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Polyphenolic profile of green/red spotted Italian *Cichorium intybus* salads by RP-HPLC-PDA-ESI-MS^n

Adele Papetti[1*], Mariarosa Maietta[1], Federica Corana[2], Giorgio Marrubini[1], Gabriella Gazzani[1]

[1]Department of Drug Sciences, University of Pavia, Viale Taramelli 12, 27100 Pavia, Italy.

[2] Centro Grandi Strumenti, University of Pavia, Via Bassi 21, 27100, Pavia, Italy

adele.papetti@unipv.it

mariarosa.maietta01@universitadipavia.it

federica.corana@unipv.it

giorgio.marrubini@unipv.it

gabriella.gazzani@unipv.it

***Corresponding author: Dr. Papetti Adele** E-mail: adele.papetti@unipv.it;

Tel.: +39 0382 987863; Fax.: +39 0382 422975

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Abstract

Today the consumption of fresh vegetables is highly recommended not only for the intake of nutrients, but also for the healthy properties of secondary metabolites involved in the prevention of many disorders. In the present work, phenolic acids and flavonoids extracted from four green (*Cichorium intybus* var. *sativus* and var. *foliosum*) and a red spotted (a cross between *C. intybus* var. *silvestre* cv "Treviso" *and C. endivia* var. *foliosum*) salads were characterized by high-performance liquid chromatography–electrospray ionization/mass spectrometry. Among the 76 compounds detected in this work, five organic acids (two malic acid derivatives, two pyroglutamic hexoside isomers, and *cis*-aconitic acid), ten hydroxycinnamic acid derivatives (ferulic, malonyl caffeoylquinic, and caffeoylmethylglutaroylquinic acids, cinnamoyl- and caffeoyl-malate, coumaroyl, sinapoyl, feruloyl, and coumaroylcaffeoyl glycosides, dicaffeoyl lactone, 3-*O*-caffeoyl-4-*O*-(3-hydroxyglutaroylquinic acid), four flavonols (quercetin-di-*O*-glucoside, quercetin-3-*O*-malonylhexosyl-7-*O*-hexoside, myricetin-3-*O*-glucoside, and isorhamnetin-3-*O*-glucoside), and cyanidin-3-*O*-glucuronyl-5-*O*-hexoside have been identified in chicory salads for the first time. These data, together with the results obtained in our previous investigations on *Cichorium* genus Italian salads, provide a contribution to a more exhaustive identification of the secondary metabolites profile of each considered plant, that could be also useful in building/selecting hybrids with agronomic and peculiar healthy features, even using traditional methods of cultivation.

1. Introduction

Asteraceae family includes both different edible plant genera which are consumed as fresh or cooked food and also some plants used in folk medicine (Bremer, 1994; Gurib-Fakim, 2006; Randrianarivony et al., 2017). About one hundred of Cichorieae genera and several hundreds of species are known, being *Cichorium intybus* L. and *Cichorium endivia* L. the two most cultivated species. *C. intybus* includes different varieties and cultigroups grown in north-western Europe, constituted by young leaf and flower shoots in the case of loose leaf chicories or by cone-shaped heads in the case of heading chicories, commonly used as fresh salad or for industrial production (Bais & Ravishnkar, 2001; Innocenti et al., 2005; Lucchin et al., 2008). In particular, *C. intybus* var. *silvestre* in its different cultivars, commonly named red "Radicchio" for their leaves characterized by an intense red color, have long been widespread and popular in northeastern Italy and, in the last decades, have become common also across Europe and USA. All these red salads are appreciated for their distinctive taste and crunchiness and for their biological properties due to their chemical composition.

Chicory vegetables are rich not only in micronutrients, i.e. vitamins and minerals, but also in phytochemicals (Terahara, 2015), above all in polyphenols, such as chlorogenic acids in their different isomeric forms (mono- and di-caffeoylquinic acids and mono- and di-feruloylquinic acids), flavonol derivatives (kaempferol and quercetin derivatives), anthocyanins (in red chicories), and sesquiterpens (Zidorn, 2008; Papetti et al., 2008; Mascherpa et al., 2012; Carazzone et al., 2013; Wulfkuehler et al., 2013; D'Antuono et al., 2016). In the two last decades many protective properties have been attributed to these components: antimicrobial, anthelmintic, antimalarial, hepatoprotective, antidiabetic, anti-inflammatory, antioxidant, tumor-inhibitory activities have been ascribed to the *C. intybus* chicories (Papetti et al., 2002; Wang et al., 2011; Street et al., 2013; Williams et al., 2016; D'Acunzo et al., 2017; Malik et al., 2017). The capacity of interfering with growth and virulence-related traits of the most important oral pathogens was also found for "Treviso" cultivar (Papetti et al., 2013). The *C. intybus* hydro-alcoholic extract inhibits xanthine oxidase enzyme dose-dependently, hydrogen peroxide, and ferrous ion chelation (Pieroni et al., 2002; El & Karakaya,

2004). Moreover, it was demonstrated that *C. intybus* var. *silvestre* cv "Chioggia" extract could be used as an additive for replacing synthetic antioxidants and that it possesses a pleiotropic effect on yeast stress response (Lante et al., 2011).

In our previous research, the polyphenolic profiles of *C. intybus* var. *silvestre* (cv "Chioggia", "Treviso", "Treviso tardivo", and "Verona") (Carazzone et al., 2013), *C. endivia* var. *latifolium* and var. *crispum* (Mascherpa et al., 2012) were defined.

The aim of this study is to complete our investigations on chicory salads by evaluating the polyphenolic profiles of *C. intybus* green and red spotted varieties by high-performance liquid chromatography (HPLC) coupled with electrospray ionization mass spectrometry (ESI-MS) which is generally the most used method for the investigations of complex matrices such as vegetable extracts and is nowadays widely applied (Abu-Reidah et al., 2013; Santos et al., 2014; Feng et al., 2017; Zhu et al., 2017). Using data-dependent acquisition approach, single-stage MS provides the putative molecular weight (MW) that can be used in combination with UV detection for a tentative screening structure assignment; then tandem MS analysis via the fragmentation pathway gives the structure prediction (Mascherpa et al., 2012; Carazzone et al., 2013).

The definition of the polyphenolic fingerprint of such vegetables and the comparison between the different profiles of all typical Italian chicory salads selected in our research will help to draw a complete picture of their composition not only in nutrients, but also in minor compounds, well known for possessing healthy properties; these considerations could influence the choice of consumers in the food selection. Moreover, our results highlight molecules that could be useful for discriminating the different varieties and contribute to define their whole biological properties and quality.

2. Materials and methods

2.1. Reagents.

HPLC–MS grade water, all organic solvents, *cis*-aconitic acid, *p*-coumaric, ferulic acid, *p*-hydroxybenzoic acid, malic acid, 5-*O*-caffeoylquinic acid (chlorogenic acid, 5-CQA), kaempferol,

and quercetin-3,7-di-*O*-glucoside were purchased from Sigma–Aldrich (Saint Louis, MO, USA). Quinic acid was purchased from Acros Organics (Geel, Belgium), while 1,3-di-caffeoylquinic acid (1,3-di-CQA), kaempferol-7-*O*-glucoside, apigenin-7-*O*-glucoside, myricetin-3-*O*-glucoside, and cyanidin-3-*O*-glucoside were obtained from Extrasynthese (Genay Cedex, France). HPLC-grade water was obtained with a Milli-Q water purification system (Millipore, Billerica, MA, USA).

2.2. Plant material and sample preparation.

Four types of green *C. intybus* L., i.e. var. *sativus* Bishoff, namely "Cicoria Belga"-CB (grown at latitude: 43°46′ N, longitude: 10°26′ E, altitude: 4 m), var. *foliosum* Bishoff cv "Catalogna"-CA (grown at latitude: 41°13′ N, longitude: 16°17′ E, altitude: 157 m), var. *foliosum* Bishoff cv "Pan di zucchero"-PZ (grown at latitude: 45°13′ N, longitude: 11°21′ E, altitude: 16m), var. *foliosum* Bishoff cv "Grumolo Verde"-GV (grown at latitude: 45°40′ N, longitude: 9°49′ E, altitude: 236m), and one red-spotted vegetable resulting from a cross between *C. intybus* var. *silvestre* cv "Treviso" and *C. endivia* var. *foliosum*, namely "Variegato di Castelfranco"-VC (grown at latitude: 45°40' N, longitude: 11°55' E, altitude: 46m) (a typical late winter vegetable which has earned Protected Geographical Indication - PGI), were used for metabolic profiling.

Five clumps of each selected salads were purchased at a local market in December–March (a plant every three weeks). All vegetables were examined by a Botanist of our University who confirmed the varieties indicated by the suppliers.

Forty grams of fresh leaves were washed, cut into small pieces, and suspended in 25 mL of acidified (1% formic acid, v/v) aqueous methanol (80%, v/v); the mixture was shaken for 1 h in an ice bath in the dark and centrifuged for 5 min at 8750g; the insoluble residue was re-extracted 3 times with a fresh aliquots of the same mixture. The extracts were frozen at -40 °C and stored until the end of the sampling process. Finally, the extracts collected were pooled, filtered through a 0.45-µm membrane (cellulose acetate/cellulose nitrate mixed esters, purchased from Millipore) and directly analyzed.

131 *2.3. RP-HPLC-DAD-ESI/MS^n analysis.*

132 A Thermo Finnigan Surveyor Plus HPLC apparatus, equipped with a quaternary pump, a Surveyor
133 UV−vis PDA detector, a Surveyor Plus autosampler, and a vacuum degasser connected to an LCQ
134 Advantage Max ion trap mass spectrometer (all from Thermo Fisher Scientific, Waltham, MA, USA)
135 through an ESI source, was used for LC-MS analyses.

136 The metabolites were separated on a Gemini C18 analytical column (150 mm × 2.0 mm i.d., 5 μm)
137 with a Hypersil Gold C18 guard column (10 mm × 2.1 mm i.d., 5 μm; both from Phenomenex,
138 Torrance, CA, USA), using 0.1% aqueous formic acid and methanol as eluting solvents and a
139 multistep gradient, as previously reported in our works (Papetti et al., 2008; Mascherpa et al., 2012;
140 Carazzone et al., 2013).

141 The diode array detector recorded spectra from 200 to 600 nm, and every run was simultaneously
142 monitored at 280 nm (phenolic acids), 320 nm (hydroxycinnamic acids), and 370 nm (flavonols). The
143 ion trap operated in data-dependent, full scan (100−2000 m/z), zoom scan, and MS^n mode to obtain
144 fragment ion *m/z* with collision energy of 35% and isolation width of 3 *m/z*. When greater
145 discrimination was required, additional targeted MS^n experiments were performed on selected
146 deprotonated or cationized molecules.

147 The negative- and positive-ion mode ESI source parameters had previously been optimized by flow
148 injection analysis using 5-CQA and kaempferol (5 ppm in 0.1% formic acid−methanol solution,
149 50:50, v/v) to a ionization voltage of 3.5 kV, a capillary temperature of 260 °C, a sheath gas (nitrogen)
150 flow rate of 50 arbitrary units, and an auxiliary gas flow rate of 20 arbitrary units; helium has been
151 used as collision gas.

152 The Thermo Fisher Scientific Xcalibur 2.1 software was used for data acquisition and processing.
153 Three independent assays were performed to analyze each extract from salad leaves by HPLC-PDA-
154 ESI/MS^n; no relevant variations attributable to the nature of the detected fragments or their relative
155 intensities were observed.

Whenever possible, the HPLC retention time, UV and mass spectra of detected compounds were compared with reference standards. Since only few reference compounds were available, structures of unknown compounds were sketched mainly comparing their MS^n fragmentation behavior, retention time and UV spectra with data reported in literature. The UV profile and spectral similarities were useful characteristics for the prediction of detected classes of compounds.

2.4.Statistical analysis

The analytes identified in each type of salad (all listed in 2.2. Section plus those previously analyzed in our published papers, i.e. *C. intybus* var. *silvestre* cv ‘‘Chioggia’’-CH, cv ‘‘Treviso’’-TR, cv ‘‘Treviso tardivo’’-TRt, cv ‘‘Verona’’-VR, *C. endivia* var. *crispum*–Cr, and var. *latifolium* -La) were listed in a comprehensive table accounting for 11 rows (one row for each type of salad) and 118 columns. Each column of the table reported the presence or the absence of the given analyte in a binomial code. The table thus contained 118 qualitative variables at two levels (1 = detected, 0 = not detected). The data were studied applying Multiple Correspondence Analysis with the goal to assess whether the collection of the data of all the identified analytes could be a tool to classify in groups the studied salads considering their secondary metabolites as a whole. By using the first 10 components (which read almost 100% of the total variance), the salads were clustered by the scores according to Ward's method.

The statistical software used was R version 3.3.2, R Core Team (2016). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.r-project.org. Multiple correspondence analysis and the resulting clustering and graphs were computed in R using the MCA() and HCPc() functions of the FactoMineR package version 1.34.

Eliminato: R

3. Results and discussion

A RP-HPLC-DAD method previously applied to *C. intybus* var. *silvestre* and to *C. endivia* var. *foliosum* and var. *latifolium* was used for the separation of the polyphenolic metabolites in green

and red-spotted Italian *C. intybus* salads (Mascherpa et a., 2012; Carazzone et al., 2013). According to the hydrophobicity of the compounds and in agreement with literature (Abu-Reidah et al., 2013), a general tendency in the elution order registered for all extracts was: organic acids, phenolic acids, and flavonoids. MS^n experiments provided information on the molecular mass and fragmentation pattern of each compound, ranging from simple structure to more complex molecules; furthermore, the number and type of sugars and of functional derivatives conjugated to aglycones were elucidated (Carazzone et al., 2013; García-Salas et al., 2013).

Figure 1 shows the PDA chromatogram acquired in the range 200-600nm of GV extract. Figure 2 shows the TIC plot for the other analyzed extracts.

3.1. Organic acids.

Seven organic acids or their hexoside derivatives were identified by the fragmentation pattern, similar to the one described in literature and consisting in the generation of a neutral loss of CO_2 from carboxylic groups and/or water, and of 162 amu (corresponding to a hexose) for the hexoside derivatives (Table 1). Only two acids were present in all tested salads, namely malic acid (**1**) (*m/z* 133), and quinic acid (**2**) (*m/z* 191), both identified by comparing their mass fragmentation pattern and UV-vis spectra with standard compounds. Citramalic (**4**) (*m/z* 147), pyroglutamic hexoside (**3**) (*m/z* 290), *cis*-aconitic (**7**) (*m/z* 173), and isopropylmalic (**15**) (*m/z* 175) acids were found only in GV, while a second isomeric form of pyroglutamic hexoside (**5**) characterized CA and VC. The presence of some organic acids was previously found in other vegetables belonging to *C. intybus* and *C. endivia* (Mascherpa et al., 2012; Carazzone et al., 2013), and also to *L. sativa* (Abu-Reidah et al., 2013); some of these have a physiological role and others are responsible for organoleptic properties (Cheng et al., 2016; Mikulic-Petkovsek et al., 2016).

3.2. Phenolic acids.

208 Four hydroxybenzoic and twenty nine hydroxycinnamic derivatives eluting between 13 and 60 min
209 were detected. Simple *p*-hydroxybenzoic acid (**10**) (*m/z* 137) was found only in CA, while its
210 glycoside ester (**9**) (*m/z* 299), found in GV and VC, was identified by the neutral loss of the glycosidic
211 moiety; the same fragmentation pattern was also shown by the two isomers of dihydroxybenzoic acid
212 hexose (**11**, **13**) (*m/z* 315) of which compound **13** was found only in PZ. The presence of these simple
213 acids was previously reported in literature (Schütz et al., 2006; Albayrak et al., 2010) as well as the
214 presence of several hydroxycinnamic acid derivatives in *L. sativa* cultivars and in different
215 *Asteraceae* species (Innocenti et al., 2005; Mascherpa et al., 2012; Carazzone et al., 2013; Santos et
216 al., 2014). *p*-coumaric (**6**) (*m/z* 163) and ferulic (**8**) (*m/z* 193) acids were found in CA and PZ,
217 respectively, and both fragmented losing the carboxylic group $[M\text{-}H\text{-}COO]^-$; their structure was
218 confirmed by the analysis of standard solutions in the same conditions. Product-ion scan experiments
219 of esterified hydroxycinnamic acids disclosed characteristic fragmentations involving the cleavage of
220 acyl moiety thus leading also to the identification of the different positional isomers. Following this
221 strategy, several esters formed between cinnamic acids and other organic acids were characterized.
222 The presence of high abundance signals for MS^2 fragments at *m/z* 149 (corresponding to tartaric acid),
223 *m/z* 179 (corresponding to caffeic acid), and a low abundance signal at *m/z* 135 (corresponding to the
224 decarboxylated caffeic acid) led to the identification of caffeoyltartaric acid (**12**) (caftaric acid, *m/z*
225 311). A very intense peak eluting as compound **44** was found in all extracts; it gave also a ionization
226 dimer at *m/z* 947 and in MS^2 spectrum produced a base peak at *m/z* 311 $[M\text{-}H\text{-caffeoyl}]^-$, and other
227 fragments at *m/z* 293 $[M\text{-}H\text{-caffeoyl-}H_2O]^-$, *m/z* 179 $[M\text{-}H\text{-caffeoyltartaric}]^-$, and *m/z* 149 [M-H-
228 dicaffeoyl]$^-$; this fragmentation led to identification of di-*O*-caffeoyltartaric acid (chicoric acid). The
229 occurrence of these acids has been reported in different members of the Asteraceae family, including
230 "Treviso" and "Treviso tardivo" cultivars of *C. intybus* var. *silvestre* (Carazzone et al., 2013). Another
231 isomeric form of di-*O*-caffeoyltartaric acid (**54**) was detected in CA, PZ, and GV; its presence has
232 never been reported before in chicory salads, but it has been found in lettuce (Ribas-Agusti, 2011;
233 Lin, 2012). Following the same approach, cinnamoylmalic acid (**14**) (*m/z* 263), the two isobaric

compounds coumaroyl-*O*-pentoside (**16**) (*m/z* 295) and caffeoylmalic acid (**33**) (*m/z* 295), malonylcaffeoyl quinic acid (**25**) (*m/z* 439), and caffeoyltartaric-*p*-coumaroyl acid (**39**) (*m/z* 457) were detected. Several other derivatives containing quinic acid moiety were detected determining the linkage position of the acyl group/s (caffeoyl or feruloyl or *p*-coumaroyl) on quinic acid skeleton according to the different MS^2 fragmentation behavior of the precursor ion, and in some case to the different fragmentation pattern obtained in MS^3 experiments (Mascherpa et al., 2012; Carazzone et al., 2013; Jaiswal et al., 2014; Boeza et al., 2016). Thus, it was possible to identify: three isomers of mono-caffeoylquinic acid (CQA, *m/z* 353), 3-*O*-CQA (**17**), 5-*O*-CQA (**26**), *cis*-5-*O*-CQA (**32**); three isomers of mono-feruloylquinic acid (FQA, *m/z* 367), 5-*O*-FQA (**27**), 4-*O*-FQA (**41**), and *cis* 5-*O*-FQA (**45**); two isomers of mono-*p*-coumaroylquinic acid (*p*-CoQA, *m/z* 337), 5-*O*-*p*-CoQA (**35**) and *cis* 5-*O*-*p*-CoQA (**43**); and four isomers of di-*O*-caffeoylquinic acid (di-CQA, *m/z* 515), 1,3-di-*O*-CQA (**20**), 3,4-di-*O*-CQA (**36**), 3,5-di-*O*-CQA (**40**), and 4,5-di-*O*-CQA (**49**). The discrimination between *cis* and *trans* isomers was only speculative and based on the different hydrophobicity of the two molecules, since their UV-vis spectra and MS fragmentation patterns were the same. Actually, it has been reported that the *cis*-acyl isomers are appreciably more hydrophobic than their counterparts (Clifford et al., 2008). The presence of *cis*-isomers originating from the exposure of plant tissue to UV light is not surprising as it was previously described for compound **32** (*cis*-5-CQA) in *C. intybus* var. *silvestre* cv "Verona" (Carazzone et al., 2013), and for compound **45** (*cis*-5-FQA) in *C. endivia* (Mascherpa et al., 2012). The presence of 4,5-di-*O*-CQA acid is characteristic of PZ. Other two esters of quinic acid were identified in CA by comparison with the literature: compound **37** and compound **55**. The former produced a precursor ion at *m/z* 497 and fragments *m/z* 353 for the loss of the (3-hydroxy, 3-methyl)-glutaroyl residue, *m/z* 335 for the loss of a caffeoyl moiety (base peak), and *m/z* 191 [quinic acid $-H]^-$. It was putatively identified as 3-*O*-caffeoyl-4-*O*-(3-hydroxy, 3-methyl)glutaroylquinic acid (Clifford et al., 2010). The other ester (**55**) (*m/z* 499) produced the MS^2 base peak at *m/z* 353 $[M\text{-}H\text{-coumaroyl}]^-$ and secondary peaks at *m/z* 337 $[M\text{-}H\text{-caffeoyl}]^-$ and *m/z*

299; since the cinnamoyl residue at C5 is lost more easily than that at C4, the compound can be assigned as 4-*O*-caffeoyl-5-*O*-*p*-coumaroylquinic acid (Zhu et al., 2016).

Finally, several glycosylated hydroxycinnamates were detected and their fragmentation led to aglycone as base peak and generally to the decarboxylated product ions. Thus, it was possible to tentatively identify sinapoyl hexoside (**22**) (*m/z* 385), coumaroyl hexoside (**23**) (*m/z* 325), feruloyl hexoside (**24**) (*m/z* 355), and coumaroylcaffeoyl hexoside (**30**) (*m/z* 487).

The last hydroxycinnamic acid derivative detected in this work characterizing CA was compound **28**, with *m/z* 497, putatively identified as dicaffeoylquinic lactone, according to Mullen (2011). In fact, its fragmentation produced *m/z* 335 as base peak and *m/z* 161 as secondary peak.

In all the extracts, 4-hydroxyphenylacetic acid hexoside (**18**) (*m/z* 313) was detected. Its fragmentation pattern showed a base peak at *m/z* 151 due to the loss of the sugar that would probably be glucose according to the results reported by Sessa (2000) for lettuce cultivars, and a secondary peak at *m/z* 107 showing the typical decarboxylation of phenolic acids.

3.3. Flavonoids.

Flavonoids were widely spread in the tested vegetables and were distributed differently throughout the studied salads. Kaempferol and quercetin linked to sugars and organic acids were the most representative. Depending on the elimination of the sugar residue and the relative abundance of the radical aglycone ion that is more abundant then the aglycone in 3-*O* glycosilated compounds, it was possible to define the conjugate group and the glycosylation position (Ablajan at al., 2006); following these criteria, kaempferol-7-*O*-glucoside (**31**) (*m/z* 447), kampferol-7-*O*-glucuronide (**61**) (*m/z* 461), and kaempferol-7-*O*-(6”-*O*-malonyl)-glucoside (**47**) (*m/z* 533) were putatively identified in most of the tested salads. Conversely, kaempferol-3-*O*-sophoroside (**48**) (*m/z* 609) and kaempferol-3-*O*-glucosyl-7-*O*-(6”-*O*-malonyl)-glucoside (**50**) (*m/z* 695) were detected only in GV, and their presence was previously reported in red chicories (Carazzone et al., 2013). Kaempferol-7-*O*-neohesperidoside (**29**) (*m/z* 593) and kaempferol-7-*O*-rutinoside (**62**) (*m/z* 593) were present in VC. The sugar residue

285 in these two isobaric compounds only differed by the interglycosidic linkage between the
286 monosaccharides rhamnose and glucose, and the exact identification of the sugar was based on the
287 relative abundance of the ions corresponding to the loss of rhamnose (146 amu) and rhamnosyl-
288 glucose (308 amu) which was strikingly different (Shi et al., 2007).
289 Most of the quercetin derivatives identified in this work were known to be present only in red
290 chicories (Carazzone et al., 2013), i.e. quercetin-3-*O*-glucuronyl-7-*O*-(6"-*O*-malonyl)-glucoside (**65**)
291 (*m/z* 725), quercetin-7-*O*-glucoside (**63**) (*m/z* 463), quercetin-7-*O*-glucuronide (**75**) (*m/z* 477),
292 quercetin-3-*O*-glucuronide (**67**) (*m/z* 477), quercetin-7-*O*-(6"-*O*-malonyl)-glucoside (**56**) (*m/z* 549),
293 and quercetin-7-*O*-(6"-*O*-malonyl)-glucoside (**68**) (*m/z* 549). Two new compounds (**38**, **51**) were
294 putatively identified in GV and in CA, respectively, using the above mentioned approaches:
295 quercetin-3,7-di-*O*-glucoside (*m/z* 625) and quercetin-3-*O*-(6"-*O*-malonyl)-hexosyl-7-*O*-hexoside
296 (*m/z* 711).
297 Compounds **53**, **58**, **59**, **60**, **66**, **70**, and **74**, were identified as myricetin-3-*O*-glucoside (*m/z* 481, [M-
298 H]$^{+}$), isorhamnetin-7-*O*-glucoside (*m/z* 477), isorhamnetin-3-*O*-glucoside (*m/z* 477), isorhamnetin-7-
299 *O*-rhamnoside (*m/z* 461), isorhamentin-7-*O*-(6"-*O*-malonyl)-glucoside (*m/z* 563), isorhamnetin-3-*O*-
300 glucuronide (*m/z* 491), and isorhamnetin-7-*O*-glucuronide (*m/z* 491), respectively. The assignment of
301 the aglycones to isorhamnetin was based on MS^3 fragmentation leading to a peculiar intense ion at
302 *m/z* 300, differently from rhamnetin that originates an A-ring fragment ion at *m/z* 165 (Falcao et al.,
303 2013).
304 Six compounds classified as flavones were detected. The presence of apigenin derivatives
305 glycosilated in C7 position was attested by formation in the MS/MS spectrum of *m/z* 269 deriving
306 from heterolytic cleavage of deprotonated flavonoid glycosides as base peak, and of the ion
307 corresponding to radical aglycone (*m/z* 268) with lower relative abundance. Based on this
308 consideration, compounds **34** and **42** were identified as apigenin-7-*O*-glucoside (*m/z* 431) previously
309 found in "Chioggia" and "Verona" cultivars of *C. intybus*, and apigenin-7-*O*-glucuronide (*m/z* 445),
310 never identified before in this genus. Finally, chrysoeriol-3-*O*-hexoside (**73**) (*m/z* 461) was identified

311 in all extracts with the exception of CB and PZ, by the loss of a hexosyl moiety [M-H-162]$^-$; using
312 the same approach also luteolin-7-*O*-(6"-*O*-malonyl)-glucoside (**69**) (*m/z* 533), luteolin-3-*O*-
313 glucoside (**71**) (*m/z* 447), and luteolin-7-*O*-glucuronide (**76**) (*m/z* 461) were identified, the former in
314 all extracts and the latter only in CA, GV, and VC.
315
316 *3.4. Lignans and other phenolic compounds.*
317 The analysis of the MS spectrum registered in negative-ion mode for CA revealed the presence of a
318 pseudomolecular ion at *m/z* 579 yielding MS^2 fragments at *m/z* 417 (base peak), *m/z* 399 [M-H-162-
319 $H_2O]^-$ and *m/z* 384 [M-H-162-$CH_3]^-$; MS^3 spectrum showed a fragment ion at *m/z* 181 as base peak,
320 and secondary ions at *m/z* 402 and 166; according to the literature, these fragmentation is typical of
321 syringaresinol β-D-hexoside (Eklund et al., 2008) (**72**), a lignin derivative previously described in *L.*
322 *sativa* (Ablajan et al., 2006), but never identified before in *C.* salads.
323 Another metabolite characteristic of CA was compound **64** (*m/z* 177); it was putatively identify as a
324 coumarin derivative, i.e. 6,7-dihydroxycoumarin (esculetin), and hypothesized deriving from
325 hydroxycinnamic acid by cyclization; in fact, it fragmented giving a base peak at *m/z* 133 [M-H-
326 COO]$^-$ and a secondary peak at *m/z* 105 [M-H-COO-$C_2H_4]^-$. The glycosilated derivative of esculetin
327 (**21**) (*m/z* 339) was present in CA, GV, and VC; in MS^2 spectrum, the loss of the sugar moiety was
328 also observed. These compounds have been previously detected in Asteraceae (Rafsanjani et al.,
329 2011), but never reported for chicory salads.
330 Finally, a pseudomolecular ion at *m/z* 405 (**19**) was detected only in the GV extract. A 2,3,5,4'-
331 tetrahydroxystilbene-2-*O*-glucoside was tentatively proposed by comparison of our MS^2 and MS^3
332 fragmentation data with those reported in literature (Abu-Reidah et al., 2013).
333
334 *3.5. Anthocyanins.*
335 Three anthocyanins were detected in red spotted chicory (VC): cyanidin-3-*O*-(6"-*O*-malonyl)-
336 glucoside (**46**) (*m/z* 535, $[M]^+$) and petunidin-3-*O*-(6"-*O*-malonyl)-glucoside (**57**) (*m/z* 565, $[M^+]$)

were known to be present in *C. intybus* var. *silvestre* "Chioggia" cultivar, while compound **52** was identified for the first time in this work. Its molecular mass was 625 and it fragmented to yield a *m/z* 449 [M-glucuronyl]$^+$, and *m/z* 287 (aglycone); since the loss of 3-*O*-residue is more likely than that of 5-*O*-residue, the glucuronide residue should be linked at the C3 position in the aglycone; subsequent MS^3 experiments found a hexosyl moiety linked at C5 (*m/z* 287); therefore, the putatively identified compound was cyanidin-3-*O*-glucuronyl-5-*O*-hexoside.

3.6 Phytochemical markers analysis and taxonomic relationships between the salads

In summary, 76 compounds have been identified and considered for the polyphenolic profiling of five varieties of green and red spotted edible Italian chicories. Five organic acids, ten hydroxycinnamic acid derivatives, four flavonols, and one anthocyanin have been documented in *C.* salads for the first time in this work. Among the considered salads, VC and GV generally share the presence of the highest numbers of polyphenols; GV and CA are the salads richest in simple organic acids, flavones, flavonols, coumarins and lignans. c*is*-5-*O*-*p*-coumaroylquinic acid, 3-*O*-caffeoyl-4-*O*-(3-hydroxy,3-methyl)glutaroylquinic acid, dicaffeoyl lactone, 4-*O*-caffeoyl-5-*O*-*p*-coumaroylquinic acid, *p*-hydroxybenzoic acid, esculetin, and quercetin-3-*O*-(6"-*O*-malonyl)-hexosyl-7-*O*-hexoside are unique to CA, while citramalic and isopropylmalic acids, coumaroyl pentoside, coumaroyl hexoside, cinnamoylmalate, quercetin-3,7-di-*O*-glucoside, and kaemperol-3-*O*-glucosyl-7-*O*-(6"-*O*-malonyl)-glucoside could be considered markers of GV together with 2,3,5,4'-tetrahydroxystilbene-2-*O*-β-glucoside. Quercetin 7-*O*-(6"-*O*-malonyl)-glucoside was found only in VC, together with cyanidin-3-*O*-(6"-*O*-malonyl)-glucoside and cyanidin-3-*O*-glucuronyl-5-*O*-glucoside. Finally, a syringaresinol derivative characterized CA(Table 2).

Eliminato: (see Table 1 Supporting material

The green chicories considered in the present study share a lower number of polyphenols than the red *intybus* var. *silvestre* (characterized by the presence of different anthocyanins, conferring them the red color) in its four different cultivars analyzed in our previous investigation, while a similar

number was observed in VA, the salad deriving from a cross beteween *C. endivia* var. *latifolium* and *C. intybus* var. *silvestre* Treviso cultivar.

Malic, quinic, 5-*O*-caffeoylquinic and chicoric acids are common to all the tested salads, while caffeoyltartaric acid dimethyl ester and 5-*O*-caffeoylskikimic acid are unique for C. *endivia* var. *crispum* and "Treviso", respectively. Isorhamnetin-7-*O*-(6"-*O*-acetyl)-glucoside, myricetin-7-*O*-(6"-*O*-malonyl)-glucoside, malvidin-3-*O*-glucoside, and tricin-3-*O*-glucoside characterize "Verona" cultivar, while kaempferol 3,7-di-*O*-glucoside and a kaempferide derivative are unique for C. *endivia* var. *crispum* (Table 2).

Formattato: Inglese (Stati Uniti)

The data collected from the MS investigation have been studied using a multivariate approach. The resulting taxonomic classification of the salads was summarized in the dendrogram presented in Figure 3 that shows the similarities between the different cultivars in terms of whole qualitative metabolic profile. The salad samples could be grouped in three distinct clusters: the first cluster included the chicories characterized by red color of their leaves attributed to the presence of a number of anthocyanidins (black box); the vegetables with yellow-pale green leaves, particularly rich in flavonols are grouped in a second cluster (red box), while the third cluster (green box) contains green colored vegetables and the vegetable that is a cross between two varieties.

3. Conclusions

Taking into account all our studies on *C.* genus edible vegetables, our whole investigations focused on 11 different salads, 8 belonging to *intybus* species, 2 to *endivia* species, and 1 considered a cross between *intybus* and *endivia*.

Altogether, 118 compounds were characterized, among which about 70 never observed in other studied vegetables. Some of these compounds were present in all salads, whereas others are unique of a single vegetable and therefore could be useful as typical markers for discriminating that particular variety/cultivar. Our studies are relevant because they add a contribution to the definition of the secondary metabolites profile of each considered plant, and could also help in a more informed and

389 targeted selection of plants to be used to build/select hybrids with agronomic and peculiar
390 biochemical features, even using traditional methods of cultivation. In this way, sensory
391 characteristics such as the typical C. *intybus* var. *silvestre* ("Radicchio rosso") bitterness and
392 protective properties that are related to some polyphenols could be thus modulated. Moreover, these
393 results could help consumers in taking a more informed choice when selecting vegetable foods
394 particularly rich in healthy compounds.
395 However, further investigations are needed to verify the seasonal variability of the polyphenolic
396 fingerprint, as well as that of each individual compound. These further studies should be carried out
397 considering also several factors such as the harvesting season, the influence of thermal, water or light
398 stress, and the effects of different cultivation practices on the plant biosynthesis of polyphenols.
399

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404

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543

Figure captions

Figure 1. HPLC-PDA chromatogram (200-600 nm) of polyphenolic extract of *Cichorium intybus* var. *foliosum* Bishoff cv "Grumolo Verde" (GV).

Figure 2. Total ion chromatogram (TIC, *m/z* 100-2000) of *Cichorium intybus* var. *sativus* Bishoff, "Cicoria Belga" (CB) (**a**); var. *foliosum* Bishoff cv "Catalogna" (CA) (**b**); var. *foliosum* Bishoff cv "Pan di zucchero" (PZ) (**c**); cross between *C. intybus* var. *silvestre* cv "Treviso" *and C. endivia* var. *foliosum*, "Variegato di Castelfranco" (VC) (**d**).

Figure 3. Cluster dendrogram: the evidenced taxonomic classification reflects the qualitative secondary metabolites profiles of the salads.
C. intybus var. *sativus* Bishoff "Cicoria Belga"-CB, var. *foliosum* Bishoff cv "Catalogna"-CA, var. *foliosum* Bishoff cv "Pan di zucchero"-PZ, var. *foliosum* Bishoff cv "Grumolo Verde"-GV; a cross between *C. intybus* var. *silvestre* cv "Treviso" and *C. endivia* var. *foliosum* "Variegato di Castelfranco"-VA; *C. intybus* var. *silvestre* cv ''Chioggia''-CH, cv ''Treviso''-TR, cv ''Treviso tardivo''-TRt, cv ''Verona''-VR; *C. endivia* var. *crispum*–Cr, var. *latifolium* –La.