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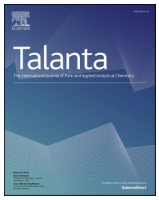

Non-aqueous capillary electrophoresis–time of flight mass spectrometry method to determine emerging mycotoxins

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ABSTRACT

Enniatins (ENN) and beauvericin (BEA) are emerging mycotoxins that have been traditionally determined by liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS). However, to the best of our knowledge, no analytical methods based on capillary electrophoresis (CE)–MS/MS have been reported so far. Due to their non-polar nature, in this work, a non-aqueous CE (NACE) method coupled to quadrupole time-of-flight–MS is proposed for the first time to identify and quantify these mycotoxins. Determination was achieved in 4 min under optimum conditions: 40 mM ammonium acetate in 80:20 (v/v) acetonitrile-methanol (buffer), 30 kV (voltage), 80 cm (capillary length), 20 °C (capillary temperature) and 50 mbar × 30 s (injection). Higher selectivity can be achieved when compared with LC due to the formation of exclusive CE adducts such as $[M + CH_3CH_2NH_3]^+$. "All Ions" acquisition mode was selected as it allows the quantification of the usual ENNs, as well as the identity confirmation of less common ENNs.

The method was validated for wheat samples, obtaining limits of quantification from 4.0 to 8.3 μg/kg depending on the emerging mycotoxin, recovery values higher than 87.4%, and intra- and inter-day precision values (RSDs) lower than 15.1% in all cases. Finally, 29 wheat samples were analyzed, finding 26 samples with concentrations of enniatin B higher than the limit of quantification (7.5–1480 μg/kg), 20 for enniatin B1 (5.2–550 μg/kg), 7 for enniatin A (10–55 μg/kg), 4 for enniatin A1 (12.6–77 μg/kg) and 5 for BEA (9.2–16.4 μg/kg). Moreover, two other ENNs were tentatively identified.

1. Introduction

Mycotoxins are toxic secondary metabolites produced by certain fungi that can contaminate food during harvesting, processing or storage. Currently, several mycotoxins are included in the European Union (EU) legislation and maximum contents have been established in different raw materials and food commodities [1]. However, there are still some mycotoxins without maximum content allowed in regulation, but with some evidence of toxicity. Those are known as "emerging mycotoxins" and include some *Fusarium* toxins such as enniatins (ENNs) and beauvericin (BEA) [2]. They are predominantly found in cereal grains and their products. The presence of these contaminants in foodstuff is a source of concern because, although adverse effects have not been attributed to these mycotoxins in humans, some studies have shown evidence of cytotoxic activity for ENNs and BEA, probably linked to their ionophoric properties [3–5].

In this sense, due to the possible toxicity of these compounds, the European Food Safety Authority (EFSA) concluded that acute exposure to BEA and ENNs do not indicate concern for human health, but there might be a concern regarding chronic exposure, although no firm conclusion could be drawn [6]. Later, the European Medicines Agency (EMA) recommended the withdrawal of fusafungine, which contained a mixture of ENN cyclohexadepsipeptides, from the market due to rare but severe allergic reactions [7].

Abbreviations: BEA, beauvericin; BGE, background electrolyte; DEAH, diethylammonium; EFSA, European Food Safety Authority; ENN, enniatin; ENNA, enniatin A; ENNA1, enniatin A1; ENNB, enniatin B; ENNB1, enniatin B1; EMA, European Medicines Agency; EP, European Pharmacopoeia; ESI, electrospray ionization; ETMA, ethyltrimethylammonium; EtOH, ethanol; EU, European Union; FA, formic acid; FBF, find by formula; FWHM, full width at half maximum; IPA, isopropanol; MeCN, acetonitrile; MeOH, methanol; MT, migration time; NACE, non-aqueous capillary electrophoresis; PCDL, personal compound database and library; RSD, relative standard deviation; SALLE, salting - out assisted liquid-liquid extraction; TEAH, triethylammonium.

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So far, 39 naturally occurring ENNs analogues have been identified [8–10], but only seven ENNs (A, A1, B, B1, B2, B3 and B4) have been found in cereals, being ENNs (A, A1, B and B1) the most frequently detected in foods and feeds [2,5].

Nowadays LC–MS/MS has become the method of choice for the quantification of mycotoxins (including BEA and ENNs) in complex matrices, allowing a reliable identification and quantification of these mycotoxins in a wide variety of cereals and derived products, with limits of quantification (LOQ) in the low ppb range [8,11].

As an alternative to LC–MS/MS, mycotoxins in food samples have also been determined by CE coupled to different detection systems, although the applications are scarce. For instance, ochratoxin A, aflatoxins or patulin have been determined in food samples by CE methods [12–15]. A recent review summarizes the applications of CE combined with different pre-concentration approaches, in the last years [16]. However, to the best of our knowledge, CE has not been used to determine ENNs and BEA so far, although this technique could be used to separate compounds with ionophore character [17], such as ENNs and BEA. Among CE modes, non-aqueous capillary electrophoresis (NACE), which uses ionic solutions in organic solvents as a background electrolyte (BGE), could be suitable for separating these non-polar compounds. BGE components can charge the compounds or interact with them, achieving a selective separation in NACE [18]. Moreover, NACE is compatible with MS due to the medium-high volatility of most organic solvents. Recently, a NACE–QqQ MS/MS method was developed to separate and determine six highly hydrophobic peptides, showing the suitability of NACE–MS instead of reverse phase LC–MS [19].

On the other hand, the use of "All Ions" fragmentation acquisition mode in a QTOF MS analyzer, which transmits precursor ions into a collision-induced dissociation for fragmentation, producing fragment ion information after each full MS scan, allows qualitative and quantitative analysis in one run. The simultaneous acquisition of both precursor and product ions makes it feasible to identify compounds, and to obtain enough data points for each chromatographic peak to perform a reliable quantification [20].

Considering the increasing interest in these emerging mycotoxins and the absence of applications of CE–MS for their determination, a first approach based on the ability of NACE–MS in "All Ions" mode to determinate ENNs and BEA is proposed in this work. To show its suitability, the method has been combined with a simple sample treatment based on a salting-out assisted liquid extraction (SALLE) procedure and applied to the analyses of wheat samples.

2. Materials and methods

2.1. Reagents and chemicals

LiChrosolv® LC–MS grade acetonitrile (MeCN, ≥99.97%), methanol (MeOH, ≥99.97%), isopropanol (IPA, ≥99.9%), formic acid (FA, 98–100%), acetic acid (100%), ammonia solution (25%), water, and ethanol (EtOH, ACS grade, ≥99.5%) were purchased from Merck (Darmstadt, Germany), while FA eluent additive for LC–MS (≥99.5%) was purchased from Fluka (St. Louis, MO, USA). These solvents were used to prepare the instrument tune solution, sheath liquid, BGE and standards.

A mixture containing LC/MS ESI low concentration tune mix and hexamethoxyphosphazine (HP-0321) from Agilent Technologies (Santa Clara, CA, USA) was used to tune and mass calibrate the instrument. A reference mixture containing purine and hexakis (1H, 1H, 3H-tetrafluoropropoxy)phosphazine (HP-0921) (Agilent Technologies) was used for online accurate mass calibration (internal reference mass correction).

Ultrapure water obtained from a Milli-Q Plus system (Millipore Bedford, MA, USA). Magnesium sulfate ($MgSO_4$, 96%) and sodium chloride (NaCl, 99–100.5%) from Panreac Química (Barcelona, Spain), sodium citrate (≥99%) and disodium hydrogen citrate sesquihydrate (≥99%) from Sigma Aldrich (Darmstadt, Germany) were used for sample treatment.

Individual chemical standards of enniatin A (ENNA), enniatin B1 (ENNB1) and BEA (all three ≥95%) from Sigma Aldrich, enniatin B (ENNB) (10 μg/mL in MeCN) from n'TOX (Saint Jean d'Illac, France) and enniatin A1 (ENNA1) (≥99%) from Caymal Chemical Company (Ann Arbor, Michigan, USA) were used for method optimization and identification. Individual standard solutions of 10 mg/L were prepared by dissolving solid standards in MeCN. Multi-mycotoxins intermediate working solutions (0.1 and 1 mg/L of ENNA, ENNA1, ENNB, ENNB1 and BEA) were prepared by combining suitable aliquots of each individual standard solution in MeCN. These solutions were stored at −20 °C.

Nylon syringe filters, 0.2 μm × 13 mm (Pall Corporation, Puerto Rico) were used for filtration of sample extracts prior to the injection into the NACE system.

2.2. Instrument and equipment

A high-speed laboratory crusher (Hukoer, China) was used for sample grinding. A vortex-2 Genie (Scientific Industries, Bohemia, NY, USA) was used for standard preparation. A multitube vortexer (Benchmark Scientific, Sayreville, NJ, USA), a Universal 320R centrifuge (Hettich ZENtrifugen, Tuttlingen, Germany) and a nitrogen dryer EVA-EC System (VLM GmbH, Bielefeld, Germany) were used for sample preparation.

Instrumentation consisted of an Agilent 7100 CE system (Agilent Technologies, Waldbronn, Germany) coupled to an Agilent 6550 iFunnel QTOF mass spectrometer. This platform was equipped with a Dual Agilent Jet Stream electrospray ion source (Dual AJS ESI). An Agilent 1260 Infinity II isocratic pump was used to deliver sheath liquid including mass reference for online reference mass calibration. This analytical instrumentation was controlled through the Mass Hunter Workstation Acquisition Software (version 10) from Agilent Technologies.

Separations were performed in a bare fused silica capillary (80 cm of total length, 50 μm i.d., 363 μm o.d.) from Polymicro Tech. (Phoenix, AZ, USA).

2.3. Samples

Twenty-nine wheat samples destined for human consumption were randomly purchased in 23 different local markets in the western region of Algeria in 2018. To obtain representative samples, several subsamples were taken from each batch, being thoroughly mixed to achieve a final 10-g sample. The samples were milled and homogenized when collected, and then stored under appropriate conditions (at −20 °C, in the dark) for two and half years until analysis. Wheat samples for human consumption purchased in a local market in Granada (Spain) were used as blank samples for validation.

2.4. Mycotoxin extraction and sample preparation

ENNs and BEA were extracted from wheat samples using a modified method previously applied by Mahdjoubi et al. [21]. Briefly, 0.5 g of milled sample were weighed in a polypropylene centrifuge tube (15 mL), 2 mL of water was added, and the mixture was vortexed for 10 s. Subsequently, 2.5 mL of 2% FA in MeCN was added to the tube and vortexed again for 2 min. Then, 1 g of $MgSO_4$, 0.25 g of NaCl, 0.25 g of sodium citrate and 0.125 g of disodium hydrogen citrate sesquihydrate were added and the tube was vortexed vigorously for 1 min. After centrifugation at 4500 rpm (3722×*g*) for 5 min, the upper supernatant layer was transferred to a 4-mL vial, evaporated to dryness under a gentle stream of nitrogen, and reconstituted to a final volume of 0.5 mL with MeCN.

For recovery studies, samples were fortified at three concentration levels (10, 100 and 1000 μg/kg of each mycotoxin), homogenized by vortexing for 10 s, and left to stand for 15 min to allow the mycotoxins to interact with the wheat matrices; then the analytical procedure described above was applied. Each analysis was carried out in duplicate

and injected twice (2 experimental and 2 instrumental replicates).

2.5. Capillary electrophoresis procedure

New capillaries were conditioned with the running buffer for 25 min at 1 bar and 20 °C. At the beginning of each day, the capillary was preconditioned also with this procedure. In order to obtain an adequate repeatability between analysis, capillary was rinsed with water for 2 min at 1 bar and 20 °C and then with the BGE for 2 min at the same conditions. Separation was performed in NACE mode using a BGE that consisted of 40 mM ammonium acetate solution in a 20:80 (v/v) MeOH: MeCN mixture. A voltage of 30 kV was applied for the electrophoretic separation, obtaining an electric current of 6 μA. The temperature of the capillary was kept constant at 20 °C. Standard solutions and samples dissolved in MeCN were hydrodynamically injected at 50 mbar for 30 s. After sample injection, a plug of BGE was hydrodynamically injected at 50 mbar for 2 s. At the end of the working day, the capillary was rinsed with water for 4 min, followed by MeOH for 4 min and afterwards, it was dried with air for 2 min at 1 bar and 20 °C.

2.6. CE–QTOF MS/MS analysis

Sheath liquid consisted of 10 mM ammonium formate in 75:25 (v/v) IPA:water, which was delivered at a flow rate of 10 μL/min by an isocratic pump.

The mass spectrometer was operated in positive ionization mode. Standards and samples were measured using "All Ions" MS/MS technique. High-resolution accurate mass data were acquired after setting up a QTOF MS/MS method with two sequential experiments at two alternating collision energies (one full scan at 0 V, followed by one MS/MS scan at 40 V). The low energy spectra (0 V) were used to obtain the precursor ions and the high-energy (40 V) spectra to obtain mainly their fragment ions. The parameters of the Dual AJS ESI source were as follows: nebulizer gas (N_2) pressure was set to 10 psi, whereas the drying gas (N_2) flow rate was set to 11 L/min at 250 °C, and the sheath gas flow rate was established at 3.5 L/min at 195 °C. The capillary, nozzle, fragmentor and 1 RF Vpp octopole voltages were set at 4000, 2000, 380 and 750 V, respectively. The nebulizer gas pressure was set to 0 psi during the injection. The instrument was calibrated and tuned according to procedures recommended by the manufacturer. MS and MS/MS data were stored in positive polarity using both centroid and profile mode at a MS scan rate of 3 spectra/s, and 2704 transients/spectrum. Accurate mass spectra in MS and MS/MS mode were acquired in the MS range 50–1000 *m/z*. Typical resolution (full width at half maximum) was 14,500 at 118.0862 *m/z* and 26,000 at 922.0098 *m/z*. To ensure the desired mass accuracy of recorded ions, continuous internal calibration was performed during analyses by using as reference mass the signals at 121.0509 *m/z* (protonated purine) and 922.0098 *m/z* [protonated hexakis (1H,1H,3H tetrafluoropropoxy) phosphazine or HP-0921] in the positive ionization mode.

2.7. Data processing

ENNs and BEA identity confirmation was performed using MassHunter Qualitative Analysis (version 10.0) software. "Find by Formula" (FBF) algorithm allowed correlating the precursor ions obtained at 0 V to the fragments generated at 40 V for a particular migration time. Each correlation was compared with those stored in the personal compound database and library (PCDL) based on its exact mass and isotopic pattern (including the exact masses of isotopes, the calculated abundances of all isotope masses of interest, and the isotopic spacings between them). Moreover, PCDL was used to select potential fragment ions, which were then compared (migration time, peak width, and peak symmetry) with the precursor ion to achieve a reliable identification.

Mycotoxins PCDL provided by Agilent contains compound information including the name, formula, accurate mass, structure, and MS/MS spectra acquired at the selected collision energy (40 V). This PCDL was improved adding data from literature [22,23] and from our own results. The final PCDL included information about 39 ENNs and BEA (see Supplementary data Table S1).

The correlation algorithm considered all ions with $z = 1$ exceeding 500 TOF counts, *m/z* error ±5 ppm and Q-score (Agilent MassHunter peak quality metric that ranges from 0 to 100 which is an estimatation of how likely a feature is in an actual molecule) ≥70%. Ions and adducts formation in positive ionization mode ($[M+H]^+$, $[M+Na]^+$, $[M + NH_4]^+$, $[M + CH_6N]^+$, $[M + C_2H_8N]^+$, $[M + C_3H_{10}N]^+$) as well as the neutral losses (H_2O) were considered to carry out the identification. The matching scores of the observed mass, isotopic abundance distribution and isotopic spacing for each analyte were determined to be higher than or equal to 100, 80 and 80 (full score: 100), respectively. Fragment ions (at least two) were confirmed when the difference in the MT was <0.1 min, S/N ratio of fragment ion was >5 and comigration score of precursor and fragment ions was >97%.

All found compounds were also visually checked for adducts (type and relative abundance), peak shape of the fragment ion, fragment-to-precursor ions abundance ratio and presence across the replicates. The compounds that complied with all the parameters previously mentioned were classified as Level 2 identity confirmation (probable structure by library spectrum match). When reference standards were available and migration order and MS/MS fragments were confirmed, identifications were classified as Level 1 confidence (confirmed structure by reference standard).

Finally, QTOF Quantitative Analysis (version B.09.00) software from Agilent was used to quantify the most well-known ENNs (ENNA, ENNA1, ENNB, ENNB1) and BEA found in analyzed samples, using the quantifier (precursor ion) and two qualifier ions (the two most abundant fragments for each ENNs and BEA, highlighted in Supplementary data Fig. S1).

3. Results and discussion

3.1. Optimization of electrophoretic separation

Main variables affecting the separation and simultaneous quantification of the ENNs and BEA were optimized to maximize sensitivity and reduce analysis time.

Electrophoretic separation was performed in a bare fused silica capillary of 80 cm, at a constant temperature of 20 °C, applying a voltage of 30 kV. Separation voltages lower than 30 kV were not tested because they increase the migration time. Shorter capillaries and higher temperatures could also reduce the analysis time but, as the analysis time was already quite short, no further values were tested.

Under these conditions, the influence of nature and concentration of BGE on the separation was investigated. Separation medium was optimized using 20 mM ammonium formate in different organic solvents (100% EtOH, 100% MeOH, 80:20 (v/v) MeCN:MeOH and 90:10 (v/v) MeOH:EtOH). Higher sensitivity for all compounds and separation time under 4 min were achieved with 80:20 (v/v) MeCN:MeOH. Increasing MeCN concentration decreases the migration time and led to higher and narrower peaks. On the other side, MeOH and EtOH have the opposite effect, they increase the migration time. In fact, baseline resolution for the five compounds was achieved with 100% EtOH, although separation time increased up to 20 min. Thus, 80:20 (v/v) MeCN:MeOH was selected as organic solvent. Then, the influence of ammonium formate concentration was investigated at 20 and 40 mM. Sensitivity for all compounds significantly increased at 40 mM. Unfortunately, we were not able to dissolve higher concentrations as ammonium formate precipitates. The type of electrolyte was also investigated, and ammonium formate was compared with ammonium acetate. Sensitivity improved again for all compounds with ammonium acetate. So, 40 mM ammonium acetate in 80:20 (v/v) MeCN:MeOH was selected as optimum BGE. Regarding injection solvent, MeCN, 80:20 (v/v) MeCN:MeOH and BGE

were tested with a 5 s injection. MeCN was the solvent that presented the best peak efficiency for all compounds. Under optimum conditions, this NACE method allowed the separation of ENNs and BEA in less than 4 min, which is faster than recent methods based on LC [8,24–26].

The separation of ionophores compounds as these ENNs and BEA by NACE might be possible because they could form charged complex with the ammonium cations from BGE, known as heteroconjugated complexes [27]. In these complexes, ENNs and BEA act as ligands. In fact, Makrlík et *al.* proposed a theoretical optimized complex (NH_4^+-ENNB), in which NH_4^+ ion is bound by three hydrogen bonds to the three carbonyl oxygens of the ENNB [28]. Also, Bondarev theoretically predicted that the stability constant (log K_{MLP}) for the NH_4^+ complexed with ENNB in acetonitrile was 4.17 [29], which proves the stability of this complex in conditions like the optimized separation medium.

3.2. Optimization of NACE-ESI–MS/MS procedure

One of the advantages of using CE as separation technique over LC is its higher selectivity due to the formation of additional adducts, not found using LC with the same ESI conditions. These new adducts could contribute to a better compound identification. Fig. 1 shows MS spectra obtained for ENNB using ammonium acetate as electrolyte by NACE–MS and LC–MS. As can be seen, MS spectrum depends on the separation technique. Under similar conditions, higher selectivity can be achieved by CE when compared to LC due to the formation of distinct ionophore cation adducts for ENNs and BEA, such as $[M + CH_3CH_2CH_2NH_3]^+$ and particularly $[M + CH_3CH_2NH_3]^+$, in addition to the common ones ($[M + NH_4]^+$, $[M+H]^+$, $[M + CH_3NH_3]^+$ and $[M+Na]^+$). Moreover, when triethanolamine was tested as electrolyte, even more additional adducts were detected such as $[M + DEAH]^+$, $[M + ETMA]^+$ and $[M + TEAH]^+$. So, the electrolyte must be carefully studied as it greatly affects the selection of the precursor ion. Potential precursor ions for studied compounds by NACE–MS using ammonium acetate as electrolyte are summarized in Supplementary data Table S2. $[M + NH_4]^+$ adducts were selected to quantify ENNB, ENNB1 and BEA because they provided the highest S/N ratio under working conditions. Also, $[M+H]^+$ adducts were selected for ENNA and ENNA1 to avoid isobaric interferences from comigrating adducts with structural isomerism. In any case, these isomers can be unambiguously identified by MS/MS as shown in Supplementary data Fig. S1. This figure also includes the most abundant fragments for each ENNs and BEA.

Ionization suppression was evaluated by a Student's t-test. A mixture of standards containing the five compounds was injected in triplicate, and the average peak area was compared with that obtained from triplicate injections of individual solutions. Calculated statistical parameter were lower than the tabulated values (P = 95%) in all cases.

Selection of the optimum values for the sheath-liquid parameters in NACE-ESI–MS/MS separation is a key step to improve the sensitivity of the method. Thus, sheath-liquid composition was optimized using a standard solution of 1 μg/mL of each analyte. The signal intensity was selected as response variable. First, the sheath-liquid composition was evaluated considering a flow rate of 10 μL/min, a dry gas temperature of 250 °C, a nebulizer pressure of 10 psi, a dry gas flow rate of 11 L/min, an ESI voltage of 4000 V and a fragmentor voltage of 380 V. The influence of nature and proportion of organic solvent (MeOH, MeCN, IPA, EtOH) and electrolyte in the sheath-liquid, as well as its flow rate, were studied (see Supplementary data Fig. S2). The response was better with IPA than with MeOH, MeCN or EtOH for all compounds. Then, percentage of IPA in water was investigated (50:50 (v/v) and 25:75 (v/v) water-IPA), providing 75% IPA better results in all cases. The nature of the electrolyte (ammonium acetate and ammonium formate) was also studied. Although the separation buffer contained acetate, slightly better results were achieved with formate in the sheath liquid. Regarding formate concentration, increasing the concentration from 5 to 10 mM increased the peak area in all cases, but when the concentration increased to 20 mM, the peak area did not change. Therefore, 10 mM was selected as the optimal formate concentration in the sheath liquid. Flow rate was also studied. It is expected that the reduction of the flow rate from 10 to 5 μL/min decreases the dilution of compounds at the capillary outlet and provides better sensitivity. However, this reduction did not increase the peak areas, except for ENNA and ENNA1. On the other hand, a flow rate of 15 μL/min provided very reproducible measurements but at the expense of lower signals. Finally, 10 μL/min was selected as compromise between precision and sensitivity.

In summary, the optimum sheath liquid composition for the detection of ENNs and BEA in NACE–MS/MS was 10 mM ammonium formate in 25:75 (v/v) H_2O-IPA at a flow rate of 10 μL/min.

Finally, the optimization of the MS/MS parameters was performed. "All Ions" acquisition mode was selected, as it allows the simultaneous acquisition of both precursor and product ions, increasing the accuracy in the identification of unusual ENNs, for which standards are not available, and allowing the quantitation of BEA and the most common ENNs, for which standards are available. Some MS acquisition parameters such as acquisition speed, number of experiments and collision energy were studied. The influence of acquisition speed on the peaks of the electropherogram was studied, comparing 3 and 6 spectra/second. Three spectra/second was preferred because it provided a two-fold improvement in the S/N ratio for all compounds. The number of experiments (different collision energies) was also studied. Two experiments (0 and 40 V of collision energy) were compared with four (0, 10, 20 and 40 V). Better balance between spectral quality and points per chromatographic peak (at least 9 points for each compound at 10 μg/L) was obtained with just two experiments. As expected, 40 V was the collision energy that provided more fragments for the Mycotoxins

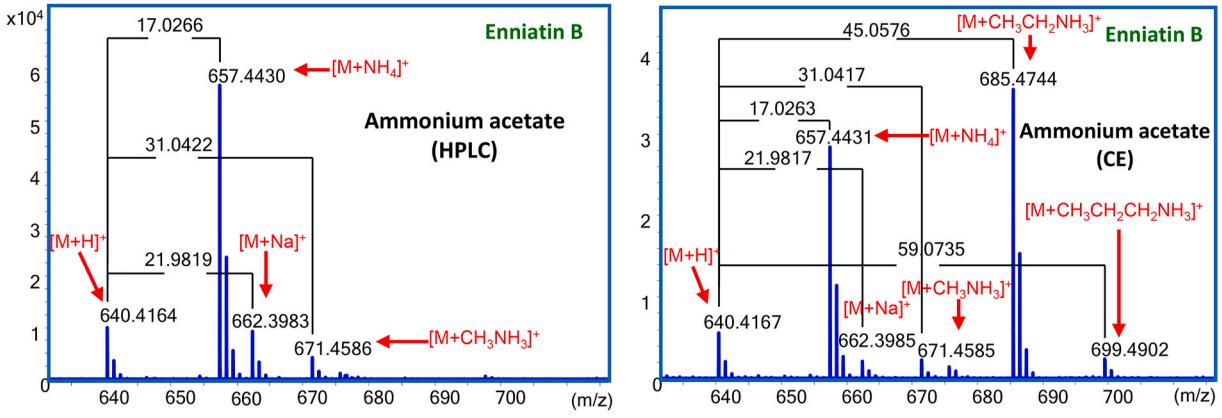

Fig. 1. MS spectra obtained for enniatin B using ammonium acetate as electrolyte by non-aqueous capillary electrophoresis–mass spectrometry and liquid chromatography–mass spectrometry.

Personal Compound Database and Library (PCDL).

3.3. Sample treatment optimization

The analytical method was applied to wheat samples, since according to literature, up to eight *Fusarium* species (*F. acuminatum*, *F. arthrosporioides*, *F. avenaceum*, *F. tricinctum*, *F. torulosum*, *F. kyushuense*, *F. poae*and *F. sporotrichioides*) have been reported to be able to produce ENNs in wheat [30].

Sample treatment consists of a previously published SALLE method [21] with some modifications. As detailed in Section 2.4, wheat samples were spiked with ENNs and BEA (10 μg/kg of each compound) 15 min before the extraction. Then, these mycotoxins were extracted by 2% FA in MeCN from the wheat sample. The FA influence on extraction was evaluated comparing three different percentages (0, 2 and 5%) of FA in MeCN. Better recoveries and lower matrix effect were obtained using 2% FA (recoveries among 92.9 and 99.7% for all compounds, n = 4, and matrix effect among −3.2 and −13.4%, being 0% absence matrix effect; n = 6).

Reconstitution solvent plays a key role because it must be able to redissolve ENNs after drying, and it should allow analyte focusing during the electrophoretic separation, as it is also the injection solvent. MeCN, MeOH, H_2O, ethyl acetate, chloroform, dichloromethane, hexane, cyclohexane, octane and dodecane were tested to reconstitute sample extracts. Dichloromethane, chloroform, and ethyl acetate provided better results than MeCN considering the number of theorical plates (N) calculated according to European Pharmacopoeia (EP). However, current disruptions were quite often. This problem could not be solved using different ratios of dichloromethane-MeCN mix. Thus, MeCN was selected as injection solvent.

The injection time was also optimized. As expected, an increase in the injection time resulted in an increase on sensitivity. However, injection times higher than 30 s led to a loss of peak efficiency. Fig. 2 shows extracted ion electropherograms of a wheat sample spiked with 10 μg/kg of each analyte. A standard solution injected in similar conditions is shown in Supplementary data Fig. S3.

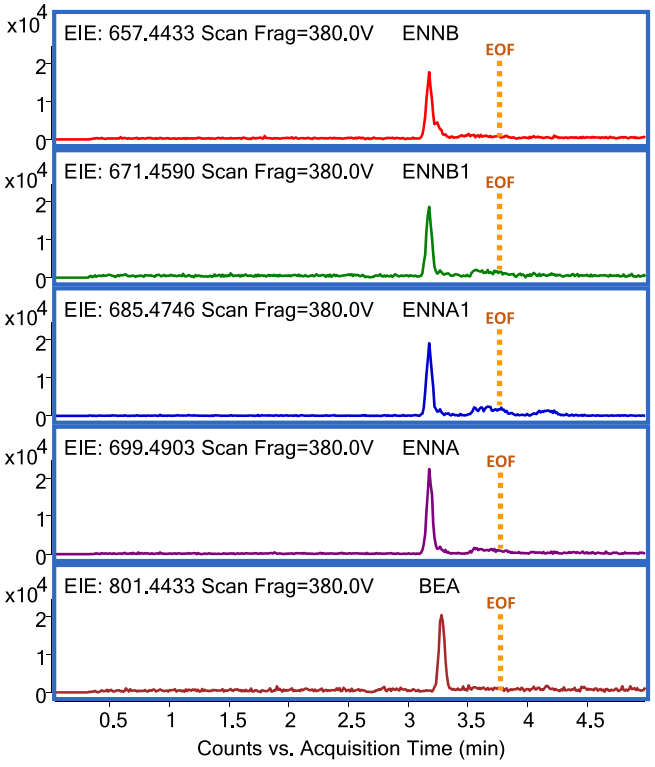

Fig. 2. Extracted ion electropherograms of 10 μg/kg of enniatin A, enniatin A1, enniatin B, enniatin B1 and beauvericin in acetonitrile (injection time: 30s), after extraction from spiked wheat. EOF: migration time of electroosmotic flow.

3.4. NACE–QTOF MS/MS method validation for quantitation of ENNs and BEA

Performance characteristics (matrix effect, linear dynamic range, recovery, repeatability, inter-day precision, LOD and LOQ) were established by a validation procedure in wheat samples spiked at different concentration levels of ENNA, ENNA1, ENNB, ENNB1 and BEA before sample preparation.

Method linearity was assessed by spiking blank samples before sample preparation at seven concentration levels (2 different samples, injected twice). All calibration curves showed a good linearity, with coefficients of determination (R^2) higher than 0.99 in all the cases. LODs and LOQs were determined as the concentration of compound giving a S/N ratio equal to 3 and 10, respectively. Table 1 shows the validation results. In the case of ENNA and ENNA1, $[M+H]^+$ adducts were used to quantify wheat samples, despite the lower sensitivity as their isomers, ENNB1 and ENNB, could otherwise interfere. Matrix effect was evaluated at three concentration levels: 10, 100 and 1000 μg/kg. In all cases, matrix effect was calculated as follow: ME = 100 × (signal of a spiked extract after sample treatment – signal of standard solution)/signal of standard solution. Low matrix effect (<I20I%) ranging from −2.8% to −18.1% was observed at three concentration levels for studied compounds in wheat (see Supplementary data Table S3).

The extraction process efficiency was evaluated by recovery studies, spiking blank samples at the same concentration levels used in the matrix effect study. Each sample was processed in duplicate and injected twice. The ratio of the peak areas of the compounds from samples spiked before the sample treatment and from samples extracts spiked after the treatment was used to calculate the recovery. The average recovery values were higher than 87% for the three levels spiked in wheat (see Table 2).

Intra-day (n = 15) and inter-day precision (n = 15) were evaluated and expressed as relative standard deviation (% RSD). Spiked blank samples (injected 3 times) at the same concentration levels mentioned above for the matrix effect were used. For the intra-day precision study, five spiked blank samples were analyzed on the same day and injected three times, while the inter-day precision was estimated through spiked blank samples analyzed on five different days and injected three times each day. Intra- and inter day precision was also evaluated at three concentration levels: 10, 100 and 1000 μg/kg. The relative standard deviation (% RSD) for intra-day and inter-day precision were lower than 15% in all cases (see Table 3).

3.5. NACE–QTOF MS/MS for identity confirmation of enniatins and beauvericin

Different wheat samples (29) from Algeria were prepared in accordance with the optimized method and analyzed by the proposed NACE–QTOF MS/MS. Their analyses allowed the identity confirmation of 6 ENNs and BEA in wheat samples. Among them, five could be confirmed as Level 1 by matching with chemical standards used in this study (ENNA, ENNA1, ENNB, ENNB1, and BEA). Due to the general lack of commercial standards, the identity confirmation of other ENNs was based on multiple identification points such as comparison of accurate mass, adducts, isotopologues distribution, characteristic fragment ions, and precursor ion-fragments comigration score. The inclusion of the ion ratio (product ion/precursor ion) calculation in the data processing method for targeted mycotoxin screening added an extra identification point. The list of identity-confirmed emerging mycotoxins is shown in Supplementary data Table S4, including supporting literature. This table includes five compounds identified as Level 1, and two compounds annotated as Level 2. As can be seen in this table, ENNs formed a [M +

Table 1
Results of the calibration curves. Statistical and performance characteristics of the proposed method for enniatins and beauvericin determination in wheat samples.

Compound	Adduct	Linear range (μg/kg)	Intercept (counts); STD	Slope (counts · kg/μg); STD	R^2	LOQ (μg/kg)	LOD (μg/kg)
ENNB	$[M + NH_4]^+$	4–5000	110,863; 29,695	1063; 21	0.990	4.0	1.2
ENNB1	$[M + NH_4]^+$	4–5000	119,200; 29,969	1166; 18	0.991	4.0	1.2
ENNA1	$[M+H]^+$	8.3–1000	5769; 3198	434; 9	0.990	8.3	2.5
ENNA	$[M+H]^+$	8.3–1000	5100; 3719	481; 10	0.991	8.3	2.5
BEA	$[M + NH_4]^+$	6.9–5000	−28,404; 24,500	1386; 19	0.994	6.9	2.1

STD: Standard deviation.

Table 2
Recovery results from wheat blanks spiked at 3 concentration levels (10, 100 and 1000 μg/kg).

Recovery[1] (%)	ENNB	ENNB1	ENNA1	ENNA	BEA
10 μg/kg	98.2	99.7	93.5	92.9	98.3
RSD (%)	2.2	6.0	5.0	7.3	9.1
100 μg/kg	92.3	89.5	87.4	90.2	94.6
RSD (%)	6.9	6.2	7.7	6.5	8.0
1000 μg/kg	90.1	95.0	95.3	90.0	99.0
RSD (%)	9.4	6.1	8.1	9.6	9.1

Table 3
Intra- and inter-day precision study in wheat blanks spiked at three concentration levels (10, 100 and 1000 μg/kg) before sample treatment.

Intra-day precision [a] RSD (%)	ENNB	ENNB1	ENNA1	ENNA	BEA
10 μg/kg	3.4	4.5	3.8	3.9	4.3
100 μg/kg	7.4	5.3	4.3	8.5	11.0
1000 μg/kg	8.4	8.1	8.0	8.1	10.3
Inter-day precision[b] RSD (%)	**ENNB**	**ENNB1**	**ENNA1**	**ENNA**	BEA
10 μg/kg	8.6	8.6	8.6	9.2	10.4
100 μg/kg	10.7	12.7	12.6	8.4	14.6
1000 μg/kg	12.2	14.9	12.6	9.1	15.1

[a] (n = 15, 5 experimental and 3 instrumental replicates/each level).
[b] (n = 15, 5 days and 3 instrumental replicates/each level).

$NH_4]^+$ ion in all cases in positive ionization mode, but also all of them were found to form $[M+H]^+$, $[M + CH_3NH_3]^+$, $[M + C_2H_5NH_3]^+$ and $[M + C_3H_7NH_3]^+$ adducts. These last adducts were used for confirmation purposes. ENNs identities were confirmed with an average absolute mass error less than 7 ppm, comigration score (this value allows to evaluate the co-migrated peak profiles between precursor ion and characteristic fragment ions, comparing their migration time, peak width, and peak symmetry) higher than 97%, at least two fragments and average score higher than 97.2%. Regarding order of migration of ENNs under applied electrophoretic conditions, most of ENNs appeared at the same average time 2.9 min while BEA appeared at 3.5 min.

Moreover, Level 2 identification was additionally supported by confirmation of fragments, which were acquired by "All Ions" acquisition mode by QTOF MS/MS working at low and high collision energy. During the method development, it was observed that low collision energies (10–20 V) led to insufficient fragmentation of analytes in comparison with higher values (40 V). Identification of mycotoxins was supported by the presence of their known fragments at high energy level (40 V). Also, most ENNs can be confirmed by detection of fragments with a difference of *m/z* of 100 (2-hydroxyisovaleric acid), 113 (aliphatic N-methylvaline) and 127 (N-methylisoleucine/N-methylleucine) in the high-energy spectrum [8], which correspond to neutral losses. There are several possibilities to explain the results for compounds 6 and 7 (Supplementary data Table S4). Compound 6, according to its formula, could be identified as ENNB2, ENNK1, ENNJ2 or ENNJ3. However, fragments obtained from compound 6 (210.1484, 228.1591 and 328.2110) were only assigned to ENNB2 and J3 by the PCDL. The neutral losses of 100.0519 (328.2110 and 228.1591 fragments, and 314.1956 and 214.1424) might correspond to 2-hydroxyisovaleric acid [31], but both ENNs contain various 2-hydroxyisovaleric acids. However, the relative abundance of the fragments of the compound 6 at 40 V corresponds to ENNB2 according to the PCDL spectrum. Moreover, ENNB2 has been previously found in wheat [31]. Compound 7, $[C_{31}H_{57}N_4O_9]^+$, could be identified as ENNB3 or ENNJ1 according to its formula. These compounds differ in group R3, R10 and R11 [31] of their structure, being $CH(CH_3)_2$, H and H for ENNB3, and CH_3, CH_3 and CH_3 for ENNJ1, respectively. However, in this case, fragments did not allow to discriminate between ENNB3 and ENNJ1, despite the additional benefit of the comigration score to confirm precursor-fragment associations. This is because the only two fragments (196.1319 and 214.1438) found and confirmed are the most abundant for both ENNs according to the PCDL spectra. As above, ENNB3 has been previously reported in wheat, while ENNJ1 has not. So, compound 7 might be ENNB3, but an assessment with analytical standards is required to provide further confirmation. An illustrative example of comigrating fragments is shown in Fig. 3.

3.6. Concentration and co-occurrence of enniatins and beauvericin in wheat samples

As stated before, ENNA, ENNA1, ENNB, ENNB1, ENNB2 and ENNB3, and BEA, were identified across the wheat samples. They are the most

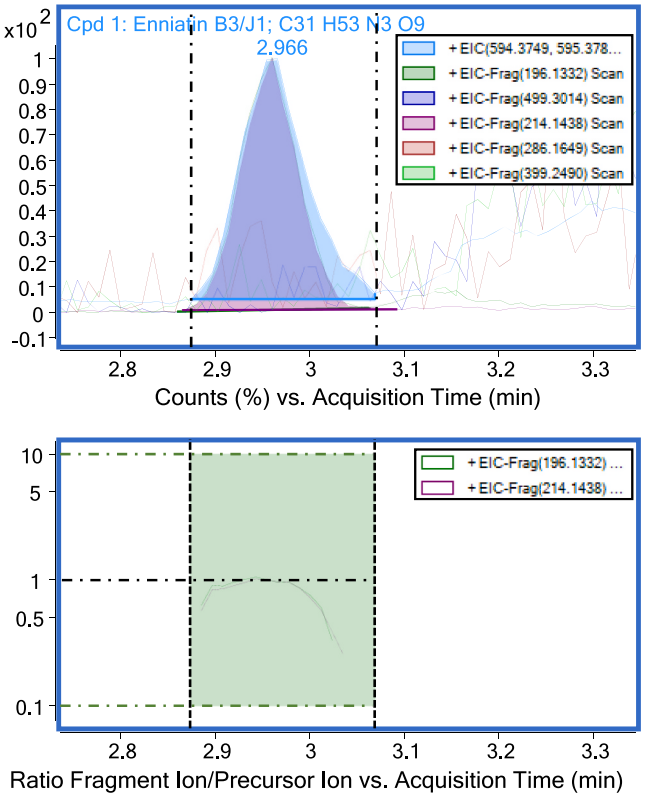

Fig. 3. Overlaid electropherogram and comigration plot of fragments of compound 7 (enniatin B3/J1) found in a wheat sample.

reported mycotoxins in wheat [32]. Supplementary Fig. S4 shows the extracted ion electropherograms of ENNs found in a wheat sample.

Compounds identified as Level 1 were quantified using calibration curves performed the same day that the sample analysis. All samples were randomly analyzed (3 experimental and 2 instrumental replicates). As can be seen in Supplementary data Supplementary data Table S5, 27 samples (93%) were contaminated with ENNB (concentrations above the LOD), while 26 showed concentrations above the LOQ (7.5–1480 μg/kg); 24 samples (83%) were contaminated with ENNB1, and 20 of them with concentrations above the LOQ (52–550 μg/kg). The incidence of the rest emerging mycotoxins was lower: 9 positive samples (28%) for ENNA, 7 of them with concentrations above the LOQ (10–55 μg/kg); 6 positive samples (21%) for ENNA1, 4 of them with concentrations above the LOQ (12.6–77 μg/kg); and finally, 5 positive samples (17%) for BEA, with concentrations above the LOQ (9.2–16.4 μg/kg). Those results are in accordance with recent studies where ENNB was the most frequent emerging mycotoxins found, and with the highest concentrations [30, 33,34].

Regarding the co-occurrence of different mycotoxins in the same sample, up to four different mycotoxins were detected in three samples, three different mycotoxins in 11 samples and two different mycotoxins in 11 samples.

4. Conclusions

This is the first time that a NACE–QTOF MS/MS platform has been used for identification and quantification of mycotoxins. Also, this is the first time that ENNs and BEA have been separated by CE. NACE allowed a fast, sensitive, and selective determination of ENNs and BEA by CE–MS/MS (QTOF), in less than 4 min, faster than UHPLC methods. All Ions mode provided quantitative and qualitative information in a single run. The satisfactory sensitivity and selectivity achieved allowed the application of the proposed method to wheat samples. Six ENNs and BEA were found in these samples, being ENNB and ENNB1 the mycotoxins with the highest incidence. Moreover, considering the suspected toxicity of these compounds, the high concentrations of mycotoxins found in some cases (more than 2000 μg/kg, as the sum of all mycotoxins found in the sample), as well as the frequent co-occurrence of more than one mycotoxin in the same sample could be a matter of concern, as additive or synergistic effect may occur.

Credit author statement

María del Mar Delgado-Povedano: Investigation, data treatment, Writing – original draft. Francisco J. Lara: Conceptualization, Investigation, Supervision, Writing – review & editing. Laura Gámiz-Gracia: Supervision, data discussion, Writing – review & editing. Ana M. García-Campaña: Supervision, Funding acquisition, project coordinator, Writing – review & editing.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.talanta.2022.123946.

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