JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

Changes in aromatic profile, sugars and bioactive compounds when purple garlic is transformed into black garlic

Lucia Martinez-Casas, Maria Lage-Yusty, and Julia Lopez-Hernandez

J. Agric. Food Chem., **Just Accepted Manuscript** • DOI: 10.1021/acs.jafc.7b04423 • Publication Date (Web): 21 Nov 2017

ACS Publications

Journal of Agricultural and Food Chemistry is published by the American Chemical Society. 1155 Sixteenth Street N.W., Washington, DC 20036

Title: Changes in aromatic profile, sugars and bioactive compounds when purple garlic is transformed into black garlic

Lucía Martínez -Casas, María Lage-Yusty*and Julia López-Hernández

University of Santiago de Compostela, Faculty of Pharmacy, Department of Analytical Chemistry, Nutrition and Bromatology, Campus Vida, 15782 Santiago de Compostela, Spain

*Corresponding author e-mail: maria.lage.yusty@usc.es

ABSTRACT

Black garlic is an elaborated product obtained from the fresh garlic (*Allium sativum L.*) at a controlled high humidity and temperature, which leads to modifications in colour, taste and texture. To clarify the physicochemical changes that occur during the thermal process, this work aimed to evaluate and contrast the antioxidant capacity and other compounds between purple garlic ecotype "Purple from Las Pedroñeras", and its black garlic derivative. Our results showed numerous differences between both, since black garlic presented a significant divergence in the volatile profile, a decreased amount of ascorbic acid, an increment in sugar and polyphenol contents, a greater antioxidant capacity and a different composition of phenolic acids and flavonoids.

KEYWORDS: purple garlic; black garlic; volatile compounds; ascorbic acid; antioxidant activity; total polyphenols content; free sugars; polyphenolic compounds; *Allium sativum;*

INTRODUCTION

Garlic (*Allium sativum* L.) belongs to the Alliaceae family and is widely used as an ingredient in traditional cooking. Moreover, garlic has powerful medicinal and healthy properties and is used to prevent different diseases, metabolic and cardiovascular[1]. Nevertheless, its use in cooking and medicine is conditioned by its organoleptic characteristics.

Black garlic (BG) is a newly processed food that is formed by aging fresh garlic for a period of time at a controlled high temperature (60-90ºC) under controlled high humidity (80-90%). In the process of manufacturing is important besides, the variety garlic[2]. As a result of the procedure, black garlic acquires a sweet and sour taste due to a significantly higher level of free sugars and chewy and jelly-like texture derived from the tissue softening caused by the degradation of cell wall polysaccharides under high temperature conditions[2,3]. The Maillard reaction takes place by the heat treatment, and this reaction also produces the dark brown colour of cloves[3]. Unlike fresh garlic, black garlic does not have the characteristic off-flavour on account of the reduced content of allicin (responsible for the pungent odour), which is converted into antioxidant compounds. Some studies reported that part of the antioxidant agents of BG that are relevant against diseases increased during the aging process[4] and that an augmentation of total polyphenols and flavonoids occur in the thermal treatment[5]. Additionally, during this process other changes take place, such as the catabolism of γ-glutamylcysteines to form S-allylcysteine (SAC), which inhibits oxidative deterioration originated by aging and various diseases[6]. Consequently, black garlic shows a significantly higher biological activity, and higher antioxidant properties than fresh garlic[7]. Another element that contributes for the "total" antioxidant power is the ascorbic acid, a water soluble antioxidant vitamin present in a great variety of vegetables, including garlic, but whose stability can be affected by factors such as high temperature, light, oxygen and other conditions of elaboration[8]. Organosulfur compounds are also an important component in garlic, as well as in the whole Alliaceae family. The garlic aroma is due to the compounds originated from the transformation of thiosulfinate derivatives of S-alk(en)yl-$_L$-cys-teine [9]. There is also evidence of the potential anticancer activity of some of its sulfide derivatives[10,11].

Spain is nowadays one of the primary garlic producers in the European Union and within Spain, Castilla-La Mancha is the leading producer area. The native ecotype "Purple from Las Pedroñeras" is the bulb of the specie *Allium Sativum* L, preserved by the Protected Geographical Indication (PGI). The bulb is spherical or round and has an average size. The outer skin of the bulb is white or slightly coloured and it is striated. The skin that protects the cloves has a characteristic violet or deep purple colour and is also striated. The cloves are small, crescent shaped and yellowish white in colour. The main production centre of the "Purple Garlic from Las Pedroñeras" is in the region surrounded by the areas of "Las Pedroñeras", all located in the province of Cuenca, in the natural region of "La Mancha Baja" [12]. The black garlic is elaborate from purple garlic ripened for a long time in controlled temperature and humidity conditions, as a result of which the cloves become darker and darker until they turn completely black

The objective of this work was to study and to compare the differences in volatile compounds, ascorbic acid, free sugars, total polyphenols, antioxidant capacity and phenolic acids and flavonoids in both commercial black and purple garlic of ecotype "Purple from Las Pedroñeras".

MATERIALS AND METHODS

Samples

Both fresh black garlic and fresh purple garlic (*Allium sativum L*. ecotype Morado de Las Pedroñeras) were kindly supplied by the enterprise JR Suárez Monedero S.L. (Las Pedroñeras, Cuenca, Spain). A few bulbs of both types of garlic were broken apart into individual cloves that were then lyophilized using a freeze dryer (Telstar LyoQuest, Spain).To carry out the analyzes in both fresh and lyophilized samples from each bulb three cloves are homogenized, constituting each of the analysed samples.

Determination of the volatile profile

The volatile profile was analyzed by a Tekmar Stratum Purge and Trap Concentrator (PTC) equipped with a probe for solid and a system of heating (Teledyne Tekmar, Mason, OH, USA), which allows the direct analysis of small quantities of samples without previous treatment. The cloves of fresh bulbs of both purple and fresh garlic

have been individually separated. Then, the four tooth cover were removed and chopped into similar-sized pieces. For the analysis were used 5 g of fresh samples in very small portions. The sample was warmed up to 35 ºC during 15 min, avoiding direct contact with the probe. Sampling conditions were as follows: sample temperature 35 ºC; purge time 15 min, purge flow 40 mL/min, dry purge time 0.5 min, dry purge temperature 20 ºC, dry purge flow of 100 mL/min, desorb pre-heat temperature 245 ºC, desorb temperature 250 ºC and bake temperature of 280 ºC.

The trapped components were coupled via a heated interface to a GC-MS system (Thermo Finnigan Trace GC ultra-chromatograph with a Trace DSQ mass detector) (Thermo Fisher Scientific, Madison, WI, USA) and directed into an in-line GC capillary column (30 m, 0.25 mm i.d., 1.40 µm film of 6% cyanopropylphenyl and 94% dimethylpolysiloxane) (DB-624, J&W Scientific). Helium was carrier gas (flow rate 0.5 mL/min). The injector temperature was 170 ºC, and the split flow was 10 mL/min. The oven temperature was programmed from 40 ºC to 200 ºC at a rate of 10 ºC/min, with the initial and the final hold times at 4 min and 0.1 min respectively. The MS detector conditions were as follows: full scan mode by scanning a mass range of *m/z* 29-400; source temperature, 200 ºC. The system was computer-controlled by the XCalibur Home Page version 1.4 SRI, Windows XP software. The spectra were compared with those in MS libraries (Xcalibur, Wiley Registry (8e Mass Spectral Library), FFNsc2 and MainLib). Diallyl disulfide (≥98% HPLC) and allyl alcohol (≥99%), the volatile compounds with the higher response in the TIC chromatogram in purple and black garlic respectively, were identified with external standards (Sigma Aldrich Chemical Co., St.Louis, MI, USA).

Measurement of ascorbic acid

The extraction procedure is a modification of the method proposed by Oruña-Concha et al[13]. Briefly, 1 g of fresh purple garlic and fresh black garlic were weighed in plastic conical tubes and 10 mL of 0.1% acetic acid were added and vortexed for 15 min. Then

the samples were centrifuged at 3935 *g* for 10 minutes and decanted into a 20 mL volumetric flask. To ensure a complete extraction, the residue was again extracted with 10 mL of 0.1% acetic; the supernatants were collected and made up to 20 mL. An aliquot of this solution was filtered through 0.5 µm PTFE Advantec Filter (Japan) prior to injection into the chromatographic column. The utilized apparatus was an Agilent 1200 chromatography system with a quaternary pump, autosampler, degassing device, thermostated column compartment, DAD (diode array detector) and Agilent ChemStation software (Agilent Technologies, Santa Clara, CA, USA). The analytical column was a Kinetex EVO C18 100A column (150 mm x 3 mm, 5 µm) (Phenomenex, California, USA) maintained at 30 ºC. A mobile phase consisting of acetic acid 0.1% *v/v* at a flow rate of 0.5 mL/min was used. The injection volume was 20 µL and the detection wavelength was 245 nm.

All reagents were of analytical grade. Ascorbic acid and acetic acid were purchased from Sigma-Aldrich. The water used for all solutions was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA).

Identification and quantification were conducted by the external standard method. Identification of ascorbic acid was conducted by comparison of retention time and ultraviolet spectra obtained by analyzing standard. The standard stock solutions were prepared in acetic acid 0.1 % v/v and the calibration line was constructed on five concentration levels between 4-100 mg/L. The limit of quantification (LOQ), calculated on the basis of signal-to-noise ratio plus ten deviations, was 0.11 mg/100 g DM.

Measurement of Sugar Content

The preparation of the sample consisted in weighing 0.5 g of lyophilized purple garlic and 0.05 g of lyophilized black garlic and then a sonication in 50 mL of Milli-Q water (Millipore, Bedford, MA, USA) for one hour. Previously to the HPLC (High Performance Liquid Chromatography) analysis an aliquot of the solution was filtered through 0.5 µm PTFE Advantec Filter (Japan) and was then injected into the chromatograph. The system consists of an Agilent 1200 chromatography system with a

quaternary pump, autosampler, degassing device, thermostated column compartment, RID (refractive index detector) and Agilent ChemStation software. The column was a Tracer Extrasil NH2 (25 cm x 0.4 cm, 5 µm) (Teknokroma, Barcelona, Spain) maintained at 30 ºC. A mobile phase consisting of acetonitrile-water (80:20 *v/v*) was used and the injection volume was 10 µL.

The standards of fructose (≥99%), sucrose (≥99.5% HPLC) and glucose (≥99.5% HPLC) were purchased from Sigma Aldrich.

Identification and quantification of sugars in garlic were conducted by the external standard method. The standard stock solutions were prepared in Milli-Q water and the calibration line were constructed between 40-400 mg/L for fructose and sucrose content and 8-160 mg/L for glucose content. The limit of quantification (LOQ), calculated on the basis of signal-to-noise ratio plus ten deviations, were 0.2 g/100 g DM for fructose, 0.16 g/100 g DM for glucose and 0.4 g/100 g DM for sucrose.

Total polyphenol content, antioxidant capacity and polyphenolic compounds

The samples were extracted according to the procedure of Ferraces-Casais et al.[14]. Briefly, 1 g of lyophilized sample was weighed in plastic conical tubes. Then 10 mL of acetic acid-water-methanol (1:69:30 *v/v/v*) were added and vortexed for 60 min covered with aluminium foil to protect them from light. The samples were then centrifuged at 1506 g for 4 min and decanted into a 20 mL volumetric flask. A re-extraction of the residue was performed by adding 10 mL of water-acetone (30:70 *v/v*). The supernatant was combined with the one from the first extraction and made up to 20 mL with water-acetone (30:70 *v/v*). Then the samples were stored at 4 ºC for a period of approximately 12 hours.

Total Polyphenols

The total polyphenol content was determined according to the method described by Ferraces-Casais et al.[14] with minor modifications. The garlic extract (500 µL) was mixed with 200 µL of Folin-Ciocalteu reagent and 4 mL of 6% Na_2CO_3. The reaction was then allowed to proceed for 30 min in the dark. After incubation, the absorbance was measured at 765 nm at room temperature. A solution composed of 500 µL of extraction solvent (acetic acid-water-methanol (1:69:30 *v/v/v*) and acetone/water (70:30 *v/v*)), 4 mL of 6% Na_2CO_3 decahydrate and 200 µL of Folin-Ciocalteu reagent was used

as a blank. The Cary 3E UV- Visible spectrophotometer (Varian, Australia) was used for all absorbance measurements. Gallic acid was used as a standard for the calibration curve. The phenolic content was calculated as gallic acid equivalents (GAE) using the calibration curve prepared by using different known concentrations (25-150 mg/L) of gallic acid in a solution composed of 50% of both extraction solvents (acetic acid-water-methanol (1:69:30 *v/v/v*) and 50% water-acetone (30:70 *v/v*). The determination coefficient was 0.9992.

To determine the total polyphenols content by the Folin-Ciocalteu assay, two parameters of the reaction were optimized: sample volumes (50 µL, 100 µL, 500 µL, 1 mL and 2 mL) and volumes of Folin-Ciocalteu reagent (200 µL, 150 µL and 100 µL). The best results were achieved with 500 µL of samples of both black and purple garlic extracts, mixed with 200 µL of Folin-Ciocalteu reagent and followed by the addition of 6% Na2CO3 decahydrate (4 mL).

Antioxidant Capacity

A modification of the method reported by Rodríguez-Bernaldo de Quirós et al[15] was used to determinate the antioxidant capacity. 3 mL of ethanolic solution that contained DPPH (2,2-diphenyl-1-picrylhydrazyl) radicals (0,08 mM) are added to garlic extract (150 µL). A solution composed of 150 µL of extraction solvent (acetic acid-water-methanol (1:69:30 *v/v/v*) and water- acetone (30:70 *v/v*) and 3 mL of methanol was used as a blank. Absorbance at t=0 and t=30 was measured at 515 nm. The sample was stored in the dark during the assay. The scavenging activity of DPPH was calculated:

$$\% \text{ DPPH scavenging} = [(Abs_{t=0} - Abs_{t=30}) / Abs_{t=0}] \times 100$$

Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was used as a standard to construct in calibration curve. Stock standard solutions were prepared in a solution composed of 50% acetic acid-water-methanol (1:69:30 *v/v/v*) and 50% water-acetone (30:70 *v/v*). Working solutions were prepared by dilution and to avoid

degradation, were stored at 4 ºC in the dark. The absorbance at 515 nm was measured at t=0 and t=30, after of 30 minutes in the darkness. The calibration line was constructed by linear regression using seven concentration levels. The determination coefficient was 0.9991 for the linear range 0.1-0.7 mM.

Polyphenolic compounds

To analyse the phenolic and flavonoid contents, 5 mL of the extraction solution were evaporated using a stream of nitrogen to 2.5 mL and made up to 5 mL with water. The solution was then injected into the chromatograph. The apparatus used was an Agilent 1200 chromatography system consisting of a quaternary pump, degassing device, thermostated column compartment, DAD (diode array detector), autosampler and Agilent ChemStation software. The analytical column was a Kinetex EVO C18 100A column (150 mm x 3 mm, 5 µm) (Phenomenex, CA, USA) maintained at 30 ºC. The mobile phase consisted of acetic acid (0.1% *v/v*) as solvent A, acetic acid-water-methanol (1:69:30 *v/v/v*) as solvent B and acetonitrile as solvent C. The gradient programme was as follows: 0 min (80% A + 20% B); 9 min (35% A + 65% B); 18 min (0% A + 100 % B), afterwards followed by a wash with acetonitrile and at 37 min the initial conditions were recovered. The flow rate was 0.5 mL/min and the injection volume 20 µL. Methanol gradient grade for liquid chromatography was supplied by Merck and acetic acid provided by Sigma-Aldrich. The DAD detector was set at 278 nm for gallic acid and epicatechin, 300 nm for chlorogenic, caffeic, coumaric and ferulic acids and finally, 360 nm for apigenin. An HPLC-PDA-MS/MS system composed of an Accela Autosampler, pump and PDA (Photodiode Array Detector) (Accela, Inc., San Ramón, CA, USA) coupled to a TSQ Quantum Access max triple-quadruple mass spectrometer controlled by Xcalibur software (Thermo Fisher Scientific, San Jose, CA, USA) was also used to identify phenolic acids and flavonoids. The mass spectrometer

operated in negative ESI (Electrospray System Ionization). The chromatographic conditions were the same as the ones used during the HPLC analysis. The gas used was nitrogen, spray voltage, 2500 V, vaporization temperature, 340 °C and capillary temperature, 350 °C. Phenolic acids and flavonoids were identified by retention time relative to external standard, PDA spectra (200-600 nm), precursor ion and fragmentation patterns.

The standards of phenolic compounds were obtained from various manufactures. Caffeic acid (≥98.0% HPLC), p-coumaric acid (≥98.0% HPLC), ferulic acid, (99%) chlorogenic acid(≥95.0%), epicatechin (≥90% HPLC) and apigenin (≥95.0% HPLC))were obtained from Sigma-Aldrich.

Previous to the identification of phenolic acids and flavonoids from the purple and black garlic extracts, the extraction was optimized. Different sample amounts (0.5, 1 and 1.5 g) were extracted with different solvents and different solvent amounts: 20 mL of metaphosphoric acid, 10 mL of acetic acid 0.1% *v/v*, 10 mL of acetic acid-water-methanol (1:69:30 *v/v/v*), 10 mL of ethanol 80% and 5 mL of acetone 70%. Different extraction times (15, 30, 45, 60 and 75 min) were also tested.

Phenolic acids and flavonoids were identified in a chromatographic system by a comparison of retention times and ultraviolet spectra data obtained for the standards. The standard stock solutions were prepared in methanol and then the calibration line was constructed for all phenolic acids and flavonoids based on five concentration levels between 0.5-5 mg/L. The quantification limit (LOQ) calculated on the basis of signal-to-noise ratio plus ten deviations and the values were: 1.5 mg/100 g DM for gallic acid, 0.06 g/100 g DM for clorogenic acid, 0.03 g/100 g DM for caffeic acid, 0.06 mg/100 g DM for epicatechin, 0.01 mg/100 g DM for coumaric and ferulic acids and 0.04 for apigenin.

Statistical Analysis

The data were examined by analysis of variance and Student's t-test using Statgraphics statistical software (version 16.1.15 for Windows, Statistical Graphics Corp., Rockville, MD). The level of significance was set at $p<0.05$.

RESULTS AND DISCUSSION

Determination of volatile profile

The 20 identified compounds are classified into 8 groups: derivatives from S-alk(en)-yl-L-cysteine, other sulfur-containing compounds, alcohols, aldehydes, furans, acids, ketones and other compounds. BG presented significant differences on the volatile profile regarding purple garlic (Table 1). The relative concentration of derivatives of S-alk(en)-yl-L-cysteine, the precursor of the lachrymatory and flavour compounds in the genus *Allium*[16] was decreased in black garlic compared with purple garlic. Furthermore, some of the sulfur volatiles identified in purple garlic were not detected in black garlic (allyl mercaptan) or were greatly reduced (dimethyl disulfide, allyl methyl disulfide) (Figure 1). Diallyl disulfide was the major sulfur volatile in purple garlic and its area represents about 45 % of the area of all volatile compounds detected in purple garlic samples, but in black garlic its area is only of 7 %. Another derivative, 1,3-dithiane was not detected in black garlic. A possible explanation of this fact is that this compound could be thermolabile or could lead to other volatile or non volatile compounds during the heating process. On the other hand, dimethyl trisulfide was present in black garlic but was not detected in purple garlic and both diallyl trisulfide and allyl methyl trisulfide suffered a significant decrease in purple garlic compared with black garlic. The compound that experimented the highest increase in black garlic as compared to purple garlic was the allyl alcohol (2-propen-1-ol). While in purple garlic this compound was not detected, in black garlic it was, by far, the most abundant volatile compound. Allyl alcohol (2-propen-1-ol) is known to be a contributing agent to the flavour of heated garlic and was found to be formed in quite considerable amounts when garlic or alliin are heated at cooking temperatures[17]. It is believed that this compound is formed from alliin during heating process of purple garlic[18]. Another study about this alcohol reported that it was the principal volatile compound found in degradation of alliin at pH 3, 7, and 9[19]. The process that leads to the formation of allyl alcohol is the thermal decomposition of alliin producing this alcohol as one of the dominant volatile compounds[20]. The formation of allyl alcohol from alliin could be through [2,3]-

sigmatropic rearrangement of alliin that may lead to intermediate sulfenate. Then, the reduction of sulfenate will yield allyl alcohol and cysteine. The aging process of BG, decreases the concentration of derivatives from S-alk(en)-yl-L-cysteine. The compounds that provide sweet flavour were clearly characteristic of BG since none were found in purple garlic meanwhile in black garlic, the area of furfural represents around 4.3% of the total area of volatile compounds. During the aging process of BG, the Maillard reaction causes the degradation of a pentose sugar to form furfural[21]. Therefore, furfural is a result of a thermal process and that is the reason why it is absent in purple garlic. One derivative of furfural, 2-acetylfuran, was also identified in the BG samples. Concerning acids, acetic acid was identified in black garlic and was not detected in purple garlic. This absence in purple garlic may be due to the fact that, as with furfural, acetic acid can be originated through the Maillard reaction in the aging process.

Ascorbic acid content

The results of the study regarding the ascorbic acid content by HPLC show that the average ascorbic acid content was decreased 4.65 times in BG compared with purple fresh garlic (n=6). The values ranged from 225.9±50.19 mg/100 g DM in purple garlic to 48.57±8.910 mg/100g DM in BG. The amounts of ascorbic acid in purple garlic are similar to those obtained previously in fresh garlic, who also analyzed the differences in the content of ascorbic acid between fresh purple garlic and black garlic under different thermal processing steps[23]. Their results were consistent with ours, the higher levels of ascorbic acid content were found in the samples of fresh garlic. In addition, they found that the minimum ascorbic acid content corresponded to BG samples that had been under high temperature and high humidity conditions for longer. These results are due to instability of ascorbic acid that is easily decomposed under certain conditions, being one of the most susceptible vitamins to be lost during the processing of vegetables[24]. The main factors that influence ascorbic acid degradation are heat and storage time which are precisely two of the conditions that occur during the production of BG from fresh garlic.

Sugars content

The comparison of the contents of sugar between purple fresh garlic and black garlic is shown in Table 2**.**

The contents of monosaccharides were remarkably increased in black garlic. The values of fructose and glucose ranged from 0.38 ± 0.06 g/100 g DM and 0.21 ± 0.02 g/100 g DM respectively in purple garlic to 44.73 ± 4.41 g/100 g DM and 2.51 ± 0.24 g/ 100 g DM in BG. Recent studies reported that during the heating process, polysaccharides are degraded to monosaccharides or oligosaccharides[25,26]. The significant increase of this compounds leads to the characteristic sweet taste of BG. The ratio fructose: glucose is 14:1 in garlic fructan and this ratio explains why there was almost a 10-fold increase in fructose contents compared with glucose contents in black garlic samples[26]. With regard to sucrose, the content of this disaccharide decreased extremely in black garlic compared with fresh purple garlic (4.41 ± 0.49 g/ 100 g DM).

Determination of total polyphenols and antioxidant activity

Folin-Ciocalteau assay trough approximation of total phenolic content in most cases, although some compounds that are present in vegetable foods can interfere with the Folin-Ciocalteu reagent in an inhibitory or additive manner[27]. The additive effects occur from compounds such as fructose, glucose or ascorbic acid, present in the plant extracts and which can alter the results of total phenolic content. Therefore, the Folin-Ciocalteu assay was also performed with the amounts of ascorbic acid and sugars (fructose, glucose and sucrose) previously determined in both garlic varieties and the values expressed as gallic acid equivalents (GAE) have been subtracted from the value of total phenolic content initially estimated in each sample.

When comparing the differences between both garlics (Figure 2) a considerable increase can be observed in the total polyphenols content and the antioxidant capacity. Our results show that the total polyphenols content of black garlic (820.4 ± 215.90 mg GAE/100 g DM) is higher than those of purple garlic (77.86 ± 37.33 mg GAE/100 g DM), with a 10-fold increase in BG. These results are in agreement with those obtained by other authors[4]. This increase can occur in the later phase of the browning reaction [28]. In addition, the antioxidant power of polyphenols has been demonstrated so it seems logical to assert that this property of polyphenols is one of the reasons why the antioxidant activity increased by 78% in BG (2089.6 ± 236.48 μMTrolox/100 g DM) compared to purple garlic (449.77 ± 115.39 μMTrolox/100 g DM). Furthermore, in the thermal process physicochemical changes occur, originating stable compounds with high antioxidant power from unstable compounds[29]. Therefore,

the increment in BG antioxidant capacity may not only be due to the increase in polyphenols, but also as a result of an increase of compounds such as S-allyl cysteine (SAC), an alliin derivative[30].

Determination of polyphenolic compounds

The transformation process of fresh purple garlic into black garlic caused significant changes in the phenolic acid and flavonoid levels. Four phenolic acids, caffeic, clorogenic, coumaric, ferulic and two flavonoids, epicatechin and apigenin and were determined (Table 3). The confirmation of the presence of the phenolic acids and flavonoids in the garlic samples was performed by an HPLC-PAD-MS / MS system (Table 4).

In BG samples there was a complete loss of two phenolic acids (caffeic, chlorogenic) compared to purple garlic. However, the levels of coumaric acid and the flavonoid epicatechin were significantly increased in black garlic compared to purple garlic. Finally, no differences were found in the amount of ferulic acid and the flavonoid apigenin between black garlic and purple garlic. The increased concentration of coumaric acid in BG can be due to that some phenolic acids are more stable than others when they undergo a thermal process. Another fact that could explain this increase is that some phenolic acids in vegetables are obtained from the breakdown of supramolecular structures that store phenolic groups[31].

The heat treatment also produces changes in the extractability of flavonoids because high temperatures cause the disruption of the cell wall. The consequence is a release and therefore an increase of flavonoid compounds in the product that underwent the heat process[32].

In our samples, although we did not find differences in the amount of apigenin between both types of garlic, what we did find was a greater amount of epicatechin in the product that experienced a thermal process, BG, compared with fresh garlic. Our results are also consistent with those obtained by other authors who reported that the heat treatment in a mushroom species caused a raise on the overall content of flavonoid compounds[33]. Regarding the decrease in phenolic acids, there are some studies whose results are in accordance with ours. Studies of citrus peel extract showed the effect of temperature and heating time on the decrease of the total phenolic acids content[34].

Sometimes, the hydrolysis of chlorogenic acid during the thermal processing causes a decrease of its levels and an increase of caffeic acid[35]. However, we suspect that in this work the decrease of the caffeic acid content in BG compared to purple garlic can be due to the fact that during the thermal process, the loss of caffeic acid surpassed the release from the chlorogenic acid. That evolution of phenolic compound levels during the thermal processing is ambiguous, with great differences depending on the product and cannot be subject of a unique explanation.

In conclusion, different analytical procedures were performed in order to study the antioxidant activity and other compounds in purple garlic and black garlic, both part of the ecotype "Purple from Las Pedroñeras". 20 volatile compounds were identified in the samples and several differences were found between the two varieties; while purple garlic provided a higher concentration of S-alk(en)-yl-L-cysteine derivatives, in black garlic the allyl alcohol and other volatiles related with sweet taste were the dominant compounds. Our results also show that during the aging process of purple garlic to produce black garlic, there is a significant loss of ascorbic acid, probably because it is an unstable compound that can be easily decomposed under conditions such as heat treatment and storage time. Moreover, the monosaccharide content is increased in black garlic, which is probably related with the characteristic sweet taste of this garlic product. Black garlic has also shown higher polyphenol content and greater antioxidant properties than purple garlic. Finally, the composition of phenolic acids and flavonoids suffer a significant variation during the aging process; while some of them where incremented, others were completely lost or highly reduced.

AUTHOR INFORMATION

Corresponding autor:

***** María Lage Yusty Email: maria.lage.yusty@usc.es

Phone: +34 881 814962 Fax: + 34 981 594912.

Conflict of interest

The authors declare no competing financial interest.

Funding

This work was financed under grant GRC2014/012 for consolidating and structuring of competitive research units do Galician University System, Spain.

Acknowlegments

The authors are grateful to D. Dolores Suárez Monedero (Commercial Director of J.R. Suárez Monedero, S.L., Cuenca, Spain) for supplying the garlic samples. The authors also grateful to G. Hermelo and P. Blanco for the excellent technical assistance.

REFERENCES

1. Banerjee, S.K.; Maulik, S.K. Effect of garlic on cardiovascular disorders: a review. *Nutr. J.* **2002,** *1,* 4 DOI: 10.1186/1475-2891-1-4

2. Kimura, S.; Tung, Y.; Pan, M.; Su, N.; Lai, Y.; Cheng, K. Black garlic: A critical review of its production, bioactivity, and application. *J. Food Drug Anal.* **2017,** *25,* 62-70 DOI: 10.1016/j.jfda.2016.11.003.

3. Kang, O.J. Physicochemical Characteristics of Black Garlic after Different Thermal Processing Steps. *Prev. Nutr. Food Sci.* **2016,** *21,* 348-354 DOI: 10.3746/pnf.2016.21.4.348

4. Choi, I.S.; Cha, H.S.; Lee, Y.S. Physicochemical and antioxidant properties of black garlic. *Molecules* **2014,** *19,* 16811-16823 DOI: 10.3390/molecules191016811

5. Ioannou, I.; Hafsa, I.; Hamdi, S.; Charbonnel, C.; Ghoul, M. Review of the effects of food processing and formulation on flavonol and anthocyanin behaviour. *J. Food Eng.* **2012,** *111,* 208-217 DOI: 10.1016/j.jfoodeng.2012.02.006

6. Colín-González,A.L.; Santana, R.A.; Silva-Islas,C.A.; Chánez-Cárdenas,M.E.; Abel Santamaría,A.; Maldonado,P.D. The antioxidant mechanisms underlying the aged garlic extract- and s-allylcysteine-induced protection. *Oxid. Med. Cell. Longev.* **2012,** *2012,* 907162 DOI: 10.1155/2012/907162

445 7. Sato, E.; Kohno, M.; Hamano, H.; Niwano, Y. Increased Anti-oxidative Potency of
446 Garlic by Spontaneous Short-term Fermentation. *Plant Foods Hum. Nutr.* **2006,** *61,*
447 157-160. http://www.ncbi.nlm.nih.gov/pubmed/17075725. DOI: 10.1007/s11130-
448 006-0017-5

449 8. Machlin, L.J. *Handbook of vitamins.* Dekker: New York u.a, 1991; Vol. 40 (Chapter
450 4)

451 9. Lanzotti, V. The analysis of onion and garlic. *J. Chromatogr. A* **2006,** *1112,* 3-22
452 DOI: 10.1016/j.chroma.2005.12.016

453 10. Seki, T.; Hosono, T.; Hosono-Fukao, T.; Inada, K.; Tanaka, R.; Ogihara, J.; Ariga,
454 T. Anticancer effects of diallyl trisulfide derived from garlic. *Asia Pac. J. Clin. Nutr.*
455 **2008,** *17 Suppl 1,* 249 DOI: 10.1007/s12161-011-9321-2

456 11. Yang J. S.; Kok, L. F.; Lin, Y. H.; Kuo, T. C.; Yang, J.L.; Lin, C.C.; Chen, G.W.;
457 Huang, W.W.; Ho, H.C.; Chung, J.G. Diallyl Disulfide Inhibits WEHI-3 Leukemia
458 Cells In Vivo. *Anticancer Res.* **2006,** *26,* 219-225

459 12. IGP Ajo Morado de Las Pedroñeras. Indicación Geográfica Protegida Ajo Morado
460 de Las Pedroñeras. http://www.igpajomorado.es/. Accesed 02.06.17

461 13. Oruña-Concha, M.J.; Gonzalez-Castro, M.J.; Lopez-Hernandez, J.; Simal-Lozano, J.
462 Monitoring of the vitamin C content of frozen green beans and Padrón peppers by
463 HPLC. *J. Sci. Food Agric.* **1998,** *76,* 477-480.. DOI: AID-JSFA975>3.0.CO;2-U.

464 14. Ferraces-Casais, P.; Lage-Yusty, M.; Rodríguez-Bernaldo de Quirós, A.; López-
465 Hernández, J. Evaluation of Bioactive Compounds in Fresh Edible Seaweeds. *Food*
466 *Anal. Methods* **2012,** *5,* 828-834 DOI: 10.1007/s12161-011-9321-2

467 15. de Quirós, A.R.; Lage-Yusty, M.A.; López-Hernández, J. HPLC-analysis of
468 polyphenolic compounds in Spanish white wines and determination of their
469 antioxidant activity by radical scavenging assay. *Food Res. Int.* **2009,** *42,* 1018-1022
470 DOI: 10.1016/j.foodres.2009.04.009

16. Rose, P.; Whiteman, M.; Moore, P.K.; Zhu, Y.Z. Bioactive S-alk(en)yl cysteine sulfoxide metabolites in the genus Allium: the chemistry of potential therapeutic agents. *Nat. Prod. Rep.* **2005,** *22,* 351-368 DOI: 10.1039/b417639c

17. Lee,S.H.; Woo,Y.H.; Kyu Hang Kyung,K.H. Allyl Alcohol Found in Heated Garlic is a Potent Selective Inhibitor of Yeasts. *J. Microbiol. Biotechnol.* **2006,** *16,* 1236-1239

18. Hu, T. H.; Wu, C. M.; Ho, C. T. Volatile compounds of deep-oil fried, microwave-heated, and oven-baked garlic slices. *J. Agric. Food Chem.* **1993,** *41*, 800–805. DOI: 10.1021/jf00029a023

19. Yu, T.H.; Wu, C.M.; Chen, S.Y. Effects of pH adjustment and heat treatment on the stability and the formation of volatile compounds of garlic. *J. Agric. Food Chem.* **1989,** *37,* 730-734 DOI: 10.1021/jf00087a033

20. Yu, T., Shu, C., Ho, C. (1994). Thermal decomposition of alliin , the major flavor component of garlic, in an aqueous solution. *Food phytochemicals for cancer prevention*. **1994** Chapter 10, 144–152 DOI: 10.1021/bk-1994-0546.ch010

21. Liang, T.; Wei, F.; Lu, Y.; Kodani, Y.; Nakada, M.; Miyakawa, T.; Tanokura, M. Comprehensive NMR analysis of compositional changes of black garlic during thermal processing. *J. Agric. Food Chem.* **2015,** *63,* 683 DOI:10.1021/jf504836d

22. MacDougall, D.; Crummett, W.B. Guidelines for data acquisition and data quality evaluation in environmental chemistry. *Anal. Chem.* **1980,** *52,* 2242-2249. DOI: 10.1021/ac50064a004.

23. Kim, J.; Kang, O.; Gweon, O. Changes in the content of fat- and water-soluble vitamins in black garlic at the different thermal processing steps. *Food Sci. Biotechnol.* **2013,** *22,* 283-287 DOI: 10.1007/s10068-013-0039-3.

24. Montaño, A.; Casado, F.J.; de Castro, A.; Sánchez, A.H.; Rejano, L. Vitamin content and amino acid composition of pickled garlic processed with and without fermentation. *J. Agric. Food Chem.* **2004,** *52,* 7324-7330 DOI: 10.1021/jf040210l

498 25. Zhang, Z.; Lei, M.; Liu, R.; Gao, Y.; Xu, M.; Zhang, M. Evaluation of alliin,
499 saccharide contents and antioxidant activities of black garlic during thermal
500 processing. *J. Food Biochem.* **2015,** *39,* 39-47 DOI: 10.1111/jfbc.12102

501 26. Yuan, H.; Sun, L.; Chen, M.; Wang, J. The Comparison of the Contents of Sugar,
502 Amadori, and Heyns Compounds in Fresh and Black Garlic. *J. Food Sci.***2016,** *81,*
503 C1668 DOI: 10.1111/1750-3841.13365

504 27. Padda, M.S.; Picha, D.H. Methodology Optimization for Quantification of Total
505 Phenolics and Individual Phenolic Acids in Sweetpotato (Ipomoea batatas L.) Roots.
506 *J. Food Sci.* **2007,** *72,* C416 DOI:10.1111/j.1750-3841.2007.00448.x

507 28. Robards, K.; Prenzler, P.D.; Tucker, G.; Swatsitang, P.; Glover, W. Phenolic
508 compounds and their role in oxidative processes in fruits. *Food Chemistry* **1999,** *66,*
509 401-436 DOI:10.1016/S0308-8146(99)00093-X.

510 29. Corzo-Martínez, M.; Corzo, N.; Villamiel, M. Biological properties of onions and
511 garlic. *Trends Food Sci. Tech.* **2007,** *18,* 609-625 DOI: 10.1016/j.tifs.2007.07.011

512 30. Bae, S.E.; Cho, S.Y.; Won, Y.D.; Lee, S.H.; Park, H.J. A comparative study of the
513 different analytical methods for analysis of S-allyl cysteine in black garlic by HPLC.
514 *LWT – Food Sci. Technol.* **2012,** *46,* 532. DOI: 10.1016/j.lwt.2011.11.013

515 31. Bunea, A.; Andjelkovic, M.; Socaciu, C.; Bobis, O.; Neacsu, M.; Verhé, R.; Camp,
516 J.V. Total and individual carotenoids and phenolic acids content in fresh, refrigerated
517 and processed spinach (Spinacia oleracea L.). *Food Chem.* **2008,** *108,* 649-656
518 DOI:10.1016/j.foodchem.2007.11.056

519 32. Peleg, H.; Naim, M.; Rouseff, R.L.; Zehavi, U. Distribution of bound and free
520 phenolic acids in oranges (Citrus sinensis) and Grapefruits (Citrus paradisi). *J Sci.*
521 *Food Agric.* **1991,** *57,* 417-426 DOI: 10.1002/jsfa.2740570312

522 33. Choi, Y.; Lee, H.B.; Lee, J.; Lee, S.M.; Chun, J. Influence of heat treatment on the
523 antioxidant activities and polyphenolic compounds of Shiitake (Lentinus edodes)
524 mushroom. *Food Chem.* **2006,** *99,* 381-387 DOI: 10.1016/j.foodchem.2005.08.004

525 34. Xu, G.; Ye, X.; Chen, J.; Liu, D. Effect of heat treatment on the phenolic
526 compounds and antioxidant capacity of citrus peel extract. *J. Agric. Food Chem.*
527 **2007,** *55,* 330-335 DOI:10.1021/jf0625171

528 35. Spanos, G.A.; Wrolstad, R.E. Influence of variety, maturity, processing and storage
529 on the phenolic composition of pear juice. *J. Agric. Food Chem.* **1990,** *38,* 817-824
530 DOI: 10.1021/jf00093a049

531

532

533

534

535

536

537

538

539

540

541

542

543

544

545

546

547 **Table 1** Identified volatile compounds, areas and average areas in both purple and black garlic.

Compound	nº CAS	RT (min)	Purple garlic		Black garlic	
			Areas	% Areas	Areas	% Areas
S-alk(en)yl-L-cysteine derivatives						
Allyl mercaptan	870-23-5	5.87	1.52E+08 ± 7.20E+07	10.05 ± 5.25	-	-
Dimethyl disulfide	624-92-0	9.76	4.04E+07 ± 1.23E+07	3.94 ± 2.32	3.30E+06 ± 1.54E+06	0.68 ± 0.10
Diallyl sulfide	592-88-1	12.11	5.06E+07 ± 2.52E+07	2.9 ± 1.61	5.86E+07 ± 2.71E+07	9.55 ± 2.85
Allyl methyl disulfide	2179-58-0	13.52	3.50E+08 ± 1.96E+08	23.15 ± 14.19	8.31E+06 ± 4.64E+06	1.74 ± 0.73
1,3-Dithiane	505-23-7	13.93	1.70E+07 ± 5.45E+06	1.39 ± 0.24	-	-
Dimethyl trisulfide	3658-80-8	14.75	-	-	5.85E+06 ± 3.25E+06	1.13 ± 0.28
Diallyl disulfide*	2179-57-9	16.58	1.36E+09 ± 6.97E+08	45.08 ± 17.80	2.06E+06 ± 1.03E+06	7.06 ± 1.09
Allyl methyl trisulfide	34135-85-8	17.70	6.20E+06 ± 3.06E+06	0.65 ± 0.53	4.87E+07 ± 2.69E+07	9.92 ± 2.38
Diallyl trisulfide	2050-87-5	20.21	7.29E+06 ± 2.06E+06	0.32 ± 0.15	4.51E+07 ± 2.56E+07	0.89 ± 0.29
Other sulfur-containing compounds						
Thiophene, 3, 4-dimethyl-	632-15-5	13.30	1.97E+06 ± 5.66E+05	0.06 ± 0.01	-	-
Alcohols						
Ethanol	64-17-5	3.85	2.87E+07 ± 1.03E+07	1.18 ± 0.67	3.03E+06 ± 7.95E+05	0.42 ± 0.05
Allyl alcohol (2-propen-1-ol)*	107-18-6	5.92	-	-	2.59E+08 ± 1.36E+08	48.33 ± 4.56
Aldehydes						
Acetaldehyde	75-07-0	2.91	3.01E+07 ± 1.87E+07	1.42 ± 0.27	1.39E+07 ± 5.67E+06	2.01 ± 0.38
Propanal	123-38-6	4.15	3.83E+06 ± 2.41E+06	0.23 ± 0.06	-	-
Furans						
Furfural	98-01-1	12.51	-	-	2.24E+07 ± 9.64E+06	4.81 ± 1.46
2-Acetylfuran	1192-62-7	13.98	-	-	2.86E+06 ± 1.23E+06	0.56 ± 0.11
Acids						
Acetic acid	64-19-7	8.49	-	-	2.69E+06 ± 4.90E+04	0.41 ± 0.27
Thiphene-2,3-dicarboxylic acid	1451-95-2	22.39	-	-	1.31E+07 ± 8.58E+06	2.55 ± 0.95
Ketones						
2-Propanone	67-64-1	4.31	-	-	3.98E+07 ± 1.86E+07	8.10 ± 2.80
Other compounds						

548
549 Data are expressed as the mean ± SD, n=4.

550 *Identification of compounds confirmed by analysis of standards.

551

552

553

554

555

556

557

Table 2 Contents of sugars in purple and black garlic.

	Purple garlic	Black garlic
Sugar		
Fructose	0.38 ± 0.06[a]	44.73 ± 4.41[b]
Glucose	0.21 ± 0.02[a]	2.51 ± 0.24[b]
Sucrose	4.41 ± 0.49	-

[1] Data are expressed as the mean ± SD, n=6, in g/100 g of DM. Different superscripts within a row indicate significant difference at $p<0.05$ level.

576

577 **Table 3** Contents of phenolic acids and flavonoids in purple and black garlic samples.

578
579

	Purple garlic	Black garlic
Chlorogenic acid	11.71 ± 9.31	-
Caffeic acid	3.45 ± 1.76	-
Epicatechin	3.12 ± 1.37[a]	11.31 ± 5.34[b]
Coumaric acid	0.30 ± 0.14[a]	0.75 ± 0.27[b]
Ferulic acid	0.91 ± 0.72[a]	0.71 ± 0.18[a]
Apigenin	0.89 ± 0.56[a]	1.35 ± 0.83[a]

580 [1] Data are expressed as the mean ± SD, n=6, in mg/ 100 g of DM. Values with the same superscript
581 within a row are not significantly ($p<0.05$) different
582

583

584

585

586

587

588

589

590

591

592

593

594

595

Molecule	Retention time (min)	PDA λ max	Precursor ion	Product ions	Collision energy	Tube lens
Chlorogenic acid	10.3	324	353	191 85 127	22 44 35	-45.81
Caffeic acid	11.0	326	179	135 134 89	19 27 35	-45.31
Epicatechin	12.6	280	289	245 203 205	17 21 20	-63.32
Coumaric acid	13.8	310	163	119 93 117	18 38 38	-43.3
Ferulic acid	15.5	322	193	134 178 149	19 15 14	-46.06
Apigenin	23.3	338	269	117 151 149	37 24 27	-89.34

596 **Table 4** Retention time, λ max and MS-MS conditions of phenolic acids and flavonoids.

597

598

599

600

601

602

603

604

605

606 **Caption Figures**

607 **Figure 1.** TIC chromatogram of a black garlic sample (A) and a purple garlic sample (B).

608 **Figure 2.** Total polyphenols content and antioxidant capacity in purple and black samples. Concentrations
609 are expressed as the mean ± SD, n=6, in mg GAE/100 g of dry matter for polyphenols and µMTrolox/100
610 g of dry matter for antioxidant activity.

611

612

613

614

615

616

617

618

619

620

621

622

623

624

625

626

627

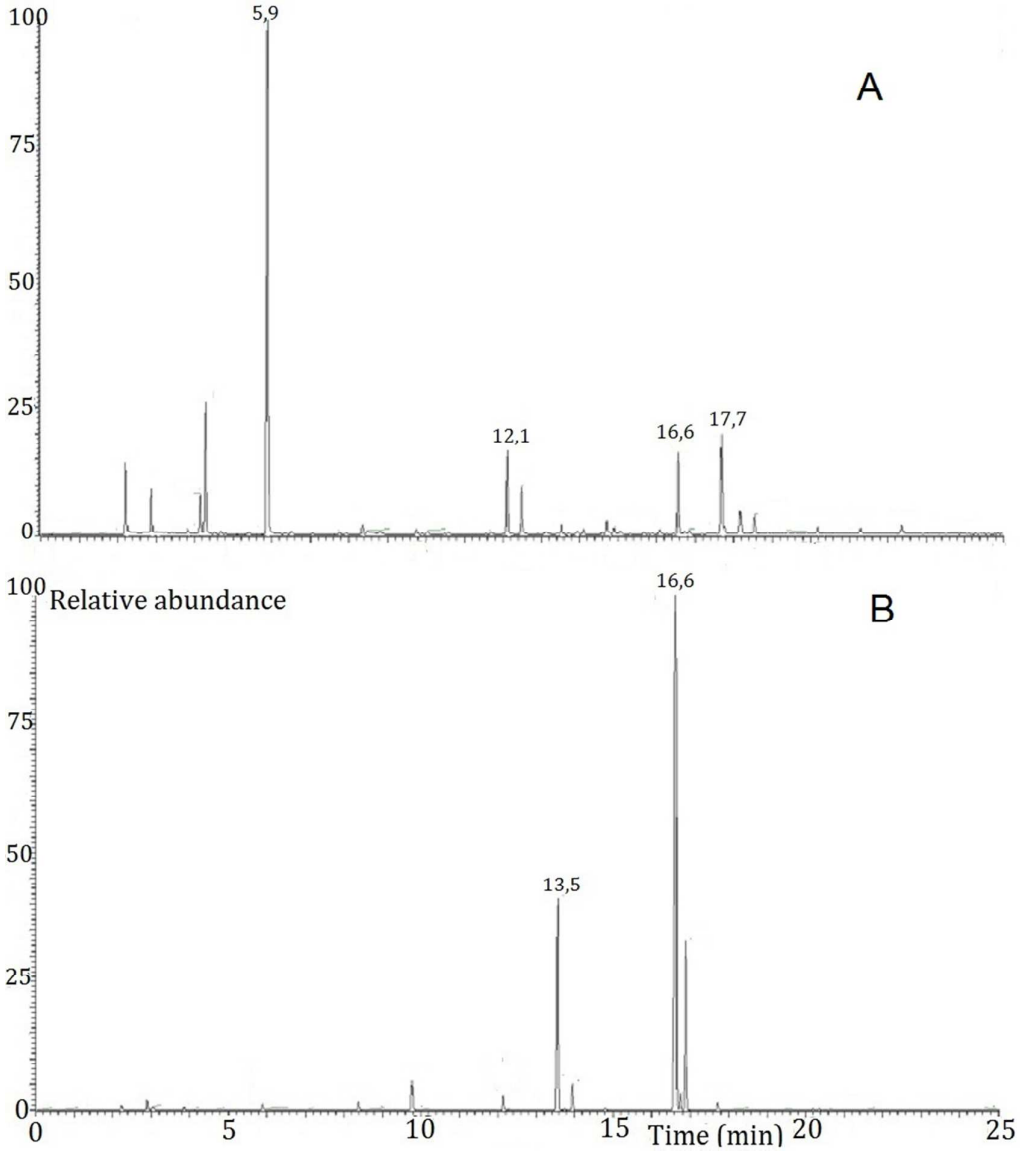
628

629

630

631

632



633

634

635 Figure1

636

637

638

639

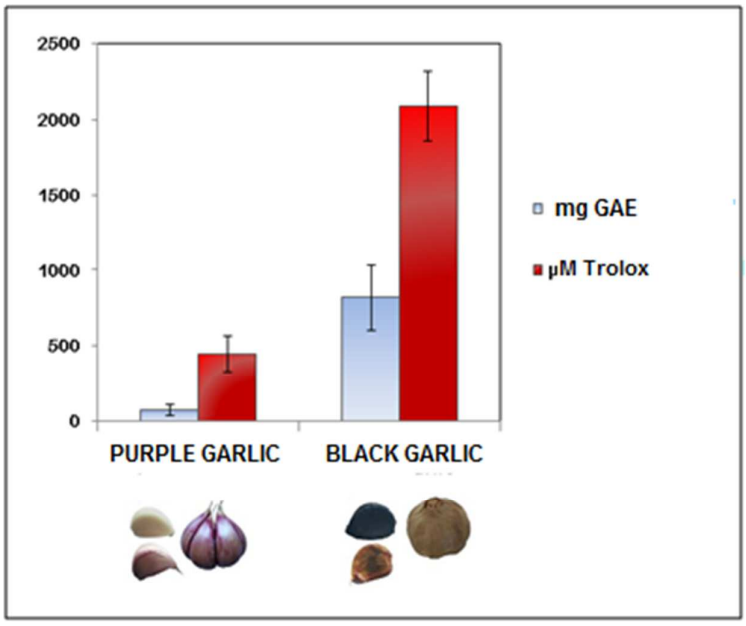
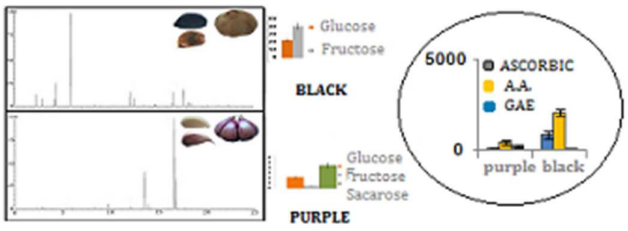

2500
2000
1500
1000
500
0
mg GAE
µM Trolox
PURPLE GARLIC
BLACK GARLIC

Figure2

654

655



656

657 TOC graphic