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Polyphenolic profile of *Cichorium intybus* L. endemic varieties from the Veneto region of Italy

Roberta Tardugno[a], Michele Pozzebon[b], Marica Beggio[b], Pierantonio Del Turco[b], Giulio Pojana[a,*]

[a] Department of Philosophy and Cultural Heritage, University Ca' Foscari of Venice, Dorsoduro 3484/D, 30123 Venice, Italy
[b] DtoLabs, via Fratta, 25, 31023 Resana, TV, Italy

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ABSTRACT

The present study was aimed at the investigation, through HPLCDAD-ESI-MS/MS, of polyphenols in seven autochthonous *C. intybus* varieties, already known from literature to contain various substances with antioxidant properties, from the Veneto region of Italy, namely 'Castelfranco', 'Chioggia', 'Rosa di Gorizia', 'Rosa di Verona', 'Treviso Precoce', 'Treviso Tardivo' and 'Verdon da Cortèl'. Thirteen polyphenols, belonging to hydroxycinnamic acid, flavone, flavonol and anthocyanin classes, were detected in most samples. The developed analytical method was validated in agreement with ICH guidelines. The total amount of polyphenols ranged from 52 to 386 (mean: 254) mg/100g fresh weight (F.W.). The results were further confirmed by Principal Composition Analysis (PCA), which highlighted peculiar features and similarities among analysed samples for each variety (except for 'Chioggia' samples). The developed method is suitable for routine analyses, as well as geographical characterization, selection of different *C. intybus* varieties and for the determination of related polyphenols dietary recommended intakes.

1. Introduction

Food vegetables are a significant part of human diet worldwide from ancient times. In this context, the Mediterranean diet is recognized as an invaluable source of health benefit constituents and represents one of most peculiar examples of traditional cuisine with many dishes rich in healthy vegetables (Guarrera & Savo, 2016, 2013).

Among traditional Italian vegetables, chicory (*Cichorium intybus* L.) represents an interesting example of a traditional plant with health benefits, as already reported from historical documents: firsts evidences were reported in I century A.D. by Pliny the Elder in his *Naturalis Historiae* (Book 19, Chapter 38), as well as by Dioscoride, and later by Galeno, who described the application of leaf infusions for various diseases (Mulinacci et al., 2001).

Chicory is a diploid plant species belonging to the Asteraceae family, including about one hundred genera according to Bischoff's classification, the most common being var. *silvestre*, var. *sativus*, and var. *foliosum* (Mulinacci et al., 2001). It is an erect fairly woody perennial herb, around 1 m in height, with a fleshy taproot of up to 75 cm in width, with large basal leaves. The name derives from both Greek and Latin: *cichorium* meaning field and *intybus* partly deriving from the Greek verb "to cut", referred to its leaves, and partly from the Latin term *tubus,* indicating its hollow stem (Al-Snafi, 2016; EMA, 2013; Street, Sidana, & Prinsloo, 2013).

Many varieties of *C. intybus* var. *foliosum*, also known as 'Red chicory' or 'Radicchio' in Italian, are widely cultivated and consumed as

* Corresponding author.
E-mail address: jp@unive.it (G. Pojana).

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a raw salad or stewed in wintertime, when most vegetables are not available, in the Veneto region of Italy, since their peculiar resistance to low temperatures (Rossetto et al., 2005). Veneto is the Italian region with most varieties and cultivars, and with the largest cultivated land, about 7800 ha (Veneto Agricoltura, 2017). Moreover, in the Veneto region the variety '*Treviso Tardivo*', a typical red leaf winter salad, earned both Protected Geographical Indication (PGI) and Protected Designation of Origin (PDO) status due to its organoleptic characteristics (Carazzone, Mascherpa, Gazzani, & Papetti, 2013).

C. intybus is currently attracting the attention of both researchers and consumers as an Italian traditional product with potential antioxidant properties, and exhibiting in the last years an increased demand in amount and quality. Many phytochemical constituents of *C. intybus* have been already identified, such as flavonoids, anthocyanins, caffeic acid derivatives, sesquiterpene lactones, coumarins, triterpenoids, phytosterols, inulins and lactucin-like guaianolides (Ferioli, Manco, & D'Antuono, 2015; Carazzone et al., 2013; Guarrera & Savo, 2013; Street et al., 2013; Mulabagal, Wang, Ngouajio, & Nair, 2009; Papetti et al., 2008). These substances are known to exhibit antioxidant, antimalarial, anti-inflammatory, antiproliferative, cytotoxic, analgesic, sedative, anti-hepatotoxic and hypoglycaemic bioactive properties (Street et al., 2013; Heimler, Isolani, Vignolini, & Romani, 2009; Rossetto et al., 2005; Costa et al., 2017).

The antioxidant and radical scavenger activity of polyphenolic compounds is well documented (Street et al., 2013; Leopoldini, Russo, & Toscano, 2011). About 60 polyphenolic constituents have been identified in *C. intybus* so far, belonging to hydroxycinnamic acids, flavonoids and anthocyanins (in red varieties) main classes (Ferioli et al., 2015; Carazzone et al., 2013; Street et al., 2013; Heimler et al., 2009; Innocenti et al., 2005).

Several analytical methods have been described in the literature for the identification and quantification of bioactive ingredients in chicory leaves. Focusing on polyphenols, they were usually extracted from fresh red leaves by acidified alcoholic solvent extraction followed by centrifugation. Various purification procedures of obtained extracts before analysis and (photo)oxidation of polyphenols were also carefully taken into consideration by various researchers (Cefola, Carbone, Minasi, & Pace, 2016; Carazzone et al., 2013; Innocenti et al., 2005; Rossetto et al., 2005). Some authors experimented also lyophilisation technique followed by ultrasonic assisted extraction (UAE) (Sinkovič et al., 2015) or reflux procedures (Zhu et al., 2015).

High-Performance Liquid Chromatography with Diode Array (HPLC-UV/DAD) and Electrospray Ionization-Mass Spectrometry detection (ESI-MS and MS-MS) are the techniques of choice for the quali- and quantitative determination of bioactive ingredients such as polyphenols with C_{18} as stationary phase, and water/methanol or water/acetonitrile acidified with formic acid as mobile phases under linear gradients followed by column purging (Cefola et al., 2016; Sinkovič et al., 2016; Carazzone et al., 2013; Innocenti et al., 2005).

Despite the large number of studies on *C. intybus* chemical composition, no method applicable to all *C. intybus* leaf varieties, such as green, green-red spotted, pink and red, with their differences in morphology and polyphenol profiles, was proposed so far, and there is no validated analytical method, to the best of our knowledge, for the simultaneous analysis of all pholyphenols from different *C. intybus* cultivars.

The aim of this study was the investigation of the major antioxidant phenolic compounds in autochthonous *C. intybus* cultivars in the Veneto region of Italy, since this is the region with the greatest variety in cultivars. Additionally, this study aims to analyse for the first time the phenolic profile of some less known endemic varieties.

Moreover, Principal Ccomponent Analysis (PCA) was employed as a chemometric tool to highlight the peculiar characteristics of each *C. intybus* investigated variety and to compare the profiles between them.

A validation of the developed method by means of HPLC-UV/DAD according to the International Conference of Harmonization (ICH) guidelines was also performed.

The proposed tool can be applied in further investigations, such as the selection of *C. intybus* varieties richer in antioxidant polyphenols, the routine quality control of raw vegetable materials, standardized extracts, dietary supplement and the evaluation of the authenticity of *C. intybus* and *C. intybus*-based products.

2. Materials and methods

2.1. Chemicals and solvents

Chlorogenic acid, chicoric acid, cyanidin chloride, kaempferol-3-glucoside and quercetin-3-glucoside, were provided by Sigma-Aldrich (Milan, Italy). Formic acid (HCOOH) and HPLC-MS grade solvents, such as acetonitrile (ACN), methanol (MeOH) and acetone, were from Sigma-Aldrich (Milan, Italy). Water for chromatographic separation was obtained from an Arium® Pro-system (Sartorius, Goettingen, Germany).

2.2. Cichorium intybus samples

Seven autochthonous varieties of chicory leaves (*Cichorium intybus* L.) cultivated in the Veneto region of Italy were selected and analyzed in this study, including four red leaf varieties ('*Chioggia*', '*Rosa di Gorizia*', '*Treviso Precoce*' and '*Treviso Tardivo*'), one pink leaf variety ('*Rosa di Verona*'), one red spotted green leaf variety ('*Castelfranco*') and one green leaf variety ('*Verdon da Cortèl*').

The '*Castelfranco*', '*Treviso Precoce*' and '*Treviso Tardivo*' varieties are cultivated according to the national PGI (Protected Geographical Indication) label disciplinary procedures.

The '*Rosa di Gorizia*', '*Treviso Precoce*' and '*Treviso Tardivo*' varieties received the traditional post-harvest blanching treatment applied in order to obtain the typical white and red veined leaves appearance.

The '*Chioggia*' variety, currently the most widespread commercial cultivar on the Italian market, was bought directly from a local supermarket, and it was also selected for the method development.

All other *C. intybus* samples were kindly provided by 'MG Radicchio' farm (Ponzano Veneto, TV, Italy). Samples were collected in winter 2016/2017 (from November to March) according to each specific variety sampling time. Freshly harvested samples were rapidly cooled at +4 °C and stored at dark for max. 12 h before extraction as described hereafter.

2.3. Extraction of polyphenols

A blend of external, medium and internal leaves was generated by manually cutting off fresh samples; 5 g of each generated blended sample were immediately extracted by maceration for 16 h at room temperature at dark with 25 mL of a MeOH:HCOOH (99:1) mixture.

Each sample was then filtered with a Whatman (Maidstone, United Kingdom) No. 1 paper filter. Each filtrate was brought to 25 mL with MeOH:HCOOH (99:1 vol:vol) into a volumetric flask. The diluted extract was then filtered again with a 0.45 μm cellulose acetate syringe filter (Whatman) into an HPLC vial and then stored at +4 °C at dark before injection (within 12 h from extraction) into the HPLC system.

2.4. HPLC-UV/DAD analysis

HPLC-UV analyses were performed with an Agilent Technologies (Waldbronn, Germany) 1290 Infinity HPLC system, consisting of a vacuum degasser, a binary pump, a thermostated autosampler compartment maintained at 10 °C, a thermostated column compartment maintained at 25 °C and a UV–Vis Diode Array Detector (UV-DAD). The chromatograms were collected and analyzed by using an Agilent Chemstation Rev. B.04.03.

The optimal HPLC separation conditions were developed by testing various stationary phases (Poroshell C18 SB 120, 2.1 × 100 mm,

Table 1
Compounds identified in *C. intybus* by HPLC-UV/DAD and HPLC-MS/MS (positive and negative mode).

Peak number	Compound name	Chemical class	t_R (min)	λ_{max} (nm)[a]	Precursor ion (*m/z*)[b]	Product ions (*m/z*)
1	caftaric acid (*cis* + *trans*)	hydroxycinnamic acid	4.05	220, 290, 330	311	179, 149, 135
2	caffeoylquinic acid (3-, 4-, 5-)	flavonol	6.71	220, 240(sh), 330	353	191, 179
3	cyanidin-3-O-glucoside	anthocyanin	8.24	280, 520	449(+)	287
4	caffeoylmalic acid	hydroxycinnamic acid	10.79	220, 240(sh), 330	295	179, 135, 133
5	cyanidin-3-O-(6″-O-malonyl)-glucoside	anthocyanin	13.75	280, 520	535(+)	287
6	chicoric acid	hydroxycinnamic acid	17.11	220, 240(sh), 330	473	311, 293, 179, 149
7	quercetin glucuronide (3-O; 7-O)	flavonol	17.61	254, 340	477	301, 179,151
8	quercetin-3-O-glucoside	flavonol	17.87	254, 340	463(+)	301, 179, 151
9	luteolin-7-O-glucuronide	flavone	19.81	256, 266(sh), 348	461	285
10	quercetin-3-O-(6″-O-malonyl)-glucoside	flavonol	20.48	266, 348	549	505, 301
11	kaempferol-3-O-glucoside	flavonol	20.81	265, 350	447	285
12	dicaffeoylquinic acid (1,4-; 3,5-)	hydroxycinnamic acid	22.91	220, 240(sh), 330	515	353, 191
13	kaempferol-3-O-(6″-O-malonyl)-glucoside	flavonol	24.15	265, 350	535(+)	287

[a] (sh): shoulder.
[b] (+):positive mode.

2.7 μm; Zorbax SB-Aq, 2.1 × 100 mm, 1.8 μm, and Zorbax Eclipse Plus Phenyl Hexyl, 2.1 × 100 mm, 1.8 μm) and mobile phases (MeOH/water/formic acid and ACN/water/formic acid mixtures).

Finally, the analyses were carried out on a Zorbax Eclipse Plus Phenyl Hexyl 100 × 2.1 mm I.D., 1.8 μm (Agilent, Waldbronn, Germany) column at a 0.3 mL/min flow rate. The mobile phase was composed of 0.1% (vol:vol) HCOOH in H_2O (A) and 0.1% (vol:vol) HCOOH in ACN (B). The separation was achieved by using a gradient elution as follows: 0–25 min from 5 to 20% B, 26–35 min from 20 to 99% B, 35–40 min 99% B. A 10 min post-run time at 99% B was then applied for cleaning the column. Three μL of sample extract were mixed with 3 μL of H_2O in the injection loop by the injector program before being injected in the HPLC column.

The UV/DAD acquisitions were carried out in the 200–600 nm range, while chromatograms were acquired at 280 nm (overall chromatogram), 340 nm (for hydroxycinnamic acids, flavonols and flavones) and 520 nm (for anthocyanins), as proposed by the literature (Cefola et al., 2016; Sinkovič et al., 2015; Heimler et al., 2009; Innocenti et al., 2005). Three injections were performed for each sample extract.

2.5. HPLC-MS/MS analysis

HPLC-MS/MS analyses were performed by using an Agilent (Waldbronn, Germany) modular 1290 Infinity II system equipped with a vacuum degasser, a binary pump, a thermostated autosampler, a thermostated column compartment, coupled with an Agilent 6470 triple quadrupole mass analyzer with a Jet Stream electrospray ionization (ESI) ion source. The HPLC column and the applied chromatographic conditions were the same as reported above for the HPLC-UV/DAD system.

The parameters in the source were set as follows: drying gas temperature: 210 °C; gas flow rate: 11 L/min; sheath gas temperature: 350 °C; sheath gas flow rate: 12 L/min; nebulizer: 35 psi; capillary voltage: 4000 V; nozzle voltage: 500 V.

The collision energy (CE) and the fragmentor voltage were optimized for each compound class by infusing standard solutions in MeOH directly into the mass spectrometer. The analyte detection was performed in both positive and negative ion modes by Multiple Reaction Monitoring (MRM).

Data acquisition and processing were performed using Agilent MassHunter Workstation software version B.07.00.

2.6. HPLC-UV/DAD method validation

The validation of the HPLC-UV/DAD method was performed in agreement with the international guidelines for analytical techniques in the quality control of pharmaceuticals (International Conference of Harmonization, 2005).

Linearity was evaluated as follows: stock standard solutions of each compound (3-caffeoylquinic acid, chicoric acid, cyanidin chloride, kaempferol-3-glucoside and quercetin-3-glucoside) were prepared by accurately weighting 1.7–2.2 mg of standard into a 5 mL volumetric flask). The standards were brought to volume with the extraction solvent (MeOH:HCOOH 99:1, vol:vol). External standard calibration curves were generated with six data points. All injections were performed in triplicate for each concentration level. The calibration curve was generated by plotting the peak area of each compound versus its concentration level.

The concentrations of analytes in *C. intybus* extracts for which standard were not available, such as hydroxycinnamic acid derivatives, and some flavonols, flavones and anthocyanins, were determined by using the calibration curves of selected standard compounds containing the same chromophore.

Limits of detection (LODs) and of quantification (LOQs) were experimentally determined for reference standards, by serial dilutions of their standard solutions until reaching a signal-to-noise (*S/N*) ratio of 3 and 10, respectively.

The accuracy of the analytical method was evaluated by means of the Recovery test. This involved the addition of a known quantity of standard compound to half of the sample weight of the reference sample (‘*Chioggia*’ variety). The fortified samples were then extracted and analyzed according to the developed method.

The precision of the extraction technique was validated by repeating six times the extraction procedure on an extract of the same chicory sample (‘*Chioggia*’ variety) and analyzing the extracts as described. The precision of the chromatographic system was tested by performing intra- and inter-day multiple injections (six injections each day for three consecutive days) of one extract (‘*Chioggia*’ variety) and then calculating the % Relative Standard Deviation (% RSD) of retention times and peak areas of each identified peak.

2.7. Matrix effect

The matrix effect (%ME) is the contribution of all components present in the extract on the chromatographic signal intensity of each analyte; it was evaluated for all reference standards employed (one standard per each chemical class). The %ME is expressed as a factor according to the following equation (Brighenti et al., 2017; Matuszewski, Constanzer, & Chavez-eng, 2003):

$$ME(\%) = B/A \times 100$$

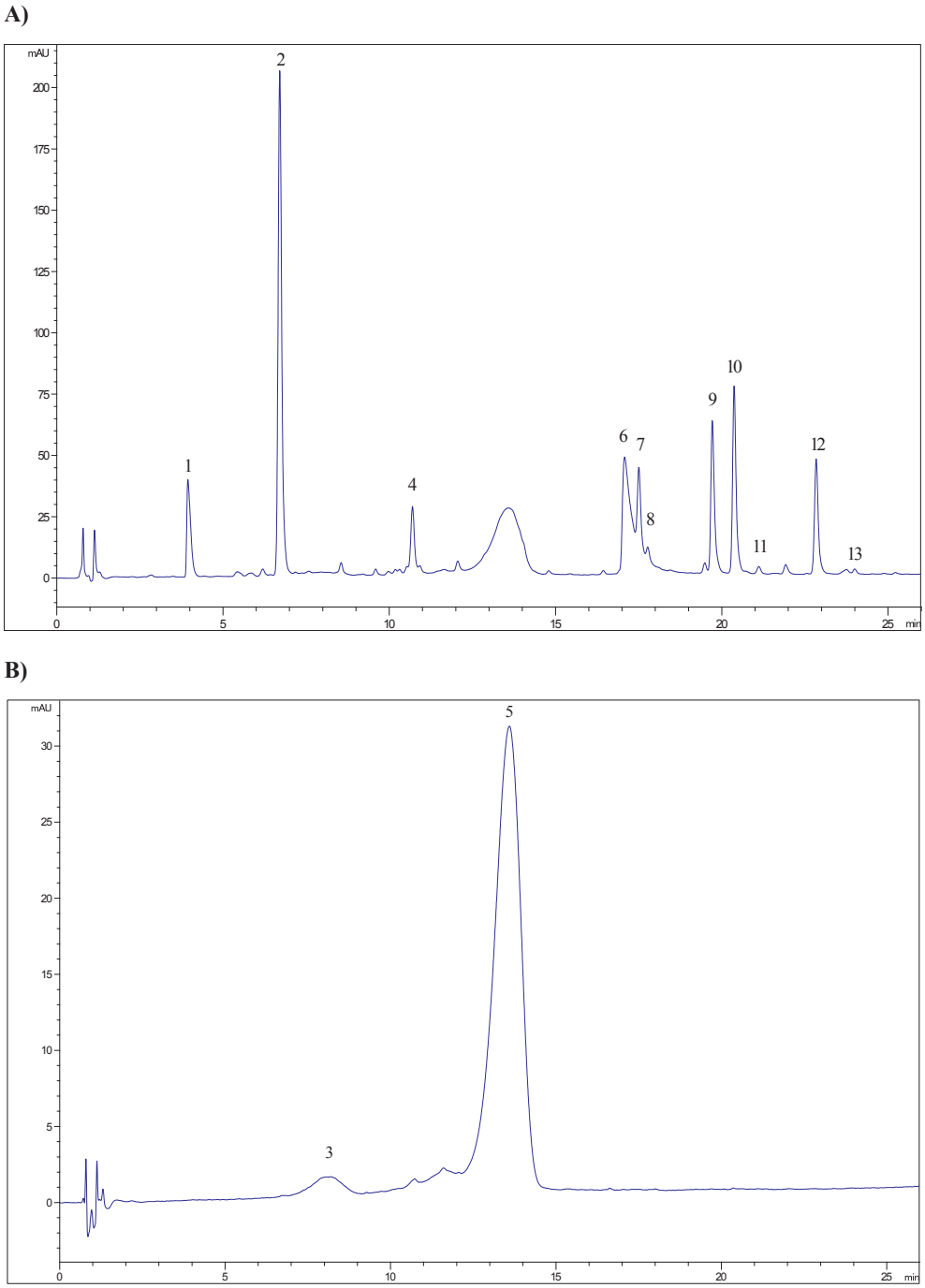

Fig. 1. Chromatograms obtained by HPLC-UV/DAD analysis of *C. intybus* var. 'Chioggia' at 280 nm (A) and 520 nm (B), respectively. See Table 1 for peak identification.

where A is the chromatographic peak area of the standard in its neat solution and B is the peak area of the (same injected amount) standard spiked into the matrix extract. According to the quali- and quantitative results obtained in this study, the sample '*Castelfranco*' was selected as the most appropriate matrix for the evaluation of matrix effect since it exhibited the lowest concentrations (< LOQ for almost all analytes) of polyphenols.

2.8. Robustness

The robustness evaluates the integrity of the developed analytical method by comparing system parameters variations obtained by employing deliberate changes. In the present study, the effect of column temperature (± 10%) and the effect of flow rate (± 10%) were considered for the HPLC method robustness as proposed by the literature (Fuad Al-Rimawi, 2014).

2.9. Statistical analysis

The results obtained by HPLC-UV/DAD analyses were submitted to multivariate statistical analysis by means of Principal Component Analysis (PCA) to obtain more information about the individual constituents contribution, by using the freeware R-based chemometric software developed by the Chemometrics Group of the Italian Chemical

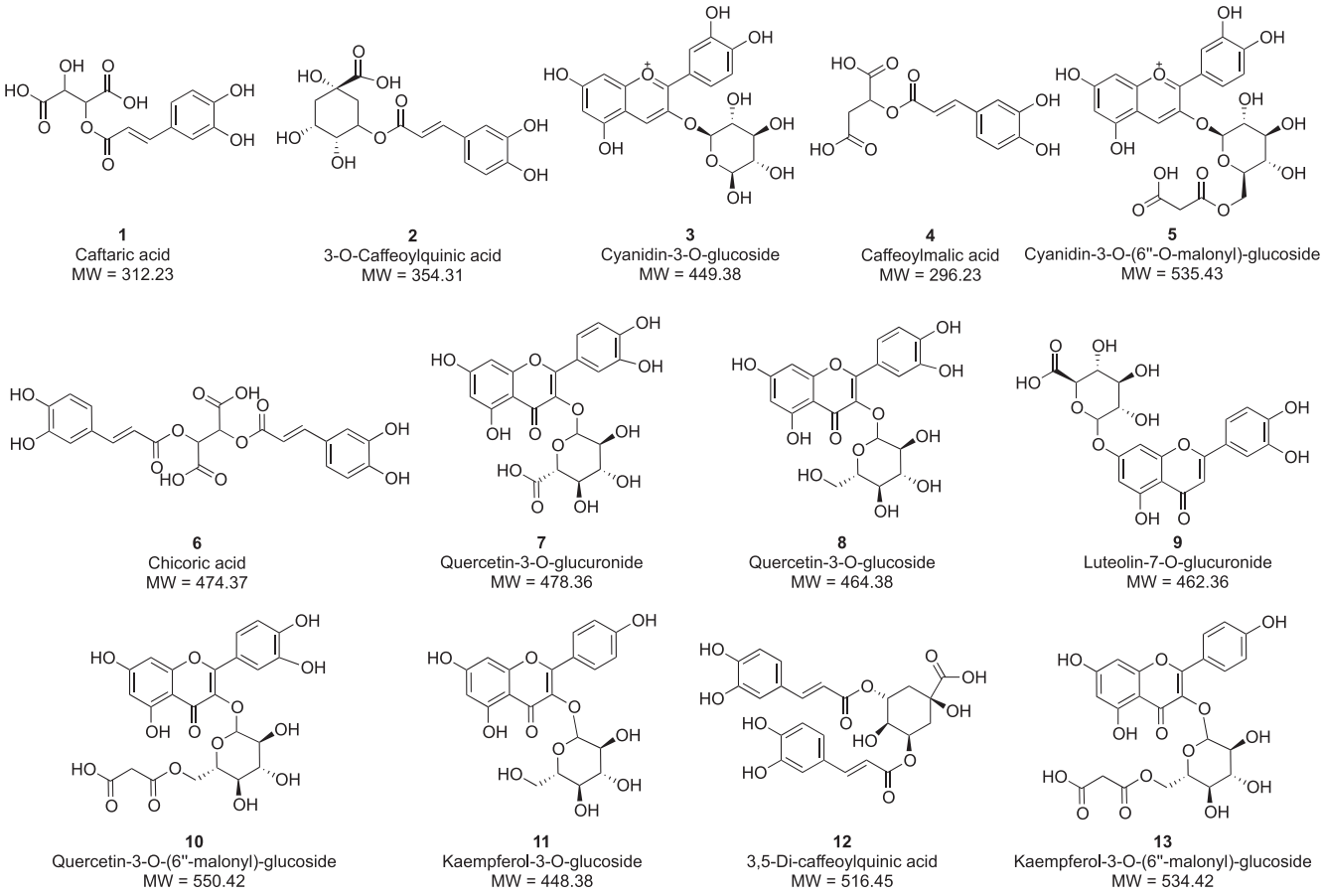

Fig. 2. Chemical structures of the 13 compounds analysed in *C. intybus* varieties in this study.

Table 2
Linearity and sensitivity data under HPLC-UV/DAD for compounds used as standards in this study. Experimental conditions as in Section 2.6.[a]

Compound	Linearity range (μg/mL)	Slope (*a*)	Intercept (*b*)	r^2	LOD (μg/mL)	LOQ (μg/mL)
3-caffeoylquinic acid	8.4–420.0	24.11 (± 0.15)	47.48 (± 28.82)	0.9994	1.20	4.01
cyanidin chloride	1.7–340.0	45.47 (± 0.28)	–120.91 (± 39.86)	0.9994	1.44	4.70
cichoric acid	18.32–440.0	41.90 (± 0.28)	–602.23 (± 58.20)	0.9993	8.39	27.97
quercetin-3-glucoside	8.8–400.0	19.31 (± 0.16)	22.32 (± 21.73)	0.9994	1.20	4.14
kaempferol-3-glucoside	6.8–340.0	18.30 (± 0.12)	–42.25 (± 19.52)	0.9993	1.42	4.74

[a] For each calibration curve the equation is $y = ax + b$, where y is the peak area, x the concentration of the analyte (μg/mL), *a* is the slope, *b* is the intercept and r^2 the correlation coefficient. Standard error (S.E.) values are given in parenthesis.

Society, Analytical Chemistry Division (Brighenti et al., 2017). For each sample, all quantified polyphenols were considered.

3. Results and discussion

3.1. Optimization of chromatographic conditions and sample preparation

The best separation was obtained using a ACN/H_20 (both with 0.1% formic acid) mobile phase on a Zorbax Eclipse Plus Phenyl Hexyl thermostated at 25 °C under a gradient elution at a flow rate of 0.3 mL/min, with clear advantages on other tested conditions in term of separations efficiency analysis time, solvent consumption reduction and ESI-MS intensity signal; it's worth noticing the different elution order of target polyphenols exhibited by the Phenyl-Hexyl column, in comparison with the C18 Poroshell and the Sb-Aq stationary phases, attributable to additional π–π interactions typical of this stationary phase (Tomaz & Maslov, 2016; Mulinacci et al., 2001).

Optimal extraction conditions were searched for, including different starting sample materials (fresh leaves, frozen leaves and lyophilized leaves), solvents (methanol, ethanol, isopropanol and acetone), extraction procedures (static maceration, dynamic maceration and ultrasonication) and extraction time (30 min, 6 h, 16 h). Formic acid was added to the extraction mixture in view of the presence of anthocyanins, which can be better extracted under acidic conditions, as reported by literature (Brighenti et al., 2017; Mulabagal et al., 2009). A static maceration of fresh leaves in a MeOH:HCOOH (99:1, vol:vol) solution for 16 h at dark proved to be the most efficient method.

3.2. Polyphenols identification

As shown in Table 1 and Figs. 1 and 2, thirteen polypenols were identified in the examined samples by comparing their retention times, UV–Vis spectra, MS-MS spectra reported by previous studies and, when possible, by co-injection of related reference compounds and sample extracts (Cefola et al., 2016; Sinkovič et al., 2015; Carazzone et al., 2013; Heimler et al., 2009; Lavelli, 2008; Heimler, Isolani, Vignolini,

Table 3
Concentration levels of polyphenols in *C. intybus* varieties by HPLC-UV/DAD, expressed as mg/100 g fresh weight (FW).[a]

Peak n.	Compound	Castelfranco[a]	Chioggia[a]	Rosa di Gorizia[a]	Rosa di Verona[a]	Treviso Precoce[a]	Treviso Tardivo[a]	Verdon da Cortèl[a]
1	caftaric acid (*cis* + *trans*)	5.49 ± 4.1	< LOQ	22.27 ± 16.8	5.57 ± 2.5	1.49 ± 1.3	10.68 ± 5.0	30.18 ± 9.3
2	caffeoylquinic acid (3-, 4-, 5-)	4.75 ± 3.0	79.75 ± 24.2	95.33 ± 29.5	51.52 ± 31.3	13.29 ± 8.3	51.41 ± 17.9	56.72 ± 16.6
3	cyanidin-3-O-glucoside	< LOD	6.05 ± 2.1	3.62 ± 0.7	n.d.	5.66 ± 1.6	3.10 ± 0.7	n.d.
4	caffeoylmalic acid	< LOD	5.85 ± 4.2	1.92 ± 1.0	9.00 ± 2.3	< LOQ	< LOD	< LOD
5	cyanidin-3-O-(6″-O-malonyl)-glucoside	< LOQ	51.15 ± 23.5	26.05 ± 7.1	3.77 ± 0.8	33.82 ± 8.0	16.87 ± 5.5	n.d.
6	chicoric acid	29.85 ± 3.7	30.37 ± 14.2	68.37 ± 13.4	97.22 ± 15.1	37.66 ± 9.5	48.03 ± 11.6	52.55 ± 27.4
7	quercetin glucuronide (3-O; 7-O)	< LOD	16.96 ± 5.6	16.72 ± 9.0	n.d.	16.92 ± 7.2	12.45 ± 4.5	26.88 ± 19.6
8	quercetin-3-O-glucoside	< LOD	7.34 ± 2.8	5.54 ± 3.6	5.48 ± 8.7	10.38 ± 2.4	4.81 ± 1.4	16.83 ± 3.5
9	luteolin-7-O-glucuronide	< LOD	44.79 ± 17.9	16.37 ± 7.2	2.78 ± 1.6	35.57 ± 6.3	9.00 ± 4.4	13.99 ± 11.0
10	quercetin-3-O-(6″-O-malonyl)-glucoside	< LOD	53.09 ± 27.2	67.67 ± 19.8	< LOQ	31.38 ± 12.2	22.70 ± 13.5	6.20 ± 4.2
11	kaempferol-3-O-glucoside	n.d.	n.d.	2.32 ± 1.5	67.55 ± 38.8	< LOQ	< LOQ	23.71 ± 14.2
12	dicaffeoylquinic acid (1,4-; 3,5-)	2.79 ± 5.2	31.49 ± 11.0	19.22 ± 12.2	5.53 ± 5.9	4.19 ± 3.4	33.05 ± 28.4	4.70 ± 2.9
13	kaempferol-3-O-(6″-O-malonyl)-glucoside	n.d.	< LOQ	8.23 ± 2.0	134.88 ± 35.5	2.68 ± 1.3	2.93 ± 0.7	12.37 ± 5.4
	Total	51.57 ± 11.66	329.86 ± 110.75	353.64 ± 81.05	386.22 ± 48.68	195.52 ± 28.72	216.12 ± 74.52	245.19 ± 93.27

n.d. = not detected.
[a] Data are expressed as mean ($n = 6$) ± SD.

Tombelli, & Romani, 2007; Innocenti et al., 2005; Rossetto et al., 2005).

The exhibited UV–Vis spectra were utilized to identify the chemical classes of eluted constituents. The selected analytes, according to their structural characteristics, were divided into four polyphenolic classes: hydroxycinnamic acids, flavonols, flavones and anthocyanins.

Since the phenolic fingerprint of *C. intybus* was deeply investigated in the literature, the analytes selected in the seven variety extracts were identified by HPLC-QqQ analysis under Multiple Reaction Monitoring (MRM) mode. The selection of these thirteen phenolic compounds was achieved investigating all MS-MS spectra, obtained by scanning in both positive and negative ion modes the extracts, of eluted peaks and comparing recorded spectral data with possible transitions reported in the literature regarding known *C. intybus* phenolic constituents (Cefola et al., 2016; Sinkovič et al., 2015; Carazzone et al., 2013; Heimler et al., 2009; Heimler et al., 2007; Lavelli, 2008; Innocenti et al., 2005; Rossetto et al., 2005). Each compound was identified by one qualification transition and by one or (when available) two confirmatory MS-MS transitions. The exhibited HPLC-QqQ fragments compared with the literature data, confirmed the presence of (numbering as in Table 1 and Figs. 1 and 2) hydroxycinnamic derivatives (caftaric acid (1), caffeoylmalic acid (4), 5-O-feruloylquinic acid (5), chicoric acid (7) and dicaffeoylquinic acid (13)), flavonols (caffeoylquinic acid (2), kaempferol glucoside (12), kaempferol malonyl glucoside (14), quercetin glucuronide (8), quercetin glucoside (9) and quercetin malonyl glucoside (11)); anthocyanins cyanidin glucoside (3) and cyanidin malonyl glucoside (6) were also identified; luteolin glucuronide was the only flavone detected (10), (Cefola et al., 2016; Sinkovič et al., 2015; Carazzone et al., 2013; Heimler et al., 2009; Heimler et al., 2007; Lavelli, 2008; Innocenti et al., 2005; Rossetto et al., 2005).

3.3. Method validation

The HPLC-UV/DAD method was fully validated by applying the internationally accepted ICH guidelines, through evaluation of linearity, sensitivity, precision, accuracy, matrix effect and robustness (International Conference of Harmonization, 2005).

The linearity over the concentration range tested was optimal, exhibiting $r^2 > 0.9992$ for all reference standards , as shown in Table 2. Exhibited LOD values ranged from 1.2 to 8.4 μg/mL, while LOQs ranged from 4.0 to 28 μg/mL (Table 2), indicating the good sensitivity of the method. The low intra- and inter-day % relative standard deviations (% RSD) for retention times (Tables S1 and S2) and standard deviation (SD) values indicated a satisfactory precision of both chromatographic separation and extraction procedure (See Supplementary Tables S1 and S2).

The accuracy of the analytical procedure, evaluated by using the recovery test, was very good, with percentage recovery values in the 85–99% range (See Supplementary Tables S3).

The matrix effect on the peak response (See Supplementary Tables S4), can be considered acceptable for vegetable extracts: enhanced signal values up to 20% were found for chlorogenic acid and quercetin glucoside, and up to 50% for chicoric acid; kaempferol glucoside exhibited instead almost no matrix influence. The cyanidin chloride signal was instead suppressed of approx. 20% at all concentration levels.

The retention time %RSD (See Supplementary Tables S5) and the area standard deviation (SD) values indicated a reasonable precision of both chromatographic parameters evaluated, being the chromatographic separation not affected by minimal changes of the operating conditions, confirming the robustness of the proposed analytical method.

3.4. Quantitative analysis of chicory samples

The developed method was applied to the quantitative determination of identified polyphenols in forty-two *C. intybus* samples from seven different autochthonous varieties cultivated in the Veneto region of Italy. Quantitative data of the exhibited polyphenolic content, are reported in Table 3, expressed as mg/100 g (Fresh Weight, F.W., mean ± RSD, Relative Standard Deviation) of six samples for each variety.

The concentrations showed a noteworthy variability among examined samples, as expected, since the differences in cultivars, as well as in soil, cultivating conditions (blanching/not blanching) and place of cultivation. The total amount of the phenolic constituents ranged from 51.6 to 386.3 mg/100 g (F.W.), with a mean polyphenolic content of 255 mg/100 g (F.W.). The following order among the examined varieties could be defined: '*Rosa di Verona*' (386 mg/100 g, F.W.) > '*Rosa di Gorizia*' (355 mg/100 g, F.W.) > '*Chioggia*' (332 mg/100 g, F.W.) > '*Verdon da Cortèl*' (245 mg/10 g, F.W.) > '*Treviso Tardivo*' (217 mg/100 g, F.W.) > '*Treviso Precoce*' (196 mg/100 g, F.W.) > '*Castelfranco*' (52 mg/100 g, F.W.). Noteworthy differences in polyphenolic profiles among the seven varieties were also observed (Table 1): caffeoylquinic acid and chicoric acid are known from the literature as the main compounds in *C. intybus*, and this behaviour was confirmed in most samples (Sinkovič et al., 2015; Carazzone et al., 2013; Heimler et al., 2009).

As regard the '*Castelfranco*' variety, most of phenolic constituents were below their LOD and/or LOQ values, such as kaempferol

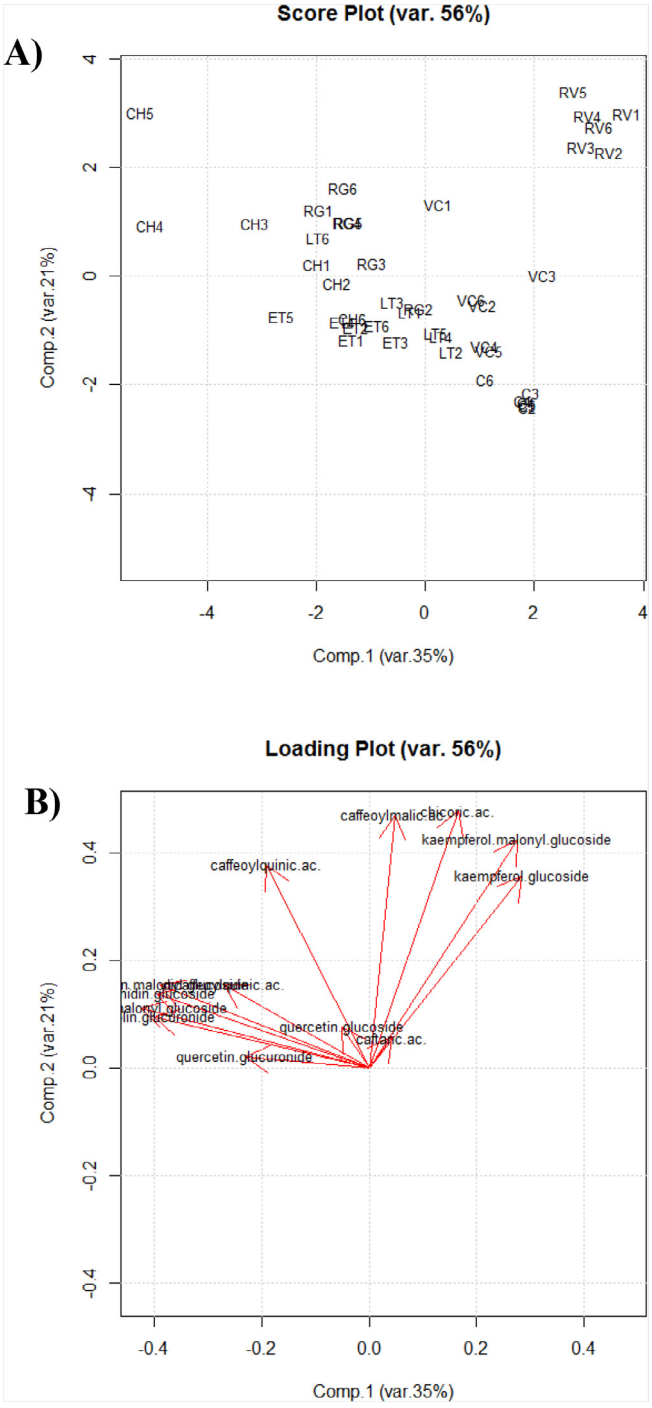

Fig. 3. Principal component analysis (PCA) applied to quantitative data of all *C. intybus* samples: (A) score plot, (B) loading plot. *Legend:* C 1–6 'Castelfranco' variety samples. CH 1–6 'Chioggia' variety samples. ET 1–6 'Treviso Precoce' variety samples. LT 1–6 'Treviso Tardivo' variety samples. RG 1–6 'Rosa di Gorizia' variety samples. RV 1–6 'Rosa di Verona' variety samples. VC 1–6 'Verdon da Cortèl' variety samples.

derivatives. Only chicoric acid (30 mg/10 g, F.W.), caftaric acid (5.5 mg/100 g, F.W.) and dicaffeoylquinic acid (2.8 mg/100 g, F.W.) could be quantified. The '*Chioggia*' variety showed caffeoylquinic acid as major polyphenolic component (80 mg/100 g, F.W.), followed by quercetin malonyl glucoside (53 mg/100 g, F.W.) and cyanidin malonyl glucoside (51 mg/100 g, F.W.). The '*Rosa di Gorizia*' cultivar exhibited the typical phenolic profile of *C. intybus* (Sinkovič et al., 2015; Carazzone et al., 2013; Heimler et al., 2009), with high concentration levels of caffeoylquinic acid (95 mg/100 g, F.W.), chicoric acid (68 mg/100 g, F.W.) and quercetin malonyl glucoside (68 mg/100 g, F.W.). On the contrary, the '*Rosa di Verona*' cultivar, although the similar overall concentration levels (386 vs. 355 mg/100 g, F.W.) exhibited a different profile, with kaempferol malonyl glucoside (135 mg/100 g, F.W.), chicoric acid (97 mg/100 g, F.W.), caffeoylquinic acid (52 mg/100 g, F.W.) as main compounds, and with low concentrations of cyanidin malonyl glucoside (3.8 mg/100 g, F.W.), the last one probably providing at such concentration level the peculiar pink colour to its leaves. The investigated '*Treviso Precoce*' variety resulted to be relatively rich in chicoric acid (38 mg/100 g, F.W.), luteolin glucuronide (36 mg/100 g, F.W.) and cyanidin malonyl glucoside (36 mg/100 g, F.W.), while in the '*Treviso Tardivo*' variety caffeoylquinic acid (51 mg/100 g, F.W.), chicoric acid (48 mg/100 g, F.W.) and dicaffeoylquinic acid (2.8 mg/100 g, F.W.) were the main compounds identified. The green leaf variety '*Verdon da Cortèl*' was characterised by the presence of caffeoylquinic acid (57 mg/100 g, F.W.), chicoric acid (53 mg/100 g, F.W.) and caftaric acid (30 mg/100, mg F.W.), and by the absence of anthocyanins.

A comparison of the phenolic profiles with available literature data about *C. intybus* varieties previously investigated was possible only for '*Castelfranco*', '*Chioggia*', '*Treviso Precoce*' and '*Treviso Tardivo*' varieties: the results obtained in this study were in good agreement, with some minor differences, with data previously reported (Cefola et al., 2016; Sinkovič et al., 2015; Innocenti et al., 2005); the other selected cultivars, namely '*Rosa di Gorizia*', '*Rosa di Verona*' and '*Verdon da Cortèl*', could not be compared since they were analysed for the first time in this study. In addition, the results obtained were comparable with respect to other species commonly employed in fresh mixed salads, such as *Lactuca sativa* L., *Plantago coronopus* L., *Eruca sativa* Mill., *Diplotaxis tenuifolia* (L.) DC. and *Chicorium endivia* L. which are known to contain polyphenols belonging to the same chemical classes as *C. intybus* cultivars analyzed in this study, in particular caffeic acid derivatives, flavones, flavonols and anthocyanins; the constituents found in this work fall into the range as total phenolic constituents from about 18 mg/100 g (F.W.) to 570 mg/100 g already reported by the literature (F.W.) (Heimler et al., 2007; Llorach, Martìnez-Sànchez, Tomàs-Barberàn, Gil, & Ferreres, 2008).

The quantitative concentration data obtained by HPLC-UV-DAD were further processed by Principal Component Analysis (PCA), using an R-based software in order to obtain a deeper overview of the polyphenolic profiles exhibited by the investigated varieties (Fig. 3). The principal components (PCs) could explain up to 56% of the total variance in examined *C. intybus* samples. The generated score plot (Fig. 3A), indicated a certain homogeneity among all analysed samples for each variety (except for '*Chioggia*' cultivar); some homogeneity was also noticed across '*Treviso Precoce*' and '*Treviso Tardivo*' cultivars, which both received the traditional post-harvest blanching treatment, with data distributed in the middle of the plot. A clear difference appeared for the '*Rosa di Verona*' variety, due to its relatively high kaempferol malonyl glucoside concentration level, with data centred on the upper right side of the plot (Fig. 3B). The '*Rosa di Gorizia*' variety, being located upper centred to the above-mentioned group, suggests its relatively high caffeoylquinic acid concentration level. Also the two green and green-red spotted leaves varieties could be distinguished: the '*Verdon da Cortèl*' variety was close to the '*Treviso Tardivo*' group on the score plot since their similar amounts in caffeoylquinic and chicoric acids, while the '*Castelfranco*' variety, since its low polyphenols concentration levels, could be located on the lower right side.

The concentration levels of kaempferol derivatives and chicoric acids mainly influenced the first principal component (the concentration levels are higher moving from the left to the right of the score plot), so the varieties on the upper and upper-right side can be considered the most interesting for their polyphenolic constituents, while anthocyanins resulted not significant, according to the PCA results (Fig. 3A).

In the light of all the above consideration, further investigations will be required in order to better understand the influence of specific

cultivation areas and soils, as well as of cultivation techniques, specifically the traditional blanching, on the polyphenolic profiles of '*Treviso Precoce*', '*Treviso Tardivo*' and '*Rosa di Gorizia*' varieties. The '*Rosa di Verona*' and '*Rosa di Gorizia*' cultivars, since their highest polyphenolic content, may find an additional application as a natural source for nutraceutical products.

4. Conclusions

An analytical tool by HPLC-UV-DAD for the identification and quantification of main polyphenol constituents in *C. intybus* leaves has been developed. The method was moreover fully validated according to the International Conference of Harmonization (ICH) guidelines. The proposed method was applied to the study of the polyphenolic profile of seven *C. intybus* varieties endemic from the Veneto region of Italy.

The results confirmed that the polyphenolic profiles are strongly affected by the examined variety. The '*Rosa di Verona*' cultivar was characterized by the highest polyphenolic concentration levels, while the '*Castelfranco*' cultivar exhibited the lowest ones.

The analytical method showed to be a reliable tool for the determination of polyphenols in *C. intybus* leaves, and could be applied to the selection of *C. intybus* varieties richer in antioxidant polyphenols, as well as of *C. intybus*-based products, also for nutraceutical dietary recommended intakes. Further investigations should be taken in consideration to better understand the influence of specific cultivation areas and soils, as well as of cultivation techniques, specifically the traditional blanching, on the polyphenolic profiles.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.foodchem.2018.05.085.

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