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Manuscript Draft

Manuscript Number: JFF-D-16-02461R2

Title: Bioaccessibility and HPLC-MS/MS chemical characterization of phenolic antioxidants in Red Chicory (Cichorium intybus)

Article Type: Full Length Article

Keywords: bioaccessibility; red chicory; HPLC-MS/MS; polyphenols; antioxidants

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Dear Editor,

I am sending you a revised copy of the Manuscript (JFF-D-16-02461R1) to answer the Editor's and Reviewers' comments.

Appended to this letter is our point-by-point response (in *italic*) to the comment raised by you and Reviewers. All requests were addressed and I hope that changes I have made resolve your concerns about the Manuscript. We hope that the Manuscript can now be accepted for publication on JFF.

Thank you once again for your time and interest.

Sincerely,

Nicola Marchetti

***Detailed Response to Reviewers**

Manuscript JFF-D-16-02461R2
Corresponding author: Dr. Nicola Marchetti

- Highlights: Each highlight must be 85 characters (including spaces).
  Third and fourth highlight were modified and their length is now below 85 characters.
- Title page: If available, please provide the e-mail address of each author.
  e-mail addresses of each author were added to the form used by submission system for generating title page. Additionally, a title page providing all author's email addresses was inserted before the Manuscript.
- Body of the manuscript: Units of "rpm" are used for centrifuging is not acceptable in this journal. Units of "rpm" must be accompanied by rotor diameter or substituted with the "g" value.
  All "rpm" units were substituted in the revised Manuscript with properly converted "g" values by means of rotor diameter (11.2 cm).
- Tables & figures: This journal frowns upon large tables. The table is taking up half a page or more, this is not ideal for the journal, please do what you can to reduce the size (even if it may involve splitting it into two tables and putting one into supplementary material).
  Font size and column text were modified in revised Manuscript and now the overall size for each table is largely reduced.

Highlights

- Partial chemical characterization of two cultivars of red chicory was obtained.
- Structural identification of target bioactives was achieved by HPLC-MS/MS.
- Bioaccessibility of polyphenols was obtained by in-vitro static simulated digestion.
- Structure-bioaccessibility relationships were evidenced for target polyphenols.

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Bioaccessibility and HPLC-MS/MS chemical characterization of phenolic antioxidants in Red Chicory (*Cichorium intybus*)

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Bioaccessibility and HPLC-MS/MS chemical characterization of phenolic antioxidants in Red Chicory (*Cichorium intybus*)

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Abstract

Two varieties of red chicory from southern Po Delta area have been characterized in terms of polyphenols content by HPLC-MS/MS. Seven target compounds were selected for determining their bioaccessibility from vegetable matrix. Simulated digestion process was employed for this purpose by means of an in-vitro static model. Salivary, gastric and duodenal phases were realized by using solutions of different physiological enzymes miming the stages of digestion process. Results show well defined behaviors associated with specific structures of aglycone or phenolic acid and position of O-glycosyl moiety. Dicaffeoyltartaric acid together with apigenin-7-O-glucoside and kaempferol-7-O-glucoside have similar bioaccessibility trend that is characterized by a higher value for duodenal phase than for gastric one. Quercetin-3-O-(6"-O-malonyl)-glucoside behaves in reverse way and gastric bioaccessibility turns higher than duodenal. Lastly, the difference between gastric and duodenal bioaccessibility is enhanced in the case of cyanidin-3-O-glucoside and the isomers of caffeoylquinic acid.

Key words: bioaccessibility; red chicory; HPLC-MS/MS; polyphenols; antioxidants.

1. Introduction

Phenolic compounds are well known natural antioxidants that occur in plants, fruits and vegetables as secondary metabolites. They have a protection role for aerial part of plants against

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UV solar radiation or pests and in the past they have been broadly characterized in their chemical structure and properties (Marais, Deavours, Dixon, and Ferreira, 2006; Shahidi and Naczk, 1995). Today it is recognized that flavonoids represent the largest group of plant phenolic compounds, accounting for over half of the 8,000 naturally occurring phenolic antioxidants (Harborne, Baxter, and Moss, 1999). Some foods, such as fruits, vegetables, cereals and edible plants, represents an important source of antioxidants in human diet with recognized health benefits (Lin and Weng, 2006; Peer and Murphy, 2006). Additionally, the chemical structure can considerably influence antioxidant activity as demonstrated in literature (Heim, Tagliaferro, and Bobilya, 2002). Hence, polyphenols may differently contribute to the nutraceutical role of specific food products and they can have variable health-related benefits (Cencic and Chingwaru, 2010). Multidisciplinary studies that involve medicine, nutraceutics, food chemistry and analytical chemistry seem today the most promising approach to obtain relevant results, information and advancement in this field (Prasain, Wang, and Barnes, 2004; Valls, Millàn, Martì, Borrás, and Arola, 2009).

Scientific investigations during last 10 years demonstrated that polyphenols are involved in prevention of chronic-degenerative diseases, such as those of the digestive tract and cardiovascular system (Serafini, Bellocco, Wolk, and Ekstrom, 2002). Today, epidemiological studies are strongly sustained by targeted investigations on digestion, intestinal absorption and metabolism of polyphenols from food (i.e., fruits, vegetables, edible plants and herbs). This might enlighten different perspectives on polyphenols intake and bioactivity (Cilla, Gonzalez-Sarrias, Tomas-Barberan, Espin, and Barbera, 2009; Heim, Tagliaferro, and Bobilya, 2002). The fundamental evidence is that the most common polyphenols in human diet are not necessarily the most active toward cells, tissues or organs: this can be related to either a lower intrinsic activity or a limited absorption. For the sake of completeness, also those compounds that are highly metabolized or rapidly eliminated might show a reduced bioactivity (Prasain and Barnes, 2007). In addition to clinical investigations, it appears clear that studies on the role of polyphenols in nutrition and nutraceutics cannot do without their determination in food matrix (Abu-Reidah, Ali-Shtayeh, Jamous, and Arráez-Román, 2015; Chen, Yu, Wu, Pan, Wang, Jin, and Zhang, 2015), bioaccessibility study (Gil-Izquierdo, Gil, Ferreres, and Tomas-Barberan,

2001) and in-vivo bioactivity determination (Etcheverry, Grusak, and Fleige, 2012).

Beyond all this there is an increasing demand for discovering new metabolites, elucidate chemical structures of unknown bioactive molecules and improving knowledge on their metabolic pathway and biochemical role. The employment of advanced, high technology analytical instruments, such as highly efficient liquid chromatographic separations and high resolution mass spectrometry (Capriotti, Caruso, Cavaliere, Samperi, Ventura, Chiozzi, and Laganá, 2015; Chiozzi, Capriotti, Cavaliere, Barbera, Piovesana, and Laganá, 2016; Piovesana, Capriotti, Cavaliere, Barbera, Samperi, Chiozzi, and Laganá, 2015) is fundamental to achieve valuable understanding in this field. Investigations of bioaccessibility and bioactivity are strongly sustained by advanced instrumentation and column technology, as well as reliable gastrointestinal models (e.g., chemical/enzymatic food matrix dissolution and cellular absorption of solubilized components) that are useful for elucidating bioactives transfer from food matrix to blood stream, and thus for the comprehension of those mechanisms that drive compounds to target tissue or organ.

The principal aim of this work is to contribute with new insights into chemical properties of polyphenols that reflect their behavior in biological processes (i.e., intake, digestion, intestinal absorption) and/or biochemical functions (i.e., trans-epithelial transport, delivery through blood circulation, metabolism, bioactivity). Highlighting possible structure-bioaccessibility relationships of polyphenols during gastrointestinal digestion is a relevant objective of these investigations. Secondly, fully chemical characterization of bioactives profile in foods can have relevant fall-out in understanding the cause of variation of polyphenols mainly in fruits, vegetables and edible plants, particularly those deviations due to environmental factors (such as pedoclimatic, agronomic, ripeness) and to genetic factors. This can have strong impact in protecting typical agrifood products and giving denomination/indication labels to preserve their authenticity. On one side, it has been evidenced that agricultural employment of genetically modified seeds can have negative and harmful effects on the environment, thus, the modern trend is to use seeds and plants that do not undergo hybridization or genetic modification. On the other side, characterization of major varieties of chicory present on the market has successfully been performed and these literature information represent a valid background

for further comparison and investigations (Carazzone, Mascherpa, Gazzani, and Papetti, 2013; Mascherpa, Carazzone, Marrubini, Gazzani, and Papetti, 2012; Papetti, Daglia, Aceti, Sordelli, Spini, Carazzone, and Gazzani, 2008; Papetti, Mascherpa, Carazzone, Stauder, Spratt, Wilson, Pratten, Ciric, Lingstrom, Zaura, Weiss, Ofek, Signoretto, Pruzzo, and Gazzani, 2013).

Main objectives of this study are the characterization of major polyphenols in two varieties of red chicory from southern Po Delta area and the investigation of bioaccessibility for selected polyphenols. These two varieties resemble two well established cultivars, largely present on the market: "Chioggia"-like (round leaves) and "Treviso"-like (long leaves) red chicory. Thus, it can be relevant to establish whether or not these local varieties are effectively individual cultivars.

2. Materials and Methods

2.1. Sampling of red chicory cultivars

The two inspected red chicory varieties (long-leaves and round-leaves) are cultivated in a well defined area in the southern part of Po Delta (see Figure 1). Lands where vegetables are produced are sited around Massenzatica (Municipality of Mesola, Province of Ferrara), as represented by dark gray circle. Sampling was undertaken between November and December 2015. Red chicory samples were collected from a randomized field and four replicates of each sample were taken from every experimental plot, then cut, mixed and stored at -20^{o}C until analyzed.

2.2. Chemicals

Methanol (HPLC grade), acetonitrile (LC-MS grade), formic acid (LC-MS grade), potassium chloride (KCl), monobasic phosphate (KH_2PO_4), magnesium chloride hexahydrate ($MgCl_2 \cdot (H_2O)_6$), ammonium carbonate ($(NH_4)_2CO_3$), calcium chloride dihydrate ($CaCl_2 \cdot (H_2O)_2$), quercetin-3-O-(6"-O-malonyl)-glucoside (Q3OMG), pepsin from porcine gastric mucosa (≥ 400 U/mg), α-amylase from Bacillus licheniformis (≥ 500 U/mg), bile salts (microbiology grade), pancreatin from porcine pancreas (USP grade) were purchased from Sigma Aldrich Co. (St. Louis, MO, USA). Dicaffeoyltartaric acid (DCTA), apigenin-7-O-glucoside (A7OG), cyanidin-3-O-glucoside chloride (C3OG), kaempferol-7-O-glucoside (K7OG), 3-O-caffeoylquinic acid (3CQA)

and 5-O-caffeoylquinic acid (5CQA) were purchased from Extrasynthese (Genay, France). sodium chloride (NaCl) and sodium hydrogen carbonate anhydrous ($NaHCO_3$) were purchased from Carlo Erba Reagents (Milan, Italy).

2.3. Instruments

ALC multi-speed refrigerated centrifuge (model PK121R) was from Thermo Scientific (Waltham, MA, USA). Ultra-turrax (model T18 basic) was from IKA (Staufen im Breisgau, Germany). Waterbath with shaking device (model WBN 22) was from Memmert (Schwabach, Germany). Linear ion trap mass spectrometer (model LTQ XL) and HPLC (model Surveyor Plus) equipped with solvent delivery system, degaser, quaternary micro-pump, thermostated auto-sampler and column compartment were Thermo Scientific.

2.4. Reagents preparation

Electrolyte stock solutions were prepared at the following concentrations: KCl 0.5 M; KH_2PO_4 0.5 M; $NaHCO_3$ 1 M; NaCl 2 M; $MgCl_2 \cdot (H_2O)_6$ 0.15 M; $(NH_4)_2CO_3$ 0.5 M; $CaCl_2 \cdot (H_2O)_2$ 0.3 M.
Simulated fluids - Simulated static in-vitro digestion uses three different electrolyte solutions prepared as reported by Minekus (Minekus, Alminger, Alvito, Ballance, Bohn, Bourlieu, Carriere, Boutrou, Corredig, Dupont, Dufour, Egger, Golding, Karakaya, Kirkhus, Le Feunteun, Lesmes, Macierzanka, Mackie, Marze, McClements, Menard, Recio, Santos, Singh, Vegarud, Wickham, Weitschies, and Brodkorb, 2014; Minekus, 2015).
Simulated salivary fluid (SSF): 15.1 mL of KCl; 3.7 mL of KH_2PO_4; 6.8 mL of $NaHCO_3$; 0.5 mL of $MgCl_2$; 0.06 mL of $(NH_4)_2CO_3$.
Simulated gastric fluid (SGF): 6.9 mL of KCl; 0.9 mL of KH_2PO_4; 12.5 mL of $NaHCO_3$; 0.4 mL of $MgCl_2$; 0.5 mL of $(NH_4)_2CO_3$; 11.8 mL of NaCl. SGF was adjusted to pH=3 with HCl 1 M.
Simulated intestinal fluid (SIF): 6.8 mL of KCl; 0.8 mL of KH_2PO_4; 85 mL of $NaHCO_3$; 0.33 mL of $MgCl_2$; 38.4 mL of NaCl. SIF was adjusted to pH=7 with HCl 1 M.
Enzyme solutions - Enzymes provided by the supplier were assayed according to reference tests as reported in literature (Minekus, Alminger, Alvito, Ballance, Bohn, Bourlieu, Carriere, Boutrou, Corredig, Dupont, Dufour, Egger, Golding, Karakaya, Kirkhus, Le Feunteun, Lesmes,

Macierzanka, Mackie, Marze, McClements, Menard, Recio, Santos, Singh, Vegarud, Wickham, Weitschies, and Brodkorb, 2014) and manufacturer's protocols: (i) α-amylase assay was based on spectrophotometric stop reaction using soluble potato starch as substrate; (ii) Pepsin activity assay was based on spectrophotometric stop reaction using hemoglobin as substrate; (iii) pancreatin activity was assayed in terms of its trypsin and chimotrypsin activity based on continuous spectrophotometric rate determination using p-toluene-sulfonyl-L-arginine methyl ester and N-benzoyl-L-tyrosine ethyl ester as substrates, respectively. Different enzymes were added to specific simulated fluid as reported below, either in case of enzymatic polyphenols extraction or for bioaccessibility experiments (simulated digestion). All enzyme solutions were freshly prepared, preincubated at 37°C before use and stored at 4°C for maximum three days. α-Amylase 1500 u/mL: 30mg of enzyme in 20 mL of SSF. Pepsin 20,000 u/mL: 600 mg of enzyme in 20 mL of SGF. Pancreatin 800 u/mL: 320 mg in 40 mL of SIF. Bile salts: 0.625 g in 25 mL of SIF.

2.5. Polyphenols extraction

Chemical extraction. 5 grams of frozen sample were dispersed in 20 mL of methanol/water/formic acid 80/20/0.1% (v/v) solution by means of ultra-turrax® for 1 minute. Then, sample was stirred for 1 hour and left overnight in the dark. Next day, sample was centrifuged (10100 g at 5^{o}C) for 5 minutes and supernatant was recovered. Successively, the following three steps were repeated in sequence for 5 times: (i) extraction with 10 mL of methanol/water/formic acid 80/20/0.1% (v/v) solution; (ii) magnetic stirring for 10 minutes; (iii) centrifuge (10100 g at 5^{o}C) for 7 minutes. Fractions were kept separate and 1 mL from each were gathered. A 1 mL aliquot of extracted sample was filtered and injected into HPLC-MS/MS. Pellet undergoes enzymatic extraction as reported in the following.

Enzyme-assisted extraction. This procedure is a modified approach described in literature (Chandrasekara and Shahidi, 2012; del Pilar Sànchez-Camargo, Montero, Stiger-Pouvreau, Tanniou, Cifuentes, Herrero, and Ibàñez, 2016; Pineda-Vadillo, Nau, G.Dubiard, Cheynier, Meudec, Sanz-Buenhombre, Guadarrama, Toth, Csavajda, Hingyi, Karakaya, Sibakov, Capozzi, Bordoni, and Dupont, 2016; Saura-Calixto, Serrano, and Goni, 2007) that exploits enzymatic hydrolysis reactions to solubilize polyphenols bound to dietary fiber. 35 mL of milliQ water, 2 mL of bile salts

solution, 1 mL of pancreatin solution and 0.5 mL of pepsin solution were added to pellet from previous chemical extraction. Sample was vortex-mixed and left under agitation at 37^{o}C for 2 hours. After that, sample was centrifuged (10100 g at 5^{o}C) for 5 minutes and supernatant was recovered, filtered and analyzed. Enzymatic extraction was repeated twice on the same pellet and supernatants analyzed separately.

2.6. In-vitro static digestion model

Bioaccessibility studies were assessed by *in-vitro* static digestion simulation (Alegria, Garcia-Llatas, and Cilla, 2015). Mouth, stomach and small intestine (duodenum) phases were considered and simulated by means of the three simulated fluids (SSF, SGF and SIF, respectively) as reported above and incubation at 37^{o}C for different durations according with physiological processes. Roughly 3 g of frozen sample were placed inside the fermenter vessel and all reagent solutions and water were warmed at 37^{o}C before use. Composition of simulated fluids and static digestion procedure were adapted from literature works as described in previous section and below (Chandrasekara and Shahidi, 2012; del Pilar Sànchez-Camargo, Montero, Stiger-Pouvreau, Tanniou, Cifuentes, Herrero, and Ibàñez, 2016; Pineda-Vadillo, Nau, G.Dubiard, Cheynier, Meudec, Sanz-Buenhombre, Guadarrama, Toth, Csavajda, Hingyi, Karakaya, Sibakov, Capozzi, Bordoni, and Dupont, 2016; Saura-Calixto, Serrano, and Goni, 2007).

Mouth - Sample was crushed and coarsely grinded then 3.5 mL of SSF, 975 μL of water, 25 μL of $CaCl_2$ and 500 μL of α-amylase solution were added. Sample was vortex-mixed for 30 seconds, then a 300 μL aliquot was taken for the analysis.

Stomach - Sample from previous phase was mixed with 7.5 mL of SGF, 295 μL of water, 5 μL of $CaCl_2$, 200 μL of HCl 1 M and 2 mL of pepsin solution. After vortex-mixing for 10 seconds, samples were incubated for 2 hours. Then, samples were centrifuged (10100 g at 5^{o}C) for 5 minutes and 1 mL aliquot was filtered and injected for analysis.

Duodenum - Sample from stomach phase was added with 11 mL of SIF, 3.61 mL of water, 40 μL of $CaCl_2$, 150 μL of NaOH 1 M, 5 mL of pancreatin solution and 0.2 mL of bile salts solution. After vortex-mixing for 10 seconds, samples were incubated for 2 hours. Then, samples were centrifuged (10100 g at 5^{o}C) for 5 minutes and 1 mL aliquot was filtered and injected for

analysis.

2.7. HPLC-MS/MS analysis

Liquid chromatographic separation has been done with a Symmetry C18 column (Waters, Milford, MA, USA) 2.1x150 mm, packed with 3.5 μm fully porous particles, thermostated at 30^{o}C, under gradient elution condition. Mobile phases were mixtures of water and formic acid 0.1% (v/v), channel A, and acetonitrile and formic acid 0.1% (v/v), channel B. Eluent composition changed from 5% to 30% of channel B in 25 minutes, followed by 5 min at 95% of B as column cleaning step. Then, column was re-equilibrated at beginning condition for further analysis. The running flow rate was 150 μL/min. Auto-sampler compartment was thermostated at 15^{o}C and the injected sample quantity was 2 μL. The chromatographic method was validated according to ICH (ICH, 2005) and resulting parameters reported in Section 3.2 (Polyphenols bioaccessibility). Validation occurred in terms of the following estimators: relative error (RE); relative standard deviation (RSD); linear fit coefficient of determination (R^2). Quantitative range of validity for target compounds were: C3OG 0.055–11 μg/mL, DCTA 0.11–22 μg/mL, 5CQA 0.0565–22.6 μg/mL, A7OG 0.0101–1.01 μg/mL, K7OG 0.053–1.06 μg/mL, Q3OMG 0.07–28 μg/mL, 3CQA 0.206–20.6 μg/mL. All analyses were repeated five times.

ESI-MS operating conditions were as in the following. Positive ESI: spray voltage = 4.5 kV; capillary voltage = 16 V; tube lens = 45 V. Negative ESI: spray voltage = 4 kV; capillary voltage = -6 V; tube lens = -47 V. Capillary temperature was 275^{o}C for both positive and negative ESI. MS^2 and MS^3 spectra were obtained by collision induced dissociation (CID). CID values (expressed as relative value) were optimized to maximize characteristic base peak intensity for each transition. Positive ESI: cyanidin-3-O-glucoside (C3OG), 449 m/z, CID=20%; quercetin-3-O-(6"-O-malonyl)-glucoside (Q3OMG), 551 m/z, CID=20%; quercetin aglycone, 303 m/z, CID=35%. Negative ESI: caffeoylquinic acid (3CQA and 5CQA), 353 m/z, CID=18%; dicaffeoyltartaric acid (DCTA), 473 m/z, CID=20%; apigenin-7-O-glucoside (A7OG), 431 m/z, CID=20%; kaempferol-7-O-glucoside (K7OG), 447 m/z, CID=20%; kaempferol aglycone, 285 m/z, CID=25%.

3. Results and discussion

3.1. Polyphenols determination

On the basis of recent literature, both positive and negative ESI-MS detection was used to identify phenolic acids and flavonoids in different fresh food samples (Hamed, Said, Kontek, Al-Ayed, Kowalczyk, Moldoch, Stochmal, and Olas, 2016; Orrego-Lagaron, Vallverdù-Queralt, Martinez-Huelamo, Lamuela-Raventos, and Escribano-Ferrer, 2016; Plazonic, Bucar, Males, Mornar, Nigovic, and Kujundzic, 2009; Quifer-Rada, Vallverdù-Queralt, Martinez-Huelamo, Chiva-Blanch, Jauregui, Estruch, and Lamuela-Raventos, 2015). Analogous compounds were already determined also in commercial chicory varieties (i.e., Chioggia and Treviso) (Carazzone, Mascherpa, Gazzani, and Papetti, 2013) and MS/MS fragmentation was employed also in this study to detect characteristic daughter ions and, thus, to confirm chemical structure of polyphenolics in our samples. A total of 36 compounds (15 and 21 in positive and negative ESI condition, respectively) were identified as most abundant and common components. Table **??** lists compounds detected under positive ESI condition, while Table **??** reports those negatively ionized. Both tables also list tentative compounds together with parent m/z values and characteristic daughter ions found in MS/MS and MS^3 spectra. Among the identified simplest phenolic acids (malic, caffeic, quinic and caftaric acids), all identified components were in the glycosilated or ester form. Acetyl and malonyl derivatives were also found as mostly diffuse.

MS/MS spectra, and in a few cases further selected precursor ion fragmentations, were useful to confirm the tentative compounds. Glycosilated derivatives were promptly recognized by the most intense MS/MS transition, usually determined by characteristic neutral loss of glycosyl moiety. Less intense MS/MS peaks (when present) can refer to typical fragmentations of involved saccharide unit or loss of malonyl and acetyl groups. MS^3 spectra of isobaric aglycones might be required as confirmation step (Cuyckens and Claeys, 2004; Fabre, Rustan, de Hoffmann, and Quetin-Leclercq, 2001; Stobiecki, 2000): in this work MS^3 fragments were used to identify kaempferol, cyanidin (both at $[M+H]^+$=287 m/z) and less common isorhamnetin ($[M-H]^-$=315 m/z) as reported in Tables **??** and **??**). Isorhamnetin aglycone shows characteristic loss of methyl group in both positive and negative ESI ($[M+H-CH_3]^+$=302 m/z] and [M-H-

$CH_3]^-$=300 m/z, respectively). Kaempferol fragmentation gives rise to characteristic ^{1,3}A ion (153 m/z) while this does not happen with cyanidin.

By inspecting MS^2 and MS^3 characteristic daughter ions in both tables it is possible to evidence typical neutral losses of 176 m/z and 162 m/z that refer to glucuronide and glycosyl (hexose) groups, respectively (see entries 1-6 in Table **??** and 9-11 in Table **??**). The presence of Y fragment as the most intense or even as the only one detected is a strong evidence of O-glycosyde derivatives. Assignment of glycosylation position (3 or 7) was tentatively given on the basis of known mostly diffuse flavonoids in chicory varieties already investigated in detail (Carazzone, Mascherpa, Gazzani, and Papetti, 2013). Analogous arguments can be done for other compounds (i.e., malonyl, acetyl, coumaroyl derivatives, O-diglycosydes, etc.): Tables **??** and **??** show MS^2 daughter ions that reveal the simultaneous loss of malonyl or acetyl and glycosyl moieties as most intense fragmentation, a mass change of 248 m/z (i.e., 162 m/z for hexose and 86 m/z for malonyl group) and 204 m/z (i.e., 162 m/z for hexose and 42 m/z for acetyl group), respectively. This can be seen for entries 7-10 in Table **??** and 14, 16 and 18 in Table **??**. Secondly, individual loss of glycosyl, malonyl or acetyl moieties can also be present in MS/MS spectra as less intense fragment ions (roughly 5%-20%). In case of malonyl derivatives, CO_2 neutral loss (44 m/z mass change) can also be detected as for kaempferide-3-O-(6"-O-malonyl)-glucoside at entry 8 of Table **??** (transition 549 m/z → 505.2 m/z). Also, a coumaroyl-glucoside derivative of quercetin (entry 20 Table **??**) was identified by typical coumaroylglucose cleavage (308 m/z mass change), 609 m/z → 301 m/z, and loss of coumaroyl moiety (146 m/z), 609 m/z → 463 m/z.

Compounds reported in entries 11-15 of Table **??** were identified as O-diglycosyl flavonoids and their malonyl derivatives. Mass peaks corresponding to neutral losses of both sugar rings were detected as well as cleavage of malonyl group (i.e., transitions for kaempferol-7-O-glucosyl-3-O-(6"-O-malonyl)-glucoside, 697 m/z → 535 m/z, 535 m/z → 449 and 449 m/z → 287 m/z).

Phenolic acids were promptly identified by their negative pseudo-molecular ion (see Table **??**, entries 1-8) and confirmed by MS^2 spectra. They show characteristic transitions that are helpful also in case of esterified derivatives: malic acid (133 m/z → 115 m/z), caffeic acid (179 m/z → 135 m/z), quinic acid (191 m/z → 111 m/z) and caftaric acid (311 m/z → 149, 311 m/z

→ 179 m/z). Esters of quinic acid give rise to specific fragmentations: loss of coumaroyl moiety (146 m/z) for 5-p-coumaroylquinic acid (337 m/z → 191 m/z); loss of caffeoyl group (162 m/z) and detection of caffeate negative ion (179 m/z) for caffeoylquinic acid (353 m/z → 191 m/z, 337 m/z → 179 m/z); losses of ferulyc group (176 m/z) and ferulyc acid (194 m/z) for feruloylquinic acid (367 m/z → 191 m/z, 367 m/z → 173 m/z).

Figure 2 reports an example of HPLC-MS/MS run for the seven selected target compounds. It can be seen that for some monitored transitions more than one peak is present in the chromatogram. Further investigations with the help of high resolution mass spectrometry has been started to investigate these aspects. This means that positional isomers or different hexose derivatives can be present in the extract. By looking at the third filtered chromatogram (K7OG, 447 m/z → 285, 255, 327 m/z), kaempferol-3-O-glucoside can be responsible for the second most intense peak. Analogously, the fifth chromatogram (C3OG, 449 m/z → 287 m/z) shows two more peaks that can be related to cyanidin-3-O-glucoside and cyanidin-3-O-galactoside.

3.2. Polyphenols bioaccessibility

Molecular complexity and intrinsic chemical properties of bioactive compounds can strongly influence the absorption process and this partially explains their limited uptake and the extremely low levels in blood and cells (Lipinski, Lombardo, Dominy, and Feeney, 2012). Other variables can negatively influence the amount of polyphenols available for absorption in the gut after digestion (bioaccessibility), such as competitive and anti-synergic effects of dietary components (Fernandez-Garcia, Rincon, and Perez-Galvez, 2008), or physical and chemical properties of food matrix (Hedren, Mulokozi, and Svanberg, 2002). Estimation of the bioaccessibility requires quantitative determination of polyphenols and phenolic acids: the total amount in exhaustive extracts (see Table **??**) and concentration in each digestion step (salivary, gastric, duodenal). For this purpose, calibration curves were required and external calibration method was applied. Area vs concentration data have been fitted to straight line equations for all standards except for 3CQA and 5CQA, where a quadratic polynomial fit resulted in higher values of correlation coefficient. Method validation resulted in very satisfying parameters for repeatability (RSD<5%), intermediate precision (RSD<10%), accuracy (RE<10%) and linearity

($R^2 \geq 0.995$).

Figures 3a and 3b report the bioaccessibility results of the seven selected target compounds (see section 2.7 and Table **??**). for both round and long-leaves Red Chicory varieties. The two varieties of Red Chicory show comparable bioaccessibility values for all target compounds in the salivary phase: C3OG and Q3OMG between 0.01% and 0.1%; DCTA, K7OG, 3CQA between 0.35% and 1%; 5CQA and A7OG between 2% and 5%. In the gastric phase round and long leaves red chicory display similar bioaccessibility for C3OG, DCTA, A7OG, K7OG and Q3OMG, but in the case of 5CQA and 3CQA bioaccessibility is much higher (more than twice) for long leaves than round red chicory (54.6% vs 15.2% and 46.8% vs 19.3%, respectively). In the case of duodenal phase, major differences between the two chicories occur for DCTA and 5CQA bioaccessibility data. DCTA has been found higher for round leaves chicory (37.9%) than for that long leaves one (16.9%); 5CQA is higher for long leaves chicory (10.8%) than for the other (2.0%). Small differences (below 4%) are noticed for A7OG (29.9% vs 26.0% for round and long leaves red chicory, respectively) and K7OG (8.3% vs 10.8% for round and long leaves red chicory, respectively). For all the other compounds variations are within the experimental error and, hence, bioaccessibility can be considered constant.

Three different behaviors can be evidenced for the target compounds with an identical pattern for the two red chicories (see Figures 3a and 3b). Firstly, a progressive, linear increase of bioaccessibility value from salivary to duodenal phase as seen for DCTA, A7OG and K7OG was observed (type-1 trend, see Figures 3a and 3b). Secondly, a clear bioaccessibility maximum value that occurs at gastric phase for C3OG, 5CQA and 3CQA was recognized (type-2 trend). Finally, slightly distinct trend where still gastric bioaccessibility is high but duodenal one is is immediately below was characteristic of Q3OMG (type-3 trend). Accordingly to these data, relationships between chemical structure and bioaccessibility seem relevant and significant. O-glycosylation at C7 brings apigenin and kaempferol (having similar aglycone structure, except for -OH moiety at C3) to the same type-1 trend. Conversely, O-glycosylation at C3 seems to enhance gastric bioaccessibility and to modify behavior in the direction of type-2 trend. Also, 3- and 5-Caffeoylquinic acids have the same behavior. However, it is difficult to establish if aglycone structure and position of O-glycosylation can have interplay in determining the bioacces-

sibility of each compound (relative amount and trend).

More detailed systematic comparison between glycosilated polyphenols has undoubtedly to be performed and this is already planned for ongoing works. A global point of view on bioaccessibility data for the studied seven target compounds shows that release of bioactives from Red Chicory matrix is larger during duodenal phase for round leaves variety, while for the long leaves variety total bioaccessibility is higher during gastric phase mainly due to chlorogenic acid and its isomer 5-caffeoylquinic acid. In absolute terms this does not closely follow the outcome of total antioxidant capacity (unpublished data) for the three phases. It is our idea that other polyphenols may be the cause for this deviation and in particular those yet unidentified in this work and that can be present in the two red chicory varieties with different abundance.

4. Conclusions

This study provides preliminary new information about the polyphenol content found in two red chicory cultivars that have never been investigated before. This study described a first tentative interpretation of bioaccessibility results from a chemical structure point of view. Relative amount of polyphenols released from red chicory matrix during each of the three investigated digestive phases are related with overall chemical structure of antioxidant compounds. Both aglycone and position of O-glycosylated moiety have been discussed and this has never been reported so far.

Further work has to be done to achieve a more detailed chemical characterization of bioactives in these vegetable matrices. High resolution tandem mass spectrometry coupled with ultra high efficient liquid chromatographic separation is fundamental to acquire precise structural information necessary for an unique identification of flavonoids and phenolic acids.

5. Acknowledgments

The authors thank the Italian University and Scientific Research Ministry (Grant PRIN 2012 ATMNJ_003) and the Laboratory Terra&Acqua Tech, Member of Energy and Environment Cluster, Technopole of Ferrara of Emilia-Romagna High Technology Network (POR-FESR 2014-2020). AM and VB also thank CUM Consortium (Massenzatica, Ferrara).

Conflict of Interest Statement

The authors whose names are listed in the first page of this manuscript certify that they have NO affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

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Figure captions

Figure 1. Map of southern Po Delta area where Red Chicory cultivars are produced (Massenzatica territory).

Figure 2. LC-MS/MS analysis of red chicory extract. Peaks were identified and confirmed by MS/MS transitions (see Tables 1 and 2) as: (1) cyanidin-3-O-glucoside (C3OG); (2) dicaffeoyltartaric acid (DCTA); (3) 5-caffeoylquinic acid (5CQA); (4) apigenin-7-O-glucoside (A7OG); (5) kaempferol-7-O-glucoside (K7OG); (6) quercetin-3-O-(6"-O-malonyl)-glucoside (Q3OMG); (7) 3-caffeoylquinic acid (3CQA). The inset figure shows enlarged chromatogram (dashed line border) to display peak 3 and its relative intensity vs peak 7. .

Figure 3. Results of bioaccessibility experiments for (a) round-leaves and (b) long-leaves Red Chicory varieties. Target compounds are: cyanidin-3-O-glucoside (C3OG); dicaffeoyltartaric acid (DCTA); 5-caffeoylquinic acid (5CQA); apigenin-7-O-glucoside (A7OG); kaempferol-7-O-glucoside (K7OG); quercetin-3-O-(6"-O-malonyl)-glucoside (Q3OMG); 3-caffeoylquinic acid (3CQA). Error bars refer to standard deviation based on five determinations.

Tables

Table 1: Identified polyphenols in positive ESI mode. Number in square brackets represents the parent ion, while those in parentheses are the relative abundance for each daughter ion.

Entry	Tentative name	m/z	MS^n (m/z)
1	Pelargonidin-3-O-monoglucuronide	447	MS^2[447]: 271(100)
2	Cyanidin-3-O-glucoside	449	MS^2[449]: 287.1(100)
3	Cyanidin-3-O-galactoside	449	MS^2[449]: 287.1(100)
4	Kaempferol-7-O-glucuronide	463	MS^2[463]: 287.1(100); MS^3[287.1]: 152.9(100); 258.1(30)
5	Quercetin-7-O-glucoside	465	MS^2[465]: 303(100)
6	Quercetin-7-O-glucuronide	479	MS^2[479]: 303(100)
7	Cyanidin-3-O-(6”-O-malonyl)-glucoside	535	MS^2[535]: 287(100); 449.2(5); MS^3[287]: 213(100); 136; 188; 231; 259; 269
8	Kaempferide-3-O-(6”-O-malonyl)-glucoside	549	MS^2[549]: 301.1(100); 505.2(15); 463.1(10)
9	Quercetin-3-O-(6”-O-malonyl)-glucoside	551	MS^2[551]: 303(100)
10	Isorhamnetin-7-O-(6”-O-malonyl)-glucoside	565	MS^2[565]: 317.1(100); 479(3); MS^3[317.1]: 302.1(100); 285.1(45)
11	Kaempferol-3-O-glucuronide-7-O-glucoside	625	MS^2[625]: 449.2(100); 287.1(10)
12	Kaempferol-7-O-glucosyl-3-O-(6”-O-malonyl)-glucoside	697	MS^2[697]: 535.2(100); 287.1(20); 449.4(10)
13	Delphinidin-3-O-(6”-O-malonyl)-glucoside-5-O-glucoside	713	MS^2[713]: 303.1(100); 465.1(95); 551.2(60)
14	Quercetin-3-O-glucuronide-7-O-(6”-O-malonyl)-glucoside	727	MS^2[727]: 479.1(100); 303.1(15)
15	Cyanidin-3,5-di-O-(6”-O-malonyl-glucoside)	783	MS^2[783]: 535.1(100); 287(30)

Table 2

Table 2: Identified polyphenols in negative ESI mode. Number in square brackets represents the parent ion, while those in parentheses are the relative abundance for each daughter ion.

Entry	Tentative name	m/z	MS^n (m/z)
1	Malic acid	133	MS^2[133]: 114.8(100)
2	Caffeic acid	179	MS^2[179]: 134.9(100)
3	Quinic acid	191	MS^2[191]: 110.8(100); 172.9(25)
4	Caftaric acid	311	MS^2[311]: 148.9(100); 178.9(55); 134.9(5)
5	5-*p*-Coumaroylquinic acid	337	MS^2[337]: 190.9(100)
6	3-Caffeoylquinic acid	353	MS^2[353]: 190.9(100); 178.9(40); 134.9(10)
7	5-Caffeoylquinic acid	353	MS^2[353]: 190.9(100); 178.9(5)
8	5-O-Feruloylquinic acid	367	MS^2[367]: 190.9(100); 172.9(3)
9	Apigenin-7-O-glucoside	431	MS^2[431]: 269.1(100); 268.1(5)
10	Kaempferol-7-O-glucoside	447	MS^2[447]: 285.7(100); 284.1(70); 255.1(15); 327(13)
11	Quercetin-7-O-galactoside	463	MS^2[463]: 301.1(100); 300.1(15)
12	Dicaffeoyltartaric acid (chicoric acid)	473	MS^2[473]: 311(100); 293(80); 178.9(5); 148.9(3)
13	Kaempferide-glucuronide	475	MS^2[475]: 299.1(100)
14	Kaempferol-7-O-(6"-O-acetyl)-glucoside	489	MS^2[489]: 285.1(100); 284.1(18); 299.1(5)
15	Isorhamnetin-7-O-glucuronide	491	MS^2[491]: 315.1(100); MS^3[315]: 300.1(100)
16	Quercetin-7-O-(6"-O-acetyl)-glucoside	505	MS^2[505]: 301(100); 300(45); 463.2(20)
17	3,5-Di-caffeoylquinic acid	515	MS^2[515]: 353.1(100); 335.1(2); 191(1); MS^3[353.1]: 190.9(100); 179(50); 135.9(10); 172.9(5)
18	Isorhamnetin-7-O-(6"-O-acetyl)-glucoside	519	MS^2[519]: 315.1(100); MS^3[315]: 300.1(100)
19	Kaempferol-7-O-rutinoside	593	MS^2[593]: 285.1(100)
20	Quercetin-7-O-p-coumaroylglucoside	609	MS^2[609]: 301.1(100); 300.1(25); 343.1(12); 463.3(3)
21	Isorhamnetin-7-O-neohesperidoside	623	MS^2[623]: 315.1(100); MS^3[315]: 300.1(100)

Table 3

Table 3: Total quantification of target compounds for bioaccessibility determination. Target polyphenols are: cyanidin-3-O-glucoside (C3OG); dicaffeoyltartaric acid (DCTA); 5-caffeoylquinic acid (5CQA); apigenin-7-O-glucoside (A7OG); kaempferol-7-O-glucoside (K7OG); quercetin-3-O-(6"-O-malonyl)-glucoside (Q3OMG); 3-caffeoylquinic acid (3CQA). Errors are reported as standard deviation of five sample extracts.

peak	compound name	t_r (min)	round-leaves (μg/g)	long-leaves (μg/g)
1	C3OG	8.4	61.0±5.9	14.6±1.2
2	DCTA	21.9	804±78	548±42
3	5CQA	8.0	3.920±0.053	1.060±0.082
4	A7OG	22.2	0.340±0.031	0.410±0.044
5	K7OG	21.3	6.55±0.61	7.00±0.63
6	Q3OMG	21.1	521±38	280±21
7	3CQA	11.2	638±18	206±14

Figure 1

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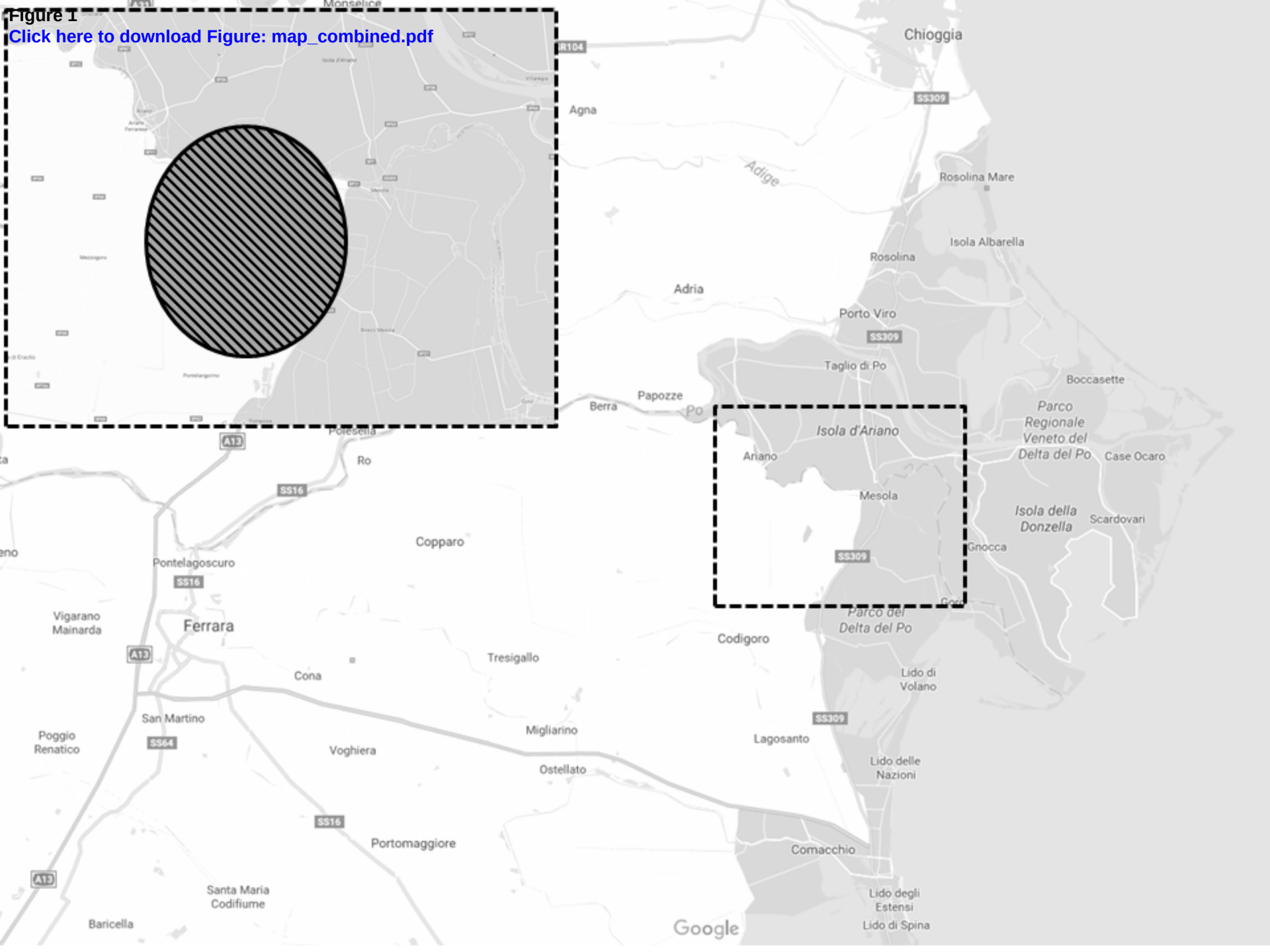

Figure 2

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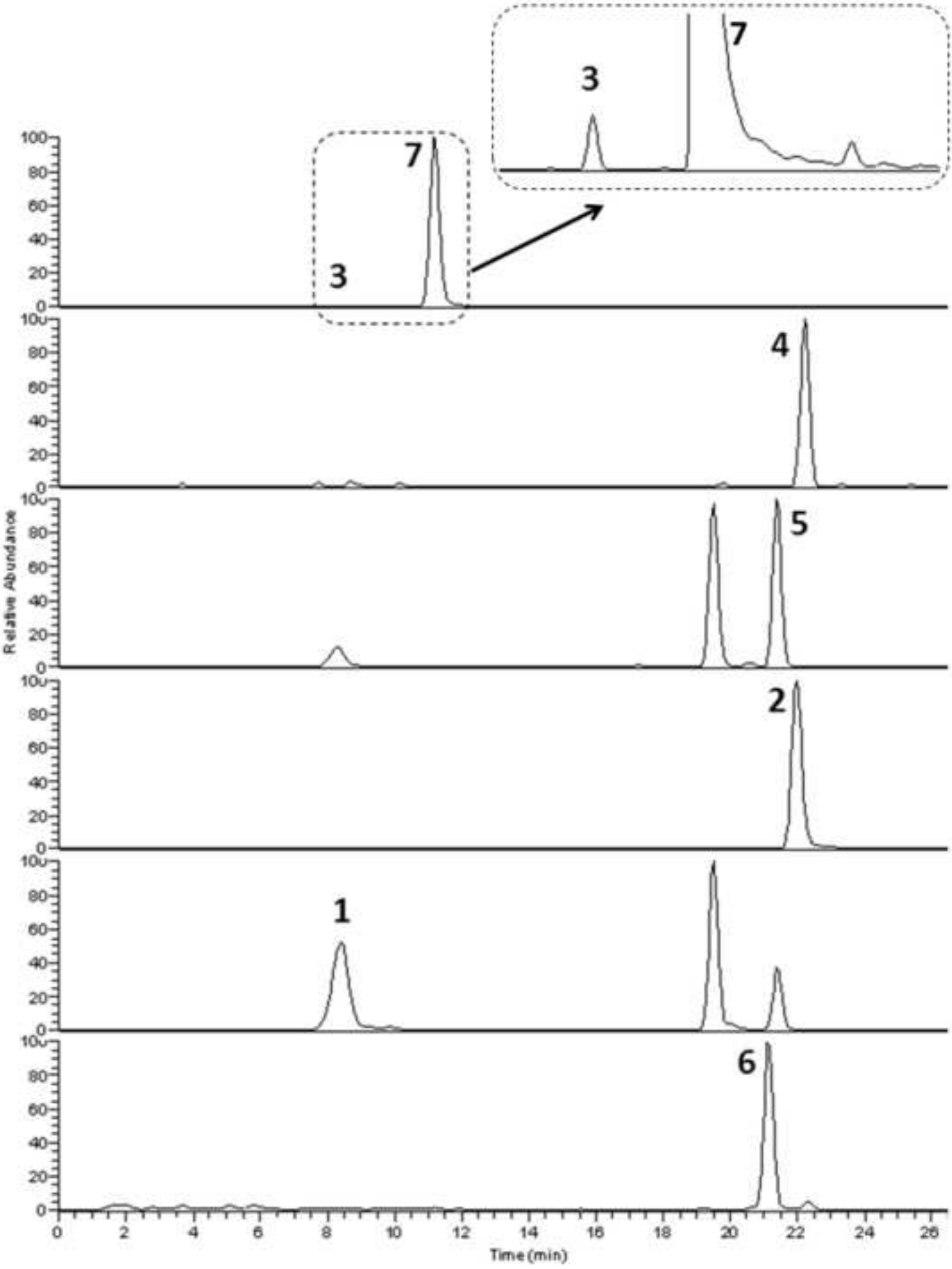

Figure 3a

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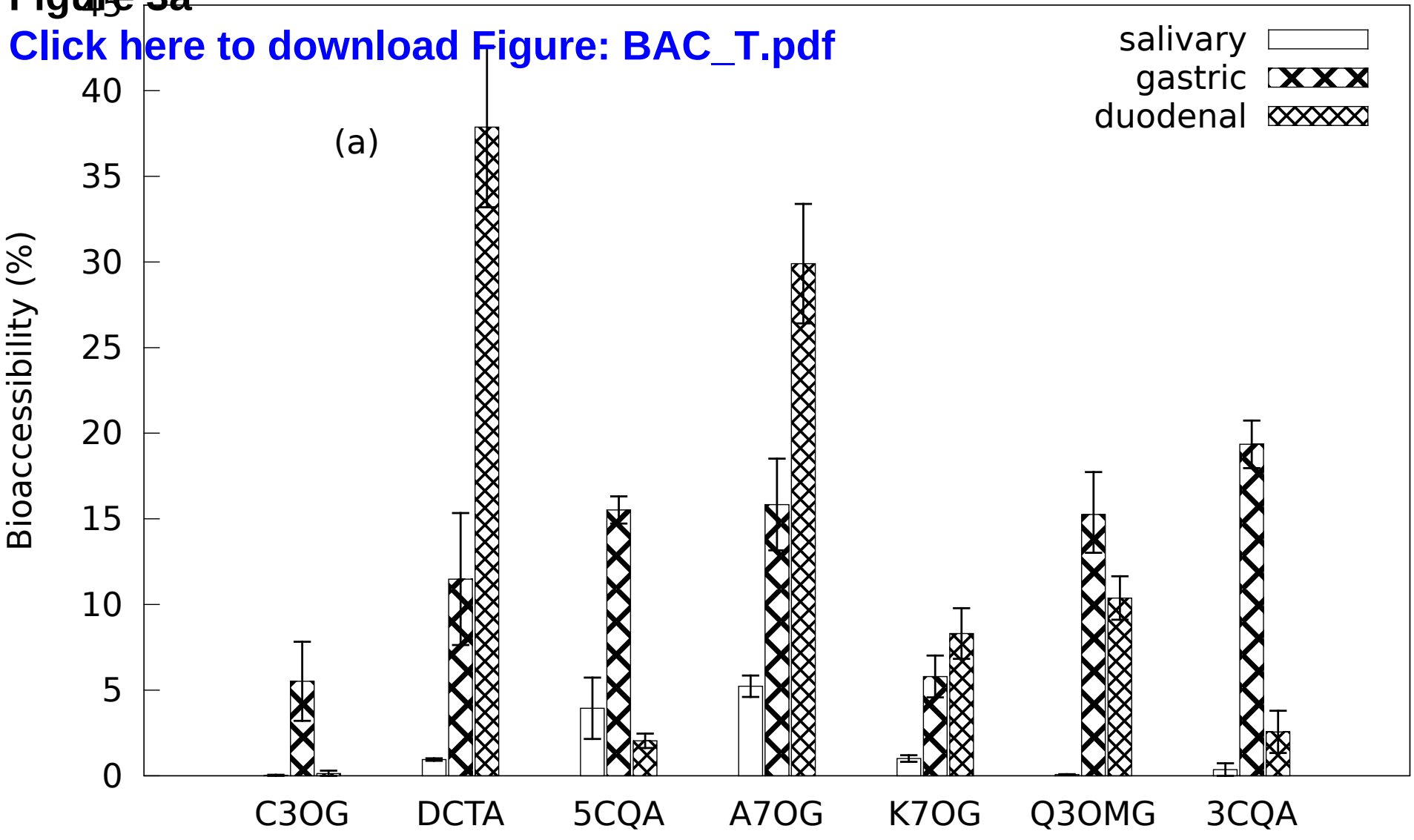

Figure 3b

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