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Antioxidants of Maltease orange peel: comparative investigation of the efficiency of four extraction methods

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ABSTRACT

The effect of four extraction methods applied at 35°C (conventional solvent extraction, CSE, ultrasound assisted extraction, UAE, microwave assisted extraction, MAE and supercritical CO_2 extraction, SC-CO_2) on the total phenol contents, total flavonoid contents, individual flavonoids, vitamin C and antioxidant activity of orange peel was examined. Neohesperidin (from 0.624±0.013 for SC-CO_2 extraction to 1.045±0.001g/100g orange peel powder for MAE) and hesperidin (from 0.407±0.008 for SC-CO_2 extraction to 0.836±0.029g/100g 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_2 extraction. However, vitamin C content is not affected by the extraction method. Antioxidant activity (DPPH method) cannot be correlated to TPC, TFC or individual flavonoids. Orange peel extracted by CSE presents the highest radical scavenging capacity compared to the other extracts obtained by MAE, UAE and SC-CO_2 extraction. Besides, no additivity on the antioxidant activity is found with the DPPH method.

INTRODUCTION

Free radicals are atoms or group of atoms that have one or more unpaired electrons (Konan *et al.*, 2016). The excessive production of these molecules induces the oxidative stress which leads to the appearance of many diseases such as diabetes, cancer, hypertension, and neurodegenerative diseases (Bairy *et al.*, 2016). The human body produces natural antioxidants, like superoxide, catalase, peroxidase-glutathione system, which neutralize and inactive the free radicals, but, its remains not enough (Rao *et al.*, 2006). Consequently, it is necessary to supply human body with a low cost source of natural antioxidants. Citrus byproduct presents rich sources of antioxidants, especially, polyphenols. Total phenol contents of the citrus peel varied from 0.67 to 7.30 g/100g dry matter (DM) (M'hiri *et al.*, 2014). Citrus peel can be used in functional foods, pharmaceutical products formulations as an ingredient for preparation of anti-diarrheal and detoxifying drugs(Liu *et al.*, 2003; Piriyaprasarth *et al.*, 2011) and also as a dietary supplement for human or animal feed (Bampidis and Robinson, 2006). Phenolic compounds of citrus peel exert numerous pharmacologically effects ranging from antioxidant to antiproliferative (Bocco *et al.*, 1998; Ghasemi *et al.*, 2009; Berim and Gang, 2015).

In fact, Xu *et al.* (2014) showed that polymethoxylaed flavones (PMF) of *citrus reticulata* extract (tangeretin and nobiletin), are the active components against the respiratory syncytial virus. In addition, the PMF of citrus can be used as nutraceutical supplement or as a therapeutic agent in pre-diabetic and metabolic syndrome conditions (Berim and Gang, 2015). Hespridin and naringin, major phenolic compounds of citrus byproduct, exhibit antidiabetic effect by potentiating the antioxidant defense system and suppressing pro-inflammatory

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cytokine production (Mahmoud *et al.,* 2012 ; Park *et al.,* 2013; Costantin *et al.,* 2014; Vinayagam and Xu, 2015). Studies showed also that PMF of citrus byproduct inhibit the growth of cancer cell types and also responsible of for the anti-inflammatory effect (Saito *et al.*, 2015; Wang *et al.*, 2014; Berim and Gang, 2015).

Antioxidants extraction constitutes a difficult step because of their sensitivity to extraction conditions such as temperature, light or food matrix, which could lead to their degradation and alteration of their antioxidant activities (Ioannou *et al.,* 2012). There are several methods of extraction of phenolic compounds in citrus peel as conventional solvent extraction (Manthey and Grohmann, 1996, Li *et al.,* 2006). These methods can cause degradation of antioxidants 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 (Chemat *et al.,* 2009; Rawson *et al.,* 2011). 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 (M'hiri *et al.,* 2014). Boukroufa *et al.* (2015) 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 and this comparison only covers the extraction efficiency (Hayat *et al.,* 2009; Khan *et al.*, 2010). A study taking into account both the efficiency 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 performance of conventional solvent extraction (CSE), ultrasound assisted extraction (UAE), microwave assisted extraction (MAE), and supercritical CO_2 extraction (SC-CO_2) performed at soft condition (35°C) on the selectivity, the total phenol contents, the total and individual flavonoids and the radical scavenging capacity of Maltease orange peel.

MATERIAL AND METHODS

Plant material and sample preparation

About 20 kg of fresh oranges (*Citrus sinensis*) of the Maltease cultivar were collected in March 2012 from Manzel Bouzalfa (Nabeul, Tunisia) at 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), 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.

Extraction methods

Conventional solvent extraction (CSE), ultrasound assisted extraction (UAE), microwave assisted extraction (MAE), and supercritical CO_2 extraction (SC-CO_2) methods were used. For the four phenol extraction methods, the temperature was set at 35°C to prevent thermal degradation of antioxidant molecules and carried out at darkness. The parameters of extraction methods were summarized in **Table 2**. The crude extract provided by each technique was cooled at room temperature, centrifuged at 8000g 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.

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 (Germany)	≥ 94.0
Sodium nitrite ($NaNO_2$)		≥ 97
Aluminium chloride ($AlCl_3$)		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_2CO_3)		-
Gallic acid monohydrate		≥ 98.0
Ascorbic acid		≥ 99.0
Hydrochloric acid	Carlo Erba-SDS (France)	-
Sodium Hydroxide (NaOH)		-
Methanol		-
Ethanol	VWR (Belgium)	≥ 95.0
Acetic acid		≥ 99.7

Table 2: Extraction conditions of CSE, UAE, MAE and SC-CO_2 methods.

Extraction method	Conditions of extraction	Characteristics
CSE	Ethanol (80%), m/v: 5g: 50ml, 30 min, **35°C**, mechanical stirring at darkness, 3 successive extractions.	-
UAE	Ethanol (80%), m/v: 5g: 50ml, 30 min, **35°C**, magnetic stirring at darkness, 3 successive extractions, **125W**.	Ultrasound sonicator (VibraCell 75115, Bioblock-Fisher, Illkirch, France)
MAE	Ethanol (80%), m/v: 5g: 50ml, **10s**, **35°C**, **170W**, 3 successive extractions.	Microwave oven (WAVEDOM LG, France)
SC-CO_2	Ethanol (80%), m/v: 5g: 50ml, 30 min, **35°C**, **22 MPa**, 3 successive extractions.	Pilot scale extractor (ENSIC, LRGP, Nancy, France)

CSE: conventional solvent extraction, UAE: ultrasound assisted extraction, MAE: microwave assisted extraction, and SC-CO_2: supercritical CO_2 extraction.

Analytical methods

Determination of total phenol and flavonoid contents (TFC_{SP}, TPC_{FC})

Total phenol contents were determined by Folin-Ciocalteu method, according to the method described by Singleton *et al.*(1988).The samples were added to Folin-Ciocalteu reagent and Na_2CO_3 solution and placed in a water bath at 40°C for 30 min. Spectrophotometric analysis (spectrophotometer Genesys 10uv screening, Thermo Electron Corporation, France) was carried out at 765 nm. Total phenols content determined by Folin-Ciocalteu method was designed as TPC_{FC} and was expressed as g of gallic acid equivalent (GAE) per 100g orange peel powder.

Total flavonoid contents were determined by spectrophotometeric method, according to the modified procedure of Zhishen *et al.* (1999). 0.5 ml of aqueous extract was placed in a 5 ml volumetric flask, then 2.5 ml of distilled water were added, followed by 0.15 ml of 5% $NaNO_2$. After 5 min, 0.15 ml of 10% $AlCl_3$ 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 flavonoid contents measured by spectrophotometeric method were designed as TFC_{SP} and were expressed as rutin equivalent per 100g orange peel powder.

Determination of vitamin C contents

Vitamin C content was titrated by a modified method described by Tabart *et al.*(2010) using dichlorophenol-indophenol 2,6 (DCPIP). 2 ml of orange peel extract was added to 23 ml of metaphosphoric acid solution (HPO_3) 5%. 5 ml was taken to which was added 5 ml of a solution of trichloroacetic acid in 20% HPO_3. 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_2= 0.9995). The vitamin C content is expressed in g per 100 g of orange peel powder.

Determination of radical scavenging activity

The determination of the radical scavenging activity was realized for the four extracts obtained by the different extraction methods and the results were expressed as micromoles of Trolox equivalent for one micromole of phenolic compounds (Rice-Evans *et al.*, 1996). The free radical scavenging activities of orange peel extracts were determined by DPPH radical cation decolorization assay, according to the method of Burda and Oleszerk (2001). 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 (OD_0) and again at 30 min (OD_t). 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 spectrofluorometer (SAFAS flx Xenius, Monaco) was calculated between OD_0 and OD_t, according to the following equation 1. Appropriate solvent blanks were run in each assay.

$$\textbf{Equation 1}: Percentage of\ inhibtion = \frac{(OD_0 - OD_t)}{OD_0} \times 100$$

with OD_0 as initial optical density and OD_t as final optical density.

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

$$\textbf{Equation 2}: TEAC = \frac{a_S}{a_T}$$

a_S: line slope for sample of percentage of inhibition (%) plotted vs. concentration (µM). a_T: line slope for Trolox reference (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) of percentage of inhibition (%) plotted *vs.* concentration (µM).

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).

$$\textbf{Equation 3}: TEACpredict = \sum_{i=1}^{10} \prod Mi \times TEACi$$

Mi: molar fraction of each phenolic compound i
TEACi: 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 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 (ThermoFisher Scientific, San Jose, CA, USA) equipped with an LTQ XL ion trap as mass analyzer (Linear Trap Quadripole). Chromatographic separation was performed on a C18 Alltima reverse phase column (150×2.1 mm, 5 µm porosity – Grace/Alltech, Darmstadt, Germany) equipped with a C18 Alltima pre-column (7.5×2.1 mm, 5 µm porosity- Grace/Alltech) at 25°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 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: electrospray positive ionization mode (ESI^+) was used; spray voltage was set at 5 kV; source gases were set (in arbitrary units min^{-1}) for sheath gas, auxiliary gas and sweep gas at 40, 10 and 10, respectively; capillary temperature was set at 300°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 $g.mol^{-1}$) at 0.1 $g.L^{-1}$ infused in the mobile phase (A/B: 50/50) at a flow rate of 5 $\mu L.min^{-1}$. Full scan MS spectra were acquired from 100 to 2000 m/z.

Analysis of flavonoids by HPLC

Quantitative analysis of orange peel phenolic compounds was performed according to M'hiri *et al.* (2015)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).Total flavonoids content determined by calculation of the sum of individual flavonoids measured by HPLC was designed as TFC_{HPLC} and was expressed as g/100g 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: TF_{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 content

As reported in **Figure 1**, the TPC_{FC} obtained by CSE was 1.968±0.003 g GAE/100 g of orange peel powder. This value is higher than that reported by Kammoun *et al.* (2011) for the same cultivar analyzed at its commercial ripening stage (1.130±0.040 gcaffeic acid/100 g DM). This difference can be due to used different extraction conditions. In fact, Kammoun *et al.* (2011) 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. If total phenol contents were compared to that of other Citrus cultivars, significant variability could be noticed. In fact, the TPC_{FC} of Maltease orange peel remains lower than that obtained by Ghanem *et al.* (2012) for fresh thompson peel (1.899 ± 0.012 g caffeic acid/100 g DM), Chen *et al.* (2011) (3.945±0.100 g GAE/100g DM for Citrus Sinensis Osbeck peel and Ghasemi *et al*. (2009) (16.03 g GAE/100g of citrus peel powder for *Citrus Sinensis* Whashington, 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 flavonoid content represents almost 50% of total phenolic of Maltease orange peel. This result was in accordance with these reported by Wang *et al*. (2011). Other studies mentioned that the total flavonoid contents can varied in a wide range: from 1.4% (Goulas *et al.*, 2012) to 80%(Sultana *et al.*, 2008). These variations can be explained by the interference of other compounds (sugars, organic acids like vitamin C) on the Folin-Ciocalteu analysis(Neveu *et al.*, 2010).

Figure 1 showed also that TPC_{FC} (2.363±0.014 g GAE/100 g) and TFC_{SP} (1.265±0.023 grutin/100 g) provided by the MAE method were higher than those obtained by UAE followed by CSE, and SC-CO_2 extraction method. Conventional solvent extraction gives low yield in comparison with UAE, and MAE. In fact, this method is accelerated by using ultrasound and microwave energy. The intensification of extraction efficiency using ultrasound has been attributed to the propagation of ultrasound pressure waves through the solvent and resulting cavitation phenomena (Rombaut *et al.*, 2014). A cavitation bubble can be generated close to the plant material surface, then during a

compression cycle, this bubble collapses and a microjet 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 (Chemat *et al.*, 2011; Rombaut *et al.*, 2014).

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 (Jawad and Langrish, 2012). 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 (Mandal *et al.*, 2007). 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 (Nayak *et al.*, 2015a). In our study, **Figure 1**shows that vitamin C contents measured in the different extracts remain constant whatever the used extraction method. The preservation of vitamin C content could be attributed to the low temperature applied during extraction (35°C).

Figure 1 indicates also that supercritical CO_2 extraction method gives the lowest TPC_{FC} and TFC_{SP} (1.204±0.019 g GAE/100 g, 0.589±0.036 grutin/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_2 extraction is more adapted to non-polar compounds(Diaaz-Reinoso *et al.*, 2006; Pereira and Meireles, 2010).

Toledo-Guillen *et al.* (2010) reported that CSE is more efficient than SC-CO_2 for the extraction of glycosylated flavanones. This result is attributed to the high molecular weight and polarity of flavonoids.

According to total phenol content, the efficiency of the examined extraction methods of phenolic and flavonoid contents from citrus peel follows the decreasing order: microwave assisted extraction followed by ultrasound assisted extraction, conventional solvent extraction and supercritical CO_2 extraction.

These results are in accordance with those found by others authors. Dahmoune *et al*. (2013) compared three methods of extraction of lemon peel phenolic compounds: CSE, UAE and MAE. The authors reported that ultrasound causes disruption of plant cells by cavitation. The particles of lemon peel powder are resistant to ultrasound energy (Aspé and Fernández, 2011). 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 dehydrates cellulose and reduces its mechanical strength, which allows an easy penetration of the solvent into the cellular channels (Mandal *et al.*, 2007). Heating by microwave causes cellular damage and a weakened microstructure that helps to quickly release the solute in the solvent.

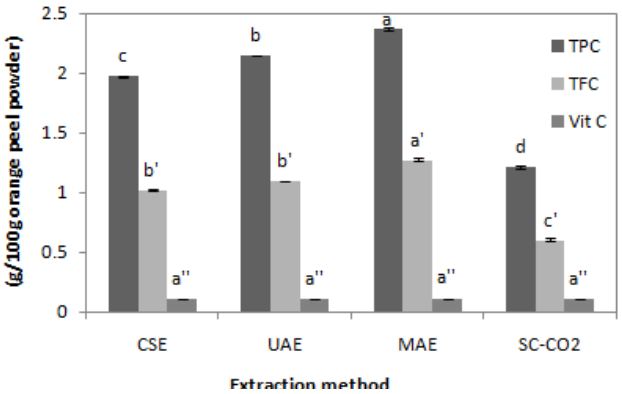

Fig. 1: TPC_{FC}, TFC_{SP} and vitamin C content of Maltease orange peel obtained by CSE (conventional solvent extraction), UAE (ultrasound assisted extraction), MAE (microwave assisted extraction), and SC-CO_2 (supercritical CO_2 extraction) methods. Results are present as means ± S.D. for triplicate analysis. Values with the same letter are not significantly different at $p < 0.05$.

Identification and quantification of orange peel flavonoids in different extracts

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

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 of the ethanolic extract. Except for narirutin and eriocitrin, a similar composition was reported by Anagnostopoulou *et al*. (2005) in Greek Navel sweet orange peel. However, this variety also contains pentamethoxyflavone. Whereas, Kanaze *et al.* (2008) 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, hexamethoxyflavoneheptamethoxyflavone). Moreover, Toledo-Guillén *et al.* (2010) identified in orange peel extracts glycosylated flavanones (hesperidin, narirutin) and the polymethoxyflavones (sinensetin, nobiletin, tetramethylscutellarein and tangeretin).

The percentages of the ten identified flavonoids reported to total flavonoids content, TFC_{HPLC} (corresponding to the sum of individual flavonoids determined by HPLC) are summarized **table 4**. The total phenol contents of Maltease orange peel extract (CSE) determined by the Folin test are equal to 1.968±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 of orange peel powder.

Table 3: R_t, pseudomolecular ions, adduct ions with Na^+, and UV_{max} 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	R_t (min)	$[M+H]^+$ (m/z)	$[M+Na]^+$ (m/z)	UV λ_{max} (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

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_2.

Compound	CSE	UAE	MAE	SC-CO_2
Hesperidin	0.551 ± 0.001^{c}	0.836 ± 0.029^{a}	0.781 ± 0.074^{b}	0.407 ± 0.008^{d}
Neohesperidin	0.860 ± 0.003^{c}	0.986 ± 0.006^{b}	1.045 ± 0.001^{a}	0.624 ± 0.013^{d}
Eriocitrin	0.019 ± 0.001^{a}	0.019 ± 0.001^{a}	0.016 ± 0.000^{a}	0.007 ± 0.001^{b}
Narirutin	0.038 ± 0.001^{a}	0.017 ± 0.001^{c}	0.002 ± 0.001^{e}	0.008 ± 0.001^{d}
Naringin	0.042 ± 0.001^{c}	0.081 ± 0.009^{b}	0.218 ± 0.001^{a}	0.043 ± 0.005^{c}
Didymin	0.026 ± 0.001^{c}	0.041 ± 0.003^{b}	0.062 ± 0.001^{a}	0.018 ± 0.001^{d}
Sinensetin	0.020 ± 0.001^{d}	0.040 ± 0.002^{ab}	0.040 ± 0.001^{b}	0.045 ± 0.002^{a}
Hexamethoxyflavone	0.006 ± 0.001^{c}	0.010 ± 0.013^{b}	0.016 ± 0.002^{a}	0.010 ± 0.006^{b}
Tangeretin	0.005 ± 0.001^{a}	0.009 ± 0.003^{a}	0.011 ± 0.000^{a}	0.008 ± 0.001^{a}
Nobiletin	0.042 ± 0.002^{e}	0.074 ± 0.003^{b}	0.084 ± 0.001^{a}	0.068 ± 0.001^{c}
TFC_{HPLC}	1.609 ± 0.013^{b}	2.113 ± 0.017^{a}	2.275 ± 0.082^{a}	1.238 ± 0.090^{c}

CSE: conventional solvent extraction, UAE: ultrasound assisted extraction, MAE: microwave assisted extraction, and SC-CO_2: supercritical CO_2 extraction. Results are presented as means ± S.D. for triplicate analysis. Values with the same letter are not significantly different at $p < 0.05$.

This difference (~18.2%) between spectrophotemtric and chromatographic methods was acceptable and it 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 (Singleton *et al.*, 1988) as reducing sugars (fructose, glucose etc.) and organic acids (vitamin C, citric acid malonic acid etc.).

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±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 ($AlCl_3$) as hesperidin which is present in large quantities in the Maltease orange peel (34.24%). The spectrophotemtric method was the main common method used by many authors for total flavonoid estimation and for comparison between process efficiency or variability between products (Sultana *et al.*, 2008; Ghasemi *et al.*, 2009). Chromatographic analysis remains more appropriate and thus should be recommended for phenols determination in citrus.

Table 4 shows the individual flavonoids content of citrus Maltease peel expressed as 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, nnobiletin, tangeretin and hexamethoxyflavone were obtained respectively by using MAE. The eriocitrin content is achieved with a maximum content by UAE, SC-CO_2 (difference not significant) while it is CSE and SC-CO_2 for the highest levels of sinensetin and narirutin. This result coincides with those found in the literature. Indeed, Hayat *et al.* (2009) compared CSE, MAE and UAE for the extraction of phenolic acids from mandarin peel. The MAE provides the highest level of ferulic acid (0.239 g/100 g DM) compared to UAE (0.235g/100 g DM) and CSE (0.205 g/100 g DM). Khan *et al.* (2010) 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/100g DM, respectively) than those obtained by CSE (0.145 and 0.051 g/100 g DM, 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 depend 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 method (**Figure 2**). Vitamin C contents were measured in the different extracts; it appears that this content remains constant (200 µM) whatever the extraction used method. So the variations observed cannot be attributed to this molecule. These data show that orange peel extract obtained by CSE presents the highest radical scavenging capacity compared to extracts obtained by other extraction methods. Moreover, it can be noticed a significant decrease of the radical scavenging activity by the DPPH method in the following order: CSE, SC-CO_2, MAE and UAE. These results are not in accordance with those previously reported for TPC_{FC} and TFC_{SP} (**Figure 1**).

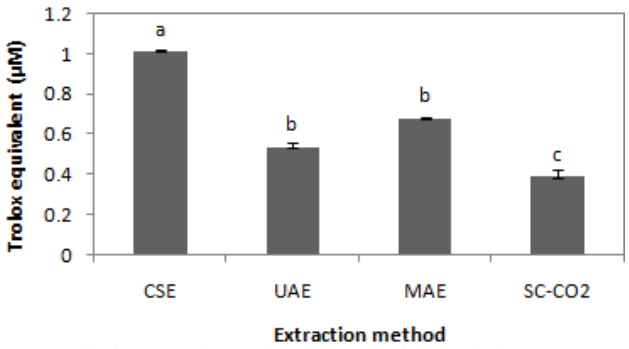

Fig. 2: Radical scavenging activity (µM Trolox equivalent, DPPH test) measured for Maltease orange peel extracts obtained by CSE, UAE, MAE and SC-CO_2 methods.

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 ($TPC_{FC,}$ TFC_{sp} and TFC_{HPLC}). 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**), (iii) the appearance of new formed compounds during MAE and interactions between the different compounds, resulting in positive or negative synergies of antioxidant activity(Hidalgo *et al.,* 2010). According to literature, compounds newly formed during the Maillard and thermo-oxidation reactions could be explained the increase of antioxidant activity of MAE extract. In our case, after the MAE, a change of extract color 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 (PMR) could be divided into different groups. During the first phase of the PMR training, small molecules such as glyoxyl, methyloxyl and others are trained dicarbonyls (Hodge, 1953; Yaylayan and Haffenden, 2003). 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, leads to higher molecular weight products and a brown color through a series of condensation and polymerization reaction (Nicoli *et al.,* 1994). PMR complexes are at the later stage antioxidant and were named collectively melanoidins (Wagner *et al.,* 2002; Liu *et al.,* 2008). These compounds can interact during extraction to form other compounds that could present different structures and properties from the original (Gil-Chavez *et al*., 2013).

Figure 2 showed also that the radical scavenging 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.* (2013). The authors have shown that the antioxidant activity of the lemon peel extract 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 (Pingret *et al.,* 2012; Pingret *et al.,* 2013;Nayak *et al.,* 2015b). In conclusion, it must also be pointed out that the radical scavenging method (DDPH test) is not sufficient to evaluate the whole antioxidant activity of the extract because some flavonoids make a minor contribution in the DPPH assay. Therefore, it is necessary to apply others methods to explain the mechanisms by which the orange peel extracts acts as antioxidant such as ferric reducing-antioxidant power (FRAP) assay, hydroxyl radical scavenging activity, nitric oxide scavenging activity. **Table 5** shows the antioxidant activity of the ten individual flavonoids of orange peel extract and the vitamin C, measured by DPPH. These results indicate that the highest antioxidant 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*.(2010) who reported that the flavanones of orange peel react very slowly with the stable DPPH radical, making, therefore, a minor contribution. 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±0.013 was obtained by DPPH against 0.332±0.001 for the predicted one. These results coincide with those found by Hidalgo *et al*.(2010) 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 vitamin E. In some cases, synergistic or antagonistic effects may occur resulting in the increase or decrease in the total antioxidant activity of the extract (Reber *et al.,* 2011).

Table 5: Antioxidant activity of individual flavonoids and vitamin C (µM Trolox equivalent, DPPH test) of Maltease orange peel extract.

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

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.* (2009). 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	TF_{HPLC}	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

TF_{HPLC}: sum of total flavonoids determined by HPLC, **Sum GF:** sum of glycosylated flavanones**, Sum MF:** sum of polymethoxylated flavones**, TEAC:** Trolox Equivalent Antioxidant Capacity**.**

Comparison of 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}, SumGF, Sum MF, TEAC) was attributed to the first two principal components (**Table 7**).The first principal component (TF_{HPLC}) explains 55.99 % of the variance and the second component (Sum GF) explains 42.20% of the variance. Based on the PCA analysis, the four extraction methods could be gathered in three groups according to the first principal component (TF_{HPLC}) and the second principal component (Sum GF). The first group was formed by CSE and the second group corresponds to SC-CO_2, whereas, the MAE and UAE could be gathered in one homogenous group. Our study showed that supercritical CO_2 extraction gives the lowest flavonoids content compared with the others methods of extraction. This result can be explained by the non-polar characteristic of CO_2, which limited its application for the extraction of polar compounds, which are the major phenolics of orange peel.

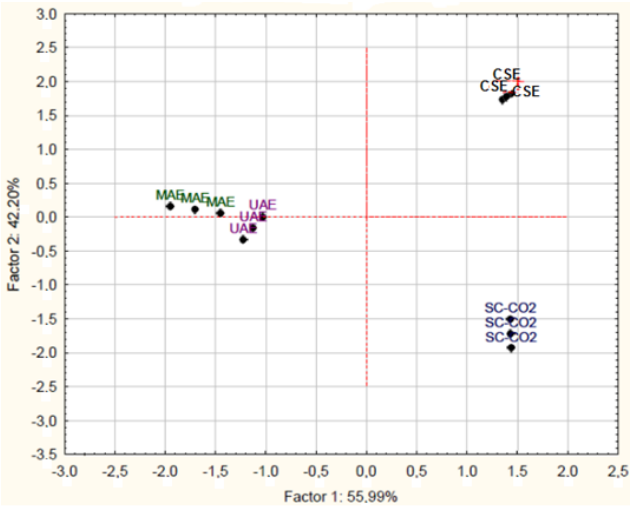

Fig. 3: Projection of the extraction methods on the factor plane according to the first principal component (TF_{HPLC}) and the second principal component (Sum GF).

Both ultrasonic and microwave extraction methods gave the highest phenolic contents. Ultrasound assisted extraction allows the breakdown of plant cells, and the improvement of solvent penetration and capillary effects of ultrasound (Mason *et al.*, 2011). Whereas, microwave assisted extraction showed obvious advantages in terms of high extraction efficiency within the shortest extraction time.

These results are in agreement with the findings of other researchers (Hayat *et al.*, 2010; Jawad and Langrish, 2012). MAE can extract bioactive compounds more rapidly and a better recovery is possible than conventional extraction processes.

It isn't possible to conclude about a single extraction method based on determined antioxidant compounds and antioxidant activity. The choice of the extraction method varied according to the objective (total flavonoids, major compounds, antioxidant activity). For example, if the objective is to obtain the highest content of total flavonoids, MAE is the best extraction method. Whereas CSE allows obtaining the highest radical scavenging activity, compared to the others investigated methods.

Table 7: Eigen values of correlation matrix and related statistics.

Variables	Eigen value	% Total variance	Cumulative Eigen value	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

TF_{HPLC}: sum of total flavonoids determined by HPLC, **Sum GF:** sum of glycosylated flavanones**, Sum MF:** sum of polymethoxylated flavones**, TEAC:** Trolox Equivalent Antioxidant Capacity**.**

CONCLUSION

The main flavonoids of Maltease orange 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 of the peel (87.69% of total flavonoids).This study is the first report comparing the efficiency of four extraction methods of Maltease 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 10s, and 3 successive extractions) was found to be the better extraction method for total and individual flavonoid than ultrasound assisted extraction, conventional solvent extraction, and supercritical CO_2 extraction. Whereas, the CSE method gives the highest radical scavenging activity. 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_2 extraction. This study showed also that there is no additivity 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.

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