Journal of Chromatography A, 1419 (2015) 37–44

Contents lists available at ScienceDirect

Journal of Chromatography A

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

Development and validation of a high performance liquid chromatographic–mass spectrometry method for the simultaneous quantification of 10 trichothecenes in ultra-high temperature processed cow milk

Myra E. Flores-Flores, Elena González-Peñas*

Organic and Pharmaceutical Chemistry Department, Faculty of Pharmacy, University of Navarra, , C/Irunlarrea 1, 31008 Pamplona, Navarra, Spain

ARTICLE INFO

Article history:
Received 3 July 2015
Received in revised form 7 September 2015
Accepted 21 September 2015
Available online 26 September 2015

Keywords:
Mycotoxin
Trichothecenes
Milk
UHT cow milk
LC–MS/MS

ABSTRACT

An LC–MS/MS (QqQ) method has been developed and validated for simultaneous determination of the following trichothecenes in UHT cow milk: nivalenol (NIV), deoxynivalenol (DON), deepoxy-deoxynivalenol (DOM-1), 3-acetyldeoxynivalenol (3-ADON), 15-acetyldeoxynivalenol (15-ADON), neosolaniol (NEO), diacetoxyscirpenol (DAS), fusarenon X (FUS-X), T-2 and HT-2 toxins. Sample treatment is simple and based on the extraction with acetonitrile (ACN), acidified with 0.2% formic acid, followed by a purification process, adding sodium acetate to the ACN/water extract in order to separate aqueous phase and, consequently, polar components of the milk. Validation of the method for all the 10 mycotoxins was successful; validation parameters taken into account were as follows: limits of detection (LOD) and quantification (LOQ), linearity, precision (within-day and between-day variability), recovery, matrix effect and stability. The LODs were 10.1, 2.5, 1.5, 1.9, 0.1, 0.5, 1.0, 0.08, 0.4 and 0.05 ng/mL for NIV, DON, DOM-1, FUS-X, NEO, 3-ADON, 15-ADON, DAS, HT-2 and T-2, respectively. Mean recovery values (obtained in intermediate precision conditions) were between 63.5 and 75.8 ($RSD_R \leq 15\%$) for all the mycotoxins. All the mycotoxins suffered from matrix effects, especially DON.

1. Introduction

Mycotoxins are secondary metabolites produced by molds and they can contaminate agricultural commodities intended for human food and animal feed [1]. In fact, approximately 20% of food products, mainly of plant origin, are contaminated with mycotoxins [2]. The presence of the mycotoxins in human food and animal feed could be a risk to human and animal health due to their toxicity [3] and could also produce economic losses due to refusal of contaminated products, reduced animal production and veterinary costs [4]. Co-occurrence of mycotoxins in food and feed is likely to appear because one type of fungus can produce different mycotoxins, and on the same substrate more than one type of mold could be present.

The *Fusarium* genus is probably the major producer of mycotoxins on temperate areas [5]. Trichothecenes are among the most frequently detected fusariotoxins in human and animal cereal-based food [6]. Trichothecenes are classified into two groups, A and B, according to their structural characteristics. Type A, including diacetoxyscirpenol (DAS), neosolaniol (NEO), HT-2 and T-2 toxins, can cause vomiting, diarrhea, leukopenia, necrotic lesions and hemorrhage. T-2 toxin is a highly toxic compound, especially for the immune system. It has been related to the Alimentary Toxic Aleukia (ATA) illness [7], to the inhibition of protein synthesis [5], to bovine infertility and to abortion [8]. The European Food Safety Authority [9] considers T-2 toxin to be one of the most dangerous contaminants, and the European Commission has recommended a tolerable daily intake for humans (TDI) of 100 ng/kg body weight (b.w.) for the sum of T-2 and HT-2 toxins [10].

With regard to type B, the trichothecenes classified into this group are as follows: deoxynivalenol (DON, vomitoxin), 3-acetyldeoxynivalenol (3-ADON), 15-acetyldeoxynivalenol (15-ADON), nivalenol (NIV) and fusarenon X (FUS-X). Their toxic effects are food refusal and vomiting, kidney problems and immunosuppression [11]. DON has emetic properties and provokes feed refusal in animal; in addition, it supposedly suppresses resistance to bacterial infections such as *Listeria* and *Salmonella* [12]. The European

* Corresponding author.
E-mail addresses: mflores.6@alumni.unav.es (M.E. Flores-Flores), mgpenas@unav.es (E. González-Peñas).

http://dx.doi.org/10.1016/j.chroma.2015.09.069
0021-9673/

Commission has recommended a TDI of 1.2 μg/kg b.w. per day for NIV [13] and a provisional maximum tolerable daily intake (PMTDI) of 1 μg/kg b.w. for DON and its acetylated derivatives (3-ADON and 15-ADON) [14].

Milk is a highly consumed food. For instance, in Europe, more than 150 kg/capita/year are consumed [15]. In 2013, the estimated milk production was approximately 159 million tons per year, approximately 15% of the European agricultural output [16]. Due to its economic impact and its importance as a human food, especially during the first years of life, it is very important to determine the presence of contaminants in this matrix in order to expand our knowledge regarding this aspect.

There is little information regarding the presence of trichothecenes in milk due to the low number of samples analyzed and their presence in this matrix has not been regulated. Ruminants are supposedly less affected for mycotoxins due to the rumen metabolism [4], and their concentration levels, if any, in milk from healthy cows are supposed to be low. However, certain cow diseases can alter rumen metabolism [17] and favors the possible presence of mycotoxins in milk. Sørensen and Elbæk detected DOM-1 in a 25% of milk samples obtained from cows with undiagnosed symptoms of disease [18].

In addition, high levels of mycotoxins in feed could also alter rumen metabolism [17]. Some trichothecenes in food intended for animal feeding are a major concern, especially DON, T-2 and HT-2. The European Union has established guidance values for DON in products intended for animal feed and has recommended the collection of more data for T-2 and HT-2 in these matrices. In order to assess co-occurrence, simultaneous analysis of diverse mycotoxins in feed has also been recommended [19].

Few studies regarding transference of trichothecenes into milk have been carried out. Only transmission of DON and T-2 to cow milk has been studied, but in few animals and with variable results due to the use of different doses or routes of administration. DON is mostly biotransformed within the rumen and detected in milk as DON and deepoxy-deoxynivalenol (DOM-1) [20–22], whereas T-2 has been detected in the milk without metabolization [23] and metabolized into HT-2, NEO, 4-deacetylneosolaniol, and 4 more unknown metabolites [24]. Metabolites could have different toxic effects than the parent mycotoxin [25,26]. More studies are needed in order to increase knowledge regarding this aspect.

Nowadays, mycotoxin detection in milk generally focuses on aflatoxin M1 (AFM1); little is known regarding the presence of other mycotoxins in this matrix. However, our previous survey [27] has found that low levels of other mycotoxins such as DOM-1 can be present in milk samples worldwide. Interestingly, Slovakia and Czech Republic have established maximum limits for mycotoxins other than AFM1 in milk [28]. In the case of different trichothecenes being present in milk, together they could have additive or synergic toxic effects on human health; however, this aspect has not been studied.

In addition, the study of the presence of mycotoxins in milk can be used as a control for animal feed contamination and as a tool to study absorption and distribution of mycotoxins in animals [29].

Validated analytical methods, especially those capable of simultaneous analysis, are needed in order to assure that contaminated milk does not reach consumers; in addition, they will be helpful in broadening our knowledge regarding toxicity, transmission from feed into milk, etc. However, due to the different physicochemical characteristics of mycotoxins and to the complexity of milk composition (lipids, proteins and sugars, among other components), the development of this type of methods is an analytical challenge.

Trichothecenes in milk have usually been analyzed using gas chromatography, in which case a step of derivatization is necessary before the chromatographic analysis. Most of these studies are based on the analysis of a single mycotoxin (usually DON or T-2) instead of the simultaneous analysis of type-A and type-B trichothecenes [18]. The use of liquid chromatography coupled with MS, in which derivatization is not necessary and in which the simultaneous analysis of different chemical structures is permitted, is an advantage. Moreover, LC–MS reaches the high selectivity and sensitivity needed in the determination of contaminants in food matrixes. In fact, methods for multimycotoxin detection in food using this technique have increased over the last years. However, methods devoted to trichothecenes determination in cow milk using LC–MS/MS are scarce.

Jia et al. validated an analytical method for the simultaneous analysis of 58 mycotoxins, including 9 trichothecenes, in commercial milk using UHPLC/ESI Q-Orbitrap and the QuEChERS extraction procedure [29]. On the other hand, after acetonitrile and hexane extraction from milk, a clean-up phase by solid phase extraction (SPE), and with the use of LC–ESI–MS/MS, Sørensen and Elbæk simultaneously determined the following trichothecenes: DON, DOM-1, 3-ADON, 15-ADON, DAS, T-2 and HT-2 [18]. In addition, Beltrán et al. determined 18 mycotoxins (nine trichothecenes) in different matrixes, including milk, using UHPLC–MS/MS [30]. Finally, Tsiplakou et al. developed a method capable of determining 11 mycotoxins in milk using LC–MS/MS, including the trichothecenes DAS, T2 and HT-2 [31].

The aim of this work is to develop and to validate a method capable of analyzing 10 trichothecenes (NIV, DON, DOM-1, 13-ADON, 15-ADON, NEO, DAS, FUS-X, HT-2 and T-2) simultaneously in ultra-high temperature (UHT)-cow milk using LC–MS/MS triple quadrupole (QqQ) mass spectrometer. Other available methods for trichothecenes determination in milk use different equipment or include a lower number of trichothecenes in a single analysis. Due to milk composition characteristics, the extraction procedure has been carefully studied, using different extraction solvent mixtures. The validated method was applied to the simultaneous analysis of 10 trichothecenes (type-A and type-B) in 13 samples of UHT cow milk collected in Navarra (Spain).

2. Experimental

2.1. Chemicals and reagents

Methanol (LC/MS grade), formic acid (mass spectrometry grade, purity >98%), ammonium formate (analytical grade), sodium acetate (anhydrous, HPLC grade >99.0%), sodium chloride (ACS reagent >99.0%) and magnesium sulfate (anhydrous reagent plus >99.5%) were purchased from Sigma–Aldrich (USA). Deionized water (>18 MΩ cm^{-1} resistivity) was purified using Ultramatic Type I system (ultrapure reagent grade water) from Wasserlab (Spain).

2.2. Mycotoxin standard solutions

All mycotoxins (reference material purity ≥98%) were supplied by Sigma–Aldrich (USA) and kept at −20 °C. Trichothecenes: nivalenol, deoxynivalenol, deepoxy-deoxynivalenol, 3-acetyldeoxynivalenol, 15-acetyldeoxynivalenol, neosolaniol, diacetoxyscirpenol, fusarenon X, T-2 and HT-2 toxins were purchased as standard solutions of 100 μg/mL in acetonitrile.

Ten milliliters of a mixed stock solution in acetonitrile was prepared by diluting appropriate volumes of the individual standard solutions. Before storing at −20 °C, the mixed stock solution was aliquoted (1 mL) into microcentrifuge tubes. Each tube was maintained at room temperature and in darkness for 30 min prior to use. The calculated concentration of each mycotoxin in the mixed stock solution (in ng/mL) was as follows: NIV 1011.4, DON 251.3, DOM-1 151.5, FUS-X 185.0, NEO 10.0, 3-ADON 50.2, 15-ADON 101.1, DAS