Lipid Profiling of the Carob Fruit (*Ceratonia Siliqua* L.) Using GC/LC/QTOF Accurate Mass Spectrometry

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Abstract

The carob tree (*Ceratonia siliqua* L.) is a native of the Mediterranean basin with several applications in the food, textile and cosmetics industries and the pods of the carob fruit have long been used as a feed for livestock and in human nutrition, including sweets, biscuits and processed drinks, because of its high sugar content and low price. This work serves as the first step towards a comprehensive metabolomics characterization of *Ceratonia siliqua* L. Gas chromatography and liquid chromatography coupled with quadrupole time of flight mass spectrometry were used to profile and quantify lipids in the whole pod/pericarp of the carob fruits. Crude lipids were extracted from the carob powder using CO₂ supercritical fluid extraction in a yield of 2.0%. A total of 14 fatty acids, 5 phospholipids, 10 triacylglycerol, 8 steroids as well as hydrocarbons and 16 head space volatile compounds were identified and quantified.

Keywords: Fatty acids; Head space; Triglycerides; Phospholipids; Unsaponifiable matters; Leguminosae

Introduction

The carob tree (*Ceratonia siliqua* L.) is a native of the Mediterranean basin with several applications in the food, textile and cosmetics industries [1]. It belongs to the Caesalpinaceae sub-family of the family Leguminosae [2]. The pods of the carob fruit have long been used as a feed for livestock and in human nutrition, including sweets, biscuits and processed drinks, because of its high sugar content and low price [3]. The world production of carob pods is 374,800 to 441,000 tones/year, the leading producer countries are Spain followed by Italy, Portugal, Morocco, Greece, Cyprus, Turkey and Algeria [4,5]. Carob pods are rich source of natural antioxidants which may, by different mechanisms, act as an effective defense against reactive oxygen species including free radicals such as superoxide anion and hydroxyl radicals, and non-free radical species such as hydrogen peroxide. The two principal components of the carob fruit are the pulp and seed. The seeds represent 10% of the weight of the fruit and the pulp represent the other 90% of the fruit, crushed

pods may be used to make a beverage, liqueur and syrup [6]. Image of the carob pods are shown in Figure 1.

Lipids are essential metabolites in cells and they fulfill a variety of functions which include structural components of cellular membranes, energy storage, cell signaling, and membrane trafficking [7]. In plants, alterations in the composition of lipids have been observed during cold/heat stress, nutrient deficiencies, aging and response to pathogen attack [8]. Lipids are divided into several classes based on their chemical structures: fatty acyls, glycerol lipids, glycerol phospholipids, sphingolipids, sterol lipids, prenol lipids, saccharolipids and polyketides [9]. In order to understand the function of lipids in biological systems, it is important to profile the diverse lipid species present in any given organism and quantify their levels. Recent technological advances in tandem chromatography and mass spectrometry have enabled high-throughput, systematic profiling of lipid species known as lipidomics [7,10]. This study aims to use accurate mass GCQTOF and LCQTOF to profile and quantify levels of lipid classes in the pods of the carob fruits as a first step towards understanding its lipidome.

Experimental

Plant material

Carob pods were collected from the seed department of

Figure 1: Image of the carob pods that was used in this investigation

the agricultural research center, Ministry of Agriculture, Giza, Egypt in the summer of 2016 and kindly authenticated by the agricultural botany department of the faculty of agriculture, university of Cairo. The pods were dried in the dark smashed and seeds were mechanically removed and the pulps were grounded using a grinder (Sunbeam Osterizer blender, Boca Raton, USA) to fine powder.

Extraction of crude lipids

CO₂ supercritical fluid extraction was done using the SFT-10 Constant Flow /Constant Pressure

Dual Piston Pump SFE unit (Proras, Rome, Italy). The extraction basket utilized in this study had an internal volume of 0.5 L and, for each test, batches of about 65 g of carob pods were placed in the basket and utilized for the extraction. Pressure and temperature were kept constant at 500 bar and a temperature of 50 °C yielding a light yellow oil in 2% by weight.

Preparation of fatty acids methyl esters (FAMES)

The transesterification was carried as we described before [11] by adding 10 mg of the lipid extract to 2 mL of 2% BF₃ in methanol, mixed by vortex and placed on the heating block at 75 °C for 1 h. After cooling down to room temperature, 1 mL of saturated sodium chloride solution was added to stop the reaction and FAMES were extracted in 2 mL of iso-octane, passed through anhydrous sodium sulfate and transferred to GC vials for GCMS analysis.

Accurate mass GC-QTOF

Agilent 7200 accurate mass GCQTOF System was used for analyzing FAMES samples. GC separation was done using a BPX90 SGE Analytical Science column 30m x 0.25 mm x 0.25µm column (SGE Analytical Science, Texas) run under average velocity of 63.9 cm/sec with a hold up time of 1 min. Temperature programming started at 75 °C held for 2 min and heated up to 200 °C at a rate of 5 °C/min, held at 200 °C for 3 min. Total run time was 30 min; solvent delay 4 min; equilibration time 3 min. Carrier gas flow 1.5 mL/min; injected volume 1µL; split mode 10:1. Mass spectral acquisitions were separately performed in EI mode and CI mode using methane as the reagent gas. The measurements and post-run analyses were controlled by the software Mass Hunter Qualitative Analysis B.07.00 SP1 [12]. FAMES was identified based on their retention times and their accurate mass data. Their electron ionization fragmentation and mass spectral data were also searched using Wiley10NIST mass spectral database.

LC-QTOF

Agilent 6530 accurate mass LC-QTOF system equipped with 1290 binary pump, Phenomenexkinetex C18 100 mm x 3.0 mm 2.6 µm columns. Solvents A was made of isopropanol; B acetonitrile run in a gradient protocol starting at 50% A, 50% B with a flow 0.2 mL/min reaching 100% solvent A at 10.0 min from the start, and held at 100% A for additional 5 min. Injection volume was 2 µL; mass range from 100 m/z to 1500 m/z, with a total run time of 30 min. The LC-QTOF instrument was operated under the following conditions: Ion source ESI + Agilent Jet

Stream Technology in positive ionization mode. Instrument parameters were set as follows: sheath gas temperature, 350°C; sheath gas flow, 8 L/min; nebulizer, 20 psi; dry gas temperature, 300 °C; dry gas flow, 5 L/min; and capillary entrance Voltage, 3500 V. Fragmentor and Skimmer1 were operated at 190 and 65 V, respectively. The MS scan data were collected at a rate of 1.02 spectra/s in the range of m/z 100–2000. All the MS data were collected with Mass Hunter Data Acquisition B.06.00 (Agilent Technologies), and Mass Hunter Qualitative Analysis B.06.00 SP1 (Agilent Technologies) was applied to identify lipid species. All EICs were obtained with 10 ppm m/z expansion. Microsoft Office Excel 2013 (Microsoft, Redmond, WA, USA) were used for statistical data analysis and data visualization [12].

LC-QTOF MS/MS experiment

MS/MS experiments were carried out as we described before [12] at energies of 50 eV, run time 15 min; ion source: positive; source gas temperature 300 °C; drying gas 3.0 L/min; nebulizer 15 psig; sheath gas temperature 125 °C; sheath gas flow 3.0 L/min; dual AJS ESI; V cap 4000 V; capillary 0.063 µA; nozzle Voltage 0 V; chamber 5.33 µA; ms/ms mass range 100 m/z to 1600 m/z; acquisition rate 1 spectrum/s; acquisition time 1000 ms/spectrum; 13,503 transients/spectrum; auto recalibration reference mass window, detection window 100 ppm; minimum height 1000 counts; polarity type: positive; offset: 15; ms/ms absolute threshold 5; reference threshold 0.01%.

Head space analysis

Head space analysis was carried out on the dry carob powder (1g) using Agilent 7890B/5975C GCMS system equipped with Agilent 7697A head space sampler. GCMS conditions were the same as those described above.

Analysis of unsaponifiable matters

Unsaponifiable matters from the lipid extract were analyzed using the standard AOCS method [13]. Oil samples were saponified by refluxing with 0.5 (N) ethanolic potassium hydroxide for 2 hours on a water bath the unsap matters were extracted with petroleum ether, washed with water, dried over anhydrous sodium sulfate and analyzed by GCMS at the same condition described above.

Results and Discussion

Crude lipids extracts were obtained from dried carob powder using CO₂ SFE extraction as shown in the experimental sections at a Yield (w/w) of 1.98 %.

Fatty acids composition

Fatty acid methyl esters (FAMES) relative percentage composition is shown in Table 1. FAMES were identified based on their retention times and their accurate mass data in the chemical ionization mode and by their electron ionization fragmentation mass spectral data by searched using Wiley10NIST mass spectral database. GCMS analysis of the fatty acids methyl esters (FAMES) was achieved with base line resolution as shown in Figure 2. 32% of the identified fatty acids were saturated fatty acid with palmitic acid (16:0) as the most abundant fatty acid contributing

more than 21% of the total fatty acids, stearic acid (18:0) was found at almost 4% of the total, all other saturated fatty acids were found as minor components contributing only 1-2% of the total fatty acids. Relative concentration of unsaturated fatty acids was 63% of the total and was made primarily of oleic acid Z-9-octadecenoic acid (18:1) with an overall concentration of 55%. Z,Z-9,12-octadecadienoic acid (18:2) was found at concentration of 6.5%. Other minor concentrations of fatty acids including Ω-3-fatty acids C18:3, C22:2 and C24:3 as well as other unidentified compounds were less than 4% of the total but no individual compound exceeded 0.05%.

Identification of triacylglycerols (TAGs)

TAGs composition in the crude lipid extract was invigilated using the accurate mass LC-QTOF system. Ten major TAGs were identified as shown in Figure 3. Ions were identified as positive ion sodium adducts with exact masses of 879.7426, 901.7251, 903.7418, 907.7743, 909.7891, 935.8055, 937.8205, 965.8523, 967.8679 and 993.8852. Although the ion [M+Na]⁺ with the accurate mass equivalent to 879.7426 represent 56 possible TAG isomers this number can be reduced to 14, by eliminating TAGs that have any fatty acid that was not detected in the oil (Table 2). Similarly, the accurate mass of 901.7256 represents 186 possible TAG isomers, this number can be reduced by eliminating TAGs that have any fatty acid that was not detected in the oil (Table 2).

Determination of regioisomers by MS/MS

There are large number of different triacylglycerols species (TAGs), which differ in the total length of acyl chains and their degree of unsaturation. Isobaric TAGs are designated by having the same number of carbon atoms and double bonds possessing acylchains with different length, position and configuration of double bonds. The acylchain may be located in three different

Table 1. Fatty acid methyl esters (FAMES) composition and relative percentage of lipid extract of the carob pods. Peak numbers are those shown in Figure 2.

Peak #	t _r (min)	Fatty acids as methyl esters (FAMES)	Short names	%
1	9.42	Dodecanoic acid	12:0	0.16
2	12.35	Tetradecanoic acid	14:0	0.30
3	13.76	Pentadecanoic acid	15:0	0.09
4	15.14	Hexadecanoic acid	16:0	21.69
5	15.86	(Z)-9-Hexadecenoic acid	16:1	1.05
6	16.37	Heptadecanoic acid	17:0	1.82
7	17.73	(Z)-9-Octadecenoic acid	18:1	54.93
8	18.33	Octadecanoic acid	18:0	3.94
9	19.29	(Z,Z)-9,12-Octadecadienoic acid	18:2	6.49
10	20.06	Eicosanoic acid	20:0	1.06
11	20.52	(Z,Z,Z)-6,9,12-Octadecatrienoic acid	18:3	2.08
12	22.29	Docosanoic acid	22:0	0.85
13	23.31	11-Eicosenoic acid	20:1	2.05
14	24.37	Tetracosenoic acid	24:0	0.26

Figure 2: GCMS Total ions chromatogram (TIC) of carob FAMES, peak numbers and identifications are shown in Table 1.

Figure 3: LCMS total ions chromatogram (TIC) of carob TAGs, peak numbers and identifications are shown in Table 2.

Table 2. Triacylglycerol (TAGs) composition and relative percentage of the carob pods, Peak numbers are those shown in Figure 2.

t _r (min)	Accurate Mass*	Identified structures (sn1, sn2, sn3)	Loss of RCOOH from		RCO+	%
			sn1 position	sn3 positions		
17.58	901.7251	18:2,18:2,18:2	621.4806 18:2	621.4806 18:2	263 18:2	0.07
19.67	903.7418	18:1,18:2,18:2	623.5020 18:2	621.4806 18:1	265/263 18:1/18:2	0.27
21.60	879.7426	16:0,18:0,18:3	623.4707 16:0	601.9316 18:3	261/239 18:3/16:0	7.26
23.47	907.7743	18:1,18:1,18:1	625.5150 18:1	625.5150 18:1	265 18:1	34.57
24.95	935.8055	18:1,18:1,20:1	653.5457 18:1	625.5152 20:1	293/265 20:1/18:1	7.11
25.26	909.7891	18:0,18:1,18:1	627.5288 18:1	625.5151 18:0	267/265 18:0/18:1	13.85
26.88	937.8205	18:1,18:1,20:0	655.5611 18:1	625.5134 20:0	295/265 20:0/18:1	10.61
28.36	965.8523	18:1,18:1,22:0	683.5962 18:1	625.5150 22:0	323/265 22:0/18:1	14.07
29.76	993.8852	18:1,18:1,24:0	711.6209 18:1	625.5151 24:0	351/265 24:0/18:1	2.92
30.02	967.8679	16:0,20:1,22:0	711.6171 16:0	627.5321 22:0	323/239 22:0/16:0	0.77

positions of TAG, known as the sn1, sn2, or sn3 positions. The region specific analysis is restricted to determine the acylchains orientation as sn1 (3) and sn2 positions. We have shown previously [13] that mass spectral fragmentation can provide information about the stereochemistry and region specific identification of the TAGs. Based on our finding on the fragmentation of standard authentic TAGs and available published work [13-15] that ions corresponding from the loss of fatty acids from the sn1 and sn3 positions are much more abundant than the ion corresponding to the loss of fatty acid at the sn2 position. In addition, the fact that each fatty acid chain produces an RCO⁺ ion, if two or three of the chains are different, two or three different RCO⁺ are found but more for the sn1 and sn3 positions. Applying that information to the TAGs of carob (Figure 3), the region isomers in the carob were identified as shown in Table 2.

Phospholipids

The LC-MS analysis of phospholipids of carob oil is presented in the Table 3 and Figure 4. On the basis of the mass fragmentation pattern of each component and the NIST library search, we found that carob oil contains one major phosphatidylcholine phospholipid (PC(O-18:1(9Z)/16:0)) representing 56.7% of the identified phospholipids, two phosphatidylinositol phospholipids; PI-18:0/0:0 (2.6%), PI-20:1,11Z/22:0 (13.5%), one phosphatidate phospholipid PA-20:0/22:0 (10.9%) and one PG-16:0/21:0 (16.3), their chemical structures are shown in Figure 5.

Unsaturation matters

The GCMS analyses of the unsaponifiable matters of carob oil are presented in the Table 4 and Figure 6. On the basis of the mass fragmentation pattern of each component, the NIST library search, it was found that carob oil contains stigmaterol, β -sitosterol, γ -sitosterol, 5 α -cholestan-3-one, betulin, β -amyrin, betulin free and squalene. Their structures are shown in Figure 7 and Figure 9.

Head space volatile compounds

The volatile emitted by fresh carob pod powder (Table 5) was analyzed by headspace followed by capillary gas chromatography and mass spectrometry (Figure 8). The headspace of carob pods is mainly constituted of monoterpenes and sesquiterpenes, and minor amounts of nonterpenoid aldehydes and hydrocarbons. The identity of each compound was determined by comparison of its retention index relative to C₆-C₂₄ n-alkanes, as well as of its

Table 3. Phospholipids composition and relative percentage of the carob pods

t _r (min)	Accurate Mass	Identified structures	Formula	%*
9.61	585.4859	PI(P-18:0/0:0)	C ₂₇ H ₅₄ O ₁₁ P	0.09 (2.6)
10.04	799.6055	PG(20:1(11Z)/18:3(6Z,9Z,12Z))	C ₄₄ H ₇₉ O ₁₀ P	0.57 (16.3)
11.35	746.5966	PC(O-18:1(9Z)/16:0)	C ₄₂ H ₈₅ NO ₇ P	1.98 (56.7)
13.22	773.6264	PA(P-20:0/22:0)	C ₄₅ H ₉₀ O ₇ P	0.38 (10.9)
14.49	949.6962	PI(20:1(11Z)/22:0)	C ₅₁ H ₉₈ O ₁₃ P	0.47 (13.5)

*Value is expressed as % in the total lipid extract and values in parentheses are relative % in phospholipids fraction.

Figure 4: LCMS total ions chromatogram (TIC) of carob phospholipids, peak retention times (t_r) and identifications are shown in Table 3.

Figure 5: Phospholipids chemical structures that were identified in the carob pods

Table 4. Composition and relative percentages of the unsaponifiable matters of carob oil identified as trimethylsilyl ethers

t _r (min)	Chemical Name	%	t _r (min)	Chemical Name	%
8.31	Stigmaterol	8.39	9.67	Betulin	8.12
8.92	β -Sitosterol	48.34	9.79	β -Amyrin	11.26
9.25	γ -Sitosterol	1.71	10.25	Betulin free	1.36
9.48	5 α -Cholestan-3-one	3.12	10.97	Squalene	16.48

spectral data with the Wiley's library spectral data bank (G1035B; Rev D.02.00; Agilent Technologies). For semi quantification purposes, the normalized peak area of each compound was used without any correction factors to establish abundances. The sesquiterpene compounds were present in higher abundance than monoterpenes.

Conclusion

This study has revealed qualitatively and quantitatively the

particular compounds that make up the bulk of lipids in the carob fruit particularly the composition of the minor fractions, i.e. the profiles of phytosterols and other compounds more distinctly define the genuineness of individual oils and fats along with their respective fatty acid compositions. Carob pods are rich source of nutritional and health components, to extract those components,

Figure 6: GCMS total ions chromatogram of the unsaponifiable matters of carob oil.

Figure 7: Chemical structures of the identified unsaponifiable matters of carob oil

Figure 8: GCMS total ions chromatogram of the head space volatile components of the fresh carob pods powder.

Figure 9: Chemical structures of the identified volatile compounds found in the head space of the fresh carob pods powder.

#	t_R (min)	Chemical names	%	#	t_R (min)	Chemical names	%
1	10.07	cis-3-methyl-2-pentene	4.0	9	14.15	2-undecanone	2.5
2	10.45	3-methyl-6-hepten-1-ol	1.5	10	15.55	3-cadinene	1.7
3	11.40	terpinen-4-ol	1.8	11	15.80	(-)-elemen-1,3,11(13)-trien-12-ol	1.2
4	11.78	α -terpineol	5.1	12	16.84	(+)-aromadendrene	1.2
5	12.97	isopropylbenzaldehyde	21.9	13	17.05	aromadendrene	1.0
6	13.08	(-)-carvone	14.9	14	17.14	α -curcumene	7.3
7	13.30	piperitone	1.8	15	17.34	isolekene	13.8
8	13.91	2-carene-10-al	6.0	16	17.69	cis- β -farnesene	3.5

both a solubility and diffusivity of the extracting agent should be investigated. In order to facilitate the use of the extracts, it is important to carry out the extraction that allows for maximum amount of a particular component while minimizing or limiting the presence of other components. For that purpose, we used supercritical fluid extraction (SCFE- CO_2) as a green technology to extract lipids from carob pods. Oils extracted by SCFE- CO_2 showed similar quality, in terms of fatty acid composition than those obtained by traditional extraction techniques such soxhlet extraction using hazardous solvents like chloroform and methanol. Our study could be considered as the first detailed document on lipids profiling of carob pods. It showed that carob

Pods are very rich in oleic acid, but does not contain sufficient amounts of Ω -3 polyunsaturated fatty acids to be considered as good nutritional oil.

Fatty acids, triacylglycerol, phospholipids, terpenoids, hydrocarbons and steroids pattern can be helpful for understanding of biosynthesis of TAG and establishing lipid profiling of plants is fundamentally important to gain insights of its lipid composition or lipidome.

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Disclaimers

The views expressed in the submitted article are the authors own conclusion and not an official position of the institution or funder.

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