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Changes in the sterol compositions of milk thistle oil (*Silybium marianum* L.) during seed maturation

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SUMMARY: In this study, the total lipid content and sterol compositions were determined during the development of milk thistle seeds. The oil content increased to a maximum value of 36±1.7% and then declined to reach a value of 30.5±0.9% at full maturity. The sterol content of milk thistle seeds was affected by the ripening degree of the seeds. At the early stages of seed maturation, Δ^7-stigmastenol was the most abundant sterol followed by β-sitosterol. However, at full maturity, β-sitosterol was the most predominant sterol (46.50±0.8%). As the seed developed, campesterol and stigmasterol amounts increased, while Δ^7-avenasterol content decreased. It can be concluded that milk thistle seed oil has a characteristic sterol pattern comparable to the ones elucidated for olive oil and corn oil. The extracted oil from milk thistle seeds is rich in phytosterols and could be used in food preparation and human nutrition.

KEYWORDS: *Maturation; Milk thistle seeds; Oil; Sterols changes*

RESUMEN: ***Cambios en la composición de esteroles del aceite de cardo mariano* (Silybium marianum *L.*) *durante la maduración de la semilla***. En este estudio se determinaron la composición de lípidos totales y esteroles durante el desarrollo de semillas de cardo mariano. El contenido de aceite incrementó a un valor máximo de 36±1,7% y posteriormente disminuyó hasta alcanzar un valor de 30,5±0,9% cuando la maduración fue completa. El contenido de esteroles de las semillas de cardo mariano se ve afectado por el grado de maduración de las semillas. En las primeras etapas de la maduración de las semillas el Δ7-estigmastenol fué el esterol más abundante, seguido de β-sitosterol. Sin embargo en plena madurez, β-sitosterol fue el esterol predominante (46,50±0,8%). A medida que las semillas se desarrollan las cantidades de campesterol y estigmasterol aumentan, mientras que el contenido Δ7-avenasterol disminuye. Se puede concluir que el aceite de semillas de cardo mariano tiene un patrón característico de esteroles en comparación con lo especificado para los aceites de oliva y de maíz. El aceite extraído de las semillas del cardo mariano es rica en fitoesteroles y podría ser utilizado en la preparación de alimentos y en nutrición humana.

PALABRAS CLAVE: *Aceite; Cambio de Esteroles; Maduración; Semillas de cardo mariano*

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1. INTRODUCTION

Phytosterols are minor components of vegetable oils and form a major proportion of the unsaponifiables (Azadmard-Damirchi *et al*., 2005). The individual sterols and their relative proportions can be used to determine the identity of the oil and to detect adulterations. It has been reported that conventional refining does not significantly affect sterol composition. Phytosterol contents in vegetables are known to vary due to different factors such as variety, season, extraction and other technological procedures (Li *et al*., 2007; Cercaci *et al*., 2007).

Furthermore, Phytosterols are known to lower serum low-density lipoprotein (LDL) cholesterol levels by reducing intestinal cholesterol absorption (Miettinenet *et al*., 1995). Clinical studies confirmed that phytosterols have hypocholesterolemy, anti-inflammatory and anti-carcinogenic effects (Awad *et al*., 2007; Ronco *et al*., 1999; Berges *et al*., 1995). Therefore, phytosterols have been added to several functional food products such as yoghurt, milk (Lagarda *et al*., 2006), and some vegetable oils (Ntanios, 2001). These types of products are now available on the market and have been scientifically proven to lower blood LDL cholesterol by around 10–15% as part of a healthy diet (Jones *et al*., 2000).

Milk thistle is an important medicinal crop in Europe and has recently become more significant in North America (Zheljazkov *et al*., 2006). The medicinal compounds of value are found in the seeds of the plant. In Tunisia, milk thistle is a common wild plant, which grows in many regions, and the people from some regions, particularly in the center areas, eat the seeds of this plant. Milk thistle seeds have been used for more than 2000 years to treat liver diseases (Karkanis *et al*., 2011). These seeds contain silymarin (Engelberth *et al*., 2008) and 25% (w/w) oil (Wallace *et al*., 2005). The oil has to be removed from the seeds prior to the extraction of silymarin. Therefore, it is a by-product of silymarin production. The oil extracted from these seeds can be used as a cure for many diseases including viral hepatitis and cirrhosis (Fadhil *et al*., 2012).

There are a very few data in the literature on the phyosterol composition of milk thistle seed oil. The sterol pattern of *Silybium marianum* has been determined in Egypt (EL-Mallah *et al*., 2003), Jordan (Dabbour *et al*., 2014) and Iran (Fathi-Achachlouei and Azadmard-Damirchi, 2009). The sterol composition can be affected by geographical growing area, difference in varieties and ripening degree of the fruits (Casas *et al*., 2004; Stefanoudaki *et al*., 2001; Harrabi *et al*., 2007). No work has been published on the sterol composition during the development of milk thistle seeds. The aim of this study was to monitor oil and sterol accumulation during seed maturation. The data obtained is important for evaluating the potential of milk thistle seeds to be exploited as a new source of oil for nutritional, industrial and pharmaceutical applications.

2. MATERIALS AND METHODS

2.1. Plant materials

Milk thistle seeds were collected from plants growing wild in Tunisia (region of Sousse), during April and June, 2012. Seeds were picked according to external color; green seeds were chosen as immature stage, mahogany brown seeds as the intermediate stage and dark brown seeds as the last stage of maturity (mature stage). 100 g of seeds were dried at 50 °C and then ground to fine powder in a grinder.

2.2. Oil Extraction

The oils were extracted using petroleum ether in a Soxhlet extractor for 4 h. The solvent was initially removed using a rotary evaporator at 40 °C. Oil samples were placed at ambient temperature (25–35 °C).

2.3. Saponification

The unsaponifiable fraction was determined by saponifying 5 g of oil extracts with 50 mL ethanolic KOH 12% (w/v) and heating at 60 °C for 1.30 h. After cooling, 50 mL of H_2O were added. The unsaponifiable matter was extracted four times with 50 mL of petroleum ether. The combined petroleum ether extract was washed with 50 mL of ethanol–water (1:1). The extracted ether was dried over anhydrous Na_2SO_4 and evaporated to dryness using a rotary evaporator. The dry residue was dissolved in chloroform for TLC analysis.

2.4. Thin layer chromatography

The unsaponifiable matter was separated into sub-fractions on preparative silica gel thin-layer plates (silica gel 60G F254) using one-dimensional TLC with hexane–diethyl ether (6:4, v/v) as the mobile solvent. The unsaponifiable fraction diluted in chloroform was applied on the silica gel plates. After development, the plate was sprayed with 2,7-dichlorofluorescein and viewed under UV light. The band corresponding to sterols was scraped, extracted three times with chloroform–diethyl ether (1:1, v/v), filtered to remove the residual silica, dried in a rotary evaporator and stored at −10 °C.

2.5. Analysis of sterols by GC-MS

GC-MS analyses were performed using a capillary HP-5MS column (30 m×0.25 mm I.D., 0.25 μm film thickness; Agilent Technologies) with gas chromatography (Agilent Technologies 7820A) coupled

directly to the mass detector (Agilent Technologies 5975 series MSD). Helium was used as carrier gas, with a constant flow rate of 1 ml/ min. The injector and detector temperatures were 230 °C. The oven temperature was programmed from 150 to 320 °C at 10 °C·min^{-1} from 150 to 250 °C and at 5 °C·min^{-1} from 250 to 320 °C. Electronimpact mass spectra were measured at acceleration energy of 70 eV. Manual injection of 1 µL of the sterol solution was performed in the split mode at a 10:1 split ratio. The phytosterol compounds were identified by comparing their relative retention times and mass spectra with those of the authentic standard. The peaks were also confirmed by comparison with the Wiley 275.L Mass Spectral Library.

2.6. Statistical analysis

A statistical analysis was performed by using the Proc ANOVA in SAS (Software version 8). Duncan's Multiple Range Test was used. For each oil sample, three determinations have been made.

3. RESULTS AND DISCUSSION

3.1. Lipid content

During seed maturation the oil content increased to a maximum value of 35.8±1.3% and then declined to reach a value of 30.5±0.9% at full maturity. More oil was synthesized during the early stage of seed development. Malekzadeh *et al*. (2011) reported that the total oil content of milk thistle seeds decreased under drought stress. In mature seeds, oil was stored in the form of oil bodies (Voelker and Kinney, 2001). The extracted oil from milk thistle seed has been suggested as suitable as an edible oil (EL-Mallah *et al*., 2003).

3.2. Unsaponifiable fraction

The results obtained showed that the amount of total unsaponifiable matter decreased during seed maturation (Table 1). The greatest change occurred during the early stage of seed development. Thus, the highest level of unsaponifiable matter (3.8±1.2%) was detected in immature seeds. At full maturity, the unsaponifiable lipid content of the studied seeds was 1.9±0.2% of the total oil. The total amount of unsaponifiable matter in olive oils ranged from 1 to 2% of the total lipids (Alonso-Salces *et al*., 2009). Overall, the level of unsaponifiable matter ranged from 0.5 to 2.5% of the total lipids (Małecka, 2002). These minor lipids greatly influence the organoleptic quality and stability of the oil (Alonso-Salces *et al*., 2009). The effectiveness of unsaponifiable matter in retarding oil deterioration has been demonstrated by many researches (Mohamed and Awatif, 1998; Gopala Krishna *et al*., 2003).

The unsaponifiable fraction is made up of minor constituents (sterols, triterpene alcohols, aliphatic alcohols, hydrocarbons, etc.), which may vary both qualitatively and quantitatively depending on genetic factor, climatic conditions, extraction and refining procedures, as well as storage conditions (Canabate-Díaz *et al*., 2007). 4-Desmethylsterols were isolated from the unsaponifiable matter and represented 55.2% of this fraction and about 1% of the total oil. This high proportion shows that milk thistle seed oil is one of the richest natural products in phytosterols versus other vegetable oils frequently used in the diet, such as olive oil (0.17%) (Weihrauch and Gardner, 1978). These results are useful for the consumer and also for the oil producer. Indeed, the unsaponifiable fraction of vegetable oils has applications in cosmetics and pharmacology due to its biological properties.

3.3. Sterols composition in immature seeds

Phytosterols are important due to their impact on health. Therefore, readily available food products have been engineered to be enriched in phytosterols and marketed to help lower serum cholesterol and reduce the risk cardiovascular disease. 4-Desmethylsterols are the major components of phytosterol matter in most vegetable oils (Azadmard-Damirchi and Dutta, 2006). Eight 4-desmethylsterols were detected in the oil analyzed (Figure 1). The changes in sterol composition during the development of seeds were

TABLE 1. Total oil and unsaponifiable matter contents of milk thistle seeds collected at three maturity stages

Maturation stage	Oil content (% of DW)*	Unsaponifable matter (% of oil)
Immature	8.4±2.1	3.8±1.2
Intermediate	35.8±1.3	2.3±0.6
Mature	30.5±0.9	1.9±0.2

*% of DW: % of dry weight.

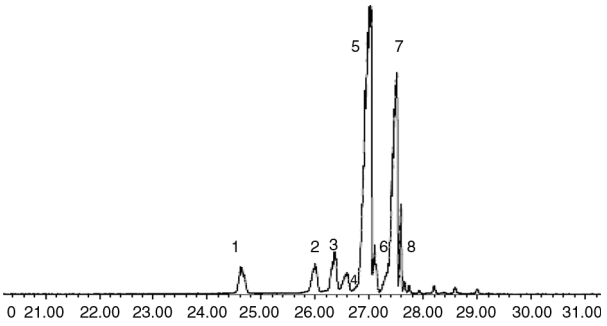

FIGURE 1. GC-MS Chromatogram of trimethylsilyl ether derivatives of 4-desmethyl sterols: (1) Cholesterol, (2) Campesterol, (3) Stigmasterol, (4) Δ^7-campesterol, (5) β-Sitosterol, (6) Δ^5-avenasterol, (7) Δ^7-Stigmastenol, (8) Δ^7-Avenasterol.

TABLE 2. Evolution of sterol composition (%) in developing seeds of milk thistle

Sterols	Immature	Intermediate	Mature
Cholesterol	1.10±0.2	1.52±0.1	3.91±0.3
Campesterol	1.83±0.3	2.30±0.5	4.20±0.5
Stigmasterol	1.34±0.1	3.60±0.3	5.47±0.4
Δ^7-Campesterol	2.15±0.2	2.27±0.1	2.88±0.2
ß-Sitosterol	32.20±1.3	37.55±1.5	46.50±0.8
Δ^5-Avenasterol	2.24±0.4	3.72±0.3	3.20±0.1
Δ^7-Stigmastenol	52.84±1.5	43.56±0.8	27.81±0.5
Δ^7-Avenasterol	6.40±0.7	5.48±0.2	3.80±0.2

summarized in Table 2. The sterol content of milk thistle seeds was affected by the ripening degree of the seeds. At the early stages of seed maturation, Δ^7-stigmastenol was the most abundant sterol followed by β-sitosterol. As the seed developed, the level of Δ^7-stigmastenol reduced from 52.84±1.5 to 27.81±0.5% of the total sterol content. This result could be explained by the high activities of Δ^7-sterol Δ^5-desaturase and Δ^7-sterol reductase during the early stages of seed development. In fact these two enzymes are involved in the conversion of Δ^7-sterols to Δ^5-sterols (Benveniste, 2002). The Δ^5-sterols are mainly accumulated in the plasma membrane, where they are believed to regulate the membrane fluidity (Grandmougin *et al*., 1989). The amounts of campesterol and stigmasterol increased gradually during seed maturation. However, the level of Δ^7-avenasterol decreased as the seed developed. The results obtained show that the levels of Δ^7-campesterol and Δ^5-avenasterol were relatively constant. The amount of cholesterol was very low in immature seeds (<2%). The rate of cholesterol accumulation was found to be greatest at the late stage of seed maturation.

3.4. Sterol composition in mature seeds

β-Sitosterol (46.50±0.8%) was the most abundant compound followed by Δ^7-stigmastenol (27.81±0.5%). The β-sitosterol content determined was much higher than that in the milk thistle cultivars grown in Iran (33–37%) (Fathi-Achachlouei and Azadmard-Damirchi, 2009), but was lower than that of the Egyptian cultivars (57.4%) (El-Mallah *et al*., 2003). The β-siosterol amount in milk thistle seeds is affected by environmental conditions and genotypes. The β-sitosterol range in olive oil is 34–66% (Mezghache *et al*., 2010). The health aspects of β-sitosterol, the most common phytosterol, have recently been reported in several studies (Awad *et al*., 1998).

The Δ^7-sterol compounds were mainly represented by Δ^7-stigmastenol (27.81%), Δ^7-avenasterol (3.80±0.2%), and Δ^7-campesterol (2.88±0.2%). The level of total Δ^7-sterols (34.49%) detected in this studied oil sample was much higher than that in the Iranian samples (19–22%) (Fathi-Achachlouei and Azadmard-Damirchi, 2009). This contrasts with the composition of corn oil where Δ^7-stigmastenol amounted to 2% of the total desmehyl sterol content (Harrabi *et al*., 2007). Important differences may occur in some plant families. For instance, many plants belonging to the order of caryophillales contain large amounts of Δ^7- sterols; spinach and chenopodiumrubrum contain almost only Δ^7-sterols such as spinasterol or stigmast-7-ene-3β-ol (Benveniste, 2002).

The campesterol (4.2±0.5%) and stigmasterol (5.47±0.4%) contents of the seed oil of this study were comparable to those of the Iranian milk thistle cultivars (Fathi-Achachlouei and Azadmard-Damirchi, 2009), but were higher than those of olive oil where the sum of these two sterols was less than 5% (Mezghache *et al*., 2010). The amount of Δ^5-avenasterol was 3.20±0.1%. In the literature, this component has been associated with antioxidant effects (Williamson, 1998; Blekas and Boskon, 1999). Yoshida and Niki (2003) reported that campesterol, stigmasterol and clerosterol exerted antioxidant effects on the oxidation of a methyl linoleate oil solution. Clerosterol was not detected in the studied seed oils and in the Egyptian samples (El-Mallah *et al*., 2003), but was detected in the Iranian samples (Fathi-Achachlouei and Azadmard-Damirchi, 2009). Moreover, sitostanol and campestanol were absent in this studied oil sample. This result agrees with these reported by Fathi-Achachlouei and Azadmard-Damirchi (2009) and El-Mallah *et al*. (2003). However, Dabbour *et al*. (2014), detected these two stanols in cold-pressed milk thistle seed oil (campestanol 0.21%, sitosanol 1.67%).

The results obtained show that cholesterol represents 3.91±0.3% of the total 4-desmethyl sterol fraction. It was lower than that in the milk thistle cultivars grown in Iran (9.5%) (Fathi-Achachlouei and Azadmard-Damirchi, 2009) and in the cold-pressed seed oil of the cultivar grown in Jordan (15.14%) (Dabbour *et al*., 2014). Consequently, it could be suggested that the unrefined oils obtained by extraction with an organic solvent had a lower level of cholesterol than the cold-pressed oils. Cholesterol is the predominant sterol in animal fats and fish oils, but is very rare in vegetable oils. It is known that cholesterol occurs in the sterol fraction of many vegetable oils as a minor component and usually amounts to 1% of the total sterol content (Phillips *et al*., 2002), significantly lower than that detected in the milk thistle seed oil. It has been recognized that plant sterols could reduce plasma cholesterol levels in humans (Ostlund *et al*., 2002). The mechanism of cholesterol reduction in the presence of phytosterols is based on the blocked absorption of it in the digestive tract. Since the level of cholesterol is low and in the presence of excessive amounts of phytosterols, it

can be expected that its absorption will be minimal and the positive effect of phytosterols will overcome it (Ostlund *et al*., 2002). Furthermore, cholesterol biosynthesis in higher plants has not been studied extensively and thus, uncertainties exist in the sequence of intermediates. The enzymatic approach to understanding and controlling the formation of the sterol structure was hampered by the low existence of sterol enzymes in higher plants. Therefore, milk thistle seeds which had a higher level of cholesterol as compared with the other seeds could be a good example for the study of the cholesterol biosynthetic pathway.

4. CONCLUSION

In summary, this study provides useful information on the sterol composition in immature milk thistle seeds. Milk thistle seeds are a rich source of phytosterols with a potential for beneficial therapeutic activities. Tunisian milk thistle oil had a very lower amount of cholesterol as compared with the Iranian and Jordanian cultivars. The results obtained can justify the important value of milk thistle seed oil as an attractive candidate for use in food preparation and human nutrition.

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