

# Comparison of the Efficiency of Different Extraction Methods on Antioxidants of Maltese Orange Peel

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## Abstract

The effect of conventional solvent extraction, CSE, ultrasound assisted extraction, UAE, microwave assisted extraction, MAE and supercritical CO<sub>2</sub> extraction, SC-CO<sub>2</sub> on the total phenols content, total flavonoids, individual flavonoids and antioxidant activity of orange peel were compared. Neohesperidin (from 0.624 ± 0.013 for SC-CO<sub>2</sub> extraction to 1.045 ± 0.001 g/100 g orange peel powder for MAE) and hesperidin (from 0.407 ± 0.008 for SC-CO<sub>2</sub> extraction to 0.836 ± 0.029 g/100 g orange peel powder for UAE) are the major flavonoids (80% of total flavonoids by MAE and 87% by CSE) of orange peel whatever the used extraction method. The method giving the highest total phenol and flavonoid contents is microwave assisted extraction (2.363 ± 0.014 g GAE/100g orange peel powder), followed by ultrasound assisted extraction, conventional solvent extraction, and supercritical CO<sub>2</sub> extraction. However, antioxidant activity (DPPH method) cannot be correlated to TPC, TFC or individual flavonoids. Orange peel extracted by CSE (ethanol (80%), m/v: 5 g:50 ml, 30 min, 35°C, mechanical stirring at darkness, 3 successive extractions) presents the higher radical scavenging capacity compared to the other extracts obtained by MAE (Ethanol (80%), m/v: 5 g: 50 ml, 10s, 35°C, 170 W, 3 successive extractions) and UAE (ethanol (80%), m/v: 5g:50 ml, 30 min, 35°C, magnetic stirring at darkness, 3 successive extractions, 125W), SC-CO<sub>2</sub> extraction (ethanol (80%), m/v:5 g:50 ml, 30 min, 35°C, 22 MPa, 3 successive extractions). Besides, no additivity on the antioxidant activity is found with the DPPH method.

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## Introduction

Citrus is the most important fruit crop in the world with a production estimated at 89 million tons in 2014<sup>[1]</sup>. Approximately 26% of Citrus fruits are industrially processed into juice. The amount of industrial Citrus waste is estimated at more than 15×10<sup>6</sup> tons<sup>[2]</sup> and it consists essentially in seeds, peels and pulp residue<sup>[3]</sup>. Numerous potential of Citrus peel valorisation are recently reported in literature. Citrus peel can be used in functional foods and even as a dietary supplement for human or animal feed<sup>[4]</sup>. Citrus peel is also used in biochemical engineering such as the production of biodegradable plastics by the copolymerization of limonene extracted from Citrus peel and carbon dioxide<sup>[5]</sup>, but also to produce bio fuels (ethanol) and biogas<sup>[6]</sup>. Pectin can be extracted from Citrus peel to use it in food industries through its thickening, texturizing, a gelling agent (making jams, jellies, fruit preparations, frozen creams and emulsified products<sup>[7]</sup>, but also in pharmaceutical industry as an ingredient for preparation of anti-diarrheal and detoxifying drugs<sup>[8,9]</sup>. Extraction of essential oils remains, actually, the main industrial valorisation of Citrus peel<sup>[10,11]</sup>. Moreover, citrus peel represents a rich source of natural phenolic compounds unique to Citrus. Total phenol contents of the peel varied from 0.67 to 7.30 g/100g dry weight<sup>[12]</sup>. Phenolic compounds of citrus peel exhibit anti-carcinogenic, anti-inflammatory, antioxidant and

anti-atherogenic properties<sup>[13]</sup> due to the presence of phenolic acids and flavonoids<sup>[3]</sup>. Antioxidants capacity of phenols could have promoting application in chemical engineering such as corrosion inhibitor in shipbuilding materials, in crude oil refining industry, in the acid pickling, industrial cleaning, descaling acid, and also in the petrochemical processes<sup>[14]</sup>. However, its extraction constitutes a difficult step because of their sensitivity to extraction conditions such a temperature, light or food matrix, which could lead to their degradation and alteration of their antioxidant activities<sup>[15]</sup>.

There are several methods of extraction of phenolic compounds in citrus peel as conventional solvent extraction<sup>[16-18]</sup>. These methods can cause degradation of phenolic compounds due to the high temperature and the extraction time. Some other methods were used to increase the efficiency of the extraction such as microwave assisted extraction, ultrasound assisted extraction, high pressure extraction and supercritical fluid extraction or subcritical water<sup>[19,20]</sup>. Some authors suggest a sequential use of two processes such as instant controlled pressure drop technology and ultrasound-assisted extraction (DIC-UAE) or combined approaches like enzyme assisted extraction in order to intensify the extraction operation and to enhance the extraction kinetic and yield<sup>[12]</sup>. Boukroufa et al.<sup>[21]</sup> combine ultrasound and microwave techniques to extract phenolics from orange peel waste, using only recycled water as solvent. This concept allowed also the recuperation of essential oils and pectin. Some comparisons of the efficiency of different extraction methods were carried out in the literature but they are incomplete because only two or three methods were compared in the same work<sup>[22-24]</sup> and of extraction and preservation of phenolics activities should help to choose the appropriate extraction method.

So, the purpose of this paper is to compare the performances of Conventional Solvent Extraction (CSE), Ultrasound Assisted Extraction (UAE), Microwave Assisted Extraction (MAE), High Pressure Extraction (HPE), and Supercritical CO<sub>2</sub> extraction (SC-CO<sub>2</sub>) on the selectivity, the total phenol content, the total and individual flavonoids and the antioxidant activity of Maltese orange peel. To reach this goal, High Performance Liquid Chromatography (HPLC), Mass Spectrometry (MS), spectrophotometer and DDPH methods were used to evaluate the criteria of the performance of these extraction methods.

## Material and Methods

### Plant Material and Sample Preparation

About 20 kg of fresh oranges (*Citrus sinensis*) of the Maltese cultivar were collected in March 2012 from Manzel Bouzefla (Nabeul, Tunisia) in their commercial maturity. All fruits were of eating quality and without blemishes or damage. On arrival at the laboratory, the orange fruits were immediately washed using tap water and peeled. The remaining orange peel accounts for approximately 40% of the total fruit.

The peels were stored at -80°C before any further treatments. They were then dehydrated by using a freeze dryer (CHRIST Alpha 1-2 LD, France) for 72h (at -50°C and 0.001 mbar) and then finely ground using a coffee grinder (Moulinex®, France) and sieving to achieve a standard size of particles of ~0.315 mm. The orange peel powder was placed in vacuum packaging bags and stored in a freezer maintained at -18°C before the experiments.

### Chemicals and Reagents

All chemicals used in the experiments carried out during this work are shown in Table 1. All chemicals were of analytical or HPLC grade purity

**Table 1.** Chemicals and reagents used in experiments

Product	Provider	Purity (%)
Phenolics standards: eriocitrin, narirutin, naringin, hesperidin, neohesperidin, didymin, sinensetin, tangeretin, nobiletin et 3',4', 5,5',6,7, hexamethoxyflavone	Extrasynthese®(Lyon, France)	≥ 95-99.0
Potassium persulfate	Fluka (Switzerland)	
Rutin	Sigma-Aldrich (Allemagne)	≥ 94,0
Sodium nitrite (NaNO <sub>2</sub> )		≥ 97
Aluminium chloride (AlCl <sub>3</sub> )		99,99
2,2-diphenyl-1-picrylhydrazyl (DPPH)		-
6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox)		≥ 97,0
Folin-Ciocalteu's phenol reagent		-
Sodium Carbonate (Na <sub>2</sub> CO <sub>3</sub> )		-
gallic acid monohydrate		≥ 98,0
Ascorbic acid		≥ 99,0
Acide chlorhydrique		-
Sodium Hydroxide (NaOH)		Carlo Erba-SDS, (France)
Methanol		-
Ethanol	VWR (Belgique)	≥ 95,0
Acetic acid		≥ 99,7

## Extraction Methods

For all extraction methods, the temperature was set at 35°C to prevent thermal degradation of antioxidant molecules. The parameters of extraction methods were summarized in Table 2.

**Table 2:** Extraction conditions of CSE, UAE, MAE and SC-CO<sub>2</sub> methods.

Extraction method	Conditions of extraction	Characteristics
CSE	Ethanol (80%), m/v: 5g: 50 ml, 30 min, 35°C, mechanical stirring at darkness, 3 successive extractions.	-
UAE	Ethanol (80%), m/v: 5g: 50 ml, 30 min, 35°C, magnetic stirring at darkness, 3 successive extractions, <b>125 W</b> .	Ultrasound sonicator (VibraCell 75115, Bioblock-Fisher, Illkirch, France)
MAE	Ethanol (80%), m/v: 5g: 50 ml, <b>10 s</b> , 35°C, <b>170 W</b> , 3 successive extractions.	Microwave oven (WAVEDOM LG, France)
SC-CO <sub>2</sub>	Ethanol (80%), m/v: 5g:50 ml, 30 min, 35°C, <b>22 MPa</b> , 3 successive extractions.	Pilot scale extractor (ENSIC, LRGP, Nancy, France)

The crude extract provided by each technique was cooled at room temperature, centrifuged at 8000 g for 10 min and the supernatant was filtered through a Millipore paper (0.22 µm). The residue was further extracted two times with 50 ml of the same solvent under the same extraction conditions. A combination of the three extracts was collected and stored at 4°C.

## Analytical Methods

### Determination of Total Phenols and Flavonoids Contents (TFC<sub>sp</sub>, TPC<sub>FC</sub>)

Total phenols content was determined by Folin-Ciocalteu method, according to the method described by Singleton et al.<sup>[25]</sup>. The samples were added to Folin-Ciocalteu reagent and Na<sub>2</sub>CO<sub>3</sub> solution and placed in a water bath at 40°C for 30 min. Spectrophotometric analysis (spectrophotometer Genesys 10 uv screening, Thermo Electron Corporation, France) was carried out at 765 nm. Total phenol content determined by Folin-Ciocalteu method was designed as TPC<sub>FC</sub> and was expressed as g of gallic acid equivalent (GAE) per 100 g orange peel powder. Total flavonoids content was determined by spectrophotometric method, according to the modified procedure of Zhishen et al.<sup>[26]</sup>. 0.5 ml of aqueous extract was placed in a 5 ml volumetric flask, and then 2.5 ml of distilled water were added, followed by 0.15 ml of 5% NaNO<sub>2</sub>.

After 5 min, 0.15 ml of 10% AlCl<sub>3</sub> were added. 5 min later, 1 ml of 1M NaOH were added and the volume made up with distilled water. The solution was mixed and absorbance was measured at 510 nm using a spectrophotometer (Genesys 10uv screening, Thermo Electron Corporation, France). Total flavonoids content measured by spectrophotometric method was designed as TFC<sub>sp</sub> and was expressed as rutin equivalent per 100 g orange peel powder.

### Determination of Vitamin C Content

Vitamin C content was titrated by a modified method described by Tabart et al.<sup>[27]</sup> using dichlorophenolindophenol 2,6 (DCPIP). 2 ml of orange peel extract was added to 23 ml of metaphosphoric acid solution (HPO<sub>3</sub>) 5%. 5 ml was taken to which was added 5 ml of a solution of trichloroacetic acid in 20% HPO<sub>3</sub>. The solution obtained is then filtered and 2 ml of the filtrate was mixed with 5 ml of a buffer solution at pH 7.1 and 1 ml of 2,6 dichloro phenol indophenol (DCPIP). The absorbance was measured at 530 nm. A standard range was performed using ascorbic acid at concentrations of 0, 5, 10, 15, 20 mg/l (R<sub>2</sub> = 0.9995). The vitamin C content is expressed in g per 100 g of orange peel powder.

### Determination of antioxidant activity

The determination of the antioxidant activity was realized on the five extracts obtained by the different extraction methods and the results were expressed as micromoles of Trolox equivalent for one micromole of phenolic compounds<sup>[28]</sup>. The free radical scavenging activities of orange peel extracts were determined by DPPH radical cation decolorization assay, following the method of Burda & Oleszerk<sup>[29]</sup>. A 46.7 mg/l of 1.1-diphenyl-2-picrylhydrazyl (DPPH) was prepared by dilution of 11.7 mg of DPPH with 250 ml of methanol incubated in dark. 80 µl of sample extract was added to 220 µl of DPPH solution. The absorbance reading was taken at 25°C, exactly 1 min after initial mixing (A<sub>0</sub>) and again at 30 min (A<sub>t</sub>). The control solution was prepared by adding 80 µl of methanol to the DPPH solution and methanol was used as blank. The inhibition percentage of absorbance at 515 nm, using a spectro fluorometer (SAFAS flx Xenius, Monaco) was calculated between OD<sub>0</sub> and OD<sub>t</sub>, according to the following equation 1. Appropriate solvent blanks were run in each assay.

$$(OD_0 - OD_t)$$

**Equation 1:** Percentage of inhibition = ----- x 100

$$OD_0$$

with OD<sub>0</sub> as initial optical density and OD<sub>t</sub> as final optical density.

Then, Trolox equivalent antioxidant capacity (TEAC) was determined according equation 2.

**Equation 2:** TEAC = ---  

$$\frac{a_s}{a_T}$$

$a_s$ : line slope for sample of percentage of inhibition (%) plotted vs. concentration ( $\mu\text{M}$ ).  $a_r$ : line slope for Trolox reference (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) of percentage of inhibition (%) plotted vs. concentration ( $\mu\text{M}$ ).

### Additivity of antioxidant capacity

To check the additivity of the antioxidant activity, a mixture containing the ten flavonoids identified in orange peel was prepared. The values obtained were compared to that predicted from the values of each compound corrected by their molar fraction (equation 3).

$$\text{Equation 3: } TEAC_{predict} = \sum_{i=1}^{10} P_i M_i \times TEAC_i$$

$M_i$ : molar fraction of each phenolic compound  $i$

$TEAC_i$ : Antioxidant activity of each phenolic compound  $i$

### Analysis of flavonoids by HPLC

Identification of phenolic compounds in orange peel was carried out in two steps: identification by mass spectrometry and confirmation by HPLC analysis with the injection of standards.

### Identification of phenolic compounds using HPLC-MS

Qualitative analysis of orange peel phenolic compounds was performed using a HPLC-MS system (Thermo Fisher Scientific, San Jose, CA, USA) equipped with an LTQ XL ion trap as mass analyzer (Linear Trap Quadrupole). Chromatographic separation was performed on a C18 Alltima reverse phase column ( $150 \times 2.1$  mm,  $5 \mu\text{m}$  porosity – Grace/Alltech, Darmstadt, Germany) equipped with a C18 Alltima pre-column ( $7.5 \times 2.1$  mm,  $5 \mu\text{m}$  porosity- Grace/Alltech) at  $25^\circ\text{C}$  and mobile phases consisted of water modified with formic acid (0.1%) for A, and methanol modified with formic acid (0.1%) for B. Phenolics were eluted using a linear gradient from 10% to 100% of B in 78 min at a flow rate of  $0.2 \text{ mL min}^{-1}$ . Photodiode array (PDA) and mass spectrometry (MS) detections were performed during the time of the run. The mass spectrometry operating parameters were set as follows: electro spray positive ionization mode ( $\text{ESI}^+$ ) was used; spray voltage was set at 5 kV; source gases were set (in arbitrary units  $\text{min}^{-1}$ ) for sheath gas, auxiliary gas and sweep gas at 40, 10 and 10, respectively; capillary temperature was set at  $300^\circ\text{C}$ ; capillary voltage was set at 48 V; tube lens, split lens and front lens voltages were set at 138 V, -38 V and -4.25 V, respectively. The ion optic parameters were optimized by automatic tuning using a standard solution of rutin ( $M = 610 \text{ g.mol}^{-1}$ ) at  $0.1 \text{ g.L}^{-1}$  infused in the mobile phase (A/B: 50/50) at a flow rate of  $5 \mu\text{L.min}^{-1}$ . Full scan MS spectra were acquired from 100 to 2000  $m/z$ .

### Analysis of flavonoids by HPLC

The quantitative analysis was performed by using an HPLC analytical system (Elite LaChrom, VWR-Hitachi, France) consisting of a Spectra System P4000 pump, a Spectra System UV 6000LP diode array detector, a Spectra System SCM 1000 degasser and a Spectra System AS3000 auto-sampler. Controlled by software (THERMO CHROMQUEST). After filtration on Millipore paper ( $0.22 \mu\text{m}$ ),  $20 \mu\text{l}$  of ethanolic extract was injected on reverse-phase C18 column ( $150 \times 4.6$  mm,  $5 \mu\text{m}$  particle size, Apollo, Grace, Belgium). The mobile phase consisted of solvent A, water-acetic acid (2%) and solvent B, methanol-acetic acid (2%). A gradient program was carried out as follows: 5 min, 10% B ; 78 min, 100% B ; 88 min, 100% B ; 90 min, 10% B ; 100 min, 10% B.

The flow rate was  $1 \text{ ml/min}$ , and the temperature of the column oven was  $40^\circ\text{C}$ . The UV spectra were recorded at 280 nm for the quantitative determination of flavanones and at 340 nm for flavones. The limits of detection of the HPLC used for the flavonoid analysis are 9000 u.a. for the detection at 290 nm and 50 u.a. for the detection at 340 nm. The limits of quantification were calculated according to each standard curve.

The standards eriocitrin, naringin, narirutin, neohesperidin, didymin, sinensetin, tangeretin, nobiletin and 3', 4', 5, 5', 6, 7-hexamethoxyflavone were prepared at a stock concentration of  $250 \text{ mg/L}$ . For hesperidin, the concentration was  $20 \text{ mg/L}$  because of its low solubility. Calibration standard samples were prepared by appropriate dilutions with a mixture of ethanol/DMSO from the stock solutions and filtered on Millipore paper ( $0.22 \mu\text{m}$ ) before use. Calibration curves obtained showed determination coefficients superior to 0.98. Total flavonoids content determined by calculation of the sum of individual flavonoids measured by HPLC was designed as  $\text{TFC}_{\text{HPLC}}$  and was expressed as  $\text{g}/100\text{g}$  of orange peel powder.

### Statistical analysis

All experiments were repeated 3 times; average and standard deviations were calculated. Statistical analysis was carried out using the software package IBM. SPSS 20.0 and the comparison of averages of each treatment were based on the analysis of variance (ANOVA) at significance level 5%. Values followed by the same letter are not statistically significant according to Duncan's multiple range test at significance level  $p < 0.05$ .

Principal component analysis (PCA) was performed on the correlation matrix of the measured parameters:  $\text{TF}_{\text{HPLC}}$ , sum of glycosylated flavanones, sum GF (neohesperidin, hesperidin, narirutin, naringin, didymin, eriocitrin), sum of polymethoxylated flavones, sum MF (sinensetin, tangeretin, nobiletin, hexamethoxyflavone) and TEAC. A measure of association between each measurement and the obtained principal components was provided.



## Results and Discussion

### Effect of extraction methods on total phenols and flavonoids contents

As reported in Figure 1, the  $TPC_{FC}$  obtained by CSE was  $1,968 \pm 0,003$  g GAE/100 g of citrus peel powder. This value is higher than that reported by Kammoun et al.<sup>[30]</sup> for the same cultivar analyzed at its commercial ripening stage ( $1.130 \pm 0.040$  g). This difference is due to different used extraction conditions. In fact, Kammoun et al.<sup>[30]</sup> have applied a single extraction with filtration of the extract, evaporation of solvent and lyophilization of the residue. Whereas, in this study three successive extractions were applied and were followed by filtration of the extract without evaporation or lyophilization of the residue. The same solvent and plant material/solvent ratio were used in both studies.

If total phenol content was compared to that of other Citrus cultivars, significant variability could be noticed. In fact, the  $TPC_{FC}$  of Maltese orange peel remains lower than that obtained by Ghanem et al.<sup>[31]</sup> for fresh thompson peel ( $1.899 \pm 0.012$  g caffeic acid/100 g dry matter), Chen et al.<sup>[32]</sup> ( $3.945 \pm 0.100$  g GAE/100g DW for *Citrus Sinensis Osbeck* peel and Ghasemi et al.<sup>[13]</sup> ( $16.03$  g GAE/100g of citrus peel powder for *Citrus Sinensis* Washington Navel variety).

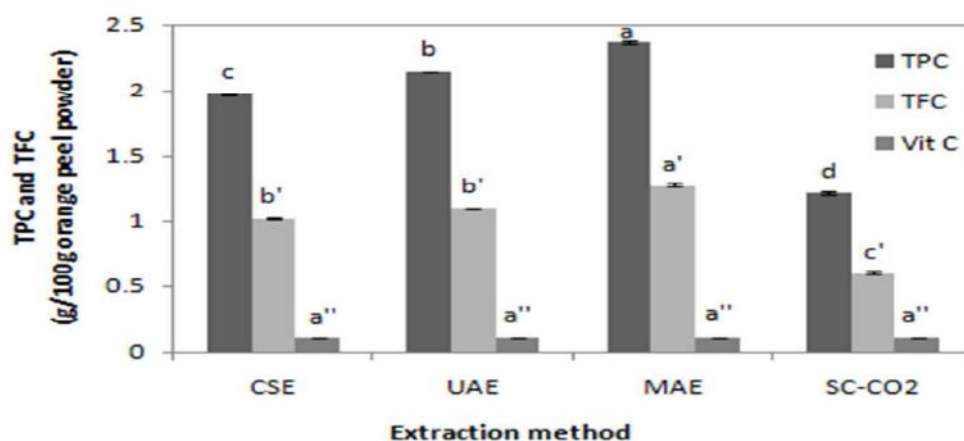
These differences can be attributed to many factors such as citrus cultivar and its stage of ripening, pedoclimatic factors (soil type, sun exposure, and rainfall), agronomic factors (biological culture, fruit yield per tree, and type of irrigation) and extraction methods used for phenolic analysis. The flavonoids content represents almost 50% of total phenolic of Maltese orange peel. This result is in accordance with results reported by Wang et al.<sup>[33]</sup>. Other studies mentioned that the total flavonoids content can varies in a wide range: from 1.4%<sup>[34]</sup> to 80%<sup>[35]</sup>. These variations can be explained by the interference of other compounds (sugars, organic acids like vitamin C) on the Folin-Ciocalteu analysis<sup>[36]</sup>.

Figure 1 showed also that  $TPC_{FC}$  ( $2.363 \pm 0.014$  g GAE/100 g) and  $TFC_{SP}$  ( $1.265 \pm 0.023$  g rutin/100 g) provided by the MAE method were higher than those obtained by UAE followed by CSE, and SC-CO<sub>2</sub> extraction method. Conventional solvent extraction gives low yields in comparison with UAE, and MAE. In fact, this method is accelerated by using ultrasound and microwave energy. The intensification of extraction efficacy using ultrasound has been attributed to the propagation of ultrasound pressure waves through the solvent and resulting cavitation phenomena<sup>[37]</sup>. A cavitation bubble can be generated close to the plant material surface, then during a compression cycle, this bubble collapses and a micro jet directed toward the plant matrix is created. The high pressure and temperature involved in this process will destroy the cell walls of the plant matrix and its content can be released into the medium. This phenomenon seems responsible for cell wall destruction and further release of the cellular content into the surrounding media<sup>[37,38]</sup>.

However, microwave irradiation accelerates the rupture of cells by causing a sudden temperature rise and internal pressure increase in the plant or fruit cell walls<sup>[39]</sup>. During microwave processing, heating causes the disruption of weak hydrogen bonds caused by the dipole rotation of the molecules. A considerable amount of pressure builds up inside the biomaterial which modifies the physical properties of the biological tissues.

This modification improve the porosity of the biological matrix, allowing better penetration of extracting solvent through the matrix, and facilitating the collection of the phenolic compounds<sup>[40]</sup>. Besides, the increase of  $TPC_{FC}$  in extract obtained by MAE can be explained by the breakdown of bigger phenolic compounds into smaller ones with their intact properties of the original molecules and which can react with Folin-Ciocalteu assay<sup>[41]</sup>. In our study, Figure 1 show that vitamin C contents measured in the different extracts remains constant whatever the used extraction method.

Figure 1 indicates also that supercritical CO<sub>2</sub> extraction method gives the lowest  $TPC_{FC}$  and  $TFC_{SP}$  ( $1.204 \pm 0.019$  g GAE/100 g,  $0.589 \pm 0.036$  g rutin/100 g respectively) compared to others methods. This result can be explained by the fact that orange peel is richer in polar flavonoids (flavanones) than non-polar ones (polymethoxylated flavones), while supercritical CO<sub>2</sub> extraction is more adapted to non-polar compounds<sup>[42,43]</sup>. Toledo-Guillen et al.<sup>[44]</sup> reported that CSE is more efficient than SC-CO<sub>2</sub> for the extraction of glycosylated flavanones. This result is attributed to the high molecular weight and polarity of flavonoids.



**Figure 1:**  $TPC_{FC}$  and  $TFC_{SP}$  of Maltese orange peel obtained by CSE (conventional solvent extraction), UAE (ultrasound assisted extraction), MAE (microwave assisted extraction), and SC-CO<sub>2</sub> (supercritical CO<sub>2</sub> extraction) methods.

Results are present as means  $\pm$  S.D. for triplicate analysis. Values with the same letter are not significantly different at  $p < 0.05$ .

According to these results, the classification of the various extraction methods taking phenolic and flavonoid contents as criteria of method efficiency is: the most efficient is microwaving assisted extraction followed by ultrasound assisted extraction then conventional solvent extraction and the least efficient is supercritical CO<sub>2</sub> extraction.

These results are in accordance with those found by others authors. Dahmoune et al.<sup>[45]</sup> compared three methods of extraction of lemon peel phenolic compounds: CSE, UAE and MAE. The authors reported that ultrasound cause disruption of plant cells by cavitation. The particles of the powder of lemon peel are resistant to ultrasound energy<sup>[46]</sup>. The rise of pressure in the cellular pores causes a faster break compared with the control. However, MAE causes more intense tissue degradation under the action of microwaves. Indeed, MAE dehydrated cellulose and reduces its mechanical strength, which allows an easy penetration of the solvent into the cellular channels<sup>[40]</sup>. Heating by microwave causes cellular damage and a weakened microstructure that helps to quickly release the solute in the solvent.

### Identification and quantification of orange peel flavonoids in different extracts

**Identification:** Ten phenolic compounds were identified in Maltease orange peel extracts. Results obtained by HPLC-DAD-MS were presented in Table 3.

**Table 3:** Rt, pseudomolecular ions, adduct ions with Na<sup>+</sup>, and UV<sub>max</sub> of orange peel phenolic compounds identified by HPLC-DAD-MS. Conventional solvent extraction: m/v:5g:50ml, 30 min, 35°C, ethanol 80%, mechanical agitation at darkness and 3 extraction cycles.

Order of appearance	Rt (min)	[M+H] <sup>+</sup> (m/z)	[M+Na] <sup>+</sup> (m/z)	UV <sub>λmax</sub> (nm)	Identification
1	22.80	597	619	284, 327	Eriocitrin
2	31.85	581	603	284, 329	Narirutin
3	31.97	581	603	280, 328	Naringin
4	33.10	611	633	284, 328	Hesperidin
5	33.95	611	633	285,327	Neohesperidin
6	40.77	595	617	226, 284, 332	Didymin
7	51.58	373	395	240, 264, 328	Sinensetin
8	52.37	403	425	237, 268,320	3',4',5,5',6',7', Hexamethoxyflavone
9	55.29	402	425	249, 271, 334	Nobiletin
10	58.54	372	395	271, 324	Tangeretin

These results showed that flavanones (eriocitrin, narirutin, naringin, hesperidin, neohesperidin, didymin) and polymethoxylated flavones (sinensetin, 3',4',5,5',6',7',-Hexamethoxyflavone, tangeretin, nobiletin) are the main compounds in the ethanoic extract. Except for narirutin and eriocitrin, a similar composition was reported by Anagnostopoulou et al.<sup>[47]</sup> in Greek Navel sweet orange peel. However, this variety also contains pentamethoxyflavone. Whereas, Kanaze et al.<sup>[48]</sup> found in Navel orange peel five flavanones (hesperidin, neohesperidin, naringin, didymin), three glycosylated flavones (leuteolin- 7-O-rutinoside, chrysoeriol-7-O-rutinoside diosmin), polymethoxylated flavones (sinensetin, nobiletin, hexamethoxyflavone heptamethoxyflavone). Moreover, Toledo-Guillén et al.<sup>[44]</sup> identified in orange peel extracts glycosylated flavanones (hesperidin, narirutin) and the polymethoxyflavones (sinensetin, nobiletin, tetramethylscutellarein and tangeretin).

**Quantification:** HPLC analysis showed that whatever the method used for extraction, the orange peel contains the following individual flavonoids: hesperidin, neohesperidin, eriocitrin, narirutin, naringin, didymin, sinensetin, 3',4',5,5',6',7'-Hexamethoxyflavone, tangeretin and nobiletin. The percentages of these compounds reported to total flavonoids content, TFCHPLC (corresponding to the sum of individual flavonoids determined by HPLC) are summarized table 4.

**Table 4:** Contents of individual flavonoid compounds (as g/100g of orange peel powder) of Maltease orange peel extracted by CSE, UAE, MAE and SC-CO<sub>2</sub>. Results are presented as means ± S.D. for triplicate analysis. Values with the same letter are not significantly different at  $p < 0.05$ .

Compound	CSE	UAE	MAE	SC-CO <sub>2</sub>
Hesperidin	0.551 ± 0.001 <sup>c</sup>	0.836 ± 0.029 <sup>a</sup>	0.781 ± 0.074 <sup>b</sup>	0.407 ± 0.008 <sup>d</sup>
Neohesperidin	0.860 ± 0.003 <sup>c</sup>	0.986 ± 0.006 <sup>b</sup>	1.045 ± 0.001 <sup>a</sup>	0.624 ± 0.013 <sup>d</sup>
Eriocitrin	0.019 ± 0.001 <sup>a</sup>	0.019 ± 0.001 <sup>a</sup>	0.016 ± 0.000 <sup>a</sup>	0.007 ± 0.001 <sup>b</sup>
Narirutin	0.038 ± 0.001 <sup>a</sup>	0.017 ± 0.001 <sup>c</sup>	0.002 ± 0.001 <sup>c</sup>	0.008 ± 0.001 <sup>d</sup>
Naringin	0.042 ± 0.001 <sup>c</sup>	0.081 ± 0.009 <sup>b</sup>	0.218 ± 0.001 <sup>a</sup>	0.043 ± 0.005 <sup>c</sup>
Didymin	0.026 ± 0.001 <sup>c</sup>	0.041 ± 0.003 <sup>b</sup>	0.062 ± 0.001 <sup>a</sup>	0.018 ± 0.001 <sup>d</sup>
Sinensetin	0.020 ± 0.001 <sup>d</sup>	0.040 ± 0.002 <sup>ab</sup>	0.040 ± 0.001 <sup>b</sup>	0.045 ± 0.002 <sup>a</sup>
Hexamethoxyflavone	0.006 ± 0.001 <sup>c</sup>	0.010 ± 0.013 <sup>b</sup>	0.016 ± 0.002 <sup>a</sup>	0.010 ± 0.006 <sup>b</sup>
Tangeretin	0.005 ± 0.001 <sup>a</sup>	0.009 ± 0.003 <sup>a</sup>	0.011 ± 0.000 <sup>a</sup>	0.008 ± 0.001 <sup>a</sup>
Nobiletin	0.042 ± 0.002 <sup>c</sup>	0.074 ± 0.003 <sup>b</sup>	0.084 ± 0.001 <sup>a</sup>	0.068 ± 0.001 <sup>c</sup>
TFC <sub>HPLC</sub>	1.609 ± 0.013 <sup>b</sup>	2.113 ± 0.017 <sup>a</sup>	2.275 ± 0.082 <sup>a</sup>	1.238 ± 0.090 <sup>c</sup>

CSE: conventional solvent extraction, UAE: ultrasound assisted extraction, MAE: microwave assisted extraction, and SC-CO<sub>2</sub>: supercritical CO<sub>2</sub> extraction.

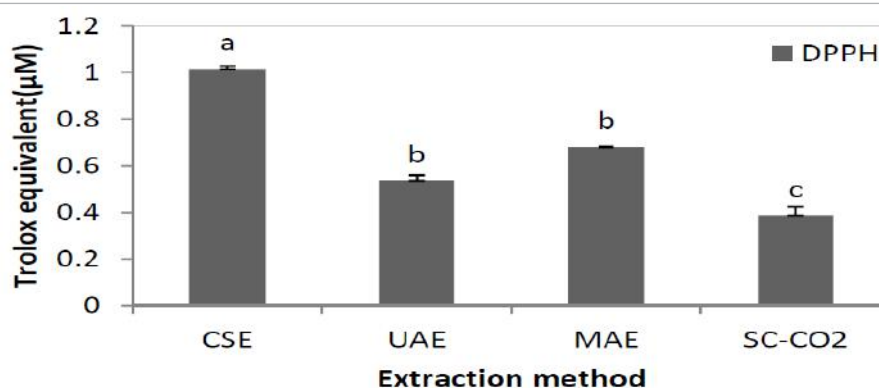
The total phenols content of Maltese orange peel extract (CSE) determined by the Folin test is equal to  $1.968 \pm 0.003$  g EAG/100 g of orange peel powder, whereas, the sum of individual flavonoids determined by HPLC and cited previously (Table 4) is equal to 1,609 g/100g orange peel powder. This difference (~18.2%) between spectrophotometric and chromatographic methods was acceptable and (18.2%) is can be explained by the fact that the Folin test overestimate the content of total phenols due to interference of the reagent with other reducing compounds which may exist in the extract<sup>[25]</sup> as reducing sugars (fructose, glucose ...) and organic acids (Vitamin C, citric acid malonic acid ...). Furthermore, the content of total flavonoids determined by HPLC (1.609 g/100 g of orange peel powder) is higher than the total flavonoids content determined by spectrometric method ( $1.012 \pm 0.003$  g rutin/100g of orange peel powder).

The spectrometric method of determination of total flavonoids underestimates the actual content of total flavonoids and this can be explained by the fact that some phenolic compounds cannot react with aluminum trichloride (AlCl<sub>3</sub>) as hesperidin which is present in large quantities in the Maltease orange peel (34.24%). The spectrometric method was the main common method used by many authors for total flavonoid estimation<sup>[13,35]</sup>. Chromatographic analysis is more appropriate and thus should be recommended for phenols determination in citrus.

Table 4 shows the individual flavonoids content of Citrus Maltese peel expressed at g/100g orange peel powder. The results obtained indicate that the efficiency of the extraction for a given method depends upon the structure of the flavonoid. The highest quantities of neohesperidin, hesperidin, didymin, naringin, nobiletin, tangeretin and hexamethoxyflavone were obtained respectively by using MAE. The eriocitrin content is achieved with a maximum content by UAE, SC-CO<sub>2</sub> (difference not significant) while it is CSE and SC-CO<sub>2</sub> for the highest levels of sinensetin and narirutin. This result coincides with those found in the literature. Indeed, Hayat et al.<sup>[22]</sup> compared CSE, MAE and UAE for the extraction of phenolic acids of mandarin peel. The MAE provides the highest level of ferulic acid (0.239 g/100 g dry matter) compared to UAE (0,235 g/100 g dry matter) and CSE (0.205 g/100 g dry matter). Khan et al.<sup>[23]</sup> reported that the contents of hesperidin and naringin of orange peel Valencia cultivar, obtained by UAE were significantly higher (0.250 and 0.070 g/100 g dry matter, respectively) than those obtained by CSE (0.145 and 0.051 g/100 g dry matter, respectively).

### Effect of extraction methods on radical scavenging activity

As has been previously reported, the amount of total and individual flavonoids of orange peel extracts depends on the used method. This variation should affect the antioxidant activity of the different extracts. To evaluate this effect, the antioxidant activity was measured by DPPH methods (Figure 2). Vitamin C contents were measured in the different extracts; it appears that this content remains constant (200 µM) whatever the extraction method used. So the variations observed cannot be attributed to this molecule.



**Figure 2:** Antioxidant activity ( $\mu\text{M}$  Trolox equivalent, DPPH test) measured for Maltese orange peel extracts obtained by CSE, UAE, MAE and SC-CO<sub>2</sub> methods. Results are presented as means  $\pm$  S.D. for triplicate analysis. Results are present as means  $\pm$  S.D. for different at  $p < 0.05$ .

These data show that orange peel extracted obtained by CSE presents higher radical scavenging capacity compared to extracts obtained by other extraction methods. Moreover, it can be noticed a significant decrease of the antioxidant activity measured by the DPPH method in the following order: CSE, SC-CO<sub>2</sub>, MAE and UAE. These results are not in accordance with those previously reported for TPC<sub>FC</sub> and TFC<sub>SP</sub> (Figure 1).

Indeed, a decrease of 15.44% was observed for antioxidant activity of MAE extract compared to CSE although that MAE extract contains the highest phenolics content (TPC<sub>FC</sub>, TFC<sub>sp</sub> and TFC<sub>HPLC</sub>). This result could be explained by (i) a slow reaction between citrus flavonoids with the stable DPPH and / or (ii) a different quantitative flavonoids composition of the extracts (Table 4), the appearance of new formed compounds during MAE and interactions different compounds thus resulting in positive or negative synergies of antioxidant activity<sup>[49]</sup>. According to literature, compounds newly formed during the Maillard and thermo-oxidation reactions could be explaining the increase of antioxidant activity of MAE extract. In our case, after the MAE, a change of extract colour from orange to brown and an odor of caramelization were noticed, but we can't identify new compounds in HPLC chromatogram. The products of the Maillard reaction (PRM) could be divided into different groups. During the first phase of the PRM training, small molecules such as glyoxyl, methyloxyl and others are trained dicarbonyls<sup>[50,51]</sup>. Since these compounds have a high oxidative potential and chemical activity, PRM trained at this stage tend to be pro-oxidant. The high chemical activity of these products between them then leads to higher molecular weight products and a brown colour through a series of condensation and polymerization reaction<sup>[52]</sup>. PRM complexes are at the later stage antioxidant and were named collectively melanoidins<sup>[53,54]</sup>. These compounds can interact during extraction to form other compounds that could present different structures and properties from the original<sup>[55]</sup>. Accordingly, it is necessary to identify, in further explorations, the structure of other antioxidants such as polymerization products and their individual contributions to the total antioxidant capacity of the extract.

Figure 2 showed also that the antioxidant activity of the extract obtained by UAE is lower than the CSE extract (29.27%). This result is similar to that reported by Dahmoune et al.<sup>[45]</sup>. The authors have shown that the antioxidant activity of the extract of lemon peel obtained by CSE is higher than that achieved by the UAE. This can be explained by the fact that ultrasound may induce the formation of free radicals in the liquid medium and improves the sonochemical reactions and polymerization/depolymerisation reactions, thus causing oxidation, degradation of bioactive compounds and appearance of off-flavours of the products<sup>[56-58]</sup>.

Table 5 shows the antioxidant activity of the ten individual flavonoids of orange peel extract and the vitamin C, measured by DPPH. Results are given in table 5.

**Table 5:** Antioxidant activity of individual flavonoids and vitamin C ( $\mu\text{M}$  Trolox equivalent, DPPH test) of Maltese orange peel extract.

	Compound	DPPH ( $\mu\text{M}$ Trolox)	Concentration in the extract ( $\mu\text{M}$ )
Glycosylated flavanones	Neohesperidin	0.095 $\pm$ 0.012	467
	Hesperidin	0.054 $\pm$ 0.002	300
	Vitamin C	1.224 $\pm$ 0.027	200
	Eriocitrin	1.009 $\pm$ 0.012	11
	Narirutin	ND at 344 $\mu\text{M}$	22
	Didymin	0.083 $\pm$ 0.019	15
	Naringin	0.056 $\pm$ 0.021	24
Polymethoxylated flavones	Hexamethoxyflavone	0.038 $\pm$ 0.009	5
	Tangeretin	0.115 $\pm$ 0.022	4
	Nobiletin	ND at 932 $\mu\text{M}$	35
	Sinensetin	ND at 1 $\mu\text{M}$	18
	TEAC predicted (Equation 3)	0.332 $\pm$ 0.001	-
	TEAC measured (Equation 2)	0.100 $\pm$ 0.013	-



These results indicate that the highest activities were observed for neohesperidin, eriocitrin and vitamin C respectively with DPPH method. However, nobiletin, sinensetin and narirutin do not present any activity. The results were confirmed by Khan et al.<sup>[23]</sup> who reported that the flavanones of orange peel react very slowly with the stable DPPH radical, making, therefore, a minor contribution<sup>[23]</sup>. To check the assumption of negative or positive synergic effects of flavonoids on the antioxidant activity, the antioxidant activity of a mixture prepared from the ten flavonoids was compared to that predicted from the values of each compound corrected by their molar fraction (Table 5). The results showed that we have a clear synergic effect. Thus, a value of  $0.100 \pm 0.013$  was obtained by DPPH against  $0.332 \pm 0.001$  for the predicted one. These results coincide with those found by Hidalgo et al.<sup>[49]</sup> which concluded that it is impossible to predict the antioxidant activity of a sample just by studying one type of flavonoid or other types of antioxidants in the extract such as vitamin C or E. In some cases, synergistic or antagonistic effects may occur resulting in the increase or decrease in the total antioxidant activity of the extract<sup>[59]</sup>.

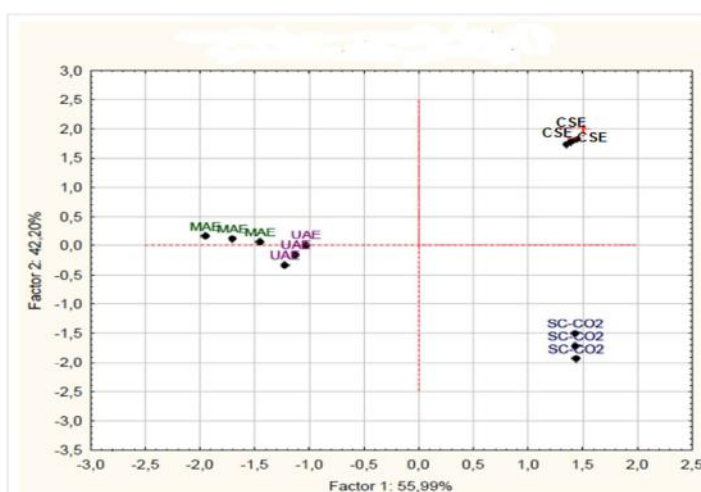
This study showed also that there isn't a correlation between radical scavenging activity (TEAC) and total flavonoids contents ( $TF_{HPLC}$ ) or glycosylated flavanones (sum GF) but significant negative correlation was observed for TEAC – and polymethoxylated flavones (Sum MF) (Table 6). This result is in agreement with those reported by Ghasemi et al.<sup>[13]</sup>. The authors explain the absence of correlation by the fact that flavonoids can act as proton donating and show radical scavenging activity, but, orange peel extract is a mix of compounds with distinct activities.

**Table 6:** Correlation matrix between variables

Variables	TFHPLC	Sum GF	Sum MF	TEAC
$TF_{HPLC}$	1.000	0.988	0.431	0.140
Sum GF	0.988	1.000	0.339	0.213
Sum MF	0.431	0.339	1.000	-0.768
TEAC	0.140	0.213	-0.768	1.000

### Comparison off the efficiency of the different extraction methods

Results of PCA (Figure 3) revealed that 98.19% of the variation among the measured parameters ( $TF_{HPLC}$ , Sum GF, Sum MF, TEAC) was attributed to the first two principal components (Table 7). The first principal component ( $TF_{HPLC}$ ) explained 55.99 % of the variance and the second component explain 42.20% of the variance.



**Figure 3:** Projection of the extraction methods on the factor plane

**Table 7:** Eigenvalues of correlation matrix and related statistics

Variables	Eigenvalue	% Total variance	Cumulative Eigenvalue	Cumulative %
$TF_{HPLC}$	2.239	55.991	2.239	55.991
Sum GF	1.688	42.203	3.927	98.194
Sum MF	0.067	1.685	3.995	99.880
TEAC	0.005	0.119	4.000	100.000

Based on the PCA analysis, three different extraction methods could be distinguished according to the first principal component ( $TF_{HPLC}$ ) and the second principal component (Sum GF): The first group was formed by CSE, the second group corresponds to SC-CO<sub>2</sub>. Whereas, the MAE and UAE could be gathered in a homogenous group. Our study showed that supercritical CO<sub>2</sub> extraction gives the lowest flavonoids content compared with the others methods of extraction. But, this technique of extraction allows the obtaining of extracts without remaining solvent traces and without having to use a cleaner. In addition, CO<sub>2</sub> is inexpensive and can easily be obtained at a high purity and is food-grade<sup>[60]</sup>. The use of supercritical fluid provides pure final extract devoid of unde-

sirable compounds such as organic pollutants, toxins and pesticides. The selectivity of supercritical fluid is higher than liquid solvent as its solvation power can be tuned either by changing temperature and/or pressure. Separation of solute from solvent in conventional extraction process can easily be passed by depressurization of supercritical fluid, which will save time<sup>[55,61]</sup>. It also allows avoiding thermal degradation and decomposition of thermolabile compounds, due to the operation at reduced temperature<sup>[42]</sup>. However, the non-polar characteristic of CO<sub>2</sub> has limited its application for the extraction of polar compounds, major phenolics of orange peel. Nevertheless, the polarity of CO<sub>2</sub> can be enhanced by adding modifiers such as ethanol which decrease extraction selectivity<sup>[62]</sup>. The cost of SC-CO<sub>2</sub> extraction process has restricted the applications to certain specialized fields such as decaffeinated coffee preparation industries<sup>[63]</sup>. The main drawbacks remain its high initial investment and difficulties to perform continuous extractions<sup>[64]</sup>.

However, ultrasonic and microwave extraction gave the higher phenolic content. Ultrasound assisted extraction is also an upcoming extraction technique which allows the extraction of thermolabile components since the operating temperature can remain low during the process, thus maintaining extract quality<sup>[65,66]</sup>. The main advantages of UAE are: improvement of mass transfer, breakdown of plant cells, improvement of solvent penetration and capillary effects of ultrasound<sup>[67]</sup>. However, it should be noted that since ultrasound generates heat, it is important to accurately control the extraction temperature. The sonication time should also be considered carefully as excess of sonication can damage the quality of extracts by the formation of free radicals<sup>[68]</sup>. Ultrasound as an extraction technique has the potential to be upscaled, at low cost and industrial potential may exist, because of possible lower operational costs<sup>[60]</sup>.

However, microwave assisted extraction showed obvious advantages in terms of high extraction efficiency and antioxidant activity of extract within the shortest extraction time. These results are in agreement with the findings of other researchers<sup>[39,69]</sup>. Several advantages of MAE have been described such as quicker heating for the extraction of bioactive substances from plant materials; reduced thermal gradients; reduced equipment size and increased extract yield. MAE can extract bioactive compounds more rapidly and a better recovery is possible than conventional extraction processes. It is a selective technique to extract organic and organometallic compounds that are more intact. MAE is also recognized as a green technology because it reduces the use of organic solvent<sup>[61]</sup>. Whereas, it is necessary to avoid high microwave power to prevent the degradation of phenolic compounds and the formation of new products that can be toxic. These results are consistent with the findings of other researchers<sup>[39,69]</sup>.

## Conclusion

The main flavonoids of Maltese citrus peel are glycosylated flavanones (neohesperidin, hesperidin, narirutin, naringin, didymin, eriocitrin) and polymethoxylated flavones (sinensetin, tangeretin, nobiletin, hexamethoxyflavone). Interestingly, neohesperidin and hesperidin were the main flavonoids constituent in the peel. This study is the first report comparing the efficiency of four extraction methods of orange peel phenolic compounds in terms of total and individual flavonoids and their antioxidant activities. MAE (80% ethanol, m/v: 5 g, 50 ml, 170 W for 10 s, and 3 successive extractions) was found to be a better approach than ultrasound assisted extraction, conventional solvent extraction, and supercritical CO<sub>2</sub> extraction in criteria of phenols, flavonoids contents and individual flavonoids but antioxidant activity is less than that of the CSE. MAE showed many advantages, such as shorter time, higher extraction rate, the saving of energy and better products with lower cost compared to supercritical CO<sub>2</sub> extraction, which need high investments. The results demonstrated that MAE can substitute the traditional CSE, which time-consuming techniques for efficient extraction of orange peel phenolic compounds. Despite a low TPC and TFC, CSE allows the obtaining of the extract with the highest antioxidant activity.

This study showed also that there is no addictivity on antioxidant activity. Consequently, the antioxidant activity of orange peel extract can be due to the synergic effect between flavonoids but also with others compounds of the extract such as vitamin C. Moreover, interactions between flavonoids or degradation products occur and can lead to positive or negative synergies on the antioxidant activity. Therefore, an identification of degradation products must be done to confirm our hypothesis and to check that these molecules are not toxic.

Although the MAE was considered the best method of extraction of phenolic compounds orange peel of the Maltese variety, more research is needed to better optimize the MAE. Indeed, in our study, a fixed temperature of 35°C was chosen to avoid degradation of phenolic compounds and to compare all extraction methods including those requiring a moderate temperature as UAE and SC-CO<sub>2</sub>.

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**Conflict of Interest:** No conflict of interest.

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