**2-Furoylmethyl amino acids as indicators of Maillard reaction during
the elaboration of black garlic**

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Abstract

This study reports the formation of 2-furomethyl-amino acids (2-FM-AA) as indicators of Maillard reaction (MR) in black garlic elaboration, followed by the determination of furosine by ion-pair RP-HPLC-UV. The method was assessed for accuracy, repeatability and detection and quantitation limits indicating its adequacy. Traditional procedure of black garlic obtainment and the inclusion of convective drying (CDP) and ohmic heating (OHP) were assayed. For comparison purposes, three commercial black garlic samples were used. Together with furosine (2-FM-lysine), 2-furoylmethyl- γ -aminobutyric acid and 2-FM-arginine were detected. Levels of furosine were higher in CDP (46.6-110.1 mg/100 g protein) than in OHP (13.7-42.0 mg/100 g protein) samples, probably due to the most severe processing conditions used in the former. These results highlight the suitability of 2-FM-AA as chemical indicators to monitor the process of black garlic elaboration in order to obtain high quality products.

Keywords: 2-Furoylmethyl amino acids, furosine, Maillard reaction, convective drying, ohmic heating, black garlic.

1. Introduction

Black garlic is an Asian product whose popularity has spread around the world due to its sweet-sour flavour, soft texture, nutrient content, as well as its health properties (Kim, Jung, Kang, Chang, Hong & Suh, 2012). In recent years, different studies on the bioactive compounds of this product have been reported. According to Choi, Cha and Lee (2014) and Toledano-Medina, Pérez-Aparicio, Moreno-Rojas and Merinas-Amo (2016) black garlic extracts have more total polyphenol content and antioxidant activity than raw garlic extracts. Furthermore, *in vivo* assays carried out in models of rats with different pathologies have shown enough evidences that the intake of black garlic exerts hepatoprotective action (Kim, Kim, Hwang, Om, Kim & Cho, 2011), antioxidant effect (Lee, Gweon, Seo, Im, Kang, Kim & Kim, 2009), improvement of the general status via regulation of lipid metabolism (Chen, Kao, Tseng, Chang & Hsu, 2014) and potential antihypertensive effect (Miao, Chen, Zhou, Xu, Zhang & Wang, 2014), being these properties related to the formation of bioactive compounds during the manufacturing process of black garlic (Li, Lu, Pei & Qiao, 2014).

Traditionally, black garlic is produced by heat treatment of the raw garlic bulbs under high temperature and high humidity conditions for 30-60 days without any additional treatment or additives (Zhang, Li, Lu & Liu, 2015), taking the name from the colour of the obtained cloves. The main characteristics of raw garlic, pungent taste and odour, are diminished during its change into a syrupy, sweet and aromatic product probably ascribed to the formation of new compounds taking place during Maillard reaction (MR). This reaction can occur between free amino groups of amino acids, peptides or proteins and carbonyl groups of reducing sugars during elaboration and

storage of foods (Gamboa-Santos, Megías-Pérez, Soria, Olano, Montilla & Villamiel, 2014; Yu, Zhang & Zhang, 2017).

Since the traditional process for black garlic elaboration takes a long time, a pre-treatment such as convective drying or ohmic heating could reduce this time. However, an exhaustive processing control is needed in order to avoid an excessive advance of MR and, consequently, a great participation of essential amino acids, such as lysine, and formation of undesirable compounds (Erbersdobler & Somoza, 2007). To date, ones of the indicators of initial steps of MR are the Amadori and Heyns compounds, which are formed before important changes in composition and functionality. In fact, global contents of these compounds formed by glucose or fructose with proline, valine or leucine have been recently, determined in commercial black garlic and the obtained values were 40-100 folds higher than those found in raw garlic (Yuan, Sun, Chen & Wang, 2016). In this sense, furosine (2-furoylmethyl-lysine, 2-FM-Lys) and others 2-furoylmethyl amino acids (2-FM-AA), which are formed after the acid hydrolysis of Amadori and Heyns compounds, have been recognised for decades as the best and more sensitive indicators of initial steps of MR in products from several origin, including processed garlic (Cardelle-Cobas, Moreno, Corzo-Martínez, del Castillo & Villamiel, 2005; Corzo-Martínez, Corzo, Villamiel & del Castillo, 2012; Gamboa-Santos, Soria, Fornari, Villamiel & Montilla, 2013). As 2-FM-AAs are formed by strong acid conditions it is difficult to distinguish if free amino acids, peptides or proteins are involved since the link between amino acids is broken and only the covalent one between the amino and carbonyl group is kept. Moreover, free amino acids and proteins (with lysine, among others) are both present in garlic (Kimura et al. 2017). To the best of our knowledge, no data are available on the determination of 2-FM-AA in black garlic during its manufacturing process.

Therefore, the aim of this study was to assess the evolution of MR in black garlic elaborated by the traditional process with the inclusion of convective drying and ohmic heating pre-treatments, paying special attention to the formation of 2-FM-AA, in order to dispose of reliable treatment indicators that allow the control of processing to obtain high quality products.

2. Materials and methods

2.1. Samples

Mexican purple garlic bulbs (*Allium sativum* L.) sets were subjected to two different pre-treatments, convective drying (CDP) and ohmic heating (OHP) (Figure 1a). CDP was carried out for 4 days at 70 °C and 9% relative humidity (RH) in a commercial oven (220 V, 2200 W, model H-82, Riossa, Mexico). For OHP, bulbs were placed inside of an acrylic cell with two stainless steel electrodes connected to a source of alternating current, an ammeter (HP multimeter), and a voltmeter (Goldstar multimeter), the samples were treated for 10 min at 70 °C and 130 V (Gaytán-Martínez, Figueroa, Vázquez-Landaverde, Morales-Sánchez, Martínez-Flores & Reyes-Vega, 2012). After both pre-treatments, CDP and OHP, samples were aged for 12 days at 70 °C and 94% RH to obtain the final product. Black garlic samples from CDP and OHP were withdrawn in duplicate at 0 (after pre-treatment), 4, 6, 8, 10 and 12 days of processing. As control group, garlic bulbs were aged in duplicate without pre-treatments for 30 days at 70 °C, 94% RH.

Additionally, pilot plant scale trials for black garlic production were carried out in a computer controlled air tray dryer (SBANC, Edibon Technical Teaching Units, Spain) (Gamboa-Santos et al., 2014) according to the diagram of Figure 1b. The SBANC equipment consists of a fan unit with air flow control, sensors for temperature,

one sensor for humidity and a weighing system for following the changes in the weight of the sample through the time. Bulbs of garlic (86 ± 2 g) were placed on the trays and a stream of hot air was passing over the sample, corresponding to air flow of 2 m s^{-1} (SP6) and 4 m s^{-1} (SP12) and a temperature inside of the drying chamber of 68°C and 56°C , respectively. The final of pre-treatments were considered when the mass loss was $\sim 40\%$, similar to the loss after the CDP pre-treatment. These times were 6 days for 2 m s^{-1} and 12 days for 4 m s^{-1} .

In addition, three black garlic commercial samples were purchased at different local markets, two in Spain and one in Mexico.

Prior to analytical determinations, all samples were lyophilized.

The pH of samples was determined using a HANNA pHmeter (H12213) with glass electrode and a sample dilution 1:10 with Milli-Q water. Water activity (a_w) was measured by an Aqualab (Water activity Meter model series 3), based on the vapour pressure principle and the dew point of the sample. Total nitrogen (TN) content was determined by the Kjeldahl method following the AOAC method (920.152) and protein level was calculated using 6.25 as conversion factor ($\text{TN} \times 6.25$).

2.2. Analytical determinations for Maillard reaction study

The determination of furosine (2-FM-Lys) and other 2-FM-AA in black garlic samples was carried out by the ion-pair RP-HPLC-UV analysis in a chromatograph Agilent Technologies 1260 Infinity LC System (Böblingen, Germany) (Megías-Pérez, Gamboa-Santos, Soria, Villamiel & Montilla, 2014) using a C_8 column (250 cm x 4.6 mm inside diameter) (Alltech furosine-dedicated, Nicholasville, KY) thermostated at 35°C ; a linear binary gradient at a flow rate of 1.2 mL min^{-1} were used, with two mobile phases: A, 0.4% acetic acid and B, 0.34% KCl in phase A. The elution program was:

100% A from 0 to 12 min, 50% A from 20 to 22.5 min and 100% A from 25 to 30 min. The UV detector was set at 280 nm. To maximize the yield of furosine formation during the acid hydrolysis of black garlic, different ratios sample amount/acid volume (62.5, 125 and 250 mg mL⁻¹) and HCl concentrations (6, 8 and 10 N) were tested at 110 °C for 24 h under inert conditions (nitrogen) in a screw-capped Pyrex vial. Following the methodology of Rada-Mendoza, Olano and Villamiel (2002) each hydrolysate was filtered through paper filter (Whatman No. 40) and 0.5 mL of filtrate was applied to a Sep-Pack C₁₈ cartridge (Millipore, Milford, MA, USA) activated with 5 mL of methanol and 10 mL of Milli-Q water. Furosine was eluted with 3 mL of 3 N HCl and 20 µL were injected in the chromatographic system. Acquisition and processing of data were obtained with Agilent ChemStation Rev. C.01.05 software. Identification and quantification was done using a commercial standard of furosine (PolyPeptide Laboratories, Strasbourg, France). 2-furoylmethyl-γ-aminobutyric acid (2-FM-GABA) and 2-furoylmethyl-arginine (2-FM-Arg) were identified employing 2-FM-AA standards synthesized in our laboratory (Del Castillo, Sanz, Vicente-Arana & Corzo, 2002). The contents of 2-FM-AA were expressed as mg/100 g protein.

The linearity of the method was evaluated with furosine standards in a concentration range of 0.023 to 6.990 mg/L and the obtained calibration curve was $y=2.9378x+0.0286$ with a R^2 of 0.999. To demonstrate the method accuracy for the determination of 2-FM-Lys in black garlic samples, a known amount of furosine standard was added to garlic hydrolysates and the % of recovery was calculated. The repeatability of the entire method (preparation of the sample, hydrolysis and chromatographic separation) was evaluated by analysing five aliquots of the same sample, whereas, the chromatographic repeatability was made by injecting one sample in different days (n = 5). Furthermore, limit of detection (LOD) and quantification

(LOQ) were established after the injection and analysis of the most diluted standard of furosine based on a signal/noise ratio of 3:1 and 10:1, respectively (Teixidó, Santos, Puignou & Galceran, 2006).

2.3. Statistical analysis

All samples were processed in duplicate and analysed two times (n=4), results were expressed as mean $\pm$ SD. Data were subjected to one-way analysis of variance (ANOVA) and significant differences ($p<0.05$) among means were determined by Tukey range test using the statistical software JMP[®] (SAS 10.0, USA).

3. Results and discussion

3.1. Set up of furosine determination in black garlic

As shown in the chromatogram corresponding to the acid hydrolysis of a commercial black garlic sample (Figure 2), among the peaks found, three 2-furomethyl-amino acids (2-FM-AA) were presumably detected. To confirm the identity of these compounds, their retention times were compared with those of 2-FM-AA standards, according to previous results of our laboratory (Del Castillo et al., 2002). The comparison showed the presence of 2-furoylmethyl- γ -aminobutyric acid (2-FM-GABA), 2-furoylmethyl-lysine (2-FM-Lys, furosine) and 2-furoylmethyl-arginine (2-FM-Arg). These compounds were also found by Cardelle-Cobas et al., (2005) in commercial dried garlic.

The most diluted standard of 2-FM-Lys was used to determine the limit of detection (LOD) and quantification (LOQ) for the proposed method and the obtained values were 17.6 ng mL⁻¹ and 59.7 ng mL⁻¹, respectively. These limits were higher than those reported by Rada-Mendoza, Villamiel and Olano (2004) who established the LOD of 6.6

ng mL⁻¹ and the LOQ of 20.2 ng mL⁻¹. These differences could be due to the different chromatographic systems applied since Rada-Mendoza and coworkers (2004) used similar preparation sample procedure but the chromatographic method was the corresponding to Delgado et al. (1992).

To maximize the yield of 2-FM-AA formation during acid hydrolysis of black garlic, three ratios sample amount/acid volume (62.5, 125 and 250 mg mL⁻¹) of a Spanish commercial black garlic (SP1) were tested. Figure 3a shows the quantity of 2-FM-AA formed and the lowest yield of furosine formation was obtained at the highest amount of sample (250 mg mL⁻¹); these decrease of yield with higher amount of sample, could be due to the fact that the ratio between sample (and, consequently, protein) and acid was too high to obtain a proper hydrolysis. This result was in agreement with the obtained in the optimisation of furosine determination for jams and fruit-based infant foods (Rada-Mendoza et al., 2002). The highest furosine content was reached using 62.5 and 125 mg mL⁻¹ and no significant differences ($p>0.05$) were found between them. For 2-FM-Arg the highest content was obtained with 62.5 mg mL⁻¹ of black garlic, while for 2-FM-GABA no significant differences ($p>0.05$) were observed. Regarding, the effect of HCl concentration (Figure 3b), the highest furosine value was obtained using HCl 10 N, in concordance with previous authors (Erbersdobler 1986; Finot & Maguenat 1981; Molnar-Perl, Pinter-Szakacs, Wittmann, Reutter & Eichner 1986). Guerra-Hernández et al. (1996) in a study on baby cereals, indicated that furosine formation by sample hydrolysis with hydrochloric acid increased with acid concentration to 10.6 M. Taking into account these results, all samples analysed in this work were hydrolysed using 62.5 mg mL⁻¹ of black garlic in 10 N HCl.

The method and chromatographic repeatability was also evaluated. The relative standard deviations (RSDs) were lower than 10% for quantitation of furosine in black

garlic. The higher RSD of method repeatability (7.6%) as compared to that of chromatographic one (5.8%) may be due to the fact that the evaluation of the entire method (preparation of the sample, hydrolysis and chromatographic separation) implies more variability than repeated injections of one sample in different days. These data, together with the high value of furosine recovery (99.6%, average of two different concentrations added (11 and 22 ng/mL), indicated the suitability of the method for the determination of furosine in black garlic. A similar behaviour was assumed for the other 2-FM-AA in these samples.

Once the method was adapted for furosine determination in black garlic, a study on the early steps of MR was carried out in both, commercial and laboratory scale production.

3.2. Study of Maillard reaction in traditional and commercial black garlic

2-FM-AA content was firstly determined in black garlic elaborated by the traditional process (TT) and in three commercial products, presumably also obtained by this process, one Mexican (MX) and two Spanish (SP1 and SP2) (Table 1). 2-FM-GABA, furosine (2-FM-Lys) and 2-FM-Arg were detected in all samples in variable amounts, being furosine the most abundant. For this compound, no significant differences ($p>0.05$) were observed in TT, MX and SP1 samples. The lowest value of 2-FM-AA, and consequently the lowest MR evolution was found in SP2 sample, indicating milder processing conditions used by the corresponding industry as compared to the other samples, who presented more similar values of 2-FM-AA. The values obtained in these samples only could be compared with the reported for commercial dehydrated garlicks. These products have, in general, minor amount of 2-FM-AA, with values between 4-34 mg/100 g protein and 2-27 mg/100 g protein for furosine and 2-FM-Arg, respectively

(Cardelle-Cobas et al., 2005; Gamboa-Santos, Soria, Corzo-Martínez, Villamiel & Montilla, 2012). However, Rufián-Henares, García-Villanova and Guerra-Hernández (2008) analysed three samples of dehydrated garlic with higher amount of furosine (95-244 mg/100 g protein), perhaps due to the fact that the samples were triturated previously to dehydration at 30-40 °C during 20-120 h, favouring the reaction. It should be considered that the dehydration processes are very different from each other commercial products and very different from the methodology to obtain black garlic.

The range of pH (Table 1) was 4.1-4.6 for all the samples; previous studies in black garlic have reported that pH decreased as a result of the ‘aging’ period of the bulbs reaching pH values below 3.8 after 35 days of processing (Choi et al., 2014; Zhang et al., 2015). It is known that pH decreased during the MR due to the formation of carboxylic acids (Villamiel, del Castillo, Corzo, & Olano, 2001). Regarding a_w values (Table 1), Gerrad (2002) reported that the maximum of non-enzymatic browning reactions preferably occurs at a_w values between 0.60 to 0.85; therefore, since all samples had a_w data above 0.85, this parameter scarcely affects the MR development.

3.3. Study of Maillard reaction in pre-treated black garlic obtained in the laboratory

Figure 4a shows the evolution of furosine in garlic pre-treated by convective drying (CDP) and ohmic heating (OHP). In both cases, a progressive increase in the furosine content was observed during the 10 days of aging of pre-treated samples at 70 °C and 94% RH. In the case of CDP, the evolution of furosine reached a maximum after 8 days of aging to decrease around a 22% after 10 days and a 79% of this content at the end of the assay (12 days). Moreover, highest levels of furosine were 2-3 fold greater in

CDP (110.1 mg/100 g protein) than those of OHP (42.0 mg/100 g protein) samples, indicating more severe processing conditions during convective drying as pre-treatment to produce black garlic. In addition, although the amounts were lower, 2-FM-Arg content followed a similar pattern than that of furosine in both types of samples OHP and CDP (Figure 4b). Nevertheless, the formation of 2-FM-GABA was detected in CDP only at day 8th and 10th of aging (9.2 ± 2.1 and 27.3 ± 3.9 mg/100 g protein) and for OHP this compound was detected only in traces at 12th day (2.6 mg/100 g protein). The progression of 2-FM-AA in CDP samples could indicate the evolution of early MR products to other more advanced products.

Additionally, pH of the pre-treated samples was monitored and it decreased with the time (Figure S1) probably due to the formation of acids, following a similar trend that pH values reported by Choi et al., (2014) for black garlic after 14 and 21 days of aging period. Comparing OHP and CDP samples, the decrease of pH was more pronounced in the former, in agreement with a more advance of MR, as indicated above. However, at the end of aging the difference was minor, although significant.

3.4. Study of Maillard reaction in pre-treated black garlic obtained in pilot plant

Two different temperatures of convective drying, 68°C (SP6) and 56°C (SP12) were evaluated and the corresponding loss of weight is shown in Figure S2. According to the figure, the drying was faster in SP6 than in SP12 sample, probably due to the effect of the higher temperature, in agreement with Gamboa-Santos et al. (2014). The changes in 2-FM-AA content were also studied. Furosine (2-FM-Lys) content (Figure 5a) was found in SP6 but not in SP12, confirming that the former was a more severe pre-treatment than the later. During the aging, furosine content gradually increased with time in both samples, SP6 and SP12, however SP6 samples reached higher contents (14-

81.7 mg/100g protein) than SP12 (11-64.7 mg/100g protein), in agreement with previous data found by Gamboa-Santos et al., (2014) during the drying of strawberries in the same system. The evolution of 2-FM-Arg (Figure 5b) followed the same trend than furosine since SP6 treatment led to a faster rate of formation than SP12. Results of furosine and 2-FM-Arg for final products were very close to the values of commercial black garlic samples showed in Table 1. 2-FM-GABA formation (Figure 5c) presented a less clear trend reaching values between 3-24 mg/100 g protein, probably due to the fact that it was the less sensitive compound and, consequently, the corresponding peak was very small with more chance to obtain more measurement errors. In agreement, 2-FM-GABA was not detected in any commercial dehydrated garlic sample studied by Cardelle-Cobas et al., (2005). The combination temperature/time had a noticeable effect in the 2-FM-AA evolution for SP6 and SP12 samples, indicating that high temperatures and long drying times could lead to high values of 2-FM-AA in black garlic.

4. Conclusions

At the sight of the obtained results it is possible to conclude that, the proposed HPLC method is suitable for determination of 2-FM-AA as quality indicators of initial steps of MR during the elaboration of black garlic. Furosine seemed to be the most sensitive indicator for samples obtained in the laboratory and in a pilot plant. Among the convective dried samples, those obtained in the pilot plant presented lower values of 2-FM-AA, even in the case of the most treated samples. Elaboration of black garlic, including ohmic heating, led to the lowest concentrations of 2-FM-AA, indicating its suitability as pre-treatment for preservation of black garlic quality. The results here obtained point out the usefulness of 2-FM-AA as chemical markers to retrospectively

know the process to which black garlic has been subjected in order to establish the processing conditions to obtain high-quality and repeatable product characteristics.

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HIGHLIGHTS

- HPLC method was adapted to determinate 2-furomethyl-amino acids in black garlic.
- Ohmic heating and convective drying were studied as pre-treatments.
- 2-furomethyl derivatives of lysine, arginine and γ -aminobutyric acid were detected.
- Furosine may be used as quality marker of black garlic elaboration.

Table 1. pH, water activity (a_w) and content of 2-furoylmethyl amino acids (2-FM-AA) in laboratory prepared and commercial samples of black garlic.

Sample ¹	pH	a_w	2-FM-AA (mg/100 g protein)		
			2-FM-GABA	2-FM-Lys	2-FM-Arg
TT	4.4±0.14 ^{a*}	0.96±0.01 ^a	2.6±0.44 ^b	81.2±1.9 ^a	41.3±0.4 ^b
MX	4.6±0.10 ^a	0.90±0.02 ^a	9.1±2.1 ^a	78.8±2.8 ^a	57.3±6.4 ^a
SP1	4.5±0.04 ^a	0.92±0.01 ^a	10.7±1.5 ^a	77.7±0.14 ^a	53.5±1.3 ^a
SP2	4.1±0.13 ^a	0.89±0.02 ^a	4.8±0.19 ^b	39.0±1.5 ^b	19.1±2.2 ^c

2-FM-GABA: 2-furoylmethyl γ -aminobutyric acid; 2-FM-Lys: 2-furoylmethyl lysine (furosine); 2-FM-Arg: 2-furoylmethyl arginine. ¹Black garlic from TT: Traditional treatment; MX: Mexican sample; SP1: Spanish sample 1 and SP2: Spanish sample 2. * Values followed by different letters in the same row were significantly different by Tukey test ($p<0.05$).

Figure 1. Scheme of black garlic production (a) by pre-treatments and traditional processing and (b) by dehydration using an air tray dryer and subsequent ‘aging period’ under controlled conditions.

Figure 2. HPLC chromatogram of 2-furoylmethyl-amino acids (2-FM-AA) during acid hydrolysis of black garlic (SP1) with HCl 10 N at 110 °C for 24 h. Peak 1: 2-furoylmethyl- γ -aminobutyric acid (2-FM-GABA), Peak 2: 2-furoylmethyl-lysine (2-FM-Lys) and Peak 3: 2-furoylmethyl-arginina (2-FM-Arg).

Figure 3. Effect on the formation of 2-furoylmethyl-amino acids (2-FM-AA) of (a) different amounts of sample (mg/mL) during acid hydrolysis with HCl 8N at 110 °C for 24 h in Spanish commercial black garlic (SP1); and (b) effect of different concentrations of HCl (b) during acid hydrolysis of 62.5 mg/mL of sample. *Same 2-FM-AA with identical letter (a-c) between different amount of sample showed no statistically significant differences for their mean values by Tukey test ($p < 0.05$).

Figure 4. Evolution of furosine (a) and 2-furoylmethyl-arginina (2-FM-Arg) (b) contents in black garlic pre-treated by convective drying (CDP) and ohmic heating (OHP) during ‘aging period’ at 70 °C/94% RH. 0* sample after heat pre-treatment.

Figure 5. Evolution of 2-furoylmethyl-lysine (2-FM-Lys) (a), 2-furoylmethyl-arginina (2-FM-Arg) (b) and 2-furoylmethyl- γ -aminobutyric acid (2-FM-GABA) (c) contents in garlic pre-treated using an air tray dryer at 68 °C (SP6) and 56 °C (SP12) during ‘aging period’ at 70 °C/94% RH. 0* samples after heat pre-treatment (in this point 2-FM-AA were not detected in SP12 sample).

Supplementary figure legends:

Figure S1. pH values in raw and black garlic pre-treated by convective drying (CDP) and ohmic heating (OHP) during ‘aging period’ at 70 °C/94% RH. 0* sample after heat treatment.

Figure S2. Weight loss in garlic samples processed at 68 °C (SP6) and 56 °C (SP12) using an air tray dryer.