Antioxidant properties of minimally processed endives and escaroles vary as influenced by the cultivation site, cultivar and storage time.

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Abtract

The influence of cultivar (CV), growth site (GS) and storage time (ST) on the quality of minimally processed endives was investigated by targeting curly and smooth-leafed cultivars, which were grown in two planting areas and bagged in modified atmosphere at fixed conditions. The changes of antioxidant properties were examined at one and seven days post-packaging by measuring both contents of total flavonols (Fol), flavonoids (Fid), carotenes (Car) and chlorophylls (Chl) and the antioxidant capacity (AOC) through chemical (ORAC) and erythrocyte-based methods (CAA-RBC and hemolytic assays). Referring to one day of storage, curly types differed from smooth ones due to the total contents of Fid (341.0 – 891.7 *vs* 312.3 – 572.3 mg kg^{-1} CE), Fol (312.0 – 452.7 *vs* 194.3 – 520.3 mg kg^{-1} QE), Car (72.4 - 110.5 *vs* 7.3 - 38.8 mg kg^{-1}) and Chl (342.7 - 824.6 *vs* 276.5 - 490.4 mg kg^{-1}). CV and GS majorly affected the content variation, whilst ST did not exert any impact on the amounts of pigments (Chl and Car). As for the AOC at one day post packaging, curly and smooth endives respectively showed ORAC mean values of 5045.8 ± 2287.6 and 4822.5 ± 573.0 mmol kg^{-1} TE, CAA-RBC units of 27.5 ± 5.4 and 21.1 ± 2.6 μmol kg^{-1} QE, and hemolysis percentage of 62.5 ± 5.9 and 57.9 ± 10.9. The three factors acted on the AOC variation at the single level and CV x GS was the most affecting interaction. The ORAC values showed positive correlations with Fid, Fol and Chl contents as well as those of CAA-RBC *vs* Fid and pigment amounts, while only the Fol raise agreed with increased anti-hemolytic effects. Positive correlations among the AOC assays were significant just for ORAC *vs* CAA-RBC units. Finally, the principal component analysis was an effective tool to point at the curly types from a specific growth site as bearing the highest antioxidant quality.

1. Introduction

The consumption of minimally processed (MP) salads has increased in western societies along with the consumer's enhanced awareness of vegetables as healthy food (Erinosho et al., 2012; Rico et al., 2007). Contextually, the EU market has been relevant in the world charts of fresh-cut sales, and the Italian industry has gained a high rate in the MP sector (Pilone et al., 2017). The "bagged salad revolution" has prompted the demand for research-supported information on product quality and effects on health (Rico et al., 2007; Sillani and Nassivera, 2015). Phase-specific processing technologies (Ghidelli and Perez-Gago, 2016; Srivastava et al., 2013; Zhang et al., 2015) have been effective to preserve product quality (e.g. inhibition of deleterious microorganisms, control of leaf physiology and chemical degradation); however some procedures (e.g. chopping, shredding, washing, drying) intrinsically affect it.

Endive (*Cichorium endivia*) leaves are used in bagged salads as mono-reference (curly- or smooth-leafed types) or as mixed products (combined with other vegetables). They naturally contain high amounts of polyphenols (Ferioli et al., 2015; Mascherpa et al., 2012), which contribute to the antioxidant capacity (AOC) and healthy properties (Chen et al., 2011; Isabelle et al., 2010; Llorach et al., 2008; Papetti et al., 2008). Phenolic content can vary with cultivar (D'Acunzo et al., 2016; Ferioli et al., 2015; Mentel et al., 2015), planting site (D'Acunzo et al., 2016), cultivation technique (Serna et al., 2013) and processing (Degl'Innocenti et al., 2008). Flavonoids are phenols that include sub-classes such as flavones, flavonols, flavanes, anthocyanins etc. (Ackar et al., 2013; Ferioli et al., 2015). Endive particularly abounds with flavonols mostly represented by kaempferol (Bhagwat et al., 2013; DuPont et al., 2000), which is efficiently absorbed in humans (DuPont et al., 2004) and exerts hepatic protection from oxidative damage (Chen et al., 2011). Carotenoids are pigments that confer yellow, orange and red colors and have healthy characteristics (Burrows et al., 2017; Giuliano, 2017); β-carotene is the major provitamin-A precursor, and zeaxanthin and lutein intake correlate with decreased risks of cataract and macular degeneration (Ma et al., 2012). Endive mainly contains these carotenoids, whose levels vary with leaf maturity, harvesting season, processing and storage (de

71 Azevedo-Meleiro and Rodriguez-Amaya, 2005; Su et al., 2002). Chlorophylls are photosynthetic
72 pigments responsible for the greenness, a crucial trait in consumer's acceptance, and endowed with
73 anti-carcinogenic/inflammatory properties (Donaldson, 2004; Lin et al., 2013) by anti-
74 mutagenic/oxidative mechanisms (Hsu et al., 2005; Kamat et al., 2000; Lanfer-Marquez et al., 2005)
75 and cell defense stimulation (Fahey et al., 2005). Leaf chlorophyll content naturally declines with
76 senescence (Lim et al., 2007) and in response to stress (Thomas and Ougham, 2014); it can be affected
77 by processing (Toivonen and Brummell, 2008) and that of endives is influenced by growing season
78 (Koudela and Petrikova, 2007) and cultivar (Adamczewska-Sowinska and Uklanska, 2010).
79 The AOC measurement is a valid tool to assess food potential beneficial effects and nutritional
80 quality (Carocho and Ferreira, 2013), and the combined use of analytical and biological methods
81 provides enhanced information (Honzel et al., 2008). Several *in vitro* and *ex vivo* assays have been
82 developed (Alam et al., 2013) and those based on erythrocytes have been successfully used for plant
83 food (Blasa et al., 2011; Frassinetti et al., 2015; Wolfe and Liu, 2007). Considering that antioxidant
84 stability is crucial in endive MP (Papetti and Marrubini, 2015), the major aim of this work was to
85 assess the quality performance of endives and escaroles (packaged in active modified atmosphere) by
86 monitoring the variation of antioxidant compounds (total flavonoids, flavonols, carotenoids and
87 chlorophylls) and of AOC, by *in vitro* and *ex vivo* assays. The study addressed the influence of
88 cultivation sites, different genotypes and storage time on these parameters, providing knowledge and
89 tools useful for the best quality selection.
90

2. Materials and methods

2.1. Plant growth conditions

'Domari' (D) and 'Cigal' (C) are curly-leafed (*Cichorium endivia* var. *crispum*), 'Kethel' (K) and 'Parmance' (P) are smooth/broad-leafed cultivars (*C. endivia* var. *latifolium*, syn.: escaroles). Enza Zaden (http://www.enzazaden.it) and Rijk Zwaan (http://www.rijkzwaan.it/) companies own D/P and C/K, respectively. These cultivars (syn.: genotypes) were grown in two areas of central Italy; info on environment/cultivation and farming practices are summarised in Table 1 and 2 (more details are available upon request). Meteorological data are available by web services of Lazio and Abruzzo regions (http://www.arsial.it/portalearsial/agrometeo/E1_2.asp; https://www.wunderground.com; station ILAQUILA4).

2.2. Product processing and sampling criteria and treatment

Sixty plants of each cultivar were delivered to the industry San Lidano (Latina, Italy http://www.sanlidano.it/). After visual selection, 45 plants were processed through standardized mechanical procedures including cut, sanitary treatment (sodium hypochlorite 20-40 mg L^{-1}) and water wash (both at 4-6 °C), active modified atmosphere packaging (initial gas flushing Oxygen 7.2 %, CO_2 8,8 %, N_2 84 %) and filling at calibrated weight (203-205 g per bag) in polypropylene bags; more details available upon request. Mono varietal bags were stored in cold rooms at 7 ± 1 °C in the dark. Bag contents were crunched in liquid nitrogen, stored at -80 °C; an aliquot of 50 g was weighed without interrupting the cold chain, lyophilized at -50 °C for 72 h (laboratory freeze dryer with stoppering tray dryer, FreeZone®, Labconco Corp., Kansas City, MO, USA) and stored at -20 °C. The content of each bag represented a replicate batch; three replicates were used in all assays and all the measurements were in triplicate. The Italian law in force (DM n ° 3746-2014) imposes rules on microbiological parameters, storage (<8 °C) and the warning on consumption date (2 d after bag opening). Industries maximally fix the sell-by date at 7 d pp on the basis that prolongation arouses

consumer's negative attitude (Stranieri and Baldi, 2017). Sampling was done at 1 and 7 d pp. Weight ratios between frozen-dried and fresh materials are in supplemental table S1.

2.3. Determination of total contents of flavonoids, flavonols, carotenoids and chlorophylls

Lyophilized material (0.5 g) was suspended in 2.5 mL of solution made of pure acetone mixed with perchloric acid solution 5 % in a ratio of 4:1 V / V, then shaken for 30 min at 4 °C and then centrifuged at 3,000 x g for 10 m (Ninfali and Bacchiocca, 2003); the supernatant was recovered and aliquoted for the content determinations. Total flavonoid amount was determined according to a method used for plum extracts (Kim et al., 2003) with minor modification (absorbance at 430 nm) and values were converted into milligrams of catechin equivalents per kilogram of fresh weight (mg kg^{-1} CE). Total flavonol content was quantified at 360 nm (Castillo-Muñoz et al., 2009; Romani et al., 1996), the quercetin was used as standard; values were in milligram of quercetin equivalents per kilogram of fresh weight (mg kg^{-1} QE). Total carotenoids, chlorophylls a and b were determined according to a slightly modified method (Lichtenthaler, 1987). The lyophilized tissue was mixed in acetone (80 %) overnight at 4 °C in the dark, hence spun at 3000 x g for 20 m; the supernatant was collected and diluted in 80 % acetone. The extract absorbance was spectrophotometrically measured at 663, 647 and 470 nm and amounts calculated by these formulas: Chl-a = 12.25 x A_{663} - 2.79 x A_{647}; Chl-b= 21.50 x A_{647} - 5.10 x A_{663}; Total Chl = 7.15 x A_{663} + 18.71 x A_{647}; Carotenoids = (1000 x A_{470}) – (1.82 x Chl-a + 85.02 x Chl-b)/198. Contents were in mg kg^{-1} of fresh weight.

2.4. Oxygen radical absorbance capacity (ORAC) assay

The ORAC assay followed the original method (Cao et al., 1997) with minor modifications (Ninfali et al., 2005). Briefly, 100 μL of the above described extract was used to prepare dilution series (1:10, 1:100: 1:1000) and added to a final reaction mixture (final volume 1 mL) containing 800 μL sodium phosphate buffer (75 mM, pH 7.0) with fluorescein sodium salt (0.05 μM) plus a 100 μL solution of 2,20-Azobis(2-amidinopropane) dihydrochloride (400 mM). As for standard solution, 100 μL of 50

142 μM 6-hydroxy-2,5,7,8-tetramethyl-2-carboxylic acid (Trolox) was added instead of sample; the
143 control consisted of sodium phosphate buffer (75 mM, pH 7.0). Fluorescence was recorded every 5
144 min at 37 °C at 485 nm excitation, 520 nm emission for 60 cycles using a Perkin-Elmer Victor^{TM} X^{3}
145 apparatus (Waltham, MA). The ORAC values were computed as follows:

146 $$\frac{As - Ab}{At - Ab}KA$$

147 As, area subtended by the curve (AUC) of fluorescein in the sample (calculated by the program Perkin
148 Elmer 2030 Work Station). At and Ab, trolox and control AUCs, respectively. K, dilution factor; A,
149 trolox concentration (μM). The ORAC unit was expressed in micromoles of trolox equivalents per
150 kg of fresh weight (μmol kg^{-1} TE).
151
152 *2.5. Antioxidant activity in red blood cells (CAA-RBC and haemolysis test)*
153 The methods employ the probe dichlorodihydrofluorescein diacetate (DCFH-DA), a non-fluorescent
154 molecule that enters cells, is hydrolyzed to dichlorofluorescein (DCFH) and interacts with peroxides
155 induced by 2'-Azobis,2-amidinopropane-dihydrochloride (AAPH), eventually producing fluorescent
156 29,79-dichlorofluorescin (DCF), which is detected by spectrophotometers. Compounds that
157 antagonize AAPH effect exert antioxidant activity, which is measured (Wolfe and Liu, 2007).
158 Regarding RBC, human samples from healthy volunteers were collected in
159 ethylenediaminetetraacetic acid (EDTA)-treated tubes and centrifuged for 10 m at 2300 x g at 4 °C.
160 Plasma and buffy coat were discarded and erythrocytes were washed twice with PBS buffer pH 7.4
161 (phosphate buffered saline; NaCl 8.0 g L^{-1}, KCl 0.2 g L^{-1}, Na_2HPO_4 1.42 g L^{-1} KH_2PO_4 0.24 g L^{-1}).
162 As for endives, 100 mg of lyophilised leaves was added to 10 mL of PBS, shaken at 4 °C overnight
163 in the dark; after debris removal (3000 x g for 20 m), the supernatant was stored at 4 °C. The CAA-
164 RBC assay followed a described protocol (Blasa et al., 2011) with minor modifications. The test
165 reaction contained RBC (final cell n. 10^5 in PBS at pH 7.4), DCFH-DA (final concentration 15 μM)
166 and leaf extracts at various dosages (see below); the reference reaction consisted of RBC, DCFH-DA

and quercetin; the control reaction was RBC plus DCFH-DA. The samples were incubated for 60 m, at 37 °C in 2.5 mL, washed twice with PBS and re-suspended in 1 mL of cold PBS. An aliquot of 180 µL was in a 96-well microplate, and, after adding 20 µL AAPH 12 mM, fluorescence was read every minute at 485 nm excitation and 535 nm emission (Victor TM X 3 Multilabel Plate Reader - Waltham, MA). Leaf extract dilutions (5, 10 and 20 g L^{-1}) were made and the dosage of 10 g L^{-1} was set for the presented experiments. Each independent experiment was in biological triplicate and measured three times. The CAA units were computed as follows

$$\text{CAA unit} = 100 - (\int \text{SA} / \int \text{CA}) \times 100$$

where ∫SA is the integrated area of the sample curve and ∫CA is the integrated area of the control curve (Wolfe and Liu, 2007) and subsequently expressed as micromoles of quercetin equivalent per kilogram of fresh weight (µmol kg^{-1} QE) using the quercetin dosage curves (4, 8, and 10 µM).

The haemolysis assay (Hem) was based on a previously developed method (Tedesco et al., 2000) and subsequent modifications (Mikstacka et al., 2010). Briefly, reactions consisted of RBC (5 % suspension) and PBS pH 7.4 (blank reaction, B), RBC plus PBS plus 1 mg endive leaf extracts (test reaction, T) which were incubated at 37 °C for 1 h in a final volume of 0.5 mL. AAPH (final concentration 50 mM) was added to the test reaction and control (C) reaction made of erythrocyte and PBS, and incubated at 37 °C for 4 h. After centrifugation (1,000 x g, 5 m), haemoglobin released in the supernatant was diluted with PBS (1:9, V/V) and haemolysis was determined by measuring the absorbance at 540 nm. Each value was normalized *vs* the blank reaction (producing less than 15% haemolysis) and expressed as percentage with respect to control reaction, which produced 100% haemolysis (both not shown in Figure 2C). A supplemental positive control reaction was carried out using trolox at 500 µM, which showed 10.1± 1.6 % of haemolysis compared to controls (not shown in Fig. 2 C).

191 *2.6. Statistical analysis*

192 All parameters were analyzed according to a strip-split plot design, where Storage Time (ST) was
193 considered as a Strip factor, orthogonally disposed towards the factor Cultivar (CV), repeated in two
194 different Growing Sites (GS) with experiment replications nested within GS (Fiumincino and Fucino
195 locations). The analysis of variance (ANOVA) was applied by a General Linear Model (GLM, SAS
196 Software, Cary, NC, USA). The separation of means was obtained by Least Significant Difference
197 (LSD) test. Proc CORR in SAS carried out the correlation analysis on the variables. Principal
198 Component Analysis (PCA) was performed (PRINCOMP procedure, SAS Software, Cary, NC, USA)
199 on mean centered and standardized data (unit variance scaled). The data matrix submitted to PCA
200 was made of 16 observations (2 GS x 4 CV x 2 ST) and 8 variables (Fid, Fol, Car, Chl-a, Chl-b,
201 ORAC, CAA-RBC, and Hemolysis). The results were shown as bi-plots of scores (treatments) and
202 loadings (variables) (XLStat Pro, Addinsoft, Paris, France).
203

204 **3. Results**

205 *3.1. The antioxidant compound content variation is majorly affected by the cultivar and growth site*

206 Plants were cultivated in two different sites, Fiumicino (S1) and Fucino (S2), and analyses were
207 carried out. The Table 3 includes the total contents of flavonols (Fol), flavonoids (Fid), carotenoids
208 (Car) and chlorophylls (Chl-a, Chl-b) of curly- and smooth-leafed endives at 1 and 7 d post packaging
209 (pp). To give an overview, we report the min and max mean values (referred to fresh weight) of
210 products at 1 d pp. As for curly endives, Fid values were 341.0-891.7 mg kg^{-1} CE; Fol were 312.0-
211 452.7 mg kg^{-1} QE; Car were 72.4-110.5 mg kg^{-1}, and Chl-a + Chl-b (Total Chl) contents were 342.7-
212 824.6 mg kg^{-1}. Referring to escaroles, Fid were 312.3-572.3 mg kg^{-1} CE; Fol were 194.3-520.3 mg
213 kg^{-1} QE; Car 7.3-38.8 mg kg^{-1}, and Total Chl were 276.5-490.4 mg kg^{-1}.

214 Overall, the ANOVA results (Table 4) showed that the growth site (GS), the cultivar (CV)
215 and the storage time (ST) influenced the variation of most parameters. Independently of the genotype,
216 the GS variation caused the major differences. Specifically, the contents of flavonols, flavonoids and
217 chlorophylls decreased over 30 % in S2 with respect to S1 (compare mean site values in Table 3).
218 Focusing on CV effect, we report average values extrapolated from leaf-type means (black-bolded in
219 Table 3) of curly *vs* smooth cultivars, regardless of GS. In detail, curly endives showed higher
220 amounts than escaroles regarding these variables: Fid 492.6 *vs* 366.5 mg kg^{-1} CE; Car 85.5 *vs* 20.0
221 mg kg^{-1}; Total Chl 490.3 *vs* 389.1 mg kg^{-1} (namely, Chl-a 346.7 *vs* 272.1 mg kg^{-1}; Chl-b 143.6 *vs*
222 117.1 mg kg^{-1}). Looking at ST incidence (regardless of both GS and CV) Fid and Fol contents
223 respectively decreased by 32.8 and 25.5 %, from 1 to 7 d pp, while the changes of chlorophylls and
224 carotenoids were not significant (Table 4). The GS x CV interaction is detailed hereafter (Table 3 for
225 mean values; Table 4 for significance), while that of GS x CV x ST is briefly reported. As for Fid, all
226 genotypes from S2 showed lower values than those from S1; curly endives showed the highest Fid
227 means among cultivars grown in S1. For each GS x CV combination, Fid values decreased from 1 to
228 7 d pp, and the highest decline occurred in the curly 'Domari' from S1. With regard to Fol, a different
229 trend from Fid occurred due to cultivar-specific responses to site variation; e.g., the smooth

'Parmance' from S1 showed the highest Fol values, while both curly cultivars contained more Fol than the smooth ones in S2. During storage, content drops of 20-30 % were significant in endives from S2, but irrelevant in those from S1. Carotenoid amounts were higher in endives than escaroles though they decreased in all cultivars from S2 compared to S1, except for 'Cigal'. The highest negative variation of total chlorophyll contents occurred in 'Cigal' by comparing values from S1 with those from S2. As for storage effects, the highest decrement of total Chl content (ca. -18 %) was measured in 'Cigal' from S1, while this and the other cultivars from S2 did not show significant changes.

3.2. Antioxidant capacity by in vitro and ex vivo assays during storage time

The AOC of curly and smooth types were measured by *in vitro* (ORAC) and *ex vivo* erythrocyte-based assays (CAA-RBC and Hem) during storage. As for the ORAC assay (Fig. 1A), mean values of curly and smooth types at 1 d pp (independently of GS) were respectively 5045.8 and 4822.5 ORAC units (μmol kg^{-1} TE). Independently of CV and ST, the site variation caused significant value drop (histograms of the S1 were higher than S2; mean values of 5977.1 *vs* 3247.9 ORAC units). One-week storage also led to ORAC decrease in all cultivars (gray histograms tend to be higher than black ones; mean values 4929.2 vs 4295.8 ORAC units from 1 to 7 d pp). The GS x CV interaction was significant (Table 4), for instance in cigal 6725.1 3025.1; Kethel 5453.5 and 3708.3 delta 55% and 32% and was mainly due to the GS incidence.

Regarding the CAA-RBC assay at 1 d pp (Fig. 1B), the CAA units (μmol kg^{-1} QE) were higher in endives than escaroles (27.5 *vs* 21.1), a trend that was significantly conserved after 7 d pp (25.7 *vs* 17.5 CAA units). Regardless of CV and ST, plants from S1 had higher antioxidant activity than those from S2 (Fig. 1 B, gray histograms of S1-Fiumincino group were higher than those of S2-Fucino) with significant differences of mean values of 26.6 *vs* 19.6 CAA units. After 7 d pp ca. 11 % drop of AOC (from 24.5 to 21.7 CAA units) was observed for all cultivars (Table 4). Regarding GS x CV interaction, minimal and maximal effects were observed for 'Domari' and 'Parmance' (CAA unit drop

255 was 16-19%), and 'Kethel' and 'Cigal' (ca 33% CAA unit drop). Further grades of interactions were
256 not significant (Table 4)

257 As for the AAPH-induced oxidative hemolysis at 1 d pp (Fig. 1C), values of curly *vs* smooth
258 leaf extracts were 62.5 and 57.9 %, and, overall, curly types had a slightly less protective effect than
259 smooth ones (63.3 *vs* 66.6 %; Table 4). Products from S1 had higher inhibitory capacity than those
260 from S2 (histograms of Fiumicino-S1 group were lower than Fucino-S2; statistically significant mean
261 values: 60.2 *vs*. 69.7 %), confirming the occurrence of a relevant GS effect (Table 4). Finally, the
262 hemolytic protection decreased during storage independently of cultivar (Fig. 1C; Table 4). Referring
263 to GS x CV interaction, 'Cigal' from S2 showed the highest capacity to contrast hemolysis (Hem.
264 value 58 %) within the curly group ('Domari', 72 %), while both smooth 'Parmance' and 'Kethel' from
265 S2 (71.2 and 61.2 %) were less effective than the curly ones. Finally, looking at each GS x CV x ST
266 combination, the hemolysis inhibition capacity declined after 7 d pp (higher values of black
267 histograms), with more intense drop in plants from S1 than from S2, with few exceptions (e.g.: curly
268 'Domari'/S1 *vs* smooth 'Parmance'/S2).

269

270 *3.3. Antioxidant capacity and metabolite content correlations.*

271 Pearson's correlation analyses (Table 5) between metabolite contents and antioxidant capacities
272 showed very strong ($r \geq 0.8$, in bold) positive correlations between ORAC units against Fid, followed
273 by strong ones ($0.6 \leq r < 0.8$, in italics) versus Chl-a/b and Fol contents. CAA-RBC values showed
274 strong positive correlations with pigments (Chl-a/b and carotenoids) and Fid levels. Hem values had
275 strong negative correlation (r=-0.63) just versus total flavonol contents, indicating that the raise of
276 these was in parallel with increased anti-hemolytic effects. Finally, positive correlation occurred only
277 for the ORAC *vs* CAA-RBC comparison.

278

279 *3.4. Principal component analysis (PCA) and separation of curly from smooth types.*

280 The PCA biplot picture (Fig. 2) showed that the first two principal components PC1 and PC2
281 explained 62.65 % and 17.22 % of the total variance. They were effective to separate neatly the
282 growth site from genotypes effects in most cases. More specifically, all products from S1 and S2 fell
283 respectively in the positive and negative quadrants of PC1 (with the exception of 'Parmance'-S1/7 d
284 pp that stood in the negative quarter). Fid, Chl-a, ORAC, and CAA-RBC were positively correlated
285 with PC1 at the highest levels. Cultivar separation took place along the PC2; indeed, most of the curly
286 (C and D) and smooth endives (P and K) fell respectively in the upper and bottom quadrants (positive
287 *vs* negative correlation to PC2), with a few exceptions ('Parmance'-S2/7 and 'Kethel'-S2/7 above the
288 PC2, and 'Cigal'-S2/1 below).

289

290 **4. Discussion**

291 This work focuses on the variation of antioxidant compound contents and antioxidant capacity of
292 packaged endives to monitor the quality as influenced by growth site, genotype and storage time.

293

294 *4.1. Contents of flavonoids and flavonols.*

295 The variation of total flavonoids (140-890 mg kg^{-1} CE) was comparable with that of chicory (110-
296 660 mg kg^{-1} CE) and lettuce (ca. 780 mg kg^{-1} CE) leaves (Preti and Vinci, 2016; Srivastava et al.,
297 2013). As for total flavonols (a sub-class of flavonoids), endive is reported to contain mainly
298 kaempferol in diverse glycosylated forms, ranging from 18 to 248 mg kg^{-1} of edible portion
299 (www.ars.usda.gov/ARSUserFiles/80400525/Data/Flav/Flav_R03-1.pdf), though quercetin traces
300 were found (Ferioli et al., 2015). Spectrophotometric detection at 360 nm was applied to target
301 flavonols (Castillo-Muñoz et al., 2009; Romani et al., 1996) and ranges of 140-520 mg kg^{-1} QE were
302 measured. The content variation of both Fid and Fol was influenced by each main factor (GS, CV and
303 ST) and by their interactions. As for genotype incidence, total Fid and Fol were reported to be higher

304 in curly than smooth types, based on 32 cultivars grown in the same site (Ferioli et al., 2015). Fid
305 values of this work were in agreement (curly *vs* smooth types were 493 *vs* 367 mg kg^{-1} CE calculated
306 from means of leaf-type groups in Table 3). Fol contents did not separate neatly the two leaf-groups
307 (322 *vs* 311 mg kg^{-1} QE), mainly because contents of the smooth 'Parmance' (341 mg kg^{-1} QE), rather
308 than those of 'Kethel' (282 mg kg^{-1} QE), were similar to the curly counterparts. Regarding the planting
309 area effects, the content drop of Fid (from 603 to 256 mg kg^{-1} CE) and Fol (from 400 to 233 mg kg^{-1}
310 QE) of samples growth in S1 and S2 confirmed the widely studied effect of environment on flavonoid
311 synthesis and metabolism in leaves (Jaakola and Hohtola, 2010). Targeting GS x CV interaction on
312 Fol contents, the content loss in smooth (from 431 to 193 mg kg^{-1} QE, -54 %) was higher than that in
313 curly types (from 371 to 274 mg kg^{-1} QE, -27 %) in S1- and S2- derived endives. As for storage
314 effects, curly endive and escarole respectively showed kaempferol losses of 54 and 44 % at 7 d pp in
315 non-MAP conditions and under the light (DuPont et al., 2000). In this work, Fid and Fol decreased
316 of 32.8 and 25.5 %, respectively from 1 to 7 d pp, suggesting that MAP processing and dark storage
317 could preserve the loss more efficiently, although not all the cultivar maintained the same behavior
318 due to GS x CV x ST interactions (Table 4).
319
320 *4.2. Contents of pigments*
321 As for total chlorophyll, the content oscillation was from 276 to 824 μg g^{-1}, which was consistent
322 with that (300-1200 mg kg^{-1}) of unprocessed endives (Adamczewska-Sowinska and Uklanska, 2012;
323 Bonasia et al., 2008; Koudela and Petrikova, 2007) and higher (ca. 170 mg kg^{-1}) than that of bagged
324 ones (Hagele et al., 2016). Moreover, curly types had higher amounts than escaroles in site 1 (651 *vs*
325 421), indicating a strong genotype effect, counterbalanced by a strong environmental effect that led
326 to comparable contents in site 2 (329 *vs* 342 mg kg^{-1}, Table 3). A ca. 3 % higher content was reported
327 for curly *vs* smooth types (Adamczewska-Sowinska and Uklanska, 2010), however analyses on six
328 cultivars did not score any significant difference in two years of cultivation (Koudela and Petrikova,
329 2007). As for storage, no significant reductions of chlorophyll contents occurred in both curly and

330 smooth endives (Table 3). In bagged salads, chlorophyll degradation depends on several storage
331 parameters, but also on the species response. For instance, storage in darkness at 4-5 °C was effective
332 to preserve chlorophyll loss in rocket and leaf chicory (Ferrante et al., 2004) but not for lamb's lettuce
333 (Ferrante and Maggiore, 2007). Moreover, the chlorophyll content of fresh-cut endive was unaltered
334 by warm washes and yet unvaried within 7 d post packaging (Hagele et al., 2016), suggesting an
335 intrinsic species-specific capacity of chlorophyll preservation. Our results indicate that the storage
336 conditions (dark, <8 °C, MAP) were optimal to prevent endives from chlorophyll degradation.
337 The average total carotenoid content range was 7.3-110 mg kg^{-1}, curly cultivars had mean
338 values over three fold higher than smooth ones (83-87 *vs* 26-13 mg kg^{-1}) regardless of the growth site
339 (Table 3). These values were minor than those measured for fresh (ca. 110 mg kg^{-1}) or bagged (ca
340 80-99 mg kg^{-1}) endives (de Azevedo-Meleiro and Rodriguez-Amaya, 2005; Su et al., 2002), whose
341 cultivars (leaf-type) were not reported. Significant loss of β-carotene and lutein, but not of other
342 carotenoid forms, were found under non-optimized conditions and the authors suggested that
343 carotenoid retention could be improved by modified atmosphere packaging and lower storage
344 temperature (de Azevedo-Meleiro and Rodriguez-Amaya, 2005). In this work, the storage time had
345 no effect on carotenoid contents, supporting that the MAP and storage temperature were effective to
346 preserve this antioxidant class. Finally, inner physiological variations were proposed to cause β-
347 carotene degradation in endive during storage regardless of processing line (Hagele et al., 2016). In
348 this context, our results support that carotenoid conservation can significantly depend on the genotype
349 in MP endives.
350
351 *4.3. Antioxidant capacity variation.*
352 ORAC ranges of bagged endives (2620-5470 and 2800-7070 μmol kg^{-1} TE for smooth and curly
353 types) were lower than those (5840-13940 and 8930-15710) reported for fresh cultivars (D'Acunzo
354 et al., 2016), supporting that minimally processing affects anti-oxidant properties (Papetti and
355 Marrubini, 2015). The change of planting area caused ORAC drop of ca. 45 %, consistently with the

356 GS influence on AOC in fresh endive produce (D'Acunzo et al., 2016). Regardless of GS, one week
357 storage lead to an ORAC fall of ca. 9 % and 16 % in curly and smooth cultivars, respectively, and the
358 former percentage was close to the AOC loss (over 6 %) measured chemically in packaged curly
359 endives (Preti and Vinci, 2016). The smooth 'Parmance' had slightly (but significant) lower ORAC
360 values than the other cultivars, supporting a genotype modest incidence on this parameter as observed
361 for fresh endives (D'Acunzo et al., 2016). The mean ranges of CAA-RBC units were 9 to 34 μmol
362 kg^{-1} QE, which were consistent with those measured for lettuce cultivars (8-27 μmol kg^{-1} QE) using
363 HepG2 cell - based assays (Song et al., 2010). CV, GS, ST and CV x GS affected this biological
364 assay, and, independently of GS and ST, the average values of curly types were higher than smooth
365 ones (28 *vs* 21 μmol kg^{-1} QE). The endive extracts were able to decrease blood cell hemolysis in a
366 range of 22-56 % and the three main variation sources had influence on the Hem assay. Looking at
367 the genotype effect, curly endives had a slightly lower (but significant) capacity than escaroles
368 (hemolysis percentage was 66.6 *vs* 63.3, respectively). To our knowledge, there has been no data on
369 salads by the same method as this work; however, the same Hem assays showed that comparable
370 amounts of bean extracts had higher protective capacity, ranging around 70% (Frassinetti et al., 2015).
371 Finally, we observed two-way interaction effects among the primary factors, though they were not
372 "maintained" in all AOC assays (e.g. GS x ST was significant in ORAC and not in Hem and CAA-
373 RB assays). Comments on each combination go beyond the scope of discussion, however, the use of
374 multiple assays are highly likely to bring out such complexity because they are based on intrinsically
375 (e.g. chemical *vs* biological) different methodologies.
376
377 *4.4. Correlations among metabolite contents and AOC*
378 Total phenol content and ORAC did not significantly correlate in fresh endives (D'Acunzo et al.,
379 2016), however, in this survey, Fol (a phenol sub-class) amounts and ORAC showed positive
380 correlation. This reinforces the finding that Fol, and specifically kaempferol in endive, mainly
381 contribute to ORAC (Chen et al., 2011; Ninfali et al., 2005). As for phenolic compounds, positive

correlations occurred specifically in these combinations: both Fid and Fol *vs* ORAC, only Fid *vs* CAA-RBC and only Fol *vs* anti-hemolytic properties, suggesting that different sub-classes of compounds may respond differently in different assay systems. As for pigments, the chlorophyll contents showed strong positive correlation with both ORAC and CAA-RBC values, while carotenoids levels just correlated positively with CAA units. Contextually, chlorophylls have antioxidant properties ascertained by several tests other than those applied here (Lin et al., 2013). Correlations among AOC assays were significant just for ORAC and CAA-RBC data (Table 5). It has been reported that chemical and cellular assays often fail to correlate (Blasa et al., 2011; Murador et al., 2016), and the reliability of AOC values from ORAC assays as direct indicators of healthy effects has been debated (Schauss, 2012). In this context, selection criteria for quality point at those combinations that favor simultaneity of highest values form different AOC assays.

4.5. PCA as a tool to score quality performance

PCA pictured the positive correlation of both CAA-RBC and ORAC with chlorophylls and Fid, indeed all these variables grouped on the PC1 positive values. The Hem assay fell in top of left quadrant, showing a feeble dependence on the other variables and maximal distance from Fol, indicating inverse correlation of their values. Finally, the group of curly endives ('Cigal' and 'Domari') from S1 clustered in the PC1 positive ranges and were characterized by high values of CAA-RBC, ORAC, chlorophylls, Fid and Fol (and anti-hemolytic ones). In this observation, PCA rapidly displayed that curly types from Fiumicino bore the highest quality for consumption.

5. Conclusions

The quality of endives in MAP minimal processing was influenced by cultivar, growth site and storage time when antioxidant properties were examined by measuring both contents of total flavonols, flavonoids, pigments and the antioxidant capacity. The cultivar, the planting area and their

interaction majorly affected the variation of antioxidant compound contents and antioxidant capacity. The observation that correlations between antioxidant molecules and assays were compound-specific and that correlations between chemical and biological assays were significant only for ORAC *vs* CAA-RBC comparisons go in support that the combined use of these latter widens the information on quality product. PCA can overview the antioxidant quality of bagged endives, providing info for decision making on the best performance.

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570

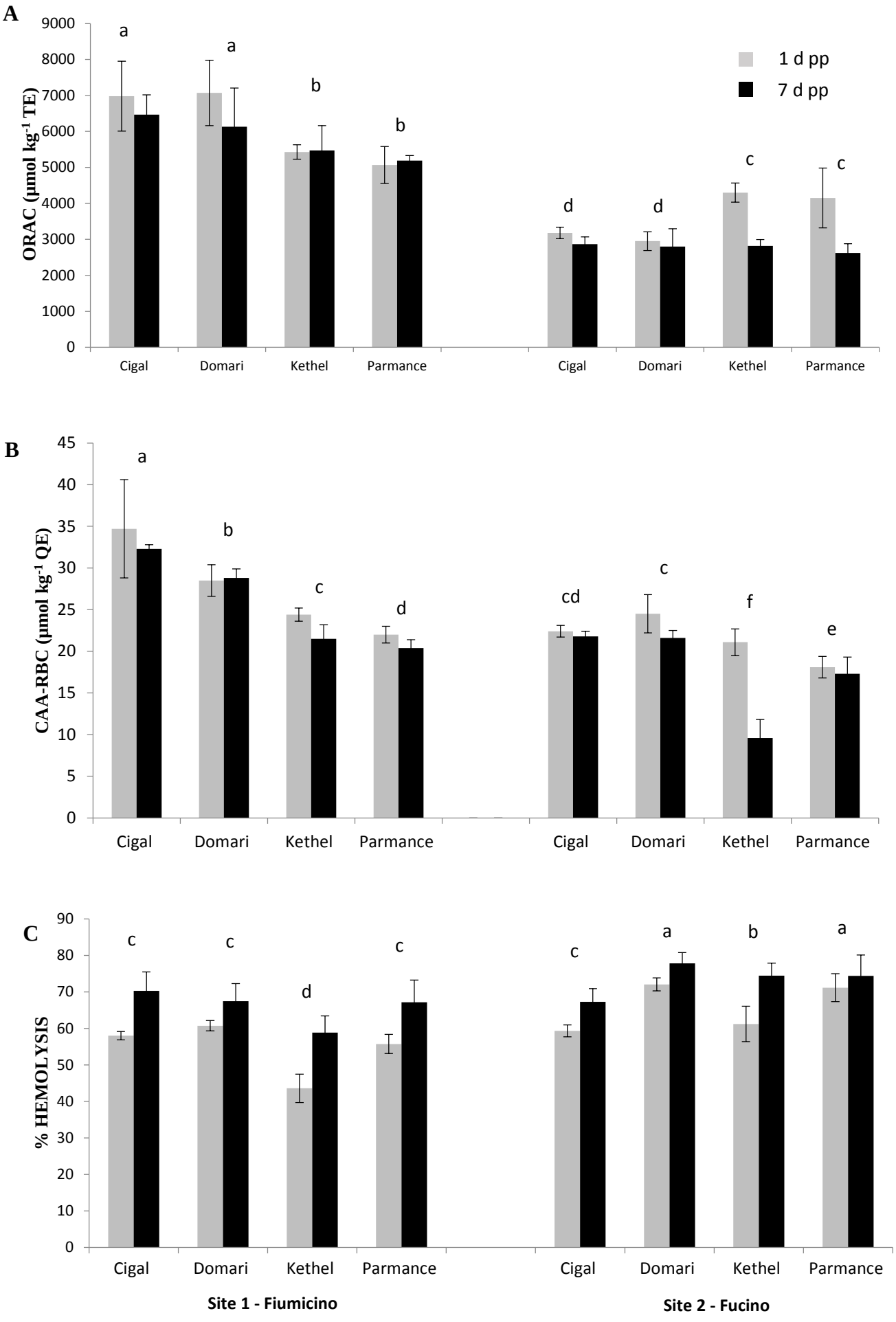
A
ORAC (µmol kg-1 TE)
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
1 d pp
7 d pp
a
a
b
b
d
d
c
c
Cigal
Domari
Kethel
Parmance
Cigal
Domari
Kethel
Parmance
B
CAA-RBC (µmol kg-1 QE)
45
40
35
30
25
20
15
10
5
0
a
b
c
d
cd
c
f
e
Cigal
Domari
Kethel
Parmance
Cigal
Domari
Kethel
Parmance
C
% HEMOLYSIS
90
80
70
60
50
40
30
20
10
0
c
c
d
c
c
a
b
a
Cigal
Domari
Kethel
Parmance
Cigal
Domari
Kethel
Parmance
Site 1 - Fiumicino
Site 2 - Fucino

Figure 1. Antioxidant capacity variation as measured by ORAC (**A**), CAA-RBC (**B**) and Hemolysis (**C**) assays in curly ('Cigal', 'Domari') and smooth endive cultivars ('Parmance', 'Kethel') derived from Fiumicino (growth site, S1) and Fucino (S2) at one and seven days (gray and black colors) of storage time. Values are mean ± standard deviation. In figure (**C**), the control reaction (red blood cells plus the peroxide inducer AAPH) caused 100% hemolysis (not shown). Letters refer to statistical significance of the GS x CV interaction and the same letters indicate that mean values are not significantly different ($P \geq 0.05$)

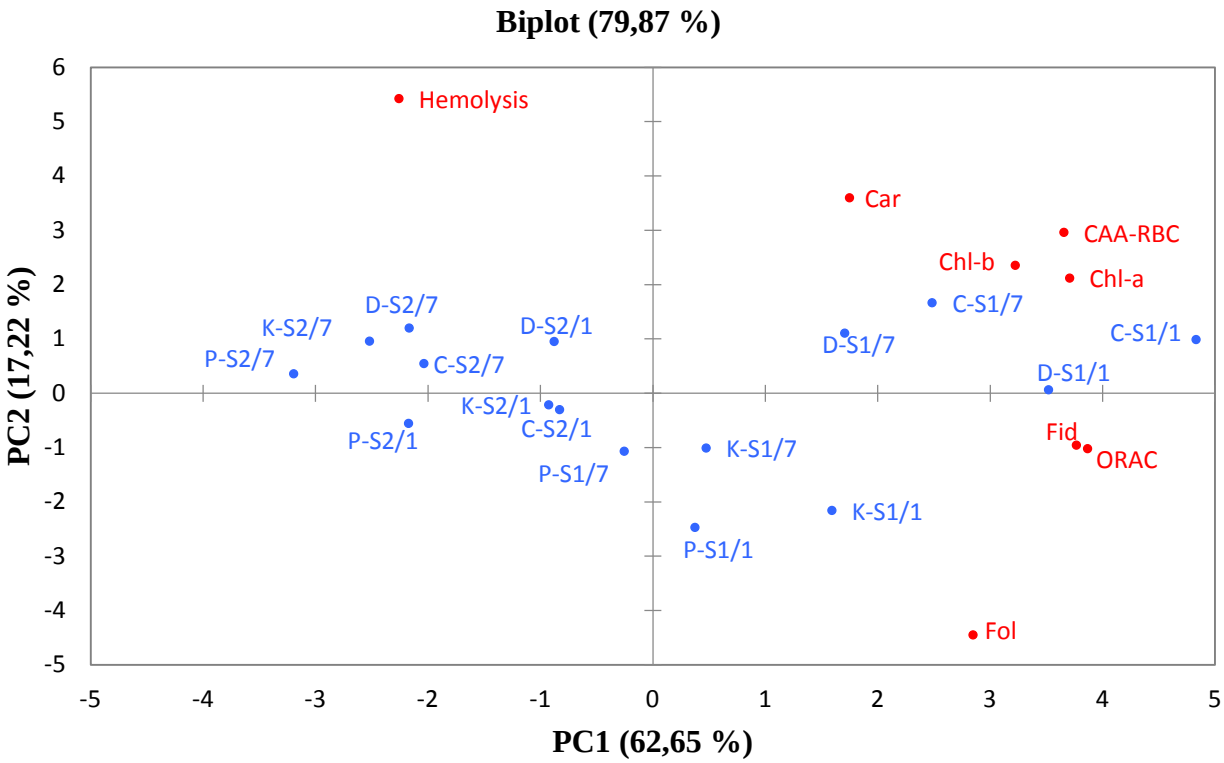

Figure 2. PCA biplot describing the spatial distribution of the measured parameters on 'Cigal' and 'Domari' curly types (C and D), and 'Kethel' and 'Parmance' (K and P) smooth cultivars, grown in the planting areas of Fiumicino (S1) and Fucino (S2) and tested one and seven days post packaging (reported as 1 and 7). ORAC, Hemolysis and CAA-RBC refer to values from in vitro and ex vivo assays respectively. Fid, Fol, Car, Chl-a, Chl-b: total flavonoid, total flavonols, total carotenoids, and chlorophyll a and b contents, respectively.

Table 1. Cultivation environment and techniques.

	Fiumicino (S1)	Fucino (S2)
Coordinates		
Latitude and longitude	41°49'05.9"N 12°14'36.5"E	41°56'26.8"N 13°35'42.8"E
Altitude (m asl)	3	700
Climate[a]		
Temperature (°C)	14.1±2.7	17.7±2.8
Relative humidity (%)	42.3±27.4	60.1±9.2
Total rain (mm)	249.1	183.8
Soil		
Clay (<0.002 mm) (%)	3	9.8
Silt (0.05-0.002 mm) (%)	4	68.7
Sand (2-0.05 mm) (%)	93	21.4
Total nitrogen (%)	0.058	0.111
Organic matter (%)	0.85	1.77
P_2O_5 available (mg kg^{-1})	49	23
K_2O exchangeable (mg kg^{-1})	234	277
E.C. (mS cm^{-1})	0.395	0.264
pH	8.00	8.28
Cation Ex. Cap. (meq 100 g^{-1})	11.32	18.19
Cultivation		
Sowing date	27/01/2014	08/05/2014
Transplant date, leaf number	5/3/2014, 3-4	26/05/2014, 3-4
Field density (plants m^{-2})	6.5	6.5
Harvest date	05/05/2014	08/07/2014

a, data refer to the period that spans from transplant to harvest
b, USDA classification (https://www.nrcs.usda.gov/)

Table 2. Major farming procedures

Fiumicino			
Operation	**Product-type**	**Dosage**	**Timing**[a]
Basal dressing	Nitrophoska special, EuroChem Agro, IT	500 kg ha^{-1}	7 d bt
Protection	Signum, BASF, UK (a.i. boscalid +pyraclostrobin)	1.00 kg ha^{-1}	2 d pt
	DecisEvo Bayer, IT (a.i. delthametrin)	0.50 L ha^{-1}	2 d pt
Fertirrigation	Calcium nitrate	75.0 kg ha^{-1}	15 d pt
	Hydrofert 14.22.11	100 kg ha^{-1}	25 d pt
	Hydrofert 14.22.11	100 kg ha^{-1}	40 d pt
Fucino			
Operation	**Product-type**	**Dosage**	**Timing**
Basal dressing	Manure	500 kg ha^{-1}	7 d bt
Protection	Signum, BASF, UK (a.i. boscalid +pyraclostrobin)	1.00 kg ha^{-1}	2 d pt
	DecisEvo Bayer, IT (a.i. delthametrin)	0.50 L ha^{-1}	2 d pt

a, bt, before transplant; pt, post-transplant

Table 3. Variation of flavonoid, flavonol, carotenoid, chlorophyll contents.

Site	Cultivar	Time	Fid	Fol	Car	Chl-a	Chl-b	Total Chl
		d pp	mg kg^{-1} CE	mg kg^{-1} QE	mg kg^{-1}	mg kg^{-1}	mg kg^{-1}	mg kg^{-1}
1	'Cigal'	1	782.3±34.6	410.0±19.3	72.4±10.8	540.3±14.6	284.4±10.7	824.6±25.3
		7	569.0±23.6	296.7±25.1	60.4±5.5	484.6±31.0	195.7±13.0	680.3±44.0
	'Domari'	1	891.7±33.6	452.7±6.7	110.5±9.4	412.1±28.7	169.5±13.5	581.6±42.2
		7	572.3±122.0	323.3±69.7	105.0±13.3	376.3±11.2	142.6±1.7	518.9±9.5
	Means curly		***703.8±155.6***	***370.7±73.7***	***87.1±24.0***	***453.3±70.2***	***198.1±57.6***	***651.4±125.9***
	'Kethel'	1	527.7±26.7	440.0±44.0	38.8±13.1	364.0±29.6	126.5±18.9	490.4±47.6
		7	452.3±4.7	348.7±1.5	12.3±3.2	319.1±31.9	172.4±17.6	491.4±14.3
	'Parmance'	1	572.3±43.0	520.3±10.7	33.5±6.9	259.1±19.1	62.0±2.6	321.0±21.5
		7	453.3±48.4	414.3±60.7	20.0±7.7	257.0±47.0	127.1±16.4	384.1±51.2
	Means smooth		***501.4±61.2***	***430.8±71.9***	***26.2±13.2***	***299.8±54.7***	***122.0±43.1***	***421.7±82.3***
	Means site		***602.6±155.1***	***400.8±77.6***	***56.6±35.3***	***376.6±97.5***	***160.0±61.3***	***536.6±151.9***
2	'Cigal'	1	341.0±18.7	315.7±13.0	82.3±6.4	245.0±16.9	97.7±17.0	342.7±33.8
		7	169.7±10.7	228.0±10.1	78.1±2.2	219.9±11.2	81.8±6.1	301.7±17.3
	'Domari'	1	360.0±17.1	312.0±10.4	94.3±0.9	273.1±1.8	98.0±2.2	371.0±4.0
		7	254.3±13.6	239.3±27.5	81.2±0.8	222.4±4.5	78.4±4.3	300.8±8.8
	Means curly		***281.3±80.2***	***273.8±44.5***	***84.0±7.1***	***240.1±24.2***	***89.0±11.9***	***329.1±34.9***
	'Kethel'	1	322.3±17.0	194.3±5.7	19.6±14.4	302.6±34.4	126.8±12.4	429.3±44.1
		7	145.3±3.2	145.7±12.6	16.4±5.0	292.9±71.8	119.4±29.1	412.2±100.5
	'Parmance'	1	312.3±5.0	261.0±19.1	7.3±3.9	176.3±33.2	100.2±28.2	276.5±50.7
		7	146.7±17.4	169.7±13.3	11.4±5.3	205.4±52.1	102.0±25.6	307.4±76.0
	Means smooth		***231.7±90.2***	***192.7±46.4***	***13.7±8.6***	***244.3±71.3***	***112.1±24.2***	***356.4±91.7***
	Means site		***256.5±87.2***	***233.3±60.8***	***48.8±36.2***	***242.2±56.3***	***100.5±22.9***	***342.7±74.2***

Fid, total flavonoids; Fol, total flavonols; Car, total carotenoids; Chl-a, chlorophyll a; Chl-b, chlorophyll b, Total Chl, total chlorophyll (Chl-a + Chl-b); pp, post packaging; site 1, Fiumicino; site 2, Fucino. All the contents refer to fresh weight.

Table 4. Significance overview from ANOVA results relative to the parameters affected by the growing site, cultivars and storage time.

Variable factors[a]	Fid	Fol	Car	Chl-a	Chl-b	T-Chl	ORAC	HEM	CAA-RBC
Growth Site	***	***	*	**	***	**	***	**	***
Cultivar	***	***	***	***	***	***	*	***	***
Storage Time	***	***	n.s.	n.s.	n.s.	n.s.	**	***	*
GS x CV	***	***	*	***	***	***	***	***	***
GS x ST	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	*	n.s.	n.s.
CV x ST	n.s.	**	n.s.	n.s.	*	n.s.	n.s.	*	n.s.
GS x CV x ST	**	***	n.s.	n.s.	**	n.s.	n.s.	n.s.	n.s.

a, Fid, total flavonoids; Fol, total flavonols; Car, total carotenoids; Chl-a, chlorophyll a; Chl-b, chlorophyll b; T-Chl, Total chlorophylls; ORAC, Hemolysis and CAA-RBC antioxidant capacity assays; GS, growing sites, Fiumicino or Fucino; CV, cultivars 'Domari', 'Cigal', 'Parmance', 'Kethel'; ST, storage time (1 and 7 d post packaging); n.s.: not significant; * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$.

Table 5. Pearson correlation coefficients (*r*).

	Fid	Fol	Car	Chl-a	Chl-b	T-Chl	ORAC	HEM	CAA-RBC
Fid	1.00	**0.80**/***	0.45/**	*0.71*/***	0.57/***	*0.69*/***	**0.89**/***	-0.50/***	0.72/***
Fol		1.00	0.26/n.s.	0.38/*	0.20/n.s.	0.33/*	*0.66*/***	*-0.63*/***	0.39/**
Car			1.00	0.36/*	0.14/n.s.	0.29/n.s.	0.20/n.s.	0.013/n.s.	*0.60*/***
Chl-a				1.00	**0.84**/***	**0.98**/***	*0.76*/***	-0.37/*	**0.82**/***
Chl-b					1.00	**0.93**/***	*0.69*/***	-0.21/n.s.	*0.67*/***
T-Chl						1.00	*0.76*/***	-0.32/*	**0.80**/***
ORAC							1.00	-0.46/**	0.67/***
Hem								1.00	-0.23/n.s.
CAA-RBC									1.00

n.s., not significant; * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; very strong ($r \geq 0.8$) and strong ($0.6 \leq r < 0.8$) correlations are in bold and in italics, respectively

Table S1. Variation of lyophilized *vs* fresh ratio during storage

	Percentage [a]			
Site	'Cigal'	'Domari'	'Kethel'	'Parmance'
Fiumicino	7.96 ± 0.34	7.31 ± 0.22	6.67 ± 0.22	6.53 ± 0.27
Fucino	6.41 ± 0.61	6.29 ± 0.40	6.25 ± 0.35	5.75 ± 0.32
Significance	**	**	**	**

a, mean ± SD; values were calculated on biological triplicates at 1 and 7 d post packaging. ** $P \leq 0.001$ (Student's test);