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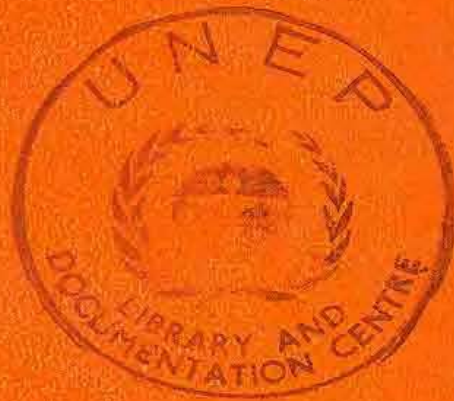
«TRAINING ACTIVITIES ON FOOD CONTAMINATION CONTROL  
AND MONITORING WITH SPECIAL REFERENCE TO MYCOTOXINS»

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**GAS-LIQUID CHROMATOGRAPHY  
AND ITS APPLICATION  
IN THE ANALYSIS OF MYCOTOXINS**



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Moscow, 1984

GAS-LIQUID CHROMATOGRAPHY AND ITS APPLICATION IN  
THE ANALYSIS OF MYCOTOXINS

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Gas-liquid chromatography (GLC) is one of most effective and widely applied methods for the separation, identification and quantitative analysis of complex multi-component mixtures. It has the advantages of high separation efficiency, considerably exceeding the possibilities of liquid chromatography (thin-layer chromatography (TLC), high-resolution liquid chromatography (HRLC)); good reproducibility of GLC chromatographic parameters, facilitating identification and acquisition of precise quantitative results; high sensitivity; universality of the applied detection techniques, making it possible to identify with a low detection limit all classes of organic compounds; lasting quality of analytic columns (compared to that of HRLC) used in GLC for separation (up to two years of continuous work).

At the same time, the GLC method has its drawbacks, of which the major ones are the limited number of compounds allowing direct GLC analysis (volatile thermostable substances) and the necessity of obtaining volatile derivatives (derivatization) for most of the analyzed classes of compounds.

Along with HRLC, used for the analysis of mycotoxins characterized by intensive fluorescence or absorbing UV radiation, and TLC densitometry, GLC serves as one of the basic instrumental methods of monitoring food contamination with mycotoxins. GLC is most widely used for the analysis of trichothe-

cene mycotoxins - a group of toxins including over 40 substances similar in their physico-chemical properties. The absence of fluorogenic and chromophore groups in trichothecenes, as well as the insufficient effectiveness of TLC for the separation of some of the trichothecene representatives, makes GLC practically the only method for the identification and quantitative assessment of this very common group of toxins. GLC and a combination of GLC with mass spectrometry (MS) are also used to identify with a very high sensitivity such mycotoxins as zearalenone, patulin, penicillic acid, etc.

#### The principle of GLC method

In GLC the separated components move along the column together with the flow of inert carrier gas (argon, helium, hydrogen and, most frequently, nitrogen). This is accompanied with the distribution of the mixture components between the mobile gaseous phase and the stationary liquid phase applied either on inert powdered material (packed columns) or on the column inner walls (open capillary column). The basic parameter determining the rate of substance, migration through the column is the distribution coefficient,  $K$ , equal to the ratio of substance concentration in the liquid and the gaseous phases:

$$K = \frac{\text{substance concentration in the liquid (stationary) phase}}{\text{substance concentration in gaseous (mobile) phase}}$$

Coefficient  $K$  depends only on the nature of the substance analyzed and that of the liquid phase, and on column temperature. The mixture components having different  $K$  values move through the column, at a different speed, forming separate chromatographic zones.

The chromatographic separation process is realized in an instrument - gas - liquid chromatograph, whose block diagram is shown in Fig. 1. The analyzed mixture is injected with a microsyringe into a vaporizer whose temperature should be sufficient to transform all the components into vaporous state. Usually the vaporizer temperature is by 50-100°C higher than that of the column. Vapour of the analyzed substances together with the carrier gas flow move through the column, where the components are being separated in accordance with the distribution coefficients. Separated chromatographic zones reach a detector, whose signal is amplified and automatically recorded by a potentiometer as the chromatographic peaks.

#### Separation in GIC (columns)

The basic unit of a chromatograph ensuring the necessary separation is the chromatographic column. In modern chromatographs two types of columns are used: packed and open capillary columns, whose main parameters are presented in Table 1.

The chromatographic process is characterized by the following parameters (Fig. 2):

$t_R$  is retention time of the substance, corresponding to the time interval from the moment of sample injection to the elution of the chromatographic zone centre;

$t_M$  is time of elution of the unretained component (e.g., methane), corresponding approximately to that of the low-boiling solvent;

$t'_R$  is corrected retention time, equal to  $t_R - t_M$ ;

$W_{0.5}$  is the peak width at its half-height;

$h$  is the peak height.

The narrower the chromatographic zone and its corresponding peak the less is the smearing of the zone in the course of GIC and the higher the column efficiency. The smearing of the chromatographic zone naturally increases with the residence time of substance in the column, and, therefore, the column efficiency depends both on  $t_R$  and  $W_{0.5}$ . It is quantitatively determined by the number ( $N$ ) of the so-called theoretical plates (t.p.) and is calculated from formula:

$$N_{t.p.} = 5.54 \left( \frac{t_R}{W_{0.5}} \right)^2 .$$

This formula usually yields overestimated efficiency ( $N_{t.p.}$ ) values, particularly at short retention times,  $t_R$ . In actual fact, the value characterizing the "dead gas volume",  $t_M$ , does not improve the chromatographic process. For example, one can attach a long narrow tube to the column inlet and get a very high value of  $t_M$  and, accordingly, very high  $N_{t.p.}$  values, exceeding by far the actual efficiency of the column. Therefore, in practice "the number of effective t.p.",  $N_{t.p.}^{eff.}$ , is used.

$$N_{t.p.}^{eff.} = 5.54 \left( \frac{t_R - t_M}{W_{0.5}} \right)^2 \quad \text{or}$$

$$N_{t.p.}^{eff.} = 5.54 \left( \frac{t'_R}{W_{0.5}} \right)^2 .$$

Packed chromatographic columns (PC) are made of glass or metal tubes with the inside diameter of 2-4 mm. The tubes are packed with inert solid carrier (infusorial earth or kieselguhr) with the grain size of the order of 0.1-0.25 mm; the carrier is pre-

impregnated with a stationary liquid phase. The efficiency of such columns is about 500-1000 t.p./m PC are characterized by a high resistance to gas flow, and their length does not usually exceed 2-4 m. Longer PC require very high carrier gas pressures, unacceptable in modern GIC equipment. The overall PC efficiency does not, therefore, exceed 5000 t.p.

A high separation efficiency is achieved in open capillary tubular columns (OCTC) - narrow glass tubes with the inside diameter of 0.2-0.5 mm. In OCTC the stationary phase is applied in the form of a 0.1-1.5 mm thick film directly onto the inner wall of a capillary. There is no packing of the column with inert solid support in OCTC. The liquid phase can be deposited directly on the smooth walls of the capillary (wall coated open tubular columns, WCOT) or retained by an intermediate porous layer of solid support previously applied on the capillary walls (support coated open tubular columns, SCOT). Specific efficiency of the WCOT type of columns is up to 3000 t.p./m, that of the SCOT type - up to 1200 t.p./m. The absence of a solid carrier filling the column section accounts for an important characteristic feature of OCTC: their low resistance to the mobile gas phase flow. This feature makes it possible to increase the column length to 50-100 m without using high pressure drops at the column inlet and outlet. The overall OCTC efficiency thus reaches the level of the order of 200,000 t.p. in the case of WCOT and of 100,000 t.p. in the case of SCOT, which is 20 and more times higher than the PC efficiency (see Table 1). High OCTC separation efficiency in combination with a higher, in comparison with PC, chemical inertness have caused an ever increasing application of glass and quartz capillary columns in the practice of

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organic analysis.

Separation of two substances, A and B (see Fig. 3), in GLC is characterized by the criterion of resolution ( $R_s$ ).

$$R_s = 1.18 \frac{t_{R_A} - t_{R_B}}{W_{0.5_A} - W_{0.5_B}}$$

Separation is regarded as satisfactory (peaks are separated by more than 98% of their area) if  $R_s$  exceeds 1.5. The value of  $R_s$  is a function of 2 quantities: selectivity of the liquid phase, measured by the distance between the vertices of the peaks,  $t_{R_A} - t_{R_B}$ , and the column efficiency characterized by the peak width,  $W_{0.5}$ .

Figure 3 clearly shows the influence of these factors on the separation of two peaks. An ideal variant is a combination of high efficiency and selectivity.

When choosing a liquid phase, one should take into account, along with its selectivity towards the components to be separated, such factors as adequate solubility of the mixture components in the phase, the low volatility, thermostability and chemical inertness of the liquid phase at the column working temperature. In the analysis of mycotoxin derivatives, nonpolar and low-polar silicone liquid phases are mostly used: nonpolar polymethylsiloxanes OV-101, OV-1 and SE-30 and a moderately polar phase - polymethylphenylsiloxane OV-17. These liquid phases are stable at temperatures up to 300-350°C, which ensures the possibility of prolonged column operation at working temperatures not exceeding 300°C. High OGC efficiency makes decisive the contribution of this parameter into the resolution criterion,  $R_s$ , and reduces considerably the requirements to the selectivity of the liquid phase.

Systems of sample injection into packed and open capillary tubular columns

The analyzed mixture must enter the column in the form of a narrow vapor zone. Most often 1-5  $\mu$ l solution of the analyzed mixtures is injected into a chromatograph by means of a microsyringe piercing a thermostable rubber membrane (see Fig. 4). The introduced substances then enter the vaporizer where they are completely transformed into a vaporous state and transported into the column together with the carrier gas flow. The vaporizer (Fig. 4) is a heated metal unit with a channel for inserting and evaporizing the liquid sample. For evaporation to be rapid the temperature in the vaporizer should exceed by 30-50°C the boiling point of the most high-boiling component of the sample; in practice the vaporizer temperature exceeds the working temperature in the column thermostat by 50-100°C. Low vaporizer temperature can result in the broadening of chromatographic zones caused by a slow evaporation of the sample, while an extremely high temperature - in thermal decomposition of the analyzed mixture components. To avoid strong dilution of the sample at injection, the volume of the vaporizer channel should be minimal; on the other hand, an inordinately small vaporizer channel volume results in an excessive rise of pressure in the system at sample injection. The evaporator "dead volume" is reduced by using a glass insert as the evaporation channel. Besides reducing the dead volume this insert ensures a more uniform heating-up of the evaporation zone and reduces the possibility of thermodestruction because of the high chemical inertness of glass.

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Sample injection into OCTC has a number of specific features. Smooth walls of WCOT columns retain a very small amount of liquid phase and, therefore, an increase in the sample mass drastically reduces the separation efficiency. The maximum size of the sample should not usually exceed  $0.1 \mu\text{g}$ . Columns of the SCOT type have a more developed inner surface and so retain larger amounts of the liquid phase, but even in case of SCOT columns the sample mass should not exceed  $1 \mu\text{g}$ . This makes it necessary to inject very small volumes of the analyzed solution (less than  $0.1 \mu\text{l}$ ) that cannot be reliably and reproducibly measured and introduced into the column. Moreover, the low carrier gas volume flow rate in OCTC increases considerably the time necessary for all of the sample to be completely blown out from the evaporation channel. All this, when the sample is injected in the conventional way, results in a considerable broadening of chromatographic peaks. That is why in capillary GLC the injection systems with the splitting of gas flow are used. This splitting allows a fast expulsion of the separated substances from the evaporation chamber, providing at the same time a comparatively low gas flow rate in OCTC. Another function of the splitting of flow is to permit only a part of the injected sample to enter the column. The gas flow is split with the help of a pneumoresistance operating parallel to the column and making it possible to control the splitting ratio from 1:50 to 1:500. The flow splitter must be linear, which means, that there should be no discrimination of separated components, i.e. no difference in the splitting of the high- and the low-boiling components of the analyzed mixture. Figure 5 shows the best possible injector

schemes with flow splitters and good linearity.

The shortcomings of inlet systems with flow splitting are their lowered detection limit for small impurities, because only an insignificant part of the substance enters the detector, as well as lower accuracy of quantitative results. That is why different systems for injecting microquantities of samples without the splitting of the flow are being now introduced into the practice of capillary GLC.

#### Detection of separated substances

To detect mycotoxin derivatives separated on a GLC column two types of detectors are used: flame-ionization detector (FID) and electron capture detector (ECD).

The most universal and widely used is FID. Figure 6 shows the FID schematic. The gas leaving the column is mixed with hydrogen and is burnt in air. When the carrier gas leaving the column is clean the number of ions in the flame is insignificantly small, and a very low detector background current is recorded between the collector electrode and the jet. When vapour of organic substances enters the flame the ions formed in it reduce the resistance of the inraelectrode space, which raises the current in the detector chain. The detector current is amplified and registered by an automatic recorder in the current - time coordinates. The area of the peak corresponding to the substance coming out of the column is linearly dependent on the mass of the substance. The concentration range within which a linear dependence of detector signal on the analyzed substance mass is preserved is extremely broad for FID, amounting to  $10^6$ - $10^7$ . The detector has a very low detec-

tion limit: no more than  $10^{-12}$  g/sec. The advantage of FID in the case of OCTC is that hydrogen, along with its function of maintaining the flame, provides for a rapid transfer of substances separated in the column through the detector supply lines to the flame, thus playing the role of the so-called auxiliary gas. This prevents the deterioration of total separation caused by the smearing of separated zones in FID supply lines. Less universal, but much more sensitive and selective for some classes of substances, is ECD. Figure 7 shows the ECD schematic. Fast electrons emitted by a  $\beta$ -radiative source ionize the carrier gas molecules (nitrogen). Slow electrons formed in this way are collected on a positively charged anode, which gives rise to the detector direct (background) current. When the vapour of substances having a high affinity to the electron, (halogen-containing compounds, nitrocompounds, nitriles, policonjugated carbonyl compounds, etc.) leaves the column, slow electrons are bound into negative ions with a comparatively low mobility leading to a sharp decrease in the ECD background current. The characteristic volume of ECD is sufficiently large, and working with it requires the presence of auxiliary gas flow, ensuring a rapid transfer of the components from the column through the detector, which facilitates the attainment of maximal sensitivity without increasing the carrier gas flow rate.

ECD is practically insensitive to hydrocarbons, alcohols and esters. The sensitivity of ECD rapidly increases with an increase in the atom number and the number of halogen atoms in the molecule. For polyhalides the detection limit can amount to  $10^{-14}$  g/sec, which exceeds FID sensitivity by a fac-

tor of  $10^2$ . The range of ECD signal linearity is not large and is usually equal to 50-1000; the width of this range decreases with an increase in sensitivity. The sensitivity of ESD can change very strongly even for substances with a similar structure, and the obtaining of quantitative results in the case of ECD application requires a preliminary calibration for each determined compound. In the mycotoxin analysis, ECD is used for the identification of nano- and even picogram amounts of halogen-containing derivatives of trichothecene toxins, zearalenone and a number of others.

Despite the high sensitivity and selectivity of ECD, it is not used as frequently as FID, due to considerable operational problems and the difficulty of obtaining precise quantitative results, particularly in the analysis of multi-component mixtures.

#### Identification of separated compounds by retention parameters

The principal method of the identification of substances in GLC is the comparison of absolute or relative retention parameters. Absolute retention time,  $t_R$  (Fig. 3), is not used for identification, because it strongly depends on the length and diameter of the column, dead volume of the instrument, carrier gas flow rate and other difficult to reproduce factors, and cannot, therefore, be compared with similar  $t_R$  values obtained with other instruments and columns. Absolute corrected retention time,  $t'_R$  (Fig. 2), allows for the dead volume of the instrument and, hence, is a better reproducible quantity. In practice, however, both  $t'_R$  and  $t_R$  can be used for identification only when GLC analyses are performed on one and the

same instrument in completely identical conditions. Much more reliable results, comparable with those obtained on a similar liquid phase in other instruments, are obtained by using relative retention parameters. In this case the retention of a given analyzed substance is expressed relative to that of a certain standard substance, added to the sample (the so-called "internal standard"). The substances used as internal standards should be as close as possible in their properties to the analyzed compounds, they should not yield peaks overlapping those of the analyzed compounds. N-alkanes with a varying length of the carbon chain are frequently used for this purpose.

Table 2 shows the data of 3 tests to determine the retention parameters of T-2 toxin trifluoroacetyl (TFA) derivative with respect to n-tricosane (n-C<sub>23</sub>) on a glass capillary column with liquid phase OV-101 at column temperature 240°C, performed in our laboratory.

Table 2

Test No.	$t_R$ (min)		$t_M$ (min)	$t'_R$ (min)		Relat. retention time	Corrected relative retention time
	T-2 toxin TFA	n-C <sub>23</sub>		T-2 toxin TFA	n-C <sub>23</sub>		
						$\frac{t_R(T-2)}{t_R(C_{23})}$	$\frac{t'_R(T-2)}{t'_R(C-23)}$
1	16,575	8,825	3,083	13,492	5,742	1,878	2,350
2	18,925	10,158	3,683	15,242	6,475	1,863	2,354
3	17,058	9,192	3,375	13,683	5,817	1,856	2,352

As seen from the data in Table 2, the most reproducible and suitable parameter for the identification of T-2 toxin (in the form of its TFA derivative) is its relative corrected retention time with respect to n-tricosane, equal to ratio

$$\frac{t'_R \text{ (TFA T-2)}}{t'_R \text{ (n-C}_{23}\text{)}}$$

Even more accurate and reproducible data can be obtained by means of logarithmic interpolation, interrelating the corrected retention time of the analyzed substance with those of a series of normal alkanes. This interpolation lies at the basis of the Kovacs system of retention indices (RI). The retention index (RI) for substance X is determined from the following formula:

$$RI = 100N + 100n \frac{\lg t'_R(X) - \lg t'_R(N)}{\lg t'_R(N+n) - \lg t'_R(N)}$$

where X is the analyzed substance;

N is n-alkane with N carbon atoms eluting in GLC before substance X;

N+n is n-alkane with (N + n) carbon atoms eluting in GLC after substance X.

When determining Kovacs indices, n-alkanes are chosen, that are the closest to the substance identified by GLC.

There also exists a method of calculating Kovacs indices with GLC performed not at a constant column thermostat temperature, but in a mode with its programming. In this case, however, the reproducibility of indices decreases from test to test.

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Retention indices of a given substance are well reproduced when the same liquid phase and column temperature are used both on one instrument and on instruments in other laboratories. Retention indices of various mycotoxin derivatives have been published in literature and, in the absence of pure standards, can be used as a means of their identification.

One should be cautious with mycotoxin identification by the retention parameters alone. For instance, one cannot exclude a possibility of the chromatographic characteristics of a mycotoxin coinciding with those of some other substance present in the sample, which will result in either the superposition of two peaks or in a pseudopositive mycotoxin detection. Such cases are not infrequent when one works with PC, there are, e.g., data on the superposition of the peaks of T-2 toxin BMS ethers and I-oleyl glycerine in GLC on PC with liquid phase OV-17 on a solid support - Chromosorb.

High resolution of glass OCTC considerably decreases the probability of peak overlapping and raises the reliability of identification by retention indices.

Some additional information on the separated peaks can be obtained by using selective detectors, e.g., ECD. In the GLC of perfluoroacyl derivatives of some fusariotoxins (trichothecenes, zearalenone) from the extracts of a number of food products, the chromatograms obtained by ECD contain fewer peaks of foreign substances than in the case of FID, which facilitates considerably the identification of analyzed compounds.

Mass-spectrometry is undoubtedly the most reliable method of identifying the substances coming out of the chromatographic column.



In the instruments where a mass spectrometer performs the function of a detector (chromato-mass-spectrometers) the gas flow from the column passes through the separator, isolating the carrier gas from the vapour of separated substances, into the ion source. In the ion source the substance is ionized. The analyzed substances are ionized by their molecules colliding with fast electrons whose energy is of the order 70 eV (ionization by "electron shock") or with the ions of a preionized reagent gas "chemical ionization"). Methane, isobutane, ammonia, etc. are used as reagent gases in chemical ionization. In the "electron shock" ionization the energy of ionizing electrons is much higher than the ionization potential and, therefore, not only the molecular ion characterizing the molecular mass of the substance, but also a whole number of fragmented ions are formed, while the intensity of the molecular ion can be very low. In chemical ionization, with methane serving as the reagent gas, a proton is attached to the analyzed molecule, forming a quasimolecular  $MH^+$  ion; with ammonia it is an  $NH_4^+$  ammonium ion, and, correspondingly, a quasimolecular ion with the  $M + 18$  mass is formed. In chemical ionization the ionizing energy is comparatively small, and so the mass spectrum contains few fragmented ions, with the quasimolecular ion having the most intensive peak. From this point of view, mass spectrometry with chemical ionization is more suitable for the purposes of mycotoxin identification than mass spectrometry with electron shock ionization. This is manifested especially clearly in the chromato-mass-spectrometric analysis of trichothecene toxin derivatives, which, usually, yield no molecular ions in electron shock ionization.

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Ions leaving the ion source enter a mass analyzer where they are separated depending on the value of their mass to charge ratio ( $M/e$ ). When the magnetic field in the magnetic mass analyzer changes, ion beams with a successively growing  $M/e$  ratio are focused at the outlet slit of the mass-spectrometer; the ion current thus obtained is amplified by an electron multiplier.

A recording of the signal from an electron multiplier, depending on the deflecting field voltage, is called a mass-spectrum of the analyzed substance. Possibility of unambiguous mass-spectrometric identification of GLC-separated peaks makes the method of chromato-mass-spectrometry the most reliable, arbitrary method of confirming the presence of mycotoxins in analyzed samples. A factor limiting its wide application is the high cost of chromato-mass spectrometers, which does not make them readily available for laboratories performing routine analyses of food products.

The method of chromato-mass spectrometry has been used by a number of investigators to analyze B-2 toxin TMS derivatives and some other fusariotoxins (diacetoxyscirpenol, necosolaniol, zearalenone). Mirocka et al. used chromato-mass spectrometry with detection by characteristic fragmented ions (mass fragmentography) to analyze mixed feed extracts for the presence of various trichothecenes and concluded that mass fragmentography is the most sensitive and selective technique for the identification of these toxins.

#### quantitative interpretation of GLC data

In the quantitative analysis by means of GLC one should remember that GLC does not yield data on the entire composition

of the analyzed sample, but only on the composition of its volatile components. Moreover, not all substances introduced into a column are stable in the conditions of GLC process. The necessity to obtain in advance the volatile derivatives of analyzed mycotoxins (derivatization) may also contribute to the distortion of quantitative results. In the FID or ECD analyses of the amounts of a sample lying within the linearity range of these detectors, the area of automatically recorded peaks is directly proportional to the mass of a substance coming out of the column. For quantitative assessment of GLC data two methods are used: the method of absolute calibration and the internal standard method. The principle of the method of absolute calibration is based on introducing separately certain amounts of a sample and known quantities of a standard substance into the chromatograph, followed by comparing their chromatographic peak areas. If the substance used as the "external standard" in absolute calibration differs from the one to be determined, then to calculate the mass of the analyzed substance from the ratio of peak areas one has to introduce corresponding correction factors. This is necessary because carbon atoms entering into the composition of different functional groups make a different contribution to the overall sensitivity of FID with respect to a given substance. For instance, the carbon atom bound with the hydroxyl group will increase the detector signal approximately 2 times less than the methylene group carbon atom. The necessity of a preliminary determination of correction factors is still more important in ECD whose sensitivity can change radically even with very slight variations in the composition of the standard and the analyzed substance. To obtain precise quantitative results when using the absolute calibration

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method strict requirements have to be met regarding complete similarity of the conditions in which the GLC process is conducted, reproducibility of the volumes of the sample and the standard injected by the microsyringe, and in the case of OCTO - the constancy of the gas flow splitting ratio at the column inlet. All these conditions make the analyses considerably more complicated, and in practice the "internal standard" method is much more frequently used.

This method involves adding to a certain amount of the analyzed mixture a precise quantity of a substance which this mixture does not contain - the internal standard.

The content of the analyzed component in the mixture is calculated from formula:

$$M_X = M_{\text{int. stand.}} \frac{S_X}{S_{\text{int. stand.}}} \times K,$$

where  $M_X$  is the mass of analyzed substance X;

$M_{\text{int. stand.}}$  is the mass of the introduced internal standard;

$S_X$  is the peak area of analyzed substance X;

K is the correction factor of substance X with respect to the internal standard.

When the internal standard method is used it is unnecessary to introduce a precise amount of sample into the chromatograph. Furthermore, the errors associated with accidental changes in column temperature, carrier gas flow rate, detector operating conditions are reduced to a minimum since both the standard and the analyzed substance are in equal conditions. Precision and, particularly, the reproducibility of quantitative data in the internal standard method are as a rule higher than in the

method of absolute calibration, while the labouriousness of analyses is considerably lower.

The internal standard introduced into the sample must meet the following requirements: its peak should not overlap those of the analyzed mixture; its peak should not significantly differ from the mixture components in retention times; the amount of the introduced internal standard should yield a peak whose area were close to those of the peaks from the analyzed substances.

According to our data, n-tricosane (Fig. 8) meets these requirements in the analysis of trichothecene TFA derivatives. The correction factor of T-2 toxin with respect to n-tricosane amounts to 2.2-2.3 when FID is used.

In determining zearalenone in the form of its TMS derivative, both in FID and ECD, a coprostanol TMS derivative is used; the use of other sterols is also possible.

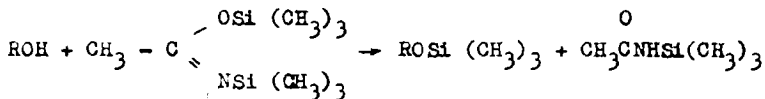
#### Methods of obtaining volatile derivatives (derivatization)

Most mycotoxins are complex multi-functional organic compounds containing a number of polar groups (most frequently - hydroxyl groups) which limit their volatility and make direct GLC analysis impossible.

Silylation and acylation of hydroxyl groups in mycotoxin molecules increases their volatility and makes these derivatives convenient for GLC analysis.

The derivatization reagents should meet the following basic requirements: unambiguous course of reaction, quantitative reaction yield, sufficient reaction rate in mild conditions excluding even a partial destruction.

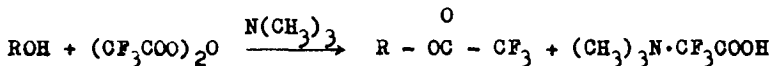
To obtain volatile derivatives of trichothecene toxins, zearalenone and patulin, the corresponding trimethylsilyl ethers are used. Bis-trimethylsilylacetamide (BSA) is the reagent most frequently used to obtain these derivatives. In the general case the reaction proceeds as follows:



Also used are: bis-trimethylsilyltrifluoroacetamide (BSTFA) producing highly volatile byproducts not superimposed on the peaks of the main substances, n-trimethylsilylimidazole (TSIM), which is less sensitive to the presence of water in derivatized samples.

Despite the simplicity of obtaining TMS ethers and their wide application, utilization of perfluoroacyl derivatives appears to be more promising because of their higher volatility, greater stability, the presence of characteristic ions in chromatomass spectrometry, and much lower detection limits in ECD.

Used for the acylation of hydroxyl groups in mycotoxins are: trifluoroacetic, pentafluoropropionic, heptafluorobutyric anhydrides, in the presence of bases (pyridine, trimethylamine) necessary for the binding of the acid evolving in the reaction:



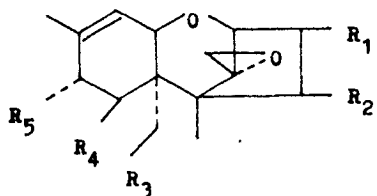
The acid evolving in the acylation with anhydrides inhibits derivatization of mycotoxins containing phenol (zearalenone) and semi-acetal (patulin) hydroxyl groups. In this case, acylating reagents which form no acid byproducts in the reaction, e.g., perfluoroacylimidasoles, are more effective.

The use of GLC in the analysis of mycotoxins

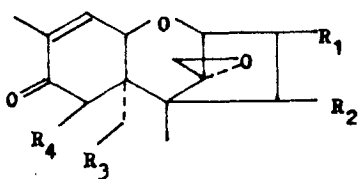
As already mentioned, GLC is most frequently used in the analysis of trichothecene mycotoxins.

By its chemical composition this group of toxins belongs to 12,13-epoxytrichothecene derivatives. The structure of the basic compounds of the trichothecene group found in natural conditions is presented in Table 3.

Table 3.



( A )

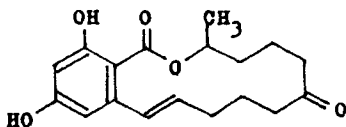


( B )

Ser. No.	Toxin	Structural type	R <sub>1</sub>	R <sub>2</sub>	R <sub>3</sub>	R <sub>4</sub>	R <sub>5</sub>
1	Diacetoxyscirpenol	A	OH	OAc	OAc	H	H
2	T-2 toxin	A	OH	OAc	OAc	H	i-VAL <sup>1)</sup>
3	HT-2 toxin	A	OH	OH	OAc	H	i-VAL
4	Neosolaniol	A	OH	OAc	OAc	H	OH
5	Nivalenol	B	OH	OH	OH	OH	-
6	Pusarenol - I	B	OH	OAc	OH	OH	-
7	Deoxynivalenol	B	OH	H	OH	OH	-

1) i-VAL = isovaleryl





Zearalenone

In most cases the GLC of trichothecene TMS derivatives was conducted on PO and OCTG with nonpolar liquid phase OV-101 or liquid phase of medium polarity OV-17. A characteristic example of the separation of TMS derivatives of a number of toxins belonging to this group and of zearalenone is shown in Fig. 9.

Detection limit in the methods using TMS derivatives with FID at the final GLC stage is on the average from 100 to 1000  $\mu\text{g}$  trichothecenes per 1 kg of the product sample. ECD is more sensitive than FID for the identification of trichothecene TMS ethers, groups A and B. Thus, with the use of ECD a five-fold increase in sensitivity was observed for TMS derivatives of group B and a two-fold increase - for the trichothecenes of group A.

More promising appears to be the use of GLC of perfluoro-acyl derivatives characterized by a higher volatility, which permits the analysis to be performed at a lower temperature. Comparison of retention indexes for TMS and TFA derivatives of a number of trichothecenes and of zearalenone on liquid phase OV-101, described in the work of Hungarian investigators and also in our work, shows a decrease in the index for TFA derivatives on the average by 400-800 units (Table 4).

Table 4. Retention indices for TMS and TFA derivatives of trichothecenes and zearalenone in OCTG with liquid phase OV-101

Toxin	Retention index	
	TMS derivatives	TFA derivatives
Deoxynivalenol	2404	1982
Diacetoxyscirpenol	2717	2235
Neosolaniol	2817	2210
T-2 toxin	3133	2593
Zearalenone	3199	2374

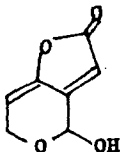
The use of perfluoroacyl derivatives makes it possible to decrease substantially the ECD detection limit. For example, in the analysis of deoxynivalenol in the form of heptafluorobutyryl derivative the use of ECD permitted the overall identification limit to be decreased to 10  $\mu\text{g}$  toxin per 1 kg grain. Table 5 shows some examples of the use of GLC on PC and OCTG for the analysis of TMS and perfluoroacyl derivatives of trichothecenes and zearalenone in the course of FID and ECD detection.

The use of highly effective capillary GLC, both for the purposes of qualitative identification by well reproducible retention indexes and precise quantitative operations, seems to be the most promising trend in the analysis of trichothecene toxins.

GLC is also widely used for reliable detection of another fusariotoxin - zearalenone, possessing a clearly expressed estrogenic effect. Zearalenone is usually transformed into di-TMS derivatives. GLC of zearalenone di-TMS derivative on PC

with 3% OV-17 on Gas-Chrom Q, with di-TMS derivative or epico prosthanol used as the internal standard, permitted to identify zearalenone in biological liquids with a sufficiently good sensitivity (up to 100  $\mu\text{g}/\text{kg}$ ). It becomes possible to raise the sensitivity of determination 10-fold by using GLC of di-pentafluoropropionyl derivatives with heptachloroepoxide as internal standard on PC and with liquid phase Dexyl on Gas-Chrom Q in ECD detection.

Over the period of the recent 12 years numerous attempts have been made to use GLC for the analysis of patulin - a mycotoxin contaminating fruits and vegetables and possessing carcinogenic and mutagenic effects. The derivative most frequently used in GLC is trimethylsilyl patulin ether.



Patulin

Acetyl and monochloroacetyl patulin derivatives were obtained earlier. In the FID detection of TMS patulin one can detect up to 10 ng patulin, while in the case of ECD the sensitivity increases to 0.1 ng. Despite the obvious advantages of GLC in the sensitivity and efficiency of separation this method has as yet been used comparatively seldom for the analysis of patulin. One of the reasons for this is the relative instability of patulin derivatives and the "tails" they form when subjected to GLC, which reduces the quality of separation. At the same time, GLC in combination with mass spectrometry is increasingly used for reliable confirmation of the presence of patulin in food products.

Table 5.

Toxin	Type of derivative	Detector	Type of column and liquid phase	Detection limit ( $\mu\text{g}/\text{kg}$ )	Reference
Nivalenol				100	
Deoxynivalenol				100	
Fusarenon-X				100	Kamimura H. et al. (1981)
Diacetoxyscirpenol				200	
Neosolaniol	TMS	FID	PC with OV-17 on Gas-Chrom	200	
HT-2 toxin				200	
T-2 toxin				200	
Zearalenone				100	
Deoxynivalenol					
Diacetoxyscirpenol	TMS	FID	OCTC with OV-101	50-100	Szathmary Cs. et al. (1980)
Neosolaniol					
T-2 toxin					
Zearalenone					
Diacetoxyscirpenol	TFA	FID	OCTC with OV-101	100	Eller K.I. et al. (1983)
T-2 toxin					
HT-2 toxin					
Deoxynivalenol	Heptafluorobutryl	ECD	PC with OV-3 on Chromosorb	10	Scott P.M. et al. (1981)
T-2 toxin					
HT-2 toxin					




GIC thus serves as one of the principal methods in the analytical chemistry of mycotoxins, its main advantages being a higher, in comparison with TLC and HPLC, separation efficiency, a low detection limit (down to 10 and less  $\mu\text{g}/\text{kg}$ ) and broad possibilities for identification by retention parameters.

An especially high information content and reliability are obtained by GIC in combination with mass spectrometry. The development of perfected injection systems, reducing losses and partial destruction of analyzed mycotoxins, as well as the simplification and improvement of derivatization methods, will make it possible to broaden the range of mycotoxins identified by means of GIC and to raise the sensitivity and reproducibility of analyses.

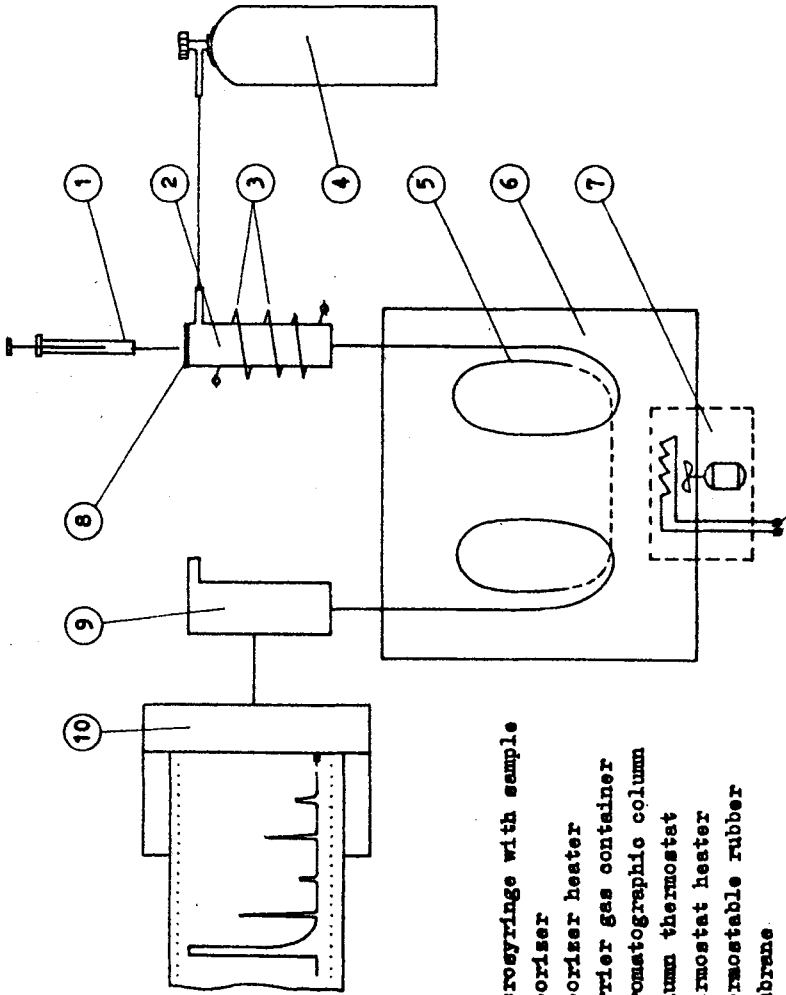
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5. Kamimura et al. "Simultaneous detection of several Fusarium mycotoxins in cereals, grains and foodstuffs" J. Assoc. Off. Anal. Chem., v. 64, No. 5, pp. 1067-1073 (1981).
6. Scott P.M. "Gas chromatography with electron capture and mass spectrometric detection of deoxynivalenol in wheat and other grains" J. Assoc. Off. Anal. Chem., v. 64, No. 6, pp. 1364-1371 (1981).
7. Szathmary Cs. et al. "Capillary gas chromatographic - mass spectrometric determination of some mycotoxins causing fusariotoxioses in animals" J. Chromatogr., v. 191, pp. 327-331 (1980).
8. Eller K.I., Sobolev V.S. "Identification and quantitative detection of trichothecene mycotoxins by the method of capillary GLC", Zh. Anal. Khim., No. 4 (1983).

Table 1.

Type of column	Wall coated open capillary tubular columns (WCOT)	Surface coated open capillary tubular columns (SCOT)	Packed columns (PC)
Schematic of column cross-section			
Inside diameter (mm)	0.2-0.5	0.5	2.4
Column length (m)	20-100	20-100	0.5-4.0
Efficiency (t.p. per 1 m column length)	1000-3000	600-1200	500-1000
Overall column efficiency (t.p.)	20000-200000	10000-100000	800-4000
Mass interval of separate sample (ng)	10-100	10-1000	10-10 <sup>6</sup>
Linear flow rate of carrier gas (nitrogen) (cm/sec)	10-15	10-15	2-5
Volume flow rate of carrier gas (ml/min)	0.5-4.0	1.5-3.0	15-50





1. Microsyringe with sample
2. Vaporizer
3. Vaporizer heater
4. Carrier gas container
5. Chromatographic column
6. Column thermostat
7. Thermostat heater
8. Thermostable rubber membrane
9. Detector
10. Automatic recorder

Fig. 1

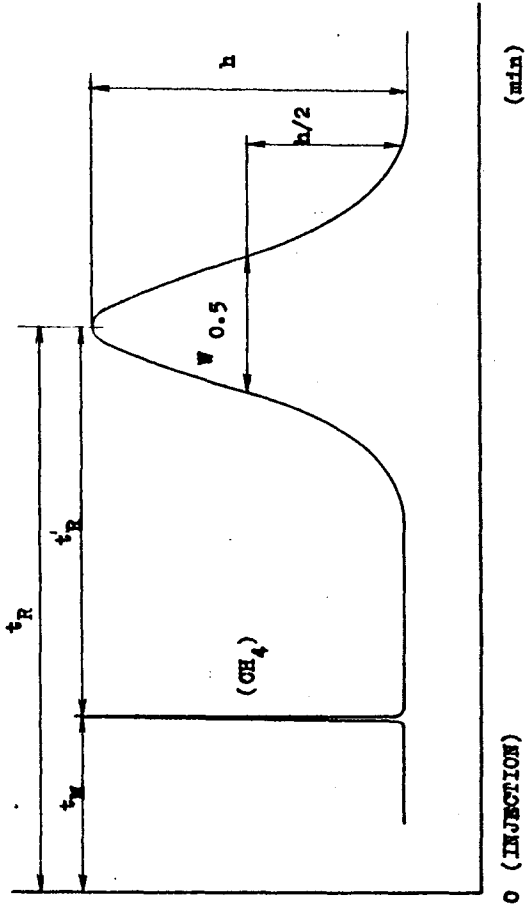
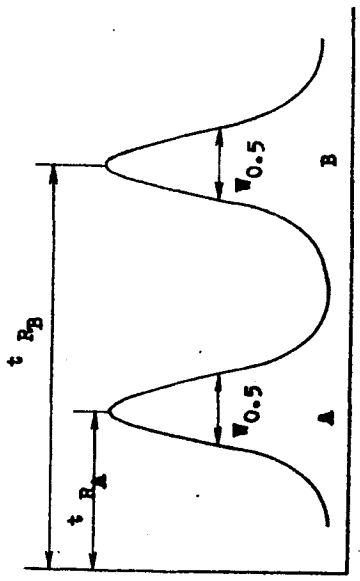
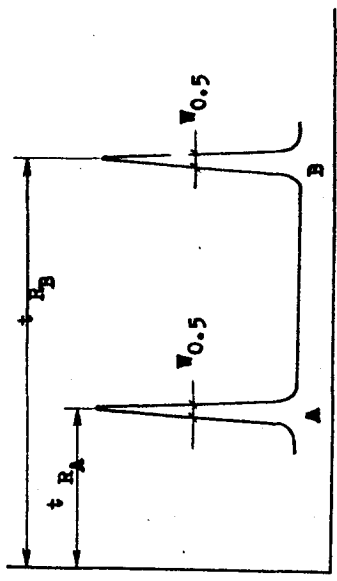


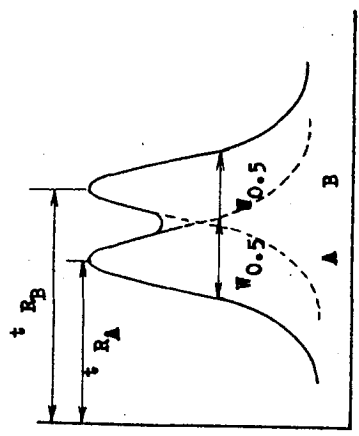
Fig. 2.



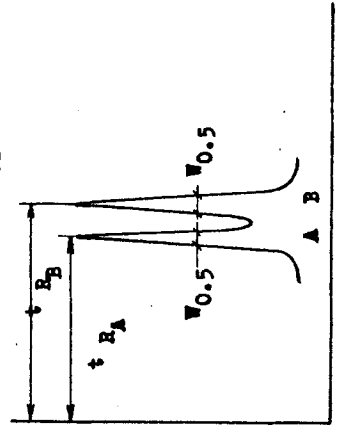
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LOW EFFICIENCY



HIGH SELECTIVITY,  
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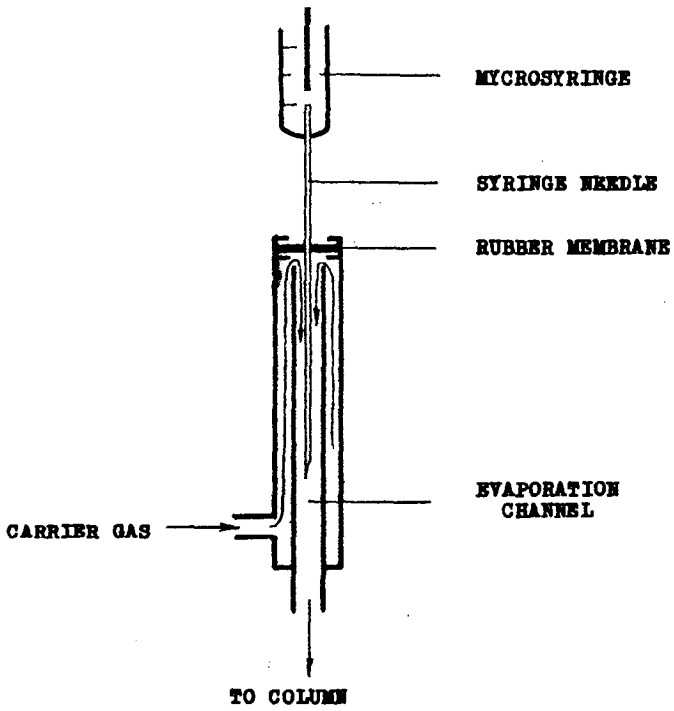


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Fig. 3.



**Fig.4.**

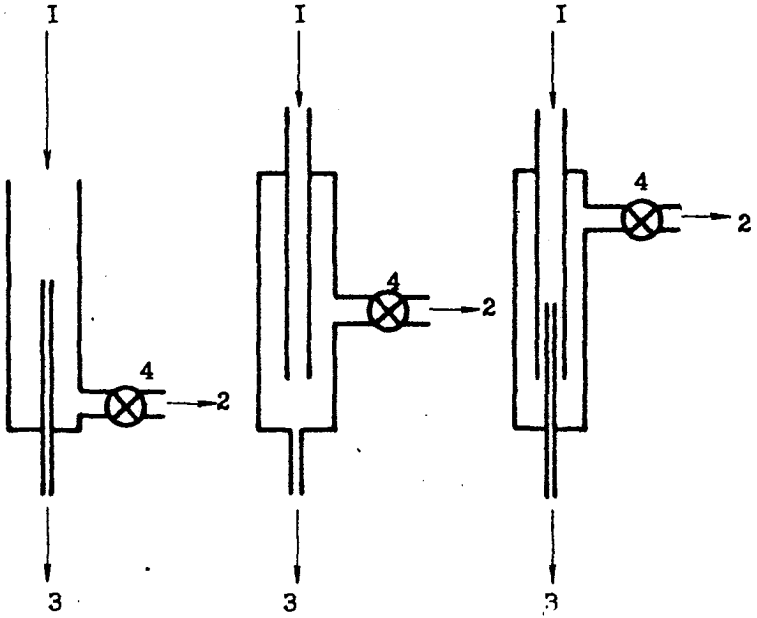


Fig.5.

- 1. Gas flow from vaporiser
- 2. Gas discharge into atmosphere
- 3. Gas inlet to column
- 4. Waste-gas valve

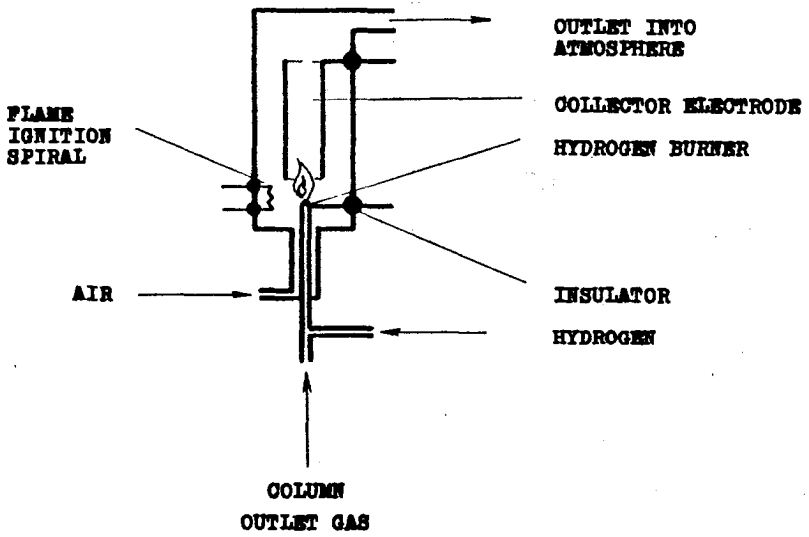


Fig.6.

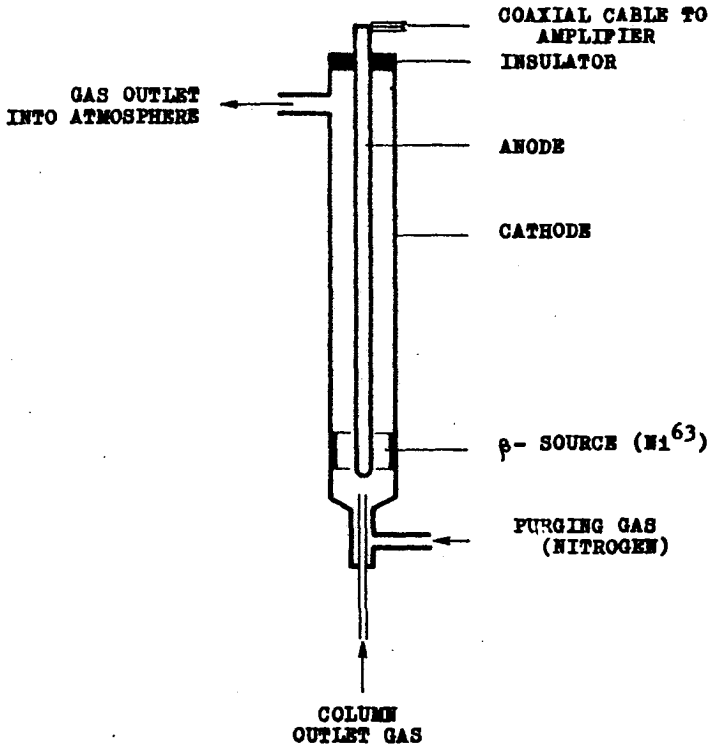


Fig.7.



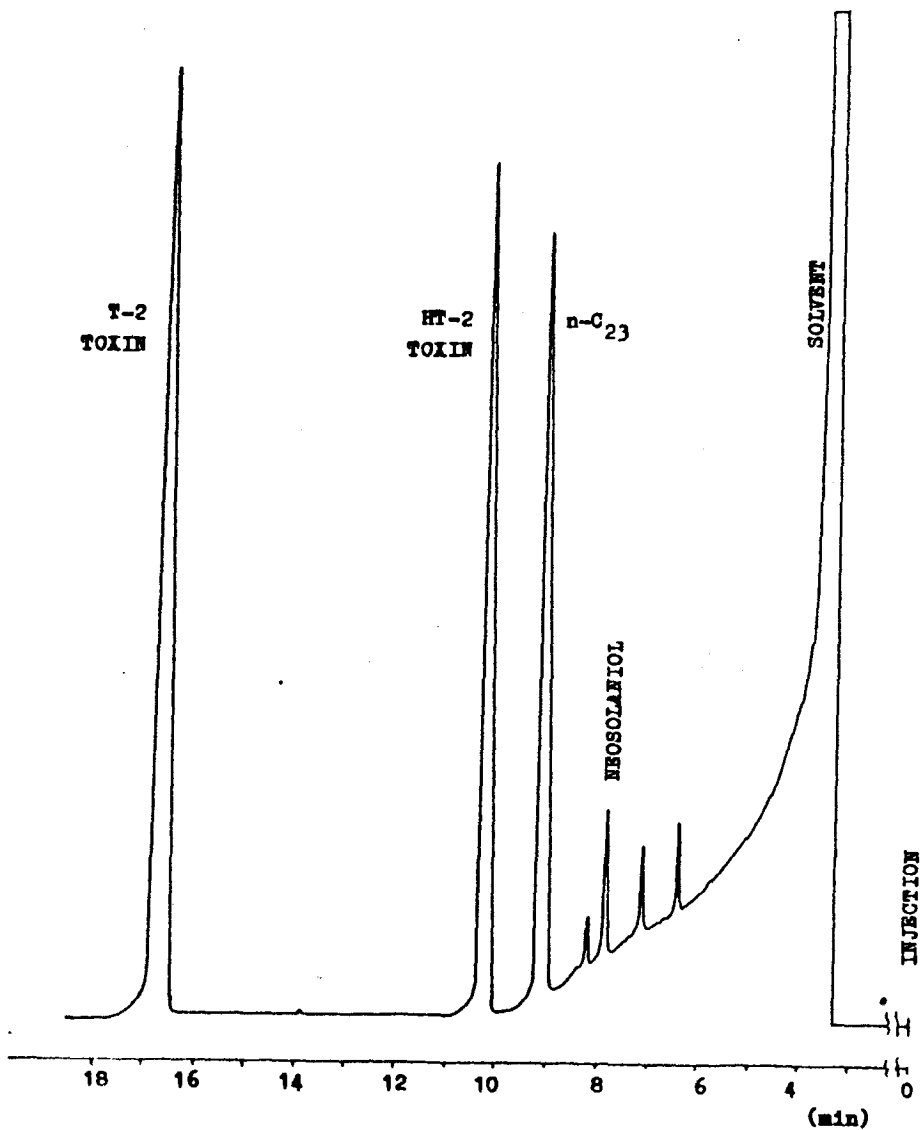


Fig.8.

GLC of TPA derivatives. Eller, Sobolev (1983). OCTC with  
OV-101,  $t_{\text{column}} 240^{\circ}\text{C}$ .

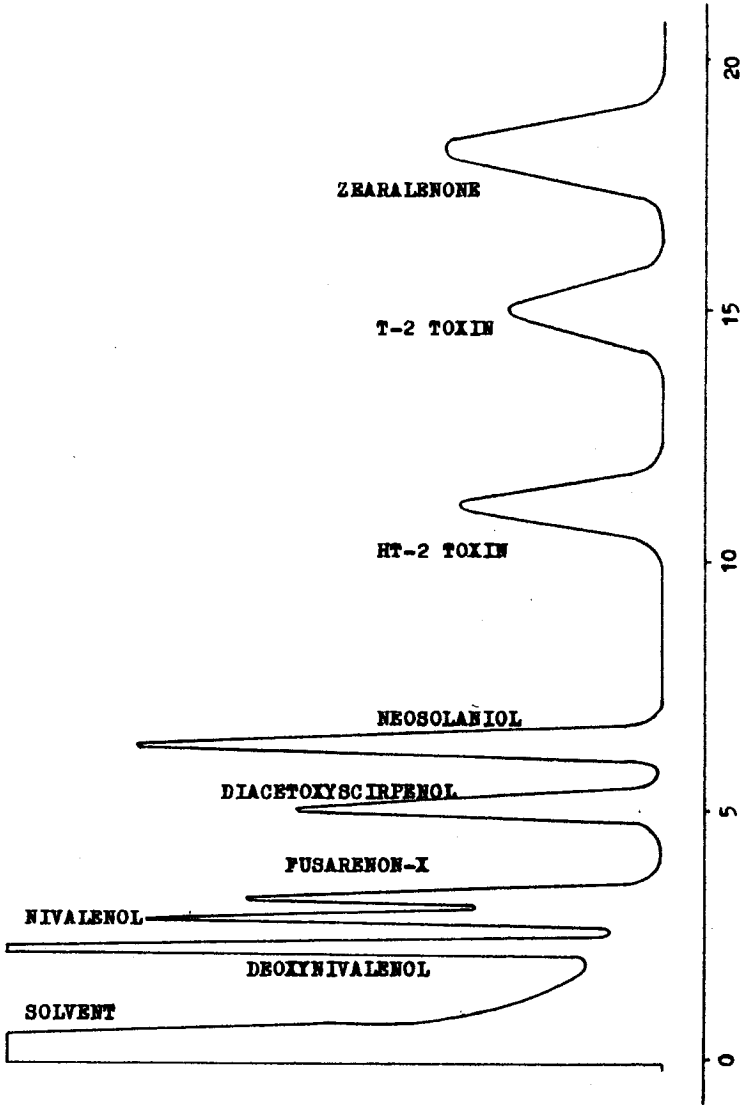


Fig. 9.  
Kamimura et al. (1981). Packed column, 2% OV-17 on  
Gas-Chrom Q, t column 250° C.

Зах. 1454 ЛИК ВИНТИ