Short note

Changes in Fatty acids and 4-Desmethylsterols Content During walnut (*Juglans regia L.*) fruit Development

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The evolution of fatty acids and 4-desmethylsterols composition during the maturation of two fruit of walnut varieties (Franquette and Local gd) was investigated. Fruit samples were collected at regular intervals period from 7 to 35 days after fruiting date (DAFD). Qualitative and quantitative analyses were made by GC-MS. Oil content showed increasing trends. During fruit development, unsaturated fatty acids are the major component, among which Linoleic acid (C18:2) was the most predominant, followed by Linolenic acid (C18:3), and Oleic acid (C18:1). The evolution of the 4-desmethylsterols content was marked by the predominance of β-sitosterol during all ripening stages, with a maximum value at 7 DAFD (269.56mg / 100g of oil) in Franquette variety and at 21 DAFD (122.17mg / 100g of oil) in Local gd variety.

Keywords: *Fatty acids; 4-Desmethylsterols; Walnut; Development; β-sitosterol*

1. INTRODUCTION

Walnut (*Juglans regia* L.) is a crop of high economic interest to the food industry. The edible part of the fruit (the seed or kernel) is globally popular and valued for its nutritional, health and sensory attributes. The high oil and essential fatty acid contents of the walnut kernel make it a good source for the commercial production of edible oil. Oil contents as high as 740 g/kg kernel (Soxhlet extraction, *n*-hexane) have been reported for some commercial walnut varieties [1].

Walnut oil (WO) can be extracted easily by screw pressing [2]. Employing a pilot plant screw-press, the highest oil recovery (660 g/kg kernel) was achieved at 7.5 g/100 g kernel moisture and 50°C pressing temperature. Fresh WO is very low in free fatty acid concentration, peroxides and phosphatides [3] because of which it may be consumed directly, without refining. Walnut oil is composed mainly of triglycerides, in which monounsaturated (oleic acid mainly) and polyunsaturated fatty acids (PUFAs, linoleic and ?-linolenic acids) are present in high amounts [4]. According to [5] WO has a perfect balance of *n*-6: *n*-3 PUFAs, a ratio of 4:1, which was showed to decrease the incidence of cardiovascular risk [6].

Walnut fruit is also rich in phytosterols [7]. Several epidemiologic and experimental studies report that dietary phytosterols may offer protection from cancers such as colon, breast, and prostate cancers. Phytosterols are the major fraction of unsaponifiable in vegetable oil, being the most relevant the 4-desmethylsterols [8]. The most frequent 4-desmethylsterols are ?-sitosterol, campesterol, and stigmasterol [8].

The objective of this work was to study, fatty acids and 4-desmethylsterols composition of two walnuts varieties (Franquette and Local gd) during fruit ripening, to determine their optimal period of harvest.

2. MATERIAL AND METHODS

2.1. SAMPLES

Juglans regia L. fruits were obtained from the National Institute for Research in Rural Engineering, Water and Forest (*INRGREF*) of Tunisia. Walnut trees were grown on the Agronomy farm in the north of Tunisia (Mateur, Bizerte), in a wet zone (rainfall average of 600 mm per year). Two varieties of walnuts, Local gd and Franquette were studied during seed development. The harvest period was stretched from 7 to 35 days after fruiting date (DAFD, 1-week intervals), from the middle of August 2011 until the end of September 2011, when the fruit reached complete maturity.

2.2. REAGENTS AND STANDARDS

Acetone, chloroform, diethyl ether and petroleum ether were purchased from Fisher Scientific SA. (Loughborough, UK). Ethanol was obtained from Scientific Limited (Northampton, UK). Fatty acid methyl ester (FAME) standards were purchased from Nu-Chek-Prep (Elysian, MN, USA). Sterols including 5-α-cholestanol (I.S) were purchased from Sigma (St. Louis, MO, USA), The individual phytosterols standards used for peak identification: cholesterol, fucosterol, stigmasterol, sitosterol, and campesterol were purchased from Sigma (Sigma–Aldrich Corporation, St. Louis, MO). *N*-Methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA), from Pierce (Rockford, USA), was used as derivatisation reagent. TLC silica plates (silica gel 60 G, F254, 20 - 20 cm, 0.25 thicknesses), potassium hydroxide pellets, pyridine and anhydrous sodium sulphate were obtained from Merck (Darmstadt, Germany).

2.3. DETERMINATION OF OIL CONTENT

Walnut kernels (about 30 g) from each variety were ground into a fine powder using a hand mortar. Then the oil was extracted using a Soxhlet extractor with petroleum ether as a solvent. After 6 h of extraction, samples were evaporated under vacuum, weighted and their oil yield was determined

2.4. DETERMINATION OF FATTY ACIDS BY GC-MS

Fatty acids were converted to their corresponding methyl esters according to the method described by Carreau and Dubacq (1978). The fatty acid methyl esters analysis of samples was performed using a Hewlett Packard HP 6890 Series Gas Chromatographer (GC) coupled with a Hewlett Packard 5975 Mass Selective Detector mass spectrometer (MS). For the quantitative determination using a calibration factor of 1.0 (external standard Dodecanese). Separation was done in a HP-5 MS 30 m length, inner diameter 0.25 µm, 0.25 µm film thickness; oven temperature, isotherm at 180°C for 1 min, from 180 to 200°C at a rate of 15°C/min, from 200 to 220°C at a rate of 3ºC/min and isotherm at 300°C for 2 min. Injector and detector temperatures were 250 and 230°C, respectively. Injection volume: 2 µL; split ratio 1:100. Helium was used as carrier gas at 0.9 mL /min. For MS detector source temperature at 230°C, scan range 50-550 U and the spectra obtained were compared with the NIST/EPA/NIH Mass Spectral Library W9N11.L database.

2.5. SAPONIFICATION

Unsaponifiable lipids were determined by the saponifying 5 g of lipid extracts with 50 mL ethanolic KOH 12% (w/v) mixed with both 500 ml 5-α-cholestanol solution (internal standard: 0.2% (w/v)) and heating at 60°C for 1.30 h. After cooling, 50 mL of H_2O was added and the unsaponifiable matter was extracted four times with 50 mL petroleum ether. The combined ether extract was washed with 50 mL of ethanol – H_2O (1:1). The ether extracted was dried over anhydrous Na_2SO_4 and evaporated. The dry residues were dissolved in chloroform for TLC analysis.

2.6. THIN-LAYER CHROMATOGRAPHY

The unsaponifiable matter was separated into subfractions on preparative silica gel thin-layer plates, using 1-dimensional TLC with hexane -diethyl ether (65:35 v:v) as the developing solvent. The unsaponifiable (4 mg in l00 mL $CHCl_3$) was applied on the silica gel plates in 3 cm bands. To correctly identify the bands 5-α-cholestanol was applied on the left and the right sides of the TLC plates. After development, the plate was sprayed with 2, 7-dichlorofluorescein and viewed under UV light. The sterol bands were identified based on the reference spots. Those bands corresponding to 4-desmethylsterols were scraped off separately and extracted three times with chloroform - diethyl ether (1:1), filtered to remove the residual silica, dried in a rotary evaporator and stored at 10°C for further analysis.

2.7. QUANTITATIVE DETERMINATION OF STEROLS BY GC-MS

GC-MS analysis of sterol TMS derivatives was performed on an Agilent 6890 N Network GC system equipped with a capillary column Agilent Ultra 1 column (length 16. 5 m, i.d. 0. 2 mm, film thickness 0.11 µm) and coupled to an Agilent 5973 Network mass selective detector. Helium was used as a carrier gas at 1 mL /min. The injector temperature was set at 280°C. The oven temperature was kept at 200°C, 2 min, increased at 5°C/min until 270°C and at 20°C/min until 300°C. The sterol TMS derivatives were immediately injected separately into a GC (Hewlett Packard 7683) performed in the split mode. The electron impact (EI) mass spectra were recorded at ionisation energy of 70 eV and the ion source temperature was set at 280°C.

2.8. STATISTICAL ANALYSIS

All extractions and determinations were conducted in triplicate. The data were analysed using the analysis of variance (Anova). Comparisons of means were achieved using the Statistical Analysis System XLSTAT (version 2013). Differences between varieties were assessed using Duncan test. Differences at $p < 0.05$ were significant.

3. RESULTS AND DISCUSSION

3.1. CHANGES IN TOTAL LIPID CONTENT

The evolution of oil content of two walnuts varieties during maturation stages (expressed as % of dry weight) showed that oil accumulation followed a similar pattern in all varieties, increasing the amount of oil between the 7th and the 14th DAFD and reached 47.2% and 42.4% for Franquette and Local gd varieties, respectively (Fig. I). This increase can be explained by the fact that between 7th and 14th DAFD the lipids synthesized by immature walnuts are used for the development of new fruit tissues. Then, between 14th and 28th DAFD the lipid biosynthesis was much slower, there was a stationary phase where the rate of lipid accumulation remained constant. In the last stage, the oil content was maximal 49.70% for Local gd and 56.91% for Franquette. The quantitative characterisation of the oil content at complete maturity of our samples agreed with that reported by [4]. It was reported that the total lipid content increased with maturity [9]. The study of the lipids accumulation during linseed development is important to decide the best moment to harvest walnut fruits.

3.2. DYNAMIC ACCUMULATION OF FATTY ACIDS CONTENT DURING MATURATION

The proximate composition of fatty acids of two walnut varieties through the maturation stages are summarised in Table I. In all stages of maturity, five major fatty acids were identified. Their variation in content followed the same pattern. Results showing that linoleic and linolenic acids, major fatty acids detected tend to decrease their percentages during fruit ripening. These results match the ones obtained by[10]. This reduction of linolenic acid concentration during ripening has been produced by genetic modifications in the desaturation step from linoleic acid to linolenic acid controlled by linoleate or omega-3 fatty acid desaturases [11]. The fall in the linoleic acid percentage content was accompanied by a concomitant reduction in that α-linolenic acid. As expected, total PUFA was the main group of fatty acids ranging from 87.63 to 95.54% in Franquette and from 86,36 to 90,86% in Local gd through the maturation stages. Similar results in the fatty acid composition were obtained for different walnut selections and varieties [12]. In fact, as ripening advances certain metabolic processes, which involve changes in the profile of certain compounds such as fatty acids and phytosterols, occur. These variations are reflected in the oxidative stability and the nutritional value of the final product.

3.3. DYNAMIC ACCUMULATION OF 4-DESMETHYLSTEROLS

The accumulation of 4-desmethylsterols during maturation of Walnuts is shown in Table II. β- Sitosterol is the major compound at every stage of maturity that accounted for over 90% of the total of 4-desmethylsterols, with a maximum value (269.56 mg / 100g of

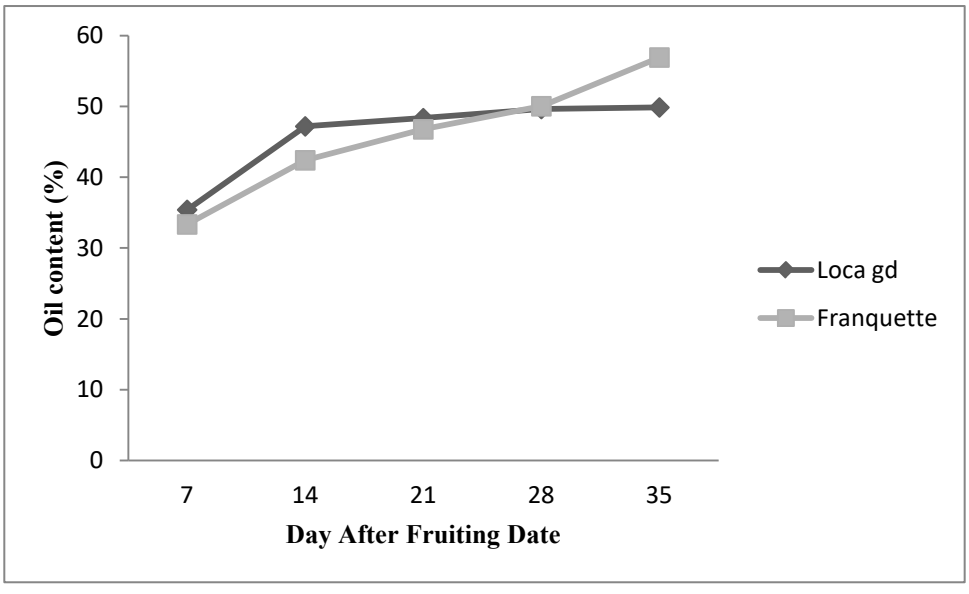

Fig1. Changes in oil content (% of dry weight) during walnut maturation.

Table I - Changes in Major fatty acids composition (g/100g of oil) during walnut maturation.

Fatty acid composition g/100g of oil	Franquette						Local gd				
	DAFD						DAFD				
	7	14	21	28	35		7	14	21	28	35
C16:0	0.05±0.01	6.60±0.28	6.95±0.00	7.03±0.01	8.38±0.01		6.16±0.03	6.62±0.03	5.65±0.00	5.63±0.28	6.60±0.28
C18:0	4.90±0.07	2.94±0.00	2.89±0.14	2.92±0.03	3.66±0.00		3.70±0.14	3.85±0.07	3.44±0.00	3.63±0.04	3.65±0.01
C18:1	21.21±0.14	24.04±0.06	25.06±0.04	23.92±0.01	22.72±0.14		23.00±0.24	27.20±0.14	27.65±0.07	26.16±0.01	24.78±0.03
C18:2	63.35±0.14	66.27±0.14	64.99±0.00	65.95±1.41	60.90±0.00		63.00±1.41	62.01±0.01	64.04±0.03	64.41±0.00	61.37±0.01
C18:3	30.14±0.01	29.27±0.03	29.03±0.04	27.62±0.03	26.73±0.03		26.95±0.01	26.93±0.00	26.82±0.03	25.04±0.03	24.99±0.00
SFA	4.95±0.08	9.54±0.28	9.84±0.14	9.95±0.04	12.04±0.01		9.86±0.17	10.47±0.10	9.09±0.01	9.26±0.32	10.25±0.29
MUFA	21.21±0.28	24.04±0.06	25.06±0.04	23.92±0.01	22.72±0.14		23.00±0.24	27.20±0.14	27.65±0.07	26.16±0.01	24.78±0.03
PUFA	93.49±0.15	95.54±0.17	94.02±0.04	93.57±1.44	87.63±0.03		89,95±1.42	88,94±0.01	90,86±0.06	89,45±0.03	86,36±0.01
UFA	114.7±0.43	119.58±0.23	119.62±0.08	117.49±1.45	110.35±0.17		112.95±0.66	116.14±0.15	118.51±0.13	115.61±0.04	111.14±0.04
MUFA/SFA	4.28	2.52	2.54	2.40	1.88		2.33	2.60	3.04	2.82	2.41
PUFA/SFA	18.90	10.01	9.55	9.40	7.28		9.12	8.50	10.00	9.66	8.42
UFA/SFA	23.17	12.53	12.15	11.80	9.16		11.45	11.10	13.03	12.48	10.84

DAFD: Days after Fruiting date
Results were given as means ± SD from triplicate estimations.
SFA: saturated fatty acid, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids, UFA: unsaturated fatty acids.

Table II - Changes in 4-desmethylsterols composition during walnut maturation

Phytosterol composition mg/100g of oil	Franquette						Local gd					
	DAFD						DAFD					
	7	14	21	28	35		7	14	21	28	35	
Cholesterol	0.76±0.03	0.30±0.01	0.87±0.00	0.42±0.01	0.90±0.07		0.79±0.01	0.78±0.00	0.67±0.03	0.96±0.44	0.90±0.07	
Fucosterol	2.78±0.01	9.00±0.00	7.27±0.04	1.86±0.01	1.58±0.01		19.74±0.01	26.91±0.03	1.03±0.03	2.27±0.01	0.09±0.03	
β-Sitosterol	269.56±0.03	146.55±0.14	163.17±0.14	171.30±0.00	152.80±0.28		102.10±0.00	105.43±0.03	122.17±0.01	117.35±0.00	95.15±0.14	
Campesterol	11.04±0.03	15.09±0.07	23.29±0.00	5.76±0.03	5.59±0.01		4.07±0.03	4.15±0.00	0.58±0.42	4.59±0.01	1.70±0.07	
Total (mg/100g of oil)	284.14±0.1	170.94±0.22	194.60±0.18	179.34±0.05	160.87±0.37		126.70±0.05	137.27±0.06	124.45±0.49	125.17±0.46	97.84±0.31	

DAFD: days after Fruiting date
Results were given as means ± SD from triplicate estimations.

oil) at 7 DAFD in the Franquette variety and (122.17 mg / 100g of oil) at 21 DAFD in the Local gd variety. At complete maturity, Franquette and Local gd had β-sitosterol contents of 152.80mg /100 g of oil and 95.15mg /100 g of oil, respectively followed by the campesterol and the fucosterol, the cholesterol represents the minor compound of 4-desmethylsterols. Generally, the sterol composition of walnut cultivars has been indicated to depend on the ripening stage of the fruit. The qualitative characterisation of our samples agreed with those listed in the literature [13]. The differences in individual contents of phytosterol when compared to literature, may be due to the cultivars used and to cultivation and/or environmental factors. Table II show that the change in profiles of campesterol, fucosterol and cholesterol was very similar during ripening of walnut. This result could be explained by the fact that these compounds had the same biosynthetic precursor [14].

β-Sitosterol was the major sterol to accumulate, campesterol and fucosterol were also present at low amounts. The essential roles that sterols perform in plant tissues as structural components of membranes ensure that they must be present during all stages of the fruit development [15]. There was a link between sterol accumulation and enzyme activities in developing fruit. However, during the fruit development there are periods of increased sterol accumulation that correlated with the increased activity of key enzymes in the sterol biosynthetic pathway [16].

In the literature, there are no available studies on the changes in the sterol composition of walnut during fruit development. Total sterol content was reported to decrease gradually during linseed development as well [14].

A decline in the total sterol content during fruit development may be related to four reasons: (1) The biosynthesis of sterols occurs in the early stage of fruit ripening [17]. (2) The downregulation of enzymatic synthesis results in a decline in the sterol accumulation at the end of ripening [16]. (3) Sterols become more diluted as the oil content of the fruit increases with ripening [17]. (4) Existed sterols may be converted to steroidal hormones, and vitamins, which regulate the growth and development of immature tissues [16].

4. CONCLUSION

Based on these results, it can be concluded that variety and ripening impact on the fatty acid and phytosterol composition of Walnut (Juglans *regia* L.). The higher level of fatty acid was detected at the 14th DAFD for Franquette and 21st DAFD for Local gd. 4-desmethylsterols, the higher level was detected at the 7th DAFD for Franquette and 14th DAFD for

Local gd. an immature walnut was a good source of 4-desmethylsterols just as a mature walnut. Therefore, immature walnut may be proposed as a source of phytosterols and the essential linoleic fatty acid for functional food and nutraceutical applications.

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