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Robotic automated clean-up for detection of fumonisins B_1 and B_2 in corn and corn-based feed by high-performance liquid chromatography

Paulo Dilkin[a,b], Carlos A. Mallmann[a,*], Carlos A.A. de Almeida[a], Benedito Corrêa[b]

[a]*Departamento de Medicina Veterinária Preventiva – Universidade Federal de Santa Maria, 97105-900 Santa Maria, RS, Brazil*
[b]*Departamento de Microbiologia, Instituto de Ciências Biomédicas – Universidade de São Paulo, 05508-900 São Paulo, SP, Brazil*

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Abstract

A high-performance liquid chromatography (HPLC) system with fluorescence detection and an automated on-line solid-phase extraction procedure for fumonisins B_1 and B_2 in corn and corn-based products is described. Different amounts of strong anion-exchange, C_{18} and end-capped C_{18} ($C_{18\ ec}$) silicas were tested for sample clean-up. Various HPLC parameters were analyzed. The best methodology was found to be extraction with acetonitrile–water and clean up on $C_{18\ ec}$ disposable extraction cartridges. The system has the advantage of running in an unattended mode of operation and allows processing of 40 samples without system refuel, performing clean-up, *o*-phthaldialdehyde derivatization, injection and fumonisin detection by fluorescence detection linked to a computer integrator for automated data processing. Recoveries were performed with corn and corn-based feed samples ($n=3$) spiked with 0.1, 0.5, 1.0, 5.0 and 10 μg/g. Average recoveries for corn and corn-based feed were, respectively, 92.6 and 88.3% with relative standard deviations (RSDs) of 5.04 and 6.22%, for fumonisin B_1 and 91.2 and 89.0% with RSDs of 5.84 and 7.88% for fumonisin B_2. Detection limits ($S/N=3$) for corn and corn-based feed were approximately 0.03 μg/g for fumonisin B_1 and 0.05 μg/g for fumonisin B_2. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

Fumonisins belong to a large group of mycotoxins produced by fungi of the genera *Fusarium* [1,2] and *Alternaria* [3]. These are natural contaminants of cereals worldwide and are mostly found in corn and corn-based products [4,5].

The most abundant naturally occurring fumonisins, B_1 (FB_1) and B_2 (FB_2), are also the main etiologic agents of fumonisin-elicited toxicoses. Their action is characterized by inhibition of de novo sphingolipid biosynthesis and consequent elevation in the ratio of sphinganine and sphingosine in serum of exposed animals [6].

Fumonisin-related toxicoses have been described in different animal species. Examples are: liver tumors in rats [7], hepatic toxicosis in poultry [8], pulmonary edema in swine [9] and leukoencephalomalacia in horses [10]. Additionally, some epidemiological studies have shown a positive as-

*Corresponding author. Tel.: +55-55-2208-445; fax: +55-55-2262-166.
E-mail address: mallmann@ccr.ufsm.br (C.A. Mallmann).

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sociation between exposure to dietary fumonisin and increased risk of human esophageal cancer [11,12].

Several analytical methods have been developed for determining fumonisins in corn, corn-based foods and feeds, milk and *Fusarium* culture material. These methods include capillary gas chromatography, thin-layer chromatography, competitive enzyme-linked immunosorbent assay (ELISA), capillary electrophoresis with mass spectrometry and high-performance liquid chromatography (HPLC) [13–17].

Analyzing fumonisins in food matrixes usually involves extraction with organic solvent mixtures that contain methanol or acetonitrile and clean-up with strong anion-exchange (SAX) [14], silica C_{18} [15,18], end-capped C_{18} silica ($C_{18\ ec}$) [17], as well as an immunoaffinity clean-up column [13,19]. Numerous published methods on fumonisin analysis use conventional and laborious manual procedures for sample clean-up and *o*-phthaldialdehyde (OPA) derivatization [13,15,17]. An automated procedure for sample preparation and derivatization of corn extract for FB_1 analysis was proposed by Jordan et al. [19]. The most applied analytical method for quantification of fumonisins in foods and feeds is liquid chromatography with fluorescence detection [13].

The mobile phase may be composed of solvents at different concentrations, such as methanol–0.1 *M* sodium phosphate, pH 3.3 (80:20, v/v) [14], (68:32, v/v) [16] and acetonitrile–water–acetic acid (50:50:1, v/v) [20] or a gradient system using two mobile phase solutions of acetonitrile–water–acetic acid: solution A (39:60:1, v/v) and solution B (60:39:1, v/v) [12].

Presence of FB_1 and FB_2 in corn and mixed horse feed samples can be detected in approximately 16 min by a chromatographic system such as HPLC, with detection limits close to 0.05 μg/g for FB_1 and 0.1 μg/g for FB_2 [14].

The present work describes an automated clean-up and derivatization methodology for the quantification of FB_1 and FB_2, utilizing a commercially available automated sample preparation system (ASPEC) coupled on-line to a HPLC system with fluorescence detection. The primary objective of the automated approach described herein was to improve laboratory productivity, in order to meet the increasing demand for product analyses by agriculture and stock raising companies in Brazil. To validate what we propose to be a more efficient, fast and economic method for the analysis of fumonisins in large numbers of samples, several different factors known to interfere with analytical quality were also investigated, such as type and amount of silica to be employed for sample clean-up, amount of eluent, and mobile phase.

2. Experimental

2.1. Material, solvents and reagents

All solvents and reagents were analytical grade. Acetonitrile, methanol, OPA, 2-mercaptoethanol, orthophosphoric acid, monobasic sodium phosphate, solid-phase extraction C_{18}, (microparticle media, catalog No. 19831-1), $C_{18\ ec}$ (microparticle media, catalog No. 1198500100) and SAX silica (microparticle media, catalog No. 5.7203) were from Merck (Darmstadt, Germany). The chromatographic tube (3 ml, catalog No. 730160) and frits (catalog No. 730160) were from Applied Separations (Allentown, PA, USA). Water was purified by passing through a Milli-Q treatment system (Sanyo, Barstead Strum, UK). Acetic acid was from Quimibras (Rio de Janeiro, Brazil).

2.2. Preparation of clean-up disposable extraction cartridges (DECs)

The DECs were prepared by placing a frit at the end of the chromatographic tube (3 ml), weighing the absorbent (200, 300, 400 or 500 mg), packing it into the tube and placing another frit on top of the packing. The DECs were then compacted by hand-pressing with a glass rod.

2.3. Standards

FB_1 and FB_2 standards were from Sigma (St. Louis, MO, USA). Stock standard solutions were prepared in acetonitrile–Milli-Q water (50:50, v/v) at 1–5 mg/ml. Working standard solutions were prepared in acetonitrile–Milli-Q water (50:50, v/v) at 5 μg/ml. All standard solutions were stored at −18°C until use.

2.4. Apparatus

2.4.1. Automated sample preparation system

The ASPEC system (Gilson, Villiers le Bel, France) was coupled on-line to the HPLC system. It includes racks for tubes and for DECs. The ASPEC standard software is task-oriented and suitable for the application of a straightforward clean-up using DECs. The sample collection rack was on-line with a water-bath equipped with a water circulator and a temperature controller set at 30°C, to keep fumonisin derivatives at a constant temperature. The software of the system was adapted for fumonisin analysis and permitted: conditioning; loading DECs, elution, sample and reagents addition, mixing, derivatization time and injection into the HPLC system. The ASPEC system also allows the decontamination through the clean-up of the different components, i.e., DECs, needle, and injection port.

2.4.2. HPLC equipment

The HPLC system consisted of a degasifier on-line (Licht Concept, Hamburg, Germany), a GBC Scientific Equipment pump Model LC 1150 and oven for columns (ICI Instruments, Dingley, Australia), a chromatographic column (150×4.6 mm) packed with Nucleosil 100, 5 μm (Macherey–Nagel, Düren, Germany), pre-column (20×4.6 mm, 5 μm), and a Merck fluorescence detector Model F100 (Hohenbreunn near Munich, Germany) on-line with a Pentium computer that included Borwin chromatography integrator software, version 1.20 (Le Fontanil, France).

2.5. Optimization of fumonisin extraction, clean-up and derivatization

2.5.1. Samples

Extractions to determine efficiencies were repeated ($n=5$) with fumonisin-negative ground corn samples spiked with FB_1 and FB_2 at 2.5 and 3.2 μg/g, respectively.

2.5.2. Extraction

Two extraction solvents and clean-up systems previously described were tested, with slight modifications: (A) 10-g aliquot of sample in 50 ml of acetonitrile–water (50:50, v/v) for use with C_{18} DECs [15,17,18], and (B) 25 g of sample in 50 ml of methanol–water (75:25, v/v) [14] for clean-up on SAX DECs. All extractions were done with a blender (Walita, São Paulo, Brazil) at high speed for 5 min. Next, each mixture was filtered through Whatman No. 4 filter paper. A 2-ml volume of acetonitrile–water extract was mixed with 6 ml of water for clean-up with C_{18} or $C_{18\ ec}$ silica DECs. Before clean-up, sample pH was adjusted to 5.8–6.5 with 1 *M* NaOH when necessary.

2.5.3. Clean-up

The solid-phase clean-up, derivatization and injection procedures were performed by ASPEC. All clean-up DECs (C_{18}, $C_{18\ ec}$ and SAX) were tested in five repetitions and were composed of 200, 300, 400 or 500 mg of absorbent.

The sequence of operations for the automated clean-up of samples using ASPEC with C_{18} and $C_{18\ ec}$ silica DECs was as follows: (1) condition the DEC with 2 ml of acetonitrile. (2) Condition the DEC with 2 ml of water. (3) Push the 8 ml sample through the DEC (2 ml sample and 6 ml of distilled water). (4) Rinse needle. (5) Wash the DEC with 5 ml of water. (6) Elute mycotoxins with 2 ml of acetonitrile–water (70:30, v/v), pH 5.8–6.5.

The clean-up employing SAX DECs was performed according to the following procedure: (1) condition the DEC with 8 ml of methanol. (2) Condition the DEC with 8 ml of methanol–water (3:1, v/v). (3) Push the 5 ml sample through the DEC. (4) Rinse needle. (5) Wash the DEC with 8 ml of methanol–water (3:1, v/v). (6) Wash the DEC with 3 ml of methanol. (7) Elute mycotoxins with 5 ml of methanol–acetic acid (99.5:0.5, v/v).

Flow-rates through these DECs were set at 2 ml/min; however, pushing of sample and eluting procedures were performed at a flow-rate of 1 ml/min.

2.5.4. Derivatization

The ASPEC was programmed to advance to derivatization and injection of samples after each elution, according to the following procedure: (1) rinse needle. (2) Dispense 200 μl of OPA solution (dissolve 40 mg of OPA in 1 ml of methanol and dilute with 5 ml 0.1 *M* sodium tetraborate; add 50 μl of 2-mercaptoethanol) into a clean sample tube

conditioned in a temperature-controlled rack at 30°C. (3) Rinse needle. (4) Add 50 μl of test solution. (5) Mix derivative solution (by aspersion and dispense). (6) Rinse needle. (7) Wait for a period of 2 min. (8) Inject 100 μl in the chromatography system. (9) Rinse needle. (10) Rinse injection port. (11) End.

A second derivatization program was used to set up the standard injection and construct a calibration curve. Aspiration and dispensing of OPA and test solutions were at 10 μl/s.

2.6. HPLC determination

2.6.1. Mobile phase

HPLC was performed on-line with ASPEC. The mobile phases tested were: one composed of methanol–0.1 *M* monosodium phosphate buffer (pH adjusted to 3.3–3.35 with orthophosphoric acid) according to Shephard et al. [14] and Sydenham et al. [16]; and another consisting of acetonitrile–water–acetic acid (50:50:1, v/v), named solution A, with a linear gradient of acetonitrile (solution B) according to Chu and Li [12] and Stack and Eppley [20], with some modifications. For the first 8 min of the chromatographic run, the mobile phase consisted of 100% of solution A, at the end of this period, it was changed to A–B (85:15). The mobile phase then returned to 100% of solution A by means of a linear gradient over a period of 4 min. These mobile phases were filtered through a 0.45-μm Waters HV membrane and pumped at a 1 ml/min flow-rate over the entire chromatogram. The chromatographic column was maintained at a constant temperature of 35°C. Fumonisins were detected by a fluorescence detector with wavelengths set at 335 nm for excitation (ex) and 440 nm for emission (em), in accordance with several published reports [12,18,20]. Calculation of fumonisin concentrations in test samples was based on peak areas compared with those of the standards as follows: FB_1 or FB_2 (ng/g)=$(ABC)/(DE)$. Where A=peak area of FB_1 or FB_2 from test solution, B=concentration of FB_1 or FB_2 (ng/μl) in standard solution, C=final volume of test solution (μl), D=peak area of FB_1 or FB_2 from standard solution, and E=mass of test sample represented by test solution (g).

2.7. Regeneration and re-use of $C_{18\ ec}$ clean-up DECs

Control fumonisin-free (detection limits 0.03 and 0.05 μg/g for FB_1 and FB_2, respectively) ground corn was spiked with FB_1 and FB_2, at 5.0 μg/g, and submitted to the extraction procedure; clean-up was performed in five separate $C_{18\ ec}$ DECs, each with 300 mg of silica. After the first extraction, the used DECs were treated by passing through them 15 ml of acetonitrile followed by 10 ml of water and then 10 ml of air. All reconditioning procedures were performed by ASPEC with the flow-rate set at 2 ml/min. Additional aliquots of the same extract were then applied to the regenerated DECs, and the fumonisin concentrations were once more determined according to the method proposed in this work.

2.8. Statistical evaluations

Descriptive statistics (mean, standard deviation and relative standard deviation, RSD) were first applied to the data, followed analysis of variance (ANOVA). Tuckey's test ($P<0.5$) was used for comparison of the means. The statistical analyses were done by computer with the software Statgraphics, version 3.0 (Statgraphics Manugistics, Rockville, MD, USA).

3. Results and discussion

3.1. Clean-up results

The influence of the extraction method and amount of C_{18}, $C_{18\ ec}$ and SAX on clean-up is shown in Table 1 (FB_1) and Table 2 (FB_2). Results are expressed as average percent recovery from five analyses of ground corn spiked with FB_1 at 2.5 μg/g and FB_2 at 3.2 μg/g.

Our results showed that the best performance was obtained by extraction with acetonitrile–water (50:50, v/v) and clean-up with C_{18} DECs, especially $C_{18\ ec}$, when compared to extraction with methanol–water (75:25, v/v) and clean-up with SAX DECs. These differences may be due to the short extraction time when employing shaking extraction with metha-

Table 1
Average recovery of FB_1 from corn samples spiked with 2.5 μg/g, performed with clean-ups on different DECs

DEC (mg)	FB_1 recovery (%)*		
	Silica C_{18}	Silica $C_{18\ ec}$	SAX
200	74.9^{de} (6.0)**	73.8^{e} (3.6)	1.6^{j} (5.6)
300	81.3^{c} (5.3)	94.8^{a} (1.7)	33.3^{h} (9.8)
400	68.5^{f} (3.0)	86.3^{b} (5.4)	40.4^{g} (4.9)
500	65.9^{f} (5.3)	79.8^{dc} (2.0)	28.3^{i} (4.6)

*Average percent recovery from five repetitions. **RSD (%).
$^{a-j}$Different letters represent statistically significant differences ($P<0.05$).

nol–water and the small volume of solvent used for toxin elution on SAX DECs, according to previous reports [14,16,17]. However, since our purpose was to establish a rapid methodology, and because ASPEC does not have an option for sample concentration, we eliminated methanol extraction and clean-up with SAX DECs.

The $C_{18\ ec}$ DECs, performed better for clean-up of both FB_1 (Table 1) and FB_2 (Table 2) and maximum results were obtained when packing 300 mg of silica. Additionally, reproducibility with these DECs was good, with the lowest RSD. Our results thus show that the type and amount of silica used for clean-up can greatly influence analytical quality. Hence, we suggest that clean-up by manual methodologies (MMs) which employ 500 mg of silica [18] require further consideration and may not be suited for automated methods. Rice et al. [17] reported good results with MM clean-up when using 360 mg of $C_{18\ ec}$, an amount of silica very close to our optimized one.

Table 2
Average recovery of FB_2 from corn samples spiked with 3.2 μg/g, performed with clean-ups on different DECs

DEC (mg)	FB_2 recovery (%)*		
	Silica C_{18}	Silica $C_{18\ ec}$	SAX
200	49.3^{cd} (8.8)**	81.5^{a} (19.7)	15.2^{f} (48.6)
300	55.4^{bcd} (14.0)	91.1^{a} (4.7)	46.4^{cde} (10.7)
400	41.7^{cde} (38.8)	72.3^{ab} (13.7)	57.5^{bc} (19.6)
500	37.2^{de} (41.9)	54.3^{bcd} (28.7)	28.5^{ef} (39.2)

*Average percent recovery from five repetitions. **RSD (%).
$^{a-f}$Different letters represent statistically significant differences ($P<0.05$).

3.2. HPLC parameters

Two mobile phases were tested to evaluate which would have the best characteristics for routine work. The first consisted of methanol–0.1 *M* monosodium phosphate buffer (pH adjusted to 3.3–3.35 with orthophosphoric acid). However, it caused excessive wear of the pistons and seals of the pump as evidenced by several mechanical problems that we encountered while using it with this solution. An additional inconvenience was the saturation of the chromatographic column, detected by an increase in pressure after prolonged use (3 months). None of these problems were observed when testing acetonitrile–water–acetic acid (50:50:1, v/v) and using a linear gradient of the acetonitrile phase. This latter phase permitted the elution of FB_1 and FB_2 at approximately 8 and 15 min after injection, respectively (Fig. 1).

The reason for good reproducibility and higher recoveries is presumed to be the ability of the automatic workstation to maintain slow and constant flow-rates, solvent volumes and time periods for conditioning, clean-up derivatization and injection of all samples.

3.3. Re-use of $C_{18\ ec}$ clean-up DECs

Bearing in mind the possibility of re-utilizing DECs, an extract of fumonisin-contaminated corn sample was prepared and applied to five separate DECs with a 300 mg content of silica $C_{18\ ec}$. No significant difference was observed between the fumonisin concentrations recorded before and after DEC regeneration. RSDs were 3.7% for FB_1 and 4.3% for FB_2 for the first determination, and 4.4% for FB_1 and 4.7% for FB_2 for the determination with re-used DECs. No increase in chromatographic interference was detected. The results thus indicate that DECs with a 300 mg content of silica $C_{18\ ec}$, can be regenerated and re-used for fumonisin purification without loss of analyte recovery. The regeneration was carried out only once, because of the increase of impurity concentration on the DECs. This fact has already been noted previously, when we tried to regenerate them more than one time: some partial cloggings in the DECs occurred and made the passage of solvents through them difficult. This

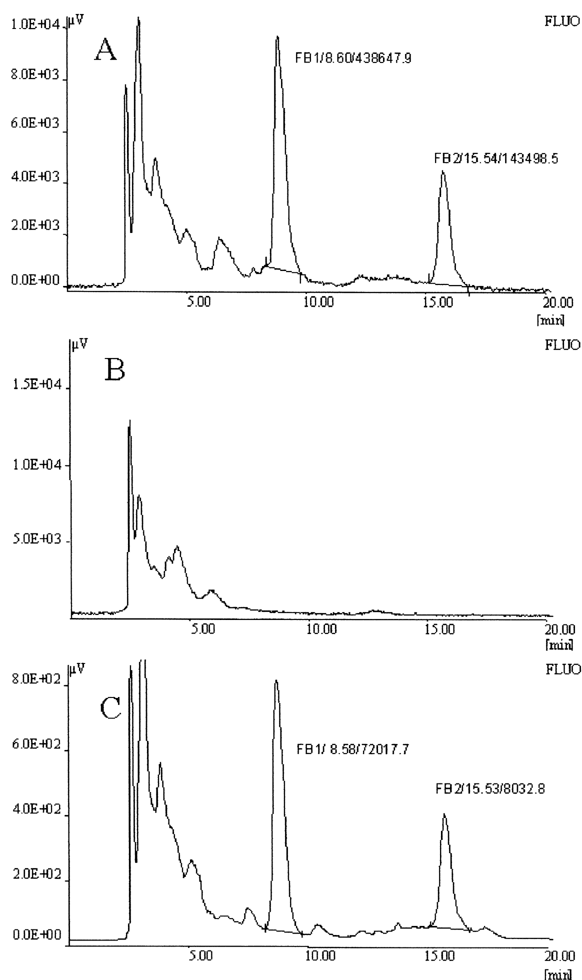

Fig. 1. Liquid chromatograms obtained by use of the automated system: (A) FB_1 and FB_2 standards, each at 5.0 μg/ml; (B) ground corn (control) cleaned-up by employing a $C_{18\ ec}$ DEC, with no detection of fumonisin; (C) ground corn cleaned-up by employing a $C_{18\ ec}$ DEC, with detection of FB_1 and FB_2 at 4.1 and 1.4 μg/g, respectively.

demands a higher pressure of the ASPEC system for the passage of solvents through the DECs and may lead to mistakes in the analyses.

3.4. Quality assurance

The performance of an (unattended) automated system has to be assured by several methods besides a simple visual inspection. To this end, we evaluated the following parameters: (a) volume of solvents and reagents required by the system; (b) artificially spiked samples, during routine analyses; (c) profile of chromatographs; (d) sensors and switches built into the system for detection of solvent leak; and, (e) uninterruptible power supply equipment.

Confirmation of the identity of the peaks assigned as FB_1 and FB_2 was made by comparing test chromatograms with standards, with attention to retention, start and end time of peak elution. Samples that presented a peak at the fumonisin retention time were confirmed by addition of standard and reprocessing.

The recovery tests of FB_1 and FB_2 were performed by control ground corn and corn-based feed samples ($n=3$) spiked with these fumonisins at levels of 0.1, 0.5, 1.0, 5.0 and 10.0 μg/g. The FB_1 recovery ranges for corn and corn-based feed were from 85 to 98%, RSD 5.1% and from 83 to 95%, RSD 6.2%, respectively. Similar results were achieved by Jordan et al. [19], mainly when they employed automated methodology for FB_1 clean-up. on corn samples, obtaining an RSD of 5.09%. FB_2 recovery ranges were from 81 to 96%, RSD 5.9% and from 80 to 96%, RSD 7.9% for corn and corn-based feed, respectively. The determination limits were approximately 0.05 μg/g for FB_1 and 0.07 μg/g for FB_2. Detection limits were 0.03 μg/g for FB_1 and 0.05 μg/g for FB_2 (signal-to-noise ratio of 3). The calibration curve was obtained by applying 100 μl ($n=3$) of derivatization mixture that contained equivalent quantities of 6.25, 12.5, 25.0 and 50.0 ng of FB_1 and FB_2 ($n=3$). These graphics were linear within this range, $r=0.9998$ and 0.9996 for FB_1 and FB_2, respectively.

The method proved to be reliable and robust for routine work with more than 1000 analyses during a period of 14 months.

4. Conclusions

The preparation of samples using $C_{18\ ec}$ DECs in an automatic work station on-line with HPLC analysis is an appropriate methodology for the quantification of FB_1 and FB_2 in corn and corn-based products. Besides reducing the amount of manual work, this method presents other advantages, such as: uniformity of sample handling as evidenced by good

reproducibility; coefficients of recovery and quantification limits adequate for a simultaneous detection of FB_1 and FB_2 over a wide range of concentrations in samples.

The proposed automation of solid-phase extraction and purification as well as derivatization of fumonisins B_1 and B_2, evaluated in the present study allow us to conclude:

(1) The methodology best adapted to automated purification of FB_1 and FB_2 was extraction with acetonitrile–water (50:50, v/v), clean-up on $C_{18\ ec}$ DECs with 300 mg silica and elution with acetonitrile–water (70:30, v/v);

(2) The re-utilization of $C_{18\ ec}$ DECs with 300 mg silica for purification of FB_1 and FB_2 proved to be viable, with no significant loss of FB_1 or FB_2 upon reconditioning of used DECs with 10 ml of acetonitrile followed by 10 ml of water and 10 ml of air.

(3) The automated method for clean-up and derivatization performed by ASPEC presented good reproducibility, recovery and, especially, reliability. As one chromatography analysis is performed, the next sample is already being prepared, thus allowing resolution of one sample every 22 min. The system has the capacity to run 40 samples during approximately 15 h non-stop.

(4) The mobile phase best adapted to the analytical method developed was composed of acetonitrile–water–acetic acid (50:50:1, v/v), with a linear gradient of acetonitrile (mobile phase B) from 8 to 12 min, starting with 15% and progressing to 0% mobile phase B.

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