Food Chemistry 252 (2018) 294–302

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Analytical Methods

Simple and rapid determination of 5-nitroimidazoles and metabolites in fish roe samples by salting-out assisted liquid-liquid extraction and UHPLC-MS/MS

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ARTICLE INFO

Keywords:
5-Nitroimidazoles
Fish roe samples
Salting-out assisted liquid-liquid extraction
Ultra-high performance liquid chromatography
Mass spectrometry

ABSTRACT

A novel multiresidue method is proposed for the determination of 12 5-nitroimidazoles and their metabolites in fish roe samples using UHPLC-MS/MS. A salting-out assisted liquid-liquid extraction procedure was performed prior to sample analysis. The separation of compounds was accomplished using a C18 Zorbax Eclipse Plus column (50 mm × 2.1 mm, 1.8 μm) at 25 °C and a mobile phase consisting of 0.025% (v/v) aqueous formic acid and pure MeOH at a flow rate of 0.5 mL/min. Parameters involved in ionization and fragmentation were also optimized. The method was characterized in terms of linearity ($R^2 \geq 0.9992$), extraction efficiency (≥68.9%), repeatability (RSD ≤ 9.8%), reproducibility (RSD ≤ 13.9%) and trueness (recoveries ≥81.4%). Decision limits (CCα) and detection capabilities (CCβ) were obtained in the ranges 0.1–1.0 and 0.2–1.7 μg/kg, respectively.

1. Introduction

5-Nitroimidazoles (5-NDZs) are wide-spectrum antibiotics that are used mainly in the treatment of anaerobic protozoan and bacterial infections. Their importance in human medicine is evidenced by the inclusion of metronidazole (MNZ), the most representative 5-NDZ compound, in the 'World Health Organization (WHO) Model List of Essential Medicines' (World Health Organization, 2015). Moreover, their application has been extended to veterinary medicine, where they are the drugs of choice for treatment of *Trichomonas gallinae* in racing and wild pigeons (Rouffaer, Adriaensen, De Boeck, Claerebout, & Martel, 2014). However, the use of 5-NDZs in animals intended for human consumption is forbidden because of their carcinogenic, mutagenic and genotoxic properties (Bendesky, Menéndez, & Ostrosky-Wegman, 2002; López Nigro, Palermo, Mudry, & Carballo, 2003). This ban has been established within European Union (EU) by Regulation No. 37/2010 (European Commission, 2010) and similar legislation exists in other countries, such as the United States (US) (Food Animal Residue Avoidance Databank, 2014) and China (USDA Foreign Agriculture Service., 2011). However, despite this ban, several alerts for these compounds in food products are still notified by, for example, the Rapid Alert System for Food and Feed (RASFF), which reports food safety issues within the EU (European Commission., 2016). However, no minimum required performance limit (MRPL), regarding 5-NDZ determination, has been established. For this reason, European Union Reference Laboratories (EURLs) have recommend that any confirmatory or screening method intended for 5-NDZ analysis should have decision limits (CCα) or detection capabilities (CCβ) of 3 μg/kg (European Union Reference Laboratories., 2007). As a consequence, in order to guarantee food safety, sensitive and selective methods are required for the determination of 5-NDZ residues in food products.

In general, LC-MS methods have been proposed for 5-NDZ determination (Mahugo-Santana, Sosa-Ferrera, Torres-Padrón, & Santana-Rodríguez, 2010) although traditional HPLC-based methods have largely been replaced by UHPLC-based methods. Consequently, analysis time and reagent consumption have decreased significantly and the features of analytical methods enhanced (Cronly et al., 2010; Gadaj et al., 2014; Rúbies et al., 2015; Tamošiūnas & Padarauskas, 2009; Xia et al., 2009). LC-MS methods have been developed for the detection of 5-NDZ residues in different types of food products, such as milk (Cronly et al., 2010; Tölgyesi et al., 2012), honey (Mitrowska, Posyniak, & Zmudzki, 2014), eggs (Mohamed et al., 2008; Mottier, Huré, Gremaud, & Guy, 2006), poultry and pork (Hurtaud-Pessel, Delépine, & Laurentie, 2000; Mitrowska, Posyniak, & Zmudzki, 2010; Zeleny, Harbeck, & Schimmel, 2009). However, few methods have been reported for 5-NDZ determination in fish or seafood (Sorensen & Hansen, 2000; Wagil et al., 2015) including prawn (Gadaj et al., 2014). To the best of our knowledge, there are no methods for the analysis of 5-NDZ residues in fish roe samples. Nevertheless, EURLs have included these matrices, namely fish muscle, crustaceans and fish roe as relevant for the monitoring of 5-

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https://doi.org/10.1016/j.foodchem.2018.01.101
Received 7 December 2016; Received in revised form 3 January 2018; Accepted 13 January 2018

NDZs (European Union Reference Laboratories, 2007), therefore, new analytical methods, including sample treatments, are required for their control in these food products.

Salting-out assisted liquid-liquid extraction (SALLE) uses water-miscible solvents, such as ethanol (EtOH), methanol (MeOH) or acetonitrile (MeCN), as extraction solvents because water-miscibility is reduced when salts are added to samples and extraction media (Zhang, Wu, Kim, & El-shourbagy, 2009). The use of water-miscible solvents as extraction solvents is especially relevant because the extraction of polar compounds, such as 5-NDZs, is improved in comparison with extractions achieved using water-immiscible solvents in classical liquid-liquid extraction (LLE) methods (Majors, 2009). SALLE has been widely employed for 5-NDZ extraction from various matrices because of its simplicity and effectiveness (Cronly, Behan, Foley, Malone, & Regan, 2009; Granja et al., 2013; Mitrowska et al., 2010; Mottier et al., 2006). However, this methodology has a major drawback when MS detection is employed due to its low effectiveness for sample clean-up. As a result, high matrix effect can be observed and the analytical signal can be decreased or inhibited. In order to solve this, a clean-up stage is usually carried out after SALLE. In QuEChERS methods, SALLE is followed by dispersive-solid phase extraction (d-SPE) with the aim of achieving sample clean-up (Anastassiades, Lehotay, Štajnbaher, & Schenck, 2003). The suitability of QuEChERS as sample treatment for multi-residue determination by LC-MS has been widely demonstrated (González-Curbelo et al., 2015; Rejczak & Tuzimski, 2015). However, this methodology has been rarely employed for 5-NDZ analysis (Mahugo-Santana et al., 2010; Wagil et al., 2015).

In this work, a new UHPLC-MS/MS method was developed for the determination of 12 5-NDZ drugs and metabolites in fish roe samples. According to the literature reviewed, it is the first time that a LC-MS-based method has been reported for this purpose. Additionally, SALLE is proposed for sample extraction although QuEChERS was also investigated to reduce matrix effects.

2. Materials and methods

2.1. Materials and reagents

All reagents used were analytical grade and solvents were HPLC grade, unless otherwise specified. Sodium chloride (NaCl) and magnesium sulfate ($MgSO_4$) were acquired from Panreac-Química (Madrid, Spain). MeOH, MeCN, acetone and ethyl acetate were purchased from VWR International (West Chester, PA, USA) while EtOH was acquired from Merck (Darmstadt, Germany). Formic acid and acetic acid (both MS grade) were supplied by Sigma-Aldrich (St. Louis, MO, USA). Additionally, d-SPE sorbents, namely C18 and primary-secondary amine (PSA), were acquired from Agilent Technologies (Waldbronn, Germany) whereas Z-Sep$^+$ was purchased from Supelco (Bellafonte, PA, USA). Ultrapure water (Milli-Q plus system, Millipore, Bedford, MA, USA) was used throughout the work.

Analytical standards of dimetridazole (DMZ; 1,2-dimethyl-5-nitroimidazole), MNZ (1-(2-hydroxyethyl)-2-methyl-5-nitroimidazole), ronidazole (RNZ; 1-methyl-2-carbamoyloxymethyl-5-nitroimidazole), ornidazole (ORZ; 1-(3-chloro-2-hydroxypropyl)-2-methyl-5-nitroimidazole), tinidazole (TNZ; 1-(2-ethylsulfonylethyl)-2-methyl-5-nitroimidazole), carnidazole (CRZ; 1-(2-ethylcarbamothioic acid O-methyl ester)-2-methyl-5-nitroimidazole), hydroxyl-metronidazole (MNZ-OH; 1-(2-hydroxyethyl)-2-(hydroxymethyl)-5-nitroimidazole), hydroxyl-dimetridazole (HMMNI; 1-methyl-2-hydroxylmethyl-5-nitroimidazole) and hydroxyl-ipronidazole (IPZ-OH; 1-methyl-2-(2′-hydroxyisopropyl)-5-nitroimidazole) were supplied by Sigma-Aldrich (St. Louis, MO, USA) while ipronidazole (IPZ; 1-methyl-2-isopropyl-5-nitroimidazole), secnidazole (SCZ; 1-(2-methyl-5-nitroimidazol-1-yl) propan-2-ol) and ternidazole (TRZ; 1-(3-hydroxypropyl)-2-methyl-5-nitroimidazole) were purchased from Witega (Berlin, Germany). Stock standard solutions were obtained by dissolving the appropriate amount of each 5-NDZ drug in MeCN, to a final concentration of 1 mg/mL. Stock standard solutions were kept in the freezer at −20 °C avoiding their exposure to light. An intermediate standard solution (5-NDZ concentration ranged from 1.25 to 3.75 μg/mL, depending on the compound) was obtained by diluting the appropriate amount of each stock standard solution with MeCN. These were stored in the dark at 4 °C but equilibrated to room temperature before use. Working standard solutions were prepared by dilution of the intermediate standard solution with water or mobile phase (according to the experiment).

Clearinert™ 13 mm syringe filters (0.22 μm pore size) were supplied by Bonna-Agela Technologies (Wilmington, DE, USA).

2.2. Instrumentation

LC separations were performed on an Agilent 1290 Infinity LC (Agilent Technologies, Waldbronn, Germany) equipped with a binary pump, a degasser, an autosampler (20 μL loop), and a column thermostat. MS measurements were carried out on a triple quadrupole (QqQ) mass spectrometer API 3200 (AB SCIEX, Toronto, ON, Canada) with electrospray ionization (ESI) interface. A C18 Zorbax Eclipse Plus RRHD (50 × 2.1 mm, 1.8 μm) column from Agilent Technologies (Waldbronn, Germany) was used. Data were collected by the Analyst® Software version 1.5 using the Scheduled MRM™ Algorithm (AB SCIEX).

A Polytron® PT 2500E homogenizer (Kinematica AG; Luzern, Switzerland), a Universal 320R centrifuge (Hettich Zentrifugen; Tuttlingen, Germany), a mechanical shaker (model 384 from Vibromatic; Noblesville, USA), a nitrogen dryer EVA-EC System (VLM GmbH; Bielefeld, Germany) and a vortex-2 Genie (Scientific Industries; Bohemia, NY, USA) were used throughout sample preparation procedures.

2.3. Sample treatment

Fresh hake (*Merluccius merluccius*) roe and packed fish roe products were purchased from a local supermarket (Granada, Spain). Two packed fish products were analyzed, specifically a product consisting of *Cyclopterus lumpus* (85.5%, w/w) and *Mallotus villosus* (9.5%, w/w) roe and a product consisting of *Oncorhynchus mykiss* roes. In the case of fresh hake roe, around 500 g (two roe) was selected for analysis. The outer membrane was removed with a knife and the eggs were collected. In the case of packed fish egg products, one pack of each product (50 g) was selected and the whole pack considered as one sample.

The eggs were placed in a 50-mL conical tube, ground and homogenized with a Polytron® for 10 min at 10,000 rpm. In all cases, a portion of 1.0 g was placed in a 15-mL conical tube and fortified with the desired 5-NDZ concentration. Water (1 mL) was added to samples, which were homogenized by vortex for a few seconds before being left to stand for 15 min. MeCN (5 mL) was added to samples, which were agitated by vortex for 30 s, and 0.1 g of NaCl and 0.5 g of $MgSO_4$ added subsequently. The mixture was mechanically agitated for 10 min and centrifuged for 10 min at 5000 rpm and 25 °C. Finally, 1 mL of the supernatant was dried under a nitrogen current at 40 °C and re-dissolved in 200 μL of 5:95 (v/v) MeOH/aqueous formic acid (0.025%, v/v) and vortexed for 2 min. The final solution was passed through a syringe filter and analyzed using the proposed UHPLC-MS/MS method.

2.4. UHPLC-ESI-MS/MS analysis

UHPLC separations were accomplished on a C18 Zorbax Eclipse Plus RRHD (50 × 2.1 mm, 1.8 μm) column using a mobile phase consisting of 0.025% (v/v) aqueous formic acid (eluent A) and MeOH (eluent B) at a flow rate of 0.5 mL/min. Separations were performed by gradient elution as follows: 0.0 min, 5% (v/v) of B; 1.5 min, 5% (v/v) of B; 3.0 min, 30% (v/v) of B; 4.0 min, 95% (v/v) of B; 5.0 min, 95% (v/v) of B; and, 6 min, 5% (v/v) of B. In order to guarantee that initial