



Chemical composition and antioxidant activity of *Cichorium spinosum* L. leaves in relation to developmental stage



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ABSTRACT

In the present study, chemical composition and bioactivity of *Cichorium spinosum* leaves were examined at different growth stages for optimum harvest stage and end-use assessment. Total fresh weight and number of leaves were higher at 4th growth stage; however, at this stage the end-use is not indicated for raw consumption but mostly for pickled or dried products. Regarding chemical composition, the highest content of tocopherols and minerals was observed in the 1st growth stage, whereas sugars content was the highest in 3rd and 4th growth stage. Polyunsaturated fatty acids content was the highest during the first two growth stages, whereas antioxidant activity, phenolic acids and total phenolic compounds content in the 3rd stage. In conclusion, the results of this study indicate that chemical composition of *C. spinosum* is highly depended on development stage, and harvest stage should be considered for alternative uses of end-products with high bioactivity.

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1. Introduction

Cichorium spinosum is a wild edible green, widely appreciated for its high nutritional value and commonly used as a basic ingredient of the so-called Mediterranean diet. Wild *C. spinosum* plants are usually hand-picked for raw consumption at growth stages which depend on growing conditions, consumer needs and accessibility of growing areas, while cultivated plants can be harvested multiple times during the growing season, as soon as they reach marketable size. Moreover, the fact that the species usually grows in coastal rocky areas which limits accessibility and available harvesting time, while modern life style have shifted consumers' needs towards ready to eat food products, both are responsible for the decrease in wild edible greens consumption during the last decades (Tardío, Pardo-de-santayana, & Morales, 2006). However, nowadays there is an increasing trend for consumption of

“healthy” or “super foods” which have created a market niche for commercial cultivation of wild edible greens (Petropoulos, Ntatsi, Levizou, Barros, & Ferreira, 2016).

Harvest stage is of the highest importance for marketability of leafy vegetables, since visual quality of the final product defines consumer's acceptance. Apart from appearance, harvest may also affect chemical composition and nutritional value and harvest at the optimum stage could significantly increase quality. There are several reports regarding the effect of harvest stage on nutrients and phytochemicals content of leafy vegetables. Omezzine, Bouaziz, Simmonds, and Haouala (2014) have reported that flavonoids, flavones, flavonols and total phenolics content of *Trigonella foenum-graecum* L. was the highest during the vegetative growth stage. Moreover, Pokkaew et al. (2013) have reported significant fluctuation of phenolic compounds of *Arachis hypogaea* L. during the vegetative stage of plants, while Zeghichi, Kallithraka, and Simopoulos (2003) suggested that the optimum stage of harvest for *C. spinosum* leaves is at 40–50 days after planting.

Although the effect of multiple harvests has been reported in terms of yield for various leafy vegetables (Csizinszky, 1999) and chemical composition of *C. spinosum* in particular (Zeghichi et al., 2003), so far no reports are available regarding the effect of growth

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stage on chemical composition and bioactive compounds content of leafy vegetables, mostly due to their short growing period and the market standards which demand specific plant size. Therefore, the aim of the present study was to assess chemical composition of *C. spinosum* leaves at various growth stages, in order to evaluate the potential of other uses except for raw consumption, such as extracts, decoctions and pickles, which could increase added value of the final product and availability throughout the season.

2. Materials and methods

2.1. Plant material and growing conditions

Seedlings of *Cichorium spinosum* L. (Asteraceae) were obtained from Vianame S.A. (Timpaki, Greece). Seeds of *Cichorium spinosum* L. (Asteraceae) were sown on September 2nd 2015 in seed trays containing peat, as previously described by Anesti et al. (2016). Young seedlings were transplanted when plants reached the stage of 3–4 true leaves on December 1st, 2015 [90 days after sowing (DAS)] in 2 L pots containing peat (Klassman-Deilmann KTS2, 1.0 L) and perlite (1.0 L), and put in an unheated plastic greenhouse. Plants were fertilized through the irrigation water at regular intervals with the same amount of nitrogen (300 mg L^{-1}), starting with 50 mL of nutrient solution per pot and up to 300 mL per pot towards the end of the growth cycle. Growing conditions (mean, max and min temperature, and Relative Humidity) throughout the experiment are presented in Supplementary Material (Fig. S1).

Leaves were harvested at four different stages of plant development and up to flower initiation (133, 175, 195 and 205 DAS) from different plants each time (with no prior harvests being applied), in order to evaluate the chemical composition of the aerial parts at various growth stages. The studied harvest stages are illustrated in Supplementary Material (Fig. S2). On each harvest day, fresh and dry weight of leaves was measured. Dry weight of fresh leaves was evaluated after oven drying of samples at 72°C to a constant weight (approximately for 48 h).

2.2. Chemical composition analyses

For chemical composition, raw samples of leaves were stored at deep freezing conditions (-80°C) and freeze-dried prior to analysis.

Free sugars were determined in dried sample powder (1.0 g), spiked with melezitose as internal standard (IS, 5 mg/mL), and extracted with 40 mL of 80% aqueous ethanol at 80°C for 30 min. The resulting suspension was centrifuged (Centurion K240R refrigerated centrifuge, West Sussex, UK) at $15,000g$ for 10 min. The supernatant was concentrated at 60°C under reduced pressure and defatted three times with 10 mL of ethyl ether, successively. After concentration at 40°C , the solid residues were dissolved in water to a final volume of 5 mL and filtered through $0.2 \mu\text{m}$ nylon filters from Whatman. The analysis was performed by high performance liquid chromatography with a refraction index detector (HPLC-RI; Knauer, Smartline system 1000, Berlin, Germany), as previously described by Barros et al. (2013). The sugars were identified by comparing their retention times with standard compounds and quantification was conducted by comparison with dose–response curves constructed from authentic standards, using the internal standard (IS, melezitose) method (DataApex, Podohradská, Czech Republic).

Organic acids were determined using ultra-fast liquid chromatography coupled to a photodiode array detector (UFLC-DAD). Samples ($\sim 2 \text{ g}$) were extracted by stirring with 25 mL of metaphosphoric acid (25°C at 150 rpm) for 45 min and subsequently filtered through Whatman No. 4 paper. Before analysis, the sample

was filtered through $0.2 \mu\text{m}$ nylon filters (Pereira, Barros, Carvalho, & Ferreira, 2013). The analysis was performed using a Shimadzu 20A series UFLC (Shimadzu Corporation, Kyoto, Japan) and detection was carried out in a DAD, using 215 nm and 245 nm (for ascorbic acid) as preferred wavelengths and controlled by LabSolutions multi LC-DAD software (Shimadzu Corporation, Kyoto, Japan). Quantification was performed by area comparison with calibration curves obtained from each standard compound.

Fatty acids were analyzed with a DANI 1000 gas chromatograph (GC, Milan, Italy) coupled to a flame ionization detector (FID), after a transesterification procedure described by Barros et al. (2013). Fatty acids (obtained after Soxhlet extraction) were methylated with 5 mL of methanol:sulphuric acid:toluene 2:1:1 (v:v:v), during at least 12 h in a bath at 50°C and 160 rpm; then 3 mL of deionised water were added, to obtain phase separation; the FAME were recovered with 3 mL of diethyl ether by shaking in vortex, and the upper phase was passed through a micro-column of sodium sulphate anhydrous, in order to eliminate the water; the sample was recovered in a vial with Teflon, and before injection the sample was filtered with $0.2 \mu\text{m}$ nylon filter from Whatman. The FAMES were identified by comparing their retention time with authentic standards and the results were recorded and processed by using Clarity 4.0.1.7 Software (DataApex, Podohradská, Czech Republic).

Tocopherols were determined following a procedure previously described by Barros et al. (2013). BHT solution in hexane (10 mg/mL ; $100 \mu\text{L}$) and IS solution in hexane (tocol; $50 \mu\text{g/mL}$; $400 \mu\text{L}$) were added to the sample prior to the extraction procedure. The samples ($\sim 500 \text{ mg}$) were homogenized with methanol (4 mL) by vortex mixing (1 min). Subsequently, hexane (4 mL) was added and again vortex mixed for 1 min. After that, saturated NaCl aqueous solution (2 mL) was added, the mixture was homogenized (1 min), centrifuged (5 min, $4000g$) and the clear upper layer was carefully transferred to a vial. The sample was re-extracted twice with hexane. The combined extracts were taken to dryness under a nitrogen stream, redissolved in 2 mL of *n*-hexane, dehydrated with anhydrous sodium sulphate, filtered through $0.2 \mu\text{m}$ nylon filters from Whatman, transferred into a dark injection vial prior to the analysis. The chromatographic separation was achieved using an HPLC (Knauer, Smartline system 1000, Berlin, Germany) coupled to a fluorescence detector (FP-2020; Jasco, Easton, MD, USA), programmed for excitation at 290 nm and emission at 330 nm. The compounds were identified by chromatographic comparisons with authentic standards and quantification was conducted by comparison with dose–response curves constructed from authentic standards, using the IS (tocol) method (DataApex, Podohradská, Czech Republic).

Oven dried samples of leaves tissues were subjected to dry ashing and extracted with 1 N HCl for mineral content determination. Mg, Fe, Mn, Zn, and Cu content were determined by atomic absorption spectrophotometry (Perkin Elmer 1100B, Waltham, MA, USA) and Ca, Na and K content by flame photometry (Sherwood Model 410, Cambridge, UK).

Nitrate content was assessed colorimetrically by the nitration of salicylic acid, following the procedure previously described by Cataldo, Maroon, Schrader, and Youngs (1975), using a Perkin Elmer Model Lambda 1A spectrophotometer (Perkin Elmer, Waltham, Mass.).

2.3. Antioxidant activity assays

For methanolic/water (80:20, v/v) extraction, one gram of lyophilized material was extracted twice for 1 h in a magnetic stirrer plate (25°C at 150 rpm), with 30 mL of methanol/water (80:20, v/v), filtered through a Whatman No. 4 paper and vacuum-dried in a rotary evaporator (rotary evaporator Büchi R-210, Flawil,

Switzerland) at 40 °C to remove the methanol. The extracts were further frozen and lyophilized. Afterwards, the extracts were re-dissolved in methanol/water (80:20, v/v) for *in vitro* antioxidant activity assays, at a final concentration of 20 mg/mL and further diluted to different concentrations.

The antioxidant activity was evaluated by DPPH radical-scavenging activity, reducing power, inhibition of β -carotene bleaching in the presence of linoleic acid radicals and inhibition of lipid peroxidation using TBARS in brain homogenates (Petropoulos, Fernandes, Barros, Ferreira, & Ntatsi, 2015). The results were expressed in EC₅₀ values (sample concentration providing 50% of antioxidant activity of DPPH radical-scavenging activity, inhibition of β -carotene bleaching and TBARS assays, or 0.5 of absorbance for the reducing power assay) for antioxidant activity and Trolox was used as a positive control.

2.4. Phenolic compounds analysis

The above-mentioned extracts [methanolic/water extracts; (80:20, v/v)] were re-dissolved in methanol/water (80:20, v/v) at a final concentration of 30 mg/mL, filtered through a 0.45 μ m Whatman syringe filter, and transferred to amber color HPLC vial for phenolic compound analysis. The phenolic compounds were determined by LC-DAD-ESI/MSn (Dionex Ultimate 3000 UPLC, Hermo Scientific, San Jose, CA, USA), as previously described by Bessada, Barreira, Barros, Ferreira, and Oliveira (2016). Double online detection was performed using Diode Array Detector (DAD) with 280, 330 and 370 nm as preferred wavelengths, and a mass spectrometer (MS). The MS detection was performed in negative mode, using a Linear Ion Trap LTQ XL mass spectrometer (ThermoFinnigan, San Jose, CA, USA) equipped with an ESI source. Phenolic compounds identification was performed by comparing their retention time, UV-Vis and mass spectra with available standard compounds and by comparing the obtained information with available data reported in the literature, giving a tentative identification of the detected compounds. For quantitative analysis, a calibration curve for each available phenolic compound standard (Extrasynthèse, Genay, France) was constructed based on the UV signal, whereas when no commercial standards were available, the quantification was performed through the calibration curve of the most similar available standard.

2.5. Statistical analyses

The experiment was laid out in a Completely Randomized Blocks design with 10 pots per treatment and $n = 3$. Statistical analysis was carried out with Statgraphics 5.1.plus (Statistical Graphics Corporation). Data were evaluated by analysis of variance, while the means of values were compared by Tukey's HSD Test at $p < 0.05$. For chemical composition analyses, three samples for each treatment were analysed while all the assays were carried out in triplicate. The results are expressed as mean values and standard deviations (SD), and analysed using one-way analysis of variance (ANOVA) followed by Tukey's HSD Test at $p < 0.05$. This analysis was carried out using SPSS v. 22.0 program (IBM Corp., Armonk, NY, USA).

3. Results and discussion

The results include limited data (all the data related with the 1st growth stage) that have been presented in another study by the authors (Petropoulos, Fernandes, et al., 2017), where the effect of successive harvesting on chemical composition and bioactive properties of *C. spinosum* leaves was examined. Therefore, these

data have been excluded from all the tables and a special mention is given on the abovementioned paper.

Fresh weight and number of leaves are positively correlated and both increase with plant development, considering that leaves are formed in a rosette shaped formation, whereas dry weight showed its highest value at the 2nd growth stage (Table 1). The same results have been observed in other leafy vegetables, which in contrast to fruit vegetables they gain weight by continuously forming new leaves in the meristem apex (Palaniswamy, McAvoy, & Bible, 2004; Petropoulos, Akoumianakis, & Passam, 2005). Considering that leafy vegetables are usually intended for raw consumption, harvest at early stages is the common practice since texture and visual quality of leaves are the highest at these stages. However, late harvesting could be also an option for alternative end-uses, such as pickled or dried innovative products.

Chemical composition of leaves changed significantly during the growing period and up to flower initiation where the last harvest took place (4th harvest at 205 DAS). Total organic acids content was higher at the 3rd growth stage (195 DAS), mostly due to higher content of oxalic and quinic acids, followed by a rapid decrease at the last harvest for both organic acids (Table 2). Although Palaniswamy et al. (2004) have reported that oxalic acid decreased significantly in purslane leaves and stems at the stage of 16-true leaves comparing to 8-true leaves, this decreasing trend was only observed at late growth stages of this study (4th growth stage). However, no direct comparison between the species can be made, considering the differences in their growth cycle duration and climate requirements. Ascorbic acid content was the highest at the 3rd growth stage, indicating high nutritional value at this developmental stage (Table 2), while Zeghichi et al. (2003) have also reported a variable ascorbic acid content at different harvest stages. Leafy vegetables are usually considered rich sources of ascorbic acid and contribute significantly to the recommended daily intake; however, there is no consistent trend reported for the various vegetable species regarding the optimum harvest stage for high ascorbic acid content, since many other factors may affect this compound's content (Bergquist, Gertsson, & Olsson, 2006).

The main identified sugars were sucrose and glucose, while fructose and trehalose were detected in lower amounts (Table 2). Total and individual sugars content increased with plant development with highest values being observed in the 3rd and 4th growth stages. This increase in sugar content with plant development has been also observed for *C. intybus* var. Rosso di Chioggia by Poli et al. (2002), who reported a significant increase of sucrose and fructose, especially in the outer leaves of two selections (C2 and C3) of this variety. However, in the same study sugar content was not significantly correlated with bitter taste of leaves, since bitterness was mostly affected by guaianolide content. In contrast, according to Lin et al. (2013) sugar content is an important quality feature of lettuce leaves. In the same study and the study of Wojciechowska, Dugosz-Grochowska, Koton, and Zupnik (2015), sugar content in lettuce and lamb's lettuce leaves, respectively,

Table 1

Fresh and dry weight (expressed in g/plant and % of dry matter, respectively) and number of leaves of *Cichorium spinosum* plants (mean \pm SD) in relation to growth stage.

Growth stage	Fresh weight	Dry weight	Number of leaves
2nd*	46.4 \pm 6.5b	9.0 \pm 0.2a	38.8 \pm 7.1b
3 rd	49.3 \pm 0.6b	8.4 \pm 0.3b	44.1 \pm 7.7b
4 th	55.3 \pm 2.3a	8.5 \pm 0.5b	52.3 \pm 6.4a

Means of the same column followed by different Latin letters are significantly different according to Tukey HSD test ($P < 0.05$).

*Data regarding 1st growth stage have been previously reported by the authors (Petropoulos, Fernandes, et al., 2017).

Table 2Composition of organic acids and sugars (mg/100 g fw) of *Cichorium spinosum* leaves (mean \pm SD) in relation to growth stage.

Growth stage	Oxalic acid	Quinic acid	Malic acid	Ascorbic acid	Citric acid	Total organic acids	Fructose	Glucose	Sucrose	Trehalose	Total Sugars
2nd ^a	51.5 \pm 0.2b	38.9 \pm 0.3b	22.99 \pm 0.08a	0.030 \pm 0.001b	11.6 \pm 0.1a	125 \pm 1b	180 \pm 2b	383 \pm 1b	185 \pm 3b	52 \pm 2c	799 \pm 1b
3rd	66.0 \pm 0.1a	52.4 \pm 0.1a	23.0 \pm 0.2a	0.090 \pm 0.001a	3.1 \pm 0.1b	145 \pm 1a	137 \pm 4c	456 \pm 2a	412 \pm 9a	66 \pm 5b	1070 \pm 1a
4th	45.5 \pm 1.4c	34.4 \pm 0.5c	18.53 \pm 0.05b	0.020 \pm 0.001c	0.14 \pm 0.002c	100 \pm 2c	249 \pm 2a	351 \pm 4c	406 \pm 1a	82 \pm 2a	1087 \pm 3a

Means of the same column followed by different Latin letters are significantly different according to Tukey HSD test ($P < 0.05$).^aData regarding 1st growth stage have been previously reported by the authors (Petropoulos, Fernandes, et al., 2017).

was significantly affected by light quality and intensity, while Wojciechowska et al. (2015) have also observed a significant positive correlation between sugars content and fresh weight of rosettes, which was also the case in our study. Furthermore, sugar composition changes during plant development have been also observed in lettuce plants, with sucrose being the main reserve sugar prior to anthesis, followed by lower molecular weight sugars such as glucose and fructose (Lee & Sugiyama, 2006).

The fatty acid composition and total fatty acids content is presented in Table 3. The main detected fatty acids were α -linolenic, linoleic and palmitic acid, whereas pentadecanoic, palmitoleic, stearic, oleic and behenic acid were detected in lesser amounts. The same fatty acids have been reported in other species of *Cichorium*, e.g. *C. intybus* (Morales et al., 2012) and *C. spinosum* (Petropoulos, Chatzieustratiou, Constantopoulou, & Kapotis, 2016; Petropoulos, Levizou, et al., 2017; Zeghichi et al., 2003). Polyunsaturated fatty acids (PUFAs) were the main fatty acids

class, consisting mostly of α -linolenic and linoleic acids which showed a decreasing trend with plant development, followed by a proportionate increase of saturated fatty acids and palmitic acid in particular. This decreasing trend of PUFAs could be associated with high metabolic rates during plant development, especially prior to flower initiation and flower stalk formation, since long-chain fatty acids are essential energy sources for plant metabolic pathways (Graham & Eastmond, 2002). In addition, the gradual decrease of PUFAs could be due to lower tocopherols content (Table 3) which have been reported to have a protective role against lipid oxidation (Cao et al., 2015). The detected ratios of PUFA/SFA and n-6/n-3 fatty acids at the various growth stages of the present study can be considered as beneficial for health, since high intake of n-6 fatty acids has been associated with many chronic diseases, such as coronary heart disease, hypertension and type 2 diabetes (Simopoulos, 1999). According to Zeghichi et al. (2003), fatty acids composition of *C. spinosum* showed a great variation at successive harvests and is highly dependent on harvest stage. Moreover, in the same study a significant decrease of palmitic and linoleic acid was observed after 30 and 40 days from transplantation, whereas α -linolenic decreased after 50 days from transplantation. However, the fatty acids evaluation in that study was carried out in plants successively harvested in regular intervals, comparing to our study where plants were only harvested once at each growth stage. To our knowledge this is the first report regarding the fatty acids composition of *C. spinosum* leaves at different growth stages.

Tocopherols content was significantly affected by growth stage for both total tocopherols and individual vitamers (Table 3). The main detected tocopherols were alpha- and gamma-tocopherols which showed an inversely proportional trend between the various growth stages. The increase of alpha-tocopherol during *C. spinosum* development could be associated with more stressful conditions due to high temperatures at late growth stages (early to mid-spring), comparing to earlier stages (winter). This response of alpha-tocopherol to high temperatures has been confirmed in soybean seeds (*Glycine max* L.) by Chennupati, Seguin, and Liu (2011) who further noted that the decrease is also cultivar dependent. Similarly, Zeghichi et al. (2003) have reported a decrease of alpha-tocopherol content in *C. spinosum* plants during growing period, despite the fact that they evaluated it in successively harvested leaves and not in different developmental stages. The effect of developmental stage on tocopherols content has been reported for other species, such as members of the Brassicaceae family (Björkman et al., 2011), *Zingiber zerumbet* L. (Ghasemzadeh et al., 2016), *Momordica charantia* L. (Dhillon et al., 2016) and so forth.

Mineral content decreased significantly during plant development with the lowest content being observed prior to flower initiation at the 4th growth stage (Table 4). Moreover, mineral content values were within the range reported by Petropoulos, Levizou, et al. (2017) who evaluated different ecotypes of *C. spinosum*. Similar decreasing trends in mineral content of *C. spinosum* leaves at late growth stages have been also by Zeghichi et al. (2003), although they analysed minerals content in leaves successively harvested at regular intervals. This decreasing trend could be due

Table 3Composition of *Cichorium spinosum* leaves in fatty acids (%; mean \pm SD) and tocopherols (mg/100 g fw) in relation to growth stage.

	Growth stage		
	2nd ^a	3rd	4th
C6:0	0.061 \pm 0.001	0.094 \pm 0.005	0.060 \pm 0.006
C8:0	0.041 \pm 0.001	0.069 \pm 0.001	0.084 \pm 0.001
C10:0	0.040 \pm 0.001	0.056 \pm 0.001	0.069 \pm 0.001
C12:0	0.065 \pm 0.003	0.068 \pm 0.004	0.156 \pm 0.008
C14:0	0.229 \pm 0.006	0.401 \pm 0.003	1.00 \pm 0.03
C15:0	0.349 \pm 0.004	0.331 \pm 0.006	0.752 \pm 0.006
C16:0	8.10 \pm 0.08	10.89 \pm 0.05	14.36 \pm 0.05
C16:1	0.327 \pm 0.004	0.405 \pm 0.001	0.684 \pm 0.008
C17:0	0.105 \pm 0.005	0.183 \pm 0.003	0.328 \pm 0.006
C18:0	0.745 \pm 0.001	1.095 \pm 0.001	1.74 \pm 0.01
C18:1n9c	0.765 \pm 0.003	1.20 \pm 0.02	1.14 \pm 0.03
C18:2n6c	15.33 \pm 0.01	19.33 \pm 0.03	18.21 \pm 0.01
C18:3n3	72.16 \pm 0.09	63.46 \pm 0.06	59.08 \pm 0.08
C20:0	0.24 \pm 0.01	0.43 \pm 0.01	0.447 \pm 0.001
C20:1	0.022 \pm 0.001	0.038 \pm 0.002	0.046 \pm 0.001
C20:2	0.12 \pm 0.01	0.117 \pm 0.005	0.072 \pm 0.001
C20:3n3	0.139 \pm 0.008	0.127 \pm 0.003	0.123 \pm 0.004
C21:0	0.027 \pm 0.001	0.067 \pm 0.003	0.087 \pm 0.008
C20:5n3	0.081 \pm 0.001	0.152 \pm 0.008	0.21 \pm 0.02
C22:0	0.287 \pm 0.008	0.458 \pm 0.024	0.446 \pm 0.001
C23:0	0.092 \pm 0.008	0.196 \pm 0.007	0.225 \pm 0.009
C24:0	0.67 \pm 0.01	0.84 \pm 0.01	0.69 \pm 0.03
Total SFA (% of total FA)	11.05 \pm 0.10c	15.18 \pm 0.04b	20.45 \pm 0.13a
Total MUFA (% of total FA)	1.11 \pm 0.01d	1.65 \pm 0.02b	1.87 \pm 0.02a
Total PUFA (% of total FA)	87.84 \pm 0.10b	83.18 \pm 0.02c	77.68 \pm 0.11d
alpha-Tocopherol	0.302 \pm 0.004c	0.563 \pm 0.005b	0.717 \pm 0.004a
beta-Tocopherol	0.008 \pm 0.001c	0.011 \pm 0.001b	0.021 \pm 0.001a
gamma-Tocopherol	0.478 \pm 0.004a	0.320 \pm 0.005b	0.152 \pm 0.001c
delta-Tocopherol	0.002 \pm 0.001	tr ^b	tr
Total Tocopherols	0.791 \pm 0.008b	0.894 \pm 0.001a	0.891 \pm 0.002a

Means of the same raw followed by different Latin letters are significantly different according to Tukey HSD test ($P < 0.05$).^aData regarding 1st growth stage have been previously reported by the authors (Petropoulos, Fernandes, et al., 2017).^btr: <0.001 mg/100 g fw.

Table 4Mineral composition and nitrates content (mg/100 g fw) of *Cichorium spinosum* leaves (mean \pm SD) in relation to growth stage.

Growth stage	K	Na	Ca	Mg	Zn	Mn	Fe	NO ₃
2nd ^a	385.6 \pm 26.0a	61.5 \pm 11.8a	252.3 \pm 52.5a	37.4 \pm 3.0a	0.28 \pm 0.06a	0.64 \pm 0.02a	0.62 \pm 0.02a	121.2 \pm 26.4a
3rd	347.3 \pm 59.5c	54.2 \pm 9.7b	178.0 \pm 35.3b	33.5 \pm 6.0b	0.22 \pm 0.01b	0.60 \pm 0.02b	0.61 \pm 0.06a	70.2 \pm 12.7b
4th	362.4 \pm 32.1b	43.2 \pm 10.1c	142.6 \pm 12.5c	29.4 \pm 2.2c	0.17 \pm 0.01c	0.59 \pm 0.04c	0.54 \pm 0.01b	112.8 \pm 25.6a

Means of the same column followed by different Latin letters are significantly different according to Tukey HSD test ($P < 0.05$).^aData regarding 1st growth stage have been previously reported by the authors (Petropoulos, Fernandes, et al., 2017).

to the fact that transition of plants from vegetative to reproductive stage is probably not related with plant size (number of produced leaves) but with environmental conditions (temperature and photoperiod) which could induce anthesis despite the number of leaves. Therefore, even regularly harvested plants proceed to reproductive stage, despite the number of formed leaves. The decrease in mineral content observed at the latest growth stages of our study could be correlated with redistribution of minerals towards the tissues that are more metabolically active, since flower initiation is linked to flower stalk elongation and flowers formation (Watanabe et al., 2016); however, this assumption cannot be confirmed in the present study since flower stalks were not analysed for their mineral content.

Nitrate content fluctuated throughout the growing season, with the highest content being detected at the 2nd and 4th growth stage. This trend could be partially explained by growing conditions, especially light quality and intensity which has an important role in nitrates reduction to nitrites by nitrate reductase (Campbell, 1999), as well as by accumulation of nitrates at later stages of plant development for osmoregulation and remobilization purposes (Masclaux-Daubresse et al., 2010). According to Lin et al. (2013), nitrate content in lettuce leaves was significantly affected by light quality, while Petropoulos, Chatzieustratiou, et al. (2016) have reported the effect of harvest time, growing period and nitrogen fertilization rates on nitrates content of parsley leaves. Moreover, Gent (2012) has reported that nitrate content in leafy vegetables is inversely related with organic acids and sugar content, which was also the case in our study and especially for the 3rd growth stage where nitrate content decrease was followed by an increase in organic acids and sucrose content.

The detected phenolic compounds and their identification data are presented in Table 5. Identification of phenolic compounds was carried out and cross checked through their mass spectrum, UV absorption and chromatograms. Fourteen different phenolic compounds were detected in total for all the studied growth stages. All the detected compounds have been previously identified by Petropoulos, Levizou, et al. (2017) in *C. spinosum* leaves. Compounds 1 and 3 (5-O-caffeoylquinic acid and chicoric acid, respectively) were the main compounds in every growth stage. Moreover, total phenolic compounds consisted mostly of phenolic acids, especially in the 3rd growth stage where 80% of total phenolic compounds were phenolic acids. Both compounds have been associated with various bioactive effects such as anti-inflammatory, antioxidant and anti-obesity properties, while *in vitro* studies have confirmed their antidiabetic effects (Brieudes et al., 2016; Zhu, Wang, Du, Liu, & Liu, 2015). Therefore, considering the high content of leaves in these compounds at late developmental stages (3rd stage), alternative uses for pharmaceutical purposes can be proposed.

Phenolic composition was also affected by growth stage, with compounds 9 and 14 (3,5-O-dicaffeoylquinic acid and isorhamnetin-3-O-(6''-O-acetyl)-glucoside, respectively) being detected only in the 1st growth stage (Petropoulos, Fernandes, et al., 2017). Zeghichi et al. (2003) have also reported fluctuation of total phenolic compounds content in successively harvested *C. spinosum* leaves. According to Brieudes et al. (2016), chicoric acid

was one of the main antioxidant compounds detected in water decoctions of *C. spinosum* and *C. intybus* leaves, while Jaiswal, Kiprotich, and Kuhnert (2011) have identified the same phenolic acid in *C. intybus*. 5-O-caffeoylquinic acid has also been positively identified in *C. intybus* leaves in many studies so far (Carazzzone, Mascherpa, Gazzani, & Papetti, 2013; Jaiswal et al., 2011). In the study of Rees and Harborne (1985), who evaluated sesquiterpene lactones and phenolic acids content at various growth stages of *C. intybus*, it was supported that chicoric acid content fluctuated during the growing season, while significant differences were observed between leaves at different positions on the plant. In the same study, it was suggested that sesquiterpene lactones and phenolic acids have a protective role against herbivore insects; therefore, their content fluctuates during the growing season according to physiological stages of plants and their pests.

Antioxidant properties of *C. spinosum* leaves were determined with four different assays (Table 6), which all indicate the 3rd growth stage as the most appropriate stage of leaves for high antioxidant potency according to the obtained EC₅₀ values. The increase in antioxidant activity at the 3rd growth stage is highly associated with the increased content of bioactive compounds, such as ascorbic acid, phenolic acids and total phenolic compounds (Tables 2 and 5), whereas tocopherols content seems that is not significantly correlated with the antioxidant properties of *C. spinosum* leaves (Table 3). Similar results have been reported by Brieudes et al. (2016), who evaluated the antioxidant properties of water decoctions of *C. spinosum* leaves and suggested a significant correlation of antioxidant potency with phenolic compounds. Pereira, Barros, Carvalho, and Ferreira (2011) have also suggested phenolic compounds content to be linearly correlated with antioxidant activity of various wild edible greens, with different correlations between the various classes of phenolic compounds and the tested assays. Considering that harvests at late growth stages may have a negative effect on edibility and digestibility of leaves due to harsh texture and high content of fibres, their alternative uses as extracts, decoctions or pickles could have a great potential in the food and pharmaceutical industry due to their high content in antioxidant compounds.

4. Conclusions

The results of the present study suggest that chemical composition and bioactive compounds content of leaves is significantly modified during plant development. Although leaves intended for raw consumption are usually harvested at early stages, other end-uses could be possible for leaves harvested at later stages, especially prior and during flowering initiation where phenolic compounds content and antioxidant properties of leaves were the highest. In conclusion, apart from raw consumption, pickled products, water extracts or decoctions are also possible to be prepared ensuring better product availability throughout the year, as well as alternative end-use products with high added value for the food and pharmaceutical industry. Therefore, harvesting at the proper developmental stage should be considered as a means of manipulating chemical composition and bioactive properties of leaves.

Table 5

Retention time (Rt), wavelengths of maximum absorption in the visible region (λ_{\max}), mass spectral data, tentative identification quantification of phenolic compounds in *Cichorium spinosum* (mg/g extract, mean \pm SD) in relation to the development stage.

Peak	Rt (min)	λ_{\max} (nm)	[M–H] [–] (m/z)	MS ² (m/z)	Tentative identification	Growth stage		
						2nd ^a	3rd	4th
1	7.1	325	353	191(100),179(5),173(3),161(3),135(3)	5-O-Caffeoylquinic acid ¹	3.51 \pm 0.08	6.99 \pm 0.02	6.7 \pm 0.1
2	12.0	313	337	191(100),173(3),163(10),145(3),119(3)	p-Coumaroylquinic acid ²	0.179 \pm 0.008	0.388 \pm 0.002	0.199 \pm 0.001
3	12.4	328	473	311(100),293(94),219(3),179(7),149(3),135(3)	Chicoric acid ³	5.33 \pm 0.03	10.27 \pm 0.01	4.8 \pm 0.1
4	13.6	327	367	193(10),191(100),173(5),143(3),134(3)	5-O-Feruloylquinic acid ⁴	0.010 \pm 0.001	nd	0.434 \pm 0.001
5	16.5	328	193	178(20),134(100),117(8)	Ferulic acid ⁴	0.141 \pm 0.003	0.078 \pm 0.001	0.147 \pm 0.002
6	18.2	342	477	301(100)	Quercetin-3-O-glucuronide ⁵	0.757 \pm 0.001	0.576 \pm 0.003	1.634 \pm 0.001
7	18.7	348	461	285(100)	Kaempferol-O-glucuronide ⁶	1.77 \pm 0.01	2.20 \pm 0.01	2.833 \pm 0.005
8	20.5	360	505	463(26),301(100)	Quercetin-7-O-(6''-O-acetyl)-glucoside ⁵	0.226 \pm 0.001	0.246 \pm 0.002	0.408 \pm 0.009
9	20.9	329	515	353(100),191(97),179(48),173(5),161(3),135(7)	3,5-O-Dicaffeoylquinic acid ¹	nd ^{**}	nd	nd
10	22.2	345	461	285(100)	Kaempferol-3-O-glucuronide ⁶	0.915 \pm 0.004	0.806 \pm 0.003	2.104 \pm 0.003
11	23.4	337	447	269(100)	Apigenin-O-glucuronide ⁷	0.238 \pm 0.001	0.236 \pm 0.002	nd
12	23.7	350	491	315(100)	Isorhamnetin-3-O-glucuronide ⁵	0.233 \pm 0.001	0.229 \pm 0.001	0.364 \pm 0.001
13	25.0	345	489	285(100)	Kaempferol-3-O-(6''-O-acetyl)-glucoside ⁶	0.047 \pm 0.001	0.140 \pm 0.002	0.286 \pm 0.004
14	26.3	334	519	477(5),315(100)	Isorhamnetin-3-O-(6''-O-acetyl)-glucoside ⁵	nd ^{**}	nd	nd
Total phenolic acids						9.2 \pm 0.1c	17.733 \pm 0.008a	12.31 \pm 0.03b
Total flavonoids						4.18 \pm 0.01c	4.429 \pm 0.003b	7.63 \pm 0.01a
Total phenolic compounds						13.4 \pm 0.1c	22.162 \pm 0.004a	19.94 \pm 0.02b

^aData regarding 1st growth stage have been previously reported by the authors (Petropoulos, Fernandes, et al., 2017).

^{**}nd: not detected (detected only in the 1st growth stage). Calibration curves used: A- chlorogenic acid ($y = 168823x - 161172$; $R^2 = 0.999$); B- p-coumaric acid ($y = 301950x + 6966.7$; $R^2 = 0.999$); C- caffeic acid ($y = 388345x + 406369$; $R^2 = 0.994$); D- ferulic acid ($y = 633126x - 185462$; $R^2 = 0.999$); E- quercetin-3-O-glucoside ($y = 34843x - 160173$; $R^2 = 0.999$); F- kaempferol-3-O-rutinoside ($y = 11117x + 30861$; $R^2 = 0.999$); G- apigenin-7-O-glucoside ($y = 10683x - 45794$; $R^2 = 0.996$).

Means of the same raw followed by different Latin letters are significantly different according to Tukey HSD test ($P < 0.05$).

Table 6

Antioxidant properties of *Cichorium spinosum* leaves (mean \pm SD) in relation to growth stage.

Growth stage	Reducing power	Radical scavenging activity		Lipid peroxidation inhibition
	Ferricyanide/Prussian blue (EC ₅₀ ; mg/mL)	DPPH scavenging activity (EC ₅₀ ; mg/mL)	β -carotene/linoleate (EC ₅₀ ; mg/mL)	TBARS (EC ₅₀ ; mg/mL)
2nd ^a	0.656 \pm 0.008b	1.45 \pm 0.06a	0.74 \pm 0.03b	0.32 \pm 0.02b
3rd	0.458 \pm 0.008c	0.818 \pm 0.009c	0.69 \pm 0.02c	0.31 \pm 0.02b
4th	0.683 \pm 0.011a	1.37 \pm 0.06b	1.00 \pm 0.03a	0.44 \pm 0.01a

The antioxidant activity was expressed as EC₅₀ values, what means that higher values correspond to lower reducing power or antioxidant potential. EC₅₀: Extract concentration corresponding to 50% of antioxidant activity or 0.5 of absorbance in reducing power assay. Trolox EC₅₀ values: 41 μ g/mL (reducing power), 42 μ g/mL (DPPH scavenging activity), 18 μ g/mL (β -carotene bleaching inhibition) and 23 μ g/mL (TBARS inhibition).

Means of the same column followed by different Latin letters are significantly different according to Tukey HSD test ($P < 0.05$).

^aData regarding 1st growth stage have been previously reported by the authors (Petropoulos, Fernandes, et al., 2017).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.foodchem.2017.07.043>.

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