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# Immunoassay of low-molecular-mass physiologically active substances of plant and microbial origin encountered in plants

S K Volkov

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**Abstract.** The application of immune methods for the assay of both endogenic and exogenic low-molecular-mass physiologically active substances encountered in plants is considered. The bibliography includes 161 references.

## I. Introduction

The determination of the content of biologically active substances in plant tissue constitutes one of the most important parts of the science of medicinal plants. The content of physiologically active substances in plant raw material determines not only the economic parameters of the manufacture of a number of medicinal preparations but also their quality.

Selection of the most productive types of plants is based on the quantitative assay of biologically active substances in various parts of plants. The development of biotechnological methods for the cultivation of plant cells and tissues likewise cannot take place without the quantitative assay of biologically active substances. Since many biologically active compounds of plant origin are narcotics, their quantitative determination is important from the criminological point of view.

The most important groups of biologically active substances of plant origin are alkaloids, including a series of narcotic substances, terpenoids, flavonoids, anthrones, steroids, plant growth and development regulators, and certain others. Certain substances which are not endogenic but enter the plant material from outside frequently have to be determined in plant tissues, particularly those used as food by man and as cattle feed. These include antibiotics and mycotoxins.

This review constitutes a continuation of the previously published article on the immunoassay of alkaloids [1] and for this reason alkaloids will not be considered here except for those groups of alkaloids which were not dealt with in the previous review. For the same reason, narcotic alkaloids (opiates and cocaine group alkaloids) will not be considered here either.

The principal advantages and disadvantages of the immune methods for quantitative assay, the approaches to the immunoassay of low-molecular-mass compounds, and the commonest methods of immunoassay were also examined in fair detail in the previous review.

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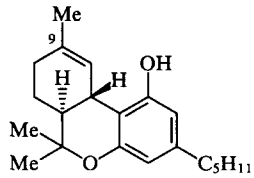
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## II. Immunoassay

### 1. Narcotic substances

Narcotics of plant origin include not only many alkaloids but also a series of nonalkaloid compounds with biological activity. Biologically active substances of hemp (cannabinoids) occupy a by no means unimportant place among them.

**Cannabinoids** constitute a group of powerful narcotics — hallucinogens. Among cannabinoids, tetrahydrocannabinols are particularly dangerous. Thus $\Delta^9$-tetrahydrocannabinol **1** (or $\Delta^1$-tetrahydrocannabinol when a different system for the numbering of atoms is used) is the principal component of hashish and marijuana, which are prepared from Indian hemp (*Cannabis sativa*). When it acts on the organism in doses of the order of $10^{-5}$–$10^{-6}$ g (kg body weight)$^{-1}$, compound **1** induces a psychiatric disorder, a decrease in blood pressure, and a decrease in sensitivity to pain.




**1**

Several methods exist at present for the immunoassay of compound **1**. The fluorescence polarisation method is simple and convenient in the assay of a large number of specimens, but its sensitivity ($5 \times 10^{-5}$ g L$^{-1}$) is not always adequate. [2] The immunofluorescence method using antibodies bound covalently to magnetic particles, which makes it possible to carry out easily the stage involving the separation of the bound and free antigens, is more sensitive; as a result of the above features of this method, its sensitivity reaches $5 \times 10^{-6}$ g L$^{-1}$. [2]

There exist also a homogeneous immunoenzyme method for the determination of cannabinoids, [3] the sensitivity of which is $(1.5-2.5) \times 10^{-2}$ g L$^{-1}$, and a solid-phase heterogeneous immunoenzyme method. [4] In the latter case, the immunogenic conjugate was obtained by oxidising compound **1** with potassium hexacyanoferrate(III) and conjugating the derivative obtained with the protein. The sensitivity of the solid-phase immunoenzyme method reaches $10^{-4}$ g L$^{-1}$.

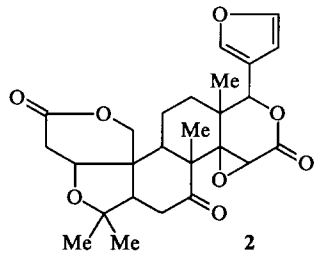
Diagnostic sets produced by the Abbot Company (UK) enable detection of **1** at a concentration of $9 \times 10^{-6}$ g L$^{-1}$.

The immunoassay methods have been used hitherto only in the determination of cannabinoids and their metabolites in human biological liquids [6, 7] but not for the quantitative assay of cannabinoids in plants.

## 2. Terpenoids

The class of terpenoids or isoprenoids, the first representatives of which were isolated from turpentine, constitute a large group of biologically active compounds of plant and animal origin. Terpenoids include certain natural glycosides (saponins), yellow and orange plant pigments (carotenoids, xanthophylls), natural rubber, products derived from pine resin, and a number of other substances.

**Limonin 2**. This triterpene is concentrated in the seeds of many types of plants of the genus *Citrus* (*Rutaceae*) and when it contaminates lemon juice it imparts an excessively bitter flavour to the latter. Since the amount of compound **2** in the plant can differ greatly from batch to batch, its content must be monitored, since the bitterness of the juice increases sharply in the range of concentrations of compound **2** from $10^{-7}$ to $10^{-6}$ g $L^{-1}$. This requirement has led to the development of a series of methods for the immunoassay of compound **2**.



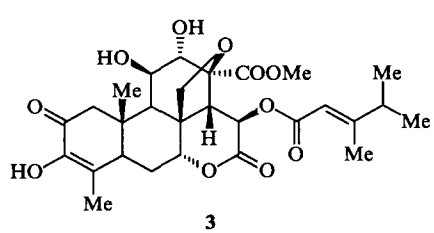
**2**

In order to obtain an immunogenic conjugate, compound **2** was attached to bovine serum albumin (BSA) with the aid of aminooxoacetic acid. The antiserum to this conjugate was used to develop radioimmunoassay methods using the conjugate of the methyl ester of 7-(*O*-carboxymethyl)limonin with the $^{125}$I-labelled aminooxo-derivative of tyrosine[8] and also $^{3}$H-labelled limonol.[9] The same antiserum has been used for the development of an immunoenzyme assay method using the 7-(*O*-carboxymethyl)oxime of compound **2** conjugated with alkaline phosphatase by the carbodiimide method.[10] Radioimmune methods using $^{125}$I- and $^{3}$H-labelled compounds make it possible to determine compound **2** in the ranges $10^{-10}-10^{-7}$ and $5 \times 10^{-10}-10^{-7}$ g respectively. Although the immunoenzyme method is somewhat less sensitive (the range of the determination is $10^{-9}-10^{-7}$ g), nevertheless it makes it possible to work with more stable substances and to carry out the assay in 1 h.

The radioimmune method has been used to investigate the distribution of compound **2** in the tissues of *Citrus paradisi*.[8] The immunoenzyme method has been applied to the assay of specimens of lemon juice and a satisfactory agreement has been observed with the results of assay by the HPLC method, but the immunoenzyme method does not require special preparation of the specimen and takes less time.[10]

A set for the immunoenzyme assay of compound **2** is manufactured by the Idetek Company (USA).[11]

**Bruceantin 3**. This secotriterpenoid, isolated from *Brucea antidisenterica* (*Simaroubaceae*), exhibits an appreciable antitumour activity. As for other medicinal drugs, immunoassay methods greatly facilitate the study of the pharmacological properties of compound **3**.

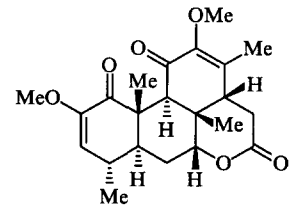


**3**

In the development of the radioimmunoassay method, compound **3** was conjugated with BSA with the aid of succinic anhydride, and an antiserum to this conjugate was obtained.[12] Radioimmunoassay with the use of [$^{3}$H]acetylbruceantin, obtained by treating compound **3** with [$^{3}$H]acetic anhydride, makes it possible to determine compound **3** in the range $5 \times 10^{-10}$ to $5 \times 10^{-8}$ g.[12]

The method has been used to study the distribution of compound **3** in mouse body tissues and for its removal from the dog; immune methods have not been applied to the assay of compound **3** in plants.

**Quassin 4**. This secotriterpenoid, which has an extremely bitter taste, has been isolated from *Quassia amara*. It is used to impart a bitter flavour in the food industry. Methods for the immunoassay of compound **4** have been developed in order to monitor the manufacture of products and also for the analysis of plant cell clones.



**4**

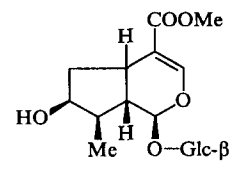
In devising an immunoenzyme method, the δ-lactone of quassin was converted into a derivative with a free carboxy-group, which was stabilised by epimerisation to form isoquassinic acid.[13] Isoquassinic acid was conjugated with BSA by the mixed anhydride method and a rabbit was immunised with the conjugate. The competing conjugate of compound **4** with the haemocyanine of the edible snail (KLH) was obtained by the carbodiimide method. The immunoenzyme method using goat antibodies to rabbit immunoglobulins G (Ig G), conjugated to alkaline phosphatase, makes it possible to determine the concentration of compound **4** in the range $2 \times 10^{-9}-2 \times 10^{-7}$ g. Appreciable cross-reactions were noted for methyl isoquassinate, α- and β-neoquassin, 14,15-dehydroquassin, 12- and 18-hydroxyquassin, and also nigakilactones and picrasin A from *Picrasma excelsa*.

Another method is based on the application of the antiserum to 18-hydroxyquassin conjugated with BSA by means of succinic anhydride. In other respects, the method is similar to the previous one and makes it possible to determine quassin in the range $5 \times 10^{-9}-10^{-7}$ g.[14] The method has been used to investigate the distribution of immunoresponsive substances in the tissues of *Q. amara*, *Q. indica*, and *P. excelsa*.[14]

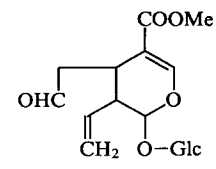
The immunoenzyme method for the determination of quassinoids has been used in the study of the tissues and cell cultures of *Ailanthus altissima*.[15]

## 3. Iridoid glycosides

The monoterpene glycosides loganin **5** and secologanin **6** are intermediates in the biosynthesis of many alkaloids.



**5**



**6**

Radioimmunoassay methods have been developed for the investigation of the biogenetic transformations of compounds **5** and **6**.[16] Loganin was converted into the 6′-carboxy-derivative involving the glucose residue and was conjugated with BSA by the carbodiimide method. The radioimmune method using the $^3H$-labelled derivative, obtained by the periodate cleavage of compound **5** and subsequent reduction by $NaB^3H_4$, makes it possible to determine compound **5** in the range $5 \times 10^{-10}$ to $10^{-7}$ g. Cross reactions with compound **6** (0.07%) and the aglycone of compound **5** (30%) have been noted.

The method was used to determine compound **5** and to investigate its conversion into compound **6** in *Weigela gigantiflora* and *Lonicera tatarica* cell cultures.[16]

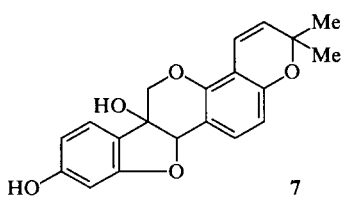
## 4. Sesquiterpenes

Sesquiterpenoid compounds are contained in various plant essential oils and resins (balsams). The group of sesquiterpenes includes substances such as juvenile hormones, perfumes, sesquiterpene lactones and phytoalexins, as well as ichthyotoxins.

## 5. Phytoalexins

The group of phytoalexins includes highly reactive compounds which protect plants against disease. They are synthesised by plants only in response to tissue trauma and for this reason they are also referred to as stress metabolites. The system for the production of phytoalexins by plants is sometimes referred to as the immune system of plants. Phytoalexins possess antimicrobial properties.

**Glyceollin I (compound 7)** is the main phytoalexin accumulating at the sites of tissue trauma or infection of soya bean (*Glycine max L. Merrill*).




7

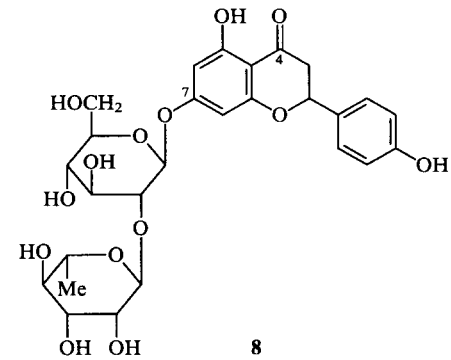
In the synthesis of the immunogenic conjugate of compound **7**, the BSA derivative of *p*-aminohippuric acid was obtained initially. For this purpose, a mixture of *p*-aminohippuric acid and BSA at pH 6.4 was treated with the hydrochloride of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide.[17] The derivative obtained was treated with $NaNO_2$ at pH 1.5 at 0 °C (the excess $NaNO_2$ was decomposed with ammonium sulfamate) and was then conjugated with compound **7** at pH 9.0 at 0 °C.[18]

The antiserum to the resulting conjugate is highly specific to compound **7**. Structural isomers of compound **7** — glyceollin II and glyceollin III — are fairly poorly recognised by the antiserum: 5.7% and 1.0% respectively. The radioimmunoassay method using [$^{125}$I]glyceollin I makes it possible to determine compound **7** at a concentration of $1 \times 10^{-5}$ M.[19] The method has been used to determine compound **7** in soya bean tissues.

## 6. Flavonoids

The group of flavonoids combines the hydroxy-derivatives of flavanone, flavanonol, catechins, flavone, flavonol, and anthocyanins. These substances are present in many plants and exhibit vitamin P activity.

A series of bitter flavonoid 7-*O*-neohesperidosides have been detected in *Citrus* (*Rutaceae*) fruits. The main one is **naringin 8**. The content of compound **8** in the grapefruit (*C. paradisi*) can reach a level such that both the fruits themselves and the juice obtained from them acquire an excessively bitter flavour. Two radioimmunoassay methods for compound **8** have been developed for the rapid monitoring of the quality of the produce. In the synthesis of the immunogenic conjugate, the amino-groups of BSA were modified with *p*-aminohippuric acid and were then attached to compound **8** by diazotisation. The radioimmunoassay method using a $^{125}$I-labelled competing conjugate makes it possible to determine compound **8** in the range $5 \times 10^{-9}-2 \times 10^{-7}$ g. The method is very sensitive to the nature of the sugar residue in the 7-position, but is insensitive to changes in the aromatic ring. All the flavonoid 7-*O*-neohesperidosides are recognised by the antiserum to the same extent.[20]



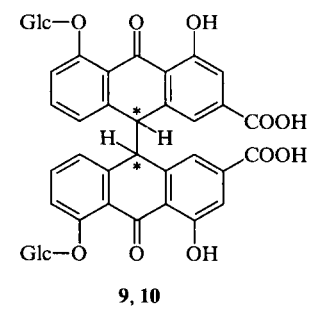

8

In another instance, the immunogenic conjugate was synthesised by activating the 4-oxo-group in compound **8** with aminooxoacetic acid and was attached to BSA by the mixed anhydride method. The $^3H$-labelled conjugate was obtained by condensing the (4-*O*-carboxymethyl)oxime of compound **8** with 5-aminolevulinic acid by the mixed anhydride method and by the reduction of the side chain with $NaB^3H_4$. The radioimmunoassay method permits the determination of compound **8** in the range $5 \times 10^{-10}-5 \times 10^{-8}$ g.[21]

Both these methods have been used to determine the content of flavonoid 7-*O*-neohesperidosides in grapefruit juice and in the study of the distribution of immunoresponsive substances in grapefruit tissues.[21] It was shown that the data obtained by the radioimmune method agree well with the results of the assay by the HPLC method. The second method has been used also to analyse grapefruit juice under the conditions of large scale production.

## 7. Anthrones

Isomeric dianthrone glucosides — **sennosides A (compound 9)** and **B (compound 10)** isolated from a series of *Cassia* (*Caesalpinaceae*) species — are highly active laxatives and constitute one of the classes of substances of plant origin most widely used in pharmaceutical practice. This stimulated the development of a method for the radioimmunoassay of dimeric glucosides.[22]




9, 10

In order to obtain the immunogenic conjugate, compound **10** was attached to BSA by the carbodiimide method. The radioimmune method using [$^3$H]-8-glucosidorheinanthrone, obtained by reducing compound **10** with $NaB^3H_4$, makes it possible to determine compound **10** in the range $4 \times 10^{-10}-8 \times 10^{-9}$ g. Cross reactions have been noted for compound **9** (75%), sennoside C (100%), 8-glucosidorheinanthrone (55%), and sennidins A (25%) and B (10%).

The method has been used to investigate the distribution of compounds **9** and **10** in various parts of *Cassia augustifolia*.

## 8. Steroids

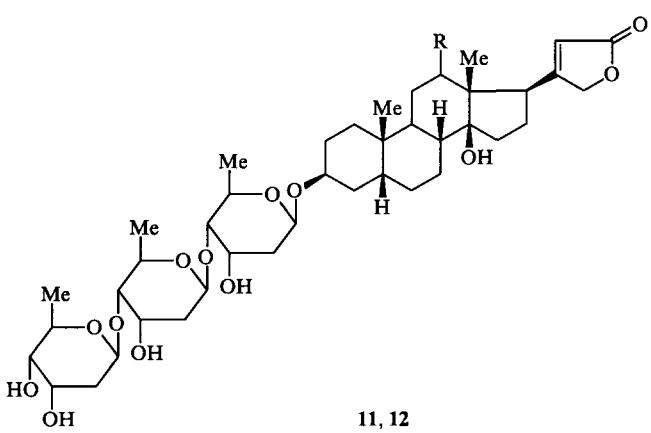
A large group of steroid glycosides of plant and animal origin is combined under the general name cardioactive or cardiotonic substances. At low concentrations, they normalise the working of the heart muscle, whilst in large doses they induce cardiac arrest in the systolic period. In contrast to their aglycones, cardiac glycosides are concentrated in heart muscle and their content in the latter is 10–40 times greater than in other tissues of the organism.

### a. Digitalis cardenolides

The myocardium-stimulating digoxin and digitoxin are isolated from the leaves of *Digitalis lanata* (*Scrophulariaceae*), where they accumulate together with related glucosides.

**Digoxin 11** (R = OH) is one of the substances of plant origin for the assay of which immune methods are used very widely.

The first attempts to devise an immunoassay method for compound **11** date back to 1967 when antibodies specific to compound **11** were obtained.[23] A method for the radioimmunoassay of compound **11** for use under clinical conditions was developed in 1970[24,25] and subsequently methods suitable for the determination of compound **11** in plant tissues were developed.[26–28] All these methods employ the rabbit antiserum to the conjugate of compound **11** with BSA or human serum albumin (HSA) obtained by the periodate oxidation method. The range of the determination of compound **11** is $8 \times 10^{-10}$ to $4 \times 10^{-8}$ g when [$^3$H]digoxin is used[25] and $5 \times 10^{-10}$ to $5 \times 10^{-9}$ g when the methyl ester of digoxin-[$^{125}$I]tyrosine is employed.[26] The study of cross-reactions with a large number of related structures showed that the serum obtained makes it possible to determine the sum of the digitoxigenin glycosides in extracts from *D. lanata*.




**11, 12**

Immune methods have been used to determine the level of compound **11** in the serum of blood from patients[25] and also for the investigation of the distribution of compound **11** in the tissues of *D. lanata*.[26,27] It was established that the main bulk of compound **11** accumulates in leaves after 8–12 weeks. The radioimmune method makes it possible to determine the optimum period for the harvesting of leaves. The same method has been applied in the selection of plants most productive as regards compound **11**.[28] The content of compound **11** in the tissues of *D. lanata* and *D. purpurea* was determined with its aid during the growth of these plants.[29]

In subsequent years, many immunoassay methods for compound **11** were developed: radioimmune,[30–32] immunoenzyme,[29,32–34] immunoluminescence,[29,35] and fluoroimmune[36] methods, a combined liquid chromatographic-immune method,[37] immune methods involving the measurement of the degree of polarisation of fluorescence,[32,38–43] an immune method using particles of polystyrene latex,[44] a chemiluminescence immunoenzyme method,[45] a heterogeneous competitive immunoenzyme method with electrochemical detection,[46] a fluorescence method with transfer of excitation energy,[32,47–49] and a number of others.[42,50] The methods developed have been used to assay compound **11** in the serum,[32,33,42,47–49,51–58] plasma,[30,39,42,49,59] urine,[30] saliva,[31] and blood of patients[60] and also for the investigation of the pharmacokinetics of compound **11**.[61] Only one of these methods has been applied to its determination in the cell culture of *D. lanata* roots.[33]

Comparison of the immune methods with one another and also with methods based on high performance liquid chromatography showed that many substances related to digoxin, frequently present in the specimen, as well as certain metabolites of compound **11** affect appreciably the results of the determination of compound **11** by immune methods.[30,32,38–40,51–57,62,63] The attempts to eliminate this influence led to the appearance of immune methods based on monoclonal antibodies[58] and to the introduction of stages involving additional purification of the specimens analysed.[37,43,44,64]

Many Companies manufacture sets for the immunoassay of compound **11**. The sets produced by Companies such as Syva Corporation, E.I. du Pont de Nemours, New England Nuclear, Amersham Corporation (USA), and a number of others have come to be most widely used. These sets are widely employed in clinical and laboratory practice for the determination of compound **11** in blood plasma and serum, in urine, and in other physiological liquids. A method for the radioimmunoassay of the total digoxigenin glycosides in the *D. lanata* plant has been developed on the basis of one of such sets — the 'Digoxin RIA kit' produced by the Farmos Diagnostica Company (Finland).[64]

**Digitoxin 12** (R = H). An antiserum to the conjugate of digitoxigenin (aglycone) with BSA, obtained by 3-*O*-succinylation and carbodiimide condensation, was isolated in 1968 from rabbit.[65] The radioimmune method using the conjugate of the methyl ether of 3-*O*-succinyldigitoxigenin with [$^{125}$I]tyrosine makes it possible to determine compound **12** in the range $2 \times 10^{-10}$–$2 \times 10^{-8}$ g. The contribution of the cross-reaction with compound **11** is 10%. The method has been used to determine the content of compound **12** in the serum of blood from patients.

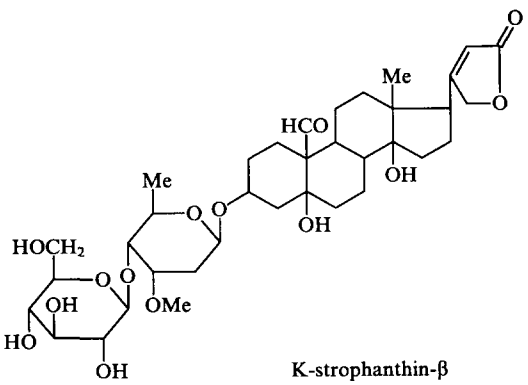
The method developed subsequently for the analysis of patients' blood, involving the measurement of the degree of polarisation of fluorescence, yielded the same results as the radioimmune method.[66]

The radioimmune method using [$^{125}$I]digitoxigenin has been applied to the assay of compound **12** in urine specimens after preliminary purification of the extract by reverse-phase HPLC.[67] Immune methods have not so far been applied to the assay of compound **12** in plants.

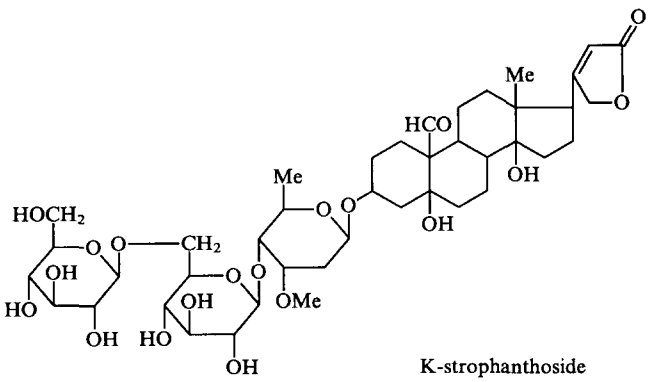
### b. K-Strophanthin

K-Strophanthin **13** is a typical representative of cardiac glycosides with a cardenolide aglycone. It is a mixture of the cardiac glycosides K-strophanthin-β and K-strophanthoside. Strophanthin is present in various *Strophanthus* species.

The immunogenic conjugate with BSA was obtained by the periodate oxidation of compound **13**.[68] The use of this conjugate made it possible to obtain antibodies specific to compound **13** and to devise an immunoenzyme assay method whereby it is possible to determine compound **13** in the concentration range $10^{-9}$–$10^{-3}$ M.[69] Subsequently immunoenzyme methods were developed for the assay of compound **13** using the antigen immobilised on AH-agarose,[70] on nitrocellulose membranes,[71] and also on activated polystyrene pellets.[72] The latter methods make it possible to determine compound **13** in the concentration range $10^{-8}$–$10^{-3}$ M; the sensitivity of the method reaches $10^{-10}$ M.[71,72] These methods were developed to assay compound **13** in the blood and other biological liquids of patients; immune methods have not so far been applied to the assay of compound **13** in plant tissues.



K-strophanthin-β



K-strophanthoside

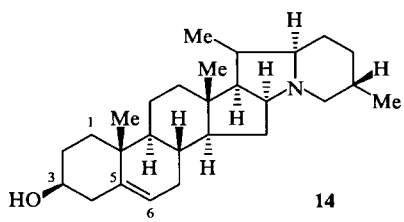
## 9. Steroid glycoalkaloids in *Solanaceae*

Plants of the genus *Solanum* (*Solanaceae*) produce a series of steroid glycoalkaloids. Certain representatives of *Solanaceae* are of greatest interest from this standpoint.

1. The potato (*S. tuberosum*) contains a number of bitter toxic derivatives of solanidine, mainly α-solanine and α-chaconine. The assay of these compounds is important for work involving selection. Furthermore, during incorrect storage of the tubers or when the potato is grown under unfavourable climatic conditions, the content of these glycoalkaloids can increase greatly, impairing the flavour of the potato.

2. The alkaloid solasodine, which serves as the starting material for the synthesis of a series of steroid preparations (progesterone and corticosteroids), accumulates in the leaves of a series of *Solanaceae* species (*S. laciniatum* and *S. aviculare*).

**Glycoalkaloids — glycosidic derivatives of solanidine 14.** A radioimmunoassay method for compound **14**, based on the use of the antiserum to compound **14** conjugated with BSA via the 3-hydroxy-group, was developed in 1979.[73] This method makes it possible to determine compound **14** in the range $2 \times 10^{-10}$ to $5 \times 10^{-8}$ g. The contribution of the cross-reaction with α-solanine is 28%.

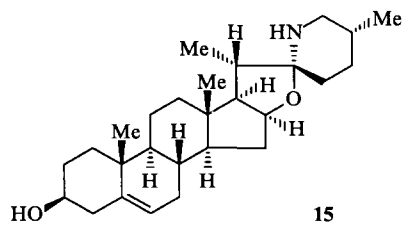


**14**

The conjugate of α-solanine with BSA was synthesised in 1983 by the periodate cleavage method.[74,75] The rabbit antiserum to this conjugate is equally sensitive to compound **14**, α-solanine, α-chaconine, and demissidine, which is very convenient for the determination of the overall content of glycoalkaloids in potato tubers. The radioimmune method using $^3$H-labelled compound **14** makes it possible to determine these glycoalkaloids in the range $2 \times 10^{-10}-8 \times 10^{-9}$ g,[76] while the immunoenzyme method permits their determination in the range $10^{-11}-10^{-9}$ g.[75]

The radioimmune method has been used to determine compound **14** in human blood serum.[76,77] The results of assays obtained by the immunoenzyme and chemical methods have been compared. It was established that they agree well, but the immunoenzyme method is simpler, rapid, and less subject to technical errors.[78,79]

**Glycoalkaloids — glycosidic derivatives of solasodine 15.** The immunogenic conjugate was obtained by subjecting a 2:3 mixture of solasonine and solamargine to periodate cleavage followed by attachment to HSA.[75] The radioimmune method using $^3$H-labelled glycoalkaloids, based on the application of the rabbit antiserum, makes it possible to determine derivatives of compound **15** in the range $10^{-9}-10^{-7}$ g. Reactions with solasonine (100%), solamargine (110%), and tomatine (104%), as well as the aglycones of tomatidine (21%), soladulcine (5%), and compound **15** (7%) have been noted.



**15**

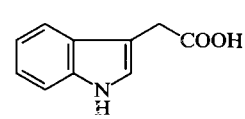
This method has been applied in the study of the content of compound **15** in the tissues of *S. laciniatum* and *S. aviculare*.[75]

## 10. Plant growth and development regulators

Plant bioregulators are low-molecular-mass products of the secondary metabolism of plants. Phytohormones, natural stimulators, and inhibitors are distinguished among them.

Phytohormones include auxins, gibberellins, abscisic acid, cytokinins, and ethylene. In contrast to other physiologically active compounds, phytohormones are bioregulators common to all plants. They are synthesised in actively dividing cells, after which they are transported to other organs and tissues where they initiate chemically physiological programs. The molecular mechanisms of the action of plant hormones are apparently associated with the regulation of protein synthesis, the activity of enzymes, and transport through a biological membrane.

**Auxins** constitute a group of natural compounds which stimulate cell division (mitosis), root formation, respiration, and the synthesis of protein in plants. The main representative of auxins is indolylacetic acid **16**.

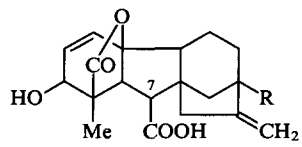


**16**

The Mannich reaction was used to synthesise the immunogenic conjugate of compound **16** with BSA; compound **16** is linked to the protein via the nitrogen atom.[76]

The formation of the bond via the carboxy-group (the mixed anhydride method) affords more stable conjugates, which induce a somewhat greater immune response both in rabbits[77] and in mice.[78]

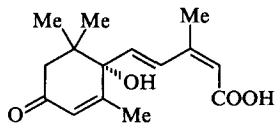
**Gibberellins** are diterpenoid tetracyclic mono-, di-, and tricarboxylic acids. The mechanism of the biological action of gibberellins has been inadequately investigated. It is known that they alter the properties of membranes and induce the synthesis of α-amylase in barley grains, while in the tissues of other plants they alter the systems comprising RNA and functioning enzymes. About 70 gibberellins have now been found in nature and antisera have been obtained and immunoassay methods have been developed for some of them [including the gibberellins $A_3$ **17** (R = OH) and $A_7$ **18** (R = H)].[79–83]



**17, 18**

The immunogenic conjugates are obtained by forming an amide linkage between the C(7)-carboxy-group of gibberellin and the amino-groups of the protein.[79] Alternatively, the mixed anhydride method is used, but in this case conjugates with a fairly low hapten density are obtained owing to steric hindrance. A spacer is introduced between the hapten and the protein in order to increase the hapten density.[82,83]

**Abscisic acid 19** is a highly specific inhibitor of the growth of higher plants. It causes a transition of the plant to the resting state and induces wilting and shedding of leaves and fruit.

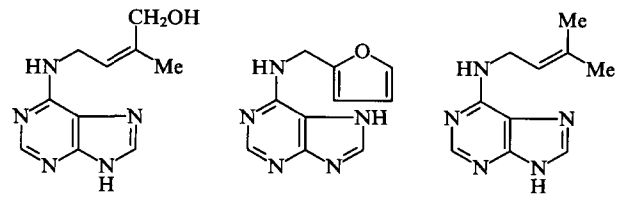


**19**

The conjugates of compound **19** may be synthesised using both the carboxy-group[84,85] and the carbonyl[85] group. In order to obtain a conjugate of compound **19** with a protein via the carboxy-group, a solution of the hapten and the protein in a mixture of dimethylformamide and water at pH 8 is treated with the hydrochloride of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide.[85] The antiserum to this conjugate interacts with the protonated form of compound **19** and its esters but hardly interacts with the deprotonated compound **19**, which is the main one actually present under the conditions of the immunoassay (pH 7–8).

The conjugate via the carbonyl group is synthesised by carrying out initially the reaction between compound **19** and tyrosine hydrazide in the presence of acetic acid in order to obtain the tyrosylhydrazone of compound **19**. BSA *p*-aminohippurate is obtained simultaneously by the reaction of *p*-aminohippuric acid with BSA in the presence of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide. The modified protein is treated with $NaNO_2$ at pH 1.5 and ammonium sulfamate and is condensed with the tyrosylhydrazone of compound **19** at pH 9.[85] The antibodies to this conjugate are preferable in those cases where it is proposed to assay compound **19** in the form of the anion. Such antibodies interact very weakly or not at all with the protonated form of compound **19**.

**Cytokinins** constitute a special group of plant growth and development regulators which stimulate cell division (cytokinesis). This group includes the *N*(6)-derivatives of adenine—zeatin **20**, kinetin **21**, and isopentenyladenine **22**. In plants, they are frequently encountered in the form of the less active ribosides and ribotides and are one of the constituents in the structures of certain transport RNA of plants.



**20** **21** **22**

The mechanism of the biological action of cytokinins is apparently associated with the intensification of the biosynthesis of DNA, RNA, and protein and also with their influence on the functioning of biological membranes.

Cytokinins have been traditionally assayed by biological methods, but the sensitivity and selectivity of these is low.[86,88] The physicochemical and immunochemical assay methods are superior to the biological methods as regards sensitivity, specificity, and reproducibility and take less time.[89,90]

The preparation of the immunogenic conjugates of cytokinins is based on the periodate oxidation of the riboside to the corresponding aldehyde, which can interact with the amino-groups of the carrier protein. For this purpose, the cytokinin riboside in methanol was treated with sodium periodate, the excess of which was decomposed with ethylene glycol. A solution of the carrier protein at pH 9.3 was treated with the dialdehyde obtained from the cytokinin riboside; the complex thus produced was stabilised by reduction with tetrahydroborate.[91]

The first radioimmunoassay methods permitting the determination of compound **22** in an amount of about $10^{-8}$ g were used in the study of plant transport RNA.[92–94] Analysis of the cross-reactions showed that the antiserum obtained to the conjugate of compound **22** with the protein interacts with the phosphate of compound **22** (100%) and with isopentenyladenine (10%).[94] The interaction of the antiserum with compound **21** is weaker by a factor of 100 and that with adenosine is weaker by a factor of 300 000 than the interaction with compound **22**.[94]

The use of the immune method for the assay of cytokinins in plants was described in 1979, but the antibodies to compound **22** interacted strongly with *trans*-zeatin riboside and dihydrozeatin riboside.[95]

A sensitive and specific radioimmune method for the determination of *trans*-**20** was developed in 1980.[96] The limit of the determination of *trans*-**20** amounted to several femtomoles. The antibodies did not interact with *cis*-**20**.

The antisera to compound **22** made it possible to attain a limit in the determination of compound **22** of $5 \times 10^{-11}$ to $10^{-9}$ g.[89,97–99] These antisera interact only weakly with cytokinins of the zeatin group, but they do interact appreciably with 6-benzylaminopurine cytokinins.

It was established that the interaction of cytokinin molecules with the corresponding antibodies depends mainly on the nature of the substituent at the N(6) atom. Owing to the appreciable cross-interactions, the accurate assay of cytokinins by the immune methods requires the introduction of a stage involving preliminary chromatographic purification.[100] Column chromatography on cellulose phosphate and Sephadex LH-20 is used for this purpose.[100,101] However, these procedures require a fairly long time, so that simpler purification procedures with the aid of thin-layer chromatography (TLC)[99] and HPLC[102] have been proposed. The choice of a suitable sorbent for HPLC makes it possible to attain the complete separation of the cytokinins assayed. As a rule, reversed-phase sorbents are used for this purpose—Bondapak C 18,[102–104] Lichrosorb RP-8,[105] Hipersil ODS,[106,107] and Sep-Pak C 18.[97,108] The preliminary purification of the specimen can be carried out with the aid of an immunoaffine column.[109]

## 11. Other compounds

Immunoassay methods for compounds which are not endogenic in plants but which it is sometimes necessary to determine in plant material will be examined in this part of the review. In the first place, these are undesirable substances in plants designed for use as food. Such substances are formed on microbial infection of the plant material.

### a. Antibiotics

In a number of instances, as a result of the infection of the plant raw material by microorganisms, such material may be contaminated with the antibiotics produced by such microorganisms.

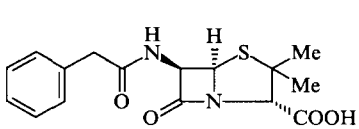
Natural substances and the products of their chemical modification, capable of suppressing at low concentrations ($10^{-6}-10^{-5}$ g $L^{-1}$) the development of bacteria, lower fungi, protozoa, viruses, and malignant tumour cells, are classified nowadays as antibiotics.

The group of β-lactam antibiotics includes penicillins, cephalosporins, cephamycins, and certain other antibiotics. The mechanism of the action of β-lactam antibiotics involves the suppression of the synthesis of the bacterial cell wall.

**Penicillins** possess a broad spectrum of antibacterial activity, exhibiting both bacteriostatic and bactericidal activities in relation to many gram-positive microorganisms (staphylococci, pneumococci, and streptococci), certain gram-negative cocci (gonococci, meningococci), Siberian ulcer bacilli, clostridia, spirochaetes, and certain fungi.

In the immunoassay of penicillins, serious problems, associated with their instability, arise: penicillins readily hydrolyse in aqueous solutions, the hydrolysis products being capable of binding to proteins. Because of this, the sensitivity, the degree of cross-interaction, and the parameters of the binding of antibodies to antigens vary as a function of the degree of hydrolysis of penicillins and the composition of the buffer solution. Hydrolysis of the β-lactam ring of penicillins in 0.1 M NaOH solution or on treatment with β-lactamase also affects these parameters. An additional source of error is the nonspecific binding of penicillins to the proteins present in the specimen assayed.[110]

Despite the complexities in the development and application of immunoassay methods for penicillins, their description is appearing with increasing frequency in the scientific literature. Thus an immunoenzyme method has been developed for the determination of penicillin G **23** and its derivatives in cow's milk and in bovine blood serum.[111]

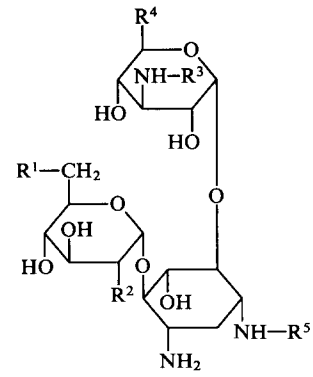


**23**

**Aminoglycoside antibiotics** represent a large family of antibiotics numbering more than 100 natural compounds produced by microorganisms of the genera *Streptomyces, Micromonospora,* and *Bacillus* as well as a large number of semisynthetic analogues. The mechanism of the action of these antibiotics has not been finally established.

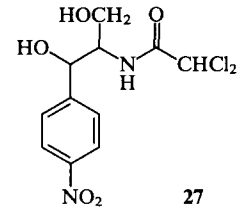
A wide range of immunoassay methods are used for the assay of aminoglycoside antibiotics.[112–114] Thus immunoassay methods have been used in the assay of gentamicin **24** ($R^1$ = OH, $R^2$ = $NH_2$, $R^3$ = Me, $R^4$ = $R^5$ = H),[115,117] amikacin **25** [$R^1$ = $NH_2$, $R^2$ = OH, $R^3$ = H, $R^4$ = $CH_2OH$, $R^5$ = C(O)CH(OH)Pr],[117,118] and tobramycin **26** ($R^1$ = $R^2$ = $NH_2$, $R^3$ = $R^5$ = H, $R^4$ = $CH_2OH$).[117,119–121] Immunofluorescence methods are also widely used in the assay of these antibiotics.[118,121,122]

The 'Optimate' automatic system, based on the immunofluorescence assay method, is used for the assay of compounds **24** and **25**.



**24–26**

**Chloramphenicol 27** occupies a special place among antibiotics. It proved to be the first preparation active against rickets and intracellular parasites which give rise to diseases such as typhus and spotted fever. Chloramphenicol suppresses also many gram-negative and certain gram-positive bacteria and is effective in the treatment of typhoid fever and infections induced by *Haemophilus influenzae, Klebsiella pneumoniae,* spirochaetes, and especially salmonellas, which are resistant to other antibiotics. The therapeutic use of compound **27** is restricted by allergic reactions and the rare instances where the erythropoiesis in the bone marrow is affected, but it is widely used for the inhibition of the biosynthesis of protein in plant chloroplasts.



**27**

Antibodies to the conjugate of compound **27** with bovine γ-globulin, synthesised by the diazotisation method, were obtained in 1966.[123] An immunoenzyme method for the assay of compound **27**, based on the use of the rabbit antiserum to the conjugate of compound **27** with KLH, obtained with the aid of succinic anhydride and carbodiimide, was developed in 1984.[124] The use of goat antibodies to rabbit Ig G conjugated with peroxidase permits the determination of compound **27** in the range $4 \times 10^{-10}-4 \times 10^{-8}$ g.

A radioimmunoassay method has been developed for residues of compound **27** in food products.[125,126] A review on the immunoenzyme and liquid-chromatographic methods for the determination of residues of compound **27** in milk and pig tissues has been published recently.[127] A set for the immunoenzyme assay of compound **27** in human blood serum is manufactured by the Aesculaap BV Company (Holland).[128]

Immune methods have not so far been used to determine antibiotics in plant raw material.

### b. Mycotoxins

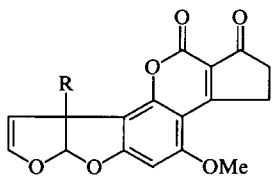
Certain mould species (*Aspergillus, Penicillium*) produce under certain conditions mycotoxins—highly toxic, sometimes carcinogenic secondary metabolites. A special effort has been made to develop methods for the immunoassay of mycotoxins. There are several reasons for this. Firstly, contaminated batches of products are frequently inhomogeneous, which requires the analysis of a large number of specimens. Secondly, in view of their high toxicity, the toxins must be assayed at a level of $10^{-12}$ g. Thirdly, owing to the presence of large amounts of chemically related compounds, the methods must be highly specific.

Although several tens of studies on the immunoassay of mycotoxins have already appeared in 1976, very often only a few of the proposed methods have found practical application. This is associated as a rule either with the low quality of the antiserum obtained (small amount, low avidity, poor specificity) or with the unsatisfactory reproducibility of the method.

However, the interest in the development of immunoassay methods for mycotoxins has not weakened owing to the large losses of food products due to their contamination by myxotoxins. According to data produced by Biotech Research Company (USA), about 50 million dollars were spent in 1985 on tests for the presence of aflatoxins in food products alone. [129]

There are at present a series of methods for the immunoassay of mycotoxins. [130, 131]

Among the large number of mycotoxins, aflatoxins, which are secondary metabolites produced by *A.flavus* and *A.parasiticus* species, constitute a special danger. [132] Several immunoassay methods for aflatoxins have been developed. [132–136]




**28, 29**

**Aflatoxin $B_1$ 28** (R = H). This mycotoxin is one of the most carcinogenic among substances known at the present time. It is produced by several *Aspergillus* species. It has been shown that compound **28** causes cirrhosis and cancer of the liver in children. When it enters the organism, aflatoxin $B_1$ is epoxidised by cytochrome P-450 and binds to RNA, which leads to the inhibition of protein synthesis. $LD_{50}$ for compound **28** is $3 \times 10^{-4} - 1.02 \times 10^{-2}$ g (kg bodyweight)$^{-1}$.

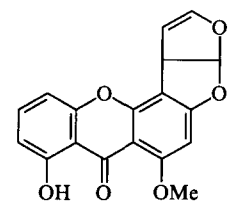
In order to obtain an antiserum aminooxoacetic acid was attached to the keto-group in compound **28** and the resulting derivative was conjugated with BSA by the mixed anhydride method. Polyclonal [138] and monoclonal [139] antisera were obtained on the basis of this conjugate. When used in the immunoenzyme method, both antisera make it possible to attain a limit of determination of $10^{-13}$ g. They are used to monitor the content of compound **28** in grain and in other food products, for example in groundnut oil. [138, 139]

In another method, the hemiacetal of compound **28** was conjugated by the Mannich reaction with methylalbumin either indirectly or via ε-aminocaproic acid. [140] An immunoenzyme method was developed on the basis of antisera to these conjugates. It makes it possible to determine compound **28** and aflatoxin $M_1$ **29** (R = OH) in the range $10^{-12} - 2 \times 10^{-10}$ g, aflatoxins $P_1$ in the range $10^{-11} - 2 \times 10^{-10}$ g, and aflatoxins $G_1$ in the range $(1-2) \times 10^{-10}$ g.

Sets for the immunoenzyme assay of compound **28** are manufactured by Neogen Corporation, Agritech Systems, Immuno Systems, Biotech Research Laboratories, Biokits (USA), and May and Baker Diagnostics (Great Britain). The URVJT Company (Czechoslovakia) manufactures a set for the radioimmunoassay of compound **28**. [11, 141]

**Aflatoxin $M_1$ 29** (R = OH). This aflatoxin is a product of the metabolic oxidation of compound **28** in the cow. On passing with cow's milk into humans, it can be the cause of cancer or cirrhosis of the liver. For this reason, almost all the methods developed for the immunoassay of compound **29** are designed particularly for the analysis of cow's milk. [142–145]

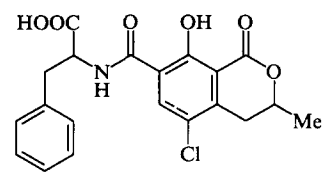
**Sterigmatocystin 30**. This substance, which is a toxic metabolite of the *Aspergillus* species, exhibits powerful hepatocarcinogenic properties. In view of the sparing solubility of compound **30** in water, a method has been developed for the assay of its hydrolysed derivative.




**30**

For this purpose, the conjugate of compound **30** with BSA was converted into a hemiacetal with which rabbits were immunised. [146, 147] The immunoenzyme method, developed on the basis of the resulting antiserum, makes it possible to determine compound **30** in the range $10^{-12} - 10^{-9}$ g.

**Ochratoxin A 31** is a powerful liver poison, frequently formed during the storage of grain in temperate climatic zones. In order to develop an immunoenzyme assay method, compound **31** was attached to protein via the carboxy-group and an antiserum was obtained, which makes it possible to detect compound **31** in amounts down to $10^{-11}$ g. A cross-reaction has been noted only for the non-toxic ochratoxin B (0.5%). [148, 149]



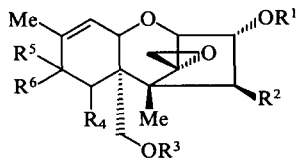

**31**

The method developed has been used in the study of specimens of the kidneys [150] and livers [151] of pigs fed with grain subjected to prolonged storage and also in the assay of compound **31** in the grain itself. [152]

The application of monoclonal antibodies made it possible to devise an immunoenzyme method for the determination of ochratoxin A in the range $5 \times 10^{-13} - 2.5 \times 10^{-10}$ g. [153, 154] Cross-reactions with the ethyl ester of compound **31** and with ochratoxin C have been noted.

Sets for the immunoenzyme assay of compound **31** are manufactured by the AFRC Food Research Institute (Great Britain) and Biokits. The URVJT Company manufactures a set for the radioimmunoassay of compound **31**. [11]

**Tricothecene mycotoxins** constitute a dangerous group of mycotoxins. They are produced mainly by the toxic fungi of the genus *Fusarium*. The most poisonous are diacetoxyscirpenol **32** ($R^1 = R^4 = R^5 = R^6 = H$, $R^2 = OAc$, $R^3 = Ac$) and the toxin T-2 **33** ($R^1 = R^4 = R^5 = H$, $R^2 = OAc$, $R^3 = Ac$, $R^6 = OCOCH_2C_4H_7$-*cyclo*). The producers of tricothecenes contaminate mainly grain, while in the animals consuming this grain the toxins induce damage to the skin, sepsis and necrosis of the mucous membranes of the intestine, kidneys, lymphatic glands, and bone marrow. In humans, the effect of tricothecenes is believed to be associated with flare-ups of the alimentary-toxic aleukia, the main symptoms of which are necrosis of the tissues of the alimentary tract and leukopenia. The reason for these infections is apparently suppression of protein biosynthesis.




**32–34**

Immune methods are used fairly widely for the assay of tricothecene mycotoxins. In the preparation of the immunogenic conjugate, compound **32** was converted into the hemiglutarate which was conjugated with BSA. The antibodies formed on immunisation of animals with this conjugate were used to develop radioimmune [155] and immunoenzyme [156, 157] methods for the assay of compound **32**.

Using similar procedures, antibodies were obtained and immunoenzyme methods were developed for the assay of compound **33** [158, 159] and 3-acetyldeoxynivalenol **34** ($R^1$ = Ac, $R^2$ = $R^3$ = H, $R^4$ = OH, $R^5$ + $R^6$ = O).[160]

## III. Conclusion

Immune methods make it possible to assay virtually any low-molecular-mass biologically active substances encountered in plants.[161] These methods are superior to the chromatographic procedures as regards sensitivity and speed of preparation of the assay sample and are hardly inferior to them as regards accuracy and reproducibility.

However, an individual approach to each specific substance is necessary for the development of immunoassay methods and in each case this requires a fairly considerable amount of preparatory work, which depends on the objects of the analysis. From this standpoint, the greatest difficulties are associated with the development of immunoassay methods for objects in which the biosynthesis or biodegradation of the substances assayed takes place, since the specimen then contains a large amount of structurally very similar compounds. Physiologically active substances of plant origin are in fact objects of this kind.

Despite the difficulties associated with the development of immunoassay methods (the main ones of which are the synthesis of the immunogenic conjugate and the preparation of highly specific antibodies), such methods are already widely used in clinical practice for the monitoring of the course of therapy and in studies on the metabolism of physiologically active substances of plant origin.

The use of immune methods in the assay of low-molecular-mass physiologically active compounds in plant cells and tissues is hindered by the presence of a large number of closely related compounds (biogenetic precursors and metabolites), which participate in intense cross-interactions with the antibodies used in the assay. However, the application of immunoassay methods is expanding to an increasing extent in biotechnology in connection with the preparation of plant cell and tissue cultures producing physiologically active substances. Immunoassay methods are also applied on an increasing scale in the cultivation of plants — in the study of the biosynthesis of physiologically active substances and in their assay in plant tissues. Methods developed for use in clinical practice, supplemented by stages involving the preliminary purification of the specimen, are frequently employed.

In recent years, the most vigorously developing field of application of immunoassay methods is the monitoring of the content of substances contaminating plant material (mycotoxins, etc.).

The appearance of automated systems for immunoassays and laboratory work suggests that the application of immune methods will expand.

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