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Milk Thistle Seed Oil Constituents from Different Varieties Grown in Iran

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Abstract In this study, fatty acids, phytosterol classes and tocopherols composition of Milk thistle seeds oil were determined at four varieties grown in Ardebil-Iran. The four varieties consisted of two modified foreign varieties—Budakalaszi (originally from Hungary) and the CN-seed variety (originally from England) and two native varieties, namely Khoreslo and Babak Castle. The oil content of the seeds ranged from 26 to 31%. Among the fatty acids, linoleic acid had the highest percentage (50–54%) followed by oleic acid (23–29%) and palmitic acid (7–8%). This is the first detailed report on the phytosterol classes of milk thistle seeds oil. The 4-Desmethylsterol class was predominant (1,800–2,200 μg/g) followed by 4,4′-dimethylsterols (50–85 μg/g) and 4-monomethylsterols (26–35 μg/g). The α-, β-, γ-, and δ-tocopherols ranged from 187 to 465, 10 to 51, 9 to 12, and 18 to 80 μg/g oil, respectively. Based on the results obtained, the extracted oil from milk thistle seeds are rich in essential fatty acids, sterols and vitamin E and can be an attractive candidate for use in food preparation mixed with other vegetable oils or alone.

Keywords Fatty acids · Milk thistle seed oil · Phytosterol classes · Tocopherols

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Introduction

The milk thistle plant, *Silybum marianum* (family: Astraceae) is an annual or biennial plant, native to the Mediterranean area and some parts of the United States, which has now spread to other warm and dry regions [1]. This plant also grows in many regions in Iran. Extracts from the mature milk thistle seeds are used as medical remedies for liver disease, liver cirrhosis and to prevent liver cancer [2, 3].

Ther milk thistle contains silymarin, which is composed of the flavanolignans silybin, silydianin and silychristine, with silybin being the most biologically active. Silymarin is found in the highest concentrations in the fruit portion of the plant but is also found in the leaves and seeds. The seeds also contain betaine, trimethylglycine and essential fatty acids, which may contribute to silymarin's hepatoprotective and anti-inflammatory effects [3].

Oil has to be removed from seeds prior to the extraction of silymarin. In fact the oil is a by-product of silymarin production. Milk thistle seeds contain a relatively high amount of oil (20–25%) [1, 4]. Extracted oil contains phospholipids and a high content of vitamin E, serving as a potential natural source of vitamin E [1, 5]. It has been reported that the extracted oil from milk thistle seed contains fatty acids such as linoleic acid, oleic acid, linolenic acid, palmitic acid, stearic acid and it has been suggested as being suitable as an edible oil [1, 6].

The aim of present study was to determine the oil composition of some varieties of milk thistle seeds grown in different part of Iran. In this paper, qualitative and quantitative characterization of 4-desmethyl-, 4-monomethyl-, and 4,4′-dimethylsterols was also carried out by saponification of oil samples, and then fractionation of the total sterols by preparative-TLC followed by GC and

GC–MS analyses. These data can help to introduce the milk thistle seeds oil as a valuable by-product of silymarin production and its potential application in food preparation.

Materials and Methods

Sample

Milk thistle seeds collected from four varieties consisted of two modified foreign varieties—Budakalaszi (originally from Hungary) and the CN-seed variety (originally from England) and two native varieties (Khoreslo and Babak Castle) grown in Ardabil-Iran. Budakalase and CN-seed variety seeds were collected from plants grown in both dry and irrigated farming. Seed samples were collected—approx. 2 kg from each variety. Each 2-kg seed sample was thoroughly mixed and 100 g of seeds from these amounts were weighed for further analysis.

Oil Extraction

Oil samples were extracted in triplicate from milk thistle seeds (100 g) according to the method described by Azadmard-Damirchi et al. [7]. In brief, chopped seeds (approx. 10 g) were processed with 30 ml hexane/isopropanol (3:2, v/v) at room temperature under vigorous shaking for 1 h in steel tubes containing four steel balls to facilitate homogenization. Ten tubes, each of them containing 10 g chopped seeds, were used to extract oil from 100 g chopped seeds. After 1 h shaking, the extracts were filtered through defatted filter papers with a Buchner funnel under vacuum, the remained defatted cake was also washed twice with 20 ml of the same solvent to extract all the possible residual oil content. Thereafter 35 ml of 6.7% sodium sulfate was added to the oil-containing solvent and thoroughly mixed. The upper organic solvent layers containing the oil were then separated and rotary-evaporated under reduced pressure at 35 °C. The extracted oil was stored at −20 °C for further analysis.

Fatty Acid Measurement

Preparation of Fatty Acid Methyl Esters

Fatty acid methyl esters (FAMEs) were prepared from the oil samples according to the method reported by Savage et al. [8]. Briefly, 2 ml of 0.01 M NaOH in methanol was added to a tube containing the oil sample (ca. 10 mg) dissolved in 0.5 ml hexane and then held in a water bath at 60 °C for 10 min. Thereafter, boron trifluoride in methanol (20% of BF3 in methanol) was added and the samples held an additional 10 min in a water bath at 60 °C. The sample was cooled under running water and 2 ml of 20% (w/v) of sodium chloride and 1 ml hexane was added. After mixing completely, the hexane layer that contained the FAMEs was separated by centrifugation.

Analysis of Fatty Acid Methyl Esters by GC

The FAMEs were analyzed by GC according to the method described by Azadmard-Damirchi and Dutta [9]. The GC instrument was equipped with a flame ionization detector and a split/splitless injector. A 50 m × 0.22 mm, 0.25 μm film thickness fused-silica capillary column BPX70 (SGE, Austin, TX, USA) was used for analysis. Injector and detector temperatures were 230 and 250 °C, respectively. Oven conditions were 158 °C increased to 220 °C at a rate of 2 °C/min and maintained for 5 min. Helium was used as the carrier gas and nitrogen as the make-up gas at a flow rate of 30 ml/min. The FAMEs were identified by comparison of their retention times with standard FAMEs and the peak areas reported as a percentage of the total fatty acids.

Phytosterol Analysis

Saponification for Sterol Analysis

Saponification for sterol analysis was done according to the method described by Azadmard-Damirchi et al. [7] after minor modification. The weighed oil sample (ca. 300 mg) was mixed thoroughly with 30 ml of 2 M KOH in 95% ethanol in a glass tube and shaken in a water bath at 90 °C for 15 min. After cooling the tubes, 20 ml of water was added and the unsaponifiable matter was extracted three times with 15 ml diethyl ether. The combined extracts were washed once with 0.5 M KOH in ethanol and again washed with distilled water. The diethyl ether layer was passed through anhydrous sodium sulfate. The solvents were removed in a rotary vacuum evaporator at 30 °C. The dry unsaponifiable matter was dissolved in 0.5 ml dichloromethane for further analysis.

Separation and Enrichment of the Phytosterol Classes

After saponification, the phytosterol classes were separated and enriched from the unsaponifiable matter by thin layer chromatography (TLC) according to the method described by Azadmard-Damirchi et al. [7]. In brief, unsaponifiable matter was applied to a TLC plate (Silica gel 60, 20 × 20 cm, 0.25 mm thickness) along with a sample containing sterol fractions as a reference to correctly identify the sterol bands. Then the TLC plate was developed with a mobile phase, hexane: diethyl ether: acetic acid (70:30:1, v:v:v). After developing, the reference band was

exposed to iodine vapor, while the sample area was covered with a glass plate. On the basis of the reference spots, phytosterol classes were identified, marked out and scraped off. After adding 20 μg 5α-cholestane as the internal standard to the scraped material, each phytosterol class was extracted three times with 2 ml dichloromethane and then the extract used for further preparation of the trimethylsilyl (TMS) ether derivatives. This preparative step was carried out in triplicate.

TMS Ether Derivatives of the Phytosterols

TMS ether Derivatives of phytosterol classes were prepared according to method described by Azadmard-Damirchi et al. [7]. Solvent containing sterol class was evaporated under stream of nitrogen and 100 μl of silylation reagent (Sigma-Aldrich Co., St. Louis, USA) was added and dispersed on the ultrasonic bath, and then held in water bath at 60 °C for 45 min and dispersing on the ultrasonic bath again. Thereafter, the solvent was evaporated under a stream of nitrogen, and the TMS ether derivatives were dissolved in 200 μl hexane for further analysis by GC.

GC Analysis of the Phytosterols

Phytosterol analysis was performed according to the method described by Azadmard-Damirchi and Dutta [9]. In brief**,** for this purpose, a fused-silica capillary column DB-5MS 30 m × 0.25 mm, 050 μm (J&W Scientific, Folsom, CA, USA) was used. The column was connected to a Chrompack CP 9002 gas chromatograph (Middleburg, The Netherlands) equipped with a flame-ionization detector. The analysis conditions were: (a) injector 260 °C, (b) oven 60 °C for 1 min, increased a rate of 40 °C/min to a final temperature of 310 °C held for 27 min (c) Helium as the carrier gas and nitrogen as the make up gas at a flow rate of 30 ml/min (d) the detector temperature was 310 °C. Quantification was done relative to the 5α-cholestane internal standard. All samples were analyzed in triplicate, and means of the results are reported.

Determination of Tocopherols by High Performance Liquid Chromatography

The tocopherols content of the oil samples was analyzed by HPLC (Cecil Instruments Ltd, Cambridge, England) according to the method described by Azadmard-Damirchi and Dutta [10]. According to the retention time of reference samples of tocopherols on chromatogram, each tocopherol in analysed oil samples was identified. Quantification was carried out using an external standard method with reference samples of tocopherols. All samples were analysed in triplicate and the results reported are the means of these.

Statistical Analysis

The statistical analyses in this study comprised two parts: descriptive statistics in which the means of triplicate values and standard deviations (SD) were calculated; and inferential statistics in which one-way ANOVA was used to compare differences between varieties. Differences were considered significant at $P < 0.05$. The statistical analysis was carried out with SPSS 15.0 for Windows software.

Results and Discussion

Fatty Acids

The oil content of four varieties of milk thistle seeds ranged from 26 to 31%. The fatty acid composition determined by GC is presented in Table 1. Nine fatty acids were detected in the extracted oils from milk thistle seeds (Table 1). Linoleic acid (18:2n-6) was predominant followed by oleic acid (18:1n-9), palmitic acid (C16:0) and stearic acid (18:0). The extracted oil samples had a high polyunsaturated fatty acid (PUFA) content (50–54%) and low saturated fatty acids (19–21%). This is important from a nutritional point of view, since it has been reported that PUFA has an influence on cellular signaling, membrane structure, gene expression, prostaglandin biosynthesis, nervous, endocrine, and immune system mediations [11]. The results obtained agree with previously published results [12]. Whether the seed were obtained using dry farming or irrigated farming did not influence the fatty acid composition in CN-seed and Budakalaszi varieties (Table 1). Native varieties (Babak Castele and Kholreslo) had lower and higher saturated and monounsaturated fatty acids content, respectively, but no differences in polyunsaturated fatty acid content were observed among varieties (Table 1). It should be mentioned that fatty acid composition of milk thistle seeds oil is similar to sunflower oil [13].

Phytosterols

Phytosterols (plant sterols) are minor components of vegetable oils and form a major proportion of the unsaponifiables [7, 14]. Phytosterols are important from a nutritional point of view because they contribute to the lowering of serum cholesterol levels in humans [14] and to the oxidative and thermal stability and shelf-life of vegetable oils [15]. Six sterols in 4-desmethylsterols could be determined in oil samples: cholesterol, campesterol, stigmasterol,

Table 1 Fatty acids composition (g/100 g) in oil extracted from milk thistle seeds from different varieties grown in Iran

Fatty acids	Babak Castle	Khoreslo	Budakalaszi[a]	Budakalaszi[b]	CN-seed[a]	CN-seed[b]
C16:0	8.4[cA]	8.0[A]	7.3A[B]	7.9[A]	7.7[A]	8.0[A]
C18:0	4.6[C]	5.6[B]	6.0[A]	6.2[A]	6.1[A]	6.8[A]
C18:1	27.7[A]	28.9[A]	22.8[B]	23.7[B]	23.1[B]	23.8[B]
C18:2	51.7[A]	49.7[A]	52.9[A]	53.6[A]	53.5[A]	51.7[A]
C18:3	0.2[A]	0.2[A]	0.3[A]	0.3[A]	0.3[A]	0.3[A]
C20:0	2.9[B]	3.3[B]	3.9[AB]	3.7[AB]	4.0[A]	4.3[A]
C20:1	0.9[A]	0.8[A]	0.9[A]	0.8[A]	0.9[A]	0.8[A]
C22:0	2.3[B]	2.3[B]	2.8[A]	2.6[AB]	2.9[A]	2.9[A]
C24:0	0.7[A]	0.6[AB]	0.7[A]	0.6[AB]	0.8[A]	0.8[A]
SAT	18.9[B]	19.8[AB]	22.8[A]	21.0[A]	20.7[A]	21.5[A]
MUFA	28.6[A]	29.7[A]	24.6[B]	24.5[B]	23.7[B]	24.0[B]
PUFA	51.9[A]	49.9[A]	52.0[A]	53.9[A]	53.2[A]	53.8[A]

SAT total saturated FA, *MUFA* total monounsaturated FA, *PUFA* total polyunsaturated FA

[a] Irrigated farming

[b] Dry farming

[c] Mean of three determinations and CV is less than 2%

[A–C] Fatty acid level within each row with different letters differ significantly ($P < 0.05$)

clerosterol, sitosterol, Δ^7-sterol (Fig. 1, Table 2). However, there were a few unknown peaks which need further study for identification (Fig. 1). Sitosterol was the predominant (33–37%) followed by Δ^7-sterol (19–22%). The results obtained concur with previously reported data [16]. Oil extracted from seeds obtained by dry farming had a higher content of total sterols (Table 2). Oil extracted from seeds obtained from CN-seeds generally had the highest content of sterols among all oil samples (Table 2).

Methylsterols are generally present at a low level in vegetable oils [7, 9]. Therefore it is necessary to separate and enrich them by TLC or SPE before analysis by GC or GC–MS [9]. In this study, methylsterols were separated and enriched by TLC and analyzed by GC. In the 4-monomethylsterol class, citrostadienol was predominant followed by cycloeucalenol and obtusifoliol (Table 3). In 4,4′-dimethylsterols, 24-methylenecycloartanol was predominant followed by cycloartenol and β-amyrin (Table 3). Results show that in milk thistle seed oil, the 4-desmthylsterol class had a higher content (1,800–2,200 μg/g) compared with 4-dimethylsterols (50–85 μg/g) and 4-monomethylsterols content (26–35 μg/g) (Tables 2, 3). In vegetable oils, methylsterols composition differ more than 4-desmethylsterols, therefore it can be used more effectively to detect vegetable oil adulteration [7, 9]. It should be mentioned that there were a few unidentified compounds in the methylsterols extract of the analyzed oil samples (results are not shown). Identification of the unidentified compounds was beyond the scope of this study. However, future work may determine the unidentified compounds.

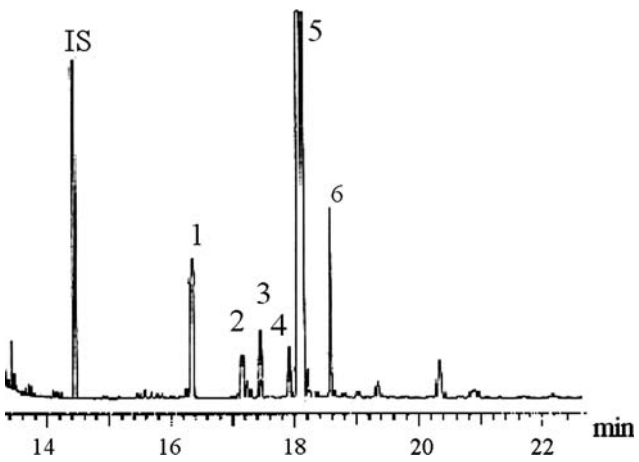

Fig. 1 Gas chromatogram of TMS ether derivatives of 4-desmethylsterols (predominant phytosterol class). Peak identification: *IS* 5α-cholestane (internal standard); *1* cholesterol; *2* campesterol; *3* stigmasterol; *4* clerosterol; *5* β-sitosterol; *6* Δ^7-sterol

Tocopherols

Tocopherols are one of the important compounds in vegetable oils. The main function of α-tocopherol is that of a radical-chain breaking antioxidant in vegetable oils and foods as well as in the body, e.g. membranes and lipoproteins [17]. Tocopherols may reduce the risk of cardiovascular diseases and of certain types of cancer because of its antioxidant properties and various functions at the molecular level [18, 19]. In this study, we determined tocopherol composition and content in all extracted oil

Table 2 4-Desmethylsterols composition and content (μg/g) in the oil extracted from milk thistle seeds from different varieties grown in Iran

Sterol	Babak Castle	Khoreslo	Budakalaszi[a]	Budakalaszi[b]	CN-seed[a]	CN-seed[b]
Cholesterol	191.0^{cD}	162.5^{E}	216.6^{C}	249.0^{B}	229.6^{BC}	314.7^{A}
Campesterol	84.4^{B}	77.7^{BC}	68.0^{C}	68.4^{C}	111.6^{A}	88.0^{B}
Stigmasterol	132.7^{A}	112.0^{B}	102.0^{C}	100.7^{C}	132.7^{A}	112.2^{B}
Cleroesterol	76.4^{B}	65.0^{C}	77.0^{B}	87.4^{A}	92.7^{A}	68.2^{C}
β-Sitosterol	689.4^{B}	644.9^{C}	619.3^{D}	617.9^{D}	777.1^{A}	728.5^{A}
Δ^7-Sterol	398.0^{B}	386.5^{CB}	386.6^{CB}	403.0^{B}	433.8^{A}	411.6^{B}
Unknown	432.1^{B}	382.9^{C}	324.0^{D}	359.3^{C}	339.1^{D}	664.7^{A}
Total	2,010.5^{C}	1,806.2^{C}	1,790.0^{C}	1,901.8^{D}	2,106.9^{B}	2,201.4^{A}

[a] Irrigated farming

[b] Dry farming

[c] Mean of three determinations and CV is less than 2%

[A–D] 4-Desmethylsterols level within each row with different letters differ significantly ($P < 0.05$)

Table 3 Methylsterol composition and content (μg/g) in oil extracted from milk thistle seeds from different varieties grown in Iran

Methylsterol	Babak Castle	Khoreslo	Budakalaszi[a]	Budakalaszi[b]	CN-seed[a]	CN-seed[b]
4-Monomethylsterols						
Obtusifoliol	5.6^{cB}	4.5^{C}	3.3^{D}	4.1^{C}	6.1^{A}	6.8^{A}
Cycloeucalenol	11.0^{C}	9.7^{D}	8.1^{C}	9.8^{D}	12.4^{B}	14.1^{A}
Citrostadienol	18.5^{A}	15.2^{B}	14.2^{C}	12.1^{D}	14.9^{B}	13.2^{DC}
4,4′-Dimethylsterols						
β-amyrin	3.0^{B}	2.5^{C}	2.4^{C}	2.0^{C}	3.6A^{B}	4.0^{A}
Cycloartenol	8.2^{C}	8.0^{C}	7.1^{C}	12.1^{B}	14.1^{A}	12.9^{B}
24-Methylenecycloartanol	50.6^{C}	45.1^{DC}	42.9^{D}	55.8^{BC}	60.1^{B}	68.1^{A}

[a] Irrigated farming

[b] Dry farming

[c] Mean of three determinations and CV is less than 2%

[A–D] Methylsterol levels within each row with different letters differ significantly ($P < 0.05$)

samples (Table 4). An HPLC chromatogram of tocopherols is given in Fig. 2.

Analyzed milk thistle seed oils had a higher amount of α-tocopherol compared with other type of tocopherols (Table 4). Oil extracted from the Khoreslo variety had the highest amount of total tocopherol as well as individual tocopherols among the oil samples analyzed (Table 4). α-Tocopherol had the highest amount among tocopherols and ranged from 187 to 465 μg/g in analyzed samples. The β-, γ-, and δ-tocopherols ranged from 10 to 51, 9–12 and 18 to 80 μg/g, respectively. The CN-seed varieties produced by irrigated farming generally had a lower amount of total

Table 4 Tocopherol content (μg/g) in the oil extracted from milk thistle seeds from different varieties grown in Iran

Tocopherol	Babak castle	Khoreslo	Budakalaszi[a]	Budakalaszi[b]	CN-seed[a]	CN-seed[b]
α-Tocopherol	378.6^{cB}	465.1^{A}	324.6^{C}	385.7^{B}	187.4^{E}	278.6^{D}
β-Tocopherol	20.9^{C}	50.9^{A}	25.4^{C}	35.4^{B}	10.1^{D}	12.6^{D}
γ-Tocopherol	18.3^{B}	35.7^{A}	12.3^{C}	30.5^{A}	8.5^{D}	10.9^{CD}
δ-Tocopherol	28.1^{D}	80.5^{A}	35.7^{C}	60.2^{B}	18.1^{F}	20.5^{E}
Total	445.9^{C}	632.2^{A}	398.0^{D}	511.8^{B}	224.1^{F}	322.6^{E}

[a] Irrigated farming

[b] Dry farming

[c] Mean of three determinations and CV is less than 2%

[A–F] Tocopherols level within each row with different letters differ significantly ($P < 0.05$)

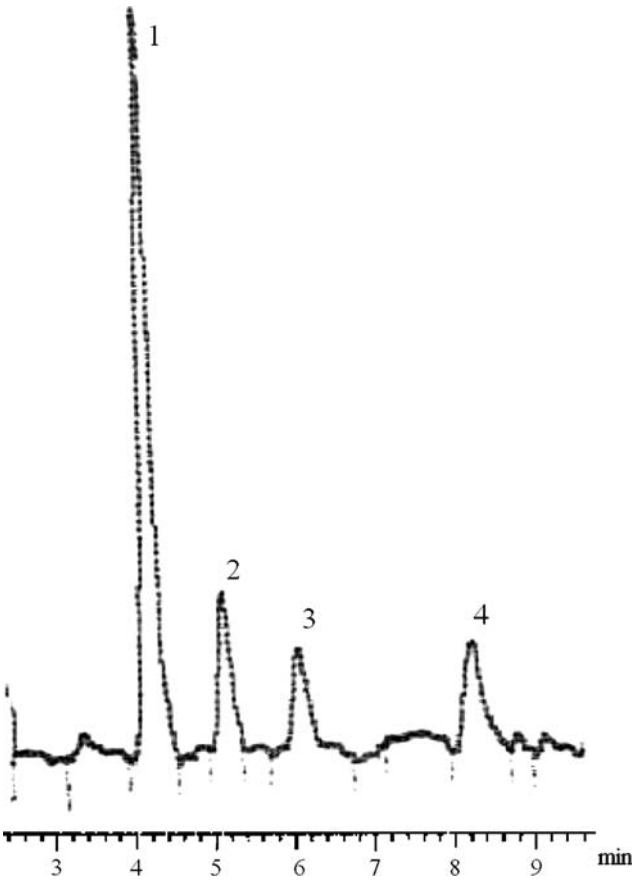

Fig. 2 High performance liquid chromatogram of tocopherols of milk thistle oil. *1* α-tocopherol; *2* β-tocopherol; *3* γ-tocopherol; *4* δ-tocopherol

and individual tocopherols compared with other varieties. In CN-seed and Budakalaszi varieties, oils from seeds obtained from dry farming had a higher amount of tocopherols compared with irrigated farming (Table 4) The results obtained for the amounts of α-, γ-, and δ-tocopherols agree with previously published results, which reported that milk thistle seeds oil has 156, 35, and 7 μg/g α-, γ-, and δ-tocopherols, respectively [12]. However, in this mentioned report, β-tocopherol was not reported**.** The amount of tocopherol in this vegetable oil is comparable with other vegetable oils such as sunflower oil [13].

Benefits to health have been claimed for milk thistle and an increasing amount of research is being undertaken on various possible medical uses. The oil is a by product in the extraction of compounds beneficial to health from the milk thistle seeds. These seeds have a relatively high oil content (26–31%) comparable with most oilseeds. In this study, oil extracted from milk thistle seeds showed that it is rich source of polyunsaturated fatty acids and α-tocopherol which makes it an nteresting candidate from a nutritional point of view. The extracted oil was similar to sunflower oil and might be used as a salad oil, as a cooking oil on its own or mixed with other vegetable oils, especially mixed with saturated oils to improve their nutritional value. This is the first detailed report on the phytosterol composition of milk thistle seed oil which can be used to differentiate this oil from other vegetable oils. It should be also mentioned that it may need further study to analyze the other minor compounds possibly present such as phenolic compounds, and also to monitor its stability during food preparation as well as during storage**.**

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