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Developmental changes in leaf phenolics composition from three artichoke cvs. (*Cynara scolymus*) as determined *via* UHPLC–MS and chemometrics

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ABSTRACT

The metabolomic differences in phenolics from leaves derived from 3 artichoke cultivars (*Cynara scolymus*): American Green Globe, French Hyrious and Egyptian Baladi, collected at different developmental stages, were assessed using UHPLC–MS coupled to chemometrics. Ontogenic changes were considered as leaves were collected at four different time intervals and positions (top and basal) during artichoke development. Unsupervised principal component analysis (PCA) and supervised orthogonal projection to latent structures-discriminant analysis (O2PLS-DA) were used for comparing and classification of samples harvested from different cultivars at different time points and positions. A clear separation among the three investigated cultivars was revealed, with the American Green Globe samples found most enriched in caffeic acid conjugates and flavonoids vs. other cultivars. Furthermore, these metabolites also showed a marked effect on the discrimination between leaf samples from cultivars harvested at different positions, regardless of the plant age. Metabolite absolute quantifications further confirmed that discrimination was mostly influenced by phenolic compounds, namely caffeoylquinic acids and flavonoids. This study demonstrates an effect of artichoke leaf position, regardless of plant age, on its secondary metabolites composition. To the best of our knowledge, this is the first report for compositional differences among artichoke leaves, based on their positions, *via* a metabolomic approach and suggesting that top positioned artichoke leaves present a better source of caffeoylquinic acids, compared to basal ones.

1. Introduction

Globe artichoke (*Cynara cardunculus* var. scolymus Hayek), formerly *Cynara scolymus* L. is an ancient perennial plant species of the Asteraceae family (Lattanzio et al., 2009), mostly cultivated worldwide for its large immature inflorescences, called capitula, with edible fleshy leaves (bracts) and receptacle (Lattanzio et al., 2009). Apart from being consumed as a food, artichoke is recognized as herbal medicine (Lombardo et al., 2010; Schutz et al., 2006b). Leaves are mostly utilized for the production of commercial extracts in nutraceuticals, being enriched in polyphenols, whereas flower heads and roots, considered as a source of inulin, are used as prebiotic ingredient in functional foods (Raccuia and Melilli, 2004). Several pharmacological experiments have demonstrated the health promoting effects of artichoke extracts including its hepatoprotective (Gebhardt and Fausel, 1997; Mehmetcik et al., 2008), choleretic (Kirchhoff et al., 1994; Matuschowski et al., 2005; Saenz Rodriguez et al., 2002), anticholestatic (Gebhardt, 2001, 2005), hypolipidemic (Shimoda et al., 2003), antioxidative (Gebhardt and Fausel, 1997), antimicrobial (Mossi and Echeverrigaray, 1999; Zhu et al., 2004) and antispasmodic effects (Emendorfer et al., 2005), as well as the antihypercholesterolemic effect (Gebhardt, 2002). In addition, artichoke extracts have shown anti-tumour (Noldin et al., 2003), apoptotic analgesic and anti-inflammatory (Trouillas et al., 2003) activities. The chemical components of artichoke have been extensively studied, found to be a rich source of caffeoylquinic acids and flavonoids (Adzet and Puigmacia, 1985; Hausler et al., 2002; Lattanzio et al., 2005; Pandino et al., 2011). Among hydroxycinnamates, chlorogenic and 1,5-dicaffeoylquinic acids are the predominant compounds (Coinu et al., 2007; Lombardo et al., 2010; Romani et al., 2006; Schutz et al., 2004). However, 1,3-dicaffeoylquinic acid was the major dicaffeoylquinic compound reported from other studies

Abbreviations: GG, American Green Globe; FH, French Hyrious; EB, Egyptian Baladi.

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(Schutz et al., 2006a; Wang et al., 2003), likely formed as an artifact from the isomerisation of 1,5-dicaffeoylquinic acid during aqueous extraction (Schutz et al., 2006a). On the other hand, main flavonoids detected in artichoke leaves and heads were those of apigenin and luteolin, as well as, their glycosides (Lombardo et al., 2010; Pandino et al., 2011). Other bioactive compounds include the anti-hyperlipedemic sesquiterpenes; cynaropicrin, as well as, the sesquiterpene glycosides; cynarascolosides A, B, C (Shimoda et al., 2003) and anthocyanins, present only in capitula (Schutz et al., 2006b).

Among artichoke secondary metabolites, chlorogenic acid and cynarin have been proven to mediate for its hepatoprotective, choleretic and antimicrobial activities (Gebhardt and Fausel, 1997; Matuschowski et al., 2005; Zhu et al., 2004). Nevertheless, lipid lowering and anti-cholestatic activities of artichoke, as well as, its ability to inhibit cholesterol biosynthesis were mainly attributed to flavonoids *i.e.*, luteolin (Gebhardt, 1998, 2001, 2005). A considerable variation in the phenolic content of artichoke extracts has been shown, throughout the literature, depending on the part used, variety, maturity stage (harvest time) and the applied methodologies, which may ultimately affect its biological efficacy (Farag et al., 2013; Fratianni et al., 2007; Lombardo et al., 2010; Pandino et al., 2011; Romani et al., 2006; Wang et al., 2003).

The developmental stage, at which leaves from medicinal plants are harvested, has been found to influence their phytochemical composition. For example, in *Mentha piperita*, it was found that the A- and B-ring *O*-methylation patterns of flavonoid aglycones differ according to leaf age (Voirin and Bayet, 1992). In sainfoin leaves, it was found that the number-average molecular weight and the degree of polymerization of proanthocyanidins increase with leaf development (Koupai-Abyazani et al., 1993). Furthermore, during leaf maturation, the influence of the developmental stage on the pools of individual flavonols, as well as, procyanidins was obvious in apple leaves (Mayr et al., 1995). Besides, in *Arabidopsis thaliana*, older leaves had lower glucosinolate concentrations than younger ones (Brown et al., 2003). In addition, Mondolot et al., 2006, found that caffeoylquinic acids content varied throughout *Coffea canephora* leaf development. In fruits, such as Loquat, high concentrations of phenolic compounds were detected in young fruits, which then decreased steadily during growth. In contrast, the concentration of chlorogenic acid increased during fruit ripening, which appeared to be a characteristic of Loquat fruit ripening (Ding et al., 2001). Moreover, immature green peppers (*Capsicum annum* L.) revealed very high phenolic content (hydroxycinnamic acids and flavonoids), *ca.* 4–5 times higher than in red ripe ones (Marín et al., 2004). It was also found that harvesting broccoli heads of over-maturity stage resulted in maximal flavonol levels (quercetin and kaempferol aglycones), compared to other stages of head development (Krumbein et al., 2007).

Regarding artichoke, young artichoke heads were reported to contain more antioxidant phenolic compounds than mature ones (Wang et al., 2003); a higher concentration of phenolics was also observed in artichoke head, as well as floral stem harvested in spring compared to the winter, revealing the influence of harvest time on phenolics composition (Lombardo et al., 2010). Although the complex network of metabolites is dramatically altered during development, most researchers have approached the problem by studying only target compounds. In this regard, metabolomics provides a better tool for analysing developmental changes due to its ability to follow a relatively large number of metabolites in a single analysis (Farag, 2014). Metabolites profiling has been increasingly applied to study the developmental changes, e.g., in tomato (Roessner-Tunali et al., 2003), strawberry (Fait et al., 2008), grape (Zamboni et al., 2010) and peach (Lombardo et al., 2011) fruits, as well as, *Vanilla planifolia* leaves (Palama et al., 2010).

In a previous report, metabolites from three artichoke cultivars: American Green Globe (GG), French Hyrious (FH) and Egyptian Baladi (EB), together with different commercial preparations, were measured using UHPLC–q-TOF-MS to reveal secondary metabolite compositional differences among cultivars and preparations (Farag et al., 2013).

Nevertheless, and to the best of our knowledge, the effect of maturity stage on artichoke leaves chemical composition has not been addressed, an issue of value for the artichoke nutraceuticals industry. In this report, we describe a simple and efficient method to determine metabolite variation in artichoke leaves of different cultivars and at different developmental stages, using UHPLC–ESI-ITMS and analyzed by chemometrics.

2. Results and discussion

2.1. Metabolite profiling of artichoke leaves using UHPLC–PDA-MS

Artichoke leaves from 3 different cultivars were harvested at different time points and positions. For each cultivar, leaves were collected at 4 different maturity stages: from 3, 5, 6 and 8 months old plants. Except for 3 months samples, two nodal positions (apical and basal) were selected for sample collections, at each time point (see Section 4.1), for a total of 21 harvested samples to provide an overview of artichoke secondary metabolite accumulation patterns in a holistic manner, using an UHPLC–MS based metabolomics approach (see Section 4.4). Different nodal positions represented different plant ages, with apical (top) leaves being the youngest ones and basal leaves the oldest, mature ones. In case of the 3 months harvest, only one nodal position (apical or top) was available due to the small size of the plants and absence of basal leaves at this plant age. Biological replicates for each sample were harvested in triplicate [(21×3), a total of 63 samples], extracted and analyzed under identical conditions via reverse-phase UHPLC coupled to electrospray negative ionization ion-trap mass spectrometry detection (UHPLC–ESI-ITMS). It should be noted that artichoke extract was initially analyzed in positive and negative ion electrospray ionization (ESI) MS modes as changes in ESI polarity can often circumvent or significantly alter competitive ionization and suppression effects revealing otherwise suppressed metabolite signals. Compared to the positive-ion ESI mode, negative-ion MS spectra revealed better sensitivity and more observable peaks than in positive mode, most notably in the elution range of phenolics acids, flavonoids and saponins (Farag et al., 2013). Consequently, all samples and metabolites identifications were made in the negative ionization mode. Chromatographic parameters described in the experimental section resulted in the separation of metabolites within *ca.* 10 min (Supplementary Fig. S1). The elution order of compounds correlated with decreasing polarity, whereby phenolic acids and flavonoid di-glucosides eluted first, followed by mono-glucosides and saponins, and finally fatty acids. Metabolites were identified based upon UV and mass spectra. A detailed description of the artichoke leaves peak identifications and strategy has been published elsewhere (Farag et al., 2013). A complete list of identified peaks, along with their characteristic UV and mass spectral data, is provided in Table 1.

2.2. Multivariate PCA and O2PLS-DA analyses of UHPLC–MS data

PCA and OPLS-DA are often used to analyze large complex data sets. Principal component analysis (PCA) is the most widely used multivariate data analyses method for chemometrics, as it is the basis of all multivariate analysis modeling regarded as an unsupervised clustering method that reduces the dimensionality of