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Influence of organic and traditional fertilization on antioxidant compounds profiles and activities in fruits of Fragaria ananassa var. Camarosa

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Full Title:	Influence of organic and traditional fertilization on antioxidant compounds profiles and activities in fruits of Fragaria ananassa var. Camarosa
Article Type:	Original Paper
Abstract:	Purpose Non-communicable diseases (NCDs) present a long duration and a slow progression. Good nutrition plays a predominant role in NCD prevention. In Chile, the cultivation of strawberry (Fragaria ananassa) has recently been adapted to volcanic soils with low available phosphorus (P), which can affect the fruit quality in terms of their antioxidant compound content and anti-inflammatory activities.Methods Here, we carried out a study using organic (OF) or traditional fertilization (TF) at 50 and 100% of the agronomical dose recommendation and without fertilization (WF) under field conditions. Identification of phenolic compounds was done by HPLC-DAD-ESI-MS/MS, whereas antioxidant activities were determined by spectrophotometric methods.Results The highest concentrations of anthocyanins and ascorbic acid were obtained in the OF treatments, showing a relationship with the CUPRAC (cupric reducing antioxidant capacity) values. The highest concentrations of total polyphenols were obtained in the TF treatments, according to the TEAC (trolox equivalent antioxidant capacity), CUPRAC and DPPH tests. The highest enzymatic activities were observed also in the TF treatments. Compared to WF, TF treatment downregulates the cytokine interleukin 10 (IL-10) significantly at a dose of 100%, but we could not show a significantly downregulation yet.Conclusion Our results suggest that enzymatic antioxidant activity responds better to a period of prolonged exposure to P fertilization. There is no direct correlation between enzymatic and non-enzymatic antioxidant activity with respect to treatments with different types of P fertilization.

Influence of organic and traditional fertilization on antioxidant compounds profiles and activities in fruits of *Fragaria ananassa* var. Camarosa

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Abstract

Purpose: Non-communicable diseases (NCDs) present a long duration and a slow progression. Good nutrition plays a predominant role in NCD prevention. In Chile, the cultivation of strawberry (*Fragaria ananassa*) has recently been adapted to volcanic soils with low available phosphorus (P), which can affect the fruit quality in terms of their antioxidant compound content and anti-inflammatory activities.

Methods: Here, we carried out a study using organic (OF) or traditional fertilization (TF) at 50 and 100% of the agronomical dose recommendation and without fertilization (WF) under field conditions. Identification of phenolic compounds was done by HPLC-DAD-ESI-MS/MS, whereas antioxidant activities were determined by spectrophotometric methods.

Results: The highest concentrations of anthocyanins and ascorbic acid were obtained in the OF treatments, showing a relationship with the CUPRAC (cupric reducing antioxidant capacity) values. The highest concentrations of total polyphenols were obtained in the TF treatments, according to the TEAC (trolox equivalent antioxidant capacity), CUPRAC and DPPH tests. The highest enzymatic activities were observed also in the TF treatments. Compared to WF, TF treatment downregulates the cytokine interleukin 10 (IL-10) significantly at a dose of 100%, but we could not show a significantly downregulation yet.

Conclusion: Our results suggest that enzymatic antioxidant activity responds better to a period of prolonged exposure to P fertilization. There is no direct correlation between enzymatic and non-enzymatic antioxidant activity with respect to treatments with different types of P fertilization.

Keywords: Strawberry, antioxidant activity, polyphenols, phenolic compounds, ascorbic acid, berries.

1. Introduction

Non-communicable diseases (NCDs), such as cardiovascular and respiratory diseases, cancer and diabetes, affect people of all age groups and are characterized by their long duration and slow progression. Currently, NCDs are recognized for their high impact on morbidity and mortality mainly in developed countries, but especially in developing countries the situation for chronic diseases is expected to worsen in the next few years (Benavente-García and Castillo 2008; Bernini et al. 2011; OMS 2010). In this context, it is important to note that insufficient consumption of fresh fruits and vegetables, in combination with a high availability of highly processed (fast) food, soft drinks and sweets in these countries will reduce the intake of vitamins, minerals and secondary metabolites, as phenolic compounds, carotenoids, sterols, glucosinolates, and saponins, among others, with antioxidative properties. The lack of these biologically active substances and a higher proportion of ingredients such as salt, sugar and fats (mainly saturated and trans) that are commonly added to processed foods should be seen as possible risk factors that can lead to serious pathophysiological consequences decreasing the antioxidative capacity of the blood and increasing the appearance of oxidative stress and the development of chronic pro-inflammatory conditions (Alothman et al. 2009; Cassileth 2008; Yang et al. 2007). Under normal conditions, cells metabolize most of the oxygen (95%) to water through a tetravalent reduction pathway, while a small percentage (approximately 5%) metabolize oxygen through a univalent reduction, forming highly toxic intermediates such as superoxide anions ($O_2^{\bullet}$), hydroxyl ($OH^{\bullet}$), and hydrogen peroxide (H_2O_2) (Montllor-Albalate et al., 2019). Antioxidant systems that cope with these reactive oxygen species (ROS) can be classified into two groups, the enzymatic and non-enzymatic one. The non-enzymatic system of plants promotes the synthesis of numerous secondary metabolites (phenolic acids, flavonoids, tannins, vitamins, and terpenoids) in the phenylpropanoid route (Karousou 2007; Yanishlieva et al. 2006). The enzymatic antioxidant system that is located in several compartments of the cell (Zheng and Wang 2001) includes superoxide dismutase (SOD), which catalyses superoxide radicals to hydrogen peroxide (H_2O_2), peroxidase (POD), which converts H_2O_2 to water, and catalase (CAT), which eliminates H_2O_2 (Oueslati et al. 2010).

The antioxidant content of several berries has been previously reported, showing that berries have high levels of flavonoids such as anthocyanins, flavonols and hydroxycinnamic acids (HCADs) (Ruiz et al. 2013; 2015). The phenolic composition of strawberry fruits cultivated under greenhouse showed high abundance of anthocyanin derivatives such as pelargonidin, reaching up to 369 µg g^{-1} under traditional fertilization (75% of the total anthocyanin content) (Crecente-Campo et al. 2012; Parada et al. 2019; Ruiz et al. 2019). Moreover, a correlation with the total antioxidant activity measured by *in vitro* tests (TEAC, CUPRAC and DPPH) was evidenced (Parada et al. 2019). However, information on the fruit quality of plants cultivated in soils with low levels of available P so far has not been reported. In addition, ecological and physiological traits have been used to explain why plants produce anthocyanins just before leaf fall.

On the other hand, inflammation as a defense response of the human body eliminates present damaged cells, irritants and pathogens and allows the tissue healing processes to begin. Chronic exposure to toxic compounds and elevated oxidative cell stress induced by sustained malnutrition and obesity are associated with a misbalanced production of immune cell-derived cytokines that enhance the development of NCDs such as diabetes and atherosclerosis (Kumar et al. 2012). Therefore, the cytokine production of *in vitro* incubated peripheral blood mononuclear cells (PBMCs) with extracts of *F. ananassa* was investigated to evaluate how different fertilization methods influence the anti-inflammatory capacity of the anthocyanins in cultivated berries. Additionally, the present study evaluated the enzymatic and non-enzymatic antioxidant activity of *F. ananassa* by means of different analytical chemistry methods to establish whether there is a relationship between these concentrations and the different types of fertilization applied to the plants during cultivation.

2. Material and methods

2.1. Samples

Strawberry cultivation was carried out under field conditions in Mahuidache locality, Freire commune (15 km south of Temuco city, 30° 50’ 32’’ S; 72° 38’ 43’’ W, 88 m a.s.l., Araucanía region). The soil (Medial, mesic, xeric Placaquands, Freire soil series, silt loam in texture for Ap

horizon at 25 cm depth) is characterized by intermediate organic matter content (8%), a moderate level of acidity in water (pH 5.5), and a low content of available P (6 ppm, as P-Olsen). The experimental unit consisted of rows of 10 m length with plants placed every 30 cm. Each unit was considered in triplicate (n=3). During fruit development, the plants were treated with different fertilization conditions: without fertilization (WF), traditional fertilization (TF) consisting of commercial triple superphosphate and potassium phosphate, and organic fertilization treatment (OF) with the use of commercial red manure acquired from ANASAC (Santiago, Chile). The last two fertilization conditions were carried out at 50% and 100% fertilization doses according to agronomic recommendations by INDAP (http://www.indap.gob.cl/extras/estrategias-por-rubros-2005/5region/11Frutillas-ExposicionEspecialista.pdf). Fruits were harvested on March 4th, 2018, and leaves (for the characterization of some other compounds of interest) were harvested at the same time and later in the year (15th May 2018).

2.2. Extraction and quantification of non-enzymatic antioxidants

Phenolic compound determinations from strawberry fruits was performed according to Parada et al (2019) whereas ascorbic acid was determined based on the reported by Ruiz et al (2019). Chromatographic analyses were carried out on an HPLC-DAD Shimadzu (Tokyo, Japan) equipped with a quaternary pump LC-20AT, a DGU-20A5R degasser unit, a CTO-20A oven and UV-Vis diode array detector (SPDM20A). Data were collected using Analyst software (v. 1.5.2) (SCIEX, Woodlands Central Indus. Estate, Singapore) for MS/MS analysis and Lab Solutions (Shimadzu, Duisburg, Germany) for DAD analysis.

Total phenols were determined by the Folin-Ciocalteu method adapted to microplates according to Parada et al (2019). Antioxidant activity was determined using the Trolox equivalent antioxidant capacity (TEAC) assay, the cupric reducing antioxidant capacity (CUPRAC) assay and the DPPH test, according to Parada et al (2019).

135 2.3. Extractions and quantification of enzymatic antioxidants

136 Fresh fruits were crushed in liquid nitrogen, and 0.3 g was transfered into a microtube with
137 900 µL 0.1 mol L^{-1} potassium phosphate buffer at pH 7.0. Samples were then centrifuged for 15 min
138 at 13.000 x g at 4°C, and the obtained supernatant was used for the determination of catalase
139 (CAT), ascorbate peroxidase (APX), glutathione reductase (GR) and superoxide dismutase (SOD)
140 activities. The tests were carried out under UV light being the extracts previously treated with PVPP
141 to eliminate the polyphenols that could interfere with the enzymatic determinations. Ten milligrams
142 of PVPP were added to 400 µL extract, vortexed and centrifuged at 12.000 x g for 10 min at 4°C.
143 The recovered supernatant was used for enzymatic determinations (n=3). Enzymatic antioxidants
144 were determined according to Donahue et al (1997).
145
146 2.4. Determination of cellular anti-inflammatory activity by cytometric bead array

147 PBMCs were isolated from buffy coats obtained from the peripheral blood of three healthy
148 donors (males, 29-59 years old). In order to determine cytotoxicity of fruit extracts, the MTT test
149 according to Mosmann (1983) was used.

150 Endotoxin detection in strawberry extracts was performed with the Kinetic-QCL™ Kinetic
151 Chromogenic Limulus amoebocyte lysate (LAL) assay according to the manufacturer's protocol
152 (assay sensitivity: 0.005 – 50 EU/mL). Quantification of cytokines in tissue culture supernatants:
153 Human inflammatory cytokines were measured by cytometric bead array (CBA; BD™ CBA Human
154 Inflammatory Cytokines Kit; BD Biosciences, San Diego, CA, USA). Human interleukin-10 (IL-10),
155 IL-6 and tumour necrosis factor α (TNFα) protein levels were determined in tissue culture
156 supernatants according to the manufacturer's protocol optimized for this sample source on a
157 FACSVerse™ flow cytometer (Becton Dickinson, Heidelberg, Germany). The data analysis was
158 conducted using FCAP Array Software v3.0 (Becton Dickinson, Heidelberg, Germany).
159
160 2.5. Statistical analyses

161 For the enzymatic and non-enzymatic profiles and activities, one-way ANOVA was
162 performed. Data sets not meeting normality and homoscedasticity requirements were transformed

using Ln, but the results are presented in their original scale of measurement. The means of all treatments were compared using Tukey's multiple range test ($p < 0.05$). The data sets were also subjected to factorial analysis with extraction of principal components (PC) and cluster analysis using Ward as agglomerative method. Statistical analyses were done using IBM SPSS Statistics software v. 23 (IBM Corp.).

Cell culture experiments were performed in triplicate (n=3). Statistical analyses were performed using JMP 14.1.0 (SAS Institute; Cary, North Carolina, USA). To differentiate the cytokine concentrations in the different treatment groups and in the control group (methanol/formic acid (95:5, v/v)), Dunnett's test was performed under normality assumptions. Error bars indicate the standard error of the mean in all figures.

3. Results

3.1. Anthocyanin, total phenol and ascorbic acid content

Chromatographic analyses were carried out for the determination of anthocyanin and ascorbic acid profiles and concentrations; both compounds are considered being important antioxidant compounds in foods. In both cases, quantification was performed by external calibration (for anthocyanin quantification, pelargonidin-3-glucoside was used as a standard) and analytical validation of the methodologies was performed (Table 1).

Pelargonidin derivatives and pelargonidin-3-glucoside were the most abundant compounds detected. Additionally, 3-rutinoside, acetylglucoside and other pelargonidin derivatives with a pseudomolecular ion *m/z* 503.2 and product ion *m/z* 271.1 were identified; tentatively assigned as pelargonidin-succinyl-arabinoside or pelargonidin-malonylrhamnoside. Cyanidin-3-glucoside was also detected. The highest concentration of total anthocyanins was observed in the 100% OF treatment, with a concentration of 0.89 mg g^{-1} fruit; the most abundant pelargonidin-3-glucoside reached 0.57 mg g^{-1}. On the other hand, the highest concentrations of ascorbic acid were detected in OF treatments reaching 0.7 and 0.6 mg g^{-1} in 100% OF and 50% OF treatments, respectively.

Quantification of total phenols was carried out using gallic acid as a standard (Table 1). The lowest total phenol concentrations were detected in treatments without fertilization (WF), reaching

191 levels near 1.9 mg g^{-1} in fruits, while in the treatment of 50% TF the highest phenol concentration of
192 2.80 mg g^{-1} was found.
193
194 3.2. Antioxidant activity
195 Concerning non-enzymatic methodologies, quantification was carried out by external
196 calibration using Trolox as the standard. Enzymatic activities were calculated using the extinction
197 coefficient and total protein concentration measured by the Bradford method. In the case of non-
198 enzymatic methods (Figure 1), correlation was observed between the total phenol concentration
199 and the antioxidant activity of the *F. ananassa* fruits. Using the TEAC method, samples with the
200 lowest antioxidant concentration (10.6 µmol $g^{-1)}$ were obtained from fruits of WF treatment, whereas
201 the highest antioxidant activity with a concentration of 14.7 µmol g^{-1} was found in fruits undergoing a
202 50% TF treatment (Figure 1B). For the CUPRAC method, the lowest antioxidant activity of 17 µmol
203 g^{-1} was found in WF, while 50% TF treatment resulted in the highest activity of 33.9 µmol g^{-1} Trolox
204 equivalents (Figure 1C). For the DPPH method, 100% TF treatment resulted in the highest level of
205 antioxidant activity, reaching a value of 16.9 µmol g^{-1} while in the WF treatment antioxidant activity
206 was only 11.7 µmol g^{-1} Trolox equivalents (Figure 1D).
207 Using enzymatic methods (Figure 2), the highest content of total proteins with 0.73 mg g^{-1}
208 was detected in the 50% TF treatment, whereas in 50% OF treatment concentration of total proteins
209 was 0.26 mg g^{-1}. According to the data obtained from the specific activity test of SOD, the lowest
210 specific activity reaching 0.46 U mg^{-1} was found after 50% OF, while 100% TF treatment resulted in
211 the highest specific activity of 1.17 U mg^{-1} (Figure 2A). For CAT activity, we found the highest
212 specific activity with 7.7 nmol min^{-1} mg^{-1} corresponding to 100% TF, while the lowest specific activity
213 (3.4 nmol min^{-1} mg^{-1} of protein) was observed with 100% OF (Figure 2B).
214 Concerning APX, the highest specific activities (3.4 µmol min^{-1} mg^{-1} of protein) were found
215 with 100% TF, while the lowest activity (0.5 µmol min^{-1} mg^{-1} of protein) was found in 50% TF,
216 treatment (Figure 2C). The highest specific activity of GR (0.52 µmol min^{-1} mg^{-1} of protein) was
217 found in 100% TF treatment (Figure 2D).
218

3.3. Phenolic composition of strawberry leaves

In order to evaluate the composition of potential bioactive compounds after fruit harvest, phenolic composition of strawberry leaves was determined since high quantities of anthocyanins (with a possible functional use) could be accumulated in the falling strawberry leaves. In postharvest tests four flavonols corresponding to glycosylated derivatives of quercetin were identified in strawberry leaves, while during the autumn period (when the colour of the leaves changed), two other quercetin derivatives were detected, matching the glucuronide-hexoside and hexoside derivatives. A further compound being detected in strawberry leaves in autumn periode was cyanidin-3-glucoside (Table 2, Figure 3). Using quantitative HPLC-DAD analysis, we found total flavonol concentrations between 1.0 and 1.7 g kg^{-1}, with the highest concentrations detected in the leaves of plants cultivated without fertilization. This also applied to the concentration of cyanidin-3-glucoside: the highest one was detected in the WF treatment and was lower in the 100% TF treatment.

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3.4. Effects of strawberry extracts on cytokine expression of human PBMCs

The detected endotoxin level was lower than 0.005 EU mL^{-1} in all strawberry extracts and therefore we considered the extracts as endotoxin free. All tested strawberry extracts were non-cytotoxic at a concentration of 10 µg mL^{-1}. The anti-inflammatory activity of strawberry extracts was tested at non-cytotoxic concentrations.

PBMCs isolated from healthy donors cultured for 18 h with different strawberry extracts at a concentration of 10 µg mL^{-1} showed no pro-inflammatory effects on the cytokine production of these cells compared to the stimulated control group, and there were no differences between the groups. Only cytokine IL-10 was down-regulated, but this effect was not significant. Future experiments should be accomplished with a larger sample size in order to show statistic significances. Possible anti-inflammatory properties of the elevated cytokine production of ConA- and LPS-activated PBMCs from the flavonoids in the extracts could not be shown.

4. **Discussion**

4.1. Phenolic compounds and antioxidant activity in strawberry fruits

The profile of the anthocyanins measured was matching (concordant) with previous results obtained by Ruiz et al (2019), in which mainly pelargonidin derivatives and pelargonidin-3-glucoside as the most abundant compound were detected. Anthocyanin concentrations results were in contrast of those of Carvajal et al (2012), where the highest concentrations of anthocyanins in Camarosa strawberry cultivar were obtained in treatments with lower levels of organic matter and higher levels of nutrient supplied, suggesting that higher concentrations of P alone may not significantly influence the concentrations of anthocyanins in the berries. Especially, the OF treatments lead to high concentrations of ascorbic acid which in general remain stable during storage at low temperatures, as previously reported (Rapisarda et al. 2008; Tavarini et al. 2008). Ascorbic acid, as a non-enzymatic compound, may contribute to the main antioxidant capacity of the fruits and especially for this reason adequate and optimal conservation of the strawberries during the post-harvest management is essential to conservate the optimal antioxidative capacity (Veliuglu et al. 1998).

Some authors reported that antioxidant activity depends on the total phenolic content (Ahmed and Hussain 2009). However, the Folin-Ciocalteu method is accepted for phenolic compound quantification despite its lack of specificity as phenolic compounds and other reducing compounds are quantified simultaneously (Santos-Buelga and Scalbert 2000). Antioxidant activity is not exclusive to phenolic compounds since in addition to flavonoids and anthocyanins it may be attributed to the presence of other secondary metabolites, such as volatile oils, carotenoids or vitamins such as ascorbic acid (Javanmardi et al. 2003). Concerning the antioxidant activity in different fertilization treatments, there are several factors that may have an effect on the presence and concentration of individual antioxidants, such as the cultivar, harvest season, environmental factors, cultural systems, storage temperatures and maturity (Ahmed and Hussain 2009; Ghasemnezhad et al. 2011; Jin et al. 2011; Oboh et al. 2004).

Regarding the enzymatic tests, it was observed that the level of total proteins in the different treatments had a strong response, being the treatments with a lower concentration of total proteins

275 those that also presented higher specific enzyme activities. The enzymatic tests with the highest
276 specific activity were SOD and CAT. It has been reported that more than other physiological factors
277 (Polata et al. 2009; Scandalios 1993), plant tolerance to lower temperatures is associated with SOD
278 and CAT activities. Since SOD and CAT activities depict the first step of cellular defence against
279 ROS (Del Río et al. 2003; Ferreira-Silva et al. 2012) an increase in enzymatic activity may be highly
280 beneficial (Alsher et al. 2002; Palma et al. 2006). Regarding the possible similar antioxidative
281 enzymatic activity of the SOD-CAT and APX-GR enzymes, our results suggest the association of
282 these conjugates enzymes as part of an oxidative chain which efficiently reduce and detoxify for the
283 stabilization of free radicals (Apel and Hirt 2004).
284
285 4.2. Phenolic composition valorization in strawberry leaves
286 The colour patterns in the senescent foliage depend, at least partially, on the composition of
287 the species and its phenology. With regard to the reddish coloration of the leaves in our study, the
288 presence of cyanidin-3-glucoside could be responsible for the red colour (Lee et al. 2018). Almost
289 all anthocyanins in senescent leaves are based on cyanidin aglycon (Lee 2002), the type that has
290 been identified in our study. According to Dalmagro et al (2018), metabolic changes in general
291 occur because plants constantly develop approaches to confront environmental adversities through
292 adaptation strategies. In this context, a set of metabolic adjustments and structural and
293 phenodynamic changes is essential. Our results suggest the environmental variables in the autumn
294 season being involved in increasing the synthesis of new secondary metabolites, which mainly
295 contribute to the total content.Location and agroclimatic season may determine the antioxidant
296 activity of the leaves, which is typically higher in plants cropped in cold zones (Iqbal and Bhanger
297 2006). These findings suggest that environmental temperature has a significant effect on the
298 generation of antioxidant activity. In turn, there are several factors as the incidence of light,
299 neighbouring species, mineral nutrition and herbivory; that can affect antioxidant activity.
300

4.3. Multivariate data anaysis

Principal component (PC) analysis reflects the formation of highly homogeneous groups of experimental variables (Figure 4), where PC1 explains 33% and PC2 explains 24.8% of the total experimental variance. PC1 was positively related mainly to metabolites such as ascorbic acid and anthocyanins, and also to the Folin-Ciocalteu result as a global measurement of phenolic compounds. Moreover, PC2 was positively associated with most of the antioxidant activities, such as CUPRAC, TEAC and SOD. PC analysis, together with cluster analysis, allowed the differentiation of three well-defined groups of experimental individuals (Figure 4). One group includes only individuals from the 50% TF treatment; one group includes all the individuals from the 100% TF treatment and some from the 100% OF treatment; and the third group includes all the individuals from the 50% OF and WF treatments. In this sense, it is clear that, irrespective of the type of fertilization, high doses of nutrients exert a protective effect by accumulation of metabolites, mainly anthocyanins, which could be of interest based on their functional characteristics. Nevertheless, a medium supply (50%) of nutrients from organic fertilization, especially P as in our case, resulted in the lowest level of plant stress represented by the overall low antioxidant activity levels. This last aspect is very important in terms of production as it is possible to manage the stress response of the strawberry fruit in soils with low P availability by means of type and dose of fertilization. Therefore, the paradigm is whether the productive aspects or the functional characteristics of the fruits are more relevant when selecting both the type of fertilization (organic or traditional) and the amount of fertilizer to be supplied.

4.4. Anti-inflammatory effect of strawberry fruits

As PBMCs were isolated from healthy donors and not from obese individuals or individuals with chronic inflammation, it could be of interest to test the potential anti-inflammatory effects of strawberry extracts on tissues or blood from patients with chronic inflammation in further experiments. Whether extracts from different strawberry cultivars show the potential to positively

affect inflammation and chronic diseases, as it has been shown for other naturally occurring substances with antioxidant properties, such as curcumin, remains to be clarified (Carocho and Ferreira 2013; He et al. 2015; Menon and Sudheer 2007).

5. Conclusions

Based on our results, we can conclude that under field conditions, the dose of fertilizer has a primary influence on the profiles and concentrations of antioxidant compounds in strawberry fruits and also in their antioxidant activity, but not on their cellular anti-inflammatory activities. It is important to consider that, at higher doses of both traditional and organic fertilizer, higher concentrations of anthocyanins were obtained that could be important in terms of antioxidant activity and in relation to the enzymatic antioxidant activities of APX, CAT and GR. At last, in view of the enrichment of the final products, the management of the strawberry crop can be addressed through changes in traditional or organic fertilization, due to the strong effect on the concentration of phytochemicals of interest, both in the fruits or in the leaves.

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Figure captions

Figure 1: Total phenols and antioxidant activity of *F. ananassa* fruits measured by chemical methods. A) Total phenols, by Folin-Ciocalteu method; B) Antioxidant activity, by TEAC method; C) Antioxidant activity, by CUPRAC method; D) Antioxidant activity, by DPPH method. Fertilization treatments: OF, organic fertilization; TF, traditional fertilization; WF, without fertilization and doses: 50% or 100% of the recommended fertilization.

Figure 2: Antioxidant activity assessed by enzymatic test in *F. ananassa* fruits. A) Superoxide dismutase; B) Catalase; C) Ascorbate peroxidase; D) Glutathione reductase. Fertilization treatments: OF, organic fertilization; TF, traditional fertilization; WF, without fertilization and doses: 50% or 100% of the recommended fertilization.

Figure 3: HPLC-DAD chromatogram of autumn leaves from *Fragaria ananassa* var camarosa. A) Anthocyanins (520 nm), B) Flavonols (360 nm). Identification was assigned by HPLC-DAD-ESI-MS/MS, according to Table 2.

Figure 4: Principal components (PC) analysis scores (left) for the respective fertilization treatments (OF, organic fertilization; TF, traditional fertilization; WF, without fertilization) and doses (50% or 100% of the recommended fertilization). Percentage values in parentheses indicate the experimental variation explained by each PCA. A cluster analysis was implemented, and groups of individuals with similar behaviour according to the analysis are grouped in circles that should be understood as a visual aid to identify the groups. The distribution plot is shown on the right between the variables examined according to the extracted PCs (PC1, PC2).

Table 1. Analytical parameters of tests performed by means of HPLC-DAD and different tests performed by means of spectrophotometry.

Method	Standard	Equation	R^2	DL	QL	LR	CV%
Anthocyanins	Pel-3-glucoside	y = 65162x - 34164	0.9995	1.63 mg L^{-1}	5.43 mg L^{-1}	5.4 - 75 mg L^{-1}	4.37
Ascorbic acid	Ascorbic acid	y = 58901x + 7035557	0.9948	34.28 mg L^{-1}	114.28 mg L^{-1}	114 - 400 mg L^{-1}	10.22
Folin	Galic acid	y = 0.0007x - 0.004	0.9965	22.7 mg L^{-1}	75.6 mg L^{-1}	75.6 - 500 mg L^{-1}	8.75
TEAC	TROLOX	y = 0.5102x + 0.0173	0.9847	0.015 mmol L^{-1}	0.051 mmol L^{-1}	0.052 - 0.500 mmol L^{-1}	4.86
CUPRAC	TROLOX	y = 2.9583x + 0.0603	0.9852	0.247 mmol L^{-1}	0.823 mmol L^{-1}	0.009 - 0.7 mmol L^{-1}	5.42
DPPH	TROLOX	y = 0.5674x - 0.0031	0.9993	0.006 mmol L^{-1}	0.020 mmol L^{-1}	0.02 - 1 mmol L^{-1}	7.14

Where: pel: pelargonidin, DL: detection limit, QL: cuantification limit. LR: linear range

Table 2: Identification of anthocyanins and flavonols in strawberry leaves by HPLC-DAD-ESI-MS/MS.

Peak	t_R (min)	Compound	λmax (nm)	Pseudomolecular ion $[M+H]^+$ or $[M-H]^-$	Product ions (m/z)	Detected in season
1	11.0	Cyanidin-3-glucoside	516	449.5	287.3	autumn
2	7.5	Quercetin-glucoronide-hexoside	352	639.6	463.4; 301.2	autumn
3	14.8	Quercetin-rutinoside	363	609.5	301.2	all year
4	15.5	Quercetin-pentoside	352	447.6	301.3	all year
5	16.0	Quercetin-glucoronide isomer	367	477.6	301.2	all year
6	16.5	Quercetin-glucoronide isomer	353	477.6	301.3	all year
7	17.0	Quercetin-hexoside	352	463.4	301.2	autumn

Figure 1

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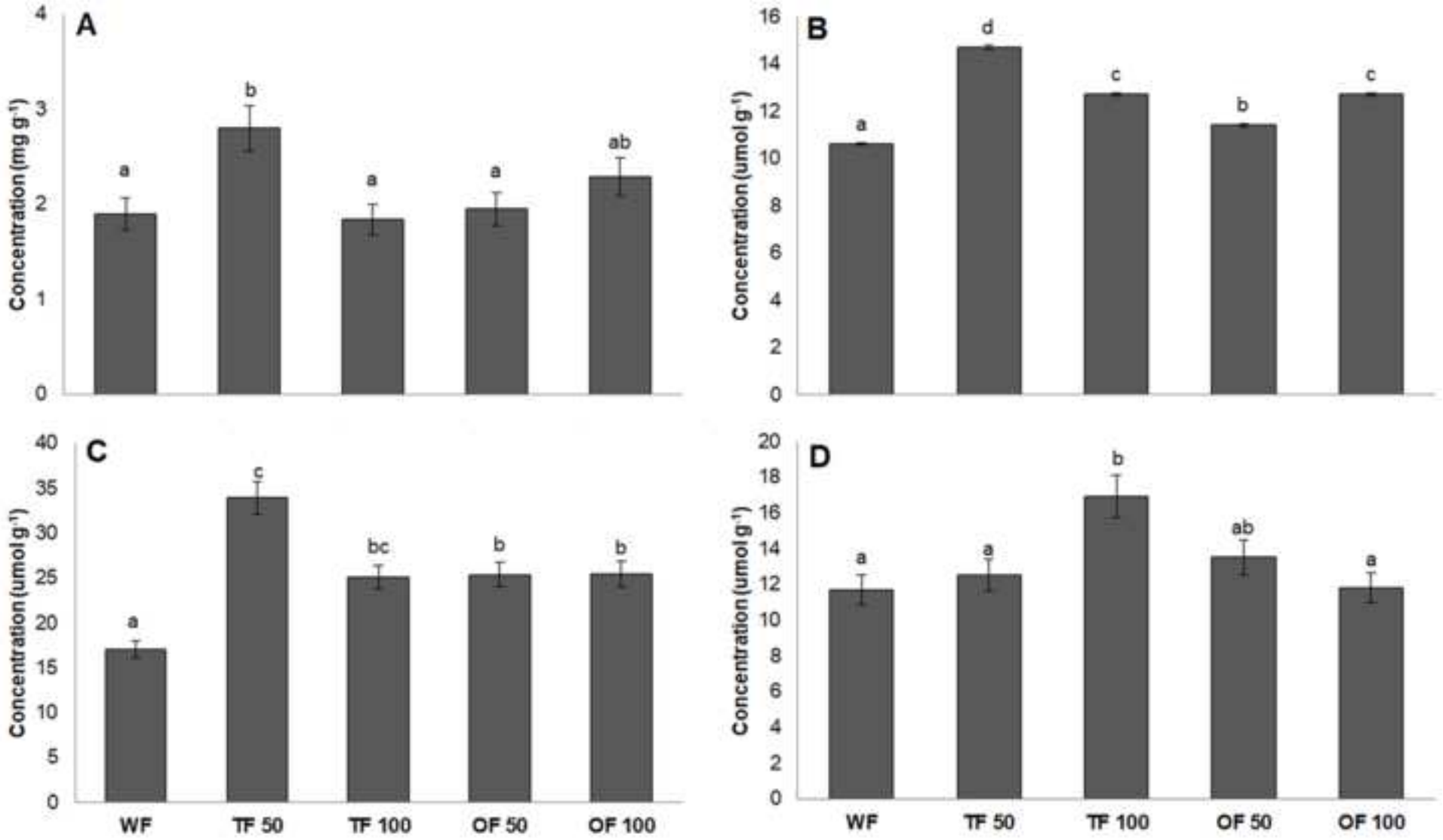

Figure 2

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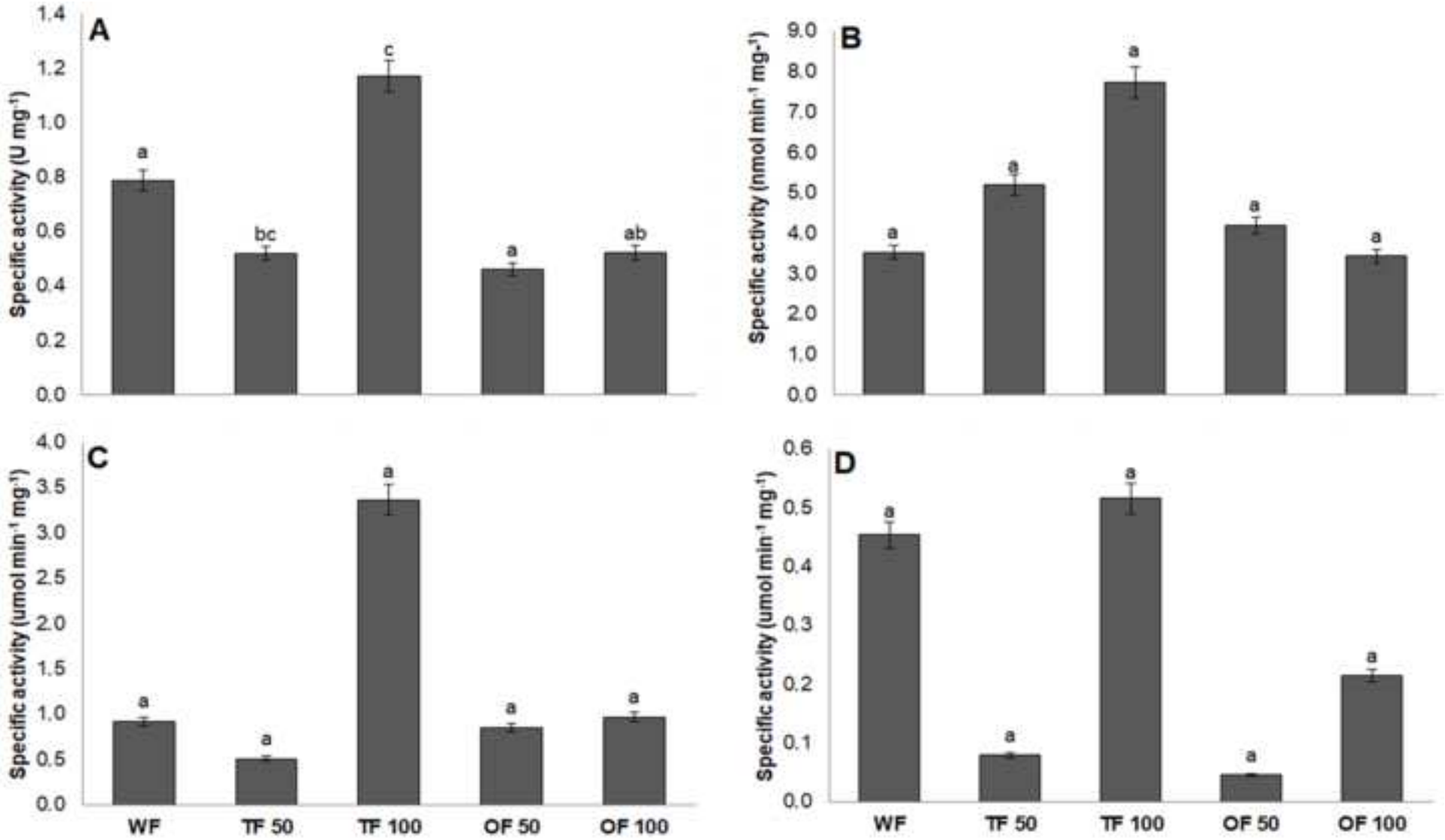

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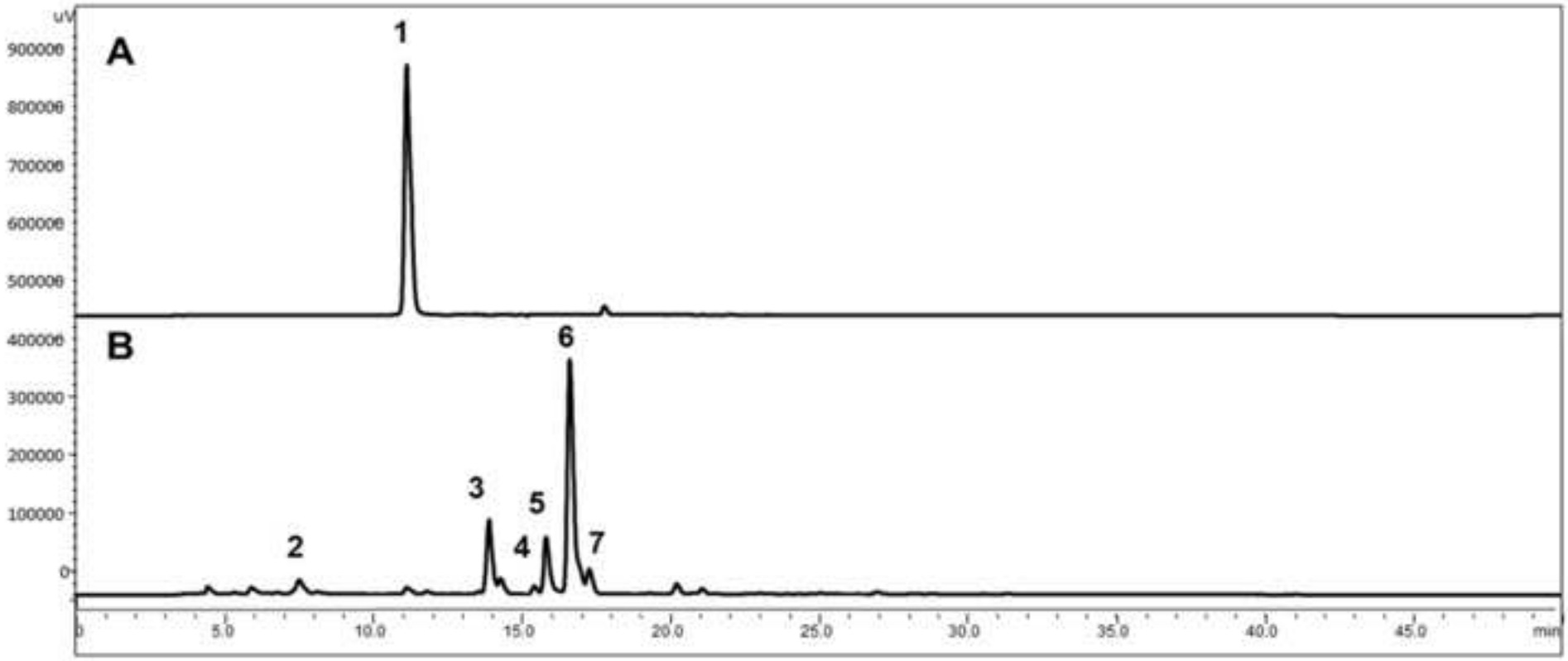

Figure 4