Influence of cultivation sites on sterol, nitrate, total phenolic contents and antioxidant activity in endive and stem chicory edible products

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ABSTRACT
Chichories produce a wide range of vegetables with important nutritional value. We determined the variation of sterol, total polyphenol, nitrate contents and antioxidant capacity (SC, TPC, NC, AC) in endive leaves and stem-chicory novel vegetables, cultivated in two Italian regions. Within a given area, the SC was similar in smooth- and curly leafed endives (106.3–176.0 mg/kg FW); sitosterol and stigmasterol were major fractions (45.6–56.2% versus 38.4–43.9%). The stem SC was independent of landrace (101.5–118.6 mg/kg FW); sitosterol prevailed on stigmasterol and fucosterol (73–76 versus 12–14% versus 8–9%); the latter reached 15.7 mg/kg FW, conferring value as potential antidiabetes food. The planting site affected the AC and TPC of endives (893.1–1571.4 μmTE/100 g FW; 30.8–76.1 GAE100/g FW) and chicory stems (729.8–1152.5 μmTE/100 g FW; 56.2–124.4 GAE100/g FW), while the NC was recurrently below dangerous thresholds. PCA showed that environment was the major cause of variation, though it modestly affected these parameters.

Introduction
A wide range of products derives from plants of Cichorium species (Fam. Asteraceae), which enrich the diet, cuisine and medicine (Conforti et al. 2009; Sünat et al. 2012; Street et al. 2013) all over the world. Vegetables of Cichorium spp. comprise leafy endives, red radicchio, Belgian endive (witloof) and bitterish tender stalks. More specifically, C. endive produces curly- (var. crispum) or smooth-leafed (var. latifolium, synonym escarole) rosettes, which are consumed as fresh or bagged salads. The C. intybus species include several cultivated groups based on the product type (e.g. roots, leaves, buds, stems) and destination, including fresh use and processing for food, feed and medicines (Lucchin et al. 2008; Cadalen et al. 2010; Raulier et al. 2015). Focusing on the “Catalogna group” (more details on classification are in the materials and methods), some landraces are specialized to yield stems (inflorescence rachises), which are consumed as raw or cooked vegetables in central/southern Italy (Renna et al. 2014). In 2014, the world market for chichories (excluding witloof) exceeded 176 million USD; Italy and Spain ranked the first two positions in the world (TrendEconomy.com 2014). Moreover, the increasing use of precut or ready-made vegetables (IBISWorld Inc. 2016) prompts the marketing of novel vegetables such as “Catalogna” stems in the processed food chain (Renna et al. 2014). The literature attributing potential health benefits to phytosterols (PSs) is rich, and spans from cholesterol lowering (Gylling et al. 2014; Ras et al. 2014) to anti-inflammatory and antidiabetic effects (Lee et al. 2004; Derdemezis et al. 2010; Jung et al. 2013b). Due to the assessed or claimed health benefits of PSs, their content has been determined in a wide range of vegetable matrices (Moreau et al. 2002; Piironen et al. 2003; Lagarda et al. 2006; Bernal et al. 2011). Intriguingly, literature data on sterol contents of Cichorium spp. products has been limited and referred to stressed young plants (Krebsky et al. 1999), wild populations of C. intybus (Conforti, et al. 2009), or...
products to be found on the market with little specification on cultivars (Jiménez-Escrig et al. 2006; Han et al. 2008). To our knowledge, the sterols content of “Catalogna” stems has not been determined so far.

Endive leaves are rich in antioxidant compounds, including vitamin C (Koudela and Petrikova 2007; Llorach et al. 2008; Mentel et al. 2015), carotenoids (Su et al. 2002; de Azevedo-Meleiro & Rodrigues-Amaya 2005), polyphenols/phenolics (Ninﬁali et al. 2005; Llorach, et al. 2008; Papetti et al. 2008; Ferioli & D’Antuono 2012; Mascherpa et al. 2012) that exert radical scavenging functions and hepatoprotective activity (Papetti et al. 2002; Chen et al. 2011). Consequently, good antioxidant capacity occurs in endives (Degl’Innoccenti et al. 2008; Llorach, et al. 2008; Isabelle et al. 2010; Serna et al. 2013; Mentel et al. 2015). To date, few works have dealt with polyphenol and flavonoid contents and antioxidant capacity in “Catalogna” stems (Renna et al. 2014; Montefusco et al. 2015), and, in light of the market attention to these products, an increasing number of studies on their nutritional properties is expected.

Endive was originally classiﬁed as a species of low nitrate content, ranging from 200 to 500 mg kg\(^{-1}\) FW (Santamaria et al. 1999), though nitrate levels were measured up to 1000–2500 mg kg\(^{-1}\) FW (Santamaria 2006). The smooth- and the curly leaved types can accumulate nitrate differently due to genetic and physiologic characteristics (Reinink et al. 1994; Koudela & Petrikova 2007). As for chicory stems, nitrate levels appeared to be lower than in leaves (Santamaria et al. 1999), however, their content assessment is mandatory for products employed in salads (Santamaria 2006).

High quality of the product at harvest is crucial in the chains of both fresh and processed vegetables (Gil et al. 2012) because it impacts on nutritional properties (Rickman et al. 2007a,b). Moreover, nutritional improvement requires the monitoring of metabolite changes in vegetable breeding programs (Bliss 1999). This study aimed at assessing the variation of beneficial (sterols and total phenols) nutrients and properties (antioxidant capacity) and critical compounds (nitrates) in response to cultivation area swaps, using different genotypes of endive and “Catalogna” chicory at commercial maturity. The study also addresses to what extent ex-situ cultivation is compatible with conserving the quality of local products.

**Materials and methods**

**Plant material and growth conditions**

The endive cultivars (Figure 1(A–F)) “Domari” and “Myrna” (C. endivia var. crisperm) are curly leaved, and “Confiance” and “Flester” (C. endivia var. latifolium) are smooth/broad-leafed (syn.: escaroles); these cultivars are registered at the Community Plant Variety Office (www.cpvo.europa.eu/) and marketed seeds were provided by the Enza Zaden Italy Srl (www.enza-zaden.com). One production cycle (Tarquinia, Lazio, Italy) included the four cultivars and a second cycle included only “Domari” and “Flester” (Conversano, Apulia, Italy). As for chicory stems, “Galatina” and “Molfettese” landraces (Figure 1(G–I)) may be ascribed to the “Catalogna” (syn. “Catalogne”) cultivated group of C. intybus subsp. intybus var. foliosum (Lucchini, et al. 2008), or alternatively to C. intybus var. spicatum or complanatum (Hammer et al. 2013). A recent and simple classification based on genetic diversity structured the C. intybus into witloof, root and leaf chicory groups (Raulier et al. 2015) and ascribed the “Catalogne” to the latter, coinciding with the minimum descriptor classiﬁcation (leaf chicory culti-group 3) of the ECPGR Working Group on Leafy Vegetables (http://www.ecpgr.cgiar.org). Seeds of “Galatina” and “Molfettese” landraces were provided by a local custodian farmer (Sempreverde Srl, Molfetta, BA, Italy) and are deposited in the germplasm bank at the Institute of Biosciences and Bioreources of the CNR of Bari, Italy (http://ibbr.cnr.it/ibbr). The plant material was identiﬁed and veriﬁed by Dr V.V. Bianco, full Professor of Horticulture at the University of Bari and is deposited in the herbarium of the Di.S.Te.B.A., Salento University, Lecce, Italy (https://www.disteba.unisalento.it). Two production cycles were carried out (Molfetta, Apulia, and Tarquinia, Lazio). Table 1 reports site coordinates, soil types, dates and cultivation parameters. Agro-techniques (basal dressing, fertirrigation, protection) were standardized for both species; details are available upon request. Meteorological data are available on the Regional Agency of Lazio for Development and Innovation in Agriculture (ARSIAL 2016) and the Regional Agency for Environmental Prevention and Protection of Apulia (ARPSAL 2016) websites.

**Sampling criteria and treatment**

As for endives, 20 external leaves were first removed from head/rosette (n = 15) of each cultivar; the following 10 leaves bearing homogeneous features were selected (Figure 1(C,F)) as representative products (for fresh consumption and processing). A replicate batch consisted of 50 leaves, which was crushed in liquid nitrogen and stored at −80 °C; three biological replicates were assayed in the experiments. As for “Catalogna” chicory (Figure 1(I,L)), marketable stems were removed from rosettes (n = 15) of each landrace. A replicate batch consisted of at least five stems,
**Figure 1.** Synopsis of experimental material from *Cichorium* spp. vegetables. (A–F) Endive cultivars bearing (A–C) smooth and (D–F) curly leaves. (A and D) Open field cultivation two days before harvest, (B,E) head types and (C,F) leaf shapes of the cultivars “Flester” and “Myrna,” respectively. (G–L) “Catalogna” landraces. (G,J) Open field cultivation, (H,K) stem bulk and (I,L) stem features of the genotypes “Molfettese” and “Galatina,” respectively. Bar sizes: B and E, 8 cm; C and F, 4.5 cm; H and K, 3 cm; I and L, 4 cm.

**Table 1.** Major features on cultivation environment and techniques.

| Geographical coordinates | *C. endivia* cultivars | *C. intybus* landraces |
|--------------------------|------------------------|------------------------|
| Latitude and longitude   | Tarquinia (Lazio)      | Conversano (Apulia)    |
| Altitude (m asl)         | 31                     | 100                    |
| Soil (USDA classification)| Clay loam              | Clay loam              |
| Clay (<0.002 mm) (%)     | 34.4                   | 37.3                   |
| Silt (0.05–0.002 mm) (%) | 22.3                   | 33.2                   |
| Sand (2–0.05 mm) (%)     | 44.3                   | 30.5                   |
| Total N (g kg⁻¹)         | 0.9                    | 0.8                    |
| Organic matter (g kg⁻¹)  | 15.9                   | 15.7                   |
| P₂O₅ available (mg kg⁻¹) | 23                     | 39                     |
| K₂O exchangeable (mg kg⁻¹) | 362.2                 | 185.3                  |
| EC (mS cm⁻¹)             | 0.16                   | 0.25                   |
| pH                       | 8.1                    | 8.2                    |
| Cation Ex. Cap. (meq 100g⁻¹) | 15.4               | 28.5                   |

**Cultivation dates/parameters**

Sowing (substrate) 23/8/2012 (peat) 10/8/2013 (peat) 10/8/2013 (peat) 12/8/2012 (peat)

Transplant (n. of leaves) 14/9/2012 (3–4) 15/9/2013 (3–4) 9/9/2013 (4–5) 15/10/2012 (4–5)

Field density (plants/m², spacing) 8.2; 0.35 × 0.35 m 9.5; 0.35 × 0.30 m 6.3; 0.4 × 0.4 m 6.3; 0.4 × 0.4 m

Harvest (cultivar/landrace) 15/11/2012; D, My, C, F 22/11/2013; D, F 8/1/2014; Mo 28/1/2014; G 14/1/2013; Mo 25/2/2013; G

D: “Domari,” My: “Myrna;” C: “Confiance;” F: “Flester;” Mo: “Molfettese;” Ga: “Galatina.”
which were sliced transversally, frozen in liquid nitrogen and stored at −80°C; three biological replicates were used in the experiments. The samples were lyophilized at −50°C for 72 h (laboratory freeze dryer with stoppering tray dryer, FreeZone®, Labconco Corp., Kansas City, MO) and stored at −20°C.

Sterols analysis

All chemicals and solvents were purchased from Sigma-Aldrich (Milan, Italy) or VWR (Milan, Italy) and used as received, unless otherwise specified. Extraction, purification and analysis procedures were adapted from available literature (Takahito et al. 2001), adding an ultrasound-assisted step as hereby described. As for extraction, freeze-dried samples were ground in liquid nitrogen and 300 mg was added to 4 mL of a MeOH/CHCl₃ (4:1 vol) mixture spiked with the internal standard (cholesterol-d₆, Medical Isotopes Inc., Pelham, NH), and placed in an ultrasound bath for 1 min at room temperature. After centrifugation (2 min at 3500 rpm, 18 °C), the supernatant was removed, and the extraction-centrifugation procedure was repeated twice more. The solvent was evaporated from the combined extracts under a nitrogen stream. The residue was diluted with 1 mL K₂HPO₄ 0.5 M, washed three times with 1 mL K₂HPO₄ 0.5 M, then treated at 90°C with 1.5 mL MeOH/KOH for 90 min (methanolation). The mixture was diluted with water (3 mL), and the analytes were extracted three times with 2 mL hexane aliquots. The organic extract was dried over Na₂SO₄, evaporated to dryness and further purified by silica gel chromatography (2.5 g) with a CHCl₃/acetate, washed three times with 1 mL K₂HPO₄ 0.5 M, and placed in an ultrasound bath for 1 min at room temperature. After centrifugation (2 min at 3500 rpm, 18 °C), the supernatant was removed, and the extraction-centrifugation procedure was repeated twice more. The solvent was evaporated from the combined extracts under a nitrogen stream. The residue was diluted with 3 mL ethyl acetate, washed three times with 1 mL K₂HPO₄ 0.5 M, then treated at 90°C with 1.5 mL MeOH/KOH for 90 min (methanolation). The mixture was diluted with water (3 mL), and the analytes were extracted three times with 2 mL hexane aliquots. The organic extract was dried over Na₂SO₄, evaporated to dryness and further purified by silica gel chromatography (2.5 g) with a CHCl₃/MeOH gradient. The sterol-containing fraction was derivatized with N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) prior to GC-MS analyses. These were performed with a Finnigan Trace GC Ultra coupled to a Trace DSQ single quadrupole MS detector operating at 760 nm according to the Folin–Ciocalteu method (Singleton et al. 1999) and the TPC was expressed as gallic acid equivalent milligrams in 100 grams of fresh weight (mg GAE 100 g⁻¹ FW). The ORAC assay followed the original method (Cao et al. 1997) plus minor modifications (Ninfali et al. 2005). Briefly, 1 mg of lyophilized material was dissolved in a final reaction mixture (2 ml) containing 1650 μl of 0.05 μM fluorescein sodium salt dissolved in sodium phosphate buffer (75 mM, pH 7.0) plus 200 μl of 50 μM 6-hydroxy-2,5,7,8-tetramethyl-2-carboxylic acid (Trolox). The control was sodium phosphate buffer (75 mM, pH 7.0). Fluorescence was recorded every 5 min at 37°C at 485 nm excitation, 520 nm emission for 60 cycles using a Perkin-Elmer Victor™ X³ apparatus (Waltham, MA). After reaching stability, the reaction was started with 150 μl of a 400 mM 2,2′-Azobis(2-aminopropane) dihydrochloride solution (Warrington, PA), and fluorescence was recorded up to the zero value. The ORAC was expressed in micromoles of Trolox equivalents per 100 grams of fresh weight (μmol TE 100 g⁻¹ FW); the value derived from the following formula (A₀−Aᵢ/Aᵢ−A₀)×10⁶ KAH. As, area subtended by the curve (AUC) of fluorescein in the sample (calculated by the program Perkin Elmer 2030 Work Station), A₀, and Aᵢ, Trolox and control AUCs, respectively. K, dilution

Table 2. Characteristic ions (m/z) for the GC-MS identification and quantification of trimethylsilyl-sterols.

| Compound          | Timea | Characteristic ions (m/z) b          |
|-------------------|-------|-------------------------------------|
| Cholesterol       | 7.24  | 459 (M+ 12%), 368 (33%), 353 (21%), 329 (54%) |
| Cholesterol-d₆    | 7.19  | 466 (M+ 12%), 375 (38%), 360 (21%), 336 (60%) |
| Campesterol       | 7.9   | 473 (M+ 9%), 382 (27%), 367 (17%), 129 (100%) |
| Stigmasterol      | 8.19  | 484 (M+ 15%), 394 (27%), 379 (12%), 355 (14%) |
| Fucosterol        | 8.7   | 484 (M+ 3%), 386 (35%), 296 (53%) |
| Sitosterol        | 8.74  | 486 (M+ 8%), 396 (34%), 357 (49%) |

aRetention time in minutes.

bCharacteristic m/z (%) as abundance relative to base peak. All sterols exhibited a base peak (100%) at m/z 129.

Total phenolic content (TPC) and oxygen radical absorbance capacity (ORAC) assay

Lyophilized material (500 mg) was suspended (1:5, w/v) in 80:20 v/v acetone/perchloric acid 5%, shaken for 30 min at 4°C and then centrifuged for 10 min at 3000 g (Ninfali & Bacchiocca 2003). Phenolic compounds were measured by spectrophotometry at 760 nm according to the Folin–Ciocalteu method (Singleton et al. 1999) and the TPC was expressed as gallic acid equivalent milligrams in 100 grams of fresh weight (mg GAE 100 g⁻¹ FW). The ORAC assay followed the original method (Cao et al. 1997) plus minor modifications (Ninfali et al. 2005). Briefly, 1 mg of lyophilized material was dissolved in a final reaction mixture (2 ml) containing 1650 μl of 0.05 μM fluorescein sodium salt dissolved in sodium phosphate buffer (75 mM, pH 7.0) plus 200 μl of 50 μM 6-hydroxy-2,5,7,8-tetramethyl-2-carboxylic acid (Trolox). The control was sodium phosphate buffer (75 mM, pH 7.0). Fluorescence was recorded every 5 min at 37°C at 485 nm excitation, 520 nm emission for 60 cycles using a Perkin-Elmer Victor™ X³ apparatus (Waltham, MA). After reaching stability, the reaction was started with 150 μl of a 400 mM 2,2′-Azobis(2-aminopropane) dihydrochloride solution (Warrington, PA), and fluorescence was recorded up to the zero value. The ORAC was expressed in micromoles of Trolox equivalents per 100 grams of fresh weight (μmol TE 100 g⁻¹ FW); the value derived from the following formula (A₀−Aᵢ/Aᵢ−A₀)×10⁶ KAH. As, area subtended by the curve (AUC) of fluorescein in the sample (calculated by the program Perkin Elmer 2030 Work Station), A₀, and Aᵢ, Trolox and control AUCs, respectively. K, dilution...
factor; A, Trolox concentration (μmoles/L); H, ratio between the extract and vegetable (volume/weight, L/g).

**Nitrate content**

Lyophilized nitrate was ground to fine powder and analyzed for nitrate content by ion exchange chromatography (Dionex DX 200, Dionex Corp, Sunyvale, CA) with a conductivity detector, using an IonPac AG14 precolumn and an IonPac AS4A separation column (Dionex Corporation). Samples (0.5 g) were extracted on orbital shaker for 20 min with 50 mL of the same chromatographic eluent composed of 3.5 mmol L\(^{-1}\) sodium carbonate/1 mmol L\(^{-1}\) sodium bicarbonate solution. Results were in mg kg\(^{-1}\) FW. The eluent comprised 50 mL of 3.5 mmol L\(^{-1}\) sodium carbonate/1 mmol L\(^{-1}\) sodium bicarbonate solution and results were in mg kg\(^{-1}\) FW.

**Statistical analysis**

All analyses and assays were performed on three distinct biological replicates and each measurement in triplicate. The ANOVA included the effects of smooth versus curly leafed types within species and Apulian versus Lazio cultivation. The Tukey’s honest significant difference test was used for multiple comparisons (GNU PSPP software version 0.8.4.). The PCA allowed a visual analysis of data (PRINCOMP procedure, SAS software, Cary, NC), based on mean centered and standardized data (unit variance scaled). The data matrix to PCA was made of six observations for each genotype and growing sites; results were shown as biplots of scores (treatments) and loadings (variables) plots (XLStat Pro, Addinsoft, Paris, France).

**Results and discussion**

**Curly and smooth-leafed endives**

**Sterol content**

The total sterol mean content (Table 3) was comparable in both endive types (106.3 to 168.3 mg kg\(^{-1}\) FW in curly cultivars; 156.9 to 176.0 in smooth ones). Value ranges (10.6–17.6 mg/100 g FW) were consistent with the average 17 mg/100 g FW reported for endive (Jiménez-Escrig et al. 2006) and “lettuce-endive” (Han et al. 2008). The most abundant fractions (Figure 2(A)) were sitosterol (45–56%) and stigmasterol (38–43%), followed by campesterol (5–10%) and cholesterol (1–2%); fucosterol traces were detected below LOQ (<2 mg kg\(^{-1}\) FW). The prevalence of sitosterol, stigmasterol and campesterol is common among plant species; moreover, cholesterol occurs in their edible products as 1–2% of total sterol content (Moreau et al. 2002). The sito/stigmasterol ratio (1.2:1) was similar to that retrievable from other endive studies (Jiménez-Escrig et al. 2006; Martins et al. 2013). As for the curly leafed “Domari,” the sitosterol and campesterol amounts varied with the cultivation site (sitosterol: 70.8 mg kg\(^{-1}\) FW in Lazio versus 40.3 mg kg\(^{-1}\) FW in Apulia; campesterol: 7.0 mg kg\(^{-1}\) FW in Lazio versus 17.7 mg kg\(^{-1}\) FW in Apulia). The lower sitosterol content in “Domari” from Apulia affected the total sterol content (106.3 mg kg\(^{-1}\) FW) and the sito/stigmasterol ratio (0.8:1). Regarding the escaroles, total and single sterol contents were comparable between cultivars, and no significant difference in sitosterol content occurred when the cultivation site changed. On the

| Table 3. Sterol content in endive (C. endivia) leaves. |
|---------------------------------------------------------|
| **Stevos (mg kg\(^{-1}\) FW)**                          |
| **Site**       | **Total**   | **Sitosterol** | **Stigmasterol** | **Campesterol** | **Cholesterol** |
|----------------|-------------|----------------|-----------------|-----------------|-----------------|
| Var. crispum (curly) |            |                |                 |                 |                 |
| "Domari"      | L           | 140.5 ± 18.8   | 70.8 ± 25.6\(^a\) | 59.1 ± 18.0    | 7.0 ± 3.2\(^b\) | 3.6 ± 4.9       |
| "Myrna"       | L           | 168.3 ± 73.5   | 93.9 ± 46.2\(^a\) | 64.6 ± 25.7    | 8.4 ± 3.1\(^b\) | 1.4 ± 1.1       |
| "Domari"      | A           | 163.5 ± 20.5   | 40.3 ± 8.8\(^b\) | 47.8 ± 15.5    | 17.7 ± 8.0\(^a\) | 0.5 ± 0.1       |
| **Significance** | ns          | *              | ns              | *              | ns              |
| Var. latifolium (smooth) |            |                |                 |                 |                 |
| "Flester"     | L           | 176.0 ± 38.9   | 97.5 ± 8.5      | 65.4 ± 3.4     | 9.3 ± 0.9\(^b\) | 3.8 ± 0.9       |
| "Confiance"   | L           | 156.9 ± 62.6   | 77.1 ± 34.9     | 68.0 ± 23.6    | 10.0 ± 4.2\(^b\) | 1.8 ± 1.9       |
| "Flester"     | A           | 163.5 ± 21.1   | 82.7 ± 12.2     | 66.9 ± 12.8    | 13.0 ± 2.9\(^a\) | 0.9 ± 0.5       |
| **Significance** | ns          | ns             | ns              | ns              | ns              |

\(^a\)Cultivation site: A: Apulia; L: Lazio.
\(^b\)Mean ± standard deviation; different letters indicate significant differences among genotypes within botanical varieties; ns: nonsignificant;
\(^*\)significant at \(p ≤ 0.05\);
\(^**\)significant at \(p ≤ 0.01\). Fucosterol was below established LOQ (2 mg kg\(^{-1}\) FW).
other hand, campesterol content was higher in “Flester” from Apulia than Lazio (13.0 versus 9.3 mg kg\(^{-1}\) FW), supporting the occurrence of environmental effects as observed for campesterol variation in “Domari.” Just to mention an example, this is consistent with the observation that tobacco leaf sterol content depends on cultivation sites (Liu et al. 2008). Being membrane components, sterols regulate fluidity and permeability (Schuler et al. 1991) and can exert functions in adaptation to temperature changes (Dufourc 2008). In this context, campesterol variation in “Domari” and “Flester” leaves may reflect adaptive stress responses in different sites. In Europe, the PS intake ranges from ca. 170 to 460 mg/day per capita (Marangoni & Poli 2010); the quality of PS sources varies, for example, vegetable oils prevail in the Mediterranean diet while cereals abound in the Northern ones (Normen et al. 2001; Valsta et al. 2004; Saura-Calixto & Goni 2009). In the Spanish diet, vegetables contribute with 24 mg of PS out of total 374 mg/day (Saura-Calixto & Goni 2009). Assuming that Italy shares similar values, 100 g of endive provides from 10–17 to 13–22 mg of PS for smooth and curly types, respectively.

**Antioxidant capacity, total phenolic and nitrate contents**

The average antioxidant capacity (AC) varied from 893.1 to 1571.4 ORAC units (μm TE 100 g\(^{-1}\) FW) in curly endives and from 584.3 to 1394.5 ORAC in smooth-leafed ones (Table 4). The differences among the four cultivars were not significant regardless of the growth area (F = 0.87, \(p = .47\)). The AC of endives is measured by several assays (Llorach, et al. 2008; Isabelle et al. 2010), hence we discuss literature data including the same methods as those used in this work. The ORAC values fell in the range of those measured on a small number of sampled endives (Isabelle, et al. 2010; Speisky et al. 2012; INTA 2015); they were comparable to those of green lettuce cultivars but lower than the red ones (Ninfali et al. 2005). As for Lazio versus Apulia cultivation, the ORAC drop in Apulia was significant in both “Domari” and “Flester,” in agreement with the observation that AC variation depends on geographical origin, harvest time and environmental conditions (Ou et al. 2002). The ORAC assay measures AC in vitro and the units can be used as indicators of antioxidative performance to monitor salad quality and safety during shelf life and minimal processing (Degl’Innocenti et al. 2008; Papetti & Marrubini 2015). ORAC trends showed that curly leafed had higher values than smooth cultivars (893.1–1571.4 versus 584.3–1394.5 μm TE 100 g\(^{-1}\) FW), suggesting that the former may better perform in minimal processing. The significance of ORAC values as direct indicators of beneficial effects of polyphenols.
on human’s health has been questioned (Schauss 2012); however, endive extracts were proven to protect liver from oxidative damage in vivo due to the high content of kaempferol and its glycosylated derivate (Chen et al. 2011). These latter had high ORAC units, likely contributing to the whole AC of endive heads; hence the screening for cultivars with highest ORAC values may turn useful in selecting products in hepatoprotective diets.

The average TPC varied from 30.8 to 76.1 mg GAE 100 g−1 FW (Table 4), and differences among the four genotypes were not significant, independently of the planting area (F = 0.75, p = .53). The variation of this latter (Apulia versus Lazio) caused significant TPC increase in both “Domari” and “Flester” (from 46.3 to 76.1 and from 39.1 to 69.7 mg GAE 100 g−1 FW, respectively). Among several environmental stresses, light intensity can affect phenolic content in lettuce (Oh et al. 2009) and may be evoked as a cause to explain the TPC raise in the two cultivars. In works adopting comparable measure methods, the TPC values of curly endives at harvest ranged from 14.9 to 23.5 mg GAE 100 g−1 FW (Serna et al. 2013), appearing lower than the “Domari” and “Myrna” counterparts (46.3 and 30.8 mg GAE 100 g−1 FW). The TPC of market-sampled curly endives ranged from ca. 30 to 99 mg GAE 100 g−1 FW depending on year of sampling and cultivar-environment interactions (Isabelle et al. 2010; Mentel, et al. 2015). Higher TP contents were measured in curly endive than escarole (Llorach et al. 2004; Llorach et al. 2008). Based on a large set of genotype accessions, the mean TPC of curly cultivars was 41% higher than the smooth ones, though the intra-cultivar variability was extremely high (Ferioli et al. 2015). In this work, curly versus smooth-type differences were not observed at fixed cultivation site, and this may be attributed to intrinsic (genetic) features of the cultivars. Finally, no significant correlation was found between ORAC values and TP contents (r = .2, p = .19) consistently with other reports in other vegetables (Schaffer et al. 2005; Conforti et al. 2009). Referring to the TPC extractable fraction (in methanol–acetone–water), the daily intake was esteemed 1171 GAE mg/day/person in the Spanish diet (Saura-Calixto & Goni 2006); the estimated value raised to 2590–3016 GAE mg/person/day when the condensed tannins plus hydrolyzable polyphenols were considered (Saura-Calixto et al. 2007). TPC from vegetables provided 230–280 mg GAE from an edible portion of 280 g/day/person, and the extractable fraction was 98 mg (all values on dry weight basis), that is 35 mg GAE from a 100 g edible portion (Saura-Calixto, et al. 2007). Data from our work indicate that a daily portion of 100 g/person of endive would contribute with 55–120 mg GAE (on dry weight basis) from TPC extractable fraction (see Table 4 and Table S1 for conversions), which per se represents a good contribution within the Mediterranean diet.

The nitrate content ranged from 693.3 to 1332.0 mg/kg FW in curly endives and from 980.0 to 1759.7 mg/kg−1 FW in smooth genotypes. Average values of smooth types were higher than curly types (F = 5.18, p = .037), consistently with another survey (Koudela & Petrikova 2007); “Flester” appeared to maximize the accumulation for the Lazio site (1759.7 mg kg−1 FW). Curly endives form more open leaf-rosettes than escaroles (Supplementary Table S1), hence the former might capture more light, which would account for lower average nitrate contents (Gruda 2005). Finally, a ca. 44% reduction of the nitrate content occurred in “Flester” comparing cultivation in Lazio versus Apulia, suggesting that variation of genotype–environment interactions can affect nitrate accumulation as observed in lettuce (Burns et al. 2011). The nitrate levels found in our work are far below the EU legislative limits assigned to lettuce (EC Reg. No. 1258/2011). The recommended Acceptable Daily Intake (ADI) for nitrate is 3.7 mg/kg body weight per day (equivalent to 222 mg nitrate per day for a 60 kg

### Table 4. ORAC, total phenolic (TP) and nitrate contents in endive (C. endivia) leaves.

| Var. crisum (curly) | Site  | ORAC μmol TE 100 g−1 FW | TPC mg GAE 100 g−1 FW | Nitrate mg kg−1 FW |
|---------------------|-------|------------------------|-----------------------|-------------------|
| “Domari”            | L     | 1571.4 ± 320.6a         | 46.3 ± 11.8b          | 941.0 ± 146.3ab   |
| “Myrna”             | L     | 1107.4 ± 246.6a         | 30.8 ± 7.4b           | 1332.0 ± 193.5a   |
| “Domari”            | A     | 893.1 ± 220.2b          | 76.1 ± 3.7a           | 693.3 ± 242.4b    |
| Significance        |       | **                     | **                    | *                 |

| Var. latifolium (smooth) | Site | ORAC μmol TE 100 g−1 FW | TPC mg GAE 100 g−1 FW | Nitrate mg kg−1 FW |
|-------------------------|------|------------------------|-----------------------|-------------------|
| “Flester”               | L    | 1273.9 ± 409.8a        | 39.1 ± 10.5b          | 1759.7 ± 148.2a   |
| “Confiance”             | L    | 1394.5 ± 314.1a        | 39.4 ± 6.9b           | 1357.3 ± 267.8ab  |
| “Flester”               | A    | 584.3 ± 105.2b         | 96.7 ± 5.7a           | 980.0 ± 92.2b     |
| Significance            |      | **                     | **                    | **                |

1 Cultivation site: A: Apulia; L: Lazio.
2 Mean ± standard deviation; different superscript letters indicate significant differences among genotypes within botanical varieties; ** Significant at p ≤ 0.05 and p ≤ .01, respectively.
adult) according to the Panel on Contaminants in the Food Chain of the European Food Safety Authority (EFSA Panel 2008). Hence, the nitrate amount coming from a serving size of 100 g “Flester” leaves grown in Lazio is far lower than the mentioned intake.

**Principal component analysis (PCA)**

All the data regarding the nutritional and chemical parameters were subjected to PCA. The first two principal components (PCs) accounted for more than 83% of the total variance, respectively, 63.12 and 20.02% in PC1 and PC2 (Figure 3). As for the PC1, positive values showed high correlation with TPC and campesterol, consistently with the ANOVA results (Tables 3 and 4), while the negative ones correlated with total sterols, sitosterol, stigmasterol and nitrates. The variables were discriminant for the two planting sites, allowing a clear distinction of the genotypes from Apulia and Lazio, respectively sited on the right and left of the PC1. Focusing on cultivars, the curly type “Domari” was separated from the smooth-type “Flester” (respectively, upon and below the PC1 axis) regardless of the site, mainly due to the higher ORAC value of “Domari” and the higher nitrate content of “Flester,” consistently with the ANOVA results (Table 4). Finally, high positive correlation occurred between nitrate versus total-, sito- and stigmasterol contents, suggesting a link between nitrate (e.g. transport/accumulation) and PS pathways (e.g. synthesis/interconversion). Nitrogen supply to rapeseed (Gul et al. 2007) and maize (Pavlík et al. 2010) could modify the content of β-sitosterol; the latter functions to reinforce membranes and control permeability in response to alterations caused by nitrate ion variations (Schaller 2004).

**Chicory stems**

**Sterol content**

The total sterol content (Table 5) was comparable in “Galatina” and “Molfettese” (101.5–111.7 versus

![Figure 3. PCA biplot describing the spatial distribution of the nutritional parameters of four endive (C. endivia) cultivars grown in Lazio (L) and Apulia (A). DM (dry matter), TPC (total phenolic content). PCA was performed on fresh basis data.](image)

**Table 5. Sterol content in stems of “Catalogna” landraces (C. intybus).**

| Landraces      | Site | Total  | Sitosterol | Stigmasterol | Campesterol | Cholesterol | Fucosterol |
|---------------|------|--------|------------|--------------|-------------|-------------|------------|
| “Galatina”    | A    | 101.5 ± 13.9 | 77.1 ± 12.4 | 14.2 ± 4.9a | 3.9 ± 0.9   | 0.4 ± 0.3   | 5.9 ± 1.9b |
| “Galatina”    | L    | 111.7 ± 16.0 | 74.6 ± 15.0 | 16.9 ± 2.8a | 4.0 ± 1.1   | 0.5 ± 0.1   | 15.7 ± 5.8a |
| “Molfettese”  | A    | 102.5 ± 20.8 | 79.6 ± 16.7 | 11.7 ± 2.0b | 3.0 ± 1.3   | 0.3 ± 0.3   | 7.7 ± 1.8b  |
| “Molfettese”  | L    | 118.6 ± 17.1 | 87.2 ± 10.2 | 17.2 ± 2.4a | 3.5 ± 0.5   | 2.9 ± 3.6   | 8.9 ± 4.1b  |

| Significance  |      |        | **   |      |      | **   |      |
|---------------|------|--------|------|------|------|------|------|
| Cultivation site: A: Apulia; L: Lazio.
| Mean ± standard deviation; different letters within the same column indicate significant differences between landraces; ns: nonsignificant; **significant at p ≤ .01.
102.5–118.6 mg kg\(^{-1}\) FW). Sitosterol (Figure 2(B)) was the most abundant fraction (ranges 73–76%), followed by stigmasterol (12–14%), fucosterol (8–9%), campestrol (3–4%) and cholesterol (1%). Different environments (Apulia versus Lazio) affected both the stigmasterol content in “Molfetta” (11.7 versus 17.2 mg kg\(^{-1}\) FW) and the fucosterol amount in “Galatina” (5.9 versus 15.7 mg kg\(^{-1}\) FW) stems. Sterol content, composition and ratio among fractions vary with organ type and growth stage within plant species (Moreau et al. 2002). Referring to literature data available, the composition and ratio among fractions vary with organ type and growth stage within plant species (Moreau et al. 2002).

Fucosterol, which has been attributed antidiabetic (Choi et al. 2015), and is remarkably abundant in macroalgae (30–200 mg kg\(^{-1}\) dry matter), which are a natural major source (Lopes et al. 2011). Interestingly, fucosterol levels in “Catalogna” stems can range from 100 to 250 mg kg\(^{-1}\) DW (conversions based on Supplementary Table S2) and represent an alternative to edible algae from a nutritional standpoint. Finally, a daily portion of 100 g of chicory stems provides an intake of 10 mg of PS, a slightly smaller value than the daily portion of 100 g of chicory stems provides an intake of 10 mg of PS, a slightly smaller value than the first two PCs explained more than 88% of the total variance, assigning 62.26 and 25.80% to PC1 and PC2, respectively (Figure 4).

**Table 6.** ORAC, total phenolic (TPC) and nitrate contents in stems of “Catalogna” landraces (*C. intybus*).

| Landraces | Site\(^1\) | ORAC \(\mu\)mol TE 100 g \(^{-1}\) FW | TPC mg GAE 100 g \(^{-1}\) FW | Nitrate mg kg\(^{-1}\) FW |
|-----------|-----------|---------------------------|-----------------|---------------------|
| “Galatina” | A         | 1152.5 ± 228.1\(^a\)       | 57.2 ± 7.5\(^c\) | 275.3 ± 39.0\(^b\) |
| “Molfetta” | A         | 953.0 ± 120.1\(^b\)       | 56.2 ± 7.3\(^c\) | 537.9 ± 20.1\(^a\) |
| “Galatina” | L         | 729.8 ± 148.3\(^b\)       | 102.3 ± 3.1\(^b\) | 213.2 ± 54.9\(^c\) |
| “Molfetta” | L         | 745.2 ± 125.8\(^a\)       | 124.4 ± 4.1\(^a\) | 143.6 ± 51.8\(^c\) |

\(^{1}\)Cultivation site: A: Apulia; L: Lazio.

**Antioxidant capacity, total phenolic and nitrate contents**

The AC ranged from ca. 729.8 to 1152.5 ORAC units (Table 6), and “Galatina” and “Molfetta” showed comparable values within a given site. Consistently, no significant differences were measured between these two genotypes by using a different AC method in Apulia (Renna et al. 2014). Planting area diversity caused changes of ORAC values, which were lower in Lazio than Apulia for both landraces (though significantly just for “Galatina,” \(F = 12.3, p < .01\), hinting at the occurrence of environment–genotype interactions.

The average TPC (Table 6) increased from 56.2 in Apulia to 124.4 mg GAE 100 g \(^{-1}\) FW in Lazio, indicating that the growth site had a significant impact. Referring to Apulian products, the contents were comparable between the two landraces sampled in January in this work (ca. 8.4–8.6 GAE g\(^{-1}\) DW, see Supplementary Table S2 for dry and fresh weight ratios), but lower than comparable stems (ca. 18 GAE g\(^{-1}\) DW) harvested from February to May (Renna et al. 2014). The value differences are consistent with the notion that the TPC of vegetables varies with environment changes (Oh et al. 2011). Finally, no correlation was found between ORAC values and TPC (\(r = .5, p < .001\)), differently from another work based on pooled material (leaves and stems) of “Catalogna” chicory (Montefusco et al. 2015). The phenols extractable fraction of chicory-stem produced 90–200 mg GAE (on dry weight basis, see Table 6 and Table S2 for conversions) for a daily serve of 100 g edible product, which significantly contributes to the Mediterranean diet (Saura-Calixto et al. 2007).

Overall, the nitrate levels ranged from 143.6 to 537.9 mg kg\(^{-1}\) FW; these values were consistent with those observed in shoots of other “asparagus chicory” and a serving size of 100 g can be considered not harmful, as it is ca. the 15% of the above reported ADI, ascribing the stems of these chicory landraces to low-nitrate content vegetables (Santamaria et al. 1999). The flowering process (induction, timing etc.) is associated with low nitrogen levels (Castro Marín et al. 2011); this may account for the low nitrate contents in “Catalogna” stems, which are shoots programed to produce flowers. “Molfetta” had a greater content than “Galatina” only in the Apulian area (Table 6) in agreement with a previous study (Renna et al. 2014), while no difference was scored in Lazio, suggesting that “Galatina” genotype–environment interactions may have been less influenced in the nitrate metabolism than those of “Molfetta.”

**Principal component analysis (PCA)**

The first two PCs explained more than 88% of the total variance, assigning 62.26 and 25.80% to PC1 and PC2, respectively (Figure 4).
separated the Apulia from the Lazio sites, respectively placed on the left and right side of PC1. Moreover, PC1 correlated positively with total sterols and TPC, and negatively with ORAC, nitrate and dry matter, placing the genotypes from Apulia in the left quadrants. The separation between “Galatina” and “Molfettese” was neat, the former and the latter showed, respectively, positive and negative correlations with the PC2 (up- and down-stream quadrants, Figure 4). The “Galatina” versus “Molfettese” differences were higher in the Lazio versus Apulia sites. Specifically, higher fucosterol in “Galatina” versus higher TPC and sitosterol levels in “Molfettese” were mostly responsible for the differences observed in Lazio (Figure 4 and Tables 5 and 6).

Conclusions
Sterols, total phenolic and nitrate contents, as well as antioxidant capacity were assessed in curly and smooth-endive cultivars and in stem-chicory landraces cultivated in central and southern Italy. As for sterols, the contents were comparable among smooth and curly endives within a given area. Sitosterol levels varied significantly in both “Domari” and “Flester” when the sites changed. The total sterol level in chicory stems was mainly independent of landrace and cultivation site, though the latter played effects on the distribution of stigmasterol and fucosterol. The high content of the latter might confer great value to these novel products diabetes-associated diets. A daily portion of 100 g of endive leaves and chicory stems provide mean intakes of 10–22 and 10 mg/day of PS, respectively. Geo-localization was also the major cause of ORAC and TPC variation in both species and the value ranges were comparable in both endive and chicory products. A daily portion of 100 g/person of endive and chicory stems contribute with 55–120 and 90–200 mg GAE from TPC extractable fraction, respectively. The examined vegetables did not show dangerous nitrate levels; smooth endives maintained higher contents than curly ones, though below the recommended thresholds. The PCA could depict a neat separation between the two cultivation sites for both endive and chicory and allowed clustering of genotypes.

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The authors report no conflicts of interest and are responsible for the content of this article.

Figure 4. PCA biplot describing the spatial distribution of the nutritional parameters of two C. intybus genotypes cultivated in Lazio (L) and Apulia (A). DM (dry matter), TPC (total phenolic content). PCA was performed on fresh basis data.
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