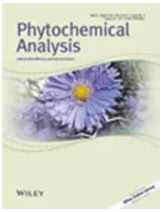

METABOLOMIC STUDY OF BIOACTIVE COMPOUNDS IN STRAWBERRIES PRESERVED UNDER CONTROLLED ATMOSPHERE BASED ON GC-MS AND DI-ESI-QqQ-TOF-MS

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METABOLOMIC STUDY OF BIOACTIVE COMPOUNDS IN STRAWBERRIES PRESERVED UNDER CONTROLLED ATMOSPHERE BASED ON GC-MS AND DI-ESI-QqQ-TOF-MS.

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ABSTRACT

Introduction.- The storage of the vegetables products in a controlled atmosphere (CA) with low O_2 and high CO_2 concentrations, reduces respiration rates and delays the ripening process, and in some cases, improves their quality and organoleptic properties.

Objetive.- To deep insight into strawberry fruit metabolic changes caused by these controlled atmospheres (CA) treatments.

Methodology.- Freshly harvested strawberries were preserved under different atmospheres enriched with 10 %, 20 % and 30 % of CO_2, for 2 days at 0ºC, containing in all the cases 5 % of O_2 and were subjected to a metabolomic analysis based on GC-MS and DI-ESI-QqQ-TOF-MS. Partial least square discriminant analysis (PLS-DA) was employed to compare control and treated samples for the identification of altered metabolites.

Results.- Several metabolites related to CA treatment could be identified by databases and literature, which are mainly sugars, organic acids and phenolic compounds (bioactive compounds).

Conclusions.- Good correlation coefficients were obtained between discriminant metabolites and fruit quality parameters. These results suggest that treated strawberries under CA could be considered as bioactive healthy compounds, suggesting that treated strawberries under CA could be used as raw material for the preparation and formulation of food supplements and nutraceutical products.

KEYWORDS: strawberry, controlled atmospheres, untargeted metabolomic profile, GC-MS, DI-ESI-QqQ-TOF-MS.

INTRODUCTION

Strawberry fruit (*Fragaria x ananassa*), is a rich source of bioactive compounds, such as phenolic acids, flavonoids and anthocyanins among others that have been demonstrated to have beneficial effects on human health (Sun, Chu, Wu & Liu 2002). Phenolic compounds are widely recognized for their pharmacological activities, for this reason phenolic-rich foods are associated with a reduced risk to cardiovascular diseases (CVD) (Zafra-Stone, *et al.,* 2007), inhibition of low-density lipoproteins (Forbes-Hernández, *et al.,* 2017), anti-inflammatory action (Joseph, Edirisinghe & Burton-Freeman 2014) and anti-carcinogenic activities (Bagchi, *et.al.,* 2004; Gullett, *et al.,* 2010). These results place strawberry fruit among the families of functional foods. Therefore, the general perception that strawberry is good for health has encouraged many researchers to look for their "magic" ingredients that contribute to pathological prevention.

For this reason, post-harvest studies of fruits are of great importance to fully understand the changes in their biochemical components during the fruit maturation in the plant, using physical, chemical and physiological analysis (Andrade, Aragão, *et al.,* 1995). Once that the bioactive compounds have been identified, and the correlation between them established, it is possible to determine the optimum time of harvest for immediate marketing and/or storage, in the case of the use for industrialized production of extracts.

The major challenge of post-harvest technology is that fresh product gets the consumer with a similar quality that than of the harvest time, but in addition with improved organoleptic and nutraceutical properties. Strawberry post-harvest techniques often focus on the use of controlled atmospheres (CA) and modified atmosphere packing (MAP) (Kader 2002). The use of controlled atmosphere storage maintain the vegetable product in lower O_2 and high CO_2 atmosphere, reducing respiration rates and delaying the ripening process.

Strawberry are especially tolerant to elevated CO_2 concentration between 10-40% during storage, that causes changes in fermentation metabolites (acetaldehyde, ethyl acetate and ethanol), sugars (Bodelón, *et al.,* 2010), organic acids (Fernández-Trujillo, Nock & Watkins 1999), and phenol content (Gil, Holcroft & Kader 1997).

These previously cited postharvest studies are mainly focused on the investigation of physicochemical changes, but failed to characterize the biological response of the fruit against to abiotic stress and to fully understand the action mechanisms triggered by the treatment (Zhang *et al.,* 2011). Therefore, metabolomics (Bowen & Northen 2010) based on the complete characterization of metabolites related to the changes suffered by the fruit after postharvest treatment is a good methodological approach to deep insight into the metabolic evolution of product during treatment, as well as its final quality and the production of compounds to be used in nutraceutical formulations and foods supplements. In this way, metabolomics has been used to elucidate metabolic composition of strawberries roots, leaves and fruits, as well as differences in the content of metabolites among species or cultivars and stages of maturity (D'Urso, *et al.,* 2015; Koehler *et al.,* 2015) and optimum growing conditions (Saia, *et al.,* 2015). Since metabolomics is the approach that better characterize the phenotypes of living organisms, it can be a good tool to develop new post-harvest strategies to improve the quality and attributes of strawberry and increase their healthy properties.

The aim of this study was to get a global understanding of the metabolomic changes of strawberry fruits in response to different short time storage conditions under controlled atmospheres and low temperatures. For this purpose, a metabolomic procedure based on the use of gas chromatography-mass spectrometry (GC-MS) and direct- infusion mass spectrometry equipped with triple quadrupole (QqQ) coupled to a time of flight (TOF) analyser with electrospray ionization source (DI-ESI-QqQ-TOF-

MS), followed by statistical analysis based on Partial Least Squares Discriminant Analysis (PLS-DA) was applied to strawberries preserved under different controlled atmospheres to identify bioactive metabolites and to determine the best CO_2 percentage treatment.

EXPERIMENTAL

Material

Methanol and hydrochloric acid were purchased from Fisher Scientific Co (Nepean, Ontario, Canada). Formic acid was supplied by Merck (Darmstadt, Germany). Pyridine and derivatizing agents, namely methoxylamine hydrochloride and N-methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA) were obtained from Sigma-Aldrich (St.Louis, MO, USA). Water was purified with a Milli-Q Gradient System (Millipore, Watford, UK).

Strawberry plants (Fragaria×ananassa. cv Fortuna) were harvested in Palos de la Frontera (Huelva, Spain) belonging to the enterprise Fresón de Palos (Santa María de la Rábida Cooperative) and were stored at 0ºC during 1 day for minimising its physiological deterioration. A previously assay of cold-storage for 24, 48 and 72 hours at 0ºC was performed, being the best results which 48 hours at 0ºC (data not show). Twelve plastic boxes containing approximately 0.5 kg of strawberries were placed in each controlled atmosphere container for 48 hours at 0ºC and exposed to a continuous flow of air (control samples) or gas mixture containing 10% CO_2 + 5 % O_2 + 85 % N_2 (Treatment 1, T1); 20 % CO_2 + 5 % O_2 + 75 % N_2 (Treatment 2, T2) and 30 % CO_2 + 5 % O_2 + 65 % N_2 (Treatment 3, T3. Strawberries from each treatment were taken for metabolic analysis, frozen in liquid nitrogen and then stored at -80ºC to further analysis.

Quality parameters

The quality parameters evaluated for sensory analysis have been appearance and flavour using different numerical scales. The analysis of appearance consists in a visual exam of the strawberries. For this, a scale from 0-5 was used, considering the degree of dehydration (tissue damage) and presence of mould, rot and bruises. The measure of strawberry flavour was carried out by tasting several pieces of fruit. The scale used

varies with the intensity in flavour, with 5 being the maximum intensity and 0 if it lacks flavour.

Total soluble solids (TSS)

The content of soluble solids was measured on strawberry juice using a digital refractometer (Milwaukee MA871 Refractometer) and reported as ºBrix.

Metabolites extraction

Strawberry samples were differently prepared depending on the metabolomic technique applied. All the strawberries samples were previously cryohomogenized using a cryogenic homogenizer SPEX Sample Prep (Freezer/Mills 6770).

For GC-MS, the extraction procedure was carried out according to the method proposed by González-Domínguez, *et al.,*(2015). For this purpose, strawberries were cryohomogenized and 30 mg of strawberry samples were mixed with 300 µL of precooled methanol. The mixture was homogenized using a pellet mixer during 2 min and then centrifuged at 10000 rpm for 10 min at 4ºC. An aliquot of the supernatant (50 µL) was mixed with 30 µL of ribitol (used as internal standard, 0.2 mg mL^{-1} in water) and dried using a SpeedVac device. Chemical derivatization before GC-MS analysis was carried out using the two-step methodology proposed by Begley et al. (Begley, *et al.,* 2009). The polar extracts were re-dissolved in 50 µL of 20 mg/mL methoxyamine in pyridine and after vortexing, samples were incubated at 80ºC for 15 min in a water bath. Samples were further derivatized by the addition of 50 µL of MSTFA (N-methyl-N-(trimethylsilyl)trifluoroacetamide) and incubated at 80ºC for 15 min.

When apply DI-ESI-QqQ-TOF-MS, the polar metabolites were extracted following the methodology proposed by D'Urso *et al.,* (2015) with brief modifications. For this, 30 mg of sample were extracted with 300µL a solution of methanol:hydrochloric acid (1% (v/v)) for 24h at 4ºC and under stirring and darkness. After the extraction, the mixture was centrifuged at 10000 rpm for 10 min at 4ºC. The supernatant was filtered through a 0.22 µm PTFE membrane syringe filter, dried using the speedvac concentrator Savant SPD111V (Thermo Fisher Scientific) and finally diluted in 1.5 mL of methanol:formic acid (0.1% (v/v)).

DI-ESI-QqQ-TOF-MS and GC-MS analysis

Chromatographic analysis was performed following the procedure described by Zhang *et al.,* (2011) with few modifications. The polar extract was analyzed in a gas chromatograph (Trace GC ULTRA) coupled to an ion trap mass spectrometer detector ITQ 900 (Thermo Fisher Scientific), using a Factor Four capillary column (30m x 0.25 mm i.d., 0.25 µm VF-5MS, Varian). Helium was used as the carrier gas with the flow rate at 1 mL min^{-1} and the injector temperature fixed to 280ºC. The column temperature was held at 100ºC for 2 min; increased to 200ºC with a temperature gradient of 5ºC/min and then held to this temperature for 3 min and finally increased to 300ºC with a gradient of 10ºC/min and then, held for 5 min. The significant MS operating parameters were as follows: ionization voltage was 70 eV (Electronic Impact Ionization), and the ion source temperature was set at 200ºC. Data were obtained acquiring full-scan spectra in the m/z range of 35-650.

The DI-ESI-QqQ-TOF-MS experiments were performed in a QSTAR XL Hybrid system (Applied Biosystems, Foster City, CA, USA) using an electrospray (ESI) source. Spectra were acquired in negative and positive ion modes, in a range of m/z 80-1100 at a flow rate of 5µl/min. The analytical parameters of the QTOF system were optimized to obtain the higher sensitivity with minimal fragmentation of molecular ions using a mixture of standard solution of rutin, ellagic acid, gallic acid, catechin, chlorogenic acid and cyanidine-3-*O*-glucoside (10 pmol μl^{-1} in methanol) in both ionization modes. In positive mode, the ion spray voltage (IS) was set at 5300 V, the source temperature was fixed at 280ºC, with a decluttering potential (DP) of 150V and a focusing potential (FP) of 210 V. In negative mode, the IS was set at -4500V, the temperature source was fixed at 500ºC and the values of DP and FP were -83V and -250V respectively.

Data processing

In order to filter the mass spectrometric data obtained from DI-ESI-QqQ-TOF-MS, and to carry out the reduction into a two-dimensional data matrix of spectral peaks and their intensities, the data were exported to MarkerView software (Applied Biosystems). For this purpose, all peaks above the noise level (10 counts, determined empirically from experimental spectra) were selected.

The raw data obtained by GC-MS was processed following the typical pipeline described by Katajamaa & Oresic (2007), which usually proceeds thought multiple stages, including, filtering, feature detection, alignment and normalization. For this

purpose, the freely available XCMS software, included in the R platform (http://www.r-project.org) was used. GC-MS files were converted into net CDF using the Thermo File Converter tool (Thermo Fisher Scientific) and subsequently, data were extracted using the matched filter method. This algorithm slices data into extracted ion chromatograms (XIC) on a fixed step size, and then each slice filtered with matched filtration using a second-derivative Gaussian as the model peak shape. The XCMS parameters were optimized according to the characteristics of datasets obtained in order to extract the maximum information as possible. Finally, the settings applied for GC-MS data were S/N threshold 2, full width at half-maximum (fwhm) 3, and width of the m/z range 0.1 (step parameter). After peak extraction, grouping and retention time correction of peaks (alignment) was accomplished in three iterative cycles with descending bandwidth (bw) from 5 to 1 s. Then, imputation of missing values was performed by returning to the raw spectral data and integrating the areas of the missing peaks which are below the applied signal-to-noise ratio threshold, using the fill Peaks algorithm. For data normalization, the locally weighted scatter plot smoothing (LOESS) normalization method was used, which adjusts the local median of log fold changes of peak intensities between samples in the data set to be approximately zero across the whole peak intensity range. Finally, the pre-processed data were exported as a .csv file for further analysis to eliminate non-significant features following multivariate procedures.

Statistical analysis

The data collected of quality parameters and TSS content were treated using MiniTab16 statistical software to provide ANOVA significance of each treatment effects. Significant differences between the means were determined using the Tukey's test at $p<0.05$.

For the analysis and visualization of multivariate statistical methods statistical software SIMCA-P (version 11.5, Umetrics AB, Umea, Sweden) was used. Principal component analysis (PCA) for an unsupervised analysis and partial least squares-discriminant analysis (PLS-DA) were performed in order to discriminate between the groups of study. All data were submitted to Pareto scaling to reduce the relative importance values, and logarithmic transformation in order to approximate to a normal distribution. The values of R2 and Q2 provided by the software, which are indicative of class separation and predictive power of the model respectively, were assessed. Finally, metabolites responsible for discrimination of treatments were selected according to the

Variable Importance on the Projection (VIP), considering only variables with VIP values higher than 1.5 as indicative of significant differences among groups. The specificity and sensitivity of the altered metabolites were evaluated according to the area under the curve (AUC value) of the receiver operator characteristic (ROC) using MetaboAnalyst 4.0 software (http://www.metaboanalyst.ca/). Also, a correlation analysis by Pearson's correlation coefficient (r_{ij}) were calculated within the discriminant metabolites and the quality parameters (appearance, flavour, and TSS content) using MiniTab16 statistical software. The level of moderate significance was set as $r_{ij} \geq 0.6$ for all correlation analysis.

RESULTS AND DISCUSSION

Quality parameters and TSS content

The results of appearance, flavour and TSS that determine strawberry fruit quality are summarized in Table 1. The appearance of strawberries decreases over the shelf-life as a result of the onset of bruises and rot. Treatments of strawberries with CA maintain better appearance respect to untreated ones, being those treated with 20 % of CO_2 during 48h at 0ºC the one which have better appearance after 7 days of shelf-life (p=0,000). Statistical analysis of strawberry flavour shows large differences depending of treatments. In this way, the treatment with 20 % of CO_2 during 48h at 0ºC produces more intense strawberry flavour. The soluble solids content presented large differences between treated and control samples. The strawberries had an initial value of 8.97 ºBrix (at 0 days of shelf-life). After 3 days of shelf-life the value of ºBrix of control strawberries decreased to 8.16 while those treated with 10 % CO_2 remained constant. Similarly, strawberries treated with 20 % of CO_2 during 48h at 0ºC reached 9.36 ºBrix and those treated with 30 % of CO_2 during 48h at 0ºC exceed 8.16 °Brix. The TSS decreased with the time in both air and CO_2 treatment and in contrast to the published experiences by other authors (Gil *et al.*, 1997; Pelayo-Zaldívar *et al.*,2007) exist a difference between treatments (p=0.001), being strawberries treated with 20 % CO_2 during 48h at 0ºC those with a higher value ºBrix along shelf-life.

Metabolite analysis

In order to investigate metabolic differences an untargeted metabolomic analysis of strawberries based on both GC-MS and DI-ESI-QqQ-TOF-MS was performed to the

three CA treatments considered and control samples. The multivariate statistical analysis by PLS-DA allowed a good classification of different sample groups (control, and treatment under atmosphere of 10 %, 20 % and 30 % CO_2 and maintaining always a concentration of 5% O_2 during 48h at 0ºC). As it can be observed in Figure 1a, 2a, and 3a, that clustering between treatment experiments was satisfactory in all cases getting a good classification among them. This cluster analysis was applied to a total of 32 samples, 8 control samples, and 8 samples under each atmosphere as previously described. Furthermore, in order to access the reliability of the methodology in terms of stability and repeatability, quality control (QCs) samples analysed thought out the sequence run were predicted in the model. QCs samples were also good clustered, which indicate results stability during analysis without significant outliers.

Thereby, QCs were closely clustered (QCs are prepared by pooling equal volumes of each type of samples), showing a low analytical variability among different runs in which also shows a good classification between treated and control samples. The most significant differences on appearance, flavor and TSS content were observed in samples treated with 20% of CO_2 enriched atmosphere during 48h at 0ºC., and for this reason, a second model was developed with data from samples treated under these conditions along with controls to understand the interclass separation and identify potential characteristic markers related to this treatment (Figure 1b, 2b, 3b). The quality of the model was evaluated by the relevant performance statistic of R^2 Y Q^2. In all cases, satisfactory values for the quality parameters were obtained suggesting that all models were valid and highly predictive. The R^2 and Q^2 values obtained from second model were 1 and 0.857 for GC-MS analysis; 0.963 and 0.802 for ESI(+)-QqQ-TOF-MS analysis and finally, 0.987 and 0.908 for ESI(-)-QqQ-TOF-MS analysis.

The variables with more influence in the model were selected on the basis of Variable Influence on the Projection (VIP) parameter, only variables with VIP values higher than 1.5 were selected and used to identify metabolites that contribute to the separation between treated strawberries and control samples.

GC-MS profile

A total of 29 components were identified in the chromatogram (Figure 2) of polar extracts using the NIST Mass Spectral Library (version 08), considering only those variables with a Similarity Index (SI) greater than 70%. In addition, tandem mass spectrometry was performed for the unequivocal identification of these compounds. The

304 most significant metabolites altered in strawberries treated with 20% of CO_2 during 48h
305 at 0ºC respect to the control samples, including sugars (glucose, ribose, inositol) and
306 organic acids (malic acid, oxalic acid, acetic acid, phosphoric acid) among others (Table
307 2).
308 In order to identify the metabolic pathways perturbed by postharvest treatments,
309 MetaboAnalyst 4.0 database was used, which reveals the perturbation of starch and
310 sucrose, inositol - phosphate, pentose - phosphate, pyruvate and glycolysis pathways as
311 well as the TCA cycle. The most altered metabolic pathways in strawberries treated
312 with 20%CO_2 during 48h at 0ºC are showed in Figure 3.
313 It can be observed over-expressed levels of inositol (fold change 33.96) and
314 glucose (fold change 6.05) in strawberries under 20% CO_2 enriched atmosphere for 2
315 days at 0ºC, but not in fructose and sucrose as observed by Sun *et al.,* (2012) and Wang
316 & Bunce (2004). These metabolites are involved in the glycolysis and inositol
317 phosphate metabolism. The conversion of glucose into pyruvate produces several
318 important precursor metabolites, as 3-phosphoglycerate which connect with the inositol
319 phosphate metabolism. Inositol and their derivates are sugar alcohols present in fresh
320 strawberry juices having positive physiological effects on humans (Sanz, Villamiel &
321 Martínez-Castro 2004). In plants, inositol is converted by oxidative cleavage to
322 glucuronic acid, which is transformed to pectin and hemicellulose, both cell wall
323 polysaccharides (Loewus & Murthy 2000), and after the storage of strawberries under
324 CA with the conditions above described, undergo down-regulation (fold change 0.15).
325 Inositol compounds are involved in phosphate storage, cell wall biosynthesis, salinity
326 stress tolerance, cell-to-cell communication, storage and transport of plant hormones. It
327 is known that myo-inositol and myo-inositol-1-phosphate among others decrease in
328 over ripening stage of the strawberry (Zhang *et al.,* 2011) which can be retarded after
329 treatments under CA due to its delaying ripening and senescence action. Another sugar,
330 in this case down-regulated by storage under CA and low temperature treatment is
331 ribose (fold change 0.25), which is involved in the metabolism of sugars through the
332 pentose phosphate pathway.
333 The two main organic acids in strawberry, citric and malic acids are important
334 products of TCA cycle. Storage under 20% CO_2 at 0ºC over-express the last one (fold
335 change 3.63), however, citric acid content was not affected by CO_2 treatment. These
336 results differ from those reported by other authors (Fernández-Trujillo, Nock & Watkins
337 1999; Pelayo-Zaldivar *et al.,* 2007; Ponce-Valadez & Watkins 2008) which report the

338 decrease of both organic acids after postharvest treatments with controlled atmospheres.
339 Nevertheless, Koyunku & Dilmacünal (2010), found an increase of malic acid in 'Dorit'
340 strawberry variety stored at 0ºC for 2 days, decreasing with storage during 10 days.
341 Other organic acids present in very small amounts are tartaric, fumaric and oxalic acid
342 (Sturm, Koron & Stampar 2003). Oxalic acid content in 'Dorit' and 'Selva' strawberries
343 decreased after the first 2 days of storage at 0ºC as was reported by Koyunku &
344 Dilmacünal (2010) but in the present study, applying 20% CO_2 enriched atmospheres
345 and cold storage at 0ºC, the content of malic and oxalic acids increased (fold change
346 1.16), decreasing acetic (fold change 0.61) and phosphoric acids (fold change 0.41).
347 Therefore, the results of the metabolomic analysis show an increase of sugars (inositol,
348 glucose) and organic acids (malic acid, oxalic acid) in strawberries treated with 20% of
349 CO_2 at 0ºC. These results are in good agreement with the values of Total Soluble Solids
350 (TSS) content of the strawberries treated, which have a higher ºBrix value in
351 comparison to fresh product.

352 **DI-ESI-QqQ-TOF-MS analysis**

353 Extracts from treated and control strawberries were analyzed by direct infusion
354 electrospray ionization mass spectrometry in positive ion mode (Fig. 4A) and negative
355 ion mode (Fig.4B) to obtain a fingerprint of the different samples. Identification of
356 compounds was performed by MS/MS, selecting precursor ions according to variables
357 (m/z) with VIP values higher than 1.5 as indicative of significant differences among
358 groups and scientific literature. Table 3 report the identified metabolites altered in
359 strawberries treated with the mixture of 20%CO_2 + 5%O_2 for 2 days at 0ºC. As shown
360 in Table 3 the compounds identified using this technique are phenolic compounds
361 belonging to flavonols class (compounds 9, 10, 11, 12, 13), flavanols (3, 7)
362 anthocyanins (6, 8) and derivatives of phenolic acids and others (1, 2, 4, and 5).
363 Flavonoids are secondary metabolic compounds responsible of colour and aroma
364 in plants that play numerous biological functions such as UV-filter, detoxify agents,
365 defence against pathogen infection among others (Falcone-Ferreyra, Rius & Casati
366 2012). Flavonol glycosides are the most numerous group of phenolic compounds
367 identified in strawberries treated with 20%CO_2 +5%O_2 in this study, including quercetin
368 3-O-glucuronide, dihydromyricetin-hexose, kaempferol-3-O-rutinoside, isorhamnetin 3-
369 O-rhamnoside-rhamnoside-glucoside, and quercetin 3-O-(6-O-feruloylglucoside)-
370 glucoside-7-O-rhamnoside, which have been previously identified in berry fruits

(Kårlund *et al.,* 2015; Tian *et al.,* 2017; Spínola, Pinto & Castilho 2015; D'Urso *et al.,* 2016). Quercetin 3-O-glucuronide, which has been described as the main flavonol in strawberries (Seeram *et al.,* 2006) is down-regulated (fold change 0.741) against other flavonols glycosides which undergo over-expression (peaks no. 10, 11, 12, 13, in Table 3). Kafkaletou et al. (2017) observed an increase in the total phenolic compounds after the storage under CA in goji berries under similar conditions (5 $\%O_2$, 15$\%CO_2$ at 1ºC for 2 days). Others authors such as Selcuk et al. (2015) and González-Gómez et al. (2017) have experienced an increase in the total flavonoid content in cherries and medlar fruit respectively after long-term treatments with CA.

Epi(catechin) hexoside and epi(gallocatechine) were identified by their typical DI-MS and MS-MS data, which are described in previous studies (González-Gómez *et al.,* 2017; Del Bubba, *et al.,* 2012). In the present work, both compounds are over-expressed displaying fold change values of 2.831 and 2.604, respectively.

Like as flavonoids, phenolic acids are secondary metabolites crucial for plant growth and reproduction (Vrhovsek, *et al.,* 2012) and whose production can be affected by the environment or post-harvest process (Carbone & Mencarelli, 2015; Mozetič, Simčič & Trebše 2006). Derivates from phenolic acids such as, caffeoylthreonate (peak no. 1) and caffeic acid hexoside (peak no. 4) are disturbed in strawberry fruit after the storage under 20%CO2+5%O2 atmosphere at 0ºC for 2 days. Unlike caffeoylthreonate (fold change 1.814), caffeic acid hexoside is down-regulated (fold change 0.688). A characteristic fragment ion at m/z 189, 190 and 171 correspond to cinnamic acid-3-O-acetyl hexoside (compound No. 5) which suffer an over-expression (fold change 2.185) in stored strawberries. This compound has being detected and identified previously in strawberry fruits by Spinola *et al.,* (2015). In addition, 4-(2-Hydroxyethyl)phenol-hexoside (peak no. 2) is a phenolic compound present in others berries like currants and chokeberries (Tian *et al.,* 2017) which undergo to an over-expression (fold change 1.203).

The main compounds responsible for strawberry colour are anthocyanins, carotenoids and chlorophylls. The main anthocyanins found in strawberry are pelargonidin-3-glucoside and cyanidin-3-glucoside that correspond to the colours red/orange and orange respectively (Nunes, *et al.,* 2006). Peaks 6 and 8 were identified as cyanidin-3-glucoside/cyanidin-3-galactoside and pelargodinin-3-O-acetyl hexoside

on the basis of the spectral data and literature and are commonly found in strawberries. The use of DI-MS in positive ion mode do not allow the distinction between cyanidin-3-glucoside or cyanidin-3-galactoside, in this case, it's necessary the use of a complementary chromatographic technique for a better identification. During the maturation of the fruit, the content of the anthocyanins increases (Jimenez, Zambrano & Aguilar 2004; Crecente-Campo, Nunes-Damaceno & Romero-Rodriguez 2012) though, the amount of these pigments varies depending on the variety, crop and can be affected after post-harvest treatments. Accumulation of anthocyanins in similar CO_2 storage was reduced in the cultivars 'Selva' (Gil, Holcroft & Kader 1997; Holcroft & Kader 1999), Northeaster and Earliglow (Shin, *et al.,* 2008). These result are in good agreement with the pelargodinin-3-O-acetyl hexoside content which experiences a down-regulation (fold change 0,7), however, the content of cyanidin-3-glucoside/cyanidin-3-galactoside (fold change 1,891) are over-expressed under the storage condition studied in 'Fortuna' and in grapes, the combination of low- temperatures storage and high CO_2 may provoke an accumulation of anthocyanins (Romero, *et al.,* 2008). The content of cyanidin-3-glucoside in strawberries is very low compared to other anthocyanins. This compound has a special interest because it has numerous beneficial properties in human health. Studies in rodents and cell lines highlight the action of this compound against infections, diabetes, metabolic syndromes and cancer among others (Olivas-Aguirre, *et al.,* 2016).

A possible increase in the concentration of this anthocyanin and the others phenolic compounds in strawberries treated with CA makes it a fruit with high nutraceutical potential.

The analysis of polar extracts using GC-MS and DI-ESI-QqQ-TOF-MS enabled the detection of a great number of compounds of different classes. The heat map based on hierarchical clustering analysis (Fig. 5) summarizes the differential distribution of altered metabolites after a controlled atmosphere treatment.

Correlation analysis between discriminant metabolites and quality parameters

In the polar extract of strawberry fruits, a total of 22 metabolites were identified from untargeted metabolic analysis and were submitted to a correlation analysis against quality parameters. Overall, positive and negative dependencies were observed. Metabolites that showed higher correlation coefficients against appearance parameters

were caffeoylthreonate, cinnamic acid-3-o-acetylhexoside, gallocatechine/epigallocatechine, dihydromyricetin-hexose, kaempferol-3-o-rutinoside, cyanidin-3-glucoside/cyanidin-3-galactoside, isorhamnetin 3-o-rhamnoside-rhamnoside-glucoside, quercetin 3-o-(6-o-feruloylglucoside)-glucoside-7-o-rhamnoside and glucose. in the case of flavour, caffeoylthreonate, gallocatechine/epigallocatechine, cinnamic acid-3-o-acetylhexoside, dihydromyricetin-hexose, kaempferol-3-o-rutinoside, cyanidin-3-glucoside/cyanidin-3-galactoside, isorhamnetin 3-o-rhamnoside-rhamnoside-glucoside, quercetin 3-o-(6-o-feruloylglucoside)-glucoside-7-o-rhamnoside, glucose and inositol. Finally, the content of soluble solids was correlated with isorhamnetin 3-o-rhamnoside-rhamnoside-glucoside, cyanidin-3-glucoside/cyanidin-3-galactoside, cinnamic acid-3-o-acetylhexoside and glucose.

Our results conclude that the use of metabolomic fingerprinting allows a better understanding of the mechanisms of action and response of 'Fortuna' strawberry cultivar after the application of controlled atmospheres and a good correlation with physical-chemical and sensory analysis. GC-MS and DI-ESI-QqQ-TOF-MS stand out as a suitable and complementary metabolomic tools for the characterization of the metabolome of strawberry fruits. The application of this analytical approach and subsequent multivariate statistics has allowed the classification three different treatments in CA and control samples. Preservation of fruits under 20% of CO_2+5%O_2 during 48h at 0ºC produces alterations in primary and secondary metabolites including sugars (glucose, ribose, inositol) organic acids (malic acid, oxalic acid, acetic acid, phosphoric acid) and phenolic compounds (flavonols, anthocyanins, phenolic acids). These compounds contribute to the strawberry flavour and are considered as bioactive compounds due to its health benefits in humans. The increase in these bioactive compounds after the CA treatment mentioned above allows producing a fruit enriched in bioactive compounds with significant added value from health point of view, that make it a good candidate for the production and formulation of food supplements and nutraceutical products.

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615

616

FIGURES

Fig. 1. Partial least squares discriminant analysis (PLS-DA) including all groups from the polar extract of (A) GC-MS analysis, (B) ESI(+)-QqQ-TOF-MS analysis and (C) ESI(-)-QqQ-TOF-MS analysis. Each replicate is one piece of strawberry.Control samples: black; T1: blue; T2: green; T3: orange; QCs samples: red. Second model comparing control samples (red) and T2 (black) of (D) GC-MS analysis, (E) ESI(+)-QqQ-TOF-MS analysis and (F) ESI(-)-QqQ-TOF-MS analysis.

Fig. 2. GC-MS chromatogram of strawberry polar extract**.**

Fig. 3. Changes of metabolites after CA treatment shown in a metabolic diagram.

Fig.4. DI-ESI-QqQ-TOF-MS Full scan (50-1100 m/z) of strawberry polar extract in (A) positive ionization mode and (B) negative ionization mode.

Fig. 5. Heatmap based on hierarchical clustering analysis of discriminant metabolites.

TABLES

Days	Appearance					Flavour					TSS				
	Control	T1	T2	T3	*p* value	Control	T1	T2	T3	*p* value	Control	T1	T2	T3	*p* value
0	5	-	-	-		5	-	-	-		8.97	-	-	-	
3	4.75b	5a	5a	5a	0.03	4.5c	4.75ab	4.95a	4.25b	0.002	8.16b	8.96ab	9.36a	8.16b	0.009
5	4.5b	4.75a	4.75a	4.75a	0.000	4b	4b	4.5a	3.5c	0.002	7.10c	7.92b	8.28a	7.82b	0.000
7	4.5b	4c	4.75a	4.5b	0.000	4b	4.25b	4.5a	3.5c	0.01	7.68bc	7.50c	7.94a	7.72bc	0.000

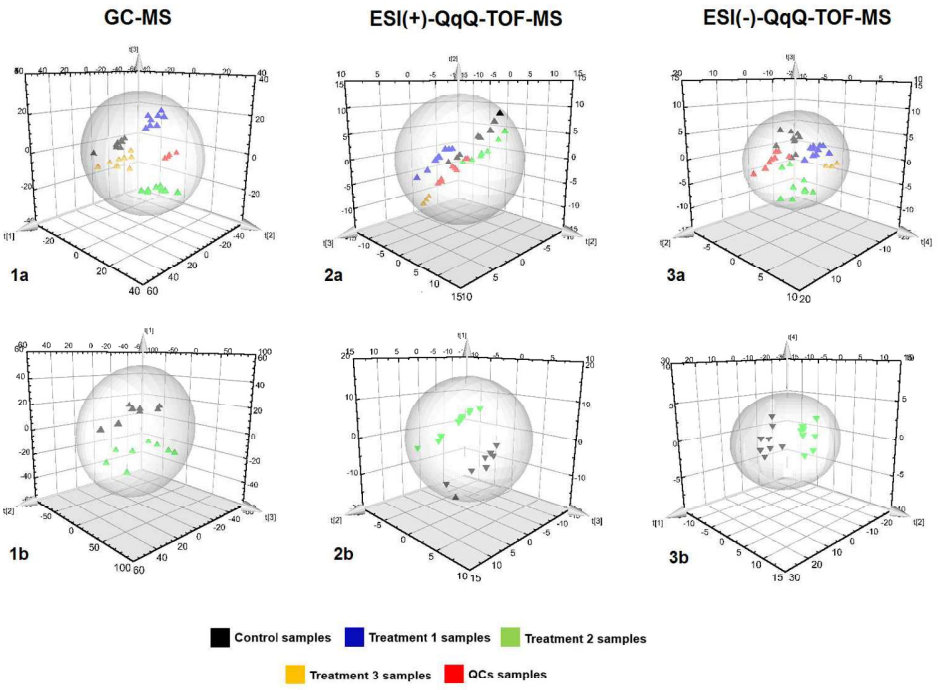
Table 1. Effects of CA on appearance, flavour and soluble solids content (TSS) of strawberry from the first harvest (control, 0 days) and after CA treatment at 3, 5 and 7 days. Appearance are means of all the strawberries content in the plastic boxes. Flavour data are means of 3 individual fruit replicates. °Brix data are means of 5 individual fruit replicates. Values with the same letters do not differ statistically.

Metabolite	VIP	Fold Change	Sense of change	AUC value
Glucuronic acid	2.291	0.15	↓	0.70
Oxalic acid	1.721	1.16	↑	0.70
Phosphoric acid	2.353	0.41	↓	0.86
Malic acid	2.164	3.63	↑	0.77
Glucose	2.825	6.05	↑	0.94
Inositol	2.251	33.96	↑	0.77
Acetic acid	2.588	0.61	↓	0.72
α-D-glucopyranoside	2.943	0.13	↓	0.94
Ribose	1.740	0.25	↓	0.78

Table 2. Discriminant metabolites identified by GC-MS profiling for differentiation of strawberry fruits treated under T2 conditions.

No.	Ionization Mode	m/z	MSMS	Tentative Metabolite	Class	Fold Change	Sense Of Change	Literature	AUC value
1	-	297	135, 89	Caffeoylthreonate	Phenolic acid derivates	1,814	↑	Karlund et al. (2016)	0.91
2	+	301	139	4-(2-Hydroxyethyl)phenol-hexoside	Other phenolic compounds	1,203	↑	Tian et al. (2017)	0.65
3	-	305	203, 123	Gallocatechine / Epigallocatechine	Flavan-3-ols	2,604	↑	Vrhovsek et al. (2012)	0.92
4	+	343	179	Caffeic acid hexoside	Phenolic acid derivates	0,688	↓	Tian et al. (2017)	0.75
5	-	351	189, 190, 171	Cinnamic acid-3-O-acetylhexoside	Phenolic acid derivates	2,185	↑	Spinola et al. (2015)	0.85
6	+	449	287	Cyanidin-3-glucoside / Cyanidin-3-galactoside	Anthocyanins	1,891	↑	Del Bubba et al. (2012);	0.92
7	-	451	245, 125, 109	Epi(catechin) hexoside	Flavanol glycoside	2,831	↑	Del Bubba et al. (2012)	1
8	+	475	271	Pelargonidin-3-O- acetyl hexoside	Anthocyanins	0,7	↓	La Barbera et al. (2017); Spinola et al. (2015)	0.76
9	-	477	301	Quercetin 3-O-glucuronide	Flavonol glycoside	0,741	↓	D'Urso et al. (2016); Tian et al.(2017); Karlund et al. (2016)	0.92
10	-	481	463, 319, 301	Dihydromyricetin-hexose	Flavonol glycoside	1,807	↑	D'Urso et al. (2016)	0.89
11	-	593	449, 285	Kaempferol-3-O-rutinoside	Flavonol glycoside	1,940	↑	Tian et al. (2017); Spinola et al. (2015)	0.99
12	+	771	625, 479, 317	Isorhamnetin 3-O-rhamnoside-rhamnoside-glucoside	Flavonol glycosides	1,745	↑	Tian et al. (2017)	0.97
13	+	949	303	Quercetin 3-O-(6-O-feruloylglucoside)-glucoside-7-O-rhamnoside	Flavonol glycosides	1,956	↑	Tian et al. (2017)	0.96

Table 3. Discriminant metabolites identified by DI-ESI-QqQ-TOF-MS in strawberry fruits treated under T2 conditions.


GC-MS
ESI(+)-QqQ-TOF-MS
ESI(-)-QqQ-TOF-MS
1a
2a
3a
1b
2b
3b
Control samples
Treatment 1 samples
Treatment 2 samples
Treatment 3 samples
QCs samples

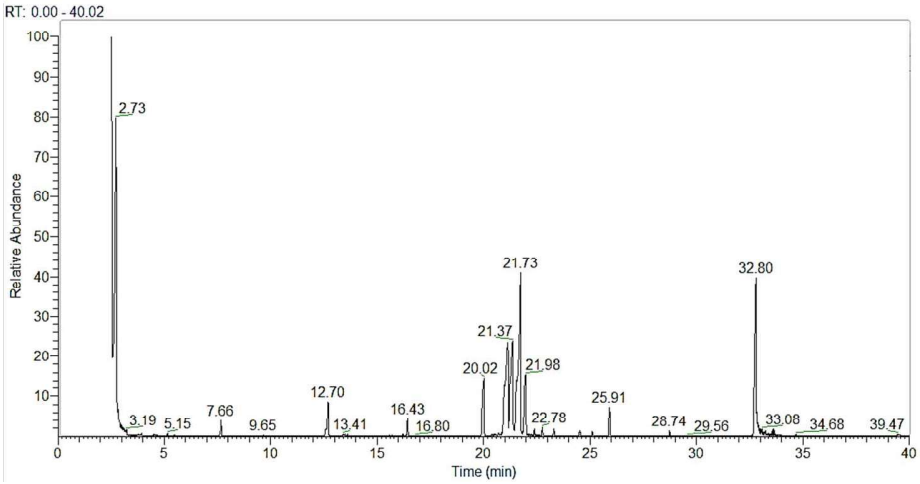


Figure 2

299x148mm (95 x 95 DPI)

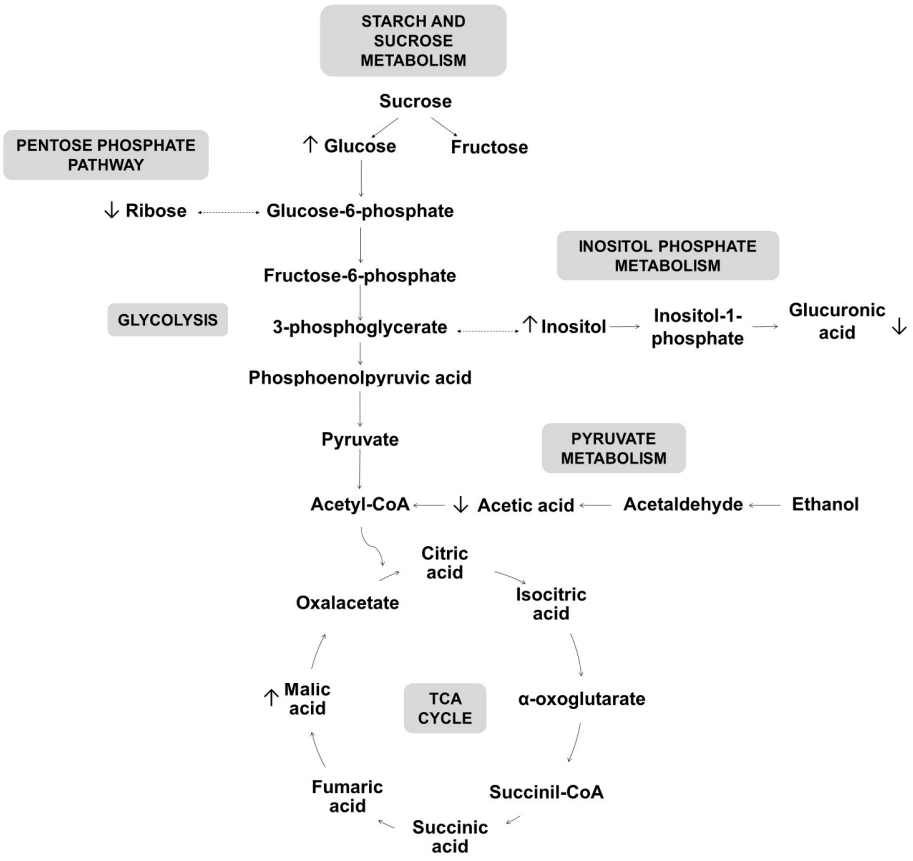


Figure 3

600x548mm (96 x 96 DPI)

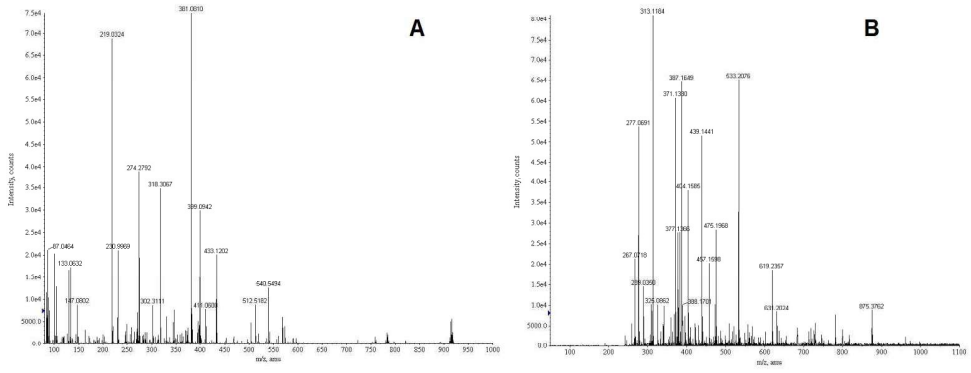
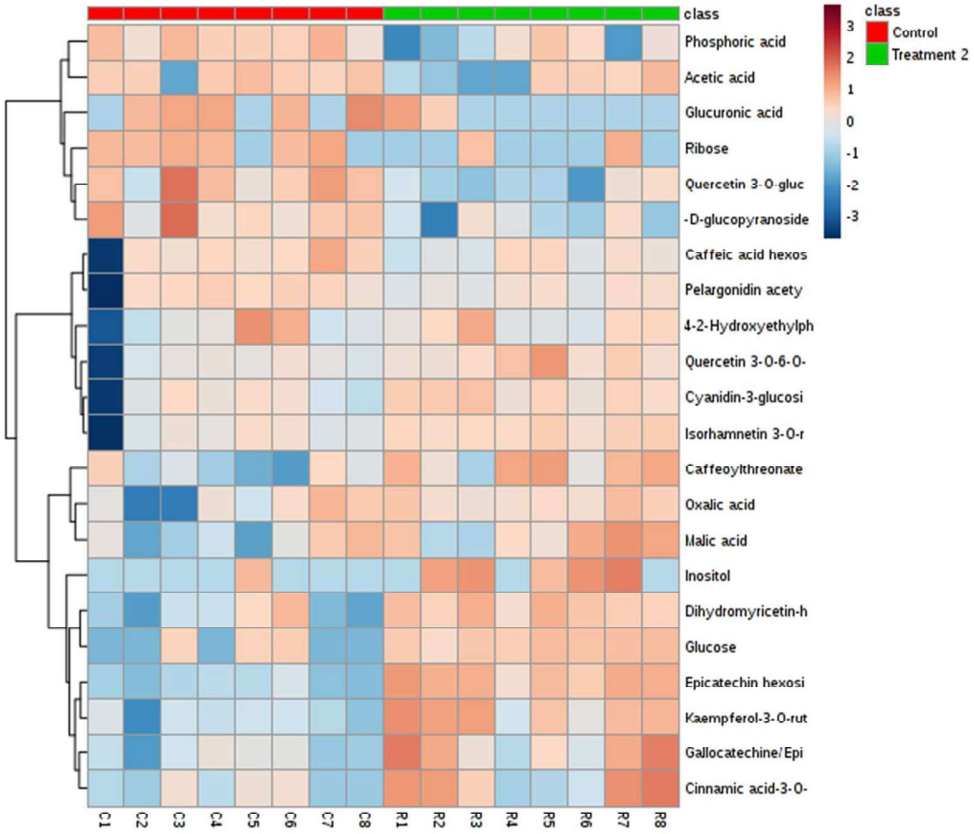


Figure 4



222x192mm (72 x 72 DPI)