Chromatographic Quantitation of Some Bioactive Minor Components in Oils of Wheat Germ and Grape Seeds Produced as By-Products

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Abstract: The objective of this work was to determine some beneficial and bioactive minor lipid components in wheat germ and grape seeds as a result of milling industry of wheat and pressing of grapes in wineries. Sterylglycosides (SG's) were isolated and fractionated into free and acylated SG's by TLC and were determined as their 1-anthrolynitriles (1-AN) by HPLC. Moreover, 4-desmethylsterols were isolated, derivatized into their trimethyl silyl derivatives and analyzed by GLC. Tocopherols and tocotrienols were directly analyzed by HPLC. In addition, fatty acids composition by GLC was accomplished. The results were compared to three conventional edible oils, namely, corn, sunflower and cottonseed. It was found that the wheat germ oil (WGO) and grape seed oil (GSO) contained reasonable amounts of whole sterols. Sterylglycosides fraction (SG), which have not been evaluated, it was found that the two by-products contained high amounts of SG's and they were rich in free and acylated campe/stigma SG as well as free and acylated β -sitosterol. Total tocopherols and tocotrienols components were found in very high amounts in WGO (1300 ppm) and GSO (380 ppm). It is noteworthy to mention that GSO contained significant amounts of α - and γ -tocotrienols which prevent cardiovascular diseases and contained reasonable amounts of α - and γ -tocopherols. On the other side, it was found that WG and GS oils were enriched in linoleic acid (ω -6), while linolenic acid (ω -3) was present in higher quantity in WGO.

Key words: by-products, sterylglycosides, vitamin E, wheat germ oil, grape seed oil, omega-fatty acids

1 INTRODUCTION

The potential and current sources of oils, not only restricted to seeds, but also are included in food industry by-products. These by-products are obtained in tonnages such as grape pressing product obtained from the wineries and wheat germ produced from milling industry. It is well known that oils obtained from these by-products can be used for pharmaceutical and nutritional purposes¹. The increase in production volume of these by-products resulted in an exponential increase of these by-products in wheat milling and grape pressing industries. These by-products contain some valuable substances such as fatty acids, tocopherols, sterols and sterylglycosides with potential applications in food industry mostly go to waste.

Subsequently, it was reported that WG constitute about 2-3% of the whole wheat kernel; it has an average moisture content of 14% and contains about 10% oil². The well

known beneficial effect of WGO is basically due to their high content of vitamin E and unsaturated FA's, mainly linoleic acid^{3,7}. WGO has been found to reduce plasma and liver cholesterol in animals and to delay aging^{8,9}. Nevertheless, because of its unfavorable baking properties and susceptibility to oxidation, the germ is removed from the endosperm during milling and is mainly used for fodder and for oil production¹⁰. On the other side, WGO is obtained either by solvent extraction, which recovers about 90% of the oil, or by mechanical pressing which recovers about 50%¹¹. The extracted WG oils usually need to be refined, owing to their high FFA content varying from 5 up to 25%, depending on the conditions of milling, germ storage, and oil extraction¹². Additionally, supercritical CO₂ was employed in extracting WGO¹³. Phytosterols esters content were evaluated in the WGO and the phytosterols were enriched in the raffinate fraction^{7,14}. Moreover,

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Accepted January 8, 2009 (received for review November 4, 2008)

Journal of Oleo Science ISSN 1345-8957 print / ISSN 1347-3352 online
<http://www.jstage.jst.go.jp/browse/jos/>

they found that alpha-, beta-, and gamma-tocopherol in crude wheat oil amount to 13.9, 1.1 and 0.08 mg/g oil respectively. Barnes J.P. found that WGO exhibits a range of sterols⁶⁾. 4-Methylsterols, β -sitosterol and campesterol are the major components. Barnes and Taylor in their study dealt with the composition of acyl lipids and tocopherols in WGO's from various sources¹⁵⁾.

On the other side, grape pressing in wineries resulted in huge amounts of grape pomace containing grape seeds that are used as a source of oil having nutritional and pharmaceutical implications. The oil has a clear, light taste that has been described as "nutty" and is safe for cooking food compared with other oils¹⁶⁾. In addition, GSO, a high linoleic acid (76.0%) product, is the only food known to raise HDL (good cholesterol) and lower LDL (bad cholesterol). Linoleic acid (omega-6) is one of two essential fatty acids which can not be synthesized in the body and it is the precursor of a family of other fatty acids such as arachidonic acid and gamma-linolenic acid^{16,17)}. Beveridge *et al.*, used two extraction methods; found that GSO is ranged from 5.85 to 13.6% and 6.64 to 11.17% with supercritical CO₂ and petroleum ether extraction respectively¹⁸⁾. Grape seed and pomace oils had nearly similar FA profile with higher linoleic acid and very low linolenic acid content that make the oil more resistant to autoxidation. Also oleic acid in grape seed and pomace oils has a great importance in terms of their nutritional implication and the effect on oxidation stability of the oil¹⁹⁻²¹⁾. EL Mallah *et al.* studied the fatty acid, triacylglycerol, tocopherol, tocotrienol and whole sterol pattern of red grape, lettuce and soybean oil²²⁾. They found that the alpha and gamma-tocotrienols amount to 54.0 and 26.0% in red grape pomace. Additionally, it was reported that the enzymatic hydrolysis prior to cold pressing had a great influence on the yield of oil²³⁾.

The objective of this work was to update the data of some bioactive and nutritional minor components of WG and GS oils, obtained from samples of freshly produced by-products resulting from wheat milling and grape pressing industries. These components comprise tocopherols, tocotrienols, and whole sterols as well as free and acylated sterylglucosides beside fatty acids profile of the oils. UV-HPLC and fluorescence-HPLC as well as capillary GLC were used for quantitative evaluation of these components. Tocopherols and tocotrienols in oils were determined by direct method, whereas sterols evaluated as TMS derivatives. In addition, sterylglucosides, as 1-anthrolynitrile derivatives whereas fatty acids were evaluated as their methyl esters. It is worthy to mention that the bioactive sterylglucosides, as far as one knows, have not been hitherto investigated particularly in local WG and GS oils. For comparison, compositions of traditional oils such as corn, sunflower and cottonseed oils are also determined. In general, the compositional data of WG and GS oils can provide additional updated information to those customers who are

concerned with health and nutritional aspects for prevention of certain diseases.

2 EXPERIMENTAL

2.1 Materials

Freshly milled wheat germ sample was supplied by the Milling Organization, North Cairo, as by-product of wheat milling process (season 2006).

Grape pomace, by-product, containing grape seeds, of grape pressing (season 2006) was obtained from the Egyptian Vineyard and Distilleries Company (Ginacelis, Alexandria season 2006). Grape seeds, sieved out, from the air dried pomace and wheat germ were ground and subsequently extracted with chloroform/methanol (2:1, v/v).

For comparison, seed oils, namely, corn, sunflower and cottonseed were extracted in the laboratory using the previous methods of analysis.

2.2 Methods

2.2.1 Oil content

The oil content was calculated on dry basis for wheat germ, grape seed, corn, sunflower and cottonseeds according to Official and Tentative Methods of the AOCS²⁴⁾.

2.2.2 Fatty acids pattern

The oil was converted into methyl esters via transesterification with 5% hydrogen chloride gas in methanol¹⁷⁾. Transesterification reaction was monitored with the help of TLC using silica gel G plates and n-hexane : diethyl ether : acetic acid (80:20:1, v/v/v) was used as a developing solvent Hewlett Packard-HP 5980-A gas chromatograph was employed for the analysis of the mixed methyl esters under the following operating conditions: column, DB-23 (0.32 mm $\times$ 30 m); temperature programming, 150-230°C, 3°C/min; injector, 230°C; detector, FID at 240°C; carrier gas, helium at flow rate of 1.3 mL/min and split ratio, 100:1. Calibration was made using standard fatty acid methyl esters and the results were recorded by an electronic integrator as peak area percent with the help of standard chromatogram representing the following esters (14:0, 16:1, 18:0, 18:1, 18:2, 18:3, 20:0 and 20:1).

2.2.3 Tocopherols and tocotrienols pattern (direct method)

HPLC analysis of tocopherols (T) and tocotrienols (T3), in the oil samples as well as the standard tocopherols mixture were carried out using Toyo-Soda-CCPM HPLC instrument. An oil sample (10 g) was dissolved in n-hexane to make 10% solution and 10 μ L was injected into silica column (YMC-A-012, 6.0 $\times$ 150 mm). Isocratic elution was conducted using n-hexane: isopropyl alcohol (100:0.5, by volume) as mobile phase, at a flow rate of 1-2 mL/min. Hitachi-650-10S fluorescence detector was used. Spectral absorption was set at excitation and emission wavelengths of 295 and 325 nm respectively. The conditions were optimized to

elute delta-T after 10 min, the results were automatically recorded as peak area percentages by electronic integrator. The HPLC pattern of the standard tocopherol and tocotrienol designated as α -T, β -T, δ -T, γ -T, α -T3, β -T3, δ -T3 and γ -T3. From the peak area and the corresponding weight of each individual standard T and T3, the weight of each individual T and T3 in the oil (ppm) can be calculated^{25,26}.

2.2.4 Whole sterols profile

Whole sterols were isolated from the prepared unsaponifiable fractions of the oils by preparative TLC on silica gel G plates (0.5 mm thickness) using chloroform/diethyl ether/ acetic acid (95/4/1 by volume) as a developing solvent²⁴. The sterol zone was located with the help of standard beta-sitosterol ($R_f = 0.16$) applied alongside the sample prior to development. The scraped zone was thoroughly extracted with moistened diethyl ether, the solvent was distilled off and the prepared sterols mixtures were converted into trimethylsilyl derivatives (TMS)¹⁷. The sterol composition of the oils were determined by the help of the standard chromatogram of cholesterol, brassica, 5-stigma, B-sito, spina, isofuco, 7-stigma, avenasterol.

Hewlett Packard-HP 5890-A gas chromatograph, was employed for analysis using the following operating conditions: column, DB-17 (0.32 mm $\times$ 15 m, 0.25 μ m coating) at 250°C; detector, FID at 260°C; carrier gas, helium (8.6 mL/min) and split ratio, 35:1. An automatic integrator was coupled directly to the detector. TMS sterols mixture of cotton seed oil (containing known percentages of sterols) was used for identification and quantification of the sterols in each oil sample. The area under each peak was measured by the automatic integrator²⁷.

2.2.5 Sterylglycosides profiles of free and acylated sterols

The procedure of separation of the sterylglycosides from the oil, derivatization into sterylglycosides 1-anthroylnitrile (SG-1-AN) and HPLC analysis were carried out according to Murui *et al.*, and El-Mallah, *et al.*,^{27,28}. The free sterylglycosides (FSG) and acylated sterylglycosides (ASG) were separated from the oil by injecting the oil solution into silica gel cartridge (sep-Pak, Waters) followed by

elution with chloroform: methanol (1:1v/v). The eluate is the mixed sterylglycosides which were subsequently fractionated into pure FSG ($R_f = 0.30$) and ASG ($R_f = 0.61$) with the help of preparative TLC. Chloroform/methanol/formic acid mixture (90/15/1, v/vv) was used as a developing solvent. Only ASG fraction was subjected to alkaline hydrolysis (0.5 N KOH in isopropyl alcohol) to obtain FSG. The original and obtained FSG after deacylation of ASG were derivatized separately, into SG-1-AN according to the same methods mentioned above. Standard FSG and ASG were kindly obtained from Dr. Tateo Murui of Nisshin Oil Mills Yokohama, Japan.

HPLC instrument, equipped with UV detector (Toso, UV 8000) and column, ODS Wakosil-5, C₁₈ (6.4 mm $\times$ 250 mm) was used for SG-1-AN analysis. Gradient elution with acetonitrile/dichloromethane (from 50/50 to 68/32 v/v) was used and absorption was measured at 254 nm. It is noteworthy to mention that this method is sensitive to 0.5 nanogram of SG. The identification of the peaks was designated with the help of standard chromatogram as a guide for identification.

All the data are the mean of two concordant replicates and the difference did not exceed more than 0.2-0.4.

3 RESULTS

3.1 Oil content

The oil contents of wheat germ, grape seed, corn, sunflower and cottonseeds were 11.0, 12.0, 15.0, 19.6 and 22.6% respectively **Table 1**.

3.2 Fatty acids pattern

Concerning the fatty acid patterns (**Table 1**) of wheat germ (WG) and grape seed (GS) oils, it was found that these oils contained appreciable amounts of linoleic acid (omega-6) i.e. 56.5 and 67.8% respectively in comparison to the three edible conventional oils, namely, corn, sunflower and cottonseed. In addition, WGO contained a reasonable amount (8.0%) of linolenic acid (omega-3), whereas GS con-

Table 1 Fatty Acids Composition of Various Oil Samples by GLC.

Oil Sample	Oil Content %	Fatty Acid Composition %					Unsaturation %
		Palmitic (16:0)	Stearic (18:0)	Oleic (18:1)	Linoleic (18:2)	Linolenic (18:3)	
Wheat germ	11.0	20.0	1.0	14.7	56.5	8.0	79.0
Grape seed	12.0	8.0	3.8	20.2	67.8	0.2	88.2
Corn	15.0	8.3	4.4	42.0	45.1	0.2	87.3
Sunflower	19.6	6.3	2.5	46.3	44.9	—	91.2
Cottonseed	22.6	25.1	4.0	20.3	50.6	—	70.9

tained a minor amount of this fatty acid (0.2%). The monounsaturated fatty acid (oleic) is incorporated in the glycerides at 14.7% for WGO and 20.2% for the oil extracted from GS however, this acid constituted 42.0% in corn oil, 46.3% in sunflower oil and 20.3% in cottonseed oil. In WGO palmitic acid was the major component of the saturated fatty acids and amounted 20.0%, whereas it constituted 8.0% of the fatty acids of GSO. In comparison with corn, sunflower and cottonseed oils, the latter contained higher amount of this acid (25.1%), while corn and sunflower contained 8.3 and 6.3% respectively.

3.3 Tocopherols and tocotrienols pattern

It is known that vitamin E consists of eight different compounds, four tocopherols and four tocotrienols (designated as α -, β -, γ - and δ)²⁹. Individual and total amounts of vitamin E (tocopherols and tocotrienols) contents of WG and GS oils compared with the three edible oils (corn, sunflower and cottonseed) are presented in Table 2).

Thus the total amount of vitamin E in WGO amounting 1300 ppm which was markedly high, while in GSO reached 380 ppm in comparison with 1017, 670 and 620 ppm for

corn, sunflower and cottonseed oils respectively. The individual components of vitamin E in WGO were 70.0, 19.0 and, 7.0% for α -, β - and γ -tocopherols respectively and 2.0% for both α - and γ -tocotrienols. While GSO contained 10.0% α -tocopherol, 2.5% γ -tocopherol, 37.5% α -tocotrienol and 50.0% γ -tocotrienols. In comparison corn oil contained γ -tocopherol as abundant component (81.0%) while it exhibited lower amount of α -tocopherol (16.2%) and a very low concentration of δ -tocopherol (2.0%). On the contrary, sunflower oil possessed the predominating α -tocopherol which reached 96.0% of the total tocopherols however; it contained minor quantities of β - and γ -tocopherols. On the other side, cottonseed oil contained 43.5% α -tocopherol and 56.5% γ -tocopherol.

3.4 Pattern of whole sterols

Prior to analysis, pure sterols mixtures were isolated from the unsaponifiable fractions via TLC of the oils and subsequently converted into silyl ethers derivatives. From the results in Table 3, it is clear that the sterols patterns of WG and GS oils included, campe, 5-stigma, β -sito, iso-

Table 2 Tocopherols and Tocotrienols Pattern of Various Oil Samples by HPLC.

Oil Sample	Tocopherol Total (ppm)	Tocopherols and tocotrienols composition (%)					
		α -Toc.	β -Toc.	γ -Toc.	δ -Toc.	α -T3	γ -T3
Wheat germ	1300	70.0	19.0	7.0	—	2.0	2.0
Grape seed	380	10.0	—	2.5	—	37.5	50.0
Corn	1017	16.2	0.8	81.0	2.0	—	2.0
Sunflower	670	96.0	3.0	1.0	—	—	—
Cottonseed	620	43.5	—	56.5	—	—	—

Toc. : Tocopherol; α -Toc. : Alpha-tocopherol ; β -Toc. : Beta- tocopherol ; γ -Toc. : Gamma-tocopherol ; δ -Toc. : Delta-tocopherol ; T3 : Tocotrienol ; α -T3 : Alpha-tocotrienol ; γ - T3 : Gamma-tocotrienol.

Table 3 Whole Sterols Patterns of Various Oil Samples by GLC.

Oil Sample	Sterol Content (%)	Whole sterol composition (%)					
		Campe sterol	5-stigma sterol	B-sito sterol	Isofuco sterol	7-Stigma Sterol	7-Avena sterol
Wheat germ	0.8	27.2	0.8	63.0	4.0	2.0	3.0
Grape seed	0.7	11.4	10.5	75.6	1.0	1.5	0.5
Corn	1.0	21.0	7.7	66.8	4.5	—	—
Sunflower	0.9	9.0	12.5	60.5	3.0	11.0	4.0
Cottonseed	0.6	10.0	2.4	80.0	7.6	—	—

Table 4 Free and Acylated Sterylglycosides Pattern of Various Oil Samples by HPLC.

Oil Sample	SG Type	Content ppm	Free and acylated sterylglycosides Composition (%)				
			Avena-SG	Isofuco-SG	Campe stigma-SG	7-stigma-SG	B-Sito-SG
Wheat germ	FSG	460	—	3.5	20.1	—	76.0
	ASG	70	—	2.0	38.0	—	60.0
	Total	530					
Grape seed	FSG	150	—	4.5	18.0	1.0	76.3
	ASG	90	—	4.5	20.0	1.5	74.0
	Total	240					
Corn	FSG	105	2.0	4.0	23.0	—	71.0
	ASG	1533	2.5	4.0	26.2	—	79.3
	Total	1658					
Sunflower	FSG	200	3.0	7.5	13.0	1.5	75.0
	ASG	174	2.0	4.0	15.0	2.0	77.0
	Total	374					
Cottonseed	FSG	230	—	5.0	9.0	—	86.0
	ASG	80	—	6.0	8.0	—	86.0
	Total	310					

AN : Anthrolylnitrile ; SG: sterylglycosides ; FSG : Free sterylglycoside and ASG : Acylated sterylglycoside

fuco, 7-stigma and 7-avenasterol at a level of 27.2, 0.8, 63.0, 4.0, 2.0 and 3.0% respectively for WGO and 11.4, 10.5, 75.6, 1.0, 1.5 and 0.5% respectively for GSO. In comparison with the three conventional edible oils, it can be noticed that sunflower oil contained the same individual sterols present in WG and GS oils. On the other side, 7-stigma- and 7-avenasterol were not detected in corn and cottonseed oils. It can be noticed that WGO contained isofuco-sterol at a level of 4.0% in comparison to 4.5, 3.0 and 7.6% in case of corn, sunflower and cottonseed oils respectively. 7-Avenasterol constituted 3.0% in WGO in comparison with 4.0% in sunflower oil.

3.5 Sterylglycosides pattern

The total sterylglycosides (SG's), free and acylated SG, amounted to 530 ppm in WGO, whereas it was at a level of 240 ppm in GSO (Table 4). WGO SG was two fold the amount found in GSO. Concerning the three conventional oils, it was found that the total amount of SG in corn oil reached 1658 ppm which was markedly higher than that of sunflower (374 ppm) and cottonseed oil (310 ppm). Dealing with free SG (FSG) of WGO, the pattern was lacking avena-SG and 7-stigma-SG. On the other hand β -sito-SG was present as a predominating compound, whereas, lower amount of isofuco-SG and reasonable amount of campe+stigma-SG were detected. On the other side, GSO was lacking only in avena-SG and the pattern was nearly the same as WGO with the exception of the presence of 7-stigm-SG at a level of 1.0%. In comparison with the three conventional edible oils, it could be noticed that corn oil as well as cottonseed

oil did not contain 7-stigm-SG; however cottonseed oil did not contain avena-SG. It is noteworthy that the sunflower profile of FSG contained the five SG components at a level of avena-SG (3.0%), isofuco-SG (7.5%), campe +stigma-SG (13.0%), 7-stigma-SG (1.5%) and β -sito-SG (75.0%). The individual distribution of ASG was the same as that of FSG in WG and GS oils as well as the three conventional edible oils.

4 DISCUSSION

In general, WG and GS oils contained essential fatty acids, namely, omega-6 and omega-3 which mean that these oils can be used as health and edible oils. In addition, the oils extracted from the two by-products contained reasonable amounts of oleic acid, a monounsaturated fatty acid. This monounsaturated fatty acid has great importance in terms of its nutritional implication and in improving the oxidative stability of oils²¹. It is known that low levels of linolenic acid are desired in edible oils, because high levels of this acid can produce unfavourable odour and taste in oil²⁰. Furthermore, the stability or shelf-life of oil rich in linolenic acid would be short because linolenic acid is easily oxidized^{30,31}. So GSO, having a low quantity of this acid, can be advantageous in terms of human consumption and shelf-life of the oil. The reasonable percentage of unsaturation in WG and GS oils may play an important role in lowering high blood cholesterol and also in the treatment of atherosclerosis³².

It seems that WGO is a rich source of alpha-tocopherol which is a high bioactive tocopherol component. Oils with high tocopherol content can be used in applications where high level of antioxidant protection is needed. On the other hand, among the tocopherols present in foods, alpha-tocopherol shows the highest vitamin E activity, thus making it the most important tocopherol for human health and biological activity^{33,34}. However, GSO was enriched with alpha- and gamma-tocotrienols which was unique among the other oil patterns under investigation. It is worthy to mention that there is a strong evidence of these tocotrienols for preventing narrowing (stenosis) of carotid artery²⁹.

Sterols play essential roles in the physiology of humans, also known to block cholesterol absorption sites in the human intestine thus helping to reduce cholesterol in humans and to provide important signals and metabolic communications eg. circadian rhythms, blood clotting.

The SG's have beneficial effect particularly it reduced the absorbability of cholesterol and fatty acids in the intestines, which resulted in reduced concentrations of plasma cholesterol and triacylglycerol³⁵. Accordingly, WG and GS oils can be used as sources of SG.

5 CONCLUSION

Laboratory extracted oils from the two by-products, namely, wheat germ (WG) and grape seed (GS) contained predominating polyunsaturated fatty acids, particularly linoleic acid (omega-6) and linolenic acid (omega-3). These acids are essential for the human body physiological function since they can not be biosynthesized by the body. Accordingly, WG and GS oils, rich in linoleic and linolenic acids, may be considered as valuable sources of dietary oils. In addition, total tocopherols and tocotrienols amounted to 1300 ppm in WGO and 380 ppm in GSO. Alpha-tocopherol was predominating in WGO while, beta-tocopherol was present in reasonable amount (19.0%) however, alpha- and gamma-tocotrienols were found as main products in GSO. It is well known that, tocotrienols have been shown to reduce plasma cholesterol levels as well as other lipids and non-lipid related risk factors for cardiovascular diseases (CVD)³⁶.

With the development of analytical tools, bioactive sterylglucosides(SG's), comprising free and acylated compounds, were able to be determined. It worthy to mention that SG's suppress the absorption of cholesterol and fatty acids in the intestine i.e. they are considered as hypocholesterolemic component³⁵.

The beneficial effects of WG and GS oils are due not only to their high polyunsaturates content, but also to their tocopherols and tocotrienols as natural antioxidant components. These components may serve as dietary sources for

natural antioxidants scavenging free radicals and thus prevent the body from diseases and promote human health. Consequently, WG and GS oils are suitable as health oils and can be generally used for in pharmaceutical purposes since they are enriched in bioactive lipid components.

ACKNOWLEDGMENTS

The authors express their gratitude to Prof. Dr. Mohamed M. Hassan EL-Mallah and Prof. Dr. Safinaz M. EL-Shami of National Research Centre, Fats and Oils Dept. for their criticism and helpful discussion during the preparation of this work. In addition, thanks are given to Dr T. Murui (Nisshin Oil Mills Yokohama, Japan), for supplying standard sterylglucosides and facilities received in many ways.

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