ELSEVIER

Journal of Chromatography A, 870 (2000) 469–472

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Effect of solvents on the fumonisins analysis by high-performance liquid chromatography with AccQ.Fluor as the derivatizing reagent

Carlos Velázquez[a,b], Monserrat Llovera[b], Jordi Plana[b], Ramon Canela[b,c,*]

[a]*Phytochemistry Department, Facultad de Ciencias Químicas, Universidad Nacional de Asunción, Asunción, Paraguay*
[b]*Chemistry Department, Centre R+D de Lleida (UdL-IRTA), Universitat de Lleida, Rovira Roure, 177, 25198-Lleida, Spain*
[c]*Area de Protecció de Conreus, Centre R+D de Lleida (UdL-IRTA), Rovira Roure, 177, 25198-Lleida, Spain*

Abstract

The effect of several solvent systems on the chromatographic response of fumonisin B_1 and B_2 derived with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AccQ.Fluor) is described. Naturally contaminated corn samples were extracted and purified by a standard method. Then, samples were dissolved in different solvents, derived with AccQ.Fluor reagent and analysed using HPLC. Results were solvent dependent, methanol being the best one among all assayed solvents for both fumonisins studied and acetonitrile the poorest. *o*-Phthaldialdehyde (OPA) reagent was used as a reference method.

Keywords: Fumonisin; AccQ.Fluor reagent; *o*-Phthaldialdehyde

1. Introduction

In recent years, growing interest arose in the study of fumonisins. They are a group of mycotoxins [1] mainly produced by the commonly occurring corn fungi, *Fusarium moniliforme* Sheldon and *Fusarium proliferatum* Nirenberg. Among fumonisins, fumonisins B_1 (FB_1) and B_2 (FB_2) are the major compounds [2]. FB_1 has been associated with several animal and human diseases including ELEM (equine leukoencephalomalacia), PPE (porcine pulmonary edema), oesophageal cancer in humans and rat liver cancer [3–6]. Improved methods are needed to determine their level in corn, corn products and other commodities so that exposure to them can be prevented. Almost all methods use HPLC-fluorometric techniques. Then, a derivatization step is necessary before the analysis by RP-HPLC due to the lack of chromophores in the fumonisin chemical structures [7]. Although several derivation reagents have been used for that purpose [8], OPA reagent is the one proposed for the different intercollaborative laboratory studies carried out until now [9,10]. The OPA method presents some problems: it has to be injected within 2 min of the derivation reaction and the derivatizing reagent has only one week of stability [9,10].

Recently, we have proposed the use of 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AccQ. Fluor) in fumonisin analysis. Cohen and co-workers had suggested the use of this reagent to determine amino acids in different matrices [11]; lately Diaz and co-workers have carried out amino sugar analysis with the same reagent, describing detection limits in the femtomole range and a high stability for the derivatives [12]. High stability is also showed by fumonisin B_1 and B_2 derivatives. They are stable for

*Corresponding author. Fax: +34-73-23-8264.
E-mail address: canela@quimica.udl.es (R. Canela)

0021-9673/00/$ – see front matter
PII: S0021-9673(99)00908-5

at least 48 h, value much greater than 2 min [13]. However, our proposed method initially showed some erratic results compared with the OPA method. Moreover, it occasionally presented back pressure problems during the HPLC analyses when a gradient program was used, starting at 50% of 0.1 *M* aqueous potassium dihydrogen phosphate at pH 7 and 50% of methanol.

In this paper, we describe the effect of different media, applied to prepare the OPA and AccQ.Fluor derivatives, on the chromatographic results. We also propose a new mobile phase for AccQ.Fluor derivative analysis, eliminating the back pressure problems observed in our former method [13].

2. Experimental

2.1. Chemicals

Sodium dihydrogen phosphate, sodium hydroxide and acetic acid, pro analysis quality, were supplied by Panreac (Barcelona, Spain). Ultrapure water was produced by a Milli-Q_{185} Plus system (Millipore Corporation, USA). HPLC grade methanol and acetonitrile were obtained from Romil (Germany). AccQ.Fluor™ Reagent Kit was purchased from Waters (Waters Cromatografia SA, Barcelona, Spain) and used according to manufacturer's instructions. OPA reagent was prepared according to Shephard [14], purchasing the product from Sigma (St. Louis, MO, USA). Three milliliter Bond-Elut SAX columns were supplied by Analytichem Bond-Elut (Varian, Harbor City, CA).

2.2. Standard solutions

Fumonisin B_1 and B_2 standards were prepared from solid corn cultures of *Fusarium moniliforme* following Cawood and co-workers' method [15]. We introduced an additional HPLC purification step, which yielded a >95% of purity in both FB_1 and FB_2. Purity was determined by comparing the isolated products with fumonisins purchased from the Division of Food Science and Technology (CSIR, Pretoria, South Africa). An independent confirmation of purity was carried out using electrospray mass spectrometry [16].

A stock solution of 1 mg/ml of FB_1 and 1 mg/ml of FB_2 in water–acetonitrile (1+1, v/v) was prepared. From this, serial standard dilutions of fumonisin B_1 and fumonisin B_2 in water–acetonitrile (1+1, v/v), methanol or acetonitrile were prepared just before analysis.

2.3. Corn samples

Ten kilograms of kernels were obtained from a local market and stored at −20°C during the whole study period. For immediate analysis 1 kg of the sample was ground and five 100 g subsamples were analysed for FB_1 and FB_2 content as described below.

2.4. Sample preparation

Corn samples were extracted and purified using a slightly modified Shephard's method [14]. Thus, 100 g of ground material were extracted using a magnetic stirrer for 60 min with 200 ml of methanol–water (3+1, v/v); the supernatant was filtered through a Whatman No. 4 filter paper, and three 10 ml portions were applied separately to Bond-Elut SAX cartridges that had been conditioned previously with methanol (5 ml), followed by 5 ml of methanol–water (3+1, v/v). Each cartridge was washed successively with 8 ml of methanol–water (3+1, v/v) and methanol (3 ml). Finally the toxins were eluted with 14 ml (7+7) of 0.5% acetic acid in methanol. The eluates were evaporated to dryness under vacuum, redissolved in 2 ml of methanol, transferred to a 4 ml screw cap vial and reevaporated to dryness under a nitrogen stream in a sandbath at 60°C. Prior to the analysis, the residue in a vial was dissolved in 1 ml of methanol, whereas the others were dissolved separately in an equivalent volume of acetonitrile or a mixture of water–acetonitrile (1+1, v/v).

2.5. Derivation

Using OPA reagent (OPA): 200 μl of OPA reagent were added to a 50 μl sample solution and mixed well. Twenty microliters of this solution were injected into the HPLC system during the first two minutes after derivation.

Using 20 μl of AccQ.Fluor reagent (AccQ-20): 50

μl fraction of sample solution was added to 2 ml screw cap vials. Then 60 μl of AccQ.Fluor borate buffer and 20 μl of AccQ.Fluor reagent were added to the vial. Reaction mixture was left for one minute at room temperature and heated for 10 min at 55°C in a sandbath. Twenty microliters of this solution were injected into the HPLC system.

Using 40 μl of AccQ.Fluor reagent (AccQ-40): 50 μl fraction of sample solution was added to 2 ml screw cap vials. Then 60 μl of AccQ.Fluor borate buffer and 40 μl of AccQ.Fluor reagent were added to the vial. Reaction mixture was left for one minute at room temperature and heated for 10 min at 55°C in a sandbath. Finally, twenty microliters of this solution were injected into the HPLC system.

3. HPLC

The HPLC system consisted of Applied Biosystem Series (ABI Analytical Kratos Division, Ramsey, NJ), model 400 pumps, model 491 Dynamic Mixer/Injector with a 20 μl loop, model 980 fluorescence detector (excitation at 335 nm and emission at 418 nm cut-off filter) and a Hewlett Packard 3396 Series II integrator (Hewllet Packard, Avandale, PA). The column was a reversed-phase Nova pak C_{18} (15× 0.39 cm) protected by a C_{18} guard column Waters (Waters Cromatografia SA, Barcelona, Spain).

For OPA derivatives a flow-rate gradient was used, flow-rate was 1 ml/min for 6 min and then switching to 1.5 ml/min for an additional 12 min. The mobile phase consisted of methanol–0.1 *M* sodium dihydrogen phosphate (75+25, v/v) adjusted to pH 3.35.

For AccQ.Fluor derivatives changes to previously published procedure [13] were use of methanol–0.1 *M* sodium dihydrogen phosphate (65+35, v/v) adjusted to pH 3.35 as mobile phase A (instead of 0.1 *M* aqueous potassium dihydrogen phosphate at pH 7), and running a different gradient program. The mobile phase B was methanol. The gradient program was started at 100% of A and kept for 5 min, then ramped to 45% B in 1 min, and finally held for 9 min. The Nova pak C_{18} column was operated at a flow-rate of 1.0 ml/min.

3.1. Statistical analysis

The data were processed using the SAS system 6.03 version (SAS Institute. Cary, NC).

4. Results and discussion

Table 1 shows the influence of solvents and reagents on fumonisin analysis from corn samples. Methanol proved to be the best solvent for both derivatives and for both fumonisins, whereas acetonitrile proved to be the poorest. AccQ.Fluor-40 in water–acetonitrile (1+1, v/v) gave results closer to OPA analysis than AccQ.Fluor-20 but it also in-

Table 1
Results of the analysis of the content of fumonisin B_1 and B_2 in corn samples using the assayed methods

Solvent	Method	n[a]	Fumonisin content (μg/g[b]±SE)	
			Fumonisin B_1	Fumonisin B_2
Methanol	OPA	5	8.1^{a}±0.4	3.0^{ab}±0.2
	AccQ-20	5	8.2^{a}±0.7	2.8^{a} ±0.2
	AccQ-40	5	9.2^{a}±2.3	3.7^{b} ±0.9
Water–acetonitrile	OPA	5	7.2^{a}±1.2	3.1^{a} ±0.4
	AccQ-20	5	4.3^{b}±1.0	0.9^{b} ±0.4
	AccQ-40	5	9.0^{a}±3.6	3.3^{a} ±0.8
Acetonitrile	OPA	5	4.8^{a}±3.7	1.8^{a} ±1.2
	AccQ-20	5	4.0^{a}±1.4	2.3^{a} ±0.2
	AccQ-40	5	5.5^{a}±0.6	3.0^{b} ±0.4

[a] n=number of analyzed samples.
[b] Numbers wearing the same letter are not significantly different ($P<0.5$, Duncan test) for each solvent and mycotoxin.

creased the standard error of the measurement. AccQ.Fluor-40 gave the best results in fluorescence response for both toxins and all assayed solvents, but the standard errors are frequently high.

To avoid residual presence of acetic acid, which could partially decompose the reagents, we added a buffer solution (pH=8) before the sample derivation. However, this procedure led to no improvement in the results but rather a significant decrease in quality (data not shown).

In spite of using the same solvent for the standards and samples, the fluorescence response to fumonisin levels was solvent dependent. Then, the corn matrix seemed to have a clear influence on the results; it may have modified mycotoxins solubility in the different solvents systems. Also, some component of the corn matrix can dissolve within the sample at different extends depending on the solvent. This component may have reacted with the reagents changing their initial concentration.

Although methanol appears to be the solvent of choice, a potential problem could arise from its use. Fumonisins are fairly unstable in that solvent [17]; consequently, methanol solutions can only be kept for a short interval. That problem could be avoided by dissolving samples in methanol just before HPLC analysis as Sydenham and co-workers suggest [9]. Stock solutions can be stored in water–acetonitrile (1+1, v/v); standard solutions in the different solvents are then prepared just before analysis.

5. Conclusions

For AccQ.Fluor reagent, only methanol seems to be the solvent of choice when the derivatizing reaction is carried out using 20 μl. Doubling the reagent amount also led to good results when water–acetonitrile (1+1, v/v) as solvent was used, but the standard error increased. Acetonitrile proved to be the poorest solvent compared to the fluorescence response for both AccQ.Fluor and OPA methods. Consequently, AccQ.Fluor reagent is a useful alternative fluorescence derivatizing reagent for determination of fumonisins B_1 and B_2 in corn when methanol is used as solvent. The derivatives are quite stable showing a good fluorescent response [13].

The new more acidic mobile phase proposed for AccQ.Fluor derivative analyses eliminates back pressure problems of our former method [13].

Acknowledgements

This research was supported by the Spanish Government (CICYT, grant ALI98-0509-C04-01). We are grateful to the Agencia Española de Cooperación Internacional (Programa de Becas MUTIS), for a grant to C.V.

References

[1] W.C.A. Gelderblom, K. Jaskiewicz, W.F.O. Marasas, P.G. Thiel, R.M. Horak, R. Vleggaar, N.P.J. Krierk, Appl. Environ. Microbiol. 54 (1988) 1806.
[2] R.D. Plattner, D. Weisleder, D.D. Shackelford, R. Peterson, R.G. Powell, Mycopathologia 117 (1992) 23.
[3] G.J. Diaz, H.J. Boermans, Vet. Human Toxicol. 36 (1994) 548.
[4] J.D. Miller, J. Stored Prod. Res. 31 (1995) 1.
[5] K.H. Plumlee, F.D. Galey, J. Vet. Intern. Med. 8 (1994) 49.
[6] T. Yoshizawa, A. Yamashita, Y. Luo, Appl. Environ. Microbiol. 60 (1994) 1626.
[7] E.W. Sydenham, G.S. Shephard, P.G. Thiel, C. Bird, B.M. Miller, J. Agric. Food Chem. 44 (1996) 159.
[8] J.G. Wilkes, J.B. Sutherland, M.I. Churchwell, A.J. Williams, J. Chromatogr. A 695 (1995) 319.
[9] E.W. Sydenham, G.S. Shephard, P.G. Thiel, S. Stockenstrom, P.W. Snijman, D.J. Vanschalkwyk, J. AOAC Int. 79 (1996) 688.
[10] A. Visconti, A. Boenke, M. Solfrizzo, M. Pascale, M.B. Doko, Food Addit. Contam. 13 (1996) 909.
[11] S.A. Cohen, D.P. Michaud, Anal. Biochem. 211 (1993) 279.
[12] J. Díaz, J.Ll. Lliberia, L. Comellas, F. Broto-Puig, J. Chromatogr. A 719 (1996) 171.
[13] C. Velázquez, C. van Bloemendal, V. Sanchis, R. Canela, J. Agric. Food Chem. 43 (1995) 1535.
[14] G.S. Shephard, E.W. Sydenham, P.G. Thiel, W.C.A. Gelderblom, J. Liq. Chromatogr. 13 (1990) 2077.
[15] M.E. Cawood, W.C.A. Gelderblom, R. Vleggaar, Y. Behrend, P.G. Thiel, W.F.O. Marasas, J. Agric. Food Chem. 39 (1991) 1958.
[16] W.R. Dantzer, E. Hopmans, A. Clark, C. Hauck, P.A. Murphy, J. Agric. Food Chem. 44 (1996) 3730.
[17] A. Visconti, M.B. Doko, C. Bottalico, M.B. Schurer, A. Boenke, Food Addit. Contam. 11 (1994) 427.