Comparison of the suitability of derivatisation agents in HPLC - fluorescence detection analysis of fumonisins

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Summary
Suitability of derivatisation agents - *o*-phthaldialdehyde (OPA), 2-mercaptoethanol (MET), *N*-acetyl-L-cysteine (*N*-AC), 4-chloro-7-nitrobenzofurazan (NBD-Cl), and dansylchloride (DC) - for determination of fumonisins in model solutions and in beers has been studied measuring UV/VIS stability of reaction complexes as well as fluorescence characteristics. While DC adducts of fumonisins showed sufficient stability, OPA and MET gave the best fluorescence responses. However, stability of their reaction complex was much lower than that of the dansylated adduct. The mixture of OPA and *N*-AC provided comparable results to OPA and MET mixtures. Under given reaction conditions, the lowest fluorescence response of derivatised fumonisins was observed with NBD-Cl. Calibration lines for both fumonisins in standard solution were linear within the range 2.2–3000 $\mu g.l^{-1}$ with the coefficient of linearity 0.9998.

Keywords
fumonisins; derivatisation; HPLC; UV/VIS; beer

Some of the mycotoxins, the secondary fungi metabolites are considered as the most dangerous natural food contaminants. Fumonisins are mycotoxins belonging to this group already within the scope of the Slovak food legislation [1]. Fumonisins are produced by fungi of the genus *Fusarium*. Species producing significant amount of fumonisins are *Fusarium verticillioides* and *Fusarium proliferatum*. There are at least other ten *Fusarium* species producing these toxins [2]. *Fusarium verticillioides* and *Fusarium proliferatum* are the major pathogens of maize all around the world [2]. That is why maize and maize based food products are the most likely to be contaminated with fumonisins. There were also reported results of the contamination of beer [3]. Among 41 beer samples analysed four samples contained fumonisin B_1 (FB_1) at the levels lower than 2 $\mu g.l^{-1}$ (the highest one 59 $\mu g.l^{-1}$) and three samples contained also fumonisin B_2 (FB_2) at the levels lower than 2 $\mu g.l^{-1}$ (the highest one being 12 $\mu g.l^{-1}$) [3]. There were also reported results of rice contamination [4]. Fumonisins were identified in beef muscle after continuous exposure of cattle to highly contaminated feed [5]. There is a very low incidence of fumonisins in milk. Fumonisins were observed (FB_1 only) in one among out of 165 milk samples [6]. Comprehensive documentation on fumonisins monitoring around the world as well as their potential health risk was published by the Joint Expert Committee on Food Additives 2001 [2]. Fumonisins belong to a group of structurally related compounds. Due to the lack of any chromophore, direct measurement are impractical. But owing to the relatively high stability of fumonisins [7], there is a great opportunity for using derivatisation under a variety of conditions and a broad range of derivatisation agents. Commonly, *o*-phthaldialdehyde (OPA) [8] or dansylchloride (DC) [9] are used for derivatisation of aliphatic amino groups. OPA was applied as a pre-column derivatisation agent [10] as well as post-column one [11]. Due to the lack of stability of OPA's derivatisation complex, 2-mercaptoethanol (MET) is added to the reaction mixture [12-14]. Because of toxic effects of MET other experiments have been done with the aim to find acceptable alternative [15, 16]. It was found that *N*-acetyl-L-cysteine (*N*-AC) is a suitable alternative to MET. Amino group in fumonisin molecule was also successfully derivatised with naphthalene dicarboxaldehyde [16] and 4-fluoro-7-nitrobenzofurazan [17].

This study was directed towards comparison of various pre-column derivatisation procedures

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of fumonisins by testing fluorescence response stability of the acquired derivatised fumonisins. The aim of this work was to study the suitability of derivatisation agents and their mixtures (OPA + MET, OPA + *N*-AC, NBD-Cl and DC) for analytical determination of fumonisins in model solution as well as in beer matrix.

MATERIAL AND METHODS

Reagents

o-phthaldialdehyde (OPA), 2-mercaptoethanol (MET), *N*-acetyl-L-cysteine (*N*-AC), 5-dimethylaminonaphthalene-1-sulfonylchloride (dansylchloride, DC), and disodium hydrogen phosphate were from Merck (Darmstadt, Germany); 4-chloro-7-nitrobenzofurazan (NBD-Cl) was purchased from Fluka (Buchs, Germany); fumonisin B_1 (FB_1), fumonisin B_2 (FB_2), acetonitrile for HPLC, and methanol for HPLC were from Sigma Aldrich (Louisiana, USA); disodium tetraborate, sodium hydrogen carbonate, sodium dihydrogen phosphate, sodium chloride, potassium dihydrogen phosphate, potassium chloride, *o*-phosphoric acid (> 85%), and acetone (all chemicals were p. a.) were delivered by Lachema (Brno, Czech Republic); glacial acetic acid p. a. was from AFT (Bratislava, Slovakia); hydrochloric acid 35% was from ITES (Vranov nad Topľou, Slovakia); immunoaffinity columns Fumoniprep were received from Biopharm Rhone (Glasgow, United Kingdom); deionised water for HPLC was prepared using Purite Select unit from Purite (Oxon, United Kingdom).

Derivatisation procedures

Model derivatisation reactions were carried out with FB_1. The stock standard solution was prepared at the concentration 1.257 mg.ml^{-1} and stored at –18 °C in a mixture of acetonitrile + water 1:1 (v/v). The working standard was prepared by dilution of the stock standard to the level 31.4 μg.ml^{-1}. Working standard was stored at 4 °C. The stock solution of FB_2 was also diluted in the same solvent as FB_1 the concentration of 0.252 mg.ml^{-1}. The working standard was prepared at the level 6.3 μg.ml^{-1}. Conditions for the storage of FB_2 standard solutions were the same as for FB_1.

Derivatisation reaction 1

Preparation of OPA and MET reaction mixture: 40 mg of OPA was diluted in 1 ml of methanol and 5 ml of 0.1 mol.l^{-1} disodium tetraborate was added. The mixture was stirred for 1 minute and 50 μl of MET was added. This mixture was stirred again for 1 minute. To the 450 μl of FB_1 standard solution with concentration FB_1 of 31.4 μg.ml^{-1} was added 50 μl of the prepared derivatisation mixture. After shaking, the mixture was ready for injection on HPLC.

For UV/VIS kinetic measurements a mixture consisting of 400 μl of FB_1 standard solution (with FB_1 final concentration of 4 μg.ml^{-1}), 2770 μl of 0.1 mol.l^{-1} disodium tetraborate, and 30 μl of derivatisation solution was prepared.

Various volumes of 60 μl, 30 μl, and 16 μl of derivatisation solutions were tested. Optimal for the purpose proved to be the volume of 30 μl. FB1 concentration 4 μg.ml^{-1} was taken with regard to the expected concentration of fumonisin in real food (beer) samples.

Derivatisation reaction 2

Preparation of *N*-AC and OPA reaction mixture: 40 mg OPA was diluted in 1 ml of methanol and 5 ml of 0.1 mol.l^{-1} disodium tetraborate was added. The mixture was stirred for 1 minute and then 50 mg of *N*-AC was added. This mixture was stirred again for 1 minute. To the 450 μl of FB1 standard solution with concentration FB_1 of 31.4 μg.ml^{-1} was added 50 μl of the prepared derivatisation mixture. Shaked, the mixture was ready for injection on HPLC.

For UV/VIS kinetic measurements a mixture of 400 μl of FB_1 standard solution (with FB_1 final concentration of 4 μg.ml^{-1}), 2770 μl of 0.1 mol.l^{-1} disodium tetraborate, and 30 μl of the derivatisation solution was prepared.

In this case various volumes of derivatisation solutions were tested, namely 60 μl, 30 μl, and 10 μl. The optimal volume of 30 μl was used. FB_1 concentration 4 μg.ml^{-1} was taken with regard to the expected concentration of fumonisins in real food (beer) samples.

Derivatisation reaction 3

DC for HPLC measurement was prepared at concentration of 1 mg.ml^{-1} in acetone. Into 250 μl of FB_1 standard solution (final concentration 1.14 μg.ml^{-1}) was added 100 μl of DC and 100 μl of the saturated solution of sodium hydrogen carbonate. The mixture was stirred for 1 minute and then tempered in a water bath at 40 °C for at least 30 minutes. Afterwards the mixture was quickly cooled to the ambient temperature. Then 100 μl of 0.1 mol.l^{-1} phosphoric acid with pH value of 3.3 was added.

The final concentration of FB_1 for UV/VIS kinetic measurement was 4 μg.ml^{-1}, created by mixing together of 2100 μl of FB_1 standard solution, 300 μl of the derivatisation solution, and 300 μl of

saturated solution of sodium hydrogen carbonate. The mixture was mixed for 1 minute and tempered in water bath at 40 °C at least for 30 minutes. Afterwards the mixture was quickly cooled to the ambient temperature. Before measurement 300 μl of 0.1 mol.l^{-1} phosphoric acid with pH value of 3.3 was added.

Derivatisation reaction 4

Methanolic solution of NBD-Cl was prepared at concentration 0.05 mol.l^{-1}. Into 250 μl of FB_1 standard solution (final concentration 1.2 μg.ml^{-1}) was added 100 μl of 0.1 mol.l^{-1} disodium tetraborate and 50 μl of the NBD-Cl solution. The mixture was stirred for 1 minute and then tempered at 60 °C for 5 minutes. After cooling, 100 μl of the mixture of methanol + 0.05 mol.l^{-1} sodium dihydrogen phosphate (pH = 5) 1:1 (v/v) was added. Kinetic measurements by UV/VIS were done again with the concentration of FB_1 as in previous cases.

Equipment

UV/VIS measurements

UV/VIS equipment Specol (Carl Zeiss, Jena, Germany) was used for kinetic measurements. A quartz cuvette of 3 ml volume was used. Absorption measurements were done at 335 nm except for NBD-Cl, which was measured at 464 nm. The reference cuvette was always filled up with derivatisation agent and to the measurement cuvette was added the standard solution of FB_1 with concentrations described in part "Derivatisation procedures". The zero level of UV/VIS equipment was set with appropriate derivatisation mixtures without FB_1 before measurement. Absorbance data were taken at regular intervals. The entire experiment took 3 hours.

HPLC measurements

Agilent Technologies 1100 Series HPLC with quaternary pump, autosampler, and fluorescence detector (Halbron, Germany) was used for assessing the stability of reaction complexes as well as for estimation of fluorescence response. The excitation wavelength was 335 nm, while emission wavelength was set on multi emission mode. This setting was used for derivatisation mixtures OPA + *N*-AC, OPA + MET, and DC respectively. For NBD-Cl derivatisation agent the following setting was applied: excitation wavelength at 464 nm and emission wavelength at the maximum available. The column Zorbax SB C-18, 4.6 × 250 mm (Agilent Technologies, Palo Alto, USA) with appropriate guard column was used. Mobile phase for all reaction mixtures except that prepared with NBD-Cl was methanol + acidic water + acetonitrile (72:23:5) (v/v/v). Acidic water was prepared by adding 10 ml of glacial acetic acid into 1000 ml of deionised water. NBD-Cl derivatives were estimated by gradient elution of mobile phase: A was methanol + 0.05 mol.l^{-1} sodium dihydrogen phosphate (at pH = 5) (1:1) (v/v); B was acetonitrile + water (4:1) (v/v). Programmed gradient elution was 5 minutes of A and afterwards 15 minutes of A + B (1:1) (v/v). Fumonisins were identified by retention times and comparison of UV spectra of eluted peaks during HPLC analysis.

Beer analysis

Samples were prepared by the method of Scott and Lawrence [3]. Immunoaffinity columns Fumoniprep were used. Dansylation was performed under conditions described above, 250 μl of beer extract was used instead of FB_1 standard solution. Derivatisation mixture with OPA + MET was prepared as mentioned above. Derivatisation of the sample was performed using autosampler which assured good reproducibility of each step of derivatisation. From 250 μl of beer extract 20 μl was taken and 10 μl of derivatisation reagents was added. After mixing and waiting (up to 3 minutes) the volume of 30 μl was injected onto analytical column.

The conditions for separation of dansylated samples were as follows: mobile phase was mixture of methanol + acidic water + acetonitrile (69:26:5) (v/v/v); for OPA + MET derivative products following gradient was applied: mobile phase of methanol + acidic water + acetonitrile (69:26:5) (v/v/v) was used for 17 minutes, afterwards the mobile phase of methanol + acidic water + acetonitrile (72:23:5) (v/v/v) from 17th minute to 27th minute of elution was used.

Calibration line for both fumonisins was linear within suitable the range 2.2–3000 μg.l^{-1} with the linearity coefficient of 0.9998.

RESULTS AND DISCUSSION

Because fumonisins exhibit no fluorescence, they have to be derivatised before HPLC-FD analysis. The fumonisin molecule contains one amino group at the end of a hydrocarbon chain (Fig. 1). This is the place where a fluorescence agent could be bound. As found, fluorescence intensity of fumonisin's adduct was closely dependent on the derivatisation reagent used (Tab. 1).

UV/VIS kinetic measurements showed that the derivatisation mixtures of OPA + *N*-AC and

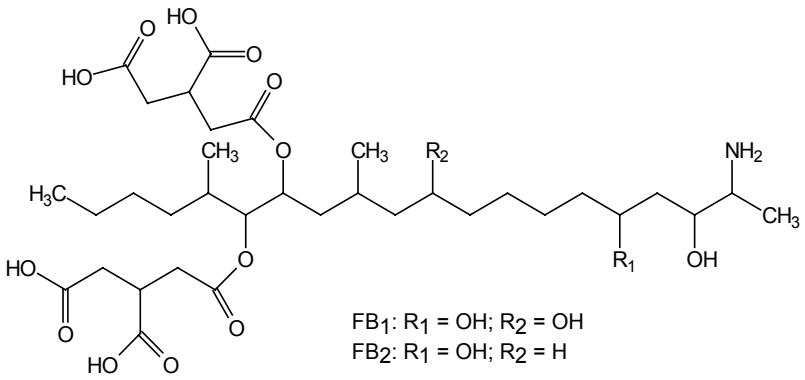

Fig. 1. Chemical structure of fumonisin FB_1 and FB_2.

OPA + MET appeared very similar in reaction kinetics as well as having reaction products of similar stability (Fig. 2, 3). A slight stability decline in time was observed in case of UV/VIS absorbance measurements at 335 nm. Similar decline was confirmed by HPLC analysis (Tab. 1).

Fig. 3 illustrates absorbance decrease of derivatised fumonisin's molecules after a few minutes of measurement in the case when mixture of OPA + MET was used for derivatisation. These absorbance values were dependent on the amount of derivatisation mixture at the beginning of derivatisation reaction (e. g. for addition of derivatisation mixture 16 μl was measured value of 0.03 absorbance units and increased after 3 minutes up to 0.035 absorbance units, for 60 μl addition was obtained initial value of 0.04 absorbance units and no additional increase was observed). It was evident that an induction period was required when derivatisation mixture was not in sufficient excess (3 minutes approximately). Similar phenomenon was also observed when OPA + *N*-AC were used for derivatisation (Fig. 2.).

Likewise, there was a slow gradual fall decrease of absorbance for both OPA + MET and OPA + *N*-AC derivatisation mixtures with similar behaviour during UV/VIS measurements for all tested additions of derivatisation reagent. Derivatisation reaction was fast enough (OPA + MET), e.g. 3 minutes were sufficient for mass transfer within the reaction vessel and for completion the derivatisation. The only difference between both derivatisation mixtures was different first stage up to 20th minute for OPA + *N*-AC.

Both reaction mixtures presumably follow a similar reaction pattern [15]. One practical implication of this fact is the recommendation to wait at least 3 minutes after adding derivatisation mixture into the sample extract before injection on the separation column when amount of fumonisins is in the sample is unknown. This showed to be true both for OPA + *N*-AC and OPA + MET mixtures. It is also recommended to add sufficient access of the derivatisation agent.

Dansylation of amines is popular since it affords relatively stable complexes. This was confirmed by FB_1 (Fig. 4). UV/VIS measurements indicated that reaction complex was stable enough for two and half hours. HPLC measurements also supported these results (Tab. 1). For successful derivatisation increased temperature (see part "Derivatisation procedures") and additional time (30 minutes) were required. The advantage of this was obtaining stable derivative complex at the end of mentioned time interval, the drawback being relatively low fluorescence response of the derivatised fumonisin (Tab. 1). On the whole, low fluorescence response is close to the detection level of fumonisin, accounting for the single greatest dis-

Tab. 1. HPLC measurements of time stability of derivatised fumonisin FB_1 in standard solution.

Derivatisation mixture	Time [h]	Peak area of FB_1 (1.2 μg.ml^{-1}) [mAUs]
OPA + MET	0*	137
	3	79.7
	6	53.8
OPA + *N*-AC	0*	35.1
	3	31.2
	6	26.2
DC	0*	5.5
	3	5.2
	6	5.4
NBD-Cl	6	4.4
	48	4.3

* - time, 0 represent the prompt injection after derivatisation reaction was completed (see part "Derivatisation procedures").

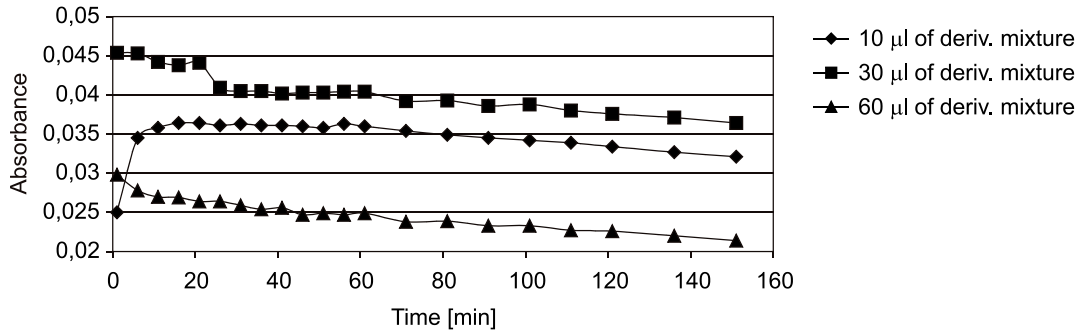

Fig. 2. Kinetic measurements of the products derivatised with various volumes of the mixture of OPA + *N*-AC.

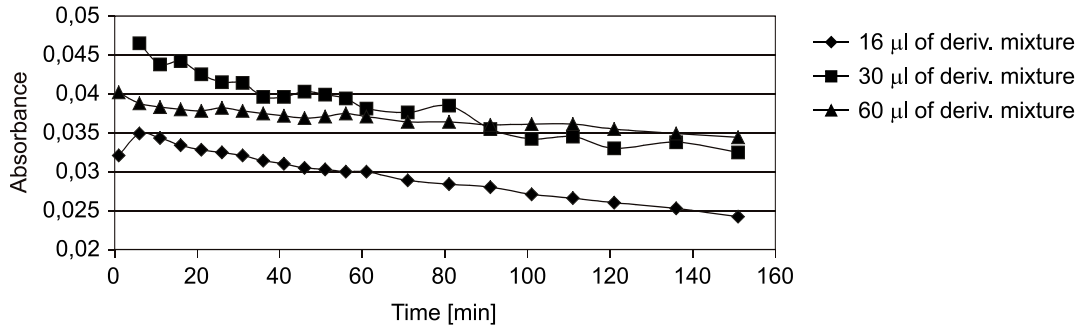

Fig. 3. Kinetic measurements of the products derivatised with various volumes of the mixture of OPA + MET.

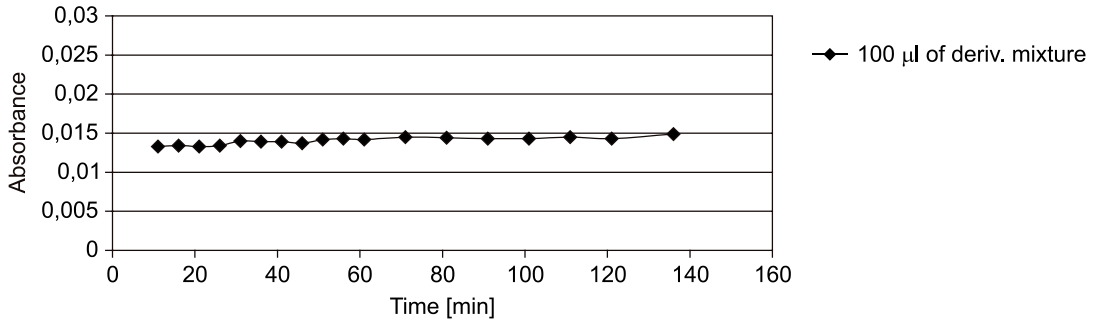

Fig. 4. Kinetic measurements of the product derivatised with dansylchloride.

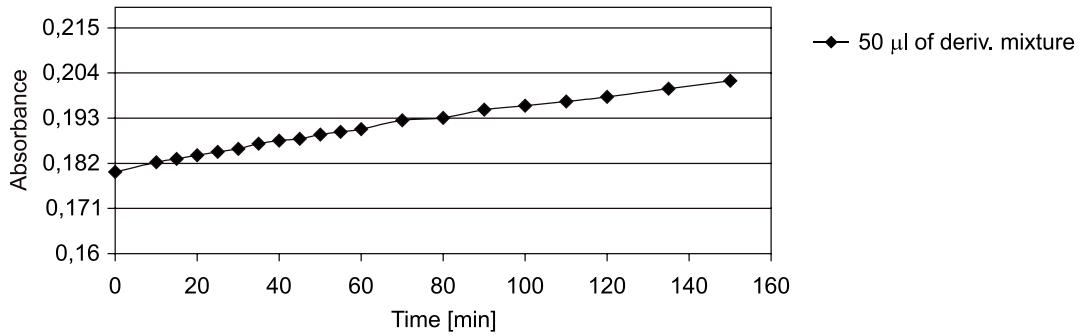

Fig. 5. Kinetic measurements of the product derivatised with NBD-Cl.

advantage of dansylated reaction products.

NBD-Cl was less reactive in comparison with 4-fluoro-7-nitrobenzofurazan [17]. Nevertheless NBD-Cl is recommended as suitable agent for derivatisation of primary amines. In our experiments, various conditions leading to completion of derivatisation were tested. Temperature was set to 60 °C and time was changed from a few minutes up to 1 hour. Under these conditions the expected reaction failed to proceed. This is documented in Fig. 5. Even more than two and half hour was not enough to complete the reaction. Applying HPLC it was recognized that after 6 hours the reaction was at last over and the peak of derivatised fumonisin did not increase any more. This fact showed that the complex was stable for at least 3 days, as confirmed HPLC.

Application of dansylation was examined in beer samples analysis. Immunoaffinity cleaning was an efficient tool for elimination of interfer-

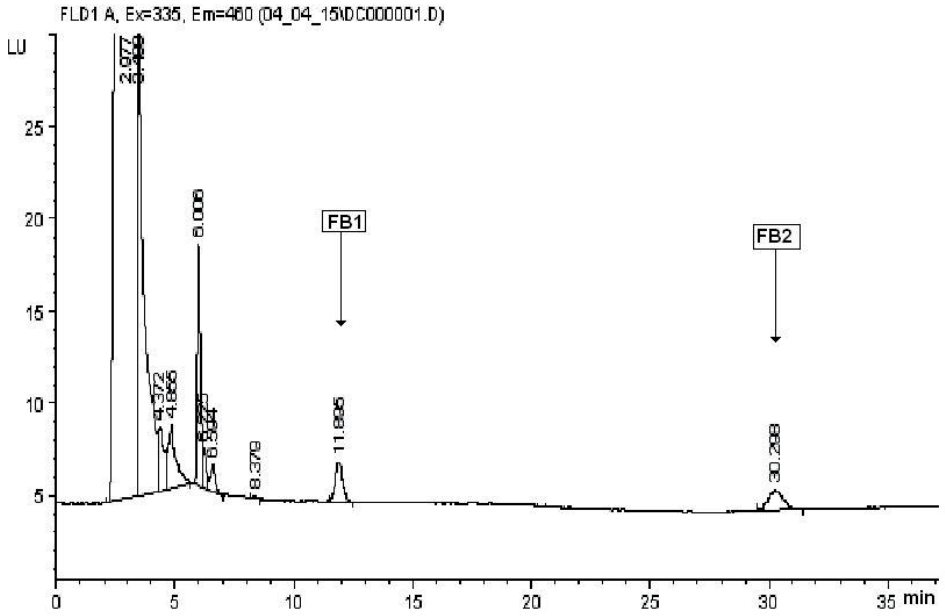

Fig. 6. Dansylated standard of fumonisins FB_1 and FB_2.

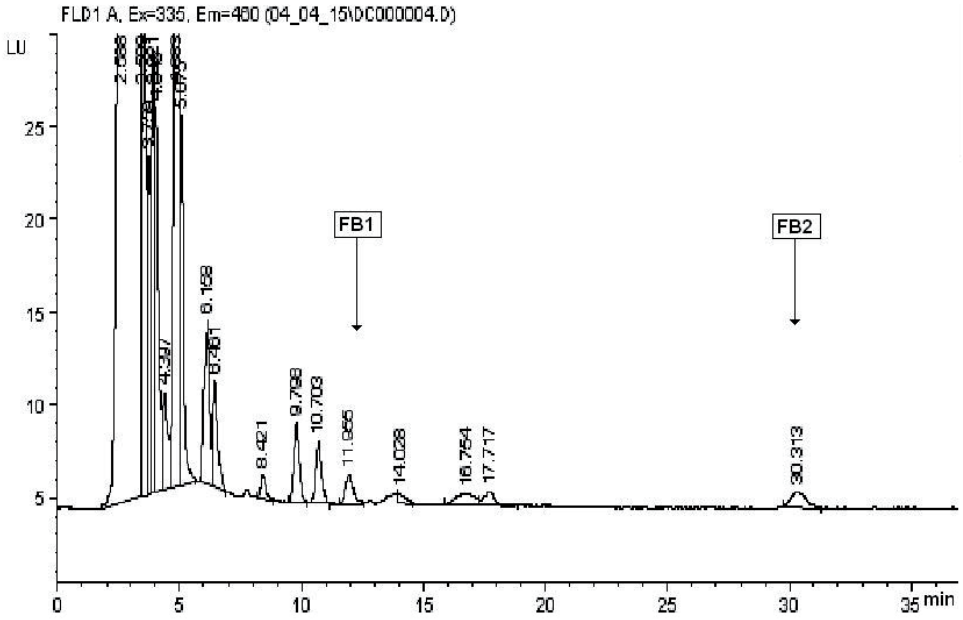

Fig. 7. Dansylated beer sample spiked with FB_1 and FB_2 to to the level of 4 $\mu g.l^{-1}$.

ing substances from beer. The chromatographic separation was satisfactory (Fig. 6, 7). No naturally contaminated beers were found, therefore beer samples had to be spiked with standard solution of fumonisins with concentration 4 $\mu g.l^{-1}$.

CONCLUSION

Comparison of four derivatisation mixtures for determination of fumonisins was studied. As far as the highest fluorescence response is concerned, our experiments showed that the best solution was the mixture of OPA + MET. Similar results were obtained using derivatisation mixture of OPA + *N*-AC. Dansyl derivatives had lower fluorescence response than the above mixtures, but it was still sufficient for FB_1 determination at levels of 4 $\mu g.l^{-1}$. NBD-Cl was not suitable for derivatisation of fumonisins under applied conditions. The best choice for obtaining stable derivatised complexes seemed to be DC. It was confirmed that 3 hours were sufficient for derivatised products to remain stable till the end of HPLC analysis. It would be especially convenient in the case when the autosampler is not accessible. The lack of stability of derivatisation complex prepared by reaction mixtures of OPA + MET and OPA + *N*-AC was compensated by the short time required for derivatisation reaction and would be eliminated by application of autosampler. Both mentioned derivatisation mixtures should be added in excess.

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