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«TRAINING ACTIVITIES ON FOOD CONTAMINATION CONTROL AND MONITORING WITH SPECIAL REFERENCE TO MYCOTOXINS»

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APPLICATION OF GAS-LIQUID CHROMATOGRAPHY IN THE FUSARIOTOXIN ANALYSIS (TRYCHOTHECENE, ZEARALENONE)

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GAS-LIQUID CHROMATOGRAPHY IN THE ANALYSIS OF FUSARIOTOXINS (TRICHOTHECENS, ZEARALENONE)

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1. Introduction

Gas-liquid chromatography (GLC) is commonly used for the identification and quantitative determination of a number of mycotoxins in foodstuffs and fodder. In particular, the techniques involving GLC have been developed for the analysis of trichothecen mycotoxins (T-2 and HT-2 toxins, diacetoxyscirpenol, deoxynivalenol, nivalenol, etc.), zearalenone, patulin and some others.

The GLC technique is characterised by high partition effect, adequate reproducibility of results and high sensitivity.

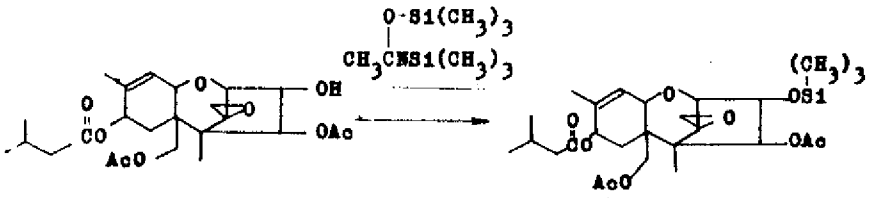
The subject of the present seminar is to discuss practical aspects of this technique for the analysis of fusariotoxins using the examples of T-2 toxin, zearalenone and a mixture of trichothecens, isolated from contaminated grain.

1.1 Production of volatile fusariotoxin derivatives: derivatization

The molecules of T-2 toxin and other trichothecens, and also zearalenone contain one or several free hydroxyl groups which reduce substance volatility and complicate direct GLC analysis. This, chemical modification of analyzed toxins with the aim to obtain volatile derivatives (derivatization)

represents a necessary preliminary stage preceding the GLC analysis.

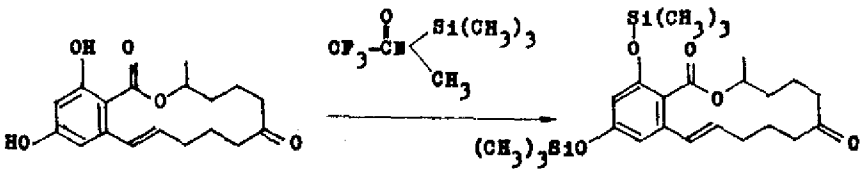
Silylation represents the most common derivatization technique, when an active hydrogen of the hydroxyl group is substituted for a trimethylsilyl group (TMS). TMS-derivatives are usually less polar, more volatile and more thermostable than the original compounds. N, O-bis (trimethylsilyl) acetamide (BSA) serves as one of the most effective reagents for the production of TMS-derivative T-2 toxin. Trimethylchlorsilane (TMCS) is used as catalyzer of silylation with BSA.



T-2 toxin BSA TMS-T-2 toxin

BSA can be used with such solvents as pyridine, dimethylsulfoxide, tetrahydrofuran, benzene, and also without solvents. A mixture of N-trimethylsilylimidazole (TSIM) with trimethylchlorsilane and a trademark mixture "TRI-SIL TBT" composed of TMCS, BSA and TSIM which is produced by the Pierce Chemical Company (USA), are also used for trichothecen silylation.

The production of di-TMS-derivatives of zearalenone is most often realized through the use of N-methyl-N-trime thylsilyltrifluoroacetamide (MSTFA) in the presence of acetone.



Zearalenone MSTFA di-TMS-zearalenone

A trademark compound "TRI-SIL BT" representing a mixture of BSA and TMCS produced by the Pierce Chemical Company (USA), is also used.

Alongside with TMS-derivatives, the production of perfluoranyl derivatives, such as trifluoracetyl (TFA), pentafluoropropionyl (PFP) and pentafluorobutyryl (PFB) ethers, represents a promicing direction in the derivatization of fusariotoxins. Perfluoracyl derivatives possess a number of advantages in comparison with TMS-derivatives: they are more volatile, are usually more stable and ensure higher sensitivity of an electron capture detector. For the perfluoracylation of fusariotoxin hydroxyl groups the corresponding anhydrides (trifluoracetic, pentafluorpropionic, gentafluorbutyric) with bases (pyridin, trimethylamine, sodium carbonate), necessary for the binding of the reaction-generated acid, are used:

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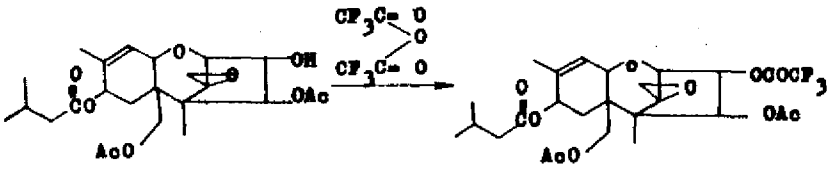

T-2 toxin TFAA TFA-T-2 toxin

The use of sodium carbonate is more preferable due to the absence of reaction-produced volatile by-products complicating GLC. In the acylation of phenyl hydroxyl groups, e.g. in the case of zearalenone, the reaction-produced acids can cause partial hydrolysis of a perfluoracid derivative and reduce the elution at derivatization. In such cases anylating means are used, which give no acid products at reaction, such as perfluoracylmidazoles and N-methyl-bis-(trifluoracetamide).

2.2. Separation and identification of fusariotoxins with the use of GLC

For the GLC separation of volatile fusariotoxin derivatives packed and open capillary columns are used. The effect of column separation is assessed by the number of the so-called "effective theoretical plates" $N_{t.p.}^{eff.}$, which is calculated by the formula:

$$N_{t.p.}^{eff.} = 5.54 \left(\frac{t_r - t_m}{W_{0.5}} \right)^2 \text{, where}$$

t_r - substance retention time, representing the interval between the moment of sample injection and that of elution of a chromatografic zone centre;

t_m - time of the unretained component elution, approximately corresponding to the time of the low-boiling solvent peak elution;

$W_{0.5}$ - chromatographic peak width at half-height.

Packed columns represent glass or metallic tubes containing solid carrier powder impregnated with the stationary liquid phase. The efficiency of such columns does not exceed 5000 t.p. A high separation effect by wall coated open tubular columns (WCOT) covered by a thin film of the liquid phase (0.5-1.5 mm). The effect rate of WCOT constitutes 50 000-200 000 t.p. which exceeds that of packed columns by a factor of 20 or more. GLC with WCOT ensures total separation of TFA- and TMS-derivatives of basic trichothecens and zearalenone.

The identification of separated compounds is performed on the basis of retention parameters. The most reproducible parameter is the relative corrected retention time $t'_{R\ rel.}$ of a substance - a so-called inner standard, injected into the sample prior to the GLC analysis n-alkanes with various chain lengths are frequently used as an inner standard. The value $t'_{R\ rel.}$ is determined by the formula:

$$t'_{R\ rel.} = \frac{t'_{R\ x}}{t'_{R\ in.\ st.}}, \text{ where}$$

$t'_{R\ x}$ - corrected retention time of the component X;

$t'_{R\ in.\ st.}$ - corrected retention time of the inner stan-

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dard.

The comparison of the above parameter with corresponding parameters of toxin standards or with literature data ensures a reliable identification of chromatographic peaks. The reproducibility of the relative corrected retention time values reduces if $t'_{R\ rel.}$ is more than 4 and less than 0.25, i.e. if the inner standard and the analyzed substance differ greatly by the retention time value. In this case more reliable results can be obtained using the retention indices (RI) of toxins on the basis of corresponding n-alkanes.

To identify RI of a substance X the latter is injected into a chromatograph together with a mixture of n-alkanes. Thus obtained chromatogramme is presented in Fig.

The RI of a substance X is determined by the formula:

$$RI_X = 100n + 100\ \frac{\lg t'_{R(x)} - \lg t'_{R(Cn)}}{\lg t'_{R(Cn+1)} - \lg t'_{R(Cn)}}\text{, where}$$

$t'_{R(x)}$ - corrected retention time of a substance X;

$t'_{R(Cn)}$ - corrected retention time of an n-alkane with the number of carbon atoms (n), eluted prior to the substance X;

$t'_{R(Cn+1)}$ - corrected retention time of an n-alkane with the number of carbon atoms (n+1) eluted prior to the substance X.

As can be seen from the formula, RI of n-alkanes are equal to the number of carbon atoms multiplied by 100, e.g. the $RI_{C_{23}} = 2300$. The needed t_m for the determination of

corrected retention time values is obtained on the basis of a known fact that when a column functions in the isothermic regime there exist a linear dependance between the logarithm of the corrected retention time of an n-alkane and a number of carbon atoms, i.e.:

$$\frac{\lg (t_{R(Cn+1)} - t_m) - \lg (t_{R(Cn)} - t_m)}{\lg (t_{R(Cn)} - t_m) - \lg (t_{R(Cn-1)} - t_m)} = 1$$

Using the above dependance it is possible to determine the value t_m by the formula:

$$t_m = \frac{t_{R(Cn+1)} \cdot t_{R(Cn-1)} - t^2_{R(Cn)}}{t_{R(Cn+1)} + t_{R(Cn-1)} - 2t_{R(Cn)}}, \text{ where}$$

$t_{R(Cn+1)}$; $t_{R(Cn)}$; $t_{R(Cn-1)}$ - absolute retention time values of n-alkanes with (n+1), (n), (n-1) carbon atoms, accordingly.

Thus, for a reliable identification of trichothecen derivatives and zearalenone both relative corrected retention time values by the inner standard (n-alkane), and retention indices (RI), can be used. It should be noted that both of the above values are reproducible when only one and the same liquid phase is used under the same column temperature.

1.3. Quantitative identification of fusariotoxins

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The most precise quantitative results in capillary GLC are obtained by the technique of inner standard, which involves

the addition of a known amount of inner standard to the analyzed mixture. The calculations are based on the fact that the mass of analyzed component is directly proportional to that of injected inner standard and to the area peak ratio of the component and the inner standard. The proportionality factor K (correction factor) of the analyzed component by the inner standard is determined on the experimental basis.

2. Experimental work

The experimental work is performed using a 25 m x 0.3 mm wall coated open tubular column (WCOT) with the liquid phase OV-101. The input separation of the gas flow is 1:50; the linear speed of the gas-carrier is 10 cm/sec. The column temperature is 240°C (for TFA-derivatives) and 250°C (for TMS-derivatives). Flame ionizing detection; amplifier sensitivity - 5×10^{-12}.

2.1 Production of volatile fusariotoxin derivatives

a) production of TMS-T-2 toxin

A 0.5 mg sample of T-2 toxin is introduced into a 2-3 ml flask, and a mixture, containing 10 ul BSA, 5 ul TMCS and 85 µl benzene, is added. The flask is sealed and is kept under 60°C during 15 min. Then it is cooled to the temperature of 20-22°C and is unsealed. The obtained solution can be directly injected into a chromatograph through a microsyringe.

b) production of the TFA-T-2 toxin and the di-TFA-zearalenone

10-15 mg of anhydrous sodium carbonate powder are added to a solution of 0.5 mg toxin (T-2 toxin or zearalenone) in 1 ml of benzene. Then 50 µl of trifluoracetic anhydride are poured in in the process of 60 min magnatic mixing at 20-22°C avoiding moisture penetration. Then 3-4 ml of benzene are added and the solution is paper-filtered into a 10-12 ml pear-shaped flask. The filtrate is evaporated to dryness using a rotation evaporator. The residue is diluted in 50-100 µl of chloroform or benzene and is microsyringed into a chromatograph.

c) production of TFA-derivatives of grain extract experimentally contaminated by a toxigenous strain Fusarium sporotrichiella

10 g of dried and ground grain, experimentally contaminated with Fusarium sporotrichiella, is extracted shaking the 70 ml methanol/water mixture (1:1) during 30 min and then paper-filtered. 40-50 ml of the filtrate is taken and is hexane-degreased in a separation funnel (2 x 20 ml). Then the hexane solution is removed while the trichothecen fraction is chloroform-extracted from the water/methanol solution (3 x 20 ml). Combined chloroform extracts are dried using anhydrate sodium sulfate, are filtered and boiled down in a rotation evaporator to the volume of 0.5-1.0 ml. The whole solution is transfered into a sealed 1.5-2.5 ml flask and is evaporated to dryness in the flow of nitrogen. 1 ml of benzene, 30-40 mg

of anhydrous sodium carbonete and 70-100 µl of trifluoracetic anhydride are added to the residue and mixed during 60 min at 20-22°C. 2-3 ml of benzene are added into the reaction mass, then the solution is cotton-filtered and is evaporated to dryness in a rotation evaporator. The residue is diluted in 30-70 µl of chloroform. The obtained solution can be micro-syringed into a chromatograph.

2.2 Determination of retention parameters of TMS- and TFA-derivatives of fusariotoxins

a) determination of a relative corrected retention time of TFA-T-2 toxin by n-tricosane on the liquid phase OV-101.

Order of work:

- to establish the following regime of work of a chromatograph:

column temperature - 240°C;

evaporator temperature - 290°C;

linear gas-carrier speed - 10 cm/sec;

flame ionizing amplifier sensitivity - 5×10^{-12};

- to prepare a benzene solution of n-tricosane with the concentration of 5 mg/ml;

- to add 50 µl of benzene solution of n-tricosane with the concentration of 5 mg/ml to a solution of TFA-T-2 toxin, obtained as described in para. 2.1 b);

- using a microsyringe to inject 0.2-0.3 µl of obtained solution (mixture of TFA-T-2 toxin and n-tricosane) into a gas chromatograph evaporator;

- identify time values of the solvent peak elution (t_m),

that of n-tricosane peak ($t_{R\ C23}$) and time of the TFA-T-2 toxin peak ($t_{R\ T-2}$);

- to calculate relative corrected retention time values of TFA-T-2 toxin by n-tricosane ($t'_{R\ rel.\ T-2}$) using the formula:

$$t'_{R\ rel.\ T-2} = \frac{t_{R\ T-2} - t_m}{t_{R\ C23} - t_m}$$

b) determination of relative corrected retention time values of di-TFA-zearalenone by n-tricosane on the liquid phase OV-101.

The order of work is similar to that described in para. 2.2 a), however, in this case instead of TFA-T-2 toxin solution a solution of di-TFA-zearalenone, obtained as described in para. 2.1 b), is used.

c) determination of the retention index (RI) of TMS-T-2 toxin on the liquid phase OV-101.

Order of work:

- to change the column temperature from 240°C to 250°C;

- to prepare a mixture solution of n-alkanes from eicosane $C_{20}H_{42}$ to octacosane $C_{28}H_{58}$ in chloroform with a concentration of about 2-3 mg/ml in each n-alkane;

- to add 100 µl of the obtained mixture solution of n-alkanes to the solution of TMS-T-2 toxin (see para. 2.1 a));

- using a microsyringe to inject 0.2 µl of the solution containing n-alkanes and TMS-T-2 toxin into a chromatograph evaporator;

- to determine absolute retention time values of TMS-T-2 toxin and n-alkanes;

- to determine the precise retention time of the unretained component (t_m) using RI values of any 3 consecutive n-alkanes according to the formula:

$$t_m = \frac{t_{R(C_{n+1})} \; t_{R(C_{n-1})} - t^2_{R(C_n)}}{t_{R(C_{n+1})} + t_{R(C_{n-1})} - 2\, t_{R(C_n)}}$$

- to determine the RI value of TMS-T-2 toxin by the formula (1).

d) T-2 toxin identification in grain extract using the value of relative corrected retention time by n-tricosane.

Order of work:

- to set the column temperature at 240°C;

- to add 10 µl of a benzene solution of n-tricosane with a concentration of 5 mg/ml to a solution of TFA-derivatives of grain extract contaminated with Fusarium sporotrichiella (see. para. 2.1 e));

- to inject 0.2-0.3 µl of the obtained solution into a gas chromatograph evaporator;

- to determine corrected retention time values of all chromatographic peaks, and also that of the inner standard - n-tricosane;

- to determine relative corrected retention time values of all chromatographic peaks and to identify T-2 toxin, comparing the above values with the value obtained in para. 2.2 a).

2.3 Determination of the calibrating coefficient of the/ TFA-T-2 toxin by n-tricosane

Order of work:

- to take 1 mg of T-2 toxin and 1 mg of n-tricosane;

- to add 2 ml of benzene, 20 mg of anhydrous sodium carbonate powder and to pore 100 µl of trifluoracetic anhydride to the above mixture and to perform T-2 toxin derivatization as described in para. 2.1 a);

- using a microsyringe, to inject the obtained solution containing TFA-T-2 toxin and n-tricosane (0.2-0.3 µl) into a gas chromatograph evaporator;

-to measure the areas of chromatographic peaks of TFA-T-2 toxin, S_{T-2} and n-tricosane S_{C-23}, multiplying the values of peak heights to peak width at half-height;

- to determine the calibrating correction factor of T-2 toxin by n-tricosane using the formula:

$$K = \frac{m_{T-2}\ S_{C-23}}{m_{C-23}\ S_{T-2}}$$; where

m_{T-2} - precise weighed portion of T-2 toxin in µg;

m_{C-23} - precise weighed portion of n-tricosane in mg.

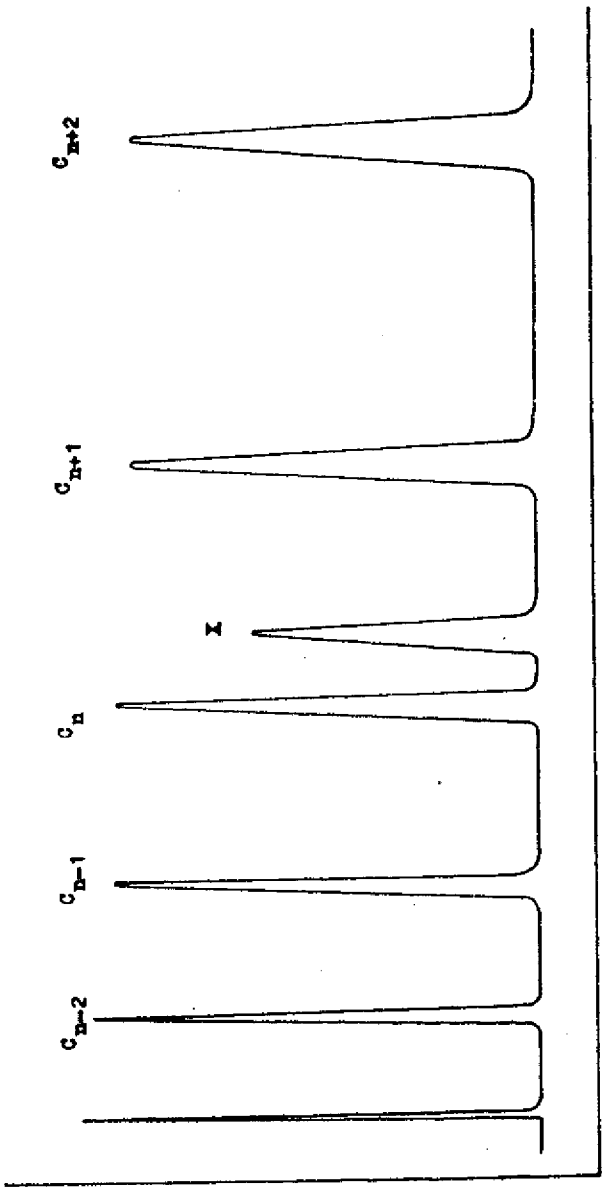

Fig. I. GLC of n-alkanes with a substance X for the identification of RI.

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