Food Chemistry 119 (2010) 408–416

Contents lists available at ScienceDirect

Food Chemistry

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

Analytical Methods

Application of an LC–MS/MS based multi-mycotoxin method for the semi-quantitative determination of mycotoxins occurring in different types of food infected by moulds

Michael Sulyok, Rudolf Krska *, Rainer Schuhmacher

Christian Doppler Laboratory for Mycotoxin Research, Department IFA-Tulln, University of Natural Resources and Applied Life Sciences, Vienna, Konrad Lorenzstr. 20, A-3430 Tulln, Austria

ARTICLE INFO

Article history:
Received 9 July 2008
Received in revised form 11 May 2009
Accepted 22 July 2009

Keywords:
Food
Liquid chromatography
Mass spectrometry
Moulds
Mycotoxins

ABSTRACT

An existing LC–MS/MS method for multi-mycotoxin determination was extended by further 19 analytes and was applied for a semi-quantitative screening of 87 mouldy food samples from private households, including bread, fruits, vegetables, cheeses, nuts and jam. In the 247 investigated sub-samples, 49 different analytes were identified, some of which were never reported before to occur in naturally contaminated food. Enniatins and ergot alkaloids occurred in all samples of (dark) bread/pastries at low μg/kg-levels. From the remaining analytes, chanoclavine, emodin, mycophenolic acid and roquefortine C were found most frequently. Regulated mycotoxins occurred less often, but the corresponding concentrations exceeded the regulatory limits up to a factor of 1000 in case of patulin. Moreover, considerable mycotoxin concentrations were observed in some sub-samples taken from non-mouldy spots of the investigated samples. Thus, it was concluded that it is not safe to remove the mouldy part and consume the remainder.

1. Introduction

Moulds are able to infect and grow on all types of food. This is usually accompanied by changes of the texture, smell and taste of the infected foodstuff due to excretion of enzymes and volatile compounds by the fungus. In some cases, these changes are desired (e.g. use of non-toxigenic strains of *Penicillium roqueforti* for the production of blue mould cheese), but most of the time, fungal infection leads to food spoilage such as off-flavours, discoloration, rotting and disintegration of the food structure (Filtenborg, Frisvad, & Thrane, 1996).

The most important aspect involved in spoilage of food is, however, the formation of mycotoxins. Although approximately 400 compounds are currently recognised as mycotoxins, only few of them are addressed by food legislation. Most of the existing analytical methods likewise focus on these regulated toxins, i.e. trichothecenes, aflatoxins, zearalenone, ochratoxin A, fumonisins and patulin. In contrast, most publications dealing with other mycotoxins produced by fungi involved in food spoilage seem to derive from the field of mycology rather than from food analysis. A typical example consists of the use of the metabolic profile of crude fungal extracts to support findings from taxonomy for the differentiation of fungal species (Nielsen & Smedsgaard, 2003).

These findings are of limited relevance concerning the occurrence of mycotoxins in naturally infected food: most mycological investigations address fungal strains that have been isolated e.g. from food and have subsequently been cultivated on synthetic culture media. However, the qualitative and quantitative mycotoxin profile, which a mould produces on a food commodity depends on the ecological and processing parameters of the particular foodstuff (Filtenborg et al., 1996) and can therefore be expected to be different from synthetic media. In addition, most of the analytical methods that are used in mycological studies such as TLC (Filtenborg et al., 1996; Freire, Kozakiewics, & Paterson, 2000; Overy, Seifert, Savard, & Frisvad, 2003) or HPLC–DAD (Andersen, Smedsgaard, & Frisvad, 2004; Andersen & Frisvad, 2004; Larsen, Gareis, & Frisvad, 2002) are sufficiently selective for the determination of fungal extracts or for single target analysis (including a dedicated procedure for sample preparation) in foodstuffs, but are incapable of dealing with a large number of analytes in complicated food matrices. Modern methods such as HPLC coupled to (tandem-) mass spectrometry offer higher selectivity, which enables multi-analyte determination without dedicated sample clean-up in principle. However, even for those methods, multi-mycotoxin analysis in food is a real analytical challenge, as it would be advantageous to work without any clean-up and analyze raw extracts instead in order not to adulterate the mycotoxin pattern by sample preparation.

* Corresponding author. Tel.: +43 2272 66280 401; fax: +43 2272 66280 403.
E-mail addresses: michael.sulyok@boku.ac.at (M. Schuhmacher), rudolf.krska@boku.ac.at (R. Schuhmacher).

0308-8146/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2009.07.042

The drawback of such a "dilute and shoot"-approach is that signal suppression due to matrix effects is far more likely to occur when crude extracts are analyzed. The existing method that covers hundreds of fungal metabolites is used rather for qualitative screening of fungal metabolites (Nielsen & Smedsgaard, 2003; Nielsen, Sumarah, Frisvad, & Miller, 2006) than for quantitative analysis, which is partially a result of the lack of availability of suitable standards. On the other hand, quantitative data on metabolites involved in food spoilage is usually restricted to a selected set of analytes that are amenable to the chosen clean-up procedure (e.g. Kokkonen, Jestoi, & Rizzo, 2005).

Despite these difficulties, there is certainly a need for fast and comprehensive methods for the analysis of toxic metabolites produced by toxigenic strains of food colonising fungi, as a simple visual inspection of food is not sufficient to exclude health hazards. It was shown that toxin concentration and visible infection may not correlate in every case (Rundberget, Skaar, & Flaoyen, 2004), and mycotoxins can be present in commodities without being able to detect fungi associated with the toxins and vice versa (Freire et al., 2000). In addition, the use of any mouldy material in the processing of food may contribute to the mycotoxin level in the final product by carry over (Filtenborg et al., 1996), e.g. use of mouldy tomatoes for the production of ketchup (Andersen & Frisvad, 2004). In such cases, the mouldy material cannot be seen in the final product. A recent report by our group (Sulyok, Berthiller, Krska, & Schuhmacher, 2006) has shown that the use of mass spectrometers of the latest generation enables a quantitative determination of mycotoxins in crude food extracts, provided that the extraction efficiencies as well as matrix effects are sufficiently characterised for all investigated analyte/matrix combinations. Only recently, we have extended the range of analytes covered by our method and have provided preliminary data on the mycotoxin pattern in mouldy food samples (Sulyok, Krska, & Schuhmacher, 2007).

In the present work, the method was further extended and applied for a semi-quantitative screening of 247 sub-samples taken from mouldy and non-mouldy spots of 87 food samples from private households in Austria. It was our goal to determine the mycotoxin pattern (including toxic metabolites that have not been reported yet to occur in naturally contaminated food), that is produced by moulds spontaneously infecting food, stored under typical conditions at the end consumer. These results might be used to identify toxins as marker substances for food spoilage and to evaluate the relevance of the related toxin concentrations for the end consumer. Furthermore, the distribution of the toxins between mouldy and non-mouldy parts of the same sample has been studied. Some of the toxins detected were only present in the moulded part of the samples whereas others were excreted by the fungus into the surrounding food tissue. There is certainly a practical relevance of this issue, as it is still common practice in case of some food products to remove the mouldy parts and to consume the remainder.

2. Materials and methods

2.1. Chemicals and reagents

Methanol and acetonitrile (both LC gradient grade) were purchased from J.T. Baker (Deventer, The Netherlands), ammonium acetate (MS grade) and glacial acetic acid (p.a.) were obtained from Sigma–Aldrich (Vienna, Austria). Water was purified successively by reverse osmosis and a Milli-Q plus system from Millipore (Molsheim, France). Mycotoxin standards were dissolved in acetonitrile and were purchased from different sources: Brefeldin A, cytochalasins A, B, C, D, J and H, HC-toxin, kojic acid, and 3-nitropropionic acid were purchased from Sigma (Vienna, Austria), penicillic acid and roquefortine C were obtained from Iris Biotech GmbH (Marktredwitz, Germany), AAL TA toxin was a gift from Prof. David Gilchrist (University of California, Davis, United States), Enniatin B3 and 2-amino-14,16-dimethyloctadecan-3-ol were a gift from Dr. Silvio Uhlig (National Veterinary Institute, Oslo, Norway), certified reference solutions of T2-tetraol and T2-triol were received from Biopure Referenzsubstanzen GmbH (Tulln, Austria). Alpha zearalenol-4-glucoside and beta zearalenol-4-glucoside were synthesised in our laboratory from zearalenone using a genetically modified yeast strain expressing a glucosyl-transferase, followed by reduction of the resulting zearalenone-4-glucoside with sodium borohydride (Krenn et al., 2007). For details concerning the other 87 toxins see Sulyok et al. (2007).

2.2. Samples

Eighty-seven spontaneously moulded foodstuffs (including 19 breads/pastries, 20 fruits, 14 vegetables, 6 cheeses, 5 jams, 6 nuts and 17 others), which had been provided by staff members of our institute, were sampled for mycotoxins in this study. Few of the samples were completely covered by mould, whereas most samples exhibited one or several – sometimes differently coloured – mouldy spots. The latter samples can be considered to be realistic in private household practice.

After visual inspection of each sample, several sub-samples per individual sample were prepared by cutting mouldy and (if available) non-mouldy spots of the individual sample using a scalpel. (Note that the term "non-mouldy" is used throughout the manuscript although we are aware that fungal mycelium may have also been present in those parts of the samples, which did not exhibit visible fungal infection. There was no fixed distance between the sampled mouldy and non-mouldy spots; one sub-sample was usually taken from the maximum distance from the fungal infection.). The surface area of the sub-samples was approximately 1 cm^2 and their thickness ranged between 0.5 and 1 cm in order to make sure that the main part of the sampled volume consisted of food matrix.

The final set of 247 sub-samples included 68 originating from bread/rolls (49 with mouldy spots/19 spots without visible infection), 49 from fruits (30/19), 34 from vegetables (22/12), 14 from cheeses (10/4), 13 from nuts (10/3), 12 from jams (6/6) and 57 from other foodstuffs (34/23).

2.3. Sample preparation and estimation of matrix effects

Extraction was carried out using a mixture of acetonitrile/water/acetic acid 79 + 20 + 1 (v + v + v), with ratios between 3 and 16 mL solvent/g sample depending on the texture of the sample. After extraction, the samples were centrifuged, diluted 1 + 1 and injected as described in detail by Sulyok et al. (2007). For the estimation of matrix effects, raw extracts of sample spots without visible fungal infections were fortified using a multi-analyte standard on one concentration level, diluted and analyzed and the corresponding peak areas were compared to a standard prepared and diluted in neat solvent.

2.4. LC–MS/MS parameters

Detection and quantification was performed with a QTrap 4000 LC–MS/MS System (Applied Biosystems, Foster City, CA) equipped with a TurboIonSpray electrospray ionization (ESI) source and an 1100 Series HPLC System (Agilent, Waldbronn, Germany). Chromatographic and mass spectrometric parameters of 87 of the investigated analytes are described by Sulyok et al. (2007). MS and MS/MS parameters of the additional 19 analytes were optimised by infusion of standard solutions into the mass spectrometer. For a detailed list of these compounds and the related parameters see Table