Degradation study of enniatins by liquid chromatography–triple quadrupole linear ion trap mass spectrometry

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Enniatins A, A1, B and B1 (ENs) are mycotoxins produced by Fusarium spp. and are normal contaminants of cereals and derive products. In this study, the stability of ENs was evaluated during food processing by simulation of pasta cooking. Thermal treatments at different incubation times (5, 10 and 15 min) and different pH (4, 7 and 10) were applied in an aqueous system and pasta resembling system (PRS). The concentrations of the targeted mycotoxins were determined using liquid chromatography coupled to tandem mass spectrometry. High percentages of ENs reduction (81–100%) were evidenced in the PRS after the treatments at 5, 10 and 15 min of incubation. In contrast to the PRS, an important reduction of the ENs was obtained in the aqueous system after 15 min of incubation (82–100%). In general, no significant differences were observed between acid, neutral and basic solutions. Finally, several ENs degradation products were identified using the technique of liquid chromatography–triple quadrupole linear ion trap mass spectrometry.

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1. Introduction

The Fusarium genus is the most prevalent toxin-producing fungi of the Northern temperate region (SCF, 2002). Several Fusarium species as avenaceum, moniliforme, proliferatum and subglutinans are producers of some minor Fusarium mycotoxins called enniatins (ENs). These bioactive compounds are cyclic hexadepsipeptides formed by alternating of the α-δ-hydroxy-isovaleric acid (HyLv) and different N-methylamino acid residues as valine (Val) and isoleucine (Ile). The ENs are classified as ionophoric compounds, forming stable molecules with a “sandwich” structure with alkali metals or alkaline earth metals, across human cell membranes (Jestoi, 2008).

In vitro studies have demonstrated that ENs evidenced cytotoxic activity in different cell lines, including rodent (V79), lepidopteran (SF-9), monkey (Vero) and human cells (Caco-2, Hep-G2, HT-29) (Behm, Degen, & Föllmann, 2009; Fornelli, Minervini, & Logrieco, 2004). Generally, the contamination levels by ENs evidenced in cereals collected in Mediterranean area is higher than the data evidenced in Central and Northern European regions, probably due to the different climatic condition of these two different parts of the continent (Santini, Meca, Uhlig, & Ritiieni, 2012). ENs have been detected in processed products containing essential cereals for adult and infant nutrition, such as breakfast cereals, rice, pasta, infant formula, bread mill and other derived products (Jestoi, 2008; Serrano, Font, Mañes, & Ferrer, 2013a).

Several studies have been published related to the mitigation strategies of mycotoxins in food, focalized principally on the reduction of the trichothecenes, fumonisins (FBs), aflatoxins (AFs), ochratoxin A (OTA), patulin (PAT) and zearalenone (ZEA) during food processing (Abramson, House, & Nyachoti, 2005; Bullerman & Bianchini, 2007; Cramer, Königs, & Humpf, 2008; Kushiro, 2008; Park, Scott, Lau, & Lewis, 2004; Ryu, Hanna, Eskridge, & Bullerman, 2003). At the moment, only two studies are available in the scientific literature on the thermal degradation of the minor Fusarium mycotoxins. In particular, Meca, Ritiieni, and Mañes (2012) studied beauvericin (BEA) stability during several heat treatments, in a model system and also in homemade crispy bread, obtaining percentages of degradation variables from 20% to 90%. Vaclavikova et al. (2013) determined ENs levels during beer and bread production, concluding that ENs concentrations were reduced during breadmaking (from 71% to 79% in milling and from 50% to 60% in baking), whereas these mycotoxins were not detected in the final beer.

Nevertheless, other studies have focused on the identification of the mycotoxins degradation products formed after the treatments, as well as in the evaluation of the toxicity of these new identified compounds. Meca, Luciano, Zhou, Tsao, and Mañes (2012) studied the stability of BEA in a solution model and in wheat flour using allyl isothiocyanate (AITC) as a reactant. Two reaction products between the bioactive compounds employed in this study were identified by LC–MS-LIT, corresponding to BEA conjugates.
containing one or two AITC molecules. Bretz, Beyer, Cramer, Knecht, and Humpf (2006) studied the DON stability in a food system elaborated with different macronutrients as sugar, starch and proteins, at temperatures ranging from 150 to 200 °C using incubation times variables from 5 to 20 min. The DON reduction was temperature and time dependent. Also three DON degradation products were identified in commercial samples. The cytotoxicity of the degradation products was compared to DON by cell culture experiments. The results evidenced that DON degradation products were less cytotoxic than DON, and the heat treatments employed reduced the risk associated to DON intake. The reduction of the fumonisins has been evidenced by many studies during food processing and cooking (baking, frying, roasting, extrusion and heating). The studies demonstrated that FBs stability depends of several factors, such as temperature, time or sugar and water content. Moreover, different FBs degradation products have been identified during food treatments (Humpf & Voss, 2004).

Considering the lack of data related to the degradation of ENs during food processing, the aims of the study were: (a) to study the thermal stability of ENA, ENA1, ENB and ENB1, at different pH, in an aqueous system and in a food model simulating pasta composition (pasta resembling system) by liquid chromatography coupled to a triple quadrupole mass spectrometer detector (LC–MS/MS QqQ), (b) to identify and characterise ENs degradation products produced during heat treatments by liquid chromatography coupled to the mass spectrometry-linear ion trap (LC–MS-LIT).

2. Materials and methods

2.1. Materials

Acetonitrile (AcN) and methanol (MeOH) were provided by Merck (Darmstadt, Germany). Ammonium formate (99%) and formic acid (>98%) were supplied by Panreac Quimica S.A.U. (Barcelona, Spain). Deionized water (<18 MΩ cm−1 resistivity) was obtained in the laboratory using a Milli-Q SP® Reagent Water System (Millipore, Bedford, MA, USA). All solvents were filtered through a 0.45 µm HV filter provided by Scharlau (Barcelona, Spain) before use.

Gluten from wheat (>80% of protein), starch from potato (PhEur) and albumin from bovine serum (99.99% purity) flow, 800 l h−1; multiplier, 650; collision gas (argon 99.995% purity) pressure, 3.83 × 10−3 mbar; interchanel delay, 0.02 s; total scan time, 1.0 s; dwell time 0.01 ms. The mass spectrometer was operated in positive ion mode. The electrospray ionisation source values were as follows: capillary voltage, 3.50 kV; extractor, 5 V; RF lens 0.5 V; source temperature, 100 °C; desolvation temperature, 300 °C; desolvation gas (nitrogen 99.99% purity) flow, 800 l h−1; cone gas 50 l h−1 (nitrogen 99.99% purity).

2.2. Sample treatment

2.2.1. Aqueous system

The model solutions were prepared in 100 ml Erlenmeyers at three different pH values (pH 4, 7 and 9). In order to adapt the experiments to the real cooking processes conditions, lemon juice and sodium bicarbonate marketed in Valencia were employed to reach the required pH. The acid (pH 4) and basic (pH 9) solutions were prepared adding 1.5 ml of lemon juice and 2 g of sodium bicarbonate to 50 ml of deionized water under continuous stirring. The experiments in the neutral solution (pH 7) were carried out using deionized water. The pH measurements were performed employing a GLP21 Crison pH-metre (Crison Instruments, S.A., Barcelona, Spain) with a Hamilton pH electrode (Fisher Scientific, Madrid, Spain). The model solutions were filtered through a 0.45 µm HV filter provided by Scharlau (Barcelona, Spain), and then 980 µl of each solution was contaminated with 20 µl of each EN (1000 mg l−1) to obtain a final concentration of 20 mg l−1. ENs reduction experiments were performed at 100 °C in a water bath SS40-2 (Gran Instruments, Cambridge, United Kingdom) at different boiling times (5, 10 and 15 min). Aliquots of each treatment were filtered through 13 mm/0.20 µm nylon filter (Membrane Solutions, Texas, USA) and injected into the LC–MS/MS and LC–MS-LIT systems.

2.2.2. Pasta resembling system (PRS)

The PRS was formulated by mixing 65 g of starch, 8 g of gluten and 2 g of albumin to obtain homogenous flour. The experiments were performed simulating the boiling process of pasta (100 g of pasta and 1 l of water) employing three aqueous solutions: acid, basic and neutral (see preparation in Section 2.2.1). For this purpose, 75 mg of PRS was contaminated with 20 µl of each EN (1000 mg l−1) individually, and 1000 µl of aqueous solution was added to the vial. The final concentration of each EN in the vials was of 20 mg l−1. Thermal experiments were performed at 100 °C in a water bath at different times (5, 10 and 15 min). Afterwards, mycotoxicin extraction was carried out as pointed out in next paragraph.

2.3. Mycotoxin extraction

Treated samples from PRS, were extracted with 10 ml of AcN using a lka T10 basic Ultra-Turrax (Staufen, Germany) for 3 min. The supernatant was evaporated to dryness by nitrogen at 35 °C using a multi-sample Turbovap LV Evaporator (Zymark, Hoptkinston, USA). After solvent evaporation, the extract was reconstituted with 1000 µl of AcN/MeOH 50/50 v/v, and filtered through 13 mm/0.20 µm nylon filter until the analysis in the LC–MS/MS and LC–MS-LIT systems.

2.4. Analysis

2.4.1. LC–MS/MS analysis

A Quattro LC triple quadrupole mass spectrometer from Micromass (Manchester, UK), equipped with an LC Alliance 2695 system (Waters, Milford, MA, USA) consisting of an autosampler, a quaternary pump, a pneumatically assisted electrospray probe, a Z-spray interface and a Mass Lynx NT software version 4.1 were used for the MS/MS analyses. The separation was achieved by a Gemini-NX C18 (150 × 2 mm i.d., 3 µm particle size) analytical column supplied by Phenomenex (Barcelona, Spain), preceded by a security guard cartridge C18 (4 × 2 mm i.d.), using gradient elution that started at 90% of A (AcN) and 10% of B (20 mM ammonium formate in MeOH), increased linearly to 50% of B in 10 min. Then, it was decreased linearly to 10% of B in 3 min. Afterwards, the initial conditions were maintained for 2 min. Flow rate was maintained at 0.2 ml min−1. The analysis was performed in positive ion mode. The electrospray ionisation source values were as follows: capillary voltage, 3.50 kV; extractor, 5 V; RF lens 0.5 V; source temperature, 100 °C; desolvation temperature, 300 °C; desolvation gas (nitrogen 99.99% purity) flow, 800 l h−1; cone gas 50 l h−1 (nitrogen 99.99% purity).

The cone voltage selected was 40 V and the collision energy selected was 35 eV for all ENs. The analyser settings were as follows: resolution 12.0 (unit resolution) for the first and third quadrupoles; ion energy, 0.5; entrance and exit energies, −3 and 1; multiplier, 650; collision gas (argon 99.995% purity) pressure, 3.83 × 10−3 mbar; interchanel delay, 0.02 s; total scan time, 1.0 s; dwell time 0.01 ms. The mass spectrometer was operated in
Multiple Reaction Monitoring (MRM) mode. According with the European Union criteria (Commission Decision, 2002), which establishes that a substance can be identified using LC–MS/MS in MRM mode by at least two transitions, the following precursor ion and product ions were selected for each mycotoxin: the precursor ion m/z 681.9 [M+H]⁺ and the product ions m/z 228.2 and 210.0 for ENA, the precursor ion m/z 667.9 [M+H]⁺ and the product ions m/z 228.2 and 210.0 for ENA1, the precursor ion m/z 639.8 [M+H]⁺ and the product ions m/z 214.2 and 196.2 for ENB, the precursor ion m/z 654.9 [M+H]⁺ and the product ions m/z 214.2 and 196.2 for ENB1 (Serrano, Font, Mañes, & Ferrer, 2013b).

2.4.2. LC–MS-LIT identification of the ENs degradation products

An Applied Biosystems/MDS SCIEX Q TRAP TM Linear Ion Trap (LIT) mass spectrometer (Concord, Ontario, Canada), coupled with a Turbo Ion Spray source, was used. This instrument is based on a triple-quadrupole path (QqQ) in which the third quadrupole can also be operated as a linear ion trap (QqLIT) with improved performance. In the QqLIT configuration the Q TRAPTM can also operate in Enhanced Resolution (ER) scan and in enhanced product ion scan (EPI) modes. Applied Biosystem/MDS SCIEX Analyst software version 1.3.2 was used for data acquisition and processing. A Gemini (150 × 2.0 mm, 5 μm) Phenomenex column was used for separation. The mobile phase (AcN/water 70/30 v/v with 0.1% of HCOOH) was delivered in an isocratic manner at a constant flow rate of 0.3 ml min⁻¹. The MS was operated 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. The analyses were carried out using: (1) the ER mode for newly formed compounds in the mass range from 200 to 800 Da; (2) the EPI mode to obtain a MS² scan of a fragment of the adducts. The mass spectrometry data obtained from these two scan modes has permitted the elucidation of the compounds of interest. Its absolute molecular structure still needs to be confirmed with other analytical methods including NMR and IR (Meca, Luciano, et al., 2012).

2.5. Statistical analysis

The experiments were carried out in triplicates. Statistical analysis were 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

3.1. Method performance

The method validation was reported previously by Serrano et al. (2013b). The detection limits (LODs) were calculated using a signal-to-noise ratio of 3. The limits of quantification (LOQs) were calculated using a signal-to-noise ratio of 10. The LOQ and LOD for ENA were 0.25 and 0.08 μg kg⁻¹, respectively. LOQ was 0.50 μg kg⁻¹ for ENA, ENB and ENB1. LOD was 0.15 μg kg⁻¹ for ENA, ENB and ENB1. ENs exhibited good linearity over the working range (from 0.05 to 20 mg kg⁻¹), and the regression coefficient of calibration curves was 0.993. The accuracy was evaluated through recovery studies at two concentration levels (LOQ and 100 × LOQ). Intra-day precision was assessed by five determinations at each addition level in the same day. Inter-day precision was assessed by one determination at each addition level during 5 days. The mean recoveries and the corresponding relative standard deviations (RSDs) are presented in Table 1 Supplementary data. RSD values ranged between 3% and 9% for intra-day precision, and between 4% and 11% for inter-day precision. Recovery ranges for the low spiked level (LOQ) and the high spiked level (100 × LOQ) were 85–95% and 88–97%, respectively. Therefore, the results were in accordance to the limits set in Commission Decision, 2002/657/EC: a mean recovery (n = 5) between 70% and 120%, and a RSD lower than 20%.

3.2. ENs reduction in the model systems

3.2.1. Aqueous system

Fig. 1 shows the percentages of ENs reduction in the aqueous solutions (acid, neutral and basic) after heat treatments at 5, 10 and 15 min.

The degradation of the tested ENs in this study was time dependent. The degradation trend of ENB was similar in the three tested solutions. The degradation trend of ENA, ENA1 and ENB1 using the acid solution (Fig. 1a) was comparable with the data observed using the neutral solution (Fig. 1b), whereas the degradation was different in the basic solution (Fig. 1c).

As is possible to observe in Fig. 1a and b, percentages of degradation of ENB and ENA1 in the acid and neutral solutions after 5 min of treatment (between 74.1% and 98.4%) were higher than those obtained for ENB, and ENA (between 16.8% and 27.7%). Percentages of reduction after 10 min of treatment were similar for all ENs using acid and neutral solutions (76.0–100.0%). After 15 min of treatment, ENs reductions were close to 100%; only the ENA reduction in the acid solution was of 80%.

Concerning reductions in the basic solution (Fig. 1c), the highest percentages of reduction after 5 and 10 min of treatment were obtained for ENB (97.1–98.8%). Percentages of reduction of ENA were lower than those obtained for ENA1 and ENB1 in the basic solution after 5 and 10 min of thermal treatment. ENs reductions after 15 min of thermal treatment were close to 100% in the basic solution.

In the last few years, several studies have been published on the evaluation of the thermal stability of other mycotoxins in aqueous systems, showing different results depending on the studied mycotoxin. Ryu et al. (2003) studied the heat stability of ZEA in aqueous model systems at different temperatures (between 100 and 225 °C) and pHs (4, 7 and 10). Percentages of reduction (23–100%) were positively related to increasing processing temperature. The highest reduction of the ZEA was observed at pH 10 below to 175 °C, whereas above 175 °C, the highest ZEA degradation was observed at pH 7. Pineda-Valdes and Bullerman (2000) evaluated the moniliformin (MON) stability in aqueous buffer solutions at different temperatures (100, 125, 150 and 175 °C), obtaining the highest MON reduction (99%) at pH 10 and 175 °C. In general, the percentages of reduction were dependent on increasing temperature, time and pH. On the other hand, it has been demonstrated that FBs stability in aqueous buffered solutions was dependent with decreasing pH and increasing temperature (Humpf & Voss, 2004). Therefore, the results are variable depending on the type of mycotoxin greatly due to the chemical diversity of the mycotoxins.

3.2.2. Pasta resembling system (PRS)

As it is possible to observe in Fig. 2, the percentages of degradation of ENs A, A1, B and B1 in the PRS were similar at the three incubation times and at the tested pHs.

In contrast to the results obtained in the aqueous system, the reduction of all mycotoxins in the PRS system reached percentages higher than 81.0% after 5 and 10 min of treatment. After 15 min of treatment, the percentages of reduction reached 95.2%, 98.3%, 100.0% and 97.7% for ENA, ENA1, ENB and ENB1, respectively. Therefore, similar results were observed between PRS and aqueous system after 15 min of thermal treatment. In general, the values produced in this study showed that the pH of the solution is not related with the ENs reduction.
Fig. 1. Degradation of the ENs in the aqueous model system during the heat treatment (100 °C) at different times and pHs: (a) acid solution (pH 4), (b) neutral solution (pH 7) and (c) basic solution (pH 9). *p ≤ 0.05, **p ≤ 0.001 and ***p ≤ 0.000 represent significant difference as compared to control values.
Fig. 2. Degradation of the ENs in the PRS during the heat treatment (100 °C) at different times and pHs: (a) acid solution (pH 4), (b) neutral solution (pH 7) and (c) basic solution (pH 9). **p < 0.000 represent significant difference as compared to control values.
Table 1
LC–MS-LIT data (MS<sup>1</sup> and MS<sup>2</sup>) of the degradation products of ENs type A obtained in the aqueous system and in the pasta resembling system.

<table>
<thead>
<tr>
<th>Mycotoxin (type of model system)</th>
<th>Degradation product</th>
<th>[M+H]&lt;sup&gt;+&lt;/sup&gt; m/z</th>
<th>Fragment</th>
<th>Structure</th>
<th>MS2 fragments m/z</th>
<th>Fragment</th>
</tr>
</thead>
<tbody>
<tr>
<td>ENA (aqueous system)</td>
<td>[ENA+K–HyLv]+ (ENA degradation product 1)</td>
<td>640.6</td>
<td>HyLv</td>
<td>+K&lt;sup&gt;+&lt;/sup&gt;</td>
<td>427.7</td>
<td>[ENA+K–2HyLv–2Ile+H&lt;sub&gt;2&lt;/sub&gt;O]&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>314.6</td>
<td>[ENA–HyLv–2Ile]&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
<tr>
<td>ENA (aqueous system)</td>
<td>[ENA–Ile]&lt;sup&gt;+&lt;/sup&gt; (ENA degradation product 2)</td>
<td>541.9</td>
<td>Ile</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ENA (pasta resembling system)</td>
<td>[ENA–HyLv–Ile]&lt;sup&gt;+&lt;/sup&gt;</td>
<td>455.2</td>
<td>HyLv+Ile</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ENA&lt;sub&gt;1&lt;/sub&gt; (aqueous system/pasta resembling system)</td>
<td>[ENA&lt;sub&gt;1&lt;/sub&gt;–Val]&lt;sup&gt;+&lt;/sup&gt;</td>
<td>541.3</td>
<td>Val</td>
<td></td>
<td>441.1</td>
<td>[ENA&lt;sub&gt;1&lt;/sub&gt;–HyLv–Ile]&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>428.1</td>
<td>[ENA&lt;sub&gt;1&lt;/sub&gt;+K–2HyLv–Val+H&lt;sub&gt;2&lt;/sub&gt;O]&lt;sup&gt;+&lt;/sup&gt;</td>
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<td></td>
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<td></td>
<td>414.1</td>
<td>[ENA&lt;sub&gt;1&lt;/sub&gt;+Na–2HyLv–Val+H&lt;sub&gt;2&lt;/sub&gt;O]&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>214.1</td>
<td>[HyLv+Val]</td>
</tr>
</tbody>
</table>

* Data non available.
Only a few studies on the thermic degradation of the minor 
_Fusarium_ mycotoxins are available in the scientific literature. 
Meca, Ritieni, et al. (2012) studied BEA reduction in model sys-
tems at 160, 180 and 200 °C and different incubation times (from 3 to 20 min). The percentages of reduction in aqueous models (between 58% and 100%) were time and temperature dependent. BEA was totally reduced at 200 °C during 20 min of treatment. In the food system, the percentage of BEA degra-
dation ranged from 20% to 90%. The results obtained by the 
authors are comparable with the data obtained in the present 
study considering that the chemical structure of BEA is very sim-
ilar with the ENs structure. Moreover, Vaclavikova et al. (2013) 
studied the ENs degradation during beer and bread production, 
achieving similar results to those obtained in the present study. 
During bread making, the impact of the thermal treatments 
causd the reduction of ENs contamination (reductions of 
50–60%). The malting and brewing processes resulted in a 
reduction of the mycotoxin concentration due to the high 
temperatures achieved. ENs levels during the malting step were 
reduced to 10–30% of their original content in barley, while ENs 
were not detected in the final beer. 

The stability of other _Fusarium_ mycotoxins during thermal food 
processing has been diversely discussed by several researchers. 
Some studies have supported that the impact of high tempera-
tures is a decisive parameter in mycotoxin reduction. Kottapalli 
and Wolf-Hall (2008) observed significant DON reductions 
(79–93%) in malts prepared from barley treated with hot water 
at 45 and 50 °C during 20 min. Visconti, Haidukowski, Pascale, 
and Silvestri (2004) studied DON degradation during durum 
heat processing and spaghetti cooking. DON degradation 
evidenced after the wheat milling, semolina and spaghetti pro-
tuction was of 23%, 63% and 67%, respectively. Cortez-Rocha, 
Trigo-Stockli, Wetzel, and Reed (2002) investigated the FB1 levels 
in corn during alkali-cooking and extrusion processing of alkali-
cooked corn, habitually used to produce several products from 
corn, such as snacks and tortilla products. The levels of FB1 were 
reduced to 17% after alkali-cooking (55 min at 95–100 °C), whereas 
the extrusion processing at 171 °C resulted in a reduction of 
99%. Furthermore, other studies have indicated low percentages 
of mycotoxins reduction after thermal treatments. Scudamore, 
Hazel, Patel, and Scriven (2009) studied the influence of bread, 
cake and biscuits production on the stability of DON, nivalenol 
(NIV) and ZEA. The concentrations of NIV and ZEA were constant 
during food production, whereas the final degradation concentra-
tion of DON ranged from 5% to 11%.

The pH influence on the ENs stability has not been observed in 
this study, but some authors have reported the importance of the 
pH on mycotoxin degradation. Abramson et al. (2005) studied 
the thermal degradation of the mycotoxin DON in feed naturally 
contaminated with the presence and absence of a water solution 
of sodium carbonate. Thermal treatments were carried out at 
80 °C during 0, 1, 3, 5 and 8 days, obtaining high percentages 
of DON degradation after 1 day when sodium carbonate solution 
was added to the feed. However, when samples were heated with-
out sodium carbonate solution, DON reduction was observed after 
8 days.

3.3. LC–MS-LIT characterization of ENs degradation products

The samples positive to ENs degradation, were also injected in 
the LC–MS-LIT in the ER scan modality (m/z = 200–900) to deter-
mine ENs degradation products produced through the heat treat-
ments employed. The abundance of ENs degradation products 
identified in this study increased with time of heating and with 
decreasing concentrations of ENs.

![Fig. 3.](image)

(a) LC–MS-LIT chromatogram of ENA treated during 10 min at pH 4 in the PRS system, (b) LC–MS-LIT spectra of ENA degradation product 1 and (c) LC–MS-LIT spectra of ENA degradation product 2.
3.3.1. ENA

In the aqueous system, two degradation products derived from ENA were formed after heat treatments at the three tested pHs (Table 1 and Fig. 3a). The abundance of degradation product 1 was positively related to increasing time of thermal treatment and decreasing ENA levels, whereas the abundance of degradation product 2, remained constant after thermal treatments. In Fig. 3a is evidenced the LC–MS-LIT chromatogram obtained in the modality of ER scan (MS$^1$) for ENA treated during 10 min at pH 4 in the PRS system. The chromatogram shows the presence of ENA and two degradation products. The degradation product 1 (Fig. 3b) was characterised as potassium adduct of ENA with the loss of a structural component of ENs structure as HyLv evidenced in the fragment with a $m/z$ of 640.6. The formation of this new degradation product was also confirmed by the fragments with $m/z$ of 427.5 and 314.5 (Fig. 3b). The first one corresponding to the potassium adduct of ENA with the loss of 2HyLv and also by the loss of another amino acid that characterised ENs structure as the Ile. The second fragment ($m/z$ 314.5) corresponding to ENA with the loss of HyLv and 2Ile groups. To obtain more information and also to confirm the structure of degradation product 1, the sample was also injected in the modality EPI scan to obtain the MS$^2$ scan of the neo forming compound using as fragmenting signal the ion with a $m/z$ of 640.6. In Fig. 4a is possible to observe the LC–MS-LIT chromatogram in MS$^2$ of the degradation product 1. Fig. 4b shows the EPI-LIT spectra that present several important fragments which confirm the structure of degradation product 1 with $m/z$ signals of 427.7 and 314.3. The fragments present the same signal identified in the MS$^1$ spectra. The fragment with $m/z$ 427.7 corresponds to the potassium adduct of ENA with the loss of 2HyLv and 1Ile, whereas the second one was identified as ENA with the loss of 1HyLv and 2Ile.

The degradation product 2 was identified as ENA with the loss of an Ile. In Fig. 3c is shown the MS-LIT spectra corresponding to the ENA degradation product 2. The structure of this new compound was also confirmed by the signals with $m/z$ of 473.8, 427.7 and 228.5. The first one was identified as ENA with the loss of HyLv and Ile, whereas the second one ($m/z$ 427.7) was identified as the potassium adduct of ENA with the loss of two groups of HyLv and one Ile group. The loss of HyLv and Ile was confirmed by the $m/z$ 228.5 corresponding to the fragment HyLv + Ile. Unfortunately no MS$^2$ EPI spectrum is available to confirm the structure of the second ENA degradation product.

In contrast to the two degradation products of ENA identified in the aqueous system, only one degradation product of ENA was identified in PRS (Table 1). The degradation products of ENA in the aqueous system were characterised as ENA with the loss of HyLv ($m/z$ 640.6) and as ENA with the loss of Ile ($m/z$ 541.9), whereas the degradation product of ENA in the PRS was characterised as ENA with the simultaneous loss of HyLv and Ile ($m/z$ 455.2). The abundance of this degradation product was positively related to increasing time of thermal treatment and the decreasing ENA.

Fig. 4. (a) LC–MS-LIT chromatogram obtained in EPI mode (MS$^2$) using as fragmenting ion the signal with a $m/z$ of 640.6 of the ENA degradation product 1 and (b) EPI mass spectrum obtained in MS$^2$ of the degradation products 1 of the ENA.

(a)

(b)
Table 2
LC–MS–LIT data (MS<sup>1</sup> and MS<sup>2</sup>) of the degradation products of ENs type B obtained in the aqueous system and in the pasta resembling system.

<table>
<thead>
<tr>
<th>Mycotoxin (type of model system)</th>
<th>Degradation product</th>
<th>[M+H]+ m/z</th>
<th>Fragment</th>
<th>Structure</th>
<th>MS2 fragments m/z</th>
<th>Fragment</th>
</tr>
</thead>
<tbody>
<tr>
<td>ENB (aqueous system/pasta resembling system)</td>
<td>([\text{ENB} - \text{Val} + \text{H}_2\text{O}]^+)</td>
<td>527.2</td>
<td>Val</td>
<td>([\text{ENB} - \text{Val} + \text{H}_2\text{O}]^+)</td>
<td>427.3</td>
<td>([\text{ENB} - \text{Val} + \text{H}_2\text{O}]^+)</td>
</tr>
<tr>
<td></td>
<td>([\text{ENB} + \text{K} + 2\text{HyLv} + \text{Val} + \text{H}_2\text{O}]^+)</td>
<td>214.1</td>
<td>([\text{HyLv} + \text{Val}]^+)</td>
<td>([\text{HyLv} + \text{Val}]^+)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ENB (aqueous system/pasta resembling system)</td>
<td>([\text{ENB} - \text{HyLv} + \text{H}_2\text{O}]^+)</td>
<td>573.2</td>
<td>HyLv</td>
<td>([\text{ENB} - \text{HyLv} + \text{H}_2\text{O}]^+)</td>
<td>427.3</td>
<td>([\text{ENB} - \text{HyLv} + \text{H}_2\text{O}]^+)</td>
</tr>
<tr>
<td>ENB&lt;sub&gt;1&lt;/sub&gt; (aqueous system/pasta resembling system)</td>
<td>([\text{ENB}_1 - \text{Val}]^+)</td>
<td>527.3</td>
<td>Val</td>
<td>([\text{ENB}_1 - \text{Val}]^+)</td>
<td>427.3</td>
<td>([\text{ENB}_1 - \text{Val}]^+)</td>
</tr>
<tr>
<td></td>
<td>([\text{ENB}_1 + \text{K} + 2\text{HyLv} + \text{Val} + \text{H}_2\text{O}]^+)</td>
<td>214.1</td>
<td>([\text{HyLv} + \text{Val}]^+)</td>
<td>([\text{HyLv} + \text{Val}]^+)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ENB&lt;sub&gt;1&lt;/sub&gt; (aqueous system/pasta resembling system)</td>
<td>([\text{ENB}_1 - \text{HyLv}]^+)</td>
<td>573.2</td>
<td>HyLv</td>
<td>([\text{ENB}_1 - \text{HyLv}]^+)</td>
<td>427.3</td>
<td>([\text{ENB}_1 - \text{HyLv}]^+)</td>
</tr>
<tr>
<td></td>
<td>([\text{ENB}_1 + \text{Na} + 2\text{HyLv} + \text{Val} + \text{H}_2\text{O}]^+)</td>
<td>214.1</td>
<td>([\text{HyLv} + \text{Val}]^+)</td>
<td>([\text{HyLv} + \text{Val}]^+)</td>
<td></td>
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</tr>
</tbody>
</table>

* Data non available.
levels. This product was identified in the neutral, acidic and basic solutions. The structure of this compound from the PRS was confirmed by the signals with \( m/z \) of 311.2 and 228.2. The first one corresponded to ENA with loss of one HyLv and two Ile, and the second one corresponded to the group HyLv and Ile. No MS\(^2\) EPI spectrum is available to confirm the structure of the ENA degradation product identified after the treatments in the PRS.

### 3.3.2. ENA\(_1\)

Similar LC–MS-LIT chromatograms and mass spectra were obtained in the aqueous system and in the PRS at the three tested pH values. Two peaks were observed in the chromatograms related to the treated solutions. One peak corresponding to ENA\(_1\) (\( m/z \) 668.3), and another peak that increased with the increment of the incubation times corresponding to a new degradation product of the ENA\(_1\) (\( m/z \) 541.3), that was characterised as ENA\(_1\) with the loss of Val (Table 1). The structure of this degradation product was confirmed by different fragments in the MS-LIT spectra obtained in ER mode. The fragment with \( m/z \) of 441.2 represents the ENA\(_1\) degradation product with a loss of an Ile and of HyLv. The loss of the amino acids Ile, Val and HyLv from the structure of ENA\(_1\) was confirmed by the presence of the fragments with \( m/z \) of 214.1 and 228.3. To confirm the structure of the ENA\(_1\) degradation product formed after the heat treatment, the sample was also injected in the modality EPI scan to obtain the MS\(^2\) scan of the degradation product isolated using a fragmenting signal ion with a \( m/z \) of 541.3 (Table 1). In particular, the fragment with a \( m/z \) of 441.1 was identified as ENA\(_1\), with the loss of a HyLv and of an Ile, whereas the signals with \( m/z \) of 414.1 and 428.1 were identified as the sodium and potassium adduct of ENA\(_1\) adduct with the loss of 2HyLv and 1Val. The loss of HyLv and Ile was confirmed in the EPI mass spectra by the presence of the signal with a \( m/z \) of 214.1.

### 3.3.3. ENB\(_2\)

The LC–MS-LIT chromatograms observed in the aqueous system were very similar to those obtained in the PRS. The chromatograms showed one peak corresponding to ENB that decreased with the increment of the incubation times. Other peaks were observed related to two degradation products of ENB that increased with the decrease of ENB. Both peaks appeared in the acidic, basic and neutral solutions.

The degradation product 1 was identified as ENB with the loss of the amino acid Val (Table 2). The formation of this new product is confirmed by the presence in the spectra of some diagnostic signals, as the fragment with a \( m/z \) of 427.3, identified as ENB with the loss of structural components HyLv and Val, and also by the fragment with \( m/z \) of 214.2 identified as ENB with the loss of 2HyLv and 2Val. The formation of this new product was confirmed by the analysis in MS\(^2\) mode of the neo forming product and in particular in the EPI (MS\(^2\)) spectra (Table 2). There are some signals that testify the formation of the reduction product as the ions with \( m/z \) of 427.3 and 214.1, present also in the MS\(^1\) spectra, and also by the fragment with \( m/z \) of 399.4 that represent ENB with the loss of two units of HyLv and one unit of Val.

The degradation product 2 was identified as ENB with the loss of the HyLv group (Table 2). The structure of this degradation product was confirmed by the presence of different fragments in the MS-LIT spectra obtained in ER mode. The fragment with \( m/z \) of 427.3 represents ENB with a loss of Val and HyLv, whereas the fragment with \( m/z \) 368.3 is related with the loss of Val and 2HyLv from the structure of ENB. The loss of amino acids Val and HyLv was confirmed by the presence of the fragment with \( m/z \) of 214.1. No MS\(^2\) EPI spectrum is available to confirm the structure of the ENB degradation product 2.

### 3.3.4. ENB\(_3\)

Similar results to those obtained for ENB were obtained in ENB\(_3\) during the different treatments applied. Two degradation products of ENB\(_3\) were identified after the treatments (Table 2). The degradation product 1 was identified as ENB\(_3\) with the loss of the aminoacid Val (\( m/z \) 527.3). This compound was confirmed by the signals with \( m/z \) of 427.3, 414.3 and 214.2. The first one was identified as ENB\(_3\) with the loss of HyLv and Ile, whereas the second one represent potassium adduct of ENB\(_3\) with the loss of 2HyLv and of a Val. The last fragment was identified as the ENB\(_3\) with the loss of the three structural components that characterise the ENB\(_3\) structure: two units of HyLv, one unit of Val, and one unit of Ile. An additional confirmation of the formation of this degradation compound was carried out with the technique of the MS-EPI, where in the MS\(^2\) spectra evidenced some fragments that confirm the formation of the ENB\(_3\) degradation product. In particular, in the MS\(^2\) spectra some ions already identified in the MS\(^1\) spectra with \( m/z \) of 427.3, 414.1 and 214.1 are shown. Also, another fragment was identified in the MS\(^2\) spectra (\( m/z \) 399.2) as the ENB\(_3\) sodium adduct with the loss of 2HyLv and 1Val (Table 2).

The degradation product 2 of ENB\(_3\) was characterised as ENB\(_3\) with the loss of the HyLv group (Table 2). The formation of this new product is confirmed by the presence in the spectra of some diagnostic signals, as the fragments with a \( m/z \) of 457.5, 427.3, 414.5 and 214.1. The first fragment represents ENB\(_3\) with a loss of Val and HyLv, whereas the second fragment represents ENB\(_3\) with the loss of Ile and HyLv. The fragment with \( m/z \) of 414.5 is related to the structure of the potassium adduct of ENB\(_3\) with the loss of two units of HyLv and one unit of Val. The loss of the amino acids Val and HyLv was confirmed by the presence of the fragment with \( m/z \) of 214.1. The confirmation of the structure of the degradation product 2 was not possible, since a MS\(^2\) EPI spectrum was not available.

### 4. Conclusions

The time of thermal treatment affected to the ENs reduction, whereas the variation of the pH not produced any effect in the ENs stability. The decrease of the ENs levels during the thermal treatments has been related to the formation of new compounds derived to the ENs. The application of the LC–MS-LIT permitted the elucidation of the new degradation products formed during the treatments. Degradation products of ENs have been identified for the first time in this pilot study. Future studies will be focused on the study of the toxicological activity of the ENs degradation products on several cell lines. This information is necessary to evaluate the potential health risk of the population to the formation of these new compounds in the processed foodstuffs.

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### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2013.05.030.
References


