

# Flavonoid Composition of Tarocco (*Citrus sinensis* L. Osbeck) Clone “Lempso” and Fast Antioxidant Activity Screening by DPPH-UHPLC-PDA-IT-TOF

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## ABSTRACT:

**Introduction** – Clonal selection and hybridisation are valid strategies to obtain fruits with enhanced sensorial and nutraceutical properties. Within *Citrus sinensis* varieties, Tarocco clone “Lempso” is a typical product of the Calabria region (Italy) characterised by its red pulp. This is the first report concerning its accurate profiling.

**Objective** – To characterise in detail the flavonoid composition of Lempso clone and to compare its antioxidant potential with other *Citrus* varieties by a fast screening method.

**Methodology** – Extracts were subjected to solid phase extraction and the qualitative/quantitative profile was elucidated through ultra-high performance liquid chromatography (UHPLC) coupled to photodiode array (PDA) and ion trap time-of-flight (IT-TOF) mass spectrometry detection, and compared to both Cleopatra mandarin (*Citrus reticulata*) and blood orange (*Citrus sinensis* (L.) Osbeck) Sanguinello varieties. The antioxidant activity was assessed by pre-column 2,20-diphenyl-1-picrylhydrazyl (DPPH) reaction coupled to UHPLC-PDA.

**Results** – Lempso is characterised by flavonoids (17) and anthocyanins (8). Flavanones content (Hesperidin:  $57.19 \pm 0.49$ , Vicenin-2:  $4.59 \pm 0.03$ , Narirutin:  $5.78 \pm 0.13$  mg/100 mL) was considerably higher than Cleopatra and Sanguinello varieties. The developed DPPH-UHPLC-PDA method provides information regarding the single contributions to antioxidant activity, highlighting how Ferulic acid, Quercetin and Cyanidin derivatives possess considerable radical scavenging activity (> 50%). The total antioxidant activity was also evaluated and compared with positive controls, showing higher scavenging activity than Cleopatra and Sanguinello (IC<sub>50</sub>:  $333.76 \pm 10.81$  µg/mL vs.  $452.62 \pm 10.81$  and  $568.39 \pm 26.98$  µg/mL, respectively).

**Conclusion** – These data evidence the nutraceutical potential. **Keywords:** Tarocco Lempso; anthocyanins; flavonoids; antioxidant; DPPH-UHPLC-PDA

## Introduction

The interest of health-conscious consumers towards foods and derived products containing bioactive compounds which can bring benefits to human health is growing considerably. These products, now widely available on the market, are better known as nutraceuticals and functional foods, which contain enriched phytochemical extracts from foods or plants (Espín et al., 2007).

Regarding nutraceuticals, functional drinks represent a sector with an impressive market (Mintel Global New Products Database, 2009), which constitutes 30% of the total market in both the United States and Europe (Menrad, 2003) whose size is increasing year by year. These formulations usually contain vitamins, minerals and enriched extracts from vegetables and fruits. Within bioactive

phytochemicals, flavonoids, are the most interesting compounds.

Numerous experimental evidences highlight their healthy properties including antioxidant, cardioprotective, anticancer and hypolipidemic (Tanaka et al., 1997; Tenore et al., 2013; Prouillet et al., 2004). Among different natural matrices, Citrus fruits are one the most abundant dietary source of flavonoids (Erlund, 2004). Several polyphenolic classes can be found in Citrus, including flavones, flavanones, flavonols, flavans and anthocyanins (Horowitz and Gentili, 1977). In the European and North American market the most popular and consumed Citrus are: grapefruits (*Citrus paradisi*), oranges (*Citrus sinensis*) and lemons (*Citrus limons*).

Since the interest in developing new functional beverages with healthy properties is increasing, Citrus are more often included in different formulations, alone or in combination with other fruits or vegetables rich in flavonoids. Tarocco blood orange is among the most consumed orange variety. In this regard, clonal selection (Bretó et al., 2001) and hybridisation (Grosser et al., 2000; Legua et al., 2014; Rapisarda et al., 2003) are common practices to improve sensorial and nutraceutical quality of these fruits. These processes are aimed not only to improve the resistance of plants and fruits to pathogens and adverse climate conditions, but also to provide fruits with high concentrations of bioactive metabolites together with higher shelf-life. Besides discrimination using genetic diversity markers or other descriptors such as those based on morphological variations (Costa et al., 2009), qualitative and quantitative secondary metabolite pattern is a useful tool to assess fruit quality. Concerning flavonoids, Citrus species contain multiple classes with different chemical behaviour and bioactivity. In this regard, before bioactivity screening and claim assessment, it is mandatory to fully characterise these matrices, to identify and quantify the different flavonoid classes contained. High performance liquid chromatography (HPLC) coupled to tandem mass spectrometry (MS/MS) represents the technique of choice for the analysis of flavonoids and, in the last years, the employment of ultra-high performance liquid chromatography (UHPLC) conditions has further extended the capability of this technique in terms of efficiency and speed (Motilva et al., 2013). The measurement of in vitro antioxidant activity of flavonoid extracts is usually carried out with spectrophotometric assays such as reactions with 2,20-diphenyl-1-picrylhydrazyl (DPPH) or with 2,2-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS). Even though these colorimetric assays can be easily performed, they are time consuming and reflect only the total antioxidant potential of the extract, without providing information regarding the single contribution of every analyte to the antioxidant activity. However, the coupling of an antioxidant assay with a separation technique such as UHPLC, can be an important parameter to appreciate the expression of a particular compound in new food matrices, such as hybrid fruits or clones. Specifically, after the reaction of an antioxidant compound with the DPPH radical, the conjugated system is destroyed, and, as a result, the analyte peak area in the UV-vis chromatogram will decrease or even disappear with respect to those of untreated sample. These methods can be divided if performed pre-column (Tang et al., 2008, Sommella et al., 2016) or post-column (Bandoniene et al., 2002), in relation to whether (prior or after the separation) the antioxidant reaction takes place. Thus, the objective of this work is focused to elucidate the qualitative and quantitative flavonoid profile of a Tarocco Lempso clone, typical of the Calabria region. Moreover, the flavonoid content was correlated with its antioxidant potential through a fast screening method based on the pre-column DPPH reaction coupled to UHPLC with photodiode array (PDA) detection, and compared with those of Mandarin (*Citrus reticulata*) var. Cleopatra and blood orange (*Citrus sinensis*) var. Sanguinello.

## Experimental

### Chemicals

Ultra pure water (H<sub>2</sub>O) was obtained by a Milli-Q Direct 8 system (Millipore, Milan, Italy), LC-MS grade acetonitrile (ACN), formic and acetic acid (HCOOH, CH<sub>3</sub>COOH) were purchased by Sigma Aldrich (Milan, Italy). For UHPLC-MS/MS analyses a Kinetex™ C18 150 mm × 2.1 mm (1. × i.d.), 2.6 µm column was employed (Phenomenex®, Castel Maggiore Bologna, Italy). Malvidin-3-O-glucoside, Narirutin, Hesperidin, Quercetin 3-O-glucoside, Apigenin 6,8 di-c-glucoside and

Ferulic acid standards were purchased by Extrasynthese (Lion, France). Unless stated otherwise all other reagents were purchased by Sigma Aldrich.

### **Sample collection**

Cleopatra mandarin (*Citrus reticulata*) and Tarocco clone “Lempso” (*Citrus sinensis* (L.) Osbeck) fruits were collected from Vivai Antonino Bertolami mother plants field (Lamezia Terme, CZ, Italy). The fruits were harvested in April. The investigation was carried out on 100 samples from the 2014–2015 fruit season. The fruits of Sanguinello (*Citrus sinensis* (L.) Osbeck) were collected in a near field.

### **Sample extraction**

Fruits were transported to the laboratory at room temperature and were carefully selected for size and absence of external defects and then stored at 8°C. The fruits were preliminarily peeled, hand-squeezed and lyophilised. Then, 1 g of lyophilised juice was subjected to magnetic stirring for 20 min at 40°C in 20 mL of hexane to remove the non-polar compounds. The supernatant was discarded and the operation was repeated three times.

Subsequently, to extract the flavonoid compounds, the precipitate was solubilised in 20 mL of methanol (CH<sub>3</sub>OH) and kept under stirring as reported earlier. The supernatant was centrifuged at 7500 rpm at 25°C for 15 min. The entire operation was repeated twice. The extract was dried under reduced pressure and  $931.4 \pm 5.35$  mg of dried extract were obtained. The same process was employed for the three juices.

### **Solid phase extraction**

To remove sugars and further purify the extracts, solid phase extraction (SPE) was carried out by employing polymeric reversed phase cartridges (Strata<sup>TM</sup>-X 100 mg/3 mL, Phenomenex®). The cartridges were equilibrated with 3 mL of CH<sub>3</sub>OH and activated with 3 mL of H<sub>2</sub>O. Three milliliters of sample were loaded. Washing and elution phase were carried out with 3 mL of 0.1% HCOOH in H<sub>2</sub>O and 3 mL ( $\times 2$ ) of CH<sub>3</sub>OH + 0.1% HCOOH, respectively. The eluted fraction was lyophilised and  $26.76 \pm 3.68$  mg were obtained. The extracts were solubilised in CH<sub>3</sub>OH in a concentration of 5 mg/mL and injected.

### **Instrumentation**

UHPLC–MS/MS analyses were performed on a Shimadzu Nexera UHPLC system, consisting of a CBM-20A controller, two LC-30 AD dual-plunger parallel-flow pumps, a DGU-20 A5 degasser, an SPD-M20A PDA detector equipped with a 2.5  $\mu$ L detector flow cell volume, a CTO-20A column oven, a SIL-30 AC autosampler. The system was coupled online to an LCMS-ion trap time-of-flight (IT-TOF) hybrid mass spectrometer through an electrospray ionisation (ESI) source (Shimadzu, Kyoto, Japan). LC–MS data elaboration was performed by the LCMS solution® software (Version 3.50.346, Shimadzu).

### **UHPLC-PDA conditions**

The optimal mobile phase consisted of 0.1% CH<sub>3</sub>COOH in H<sub>2</sub>O v/v (A) and 0.1% CH<sub>3</sub>COOH in ACN v/v (B) while trifluoroacetic acid (TFA) (0.1%) was used for anthocyanins. Separation was performed in gradient elution as follows: 0–10 min, 10–30% B; 10–10.50 min, 30–95% B; 10.50–12.00 min, isocratic at 95% B; 12.01 returning to 10% B, hold for 8 min. Flow rate was 0.5 mL/min. Column oven temperature was set to 40°C, whereas 50°C was used for anthocyanins. Injection volume was 2  $\mu$ L of extract. The following PDA parameters were applied: sampling rate, 10 Hz; detector time constant, 0.160 s; cell temperature, 40°C. Data acquisition was set in the range 190–800 nm and chromatograms were monitored at 280, 330 and 520 nm, at the maximum absorbance of the compounds of interest.

### UHPLC-IT-TOF conditions

MS detection was operated in negative ionisation mode for flavonoids whereas positive mode was employed for anthocyanins, with the following parameters: detector voltage: 1.55 kV; interface voltage: -3.5 kV (+4.5 for ESI+); CDL (curve desolvation line) temperature: 250°C; block heater temperature: 250°C; nebulising and drying gas flow (N<sub>2</sub>): 1.5 L/min and 10 L/min. Full scan MS spectra were acquired in the range 150–1500 m/z; ion accumulation time: 25 ms; IT, repeat: 3. MS/MS experiments were conducted in data dependent acquisition, precursor ions were acquired in the range 100–1500 m/z; peak width: 3 Da; ion accumulation time: 50ms; collision induced dissociation (CID) energy: 50%; repeat: 1; execution trigger base peak chromatogram (BPC) at 95% stop level. The instrument was tuned daily by a standard solution of sodium trifluoroacetate (NaTFA).

### DPPH-UHPLC method development

The determination of the total and single compound antioxidant capacity of Lempso, Cleopatra and Sanguinello polyphenolic extracts was carried out as reported previously (Tang et al., 2008). For the total antioxidant activity extracts solutions were diluted in the appropriate ratio with CH<sub>3</sub>OH and for each sample a calibration curve was built (0.250–3.75 mg/mL). The samples were added to DPPH solution (0.4–0.8 mM) in a 1:1 ratio and the mixture was briefly sonicated then left to react for 30 min in the dark at room temperature. The samples were filtered through 0.45 µm and injected in the LC system, running isocratic with mobile phases (B/A): 60:40 (for detailed conditions see earlier). The blank control was prepared by diluting the DPPH solution with CH<sub>3</sub>OH in a 1:1 ratio. In these conditions all compounds elute at the column dead time in a single chromatographic peak and DPPH peak area, monitored at 515 nm, is taken into account for the calculation. IC<sub>50</sub> values, the concentration of flavonoid extracts scavenging 50% DPPH, were calculated through linear regression analysis, by interpolation. The radical scavenging activity of the extracts was calculated with the following formula:  $\text{Radical scavenging } \delta\% = \frac{PA_{\text{control}} - PA_{\text{spiked}}}{PA_{\text{control}}} \times 100$  (1) where PA<sub>control</sub> is referring to the DPPH peak area solution diluted with CH<sub>3</sub>OH, whereas PA<sub>spiked</sub> is referring to the DPPH peak area resulting from mixing DPPH with the different extracts. Data were compared using as positive control Trolox, ascorbic acid and Quercetin. However, for the determination of single compounds contribution to the antioxidant activity, after the procedure described earlier the separation was conducted in gradient mode as reported earlier. The changes in flavonoids/anthocyanins peak areas, monitored at their maximum absorbance, between control (PA<sub>control</sub>) and DPPH spiked sample (PA<sub>spiked</sub>) were used to evaluate the antioxidant anthocyanins again according to Equation (1).

### Qualitative and quantitative analysis

Since standards for all flavonoids were not available, for the quantification Narirutin, Hesperidin, Quercetin 3-O-glucoside, Apigenin 6,8 di-c-glucoside, Dydimin and Malvidin-3-O-glucoside were selected as external standards.

Stock solutions (1 mg/mL) were prepared in CH<sub>3</sub>OH, the calibration curves were obtained in a concentration range 1.0–0.001 mg/mL with seven concentration levels run in triplicate. Peak areas were plotted against corresponding concentrations. The amount of the compounds in the sample was expressed as milligrams of standard equivalents (SE) in 100 mL of juice (as a typical volume of juice in a glass). Molecular formulas were calculated by the Formula Predictor software (Shimadzu), using the following parameters: deviation limits from mass accuracy: 5 ppm, MS/MS fragmentation data, nitrogen rule.

The data obtained were compared with online free databases such as ChemSpider (<http://www.chemspider.com>), SciFinder Scholar (<https://scifinder.cas.org>) and Phenol-Explorer ([www.phenol-explorer.eu](http://www.phenol-explorer.eu)).

### Method validation

The analytical method was validated to determine the linearity, limits of detection (LODs), limits of quantification (LOQs) and precision (repeatability and reproducibility). Linear regression was used to generate calibration curve, R<sup>2</sup> values were  $\geq 0.998$ . Method precision was assessed by triplicate injection of the sample within the same day and in a time range of three consecutive days. Coefficient of variation (CV) values relative to both retention time and peak areas were  $\leq 0.89$  and 11.32, respectively. LODs and LOQs were calculated by the ratio between the standard deviation (SD) and analytical curve slope multiplied by 3 and 10, respectively. Recovery was evaluated by spiking the juices after squeezing and prior extraction with three different concentration levels of Hesperidin, mean value was 82%. Statistical analysis was performed by paired t-test. A P-value less than 0.05 was considered significant. Results are reported in the Supporting Information Table S1.

## Results and discussion

Identification of flavonoids and anthocyanins by UHPLC-PDA-IT-TOF Figure 1(A) and (B) shows the chromatograms monitored at 280 and 520 nm for the Lempso extract. The same gradient was applied for the separation of both flavonoids and anthocyanins, whereas a different acidic additive was employed for optimal ionisation of analytes. The identification of compounds was carried out on the basis of diode array spectra, comparison with available standard retention times (rt values), and accurate MS spectra and MS/MS fragmentation patterns. The employment of a hybrid mass spectrometer, capable of high mass accuracy in both MS and MS<sup>n</sup> stages, is particularly useful for accurate profiling of this class of compounds (Sommella et al., 2013). Results are shown in Table 1 in order of peak elution. Peaks 1 and 4 (rt: 2.16, 2.61) showed loss of an hexose moiety [M-H-162]- and, from their main MS<sup>2</sup> ions at 163.0434 and 193.0376 m/z, they were tentatively identified as pcoumaric and ferulic acid hexoside derivatives. From MS<sup>2</sup> base peak ion showing the same m/z of peak 4 and accurate mass difference, peak 5 (rt: 2.81) was tentatively characterised as ferulic acid dehydroxylated dimer, while peak 6 (rt: 3.08), showing as main MS<sup>2</sup> the ion at 191.0328 m/z, of the deprotonated quinic acid moiety, was proposed as 5-p-Coumaroyl hexoside methylester. Several quercetin derivatives were found, peaks 2, 3 were characterised by similar fragmentation pattern, showing multiple losses of hexosides [M-H-162]- and rutosides [M-H-308]- so they were proposed as Quercetin 3,40-di-O- $\beta$ -hexoside and Quercetin 3-O-rutinoside-7-O-hexoside respectively, peak 3 was not clearly visible to UV thus better displayed by extracted ion chromatogram (EIC). Similarly, peaks 11 and 12 were identified as Quercetin 3-O-glucoside and Rutin. Moreover, a different structure was recognised for peak 14 (rt: 6.43), the ion at m/z 463.0867 can be attributed to loss of a methylglutaryl moiety [M-H-144]-, while the ion at m/z 505.0939 to a possible rearrangement into a 6'' acetate form, thus leading to the tentative assignment as quercetin-3-O-[6''-(3-hydroxy-3-methylglutaryl)]- $\beta$ -hexoside, this compound has been reported also in blood orange (Barreca et al., 2016). Peak 7 showed a typical fragmentation pattern of C-glucosides (Gattuso et al., 2006), along with the presence of two fragments at [M-H-120]- (base peak) and [M-H-90]- suggesting the loss of two hexose moieties. Thus it was assigned as Apigenin 6,8-di-C-glucoside. Peak 8 showed its main MS<sup>2</sup> fragment at 271.0549 m/z of the deprotonated aglycone naringenin, deriving from loss of an hexose moiety, and was thus identified as Prunin. Most abundant compounds were flavanone glucosides Narirutin and Hesperidin, whose identification was further confirmed by comparison with corresponding standards retention time. Peak 9 showed again the loss of rutinose and thus was proposed as Eriocitrin. Peak 10 showed in its MS<sup>2</sup> spectrum the deprotonated aglycone of Apigenin, and was proposed as Apigenin, the identification should be confirmed in this case by NMR and MS<sup>n</sup> data. From accurate mass and molecular formula [C<sub>15</sub>H<sub>12</sub>O<sub>7</sub>]- of the main MS<sup>2</sup> peak, derived from the loss of rutinoside moiety [M-H-308]-, compound 15 was identified as Isorhamnetin-3-O-rutinoside. Last eluting compound, Dydimin, peak 18, was identified by comparison with standard retention time. The section of Lempso fruit is characterised by a red colour due to the presence of pigments such as anthocyanins, which are absent in Cleopatra mandarin. Among anthocyanins, different Cyanidin derivatives were present (1, 2, 4, 5,

8), these compounds showed again a similar fragmentation pattern, revealing losses of mono and diglucosides [M-162-162]<sup>+</sup> together with rutosides [M-308]<sup>+</sup>. The most abundant compounds were peaks 4 and 8, the last characterised by the fragment at 491.0931 m/z amenable to the loss of malonyl moiety [M-6MG-CO<sub>2</sub>]<sup>+</sup> and tentatively identified as Cyanidin-3-O-(6'' malonyl glucoside). The anthocyanin profile reflects the traits of blood orange (Scordino et al., 2015).

Noteworthy, the identified Quercetin glycosides derivatives (2, 3, 11) as well as the Isorhamnetin derivative, were described to the best of our knowledge, for the first time in Cleopatra mandarin (Dugo et al., 2005; Gattuso et al., 2007), and now in the Lempso.

Moreover these compounds were not detected in previous approaches on blood orange (Barreca et al., 2016; Barreca et al., 2014) as well as in hybrids based on clementina (*Citrus clementina* Hort. ex Tan) and blood orange varieties (Rapisarda et al., 2009).

Quantification of flavonoids and anthocyanins by UHPLC-PDA Figure S1 (in Supporting Information) shows the comparison of profiles recorded at 280 nm of Lempso, Cleopatra mandarin and blood orange Sanguinello. It can be appreciated how the flavanone content is much higher in the Lempso variety (Table 2). In particular, the amount of both Narirutin and Vicenin-2 is almost double with respect to Cleopatra (Narirutin:  $5.78 \pm 0.13$  vs.  $4.01 \pm 0.03$  mg/100 mL; Vicenin-2:  $4.59 \pm 0.03$  vs.  $2.74 \pm 0.09$  mg/100mL), while the Hesperidin amount was roughly nine times higher ( $57.19 \pm 0.49$  vs.  $6.31 \pm 0.02$  mg/100 mL). This value is higher with respect to those reported in the literature for both mandarin and orange (Gattuso et al., 2007; Peterson et al., 2006; Barreca et al., 2016), even though the content of secondary metabolites in *Citrus* species can differ on the basis of harvesting time. From a nutraceutical point of view this aspect is highly interesting, since hesperidin and other flavanones possess antioxidant and anti-inflammatory properties (Parhiz et al., 2015; Khan et al., 2014).

Screening of antioxidant flavonoids and anthocyanins by pre-column DPPH reaction coupled to UHPLC-PDA Pre-column DPPH coupled to HPLC separation has been used for the screening of different natural matrices containing antioxidant compounds (Qiong Sun et al., 2012; Dai et al., 2013). Most of these methods rely on the employment of conventional fully porous particle columns resulting in separations that are usually longer than 40 min. In our approach we employed a core-shell C18 column under ultra-high pressure conditions, and the separation was accomplished in less than 20min with good chromatographic resolution of both flavonoids and anthocyanins. An important aspect was represented from the ratio between the concentration of DPPH and the extract, together with the reaction time. In fact, if an excess of DPPH is used, the differences in the antioxidant activity cannot be measured, since every analyte peak just disappears in the UV-vis trace. Similarly, with an inadequate concentration of DPPH, no appreciable differences can be evidenced. In this method, the best conditions were obtained using 8 mM of DPPH for flavonoids and 0.4 mM for anthocyanins, the difference depending on the relative antioxidant activity of this class of compounds. The reaction time was also optimised and set at a value of 30 min. After this point no further changes in peak areas and solution colour (stable yellow) were observed. The PDA chromatograms relative to the flavonoids and anthocyanins of untreated and spiked with DPPH are depicted in Fig. 2(A) and (B).

As can be clearly appreciated several peaks were significantly reduced, while only a few completely disappeared. Among compounds listed in Table 3, it can be appreciated how ferulic acid derivatives, such as peaks 4 and 5, possess a strong scavenging activity ( $IC_{50}$ :  $92.56 \pm 1.05$ ,  $86.10 \pm 0.32$  respectively), as confirmed by previous investigations, also compared to other hydroxycinnamic derivatives (Kanski et al., 2002). Also Quercetin glycosides (peaks 3, 12,  $IC_{50}$ :  $64.64 \pm 0.06$ ,  $57.81 \pm 0.29$ ) and Cyanidin derivatives (peaks 1, 2, 8,  $IC_{50}$ :  $63.04 \pm 3.60$ ,  $75.39 \pm 1.27$ ,  $51.89 \pm 0.40$ ) were able to scavenge  $\geq 50\%$  of DPPH (Table 3). This can be attributed to the structures of both Quercetin and Cyanidin that possess more hydroxyl groups on the B ring and thus are characterised from a higher scavenging potential (Qiu et al., 2012). Noteworthy, scavenging activity of single flavonoids in complex phytochemical mixtures can be different from their stand-alone activity, this is due to multiple effects, which can also result in synergistic activity (Stintzing et al., 2002). In this regard,

the total antioxidant activity (Table S2) evidenced that Lempso possesses the highest scavenging activity with an IC<sub>50</sub> of  $333.76 \pm 10.81$  µg/mL, significantly lower than Cleopatra and Sanguinello ( $452.62 \pm 10.81$  µg/mL and  $568.39 \pm 26.98$  µg/mL, respectively,  $p < 0.05$ ). This aspect can be related to the very high content of flavanones in Tarocco Lempso, that, together with the presence of anthocyanins, resulted in lower IC<sub>50</sub> values than Cleopatra mandarin, in which the anthocyanins are absent, but also with respect to Sanguinello blood orange, that was characterised by a lower content of flavonoids. The combination of compounds possessing both reducing behaviour and free radical scavenger activities has been previously reported as responsible for the enhancement of antioxidant activity (Brewer, 2011). The main advantage of the developed DPPH-UHPLC-PDA-IT-TOF with respect to online methods is an easier set-up since it does not require any hardware adjustment, such as post-column reactors, additional pumps and flow split. Furthermore, post-column online methods require less sample preparation and can be fully automated, but cannot take advantage of UHPLC conditions (Sommella et al., 2016) such as high efficiency and reduced analysis time, since they are subjected to deleterious band broadening effects that occur in the reactor, leading to peak overlap and thus overestimation of antioxidant activity. The developed method took only 50 min per sample, comprising reaction, separation and column reequilibration, allowing to characterise in detail the flavonoid pattern of Tarocco Lempso for the first time. Moreover, with respect to conventional DPPH assay, several information regarding the antioxidant potential of the single compounds in relation to their chemical structure can be obtained. Interestingly, higher amounts of flavanones were found in comparison to both Citrus reticulata var. Cleopatra and Citrus sinensis L. Osbeck var. Sanguinello. The DPPH-UHPLC-PDA method proved to be a fast and effective tool to highlight the antioxidant activity of single flavonoids in the Lempso clone. A higher radical scavenging activity was evidenced for the Lempso extract, with respect to both Cleopatra mandarin and Sanguinello blood orange. These results could be relevant for the employment of a new food matrix as a novel ingredient for the formulation of functional foods and beverages.

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**Table 1.** Identified flavonoids and anthocyanins of Tarocco Lempso clone by UHPLC-PDA-IT-TOF

Peak	$r_t$	$[M-H]^-$ or $[M]^+$	MS/MS	Molecular formula	Error (ppm)	Compound
<i>Methoxy-hydroxycinnamic acids and flavanones</i>						
1	2.15	325.0870	163.0434 187.0204	$C_{15}H_{18}O_8$	−2.46	<i>p</i> -Coumaroyl glucose
2	2.37	625.1331	463.0735 301.0336	$C_{27}H_{30}O_{17}$	5.00	Quercetin 3,4'-di- <i>O</i> - $\beta$ -Hexoside
3	2.56	771.1997	609.1355 301.0316	$C_{33}H_{40}O_{21}$	4.56	Quercetin 7- <i>O</i> -glucoside-3- <i>O</i> -rutinoside
4	2.64	355.1002	193.0376 217.0539 175.0413	$C_{16}H_{20}O_9$	−4.29	Ferulic acid 4- <i>O</i> -glucoside
5	2.83	385.1095	355.1003 164.0526	$C_{20}H_{18}O_8$	3.27	5-8'-Dehydrodiferulic acid
6	3.16	329.0833	191.0328 167.0259	$C_{21}H_{14}O_4$	4.22	<i>p</i> -Coumaric acid derivate
7	3.23	593.1457	383.0711 353.0628	$C_{27}H_{30}O_{15}$	−4.35	Apigenin 6,8-di- <i>C</i> -glucoside (Vicenin-2)
8	3.67	433.1104	271.0549 151.0148	$C_{21}H_{22}O_{10}$	−4.31	Naringenin-7- <i>O</i> -glucoside (Prunin)
9	4.97	595.1612	287.0476 416.0102	$C_{27}H_{32}O_{15}$	−3.41	Eriodictyol-7- <i>O</i> -rutinose (Eriocitrin)
10	5.34	563.1341	413.0806 249.0475	$C_{26}H_{28}O_{14}$	3.21	Apigenin 7- <i>O</i> -apysil glucoside (Apiin)
11	5.40	609.1401	301.0268 463.1107	$C_{27}H_{30}O_{16}$	−4.13	Quercetin-3- <i>O</i> -rutinoside (Rutin)
12	5.77	463.0963	301.0373	$C_{21}H_{20}O_{12}$	2.85	Quercetin 3- <i>O</i> -glucoside
13	6.11	579.1686	271.0579	$C_{27}H_{32}O_{14}$	−4.7	Naringenin-7- <i>O</i> -rutinoside (Narirutin)
14	6.43	607.1259	301.259 463.0848 505.0939	$C_{27}H_{28}O_{16}$	−4.54	Quercetin-3-[6''-(3-hydroxy-3-methylglutaryl)] $\beta$ -hexoside
15	6.62	623.1557	315.0475 447.1126 300.0228 255.0249 221.0199	$C_{28}H_{32}O_{16}$	−4.79	Isorhamnetin-3- <i>O</i> -rutinoside
16	6.89	609.1786	301.0690	$C_{28}H_{34}O_{15}$	−3.4	Hesperetin 7- <i>O</i> -rutinoside (Hesperidin)
17	9.47	593.1833	285.0707	$C_{28}H_{34}O_{14}$	−3.25	Isosakuranetin 7- <i>O</i> -rutinoside (Dydimin)
<i>Anthocyanins</i>						
1	4.49	611.0703	449.1709 287.0550	$C_{27}H_{31}O_{16}$	0.30	Cyanidin 3,5-diglucoside
2	4.91	611.1629	449.1609 287.0330	$C_{27}H_{31}O_{16}$	2.81	Cyanidin 3-sophoroside
3	5.09	465.2025	303.1162	$C_{21}H_{20}O_{12}$	1.94	Delphinidin-3- <i>O</i> -glucoside
4	5.25	449.1007	287.0614 231.0457	$C_{21}H_{20}O_{11}$	−0.22	Cyanidin-3- <i>O</i> -glucoside
5	5.53	595.1671	449.0769 287.0599	$C_{27}H_{30}O_{15}$	2.35	Cyanidin-3- <i>O</i> -Rutinoside
6	5.96	465.2260	303.1192	$C_{21}H_{20}O_{12}$	1.94	Delphinidin-3- <i>O</i> -Hexoside
7	6.20	463.1195	301.0703	$C_{22}H_{23}O_{12}$	4.97	Peonidin 3- <i>O</i> -glucoside
8	6.49	535.1038	287.0522 213.0315 491.0931	$C_{24}H_{22}O_{14}$	−4.22	Cyanidin-3- <i>O</i> -(6''-malonyl glucoside)

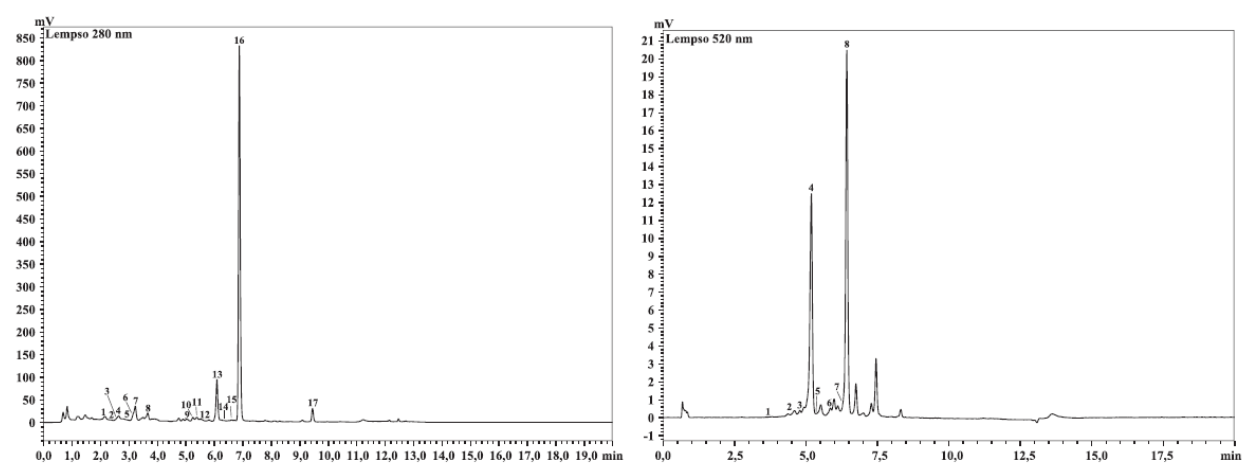
**Table 2.** Flavonoids and anthocyanins content in Tarocco Lempso clone, Cleopatra mandarin and Sanguinello blood orange ( $p \leq 0.05$ )

Peak	Compound	mg/100 mL		
		Cleopatra	Lempso	Sanguinello
Methoxy-hydroxycinnamic acids and flavanones				
1	<i>p</i> -Coumaroyl glucose	0.51 ± 0.02	0.43 ± 0.02	0.05 ± 0.03
2	Quercetin 3,4'-di- <i>O</i> -β-glucopyranoside	0.84 ± 0.03	0.73 ± 0.08	0.06 ± 0.01
3	Quercetin 7- <i>O</i> -glucoside-3- <i>O</i> -rutinoside	0.47 ± 0.01	<LOQ	ND
4	Ferulic acid 4- <i>O</i> -glucoside	0.30 ± 0.02	0.49 ± 0.02	0.03 ± 0.01
5	5-8'-Dehydrodiferulic acid	0.47 ± 0.01	0.51 ± 0.03	0.03 ± 0.01
6	<i>p</i> -Coumaric acid derivate	ND	<LOQ	ND
7	Apigenin 6,8-di- <i>C</i> -glucoside (Vicenin-2)	2.74 ± 0.09	4.59 ± 0.03	0.13 ± 0.06
8	Naringenin-7- <i>O</i> -glucoside (Prunin)	1.48 ± 0.10	2.04 ± 0.06	0.08 ± 0.06
9	Eriodictyol-7- <i>O</i> -rutinose (Eriocitrin)	0.39 ± 0.07	1.08 ± 0.01	0.03 ± 0.01
10	Apigenin 7- <i>O</i> -apyosil glucoside (Apiin)	0.32 ± 0.04	0.90 ± 0.05	0.10 ± 0.01
11	Quercetin-3- <i>O</i> -rutinoside (Rutin)	1.50 ± 0.05	2.39 ± 0.03	0.05 ± 0.01
12	Quercetin 3- <i>O</i> -glucoside	0.67 ± 0.02	1.46 ± 0.03	0.02 ± 0.01
13	Naringenin-7- <i>O</i> -rutinoside (Narirutin)	4.01 ± 0.03	5.78 ± 0.13	0.18 ± 0.09
14	Quercetin-3-[6"- (3-hydroxy-3-methylglutaryl)] β-hexoside	0.23 ± 0.02	0.45 ± 0.02	0.04 ± 0.02
15	Isorhamnetin-3- <i>O</i> -rutinoside	0.38 ± 0.04	0.67 ± 0.08	ND
16	Hesperetin 7- <i>O</i> -rutinoside (Hesperidin)	6.31 ± 0.02	57.19 ± 0.49	1.57 ± 0.74
17	Isosakuranetin 7- <i>O</i> -rutinoside (Dydimin)	0.93 ± 0.04	1.47 ± 0.01	0.05 ± 0.02
		Total: 21.55	Total: 78.71	Total: 2.42
Anthocyanins				
1	Cyanidin 3,5-diglucoside	ND	0.06 ± 0.01	<LOQ
2	Cyanidin 3-sophoroside	ND	0.05 ± 0.01	0.02 ± 0.01
3	Delphinidin-3- <i>O</i> -glucoside	ND	0.08 ± 0.01	<LOQ
4	Cyanidin-3- <i>O</i> -glucoside	ND	0.92 ± 0.02	0.11 ± 0.05
5	Cyanidin-3- <i>O</i> -rutinoside	ND	<LOQ	<LOQ
6	Delphinidin-3- <i>O</i> -hexoside	ND	0.06 ± 0.01	0.02 ± 0.01
7	Peonidin 3- <i>O</i> -glucoside	ND	0.07 ± 0.01	0.03 ± 0.01
8	Cyanidin-3- <i>O</i> -(6"-malonyl glucoside)	ND	1.20 ± 0.02	0.25 ± 0.12
		ND	Total: 2.27	Total: 0.43
Note: LOQ, limit of quantification; ND, not detected.				

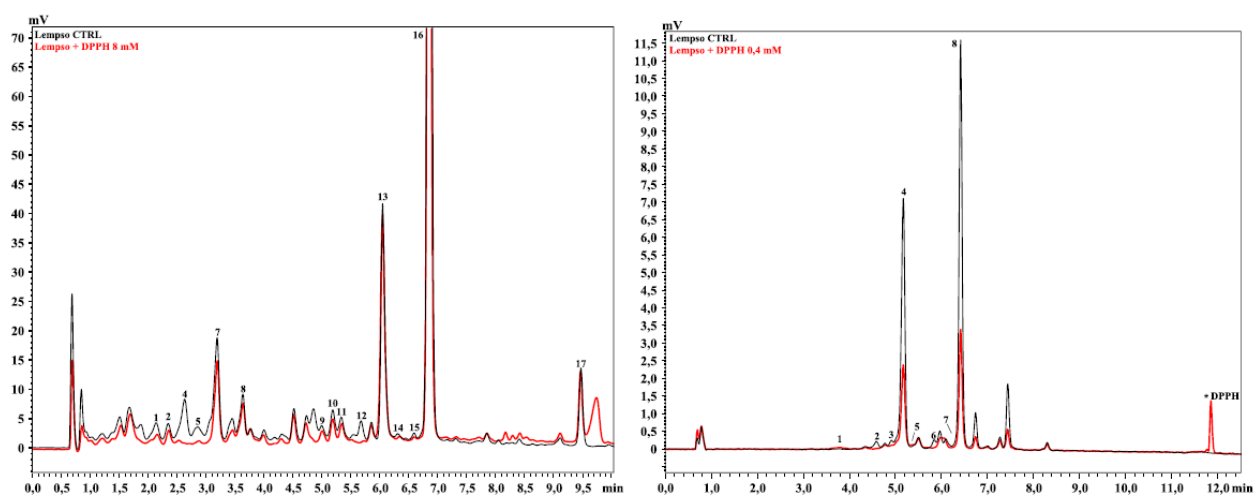
Note: LOQ, limit of quantification; ND, not detected.

**Table 3.** Single contribution of flavonoids and anthocyanins to the antioxidant activity by DPPH-UHPLC-PDA method of Lempso extract ( $p \leq 0.05$ )

Lempso					
Methoxy-hydroxycinnamic acids and flavanones			Anthocyanins		
Peak	Compound	IC <sub>50</sub> (μg/mL)	Peak	Compound	IC <sub>50</sub> (μg/mL)
1	<i>p</i> -Coumaroyl glucose	48.13 ± 0.93	1	Cyanidin 3,5-diglucoside	63.04 ± 3.60
2	Quercetin 3,4'-di- <i>O</i> -β-glucopyranoside	45.35 ± 1.29	2	Cyanidin 3-sophoroside	75.39 ± 1.27
3	Quercetin 7- <i>O</i> -glucoside-3- <i>O</i> -rutinoside	64.64 ± 0.06	3	Delphinidin 3- <i>O</i> -glucoside	37.39 ± 2.12
4	Ferulic acid 4- <i>O</i> -glucoside	92.56 ± 1.05	4	Cyanidin 3- <i>O</i> -glucoside	41.61 ± 0.78
5	5-8'-Dehydrodiferulic acid	86.10 ± 0.32	5	Cyanidin-3- <i>O</i> -rutinoside	0.91 ± 0.99
6	<i>p</i> -Coumaric acid derivate	95.08 ± 0.02	6	Delphinidin-3- <i>O</i> -hexoside	45.03 ± 1.18
7	Apigenin 6,8-di- <i>C</i> -glucoside (Vicenin-2)	28.55 ± 0.95	7	Peonidin 3- <i>O</i> -glucoside	5.88 ± 0.09
8	Naringenin-7- <i>O</i> -glucoside (Prunin)	9.27 ± 0.01	8	Cyanidin-3- <i>O</i> -(6"-malonyl glucoside)	51.89 ± 0.40
9	Eriodictyol-7- <i>O</i> -rutinose (Eriocitrin)	8.42 ± 0.01			
10	Apigenin 7- <i>O</i> -apoyosil glucoside (Apiin)	29.94 ± 1.18			
11	Quercetin-3- <i>O</i> -rutinoside (Rutin)	29.31 ± 2.25			
12	Quercetin 3- <i>O</i> -glucoside	57.81 ± 0.29			
13	Naringenin-7- <i>O</i> -rutinoside (Narirutin)	1.56 ± 1.01			
14	Quercetin-3-[6"-(3-hydroxy-3-methylglutaryl)] β-hexoside	21.38 ± 1.20			
15	Isorhamnetin-3- <i>O</i> -rutinoside	50.09 ± 4.00			
16	Hesperetin 7- <i>O</i> -rutinoside (Hesperidin)	41.35 ± 1.16			
17	Isosakuranetin 7- <i>O</i> -rutinoside (Dydimin)	1.91 ± 0.46			



**Figure 1.** UHPLC-PDA chromatograms of (A) flavonoids (280 nm) and (B) anthocyanins (520 nm) in Tarocco Lempso clone.



**Figure 2.** Overlapped DPPH-UHPLC-PDA chromatograms of (A) flavonoids (280 nm) and (B) anthocyanins (520 nm) in Tarocco Lempso clone treated and untreated with DPPH radical. [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]