



Influence of the heat treatment on the degradation of the minor *Fusarium* mycotoxin beauvericin

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ABSTRACT

Beauvericin (BEA) is a bioactive compound produced by the secondary metabolism of several *Fusarium* strains and known to have various biological activities.

This study investigated the degradation of the minor *Fusarium* mycotoxin BEA present in the concentration of 5 mg/kg in a model solution and in different crispy breads produced with different flours typologies (corn, hole, wheat, durum wheat, soy and rice) during the heat treatment carried out in an oven at three different temperatures of 160, 180 and 200 °C and at 3, 6, 10, 15 and 20 min incubation.

The concentration of the bioactive compound studied, analyzed with the technique of the liquid chromatography tandem mass spectrometry (LC–MS/MS), decreased in the experiment carried out in the model solution from 2.89 ± 0.13 mg/kg of the assay at 160 °C for 3 min until the complete degradation at 200 °C during 20 min incubation. In the experiments carried out using the crispy breads prepared with different kind of flours, as system to simulate a food preparation, the percentage of BEA degradation, resulted variable from 20 to 90%, with no a significative differences showed in the use of the different flour matrices.

Also a metabolite of the thermal degradation of the mycotoxins BEA was identified using the LC–MS in the full scan mode.

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1. Introduction

Beauvericin (BEA) is a cyclooligomer depsipeptide ionophore that transports monovalent cations across membranes as a free carrier uncoupling oxidative phosphorylation (Steinrauf, 1985).

BEA displays a diverse array of biological activities in vitro. It shows moderate antifungal and antibiotic activity (Hamill, Higgins, Boaz, & Gorman, 1969), and potentiates other antifungal agents in combination therapies (Zhang et al., 2007). Importantly, BEA displays a broad-spectrum antiproliferative activity against different human cancer cell lines by activating calcium sensitive cell apoptotic pathways (Jow, Chou, Chen, & Tsai, 2004; Meca, Font & Ruiz, 2012; Meca, Ruiz, et al., 2010). It also inhibits the directional cell motility (haptotaxis) of cancer cells at subcytotoxic concentrations (Zhan, Burns, Liu, Faeth, & Gunatilaka, 2007): haptotaxis is essential for the formation of new blood vessels in tumors (angiogenesis), invasion of other tissues by cancer cells, and metastasis (Carmeliet, 2003).

BEA is considered a contaminant of cereals and also of products composed by cereals (Zinedine, Meca, Mañes, & Font, 2011). In particular, the presence of BEA in food commodities has been recently reported during the last decade in some European countries (Finland, Norway, Spain, Slovakia, Croatia, Switzerland and Italy), in USA, in South Africa (Jestoi, 2008; Meca, Zinedine, Blesa, Font, & Mañes, 2010; Munkvold, Stahr, Logrieco, Moretti, & Ritieni, 1998; Ritieni et al., 1997; Shephard, Sewram, Nieuwoudt, Marasas, & Ritieni, 1999; Zinedine, Meca, Mañes, & Font, 2011) in higher concentrations (milligrams for kilograms) respect to the classical legislate *Fusarium* mycotoxins as the fumonisins or the trichotecens.

There is a lack in the scientific literature of data related to the thermal degradation of BEA, but the influence of the heat treatments on other mycotoxins presents in food was studied by many authors.

Considering that BEA is a molecule with a relevant toxicity on different biological models as bacteria and cells it is important to study its stability under food-processing condition and the production of new degradation products. Most of the studies on mycotoxin stability and structural elucidation of thermal breakdown products are focused on model systems based on treatments with different temperatures.

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In particular, Cramer, Keonigs, and Humpf (2008) evaluated the degradation of the mycotoxin ochratoxin A (OTA), during the coffee roasting, evidencing a reduction variable from 69 to 96%, and correlating the mycotoxin degradation with the roasting time employed. The degradation of the *Fusarium* toxin nivalenol (NIV) during the baking and cooking process was studied by Bretz, Knecht, Geockler, and Humpf (2005). The authors observed under all the conditions employed a degradation of NIV, accelerated with the increase of the temperatures used during the heat treatments.

Bretz, Beyer, Cramer, Knecht, and Humpf (2006) studied the degradation products of deoxynivalenol under food-processing conditions such as baking, using model heating experiments with compounds that mimicked the typical food constituents. The model solutions were heated at different temperatures (150–200 °C) at different time period.

The authors evidenced degradation of the mycotoxin DON, under all the temperature used, and proportionally at the increase of the temperature.

Some authors have reported that the fumonisin B₁ (FB₁) decreases during heat treatment and long cooking periods depending on temperature, exposure time, contamination level, and reducing sugar level (Jackson, Katta, Fingerhut, De Vries, & Bullerman, 1997). N-(Carboxymethyl)-fumonisin B₁ is one of the principal reaction products of the Maillard reaction between FB₁ and glucose (through the aliphatic primary amine of FB₁) in foods containing reducing sugars after heat treatment (Howard, Churchwell, Couch, Marques, & Doerge, 1998; Lu et al., 2002). The Schiff base initially produced by the reaction of FB₁ and D-glucose undergoes through the Amadori rearrangement to the β-ketoamine which is oxidized to N-(carboxymethyl) fumonisin B₁. Other derivatives such as N-(1-deoxy-D-fructosyl-1-yl)-fumonisin B₁ have been described as the first stable product of FB₁ with D-glucose (Poling, Plattner, & Weisleder, 2002). The aim of this study was to evaluate a) the thermal degradation of the minor *Fusarium* mycotoxin BEA present in a model solution and in a food preparation composed by crispy bread prepared with different of flours and b) the identification of BEA degradation products during the heat treatment using the technique of the LC–MS.

2. Materials and methods

2.1. Materials

Sodium chloride (NaCl), sucrose (C₁₂H₂₂O₁₁) and formic acid (HCOOH) were obtained from Sigma-Aldrich (Madrid, Spain). Acetonitrile, methanol and ethyl acetate were purchased from Fisher Scientific (Madrid, Spain). Deionized water (<18 MΩ cm resistivity) was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA). Chromatographic solvents and water were degassed for 20 min using a Branson 5200 (Branson Ultrasonic Corp., CT, USA) ultrasonic bath.

The BEA utilized in this study was produced and purified according to the method of Meca, Sospedra, et al. (2010).

2.2. Model solution preparation

The model solutions for the study of the BEA degradation *in vitro* during the heat treatment, were prepared introducing in a 14 mL centrifuge plastic tube, 1 mL of a mixture water–methanol (99/1 V/V) contaminated with 5 mg/L of BEA.

The solutions prepared were heated in an oven (Memmert, Büchenbach, Germany) at 160, 180 and 200 °C during 3, 6, 10, 15, and 20 min incubation. The solutions were filtered with a 0.22 μM filter (Análisis Vínicos, Tomelloso, Spain) and injected in the LC–MS apparatus. The experiments were carried out in triplicates.

2.3. Crispy breads production

For the production of the crispy breads used to simulate the BEA degradation in a food system, six different flours like corn, integral, wheat, durum wheat, soy and rice flour were employed. In particular 300 g of each flour were mixed with 180 mL of water, 3 g of sucrose and 6 g of NaCl. These suspensions were mixed during 5 min, and then the dough's obtained were spiked with 5 mg/kg of BEA. No fermentation was done; thus the toasts obtained were treated in an oven at 160, 180 and 200 °C during 3, 6, 10, 15, and 20 min incubation.

2.4. Mycotoxin extraction procedure

The method used for BEA extraction was described by Jestoi (2008). Briefly, 3 g of sample were extracted with 20 mL of a mixture of water/acetonitrile (85/15, v/v) using an Ultra Ika T18 basic Ultraturrax (Staufen, Germany) for 3 min. The extract was centrifuged at 4500g for 5 min and then the supernatant evaporated to dryness with a Büchi Rotavapor R-200 (Postfach, Switzerland) and then re-dissolved in 2 mL of extraction solvent. This final solution was filtered through 0.22 μM nylon filter purchased from Análisis Vínicos (Tomelloso, Spain) before the injection into the LC–MS system for analysis.

2.5. BEA analysis

The separation of BEA was achieved by LC Agilent 1100 (Agilent Technologies, Santa Clara, California) coupled to a mass spectrometer Applied Biosystems/MDS SCIEX Q TRAP TM linear ion trap mass spectrometer (Concord, Ontario, Canada). A Gemini (150 × 2.0 mm, 5 μm) Phenomenex (Torrance, California) column was used. LC conditions were set up using a constant flow at 0.2 mL/min and acetonitrile/water (70:30, v/v with 0.1% of HCOOH) as mobile phases in isocratic condition were used. The instrument was set in the positive ion electrospray mode using the following parameters: cone voltage 40 V, capillary voltage 3.80 kV, source temperature 350 °C, desolvation temperature 270 °C and collision gas energy 5 eV. Multiple reaction monitoring (MRM) technique was used for identification and quantification, in which protonated molecule [M + H]⁺ of the BEA *m/z* 784.50, was fragmented in the collision cell to the product-ion *m/z* 244.20. For the quantification, we used the product-ions *m/z* 244.20 (Jestoi, 2008).

The samples corresponding to the model solution experiments and to the crispy bread production were also injected in the modality full scan with a *m/z* of 150–1500, to verify in the extracts the presence of degradation products produced during the heat treatments employed (Meca, Sospedra, et al., 2010).

2.6. Calculation and statistical analysis

All experiments were performed three times. Statistical analysis was carried out using analysis of variance (ANOVA), followed by Dunnet's multiple comparison tests. Differences were considered significant if *p* < 0.05.

3. Results and discussion

In this study, the degradation of the minor *Fusarium* mycotoxin BEA, during the heat treatment operated in an oven at three different temperatures (160, 180 and 200 °C) and at several incubation times (3, 6, 10, 15 and 20 min) has been evaluated. In particular Table 1 describes the degradation of the bioactive compound studied during the heat treatments in a model solution. As is possible to evidence, BEA decreased during the heat treatments applied, and

Table 1
Degradation of the minor *Fusarium* mycotoxin BEA in the model solution at different treatment conditions.

Heat treatment (°C/min)	BEA mg/L
160-3	2.89 ± 0.13
160-6	2.73 ± 0.20
160-10	1.73 ± 0.12
160-15	1.67 ± 0.14
160-20	1.32 ± 0.25
180-3	2.52 ± 0.21
180-6	2.20 ± 0.35
180-10	1.70 ± 0.17
180-15	1.32 ± 0.12
180-20	1.22 ± 0.10
200-3	1.84 ± 0.15
200-6	1.57 ± 0.13
200-10	1.04 ± 0.09
200-15	0.40 ± 0.05
200-20	n/d

the degradation was proportionally to the increase of the temperatures employed. In particular at 160 °C BEA decreases from 2.8 ± 0.1 mg/L evidenced at 3 min incubation, to 1.3 ± 0.2 mg/L evidenced at 20 min, with a percentage of reduction of 74%.

At 180 °C, the percentage of degradation ranged from 50 to 76%. At the temperature treatment of 200 °C, as is possible to evidence in Table 1, were observed the highest reductions of the bioactive compound studied. Also in the experiments carried out in the food system, the degradation of the BEA was proportionally to the temperatures and the incubation times employed. In particular at the temperature of 160 °C, BEA decreases from 5 mg/kg introduced as start concentration to a mean value evidenced at 20 min incubation of 2.8 mg/kg, evidencing a percentage of degradation of 43%. The heat treatments that reduced the highest concentrations of the mycotoxin employed were the trials carried out with the integral flour where the BEA concentration detected after 20 min incubation was of 1.7 ± 0.1 mg/kg.

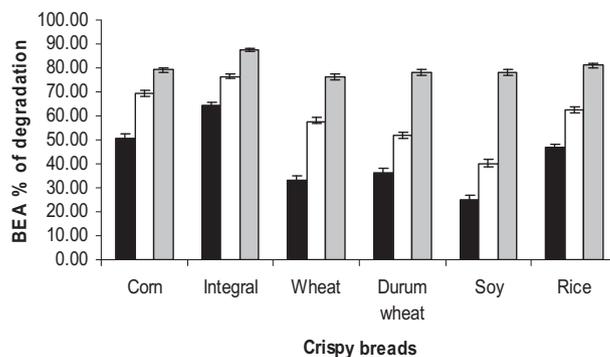


Fig. 2. Percentage of degradation of the minor *Fusarium* mycotoxin BEA present in the different crispy breads heated at 160, 180 and 200 °C during 20 min (black = 160 °C, white = 180 °C, and grey = 200 °C).

At 180 °C the percentage of reduction of the mycotoxin studied ranged from 69.3 ± 1.5 to 62.2 ± 1.8 %. Also at this temperature the highest reduction was evidenced in the trial carried out with the integral flour, whereas the lowest degradation was evidenced in the experiment carried out with the soy flour. At 200 °C, the BEA percentage of reduction was variable from 78.0 ± 1.1 to 87.6 ± 1.2 %, depending by the flour matrix employed (Figs. 1 and 2). The BEA degradation data evidenced in the alimentary system was lowest than the data evidenced in the model solutions, due probably to the protective effects that the alimentary matrices have toward the compound employed.

The extracts of the model solution and also of the trials related to the food system were also injected in the LC–MS in the modality full scan (m/z 150–1500), to detect the presence of some BEA degradation products. In Fig. 3 is shown the LC–MS chromatogram of the samples injected in the modality full scan. In the entire sample analyzed after the BEA peak was possible to detect another

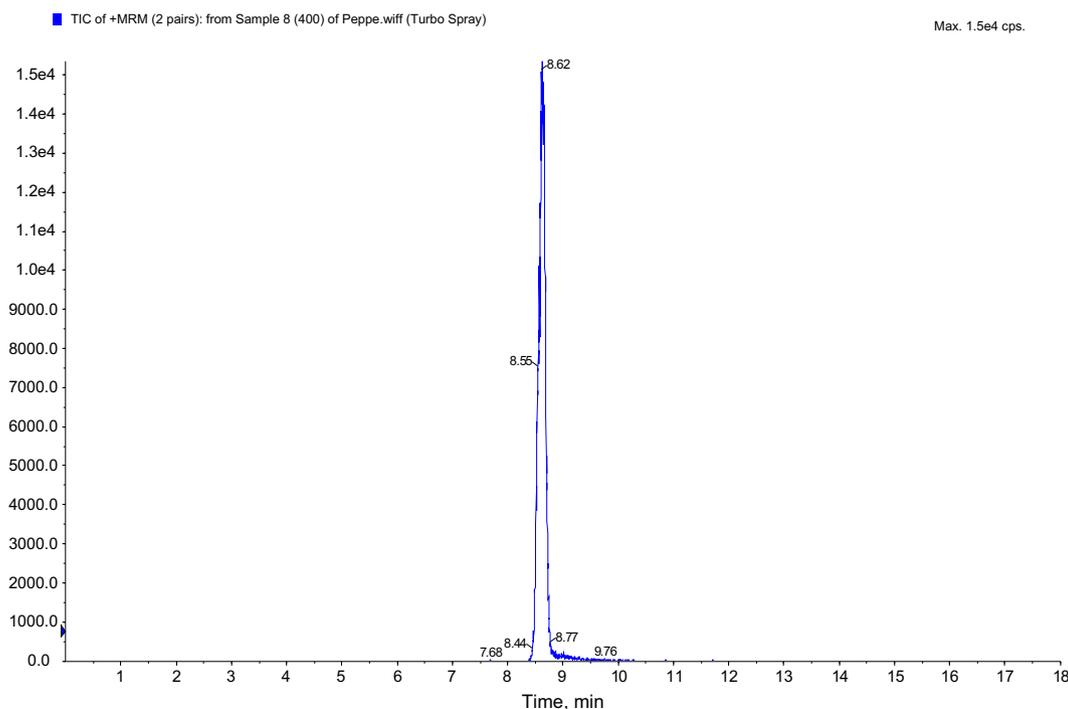


Fig. 1. LC–MS/MS chromatogram of the minor *Fusarium* mycotoxins BEA present in the wheat crispy bread contaminated with 5 mg/kg of the mycotoxin studied and heated at 180 °C during 3 min.

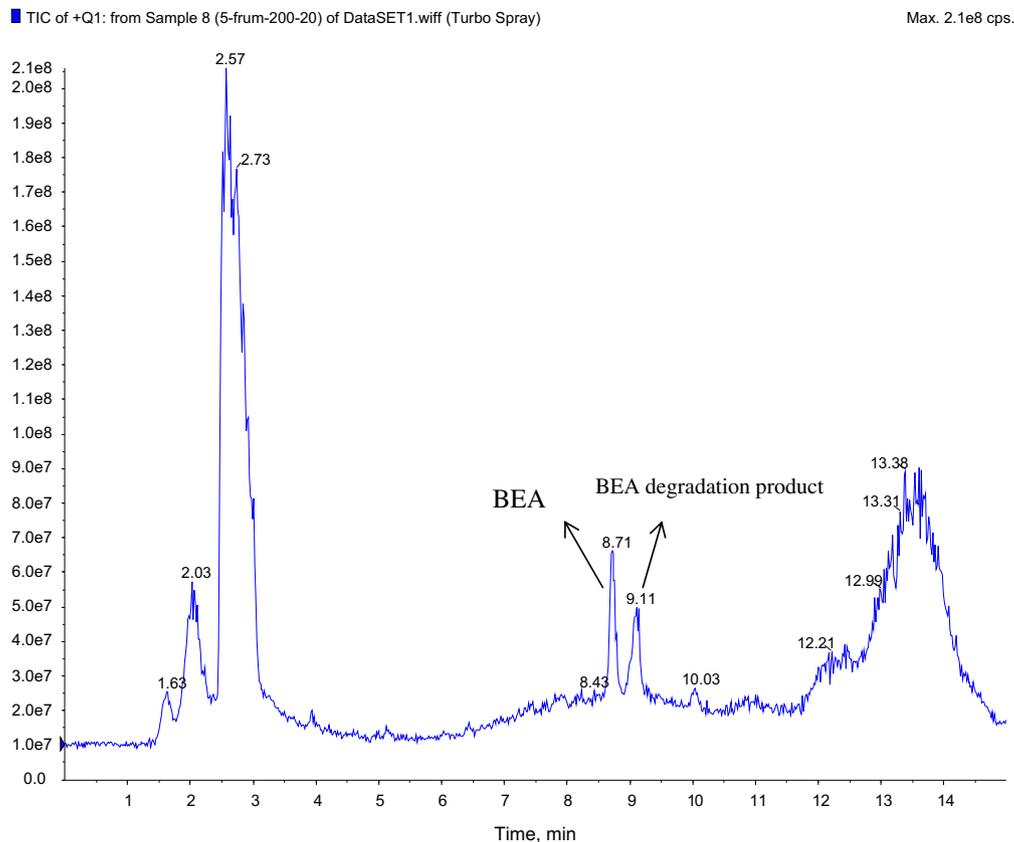


Fig. 3. LC–MS chromatogram of the minor *Fusarium* mycotoxin BEA present in the wheat crispy bread samples treated at the temperature of 180 °C during 10 min incubation. After the BEA peak is possible to evidence another compound characterized as a BEA degradation product.

peak that increased with the increment of the temperature and of the incubation times employed. The mass spectrum of the BEA degradation product is evidenced in Fig. 4. In the figure are present three main fragments that characterize the structure of this degradation product. In particular the fragment with a m/z of 485.4 represents the BEA with a lose of a two structural component of the mycotoxin, that are the phenylalanine (Phe) and the hydroxyvaleric acid (HyLv) ($\text{BEA}-(\text{Phe} + \text{HyLv})^+$). The lost of these two compounds was confirmed by the fragment with a m/z of 281.5 that correspond to the Phe + HyLv unit. Another important fragment that confirms the structure of the degradation BEA compound was the fragment with a m/z of 207.1, that represents the BEA with the lost of two $(\text{Phe} + \text{HyLv})^+$ units. The results obtained in this work demonstrate that the heat treatments applied on food contaminated with the minor *Fusarium* mycotoxin BEA can transform this compound in a degradation product that is different from the original compound for the lost of a $-(\text{Phe} + \text{HyLv})^+$ unit. Further investigation will be focused on the isolation of the degradation product and on the study of its toxicity *in vitro* on different cell models. This study can be considered the first where the thermic BEA degradation was studied, but the degradation of other mycotoxins employing different heat treatments was evaluated by many authors.

In particular Cramer, Keonigs, and Humpf (2008) determined the decrease of the ochratoxin A (OTA) in naturally contaminated green coffee, roasted under various conditions. The roasting conditions were kept within the range of commercial practice and varied from 2.5 to 10 min, and the roast color varied from light medium to dark. The authors evidenced at the end of the heat treatments used, a mean OTA degradation of 69%, with a range of variation variable from 50 to 96%.

Bretz, Knecht, Geockler, and Humpf (2005) investigated the stability of the trichothecene nivalenol (NIV) under food-processing conditions such as cooking or baking, using the technique of the gas chromatography–mass spectrometry (GC–MS).

Heating of NIV, especially under mild alkaline conditions, gave a mixture of four compounds (norNIV A, norNIV B, norNIV C, and NIV lactone), which were isolated and identified by nuclear magnetic resonance (NMR) and MS experiments. Although their formation was also demonstrated in heating experiments with spiked flour samples, only norNIV B was detectable in a screening of several commercially available samples, possibly due to the very low contamination with the mycotoxin studied.

Degradation of NIV was observed under all conditions, generally accelerating with increasing temperatures. Heating with N-acetyl-L-lysine methyl ester resulted in the fastest degradation of NIV; after 10 min at 175 °C, only 40% of NIV was left, while after 60 min, only trace amounts of NIV were detectable compared to 20–45% with the other experiments operated.

Bretz, Beyer, Cramer, Knecht, and Humpf (2006) studied the stability of DON under food-processing conditions such as cooking or baking, and also the metabolites produced during the heat treatments with the techniques of the nuclear magnetic resonance (NMR) and of mass spectrometry (MS). The results obtained by the authors evidenced that DON and 3-acetyldeoxynivalenol (3-AcDON), were transformed in a mixture of compounds known as norDON A, norDON B, and norDON C, while four new were identified and named 9-hydroxymethyl DON lactone, norDON D, norDON E, and norDON F. The significance of the DON degradation products was checked by the authors analyzing commercially available food samples. In particular norDON A, B, and C were

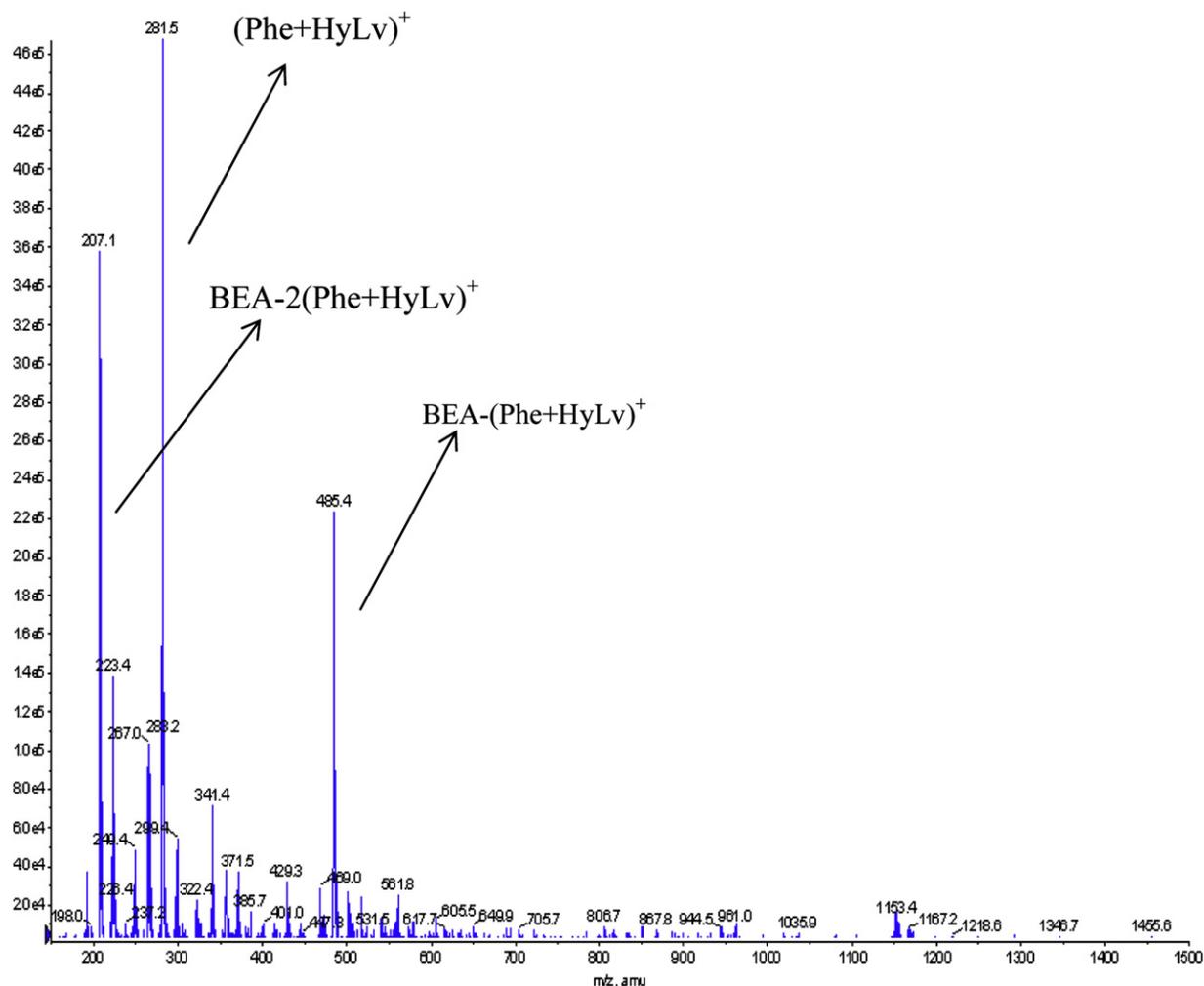


Fig. 4. Mass spectra fragments (m/z 200–1500) of the BEA degradation products produced during the heat treatments. It's possible to evidence in the mass spectra the presence of some fragments that confirm the identification of the degradation product analyzed.

detected in 29–66% of the samples in mean concentrations ranging from 3 to 15 $\mu\text{g}/\text{kg}$. DON was heated at various temperatures (150–200 °C) for different time periods (5–20 min) and with R-D-glucose (sugar model), methyl-R-D-glucopyranoside (starch model), and the amino acid derivatives *N*-R-acetyl-L-lysine methyl ester and BOC-L-cysteine methyl ester (protein models).

Degradation of DON was observed under all conditions, generally accelerating with rising temperatures. As previously reported for nivalenol (Bretz, Beyer, Cramer, Knecht, & Humpf, 2006), heating with *N*-R-acetyl-L-lysine methyl ester resulted in the fastest degradation: after 10 min at 150 °C, only 20% of DON was left, while it was 50–90% with the other model compounds. Although significant losses of DON were observed in all experiments, only in the heating experiment with the lysine derivative appreciable quantities of three degradation products (up to 40% of the total DON used) were detected.

Bullerman and Bianchini (2007) evaluated that the various food processes that may have effects on mycotoxins include sorting, trimming, cleaning, milling, brewing, cooking, baking, frying, roasting, canning, flaking, alkaline cooking, nixtamalization, and extrusion. Most of the food processes have variable effects on mycotoxins, with those that utilize the highest temperatures having greatest effects. In general the processes reduce mycotoxin concentrations significantly, but do not eliminate them completely. However, roasting and extrusion processing show promise for

lowering mycotoxin concentrations, though very high temperatures are needed to bring about much of a reduction in mycotoxin concentrations. Extrusions processing at temperatures greater than 150 °C are needed to give good reduction of zearalenone (ZEA), moderate reduction of aflatoxins (AFs), variable to low reduction of DON and good reduction of fumonisins (FBs). The greatest reductions of FBs occur at extrusion temperatures of 160 °C or higher and in the presence of glucose. Extrusion of FBs contaminated corn grits with 10% added glucose resulted in 75–85% reduction in FB₁ levels. Some FBs degradation products are formed during extrusion, including small amounts of hydrolyzed FB₁ and N-(Carboxymethyl)-FB₁ and somewhat higher amounts of N-(1-deoxy-D-fructos-1-yl) FB₁ in extruded grits containing added glucose. Feeding trial toxicity tests in rats with extruded FBs contaminated corn grits show some reduction in toxicity of grits extruded with glucose.

Cenkowski, Pronyk, Zmidzinska, and Muir (2007) studied the effects of superheated steam (SS) as a processing medium on grains contaminated with the *Fusarium* mycotoxin DON and with *Geobacillus stearothermophilus* spores. The processing temperature used by the authors ranged between 110 and 185 °C with three steam velocities of 0.65, 1.3 and 1.5 m/s for DON contaminated wheat. Reductions in DON concentration of up to 52% were achieved at 185 °C and 6 min processing time.

Meca, Fernandez-Franzon, et al. (2010) evaluated as the FB₁ content in corn products can be transformed, during the heating

process in foods containing reducing sugars, in the Maillard reaction product N-(carboxymethyl)-FB₁. In this study operated by the authors a rapid method was developed for the determination of both compounds in corn products using a high-speed blender, UltraTurrax, for solvent extraction and the liquid chromatography tandem mass spectrometry (LC–MS/MS) for the determination. The kinetics of FB₁ degradation and the formation of the Maillard adduct were studied in a model system constituted by corn bread spiked with 1 mg/kg of FB₁, heated in an oven at 160, 180, and 200 °C during 3, 6, 10, 15, and 20 min. The data evidenced by the authors demonstrated that during the treatments operated, the FB₁ decreased from 0.96 to 0.30 mg/kg and N-(carboxymethyl)-FB₁ increased until to 0.1 mg/kg.

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