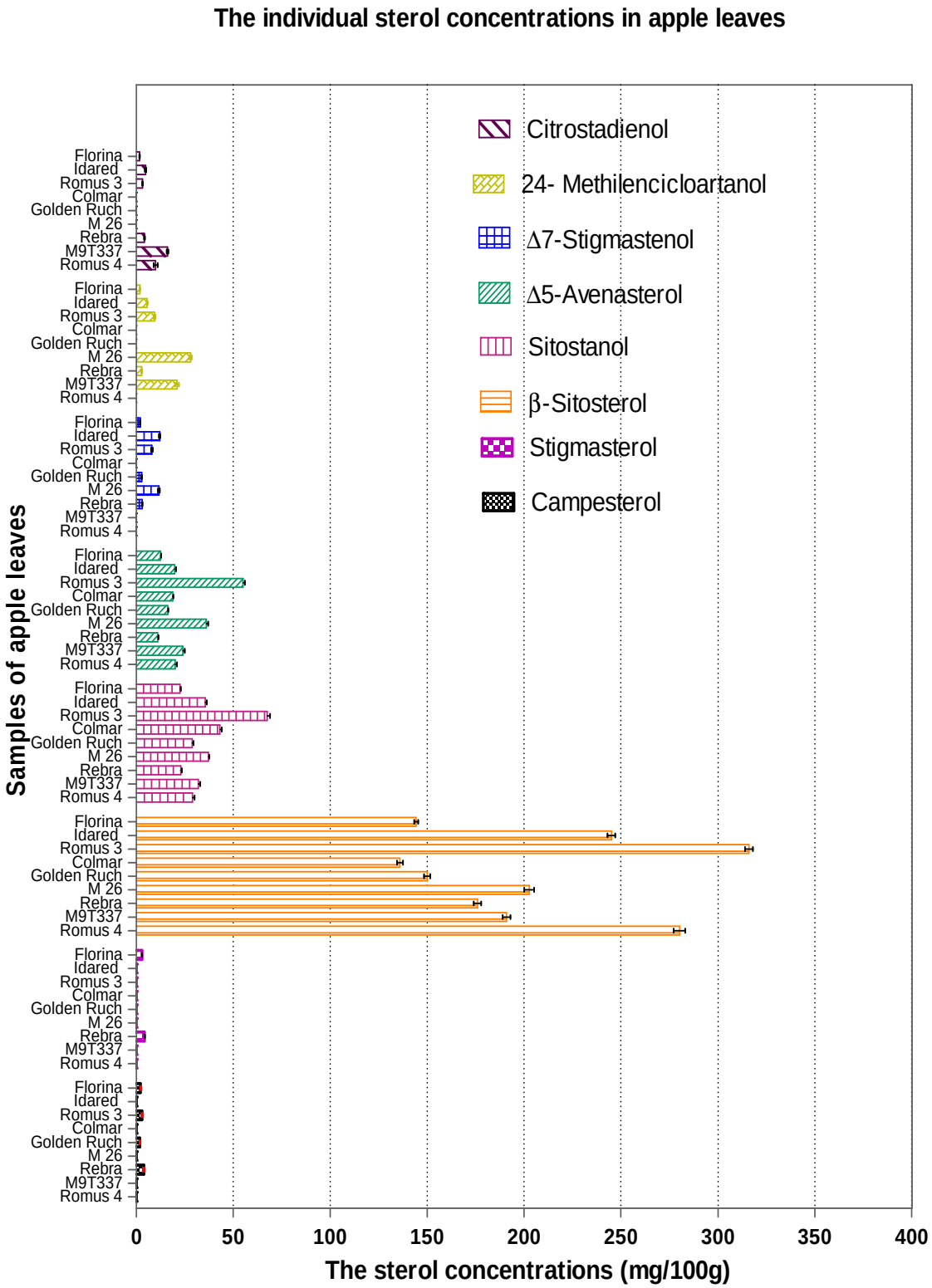

Fig. 2. Individual phytosterol content in leaves of different apple cultivars

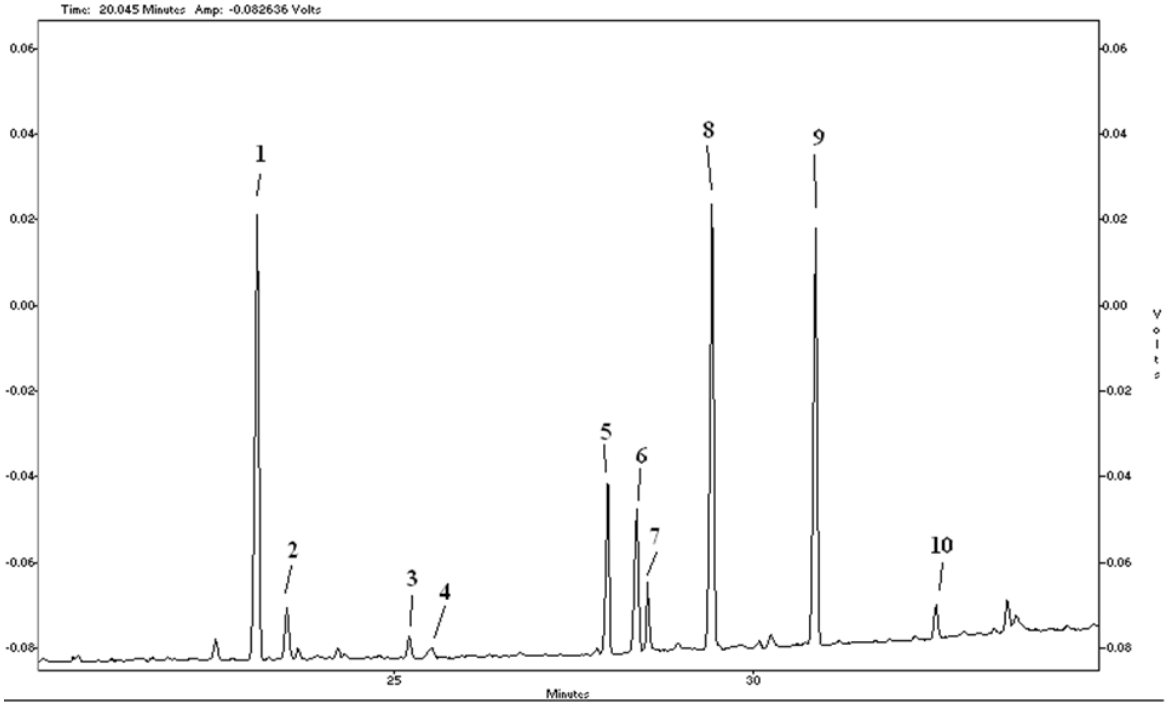

Fig. 3. GC separation of fatty acids methyl esters in apple leaves

Peak identification: 1 – Palmitic acid (16:0); 2 – Palmitoleic acid (16:1); 3 – Heptadecanoic acid (17:0); 4 – Cis-(10)-Heptadecenoic (17;1); 5 – Stearic acid (18:0); 6 – Oleic acid (18:1 *9c*); 7 – Vaccenic acid (18:1 *11c*); 8 – Linoleic acid (18:2); 9 – Linolenic acid (18:3); 10 – Arachidic acid (20:0)

Tab. 2

Fatty acids composition in leaves of different apple cultivars
(% of total fatty acids, mean of three independent analyses)

Acid gras	Romus 4	M9T337	Rebra	M26	Golden Rush	Colmar	Romus 3	Idared	Florina
16:0	20,34	24,20	21,67	24.91	23,95	17,48	21,46	19,42	20,67
16:1	4,49	3,26	3,96	2,85	3,86	2,61	2,26	2,67	3,03
17:0	0,20	0,59	0,69	0,56	0,82	0,69	0,86	1,16	1,15
17:1	0,54	0,72	0,82	0,54	0,63	0,94	1,20	1,46	1,58
18:0	4,75	4,86	3,94	4,90	4,58	3,88	4,23	4,05	3,40
18:1 (9c)	7,93	9,60	8,16	9,20	7,52	6,21	4,77	5,17	4,72
18:1 (11c)	0,35	0,32	0,25	0,30	0,27	0,45	0,36	0,41	0,28
18:2	9,98	11,86	8,26	13,99	6,97	10,59	10,35	10,45	11,48
18:3	48,90	42,25	50,47	40,41	49,38	55,27	53,07	53,77	51,98
20:0	2,46	2,29	1,74	1,96	1,96	1,82	1,39	1,14	1,68
SFA	27,75	31,94	28,04	32.33	31,31	23,87	27,94	25,77	26,90
MUFA	13,31	13,90	13,19	12.89	12,28	10,21	8,59	9,71	9,61
PUFA	58,88	54,11	58,73	54.4	56,35	65,86	63,42	64,22	63,46

SFA = saturated fatty acids
MUFA = monounsaturated fatty acids
PUFA = polyunsaturated fatty acids

The chlorophyll *a* content ranged between 1.39 – 2.3 mg/g fresh weight, while chlorophyll *b* was found at 0.6 - 0.98 mg/g (Fig. 4). The ratio chl a/chl b was close to 2.3 for all apple cultivars. The absolute values and the ration between chl a/chl b were those reported for other apple cultivars (Šircelj *et al.*, 2005).

Similar work is in progress regarding the phytosterols, fatty acids and chlorophylls in apple ex-plants cultured in vitro, before and after cryopreservation.

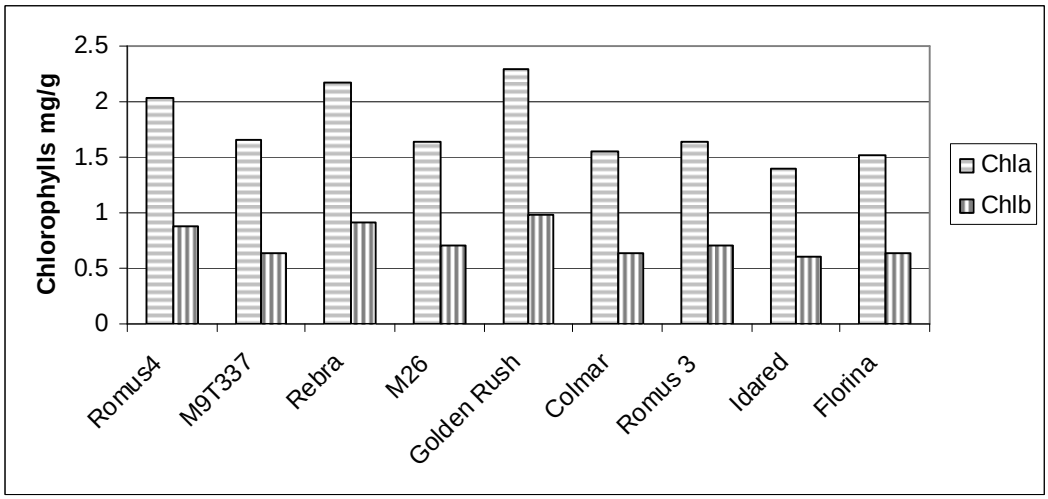

Fig. 4. Chlorophylls in leaves of different apple cultivars

CONCLUSIONS

Fatty acids and phytosterols profile was similar in all apple cultivars, which contain the same major compounds: sitosterol, Δ5-avenasterol, linolenic, linoleic and palmitic acid. However, there are differences concerning quantitative aspects of total and individual sterols and fatty acids, as well as at the level of total lipids content. Chlorophylls level did not show significant differences among apple cultivars.

Further work is necessary to identify minor sterols and fatty acids distribution in lipid classes.

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