Journal of Chromatography B 1116 (2019) 60–64

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/jchromb

GC–MS analysis of D-pinitol in carob: Syrup and fruit (flesh and seed)

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ARTICLE INFO

Keywords:
Ceratonia siliqua L.
Cyclitols
Derivatization
Polyols
3-*O*-methyl-D-chiro-inositol
Sugar alcohols

ABSTRACT

D-pinitol (3-*O*-methyl-D-chiro-inositol) is a well-known bioactive compound with anti-diabetic and anti-oxidant biological functions. A gas chromatography–mass spectrometry (GC–MS) method was developed for its quantitation in carob syrup, flesh and seed samples originated from Cyprus. The analysis was performed after derivatization of carbohydrates and polyols into trimethylsilyl ether derivatives. D-pinitol was determined in 13 carob syrup samples, in concentrations ranging 65.71 ± 4.60 – 77.72 ± 5.44 mg/g (mean: 68.58 ± 4.80 mg/g, n = 13). In two commercial samples, it was determined in relative medium-low concentrations (21.96 ± 1.54 and 44.71 ± 3.13 mg/g), revealing possible adulteration; however, this needs further investigation. Similarly, it was determined in high concentrations in carob flesh samples, in concentrations ranging 53.20 ± 3.72 – 54.58 ± 3.82 mg/g (mean: 53.81 ± 3.76 mg/g, n = 3). On the other hand, seed samples proved very poor in D-pinitol (< LOD). Therefore, bioprospecting of carob fruit and syrup is highlighted. Compared to other plants or legumes, carob appears to be the richest source of D-pinitol, highlighting carobs role as a functional organic food. The historical and cultural association of Cyprus with carobs is linked with traditional foods and habits.

1. Introduction

Carob (*Ceratonia siliqua* L.) is an evergreen tree, which is mostly cultivated in the Mediterranean region (e.g. Spain, Italy, Morocco, Portugal, Greece, Turkey and Cyprus) [1]. Pods consist 80–90% of carob fruit and seeds 10–20%, respectively [2]. A wide number of studies exist on analyzing the chemical composition of carobs. The carob pods contain approximately 40–60% sugars (sucrose, glucose and fructose), 3–4% proteins and 0.4–0.8% lipids, along with high amounts of dietary fibers, polyphenols and remarkable quantities of minerals (1–6%) [2,3].

Among the main sugars, carob fruit contains the cyclitol D-pinitol (3-*O*-methyl-D-chiro-inositol), which is considered a bioactive and effective natural health food supplement. In particular, this compound has shown anti-diabetic activity, as is related to the reduction of blood glucose level in patients with type II diabetes mellitus by increasing insulin sensitivity. The efficacy of anti-diabetic action of D-pinitol, similar to insulin, was tested in diabetic animals (mice and rats) [4,5]. Towards this, the effects of D-pinitol on insulin resistance through the PI3K (phosphatidylinositol-3-kinase)/Akt signaling pathway in experimental rats with type II diabetes mellitus was studied [6]. In addition to its anti-diabetic property, the anti-oxidant action of D-pinitol was reported along with its potential in treatment of NF-$_k$B linked pro-inflammatory diseases such as cancers, inflammatory bowel disease, arthritis, sepsis, gastritis, asthma and atherosclerosis [5]. D-pinitol was firstly isolated from the pine tree and later from many other plant sources, such as soybean, bougainvillea flower and ice plant [7,8]. Among the family *Leguminosae*, soybean and carob are considered to be the most rich pinitol-sources [8].

A number of studies investigating the presence of D-pinitol and other cyclitols have been previously performed using high performance liquid chromatography (HPLC) [7] and gas chromatography (GC) in combination with mass spectrometry (MS) [9]. Furthermore, spectroscopic techniques like nuclear magnetic resonance (NMR) and infrared spectroscopy (IR) were also employed [5]. The ultrasound-assisted extraction (UAE) of D-pinitol from carob pods using response surface methodology (RSM) by optimization of the affected parameters (temperature, ultrasonic power, dilution rate and time) was also reported [10]. Additionally, D-pinitol was isolated from carob pods using supercritical fluid extraction (SFE). The influence of SFE crucial parameters such as pressure, temperature, CO_2 flow rate and process duration were studied [11]. Chauhan et al. isolated D-pinitol from the aqueous fraction of the herb *Argyrolobium roseum* by HPLC analysis with RI/PDA (refractive index/photodiode array) detector [12]. The ethanolic extract of the latter plant was also used for the identification of D-pinitol by ^{1}H NMR, ^{13}C NMR and DEPT (distortionless enhancement by

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https://doi.org/10.1016/j.jchromb.2019.04.008
Received 4 March 2019; Accepted 2 April 2019
Available online 03 April 2019

Table 1
Determination of D-pinitol in plants or legumes.

Plant name/legume	Technique	Content (mg/g)	Ref.
Carob pods	GC–MS	112.71 (mean value for n = 3)	[18]
Carob pods	HPLC	84.59[a]	[7]
Carob pods	TLC, GC, GC–MS	50–75	[9]
Carob pods	UAE, RSM, HPLC	11.98[a] g/L	[10]
Carob syrup	HPLC	63.75 ± 2.01–95.71 ± 0.69	[17]
Chickpea	GC–MS	1.95 (mean value for n = 3)	[18]
Lentil	GC–MS	1.97 (mean value for n = 3)	[18]
Soybean	GC–MS	3.48 (mean value for n = 3)	[18]
Soybean roots	GC–MS	1.93 ± 0.29	[15]
Vegetable soybean leaves	RSM, GC	13.2 − 30.4	[21]
Argyrolobium roseum	^{1}H NMR, ^{13}C NMR, DEPT	13.8 ± 0.18[a]	[13]
Jojoba seed meal (*Simmondsia chinensis*)	GC–MS	14.1 ± 0.2, 12.0 ± 0.2	[22]
Limonium gmelini subsp. *hungarica*	GC, GC–MS	2.91 (mid-April) 15.15 (August)	[23]
Tannins from gall plant, chestnut and quebracho	GC-FID, GC–MS	0.0073 (gall plant), 0.0049 (chestnut), 0.0009 (quebracho)	[24]

[a] Maximum concentration.

polarization transfer) experiments [13]. The presence of D-pinitol was also revealed in the ethanolic extracts from the fruits of *Pithecellobium dulce* by GC–MS analysis [14]. Furthermore, D-pinitol was identified and quantified in soybean plant roots after derivatization with GC–MS analysis [15]. Additionally, it was isolated and extracted from the plant *Bougainvillea spectabilis* and then identified by different analytical techniques such as IR, NMR, differential scanning calorimetry (DSC), thin-layer chromatography (TLC), ultraviolet spectroscopy (UV) and HPLC [16]. Table 1 summarizes D-pinitol determinations in several plants and legumes with its corresponding concentrations.

In Cyprus, carob has been historically linked with the island (medieval era) and named “black gold”, since it was one of the main agriculture export products and the main source of income in the last centuries. The main varieties cultivated in Cyprus are *Tylliria*, *Koumpota* and *Kountourka*. Carob syrup is a popular traditional product (known as “teratsomelo”), which is consumed independently or as a feedstock for the production of various other traditional food and beverages. It is exported in many countries and is considered an extremely nutritious product.

The aim of the present work was to identify and quantify the concentration of D-pinitol in carob syrup (local traditional product), flesh and seed samples from Cyprus. There are no previous studies in the literature investigating Cyprus cultivars and studies focused on carob syrup are extremely limited. According to our knowledge, D-pinitol was determined only by Tetik et al. [17], in carob syrup samples from Turkey.

2. Materials and methods

2.1. Samples preparation

Sample preparation was based on a modified GC–MS method applied for soybean roots [15]. Approximately 0.4–0.5 g of carob syrup (n = 15), carob flesh (n = 3) and seed (n = 3) samples from Cyprus carob cultivars were homogenized in 25 ml MeOH (Honeywell, ≥99.9%) 80% and sonicated in a sonication bath (Transsonic 700/H) for few minutes (depending on the sample). Carob flesh samples were placed overnight in refrigerator for better homogenization. Homogenized samples were then filtered using Whatman no. 41 filter paper (Whatman, England). A volume of 1 ml of the filtrate and 1 ml of internal standard (ISTD, resorcinol, 1 mg ml^{-1}, Himedia) were transferred to a 10 ml glass tube and dried under high purity nitrogen. After the evaporation, the tube was placed in an oven (Gallenkamp) at 103 °C for 40–50 min. The residue was then silylated with 400 µl of hexamethyldisilazane (HMDS), trimethylchlorosilane (TMCS) and pyridine 3:1:9 (Sylon HTP kit, Supelco Analytical) and placed at 70 °C overnight in a vacuum oven (Jeio tech OV-11). After derivatization, 2 ml of isooctane (Merck, ≥99.8%) was added to each tube and centrifuged at 3000 rpm for 5 min (Sigma Laborzentrifugen 3K15). Finally, 1 ml of the supernatant was transferred to a vial for GC/MS measurement. Each sample was duplicate analyzed.

2.2. Pinitol standards

Pinitol standards (Sigma Aldrich, 95%) were prepared in methanol (Honeywell, ≥99.9%) 80% in concentrations approximately 1.5, 1, 0.2, 0.1, 0.01 and 0.005 mg ml^{-1}. 1 ml of each standard was placed to a 10 ml glass tube and 1 ml of resorcinol (1 mg ml^{-1}, Himedia) was added in each tube. Subsequently, drying and derivatization processes were followed similar to the preparation of carob syrup and carob flesh samples. The respective standards were then analyzed by GC/MS.

2.3. GC–MS analysis

The chromatographic analysis was performed in a Shimadzu GC–MS QP2010 Ultra gas chromatograph-mass spectrometer in the electron-ionization mode of 70 eV, at the scanning range of 30–500 *m/z*. A 1 µl sample volume was injected onto a DB-5 ms column (30 m × 0.25 mm i.d. × 0.25 µm thickness) (Agilent J&W GC columns) in the Shimadzu GC-2010 Plus with a split ratio 1:50, working under helium constant flow (1.1 ml/min). The column was heated in the oven with ramp temperature program as follows: 80 °C for 2 min, increase at a rate of 20 °C/min to 280 °C and 280 °C for 10 min. The injection port temperature was set at 275 °C. The mass spectrometer worked in full scan mode. The ion source and interface temperatures were kept at 200 °C and 250 °C, respectively. The identification of compounds was performed with NIST mass spectral library (NIST MS search 2.0).

3. Results and discussion

As indicated in Table 1, GC–MS derivatization method was previously applied in carob pods using a non-commercial kit [18]. More particular, Ruiz-Aceituno et al., optimized a yeast treatment (*S. cerevisiae*) procedure for the isolation of low molecular weight carbohydrates (including pinitol) in edible legume extracts including black-eyed peas, buckwheat, carob pods, chickpeas, grass peas, lentils and soy beans and further determination by GC–MS. For the chromatographic analysis, phenyl-*β*-D-glucoside was used as ISTD and the derivatization procedure was performed by use of hydroxylamine chloride solution in pyridine, hexamethyldisilazane and trifluoroacetic acid [18]. Therefore, in the present study, a new GC–MS method was developed using a commercial silylating agent (Sylon HTP, Supelco Analytical) based on the modification of the work of Garland et al. applied in soybean roots [15].

3.1. GC–MS analysis of sugars and polyols

Fig. 1 presents a typical chromatogram obtained from GC–MS analysis in a commercial carop syrup sample. The peaks in the chromatogram correspond to the trimethylsilyl (TMS) derivatives of the corresponding sugars and polyalcohols. Peak 1 corresponds to the TMS derivative of resorcinol (1,3-benzenediol), which was served as ISTD. The main ingredients identified were the TMS derivatives of D-pinitol, sucrose, glucose, fructose and myo-inositol. Monosaccharides (glucose, fructose) presented more than one peaks in their chromatograms, because of the presence of isomers configurations. On the other hand,

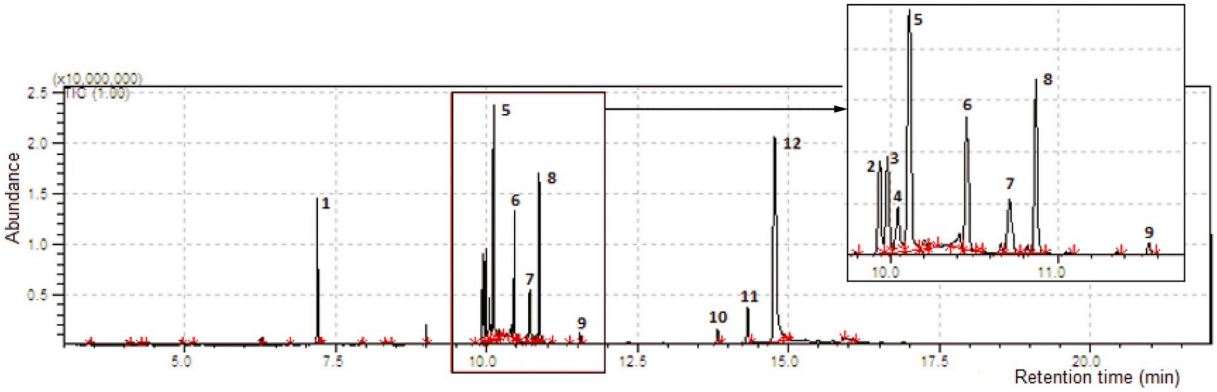
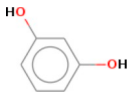
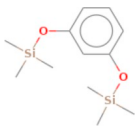
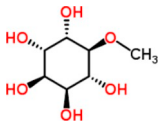
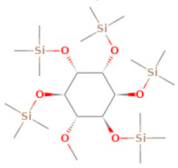
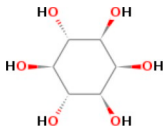
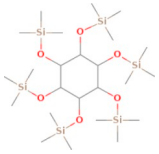


Fig. 1. Total ion chromatogram obtained by GC–MS analysis from carob syrup sample: (1) 1,3-bis (trimethylsiloxy) benzene (ISTD), (2, 3) D-(−) fructofuranose, pentakis (trimethylsilyl) ether, (4) D-fructose, 1,3,4,5,6-pentakis-O-(trimethylsilyl)-, (5) D-pinitol, pentakis (trimethylsilyl) ether, (6, 8) D-glucose, 2,3,4,5,6-pentakis-O-(trimethylsilyl)- or isomers, (7) n.i. (not identified), (9) Myo-inositol, 1,2,3,4,5,6-hexakis-O-(trimethylsilyl) ether, (10−12) sucrose, octakis-O-(trimethylsilyl) ether.

Table 2
Compounds identified by GC–MS analysis of carob syrups.

Compound (before derivatization)	Chemical structure	Compound (after derivatization)	Chemical structure
1,3-Benzenediol (recorcinol)		1,3-Bis (trimethylsiloxy) benzene	
D-pinitol		D-pinitol, pentakis (trimethylsilyl) ether	
Myo-inositol		Myo-inositol, 1,2,3,4,5,6-hexakis-O-(trimethylsilyl) ether	

sugar alcohols (D-pinitol and myo-inositol) showed one peak in their chromatograms, due to the absence of isomers [19]. Table 2 shows the structures of resorcinol, D-pinitol and myo-inositol and their corresponding TMS derivatives, which were detected by the GC–MS method. All substances showed the mass fragment 73 *m/z*, which is derived from the trimethylsilyl group (-Si$(CH_3)_3$). Peak 5 corresponds to the TMS derivative of D-pinitol and peak 9 to TMS derivative of myo-inositol. GC–MS analysis in all Cyprus carob syrup samples showed no significant differences between their chromatograms. The main components identified were appeared at identical retention times.

In the same way, the main components identified in the carob flesh samples from Cypriot cultivars expect for resorcinol (ISTD), were the polyols D-pinitol and myo-inositol and sugars sucrose, glucose and fructose at similar retention times with carob syrup samples. Fig. 2, shows a typical chromatogram of carob flesh sample from Cypriot cultivar "*Koumpota*" obtained by GC–MS analysis. On the other hand, the components identified from the analysis of carob seed samples of Cypriot cultivars, were the TMS derivatives of D-pinitol and sucrose only (Fig. 3).

3.2. Determination of D-pinitol concentrations

Quantification of D-pinitol was achieved using the ISTD method. The TMS derivative of pinitol had a characteristic ion fragment 260 *m/z*, which was not detected in any other TMS derivative besides pinitol. In the case of resorcinol, its TMS derivative presented a characteristic ion fragment of 239 *m/z*, which was the base ion detected in the mass spectrum of resorcinol and was not detected in the mass spectra of the other substances. Therefore, for the quantification of pinitol in standards as well as in carob syrup, flesh and seed samples, the ion peak areas of 260 *m/z* and 239 *m/z* were integrated at retention times t_R = 10.11 min and t_R = 7.2 min, for pinitol and resorcinol, respectively.

The linear equation resulting from the calibration curve was y = 0.428701 × + 0.002344, with a coefficient of determination R^2 = 0.998. For method validation, D-pinitol was determined 6 times in the same carob syrup sample. The minimum value was measured 73.51 mg/g, the maximum 82.51 mg/g and the mean 77.72 mg/g. The standard deviation (SD) for the six values was calculated 2.72, while the % relative standard deviation (% RSD) 3.50%. The method LOD (limit of detection) was determined 1.93 mg/g, while the LOQ (limit of quantification) 6.37 mg/g.

Table 3 shows D-pinitol concentrations in carob syrup, flesh and seed samples. D-pinitol content in carob syrup samples ranged from 65.71 ± 4.60 – 77.72 ± 5.44 mg/g (mean: 68.58 ± 4.80 mg/g, n = 13). The concentration of D-pinitol in sample 7 was determined 44.71 ± 3.13 mg/g, whereas in sample 5, only 21.96 ± 1.54 mg/g. According to literature, low amounts of D-pinitol in carob syrup may be

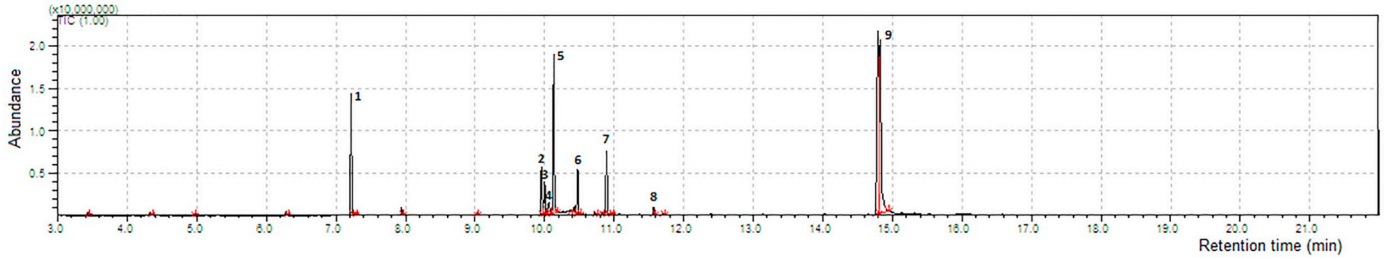


Fig. 2. GC–MS analysis of a carob flesh sample ("*Koumpota*" cultivar): (1) 1,3-bis (trimethylsiloxy) benzene (ISTD), (2, 3) D-(−) fructofuranose, pentakis (trimethylsilyl) ether, (4) D-fructose, 1,3,4,5,6-pentakis-O-(trimethylsilyl)-, (5) D-pinitol, pentakis (trimethylsilyl) ether, (6, 7) D-glucose, 2,3,4,5,6-pentakis-O-(trimethylsilyl)- or isomers, (8) Myo-inositol, 1,2,3,4,5,6-hexakis-O-(trimethylsilyl) ether, (9) sucrose, octakis-O-(trimethylsilyl) ether.

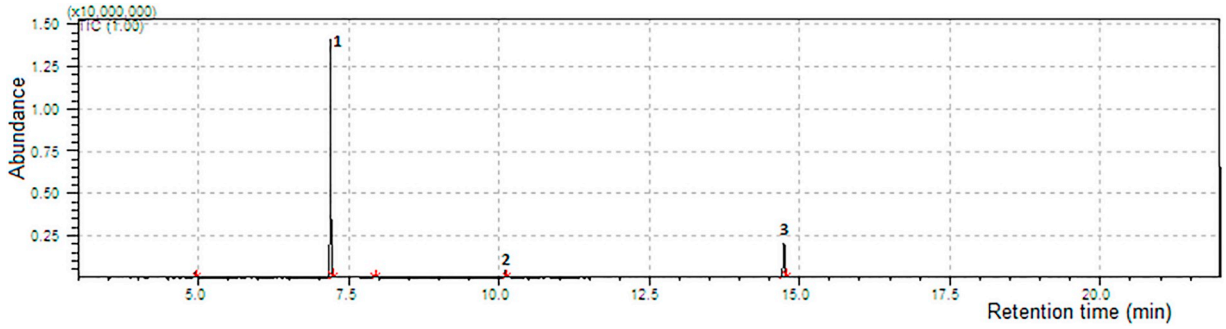


Fig. 3. GC–MS chromatogram of a carob seed sample ("*Koumpota*" cultivar): (1) 1,3-bis (trimethylsiloxy) benzene (ISTD), (2) D-pinitol, pentakis (trimethylsilyl) ether, (3) sucrose, octakis-O-(trimethylsilyl) ether.

associated with adulteration [17]. In carob flesh samples, D-pinitol concentration ranged from 53.20 ± 3.72 – 54.58 ± 3.82 mg/g (mean: 53.81 ± 3.76 mg/g, n = 3). The above concentration values of D-pinitol in carob are in agreement with literature (Table 1). Tetik et al., determined high amounts of D-pinitol in carob syrup samples using HPLC, and suggested that it can be used as indicator for the detection of adulteration in carob syrup [17]. Moreover, the correlation between sugars profile (glucose, sucrose and fructose) and D-pinitol content of different types of carob pods (wild and cultivated) was also reported. In another study, the chemical analysis of D-pinitol and other sugars carried out by HPLC revealed a maximum concentration of D-pinitol at 84.59 mg/g in wild type carob pods [7]. In the same content, TLC, GC and GC–MS were applied for the isolation and identification of sugars and cyclitols in carob powder. The major compounds identified were sucrose, fructose, glucose, D-pinitol, myo-inositol and D-chiro-inositol, along with traces of ononitol (4-*O*-methyl-myo-inositol), sequoyitol (5-*O*-methyl-myo-inositol), bornesitol (1-*O*-methyl-myo-inositol) and sorbitol [9]. In carob seed samples, the concentrations of D-pinitol were determined under the LOD of the method (≤1.93 mg/g). Therefore, from the literature, it can be concluded that carob fruit is the richest source of D-pinitol compared to other plants or legumes, such as chickpeas (1.95 mg/g), lentils (1.97 mg/g) and soy bean 3.48 (mg/g) (Table 1).

Table 3
D-pinitol content of carob syrup, carob flesh and seed samples determined by GC–MS.

Number	Sample	D-pinitol content (mg/g) (n = 2)[a]
Carob syrup		
1	Commercial	67.59 ± 4.73
2	Commercial	72.29 ± 5.06
3	Commercial	77.72 ± 5.44
4	Commercial	67.75 ± 4.74
5	Commercial	21.96 ± 1.54
6	Commercial	68.96 ± 4.82
7	Commercial	44.71 ± 3.13
8	Commercial	66.82 ± 4.67
9	Traditional[b]	68.78 ± 4.81
10	Traditional	68.62 ± 4.80
11	Traditional	65.94 ± 4.61
12	Traditional	69.36 ± 4.85
13	Traditional	66.35 ± 4.64
14	Traditional	65.71 ± 4.60
15	Traditional	65.72 ± 4.60
Carob flesh (Cyprus cultivars)		
1	"*Koumpota*"	54.58 ± 3.82
2	"*Kountourka*"	53.20 ± 3.72
3	"*Tilliria*"	53.65 ± 3.75
Carob seed (Cyprus cultivars)		
1	"*Koumpota*"	< LOD
2	"*Kountourka*"	< LOD
3	"*Tilliria*"	< LOD

[a] Data presented as mean ± 2SD.
[b] Product produce for private use by family business.

According to Narayanan et al., a sufficient amount of D-pinitol to significantly reduce the blood glucose level during 0.5–2 h after administration, was estimated to 10 mg/Kg body weight [20]. Thus, an average person weighing 60 kg should consume at least 600 mg of D-pinitol in order to achieve any beneficial health effect. In the present study, the mean value of D-pinitol in the carob syrup was 70 mg/g. This means that a consumption of about 9–10 g of carob syrup (equivalent to appr. two teaspoons) is needed in order to provide a significant benefit to the organism. However, in order to export safe conclusions for daily intake and possible beneficial effect, a cost-benefit consideration should be followed, taking into consideration the consumption of carob syrup, given its sucrose content.

4. Conclusion

Sugars (sucrose, fructose and glucose) and polyols (D-pinitol, myo-inositol) were identified in Cypriot carob syrup samples, as well as in carob flesh and seed samples from Cypriot cultivars, using GC–MS after derivatization. D-pinitol quantification revealed that Cypriot carob

syrup and carob flesh are rich sources of D-pinitol. The values in carob syrup samples were ranged from 21.96 ± 1.54 – 77.72 ± 5.44 mg/g (mean: 63.88 ± 4.47, n = 15). However, in two of the samples, D-pinitol concentration was found to be much lower compared to other samples (this needs further investigation), so for n = 13, the D-pinitol values ranged from 65.71 ± 4.60 – 77.72 ± 5.44 mg/g (mean: 68.58 ± 4.80 mg/g, n = 13). In carob flesh samples, D-pinitol values were estimated 54.58 ± 3.82, 53.20 ± 3.72 and 53.65 ± 3.75 mg/g (mean: 53.81 ± 3.76 mg/g, n = 3), respectively per variety, for the examined Cypriot cultivars. The present findings are in agreement with similar literature reports, revealing the functional richness of this natural remedy.

Acknowledgements

The authors would like to thank the Cyprus Research Promotion Foundation (CRPF Grant: INTEGRATED/0916/0019) and the University of Cyprus for funding this work. Assistance provided by the personnel of the “Food Composition, Food Quality and Nutritional Value” Laboratory of the State General Laboratory of Cyprus was greatly appreciated.

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