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Determination of the mycotoxin moniliformin in cereals by high-performance liquid chromatography and fluorescence detection

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Abstract

The development of a selective and sensitive high-performance liquid chromatography–fluorescence assay for the determination of the mycotoxin moniliformin in plant extracts is described. By means of introducing a derivatization reaction with 1,2-diamino-4,5-dichlorobenzene (DDB) we succeeded in increasing the overall determination sensitivity and the chromatographic selectivity on a RP-HPLC system. Due to the phenolic character of the derivative a bathochromic UV shift from 315 to 330 nm (phenole→phenolate) at pH 10–12 was observed. Moreover, and in contrast to the DDB reagent at pH 10–12 the moniliformin–DDB derivative showed fluorescence activity with λ_{Ex}=330 nm and λ_{Em}=440 nm, which led to the benefits that the excess of DDB did not have to be removed from the sample solution. The pH-shift was accomplished via on-line post-column effluent mixing of the RP-18 eluent using an auxiliary HPLC pump and a buffer of pH 12.00. The fluorescence detection enables the reduction of the determination limit of moniliformin from 0.05 (UV detection) to 0.02 mg/kg maize.

Keywords: Derivatization, LC; Food analysis; Moniliformin; Mycotoxins

1. Introduction

The mycotoxin moniliformin (see Fig. 1a) is a highly toxic compound, produced by different fusarium species, e.g. *Fusarium moniliforme* var. subglutinans, *F. avenaceum*, *F. acuminatum*, *F. subglutinans* [1]. It has been discovered and structurally characterized as the sodium or potassium salt of 3-hydroxycyclobut-3-ene-1,2-dione by Cole et al. [2] in 1972. In animal experiments moniliformin causes pathological changes including myocardial degeneration and necrosis [3]. Thiel [4] discovered a molecular mechanism for the toxic action, which is an inhibition of the enzymes responsible for the oxidative decarboxylation of pyruvate to acetyl CoA and of α-ketoglutarate to succinyl CoA. The first report of natural occurrence of moniliformin was from Thiel et al. [5] in 1982 who isolated it from mouldy Transkeian corn. Thalmann et al. [6] analysed 58 samples of maize, grown in Germany whereby levels lower than 0.65 mg/kg have been found. Thiel et al. [7] reported about a corn screening in the USA where moniliformin was found at levels between 0.39 and 2.82 mg/kg. In 24 samples of Canadian wheat, rye and maize, moniliformin was only found in two maize samples at levels of 0.06 mg/kg and 0.20 mg/kg, respectively [8].

Although the acute and long-term toxicity of moniliformin for human is not yet fully investigated there is a need for a selective and sensitive analysis

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method for moniliformin in cereals, food and feed samples. So far the following methods including thin-layer chromatography (TLC) [9] using N-methylbenzthiazolon-2-hydrazone as derivatization agent, gas chromatography with mass spectrometric detection of the derivatized analyte [10] and high-performance liquid chromatography [5,11,12] have been published. Thiel et al. [5], Shepherd and Gilbert [11] and Sharman et al. [12] described ion-pair chromatographic methods on RP-HPLC systems, with UV detection at 227 nm, 260 nm and 229 nm, respectively. Due to the ionic and polar nature of moniliformin (see Fig. 1) the selective isolation from different matrix compounds and the chromatographic handling of this analyte represents the main problem. The published methods showed satisfactory results with respect to compound selectivity, but the determination limit was only 0.05 mg/kg in maize samples [12]. In order to increase the selectivity and the sensitivity of the assay we introduced a derivatization reaction with 1,2-diamino-4,5-dichlorobenzene (DDB) similar to the one described for the analysis of diacetyl [13]. In this way we obtained a heterocyclic derivative of moniliformin, which showed fluorescence activity at basic pH, whereas the excess of the reagent showed hardly any fluorescence. The aim of this contribution was to implement the derivatization strategy to a total analysis method of moniliformin in cereal extract samples.

2. Experimental

2.1. Chromatography

The analyses were performed on a HP 1090 liquid chromatograph (Hewlett-Packard), equipped with a diode-array (DAD) detector (λ=330 nm) and connected in series with a Jasco FP-920 fluorescence detector (λ_{Ex}=330 nm, λ_{Em}=440 nm). The UV spectra have been recorded on-line by means of the DAD detector. The analytical column was a Beckmann ODS (15 cm×4.6 mm I.D., 5 μm) and the mobile phase was acetonitrile–ammoniumacetate (50 m*M*) (35:65, v/v) (apparent pH=7), with a flow-rate of 0.7 ml/min. The column oven was adjusted at 40°C.

For the on-line post-column treatment of the RP-18 column effluent a glycine buffer [glycine+NaCl 0.1 mol/l–aqueous NaOH 0.1 mol/l (39:61), pH 12] was used and mixed via a low dead-volume mixing-tee followed by a mixing coil (1 m×0.25 mm I.D.) by means of an auxiliary pump (Kontron LC-pump 410). To avoid pulsation of the make-up phase a polystyrene-based HPLC column Asahipak ODP-50 (125×4 mm I.D., 5 μm) from Hewlett-Packard was inserted between the auxiliary pump and the mixing tee. The flow-rate of this make-up buffer was 0.5 ml/min. The resulting mixed eluent reached an apparent pH of 11. The injection volume of the sample solution (500 μl) was 20 μl.

Data processing was performed on a HP HPLC^{3D} Chem Station.

2.2. Chemicals and solvents

Moniliformin was purchased as its sodium salt from Sigma (St. Louis, MO, USA). An amount of 4,5-dichloro-1,2-diaminobenzene (DDB) was obtained from Aldrich (Milwaukee, WI, USA) and purified by recrystallization from hydrochloric acid after charcoal treatment. Squaric acid was purchased from Sigma (St. Louis, MO, USA). The SAX solid-phase extraction columns with 500 mg adsorbent and 3 ml reservoir volume as well as the HAX (mixed mode) solid-phase extraction columns with 200 mg adsorbent and 3 ml reservoir volume were from IST (International Sorbent Technology, UK).

Glycine, acetonitrile (Lichrosolv), acetonitrile (Prepsolv), methanol (Lichrosolv) and *n*-hexane p.a. quality, were obtained from Merck (Darmstadt, Germany).

Water for HPLC mobile phases was purified in a Milli-Q system (Millipore, Bedford, MA, USA).

Several durum-wheat samples were purchased from supermarkets and tested to find also a 'blank' matrix.

The cereal samples were ground with a 'Messerschmidt Alleskörner' grain mill.

A moniliformin stock solution of 1 mg/ml in water has been prepared. From this stock solution different dilutions have been prepared: solution G (50 μg/ml), solution B (5 μg/ml), solution C (1 μg/ml). The stock solution was stored in the

refrigerator (4°C) and was stable for at least three months.

2.3. Extraction of cereal samples

A 5-g amount of finely ground cereals was extracted with 50 ml of acetonitrile–water (90:10) by stirring the suspension for 30 min, 10 ml of the supernatant of the centrifugated extract was taken and the solvent removed by rotary evaporation. The residue was redissolved two times in 1 ml methanol and once in 1 ml methanol–water (1:1) by Vortex-mixing, the combined solutions were degreased by shaking the mixture with 7 ml of *n*-hexane which was discarded. The remaining methanol–water phase (3 ml) of the cereal extract had to be further pretreated and purified by a solid-phase extraction (SPE) method.

2.4. SPE clean-up and derivatization protocol

The clean-up was accomplished best by using strong anion-exchange (SAX, 500 mg) SPE columns. These SPE columns were preconditioned by the sequential addition of methanol (3 ml), water (3 ml) and 0.1 *M* phosphoric acid (3 ml) followed by the loading of the total methanolic sample extract on the SAX column. The column was then washed with 3 ml of methanol–water (1:1), 3 ml 0.1 *M* phosphoric acid and 3 ml water. The analyte (moniliformin) was finally eluted with 2 ml 1 *M* aqueous HCl and the following derivatization was carried out directly with the total acidic eluate (2 ml).

For the SPE procedures we employed a vacuum extraction station from Supelco.

The SPE purified sample extract (2 ml) was mixed with 500 μl of the DDB reagent solution (1 mg/ml, 1 *M* aqueous HCl); the molar ratio of DDB/moniliformin ranged between 50 and 1000:1 in the course of establishing the linearity of the method, and the reaction was carried out at 60°C for 2 h in a fan-circulating oven.

After the aqueous reaction solvent was removed at 40°C under a gentle stream of nitrogen the residue was redissolved in 500 μl of HPLC-grade water and an aliquot of this acidic solution was injected onto the RP-HPLC column.

2.5. Quantitation of moniliformin in cereals

The quantitation was performed via an external standard calibration method using 'blank' durum wheat as sample matrix. A 5-g amount of ground wheat was spiked with 20 μl, 50 μl, 100 μl and 250 μl, respectively, of the moniliformin solution B (5 μg/ml), corresponding to a concentration of 0.02, 0.05, 0.10 and 0.25 mg/kg. These calibration samples were extracted, cleaned up and derivatized as described above.

3. Results and discussion

3.1. Concept of derivatization

As chemically selective derivatization reagent for α,β-dicarbonyl compounds 1,2-diamino-4,5-dichlorobenzene (DDB) [13] has been chosen; the reaction scheme is outlined in Fig. 1a. The derivatization reaction is fast and easy to perform in aqueous HCl and it seems to be quantitative (as depicted in Fig. 2) although we could not verify this statement using a preparatively prepared moniliformin–DDB derivative as calibration standard due to the limited excess of moniliformin. The derivative is stable in the aqueous HCl solution over 1–2 days.

The linearity of the method has been examined from 0.2 to 42 nmol/500 μl, the correlation coefficient was 0.999. Since moniliformin was available in amounts of only 1 mg, the derivatization reaction was feasible in the analytical scale only (see above). For comparison the described derivatization reaction was performed also with squaric acid (Fig. 1b), since moniliformin can be designated also as the sodium or potassium salt of the semisquaric acid. The structure of the reaction product of squaric acid with DDB (see Fig. 1b) was verified via IR and NMR spectroscopy. On the basis of these experiments we supposed that moniliformin reacts with DDB according to the scheme outlined in Fig. 1a.

Due to the high affinity of DDB to various α,β-dicarbonyl compounds, which may also be endobiotic 'ingredients' of the matrix, several potentially interfering compounds and peaks could be created by means of the derivatization procedure. However, this problem could be sufficiently handled by an effective

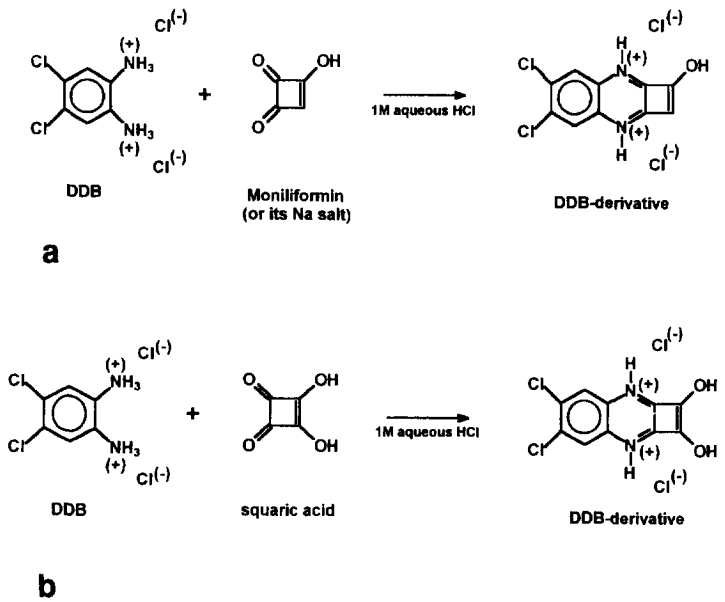

Fig. 1. Reaction scheme of (a) moniliformin and (b) squaric acid with the derivatization reagent 1,2-diamino-4,5-dichlorobenzene (DDB). The reaction was carried out in 1 *M* aqueous HCl at 60°C for 2 h.

sample preparation method and the employment of an HPLC system with high efficiency and selectivity.

3.2. Spectroscopic properties of the moniliformin–DDB product

By means of a diode-array detector the UV spectra of DDB and the moniliformin–DDB derivative could be recorded on-line. Both, the moniliformin–DDB derivative and DDB showed a UV maximum at 315 nm at a pH range from 4 to 7. At basic pH (about 11) accomplished via on-line post-column mixing (see Section 2) the moniliformin–DDB derivative showed a bathochromic UV shift from 315 to 330 nm (see Fig. 3). This is caused by the phenolic character of the moniliformin derivative, which is transformed into the phenolate between pH 9 and 11. Thus the electron density within the heterocyclic ring system gets markably enriched leading to a fluorescence phenomenon at λ_{Ex}=330 nm and λ_{Em}=440 nm.

The excess of the derivatization reagent DDB does not show this behaviour and therefore it does not appear and interfere in the chromatogram using fluorescence detection.

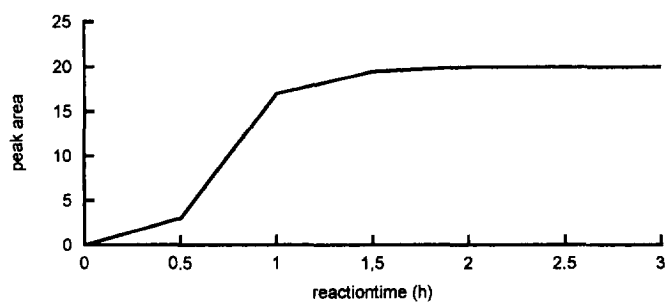

Fig. 2. Reaction kinetics of moniliformin with DDB. Reaction conditions: 1 *M* HCl, 60°C; molar ratio: DDB–moniliformin, ca. 100:1; the concentration of moniliformin was 100 ng/500 μl.

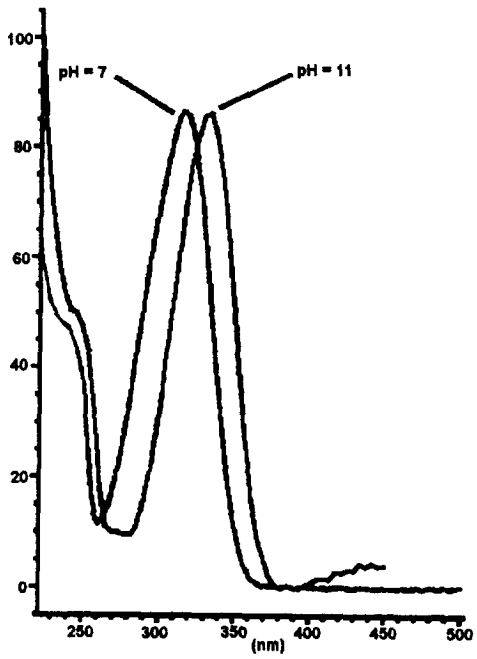

Fig. 3. UV spectrum of moniliformin–DDB derivative at pH 7, the UV maximum is at 315 nm; overlaid with the UV spectrum of moniliformin–DDB derivative at pH 11, the UV maximum is at 330 nm.

3.3. Chromatographic behaviour of the moniliformin–DDB derivative

The deprotonation of the phenolic reaction product to the corresponding phenolate at a basic pH will also change its chromatographic behaviour. The capacity factor of the moniliformin–DDB derivative as a function of pH is depicted in Fig. 4. For these studies a polystyrene-based HPLC column (Asahipak ODP-50, see Section 2) was used, but its performance was not sufficient to finally analyse matrix extracts. Within the spectrum of different silica-based RP-HPLC columns tested, the Beckmann ODS column showed the best performance for this specific application. However, such a column cannot be used at alkaline mobile-phase conditions necessary for the activation of the fluorescence activity of DDB–moniliformin (pH>11). Consequently, an on-line post-column mixing with a basic make-up buffer (pH 12) using an auxiliary HPLC pump has been introduced to reach a final pH of about 11 of the effluent entering the detector.

Based on these rather unexpected different spectroscopic properties (examined by recording on-line the UV spectra with the DAD detector) of the moniliformin–DDB derivative in comparison to the DDB reagent at the various pH conditions, the pH adjustment turned out to be an excellent tool for optimizing the detection selectivity to a certain extent.

3.4. Sample preparation

Due to the very polar character of the moniliformin-sodium salt but also of its acid form (moniliformin has been reported to have a pK_a of 1.7 [14]), the selective extraction and separation from polar and non-polar matrix compounds represented the main problem. Solid-phase extraction (SPE) using a C_{18} column to purify the sample extract from lipophilic components followed by SPE employing a strong anion-exchange (SAX) column [12] has proved to be

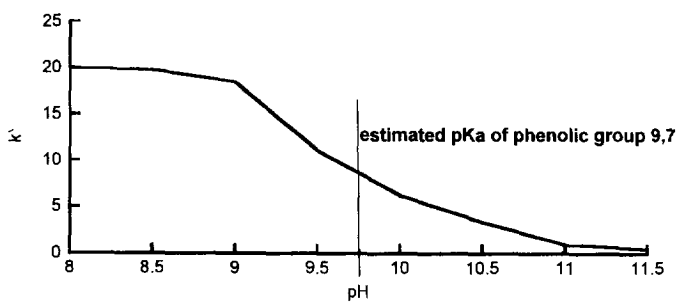

Fig. 4. Influence of the pH on the capacity factor k'. It shows the dissociation of phenolic OH of moniliformin–DDB derivative; the estimated pK_a of the phenolic group is 9.7. This experiment was carried out with a HP Asahipak ODP-50 (125×4 mm I.D., 5 μm) HPLC column.

a powerful clean-up technique. Consequently, we performed first the sample clean up with so-called HAX-SPE columns which are mixed-bed adsorbents of C_{18} and SAX material. We experienced that 200 mg adsorbent material was not sufficient to retain quantitatively moniliformin, but to exclude the potentially interfering polar and acidic matrix compounds; consequently we combined two HAX columns (in total 400 mg adsorbent) thus obtaining satisfactory recoveries. Unfortunately, 400 or 500 mg HAX-SPE columns were not available commercially; in order to simplify the solid-phase extraction and to prevent the use of two stacked HAX-SPE columns per sample, which becomes rather cost intensive, we evaluated a method with SAX columns only. The observed clean-up effect as well as the recoveries were satisfactory for the latter system (see also Section 2).

The final and redissolved sample extract (after SPE purification and derivatization) and diluted with water, had to be acidic in order to keep all derivatized components including the excess DDB in solution. Since such an acidic sample solution (pH 1) could destroy the silica-based HPLC column, we tried to buffer the injection solution to pH≅3. In this case the residue was dissolved in 200 μl of methanol and 300 μl of 0.2 *M* ammoniumacetate buffer. The pH of this solution was about 3, but sometimes a part of the components precipitated, which caused a poor repeatability due to a loss of analyte. For this reason we had to dissolve the acidic residue only in water and use commercial silica-based 4-mm RP-precolumns as guard columns but which had to be replaced after 30–40 injections; in order to elongate the system stability the use of a precolumn based on polystyrol should be recommended. Unfortunately there are none very short ones on the market yet.

3.5. Calibration and ruggedness of the method

Calibration curves of the total analysis method based on spiked durum-wheat semolina samples (range 0.02–0.2 mg/kg) have been performed; the correlation coefficients were between 0.995 and 0.999. The value for the slope, determined out of six different calibration curves, was 11.9±0.8 and for the intercept 45.8±4.0. The day-to-day repeatability for a six-day period was determined for 'blank' durum-wheat samples, which have been spiked with moniliformin corresponding to a concentration of 50 ppb; it was 12% based on the relative standard deviation of these six values. At higher moniliformin levels this value dropped to 6%. The limit of determination (smallest amount of the analyte which can be determined quantitatively) for the analysis of moniliformin in diverse cereals (e.g. durum wheat, oats, maize, wheat) was 0.02 mg/kg. The overall recoveries for spiked durum-wheat samples were constant and about 70% in the 0.02–0.25 mg/kg range. The limit of detection (smallest amount of analyte which can be qualitatively identified) was about 500 pg moniliformin DDB derivative absolute (which would correspond to 0.012 mg moniliformin/kg) using fluorescence detection; in the UV mode (330 nm) it was about 1 ng. Our efforts to find a suitable internal standard fitting to the overall analysis method and the HPLC chromatogram were not successful, so we had to rely on the external standard calibration, which leads to higher variation coefficients. Squaric acid could not be used as internal standard because in the analytical scale the reaction with DDB showed different reaction kinetics and the squaric acid–DDB product showed no fluorescence.

3.6. Moniliformin contamination of different cereal samples

Several maize and wheat samples, received from different controlled fields in Austria, have been analysed for their moniliformin content with the method described above.

The contamination ranged from 0.05 to 2 mg/kg. Examples of typical chromatograms of these samples and a blank sample are depicted in Fig. 5a–c.

4. Conclusion

By means of introducing a new derivatization reaction of moniliformin with DDB we reached the goal to increase the overall determination sensitivity of moniliformin in plant extract samples by combining chemical selectivity with chromatographic selectivity using a RP-HPLC system and fluorescence detection. The SPE sample clean-up with strong

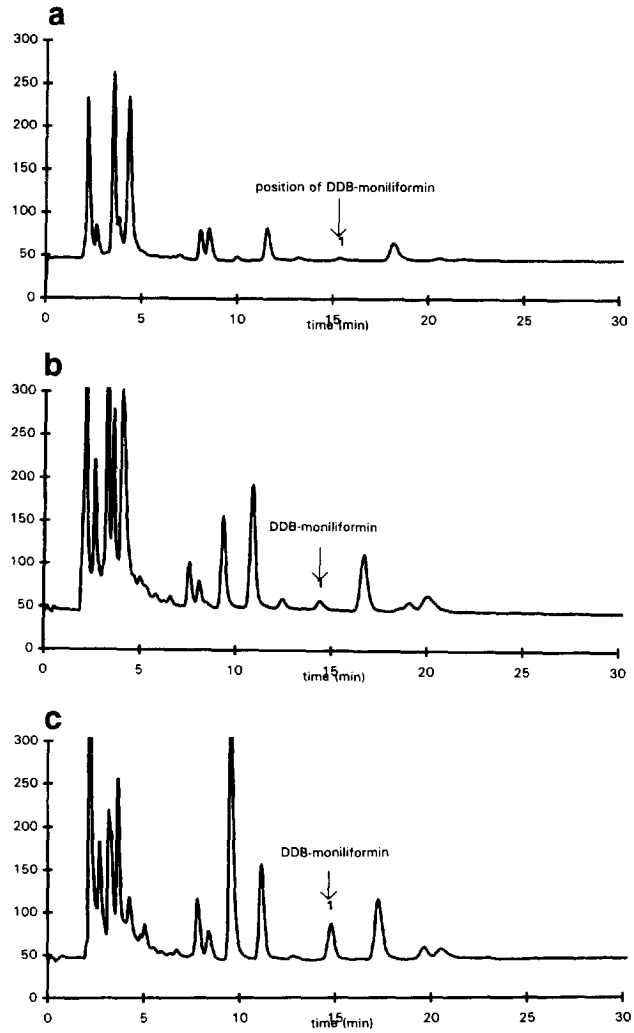

Fig. 5. Chromatogram of (a) 'blank' wheat sample, (b) wheat sample, 0.022 mg/kg and (c) maize sample, 0.079 mg/kg moniliformin (1). For chromatographic conditions see Section 2.

anion-exchange (SAX) columns (500 mg) has proved to be powerful to separate the analyte from interfering matrix compounds.

Considering the potential risk of subtoxic but long-lasting consumption of cereals contaminated with moniliformin, which is often in the range of 0.05 mg/kg, the application of a sensitive analysis method may be of significance. In contrast to the published HPLC methods, which used UV detection, we succeeded in reducing the determination limit of the method to 0.02 mg/kg by means of derivatizing the moniliformin to a fluorescence-active analyte.

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