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

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY IN THE ANALYSIS OF MYCOTOXINS

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The continuous improvement of adsorbents' quality and performance of the chromatographic equipment for their maximal adaptation to the requirements of the column chromatography theory resulted in the creation of a qualitatively new analytic technique - high-performance liquid chromatography (HPLC). During the last 10-12 years HPLC has been broadly used in the analysis of mycotoxins in foodstuffs and fodder. Compared with thin-layer chromatography (TLC), which is commonly used in routine mycotoxin analysis, HPLC is characterized by such advantages as adequate partition from interfering accompanying substances in highly efficient chromatographic columns, fast rate of partition, quantitation reproducibility of separated substances by means of flow detectors, possibility of injecting rather large sample volumes into a chromatographic system and simplicity of automation in a considerable number of analytic series. HPLC has a drawback: higher cost of basic equipment and higher requirements to the qualification of analysts.

PRINCIPLE OF THE METHOD

A principle systemic diagramme for HPLC is shown in

Fig. 1. A sample is introduced via an injector into a solvent flow (mobile phase), which is created by a solvent delivery system, consisting of one or several high-pressure precision pumps. The mobile phase flow transfers the sample into a chromatographic column containing stationary phase (5-100 μm) particles. When a sample solution passes through the column, the partition into components takes place in the form of separated chromatographic bands. Such separated chromatographic bands are eluted from the column through any one or several detectors, the signal of which is intensified and is traced by a chart recorder in the form of chromatographic peaks. When necessary the eluant containing chromatographic bands which passes out of the detector can be collected for further analysis or confirmation.

The major aim of the chromatographic process is partition of analyzed substances. The partition volume of two substances in HPLC is characterized by resolution (R) determined by the formula (1):

$$R = \frac{V_2 - V_1}{(W_1 + W_2)\ 1/2}\ , \text{ where}$$

V_2 - retention of the second substance (solvent volume necessary for the elution of the 2-nd substance or the time from the moment of the sample injection to that of its output from the column and appearance in a detector);

V_1 - retention of the 1-st substance;

W_1 and W_2 - chromatographic peak widths (see Fig. 2).

The greater is R the better is the partition effect.

Three basic parameters influence the resolution value R: column capacity factor (C), selectivity of the chromatographic process and column efficiency expressed in the so-called theoretical plate number (N t.p.) (Fig. 3).

The capacity factor K is determined by the formula:

$$K = \frac{V_1 - V_0}{V}, \text{ where}$$

V_0 - system void volume corresponding to the time of elution of the column unretained component;

V_1 - retention of the 1-st substance (Fig.3).

In other words, K characterizes the retention of the 1-st substance in units of the system void volume V_0. A value K depends on the eluting power of the solvent and properties of the stationary phase, while it remains constant with the changed solvent flow rate and column size.

The selectivity (α) value of the chromatographic process or the chemistry factor tells us where two column peaks elute in respect to each other. A value α is defined by the ratio of capacity factors corresponding to each peak or the ratio of corrected retention volums (corrected retention times):

$$\alpha = \frac{K_2}{K_1} = \frac{V_2 - V_0}{V_1 - V_0};$$

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A value α permits to assess the selectivity level of both the sorbent and the mobile phase for two given components.

Thus, K shows peak elution related to the void volume V_0, while α - counter peak elutions of the partition components.

While defining K and α, the width of peaks W or elution of a chromatographic band are not taken into consideration. Peak width depends on the efficiency of a chromatographic system, characterized by the number of theoretical plates (N t.p.), determined by the formula:

$$N = 16 \left(\frac{V}{W}\right)^2 \qquad \text{(Fig. 3).}$$

N t.p. describes the deviation of a band within the whole chromatographic system: from injection to detection. Modern HPLC devices, however, are designed so as to minimise extra-column bandspreading. Thus, a column plays the major role in the spreading of a peak. The efficiency is determined by the properties and size of sorbent particles, and also by the quality and homogeneity of column sorbent.

Mathematically the dependence of resolution R from the capacity factor, selectivity and column efficiency is expressed by the formula:

$$R = 1/4 \left(\frac{\alpha - 1}{\alpha}\right) \left(\sqrt{N}\right) \left(\frac{K}{1 + K}\right);$$

Fig. 4 graphically demonstrates the influence exerted by

each of the studied parameters on resolution R. As can be seen from Fig. 4, a more qualitative resolution can be obtained by 3 different ways. With the increase of the capacity factor K the partition improves, however, the peaks become wider. The possibilities of improved resolution due to the increased capacity factor are limited to 3-4 by K values. Further K increase results in the insignificant general resolution improvement, at the same time leading to a significant peakspreading with the corresponding loss of sensitivity at peak height.

With higher efficiency the peaks become narrower and the resolution quality increases. It is extremely important to note that with increased efficiency the technique sensitivity raises because the peaks become higher. When one and the same column is used, the efficiency grows with the decrease of the solvent flow rate and its viscosity. Lengthier columns can also raise the efficiency.

The most effective means to improve resolution consists in the increase of a selectivity value α

CHROMATOGRAPHIC COLUMN PACKINGS

HPLC is used for both analytic and preparative partitions when comparatively large substance amounts should be obtained in pure form. Preparative column packings represent relatively large fully porous particles about 50 μ (sometimes up to 100 μ).

The use of such particles for analytical HPLC will result in chromatographic bandspreading which will bring about poor resolution and long separation time. Bandspreading is due to the fact that large fully porous particles have deeper pores, while it takes more time for a sample molecules to reach the particle centre and diffuse back.

To attain higher efficiency necessary in analytic partitions 2 types of particles are used: large "pellicular" 50 μ particles representing spheres with thin porous surface layer, and small (5-10 μ) fully porous particles. Despite their high efficiency, "pellicular" particles are less frequently used due to smaller surface area (10-30 m^2/g), compared to that of 5-10 μ fully porous particles (300-400 m^2/g) and, hence, ensure less values of the capacity factor K.

When small diametre particles are used, the partition efficiency raises due to the following factors:

- the surface area of small fully porous particles is high, which results in the increase of the capacity factor;
- the diffusion way of sample molecules to the particle centre and back is reduced;
- the mass transfer rate is increased;
- the unpacked void volume between particles where mobile phase mixing can take place, is diminished.

As a rule, in the analysis of mycotoxins, the stationary phase represents silica gel with 5-10 μ particles and its various chemical modifications.

It should be noted that the mobile phase flow rate con-

siderably influences the efficiency of columns packed with small particles. With higher flow rate the effect induced by an unfavourable longitudinal molecular diffusion reduces, however, the resistance of mass transfer between liquid and solid phases increases. Thus, an optimal flow rate of the mobile phase has been established. For example, it constitutes 0.3-0.8 ml/min for columns packed with 5-10 μ silica gel particles.

PARTITION MECHANISMS IN LC

LC partition can be based on the difference in absorption of the stationary phase substance (adsorption material), on the difference in substance in mobile and stationary phases (distribution mechanism), on different charges, (ion-exchange chromatography) and molecular size (gel-permeation chromatography). In the mycotoxin analysis mainly 2 first chromatographic types are used: adsorptional and distributional.

The mobile phase in LC is liquid, while the stationary phase can be solid (liquid-solid chromatography, TLC) or liquid (liquid-liquid chromatography, LLC).

The normal phase chromatography is characterized by high polarity of the stationary phase and low polarity of the mobile phase, while the reverse-phase chromatography involves less polarity in the stationary phase than in the mobile one. In normal-phase chromatography first less polar substances move out of the column, followed by more polar ones, when

increased polarity of the mobile phase results in the decreased retention time. In reverse-phase chromatography the elution of substances takes place with the decrease of their polarity. In this case, an increase of the mobile phase polarity makes the retention time longer, i.e. slows down column substance output.

Two types of chromatographic partition deserve thorough description: liquid-liquid and liquid-solid chromatographies.

a) liquid-liquid chromatography

The columns used in TLC are packed with carrier (most often by silica gel) with the adsorbed stationary liquid phase. Passing the column the mobile phase contacts the stationary phase resulting in balanced distribution of the sample components between the two phases and their partitioning. For example, in normal-phase chromatography silica gel retains water on its surface allowing for the sample components distribution between the surface silica gel aqueous layer and chloroform - basic component of the mobile phase. (Fig. 5). Fig. 5 also demonstrates an example of a reverse-phase TLC with the sample distribution between nonpolar hydrocarbon impregnating silica gel surface and the polar mobile phase - aqueous methanol. Liquid-liquid partition on the normal polar phase is used for the partition of more polar components, while on the reverse-phase - for the partition of nonpolar components.

Theoretically TLC represents the most universal partition

technique due to its possibility of wide changes in the composition of mobile and stationary phases for the attainment of needed selectivity. However, practical application of the above technique is limited due to its serious drawbacks:

- the stationary phase always has a finite solubility in the mobile phase and its stripping from the column with the solvent is possible; hence, presaturation of the solvent with the stationary phase is required;

- the distribution reproducibility is not high, because the distribution of the stationary phase between the column and the mobile phase changes with small changes in temperature, pressure and flow rate;

- the mobile phase is contaminated with the stationary phase making the collection and identification of peaks difficult;

- solvent programming cannot be used.

In view of the above reasons liquid-solid chromatography is more frequently used.

b) liquid-solid chromatography (LSC)

In TLC solid adsorbent particles have active centres (adsorption centres) on its surface. Partition occurs due to the distribution of a sample molecules between the mobile phase and the adsorbent surface.

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In normal-phase TLC the most frequently used sorbent is silica gel - precipitated poli-silica acid, possessing ≡ Si-O-H electron accepting groups as adsorption centres. Silica gel

for analytic HPLC is produced under various trade names: Partisil ("Whatman"), Lichrosorb ("Merck"), Porasil ("Waters Associates"), Spherisorb-Si ("Spectra-Physics"), Ultrasphere-Si ("Beckman"), Zorbax SIL ("Du Pont"), etc.

The reverse-phase TLC involves the use of chemically modified silica gel (chemically bonded phase), the surface of which becomes low polar as a result of the formation of hydrolysis-resistant ≡ Si-O-Si-C bonds. Silica gel is converted into a reversed-phase sorbent by reacting it with an alkyltrichlorosilane, as a result of which the silica gel surface is modified by chemically bound C_{18}-carbon chains. Reverse bonded stationary phases of this type are marked by the addition of the sign ODS or RP-18 to a silica gel trademark. In contrast to normal-phase chromatography on silica gel, in HPLC on chemically bonded phases the partition mechanism is based not only on adsorption phenomena (resulting from hydrophobic reactions between separated components and sorbent carbon chains), but also involves certain elements of liquid-liquid partition between the mobile phase and the solvent layer, retained by nonpolar sorbent surface.

Silica gel can be modified not only by nonpolar carbon residues, but also by polar groups (bound polar phases). The above type of stationary phases results from a reaction of silica gel with organic chlorosilanes, containing cyanopropyl or aminopropyl groups:

The separation on polar bound stationary phases involves both partitional and adsorptional mechanisms. Depending on a mobile phase polarity, the adsorbents of this type can be used both for normal-phase and reverse-phase chromatographies.

The sorbents presently used in HPLC ensure the partition efficiency up to 25000-70000 theoretical plates per column meter. For most commonly used columns of 25 and 25 cm the general efficiency correspondingly constitutes from 4000 to 20000 theoretical plates, which considerably exceeds TLC plate partition properties and is inferior by this index only to capillary gas-liquid chromatography.

c) HPLC solvents (mobile phase)

The composition and properties of the mobile phase exert a considerable influence on all partition parameters. One of major solvent characteristics is the polarity index being a complex function from dipolar moment, dielectric permeation and molecular polarization. The values of polarity indices of most common solvents are presented in Table 1. The polarity characterizes an eluting capacity of the solvent, i.e. the capacity of washing a sample molecules from the adsorbent layer in normal-phase chromatography. The information about solvent polarity is necessary in the selection of optimal values of a column capacity factor K.

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Solvent viscosity also represents a significant parameter (Table 1). The use of lower viscosity solvents can, on the one hand, increase the partition efficiency, and, on the other,

to decrease the column pressure gradient, i.e. to obtain the same flow rate of the mobile phase under smaller working pressure.

Solvent purity, and, particularly, the absence of solid particles, represents a significant aspect in HPLC. Thus, before the delivery into the pumping system, solvents should be filtered.

Solvents should also meet the folloing requirements: adequacy for the used detector, capability of sample dissolving, easy removing from collected fraction (preparative chromatography).

Changing solvent composition (gradient elution) serves as an additional technique optimizing partition in HPLC. Gradient elution in HPLC performs the functions similar to temperature programming in gas-liquid chromatography. It is particularly expedient in the analysis of a multi-component mixtures having various distribution coefficients or adsorption properties, and allows to ensure a rapid combination of solvents in the partition technique.

INJECTION SYSTEMS

The purpose of an injection system is to put the sample onto the pressurized column as a sharp plug with minimal loss in efficiency. A sample can be injected into a mobile phase stream either by means of a syringe or through a valve.

The easiest way is to microsyringe a sample through the

septum ensuring minimal bandspreading.

The drawbacks of the above technique (possibility of a sample blow back, short septum life, limitation of the working pressure to 100 atm.), however, forced the investigators to use valve injection systems in modern HPLC.

Fig. 6 shows a valve injection diagramme. When the valve is in the "load" position the pump is directly connected with the column and a sample is placed into the loop via the syringe. Usually the loop volume is 20 /ul. When the valve is turned into the "injection" position, the sample is swept onto the head of the column. Such systems ensure a precise and reproducible injection and can be readily used for the automatic injection of a large number of samples.

HPLC DETECTORS

The function of a detector is to provide an electric signal to the recorder enabling to perform qualitative and quantitative analyses. In HPLC the basic detector requirements are:

- sensitivity: a detector should provide a large signal, relative to the noise level, from a small amount of solute in the effluent;

- linearity: a signal should be directly proportional to the amount of solute;

- specificity: a detector should be sensitive to solutes and insensitive to interfering admixtures, changes in the mo-

bile phase composition, temperature, flow rate and pressure.

Detectors of 3 types are used in the HPLC mycotoxin analysis: ultra-violet (UV), fluorescent and refractive index detectors.

a) UV detector

A UV detector represents a photometer with a flow microcell through which the column effuent flows. Some of UV detectors also operate in the visible wavelength range, but by far the most useful range of the spectrum is from 220 - 370 nm.

A UV detector can function under any fixed wavelength or can select the needed wavelength in the process of work.

The most common and inexpensive are the detectors with a fixed wavelength involving the use of a mercury lamp as a source of UV irradiation. Due to the fact that 90% of the radiance of this source is at 254 nm, this is the wavelength most commonly employed. Other wavelengths are blocked by corresponding filters. As can be seen from Table 2 the wavelength 254 nm does not correspond to the absorption maxima of the majority of mycotoxins, thus, sensitivity and, particularly, selectivity of detection decrease when this detector is used.

The possibilities of various mycotoxins' detection broaden considerably when detectors with variable wavelengths are applied. In this case a deuterium lamp serves as a source of irradiation, the energy of which spreads along the whole spectrum. The irradiation whithin a certain wavelength

range is isolated by means of filters. A monochromator is used for the continuous alteration of a wavelength with a narrow spectral band of passed irradiation frequencies. Filter detectors are characterized by high sensitivity due to a broader transmission band. Monochromator detectors possess high selectivity due to the possibility of selecting an irradiation wavelength, precisely corresponding to the absorption maximum of mycotoxins (Table 2).

To avoid the deterioration of partition parameters in detectors, small volume flow cells are used, the design of which excludes the possibility of eluate mixing and band-spreading. The volume of a flow cell usually constitutes 8-20 ul.

According to the Bouguer-Lambert-Beer law a detector signal is proportional to a sample concentration in a rather broad range of concentration and is described by the formula:

$$D = \varepsilon c l$$, where

D - measured optical density;

ε - molar extinction coefficient;

l - optical path length in cm;

c - sample concentration in eluate (mole/l).

The detection limit of a UV detector depends on the substance molar extinction coefficient, optical path length and the detector noise level (amplitudes of occasional fluctuations of the zero line). The greater is the value ε and the lower is the noise level, the smaller is the detectible con-

centration. For example, for aflatoxins at ε_{360} of the order of 2×10^4, cell length of 1 cm and noise level of 1×10^{-4}A the detection limit will constitute 10 nanomoles per litre of eluate or 3 ng/ml of eluate, which represents a quite satisfactory value for the majority of analytic tasks. As was mentioned already, column efficiency will also influence the level of sensitivity, because bandspreading will result in a decreased sample concentration in a flow cell.

While working with a UV detector only transparent (non-absorbing) solvents can be utilized within the working wavelength. Thus, in UV detection the mobile phase is presented by saturated hydrocarbons, cyclohexane, methanol, ethanol, water, acetonitril, chloroform and methylene chloride, but not by benzene, acetone, toluene, characterized by intensive absorption at a wavelength exceeding 250 nm.

b) fluorescent detector

As a rule, a fluorescent detector measures the intensity of visible light emitted by a sample under the effect of UV exitation. A diagramme of a fluorescent detector is shown in Fig. 7. From the source (tungsten-halogen lamp, capable of potent emission with the wavelength of 280 nm and above), passing the exitation line filters, the UV light reaches a flow cell. The sample emission light is separated by means of emission filters from the exitation UV light and is sent to the photomultiplier. Both on the exitation line and on the line of emission instead of wide band filters, monochromators

can be used, allowing for a precise selection of a needed wavelength for both exitation and emission lights. Likewise in the case of UV detection, filter fluorimeters ensure a substantially greater sensitivity, while detectors with monochromators are characterized by higher selectivity.

The basic advantage of a fluorescent detector (in contrast to a UV detector) is the increased sensitivity by 100-1000 times, and also the possibility to diminish the effect of interfering elements from concomittant substances due to the selection of optimal wavelengths of exitation and emission. Spectral absorption characteristics of UV emission (maximal value in the spectrum of fluorescent exitation) and that of several mycotoxins (maximal value in the spectrum of fluorescent emission), are shown in Table 3.

The fluorescent intensity grows with the increased intensity of exitation light and concentration of the fluorescent substance. Although fluorescent intensity represents an exponential function from a substance volume, for small concentrations it is directly proportional to the concentration of an analyzed substance in eluate. The above proportionality is impaired at larger concentrations due to the phenomena of inner fluorescence quenching.

The use of the above highly sensitive detector, however, is limited to mycotoxins with fluorogenic groups (Table 3). Fluorescent derivatives should be previously obtained for mycotoxins without natural fluorescence. This technique, however, has not yet found its broad application.

c) refractive index detector

Detectors of this type are used to measure the refractive index difference of pure solvent (mobile phase) and column effluent. Thus, they are called differential refractometers or RI detectors. RI detectors are designed for qualitative and quantitative analyses of substances possessing no property of UV absorption and having no fluorogenic groups.

The drawbacks of this universally applied detector are: low sensitivity and impossibility of using gradient elution. Thus, for example, for mycotoxins with the molar extinction coefficient of about 10^4 the sensitivity level of a RI-detector is 100 times lower than that of a UV detector. At the same time RI detectors find still greater use in HPLC of trichothecene mycotoxins, the molecules of which have no chromophore groups.

HPLC IN THE ANALYSIS OF MYCOTOXINS

HPLC is commonly used for the reproducible and precise detection of such mycotoxins as aflatoxins, ochratoxin, patulin, zearalenone, sterigmatocystin, etc. Data on the conditions of separation, detection and detection limits in HPLC of several most common mycotoxins are presented in Table 4. Let us discuss the peculiarities of HPLC in respect to some mycotoxins:

a) aflatoxins

A satisfactory separation of aflatoxins (Groups B, G and M) is attained both by means of a normal-phase, and by a reverse-phase HPLC.

In normal-phase HPLC various brands of silica gel with 5-10 μ particles are used as sorbent, which in the mobile phase is most often represented by a mixture of water-saturated choloroform, cyclohexane, acetonitril with the addition of 1-2% of ethanol or isopropanol. The mixtures of isooctane with chloroform and methanol are also used. In this case some aflatoxins are eluted in the order of their increased polarity: aflatoxin B_1, B_2, G_1, G_2 and M_1. A typical separation pattern in normal-phase HPLC is presented in Fig. 8. For the purpose of detection, either a UV detector with the wavelength of 360-365 nm, or a fluorescent detector, are used. To attain higher (than in TLC) sensitivity (i.e. to decrease the detection limit to 0.1 μg/kg and lower) a fluorescent detector should be used. It turned out, however, that aflatoxins B_1 and G_1 have a low fluorescence intensity in solvents applied in normal-phase HPLC, compared with the fluorescent intensity of the same aflatoxins adsorbed on silica gel in TLC. To raise the fluorescence level of most toxicity-significant and common aflatoxins B_1 and G_1, a flow cell packed with silica gel 30-40 μ particles (Panalaks, Scott, 1977) was proposed for fluorescent detectors, which permitted to detect up to 0.05 ng of aflatoxins. It was demonstrated that in case of fluorescent detection with

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a silica gel packed flow cell, the sensitivity level, compared to that of UV detection (with the wavelength of 360 nm) increases by 14.3 times for aflatoxin B_1 , by 31.6 times - for B_2, by 21.1 times - for G_1, and by 61.6 times - for G_2 (Pons, 1979).

In reverse-phase HPLC the stationary phase is represented by silica gel, the active centres of which are bound by octadecylsilane or octylsilane (e.g. Bondapak- C_{18} or Ultrasphere-C_8). The mobile phase represents aqueous acetonitril or a mixture of acetonitril, methanol and water. In the reversed-phase HPLC aflatoxins are characterized by a reverse elution sequence, compared to that of a normal-phase seperation: aflatoxins M_1, G_2, G_1, B_2 and B_1. The fluorescent detection in reverse-phase HPLC has a serious problem consisting in a much lower fluorescence intensity of aflatoxins B_1, G_1 and M_1 compared with those of B_2, G_2 and M_2. To intensify the fluorescence, prior to HPLC the extracts are treated with trifluoroacetic acid to transform aflatoxins B_1, G_1 and M_1 into corresponding semiacetal derivatives - aflatoxins B_{2a}, G_{2a} and M_{2a}. This permits to reduce the detection limit to 0.001 - 0.005 ng. In this case aflatoxins have the following sequence of elution: M_{2a}, G_{2a}, B_{2a}, G_2 and B_2. (Fig.9)

The use of highly efficient 25 cm columns having a diameter of 2-5 mm and a flow rate of about 1 ml/min permits to ensure separation of basic aflatoxins during 7-12 min. Lack of the necessity of preliminary preparation of intensively fluorescent derivatives is an advantage of normal-phase HPLC with

a silica gel packed flow cell. Besides, it should be noted that aflatoxins B_{2a}, G_{2a} and M_{2a} can exist as natural metabolites, e.g. in the analysis of animal tissues and organs. The drawbacks of the reverse-phase HPLC should include: an extremely long retention time for M_1 aflatoxin in view of the fact that the increased mobile phase molarity, while making the output of aflatoxin M_1 more rapid, however, drastically decreases the degree of separation of Group B and G aflatoxins. Thus, when both aflatoxin groups are present, the reverse-phase HPLC is more expedient. Another advantage of a reverse-phase HPLC consist in the use of less toxic and more available aqueous solvents as the mobile phase.

Quantitation, using either a fluorescent or a UV detector, involves the preliminary standard calibration of a detector, i.e. it is necessary to plot a calibration curve in values peak/area mass for a chromatograph-injected aflatoxin.

The general sensitivity of the above technique is influenced not only by detector parameters, but also by the degree of purification of an analyzed sample. This is due to the fact that the presence of intensively fluorescent or UV absorbing admixtures in extract at 360 nm allows to increase the background signal of corresponding detectors and results in a considerable decrease of sensitivity.

The samples adequate for HPLC of aflatoxins can be obtained by means of extraction and purification according to the so-called CB technique. The technique involves the extraction of a chloroform-impregnated semple and purification of chlo-

roform extract by means of column chromatography on silica gel. To obtain more pure extracts and higher sensitivity more complex purification patterns are used, the major stages of which are:

- sample extraction by aqueous acetone (methanol);
- purification of water-acetone (methanol) extract by means of complex formation with acetate of lead or zinc;
- defatting through liquid-liquid partition with hexane or isooctane;
- reextraction of an aflatoxin fraction into chloroform and purification of the chloroform extract by means of column chromatography on silica gel.

Recently, for more efficient and rapid purification, column chromatography on silica gel, bound with octadecylsilane in special catridges (Sep Pak C 18 catridges), has been used.

In any case, before being introduced into an injector, the analysed solution should be filtered to remove solid particles, which can plug up chromatographic lines or column filters.

Thus, HPLC combined with fluorescent detection allows to ensure a reliable detection of aflatoxins at their concentration of 0.05 - 0.1 ug/kg in food and fodder, which is considerably lesser than the MPC for aflatoxins, established in the majority of countries.

b) other mycotoxins

Besides, aflatoxins, HPLC is broadly used in the analysis of patulin, ochratoxins and zearalenone (Table 4).

For example, the use of HPLC in the analysis of fruit and vegetable juices for the presence of patulin allows to ensure an effective isolation of this mycotoxin from the bulk of its basic admixture - hydroxymethylfurfurol, and to attain higher (than in TLC) sensitivity (up to 2-5 ug/kg). The use of HPLC in the analysis of zearalenone reduces considerably the time of analysis and the detection limit (up to 5 ug/kg), compared with the same parameters in TLC.

CONCLUSION

The recent application of HPLC in the analysis of mycotoxins develops at a faster pace, than that of other chromatographic techniques. On the one hand, this is due to greater precision, reproducibility and a shorter time (than in TLC), necessary for the analysis, and to the lack of the necessity first to obtain volatile derivatives (like in the case of gas-liquid chromatography, which is still more efficient in respect to separation), on the other. In the near future one can expect to witness further progress in HPLC of mycotoxins due to the application of more universal and highly sensitive detectors and more efficient microcolumn liquid chromatography.

Table 1. Polarity indices and solvent viscosity

Solvent	Polarity index	Viscosity
Isooctane	-0,4	0,92
n-Hexane	0,0	0,92
Toluence	2,3	0,50
Benzene	3,0	0,65
Diethyl ester	2,9	0,23
Tetrahydrofuran	4,2	0,51
Methylene chloride	3,4	0,44
Chloroform	4,4	0,57
Ethyl acetate	4,3	0,47
Acetone	5,4	0,32
Acetonitril	6,2	0,37
Isopropanol	4,3	2,30
Ethanol	5,2	1,20
Methanol	6,6	0,60
Water	9,0	1,00

Table 2. Maximal UV saturation and molar extinction coefficients of several mycotoxins

Mycotoxin	Empirical formula	Maximal adsorption (nm)	Molar extinction coef. (cm^2/mole) in methanol solution
Aflatoxin B_1	$C_{17}H_{12}O_6$	360 265	21800 (in MeOH) 12400
Aflatoxin B_2	$C_{17}H_{14}O_6$	362 265	24000 12100
Aflatoxin G_1	$C_{17}H_{12}O_7$	362 265	17700 9600
Aflatoxin G_2	$C_{17}H_{14}O_7$	362 265	19300 9000
Aflatoxin M_1	$C_{17}H_{12}O_7$	375 265	21250 14150
Sterigmatocystin	$C_{18}H_{12}O_6$	326 277 246	15310 3040 32870
Patulin	$C_7H_6O_4$	276	14500
Penicillic acid	$C_8H_{10}O_4$	224	10600
Zearalenone	$C_{18}H_{22}O_5$	316 274 236	6020 12800 28900
Ochratoxin A	$C_{20}H_{18}ClO_6$	332	6400
Roridin A	$C_{29}H_{40}O_9$	263	22000
Deoxynivalenol	$C_{15}H_{20}O_6$	225	-
T-2 toxin	$C_{24}H_{34}O_9$	no adsorption at 220 nm	
HT-2 toxin	$C_{22}H_{32}O_8$	no adsorption at 220 nm	

Table 3. Fluorescent properties of some mycotoxins

Mycotoxin	Wavelength of maximal fluorescent exitation (in nm)	Wavelength of maximal fluorescent emission (in nm)
Aflatoxin B_1	365	425
Aflatoxin B_2	365	425
Aflatoxin G_1	365	450
Aflatoxin G_2	365	450
Aflatoxin M_1	365	425
Sterigmatocystin	395	520
Ochratoxin A	340	475
Citrinin	336	520
Zearalenone	313	450

Table 4. Partition conditions and detection limits in HPLC in respect to several mycotoxins

Mycotoxins	Type of partition	Sorbent	Mobile phase composition	Detector	Detection limit		Reference
					in ng per 1 injection	in µg/kg sample (ppb)	
1	2	3	4	5	6	7	8
Aflatoxins B_1, B_2, G_1, G_2	Normal-phase	Silica gel Zorbax SIL 5 µ	Water saturated chloroform-cyclohexane-acetonitril-ethanol (74:20:4:2)	Fluorescence, max. exitation (λ ex) 365 nm, max. emission (λ em) 440 nm, flow cell with silica gel	-	0,1	Miller et al. (1982)
Aflatoxins B_1, B_2, G_1, G_2	Normal-phase	Silica gel Porasil 10 µ	Water saturated chloroform-cyclohexane-acetonitril-ethanol (73:22:3:2)	UV detector max 365 nm	-	5,0	Pons et al.(1980)
Aflatoxins M_{2a}, G_{2a}, B_{2a}, G_2 and B_2	Reverse-phase	Silica gel bound with octadecyl-silane (Ultrasphere - ODS)	Water-acetonitril -methanol (66:25:9)	Fluorescence, λ ex 365 nm λ em 400 nm	0,001-0,005	0,05	Gregory & Manley (1980)

Table 4. (Cont.)

1	2	3	4	5	6	7	8
Sterigmato-cystin	Reverse-phase	Silica gel bound with octadecyl-silane (Bondapak C_{12}, 10)	Water-methanol (70:30)	UV detector λ max 246 nm	25	-	Schmidt et al. (1980)
Ochratoxin A	Reverse-phase	Silica gel bound with octylsilane (Ultrasphere-C8)	0.01 M solution KH_2PO_4 - aceto-nitril (45:55) with the addi-tion of 1 M so-lution of H_3PO_4 up to pH=3	Fluorescent λ ex 333 nm λ em 400 nm	2,5	5,0	Nesheim (1982)
Ochratoxin A	Reverse-phase	Silica gel bound with octadecylsi-lane (Sphe-risorb ODS, 5 µ)	Methanol-water-acetic acid (70:30:1)	Fluorescent λ ex 336 nm λ em 468 nm	-	5,0	Schwei-ghardt (1980)
Aflatoxin M_1 in milk	Reverse-phase	Silica gel bound with octylsilane (Zorbax C8)	Water-acetonit-ril-acetic acid (75:25:1)	Fluorescent λ ex 365 nm λ em 440 nm M_1 transformed into M_{2a}	-	0,015	Chang & DeVries (1983)

Table 4. (Cont.)

1	2	3	4	5	6	7	8
Patulin	Reverse-phase	Silica gel bound with octadecylsilane (Lichrosorb PH18)	Water-acetonitril (90:10)	UV detector λ max 276 nm	2,0	10,0	Geipel et al. (1981)
Penicillic acid	Reverse-phase	Bondapak C18	Acetonitril-water -acetic acid (45:55:2)	UV detector λ max 254 nm	5,0	-	Chan et al.(1980)
Zearalenone	Reverse-phase	Bondapak C18	Methanol-water (65:35)	UV detector λ max 254 nm	5,0	10,0	Holder et al. (1977)
Zearalenone	Normal-phase	Silica gel (Spherisorb, 5/u)	Cyclohexane-methylene chloride-methanol (75:22, 5:2, 5)	Fluorescent λ ex 310 nm λ em 470 nm	1,0	5,0	Scott et al.(1978)
T-2 toxin HT-2 toxin Diacetoxyscirpenol	Reverse-phase	Bondapak C18 10/u	Methanol-water (65:35)	Refractive index detector	1000,0	-	Schmidt et al. (1981)

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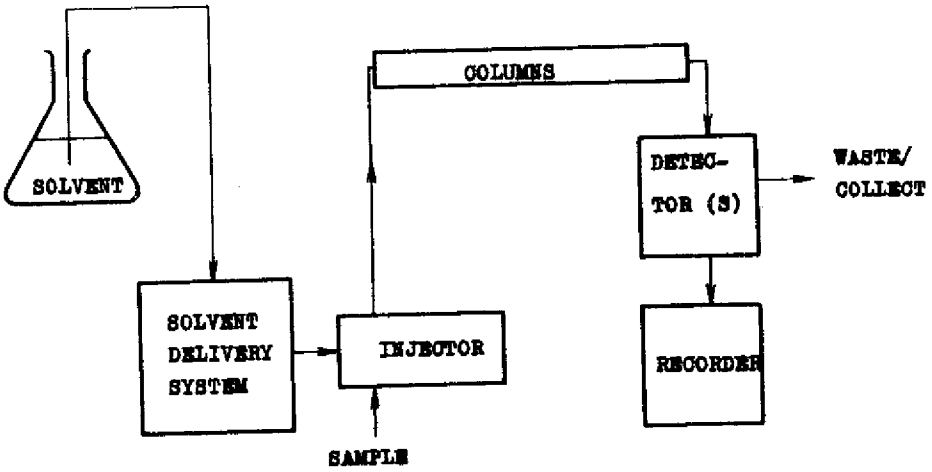
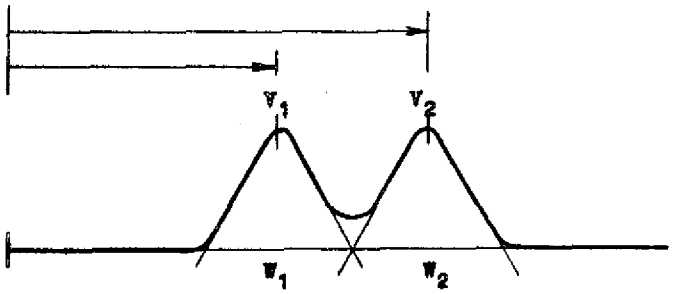


Fig.1. HPLC SYSTEM.



$$R = \frac{V_2 - V_1}{1/2\ (W_1 + W_2)}$$

Fig.2. RESOLUTION EQUATION.

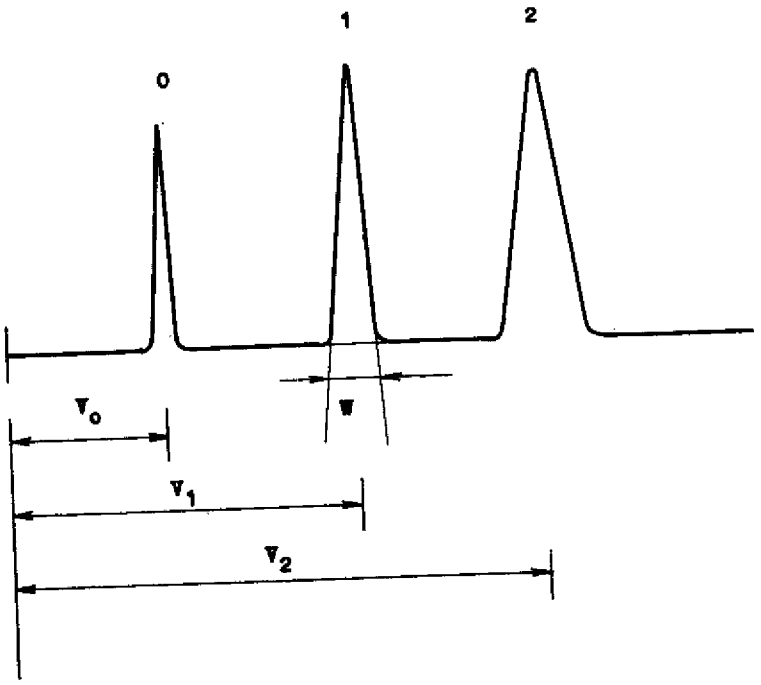


Fig.3.

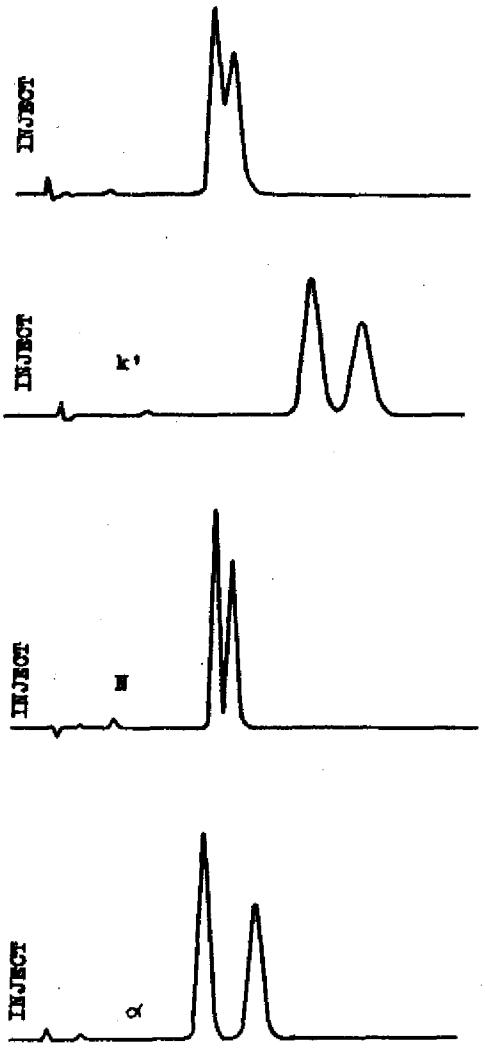


Fig.4. Effect of change in k', α and N on resolution.

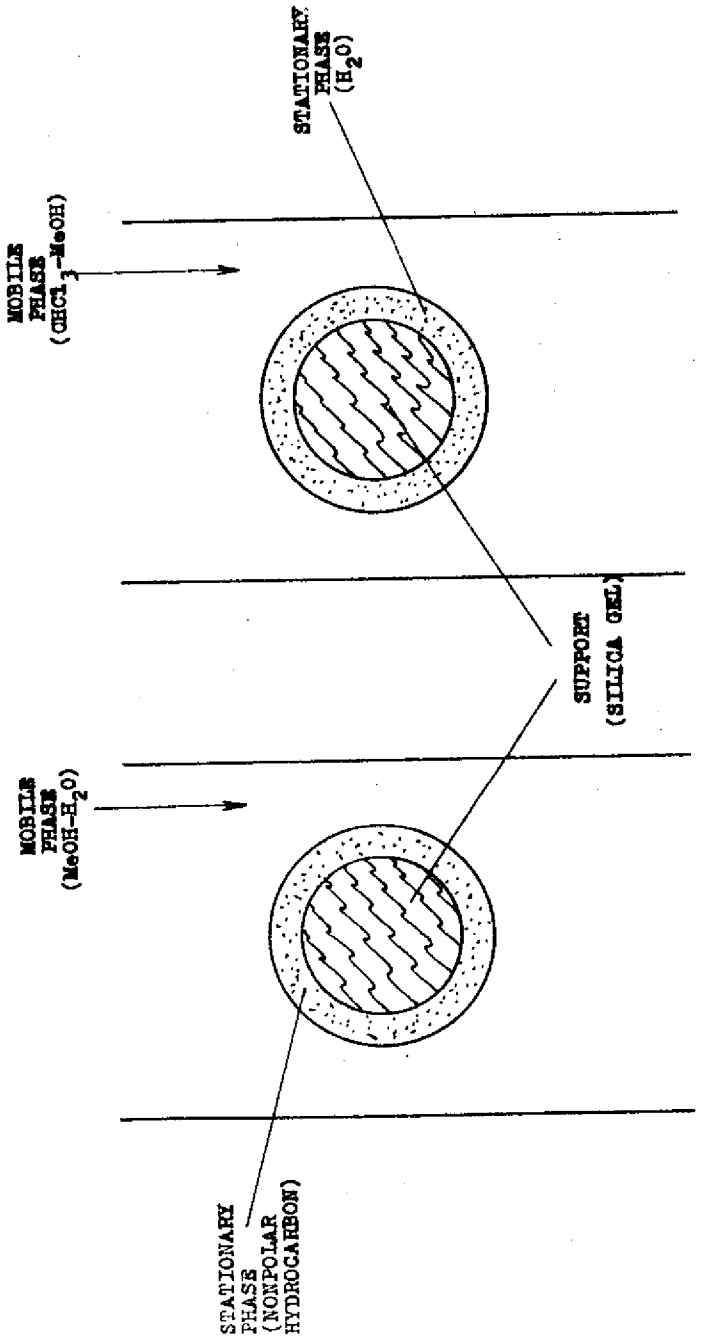

MOBILE PHASE ($CHCl_3$-MeOH)
STATIONARY PHASE (H_2O)
SUPPORT (SILICA GEL)
NORMAL PHASE MECHANISM
MOBILE PHASE (MeOH-H_2O)
STATIONARY PHASE (NONPOLAR HYDROCARBON)
REVERSED PHASE MECHANISM

Fig.5.

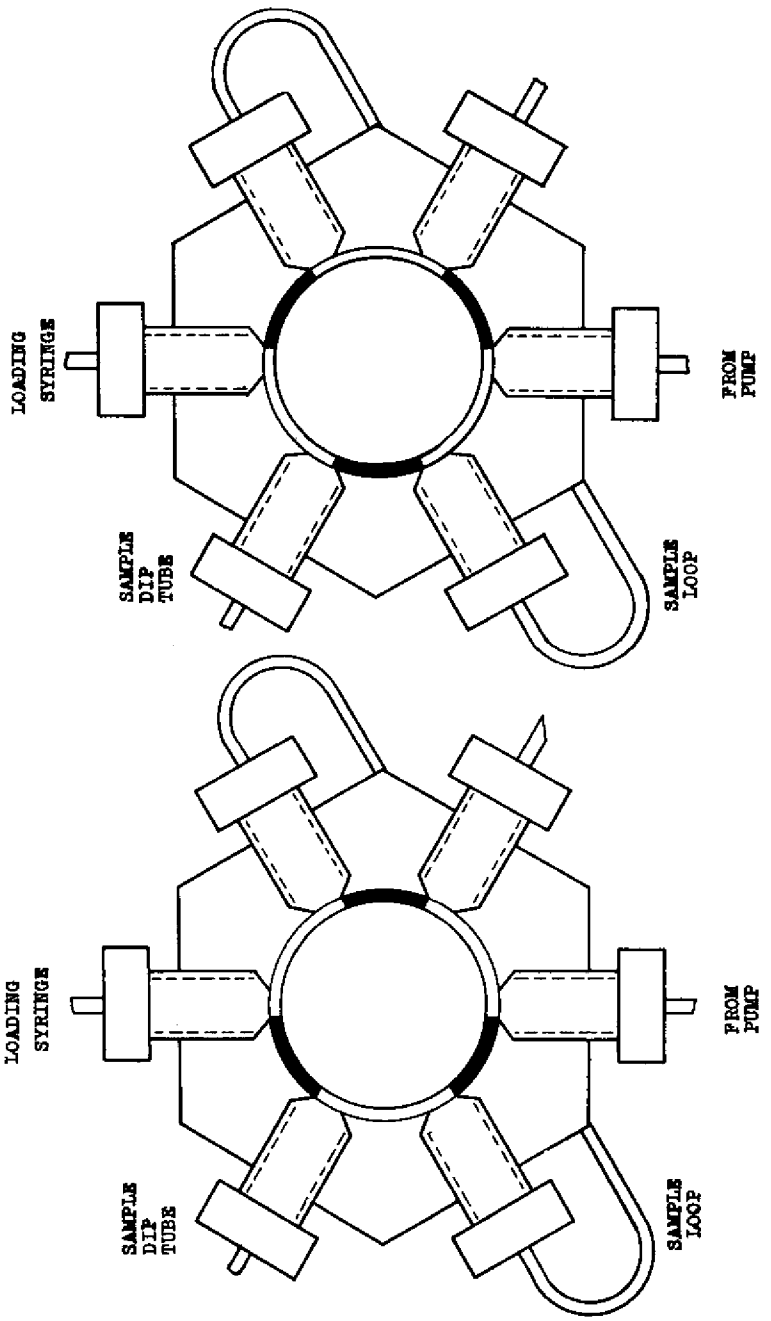

LOADING SYRINGE
FROM PUMP
SAMPLE DIP TUBE
SAMPLE LOOP
INJECTION POSITION
LOADING SYRINGE
FROM PUMP
SAMPLE DIP TUBE
SAMPLE LOOP
LOAD POSITION

Fig.6.

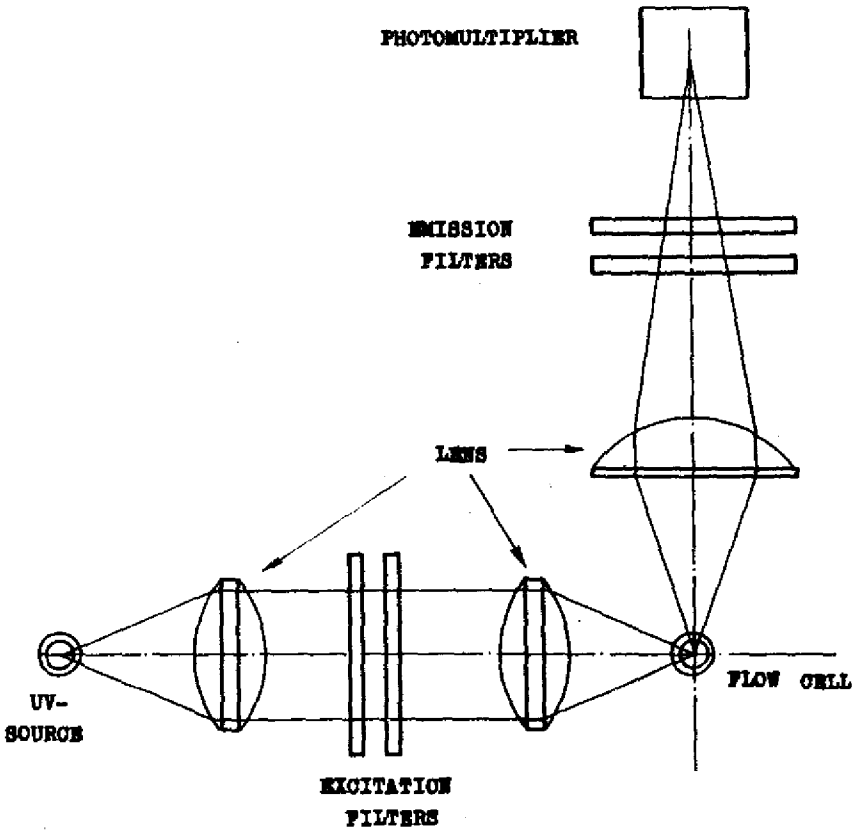


Fig.7. Optical diagram of filter fluorescence detector.

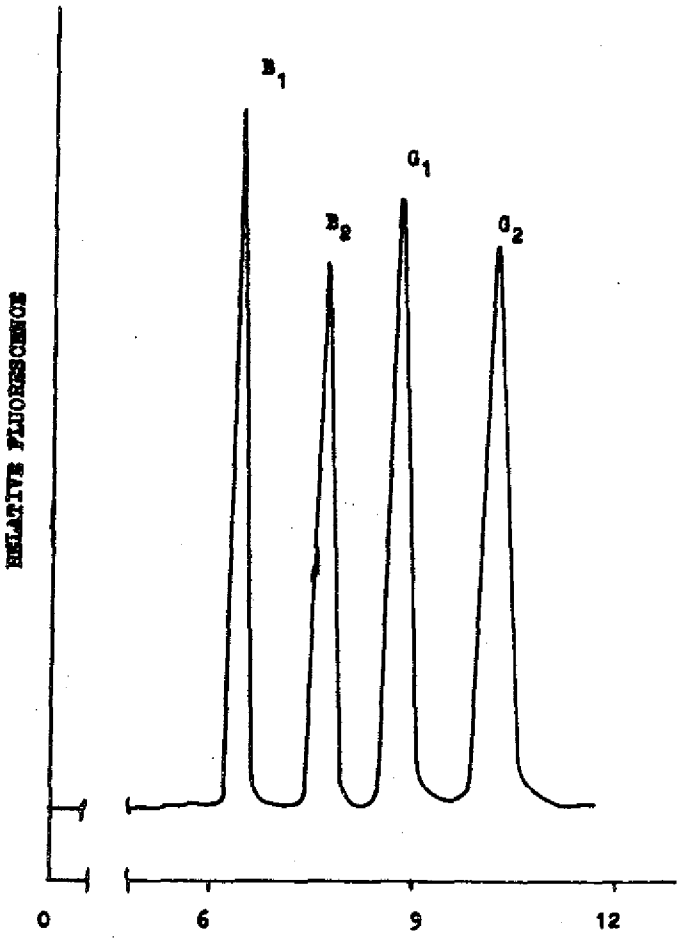


Fig.8. Normal-phase separation of aflatoxins, column - Porasil, mobile phase - water-washed $CHCl_3$-cyclohexane-acetonitrile-isopropanol (73:22:3:2).

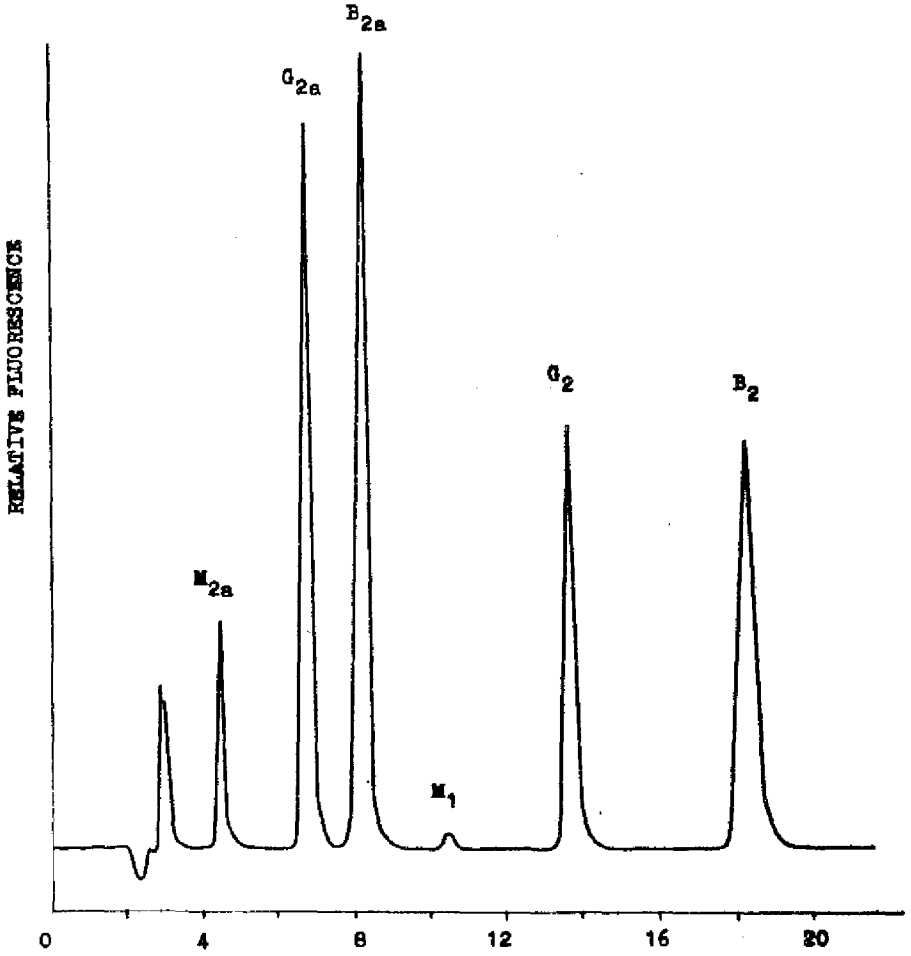


Fig.9. Reversed-phase separation of TFA-treated aflatoxins, column - Ultrasphere ODS, mobile phase - H_2O-MeOH-MeCN (66:25:9).

Зак. 2127 ПИК ВИНИТИ