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Note

High-performance liquid chromatographic determination of citrinin in cereals using an acid-buffered silica gel column

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Since the mid seventies, reversed-phase chromatography had developed to become the most dominant branch of high-performance liquid chromatography (HPLC), while normal-phase chromatography, most popular in classical column liquid chromatography, has lost its leading role. This paper describes the successful normal-phase HPLC of trace amounts of the acid compound citrinin on a acid-buffered silica gel column as recently described by Schwarzenbach[1]. By treating the silica gel with a buffer salt, virtually insoluble in the mobile phase, an environment is created on the surface of the adsorbent that permits tail-free elution of very polar compounds[1].

Citrinin (IUPAC: (3R,4S)-4,6-dihydro-8-hydroxy-3,4,5-trimethyl-6-oxo-3H-2-benzopyran-7- carboxylic acid; C.A. 518-75-2), a fungal metabolite, is produced by several Aspergillus and Penicillium species. It has been reported to demonstrate antibiotic, antifungal, antiprotozoal and bacteriostatic properties as well as to cause nephrotoxicity in several animal species. Citrinin-producing fungi grow on grains and fruits which are stored in high humidity as well as on mould-fermented sausages; however, the presence of the fungus does not necessarily indicate the presence of the mycotoxin[2,3]. In order to evaluate possible health risks, an estimate of its daily intake by the population is needed. This implies a reliable quantitative method for the determination of citrinin in food.

Several methods to determine citrinin have been published, primarily based on thin-layer chromatography (TLC), but relatively low sensitivities in the range of 10–50 ng/g at best have been reported in recent reviews[2–4]. On the other hand, HPLC has been applied to the analysis of citrinin in grains and biological fluids[5–9]. Mostly reversed-phase chromatography was performed using acidic mobile phases and fluorescence[5,8,9] or UV[6] detection; the ion-pair technique with UV detection has also been applied[7]. The stated detection limits were comparable to those reached by TLC.

Unfortunately, neither of the described HPLC conditions, except ion-pair chromatography, worked well in our hands. Seriously distorted peaks and extremely low sensitivities resulted with several commercially available reversed-phase columns using appropriate acidic eluents. However, reversed-phase ion-pair HPLC[7] provided good peak forms, but the fluorescence of citrinin was completely lost. Thereby the sensitivity and selectivity of the detection method was diminished. This drawback had

been overcome by acidifying the eluate of the HPLC column before entering the fluorescence detector.

A much easier and more elegant procedure is the buffered silica gel separation technique[1] which, as far as we know, is not widely applied at the moment. However, it seems to be very effective for the chromatographic separation of polar compounds. In any case we were very successful with citrinin at the first go.

EXPERIMENTAL

Chemicals and reagents

Citrinin was obtained from Sigma (St. Louis, MO. U.S.A.). For HPLC a working standard in chloroform (0.5 μg/ml) was used. All reagents, except chloroform, which was distilled, were p.a. grade and were used without any further treatment. Deionized water was used throughout this study.

Apparatus

The chromatographic system consisted of a Model 100A pump (Altex, Berkeley, CA, U.S.A.), a Model U6K sample injection valve (Waters, Milford, MA, U.S.A.) and a Model 650–109 fluorescence spectrometer (Perkin-Elmer, Norwalk, CI, U.S.A.) equipped with a flow-cell (excitation 360 nm, emission 500 nm, slits 10–20 nm). For the measurement of peak areas, a Model D-2000 Chromato-Integrator (Merck-Hitachi, Tokyo, Japan) was applied. All chromatographic columns (300 mm × 4.6 mm I.D.) used were commercially prepacked with microparticulates and made from stainless steel.

Preparation of buffered silica gel column[1]

A prepacked LiChrospher® Si 100 column [mean particle size 5 μm, specific surface area 420 $\pm$ 30 m^2/g, specific pore volume 1.25 $\pm$ 0.05 ml/g (ref. 10), 300 mm × 4.6 mm] supplied by Merck (Darmstadt, F.R.G.) was rinsed with about 200 ml of methanol, followed by about 200 ml of water. Then about 400 ml of aqueous buffer solution (0.2 M citric acid, adjusted to pH 2.5 with a saturated disodium hydrogenphosphate solution) were pumped through the column. It was connected to a nitrogen source and, after all the remaining buffer solution had been removed in a gentle stream of nitrogen (5–10 ml/min) at room temperature, the column was heated at 80°C for about 30 h. After cooling to room temperature, the column was equilibrated with about 100 ml of the mobile phase.

Chromatographic conditions

The mobile phase consisted of *n*-hexane–chloroform (typically 60:40, v/v), and a flow-rate of 1 ml/min was applied (room temperature). Typical injection volumes were 10 μl. The retention time of citrinin under the conditions described (buffered silica gel column) was 10 to 15 min. For quantification, peak areas were measured and compared to those of standard solutions.

Extraction and clean-up

To a 40-g ground sample of cereal (or flour), 150 ml chloroform and 20 ml 0.1 M phosphoric acid were added, mixed for 5 min, using a Polytron PCU-2 (Kinema-

tica, Luzerne, Switzerland) and then centrifuged (*ca.* 3000 *g*) for 10 min. Tapping down carefully the formed cake of flour with a spatula makes it easier to withdraw an aliquot of the chloroform extract.

A 7-g amount of Extrelut® (Merck) was uniformly impregnated with 10 ml of a an aqueous 1% sodium bicarbonate solution in a beaker. After packing the thus prepared alkaline adsorbent into an Extrelut® column (shortened by *ca.* 4.5 cm and equipped with an additional stopcock), 100 ml of chloroform extract (corresponding to 26.7 g sample) were added to the column; after elution the column was rinsed with 2 × 40 ml chloroform, and the eluates were discarded. After blowing out with air the rest of the chloroform (balloon), a mixture of 30 ml chloroform and 1 ml formic acid (100%) was added to the column (without piston, stopcock closed) and thoroughly mixed with the column material (glass rod); the drain was collected. After reinstallation of the piston and light compression of the column material, the rest of the citrinin was eluted with 2 × 40 ml chloroform. The eluate was evaporated to dryness (Rotavapor®, Büchi, Switzerland, 40°C), and the residue was dissolved in 1 ml distilled chloroform for HPLC analyses. For recovery purposes the citrinin standard solution in chloroform was distributed uniformly (syringe) on the ground sample.

RESULTS AND DISCUSSION

It might be expected that the analysis of citrinin in food be facilitated by its acid and fluorescence properties. However, several difficulties have been described in the analysis of citrinin, *e.g.*, poor repeatability and/or recovery, tendency to be retained by glassware and syringes, sensitivity to light and temperature[11-16].

Fig. 1A–D demonstrates the good peak form of citrinin, the sensitivity of the chromatographic system and the acceptable separation from coextractives. In the tested range of 1–20 ng the detector signal was linearly related to the amount of citrinin injected. Without specially optimizing the excitation and emission wavelengths the minimum detectable amount of standard citrinin was about 0.1 ng (three
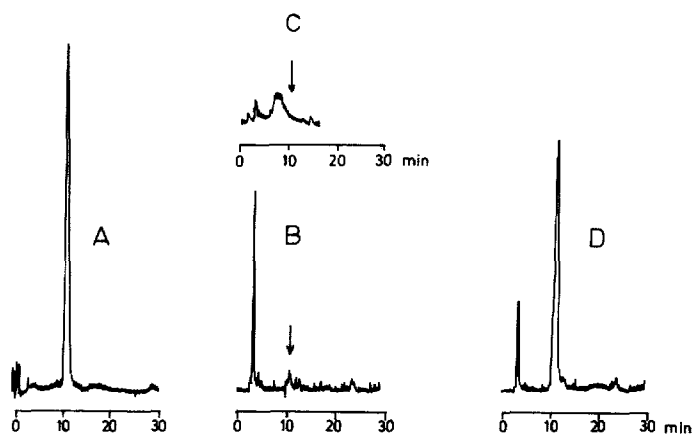

Fig. 1. Chromatograms of citrinin. (A) 5.3 ng standard; (B) brown flour (wheat naturally contaminated) *ca.* 0.8 ng/g; (C) wheat bran < 0.2 ng/g; (D) brown flour (wheat) spiked with 15 ng/g. Chromatographic conditions as described, injection volumes each 10 μl.

times the noise). Preliminary results have shown that filling the cell of the fluorescence detector with a similar acid-buffered silica gel as used in the column enhances the detector response for citrinin in about the same way as it has been demonstrated for aflatoxins[17,18].

The mean recovery of citrinin in brown wheat flour in the concentration range of 1–30 ng/g was 85.2% with a standard deviation of 7.4% ($n = 9$) and a range of 77.1–99.5%. With injection volumes of, e.g., 40 μl of the extract, corresponding to 1.07 g sample, a limit of quantification of about 0.1 ng/g is achievable. The extraction procedure and the solid phase clean-up resulted in acceptable recoveries and chromatograms. However, the efficiency of different potential extraction procedures has not been tested with naturally contaminated samples. In 14 of a total of 38 analyzed samples of cereals from the retail level, citrinin has been detected in the range of 0.2–1 ng/g; details are given elsewere[19].

Although the batch procedure to prepare a buffered silica gel column has been mentioned to be much more reliable than the in situ coating of prepacked silica gel columns[1], we have applied with acceptable success the latter procedure. Since a 0.1 M solution leads to a coating of approximately 1.5–2.5% (m/m) of silica gels with a specific surface area of about 400m^2/g (ref. 1), we can assume a load in the range of 3–5% (m/m) for our column.

Probably because the water content of the eluate mixture was not under strict control, some variation of the retention time of citrinin from one day to the next has been observed occasionally. By slightly changing the ratio of hexane to chloroform, the retention of citrinin may be adjusted.

After prolonged use of the column, the retention time of citrinin became shorter and shorter and the number of theoretical plates decreased. By pumping about 50 ml of a mixture of 5% (v/v) methanol in chloroform through the system the chromatographic properties were restored, probably by removing polar coextractives from the column. After these treatments the retention time of citrinin increased, e.g., from about 6 to 14 min.

After the column had once been accidentally eluted with about 100 ml of methanol, no chromatographic separation could be achieved. However, by repeating the coating procedure (without water, 100 ml ethanol instead of 200 ml methanol, and 200 ml buffer solution), the original properties of the column were completely restored.

CONCLUSIONS

Although reversed-phase HPLC is mostly used today because of its advantages over conventional normal-phase HPLC, our results show that the latter on modified silica gels, e.g., buffered silica gel, is a remarkable alternative, especially in trace analysis of very polar compounds.

The procedure described for the determination of citrinin in cereals, based on an alkaline/acid partition step on Extrelut and HPLC on an acid-buffered silica gel column with fluorescence detection, has been successfully applied to durum and soft wheat, wheat bran, rice, barley, corn, oat, oat groats and pastas. A limit of quantification of about 0.1 ng/g is achievable.

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