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Immunochemical methods for the assay of herbicides of the 1,3,5-triazine group

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Abstract. The review deals with studies published during the last 10 years on the development of immunochemical methods for the assay of herbicides of the 1,3,5-triazine group. Questions concerning the optimisation of methods for the immunoenzyme assay and their application in the determination of 1,3,5-triazines in various samples (water, soil, food products etc.) are discussed. The bibliography includes 75 references.

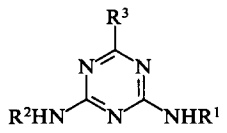
I. Introduction

The contamination of the environment as a result of man's economic activity has long become one of the contemporary global problems. An important aspect of this problem is the universal application of pesticides in agricultural practice. On the one hand, chemical protection of plants is the most radical method for combating pests, diseases, and weeds, but, on the other hand, nonrational application of pesticides can lead to their accumulation within objects in the environment and can induce the breakdown of relationships in ecosystems. For example, in the EEC member countries, norms has been established for the content of pesticides in drinking water: not more than 0.1 ng ml^{-1} for one pesticide and 0.5 ng ml^{-1} for all pesticides.[1] This is why it is important to have assay methods which permit a rapid and exact determination of residual amounts of pesticides in the soil, water, and food products. Ecological problems have in fact stimulated further development and improvement of the methods for the assay of physiologically active substances. The review deals with studies published during the last 10 years on immunochemical methods (ICM) for the assay of herbicides of the 1,3,5-triazine group within objects in the environment.

Herbicides of the 1,3,5-triazine group, used in agriculture to destroy weeds, comprise the largest group of herbicides in terms of the scale of their worldwide manufacture and consumption.[2]

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The main representatives of this group are presented in Table 1. Mainly simazine, atrazine, and prometryn are used in Russia.[3] The following limiting permissible concentrations (LPC) in water reservoirs for sanitary–domestic purposes have been adopted in our country: 0.5 mg $litre^{-1}$ for atrazine and 3.0 mg $litre^{-1}$ for prometryn; the presence of a residual amount of simazine is not allowed. Physicochemical assay methods (PCM), namely thin-layer chromatography (TLC), gas chromatography (GC) gas–liquid chromatography (GLC), and high performance liquid chromatography (HPLC), are employed traditionally for the assay of residual amounts of herbicides, including 1,3,5-triazines in air, water, soil, and food products.[4–7] These methods are irreplaceable in cases where it is necessary to identify rigorously the substance being sought, but they suffer from a number of significant disadvantages. These include their laborious nature, complex preparation of the specimen, long assay time, stringent requirements as regards the qualifications of the

Table 1. The structures of the herbicides of the 1,3,5-triazine group, their metabolites, and analogues.

Compound	R^1	R^2	R^3
Atrazine	Et	Pr^i	Cl
Simazine	Et	Et	Cl
Propazine	Pr^i	Pr^i	Cl
Terbutylazine	Et	Bu^t	Cl
Cyanazine	Et	$C(CN)Me_2$	Cl
Ametryn	Et	Pr^i	SMe
Prometryn	Pr^i	Pr^i	SMe
Terbutryn	Et	Bu^t	SMe
Aziprotryn	N_3	Bu^t	SMe
Atrazine mercaptourate	Et	Pr^i	$SCH_2CH(NHCOMe)COOH$
Hydroxyatrazine	Et	Pr^i	OH
Hydroxysimazine	Et	Et	OH
De-ethylatrazine	H	Pr^i	Cl
Deisopropylatrazine	Et	H	Cl

personnel, and the high cost of the equipment used. The technical equipment employed in physicochemical assay is being constantly improved, which increases its sensitivity and accuracy, but at the same time it also raises the cost of such equipment. It is therefore necessary to recognise that physicochemical assay methods are completely unsuitable for routine analyses and operational monitoring. The endeavour to create more effective methods for the analysis of air, soil, and food products for residual amounts of pesticides to reduce the cost of the determination has led to the development of immunochemical assay methods.

The possibility of employing immunochemical methods for the assay of pesticides in water, soil, and food products has been justified in detail in a number of reviews.[8-15] The advantages of immunochemical assay methods include specificity, sensitivity, accuracy, ease of application, high speed, the possibility of automation and use in routine analyses under field conditions, and easy preparation of the specimen (if it is required at all) which rules out the degradation of the test substance. Immunochemical methods are also characterised by a relatively low cost of the determination; for example, a single determination of the herbicide diflubenzuron by the immunoenzyme assay (IEA) method is 20 times cheaper than the determination by the HPLC method and 55 times cheaper than the determination by gas chromatography.[16] The disadvantages of the immunochemical assay methods include the following: the need for appropriate qualifications of the personnel and for the ability of antibodies to react with substances having related structures, which in many instances renders a rigorous qualitative determination of the test substance problematical. At the present time, immunochemical assay methods have been firmly introduced into analytical practice and are used in various branches of medicine, agriculture, and the microbiological and food industries and also for the monitoring of the environment.

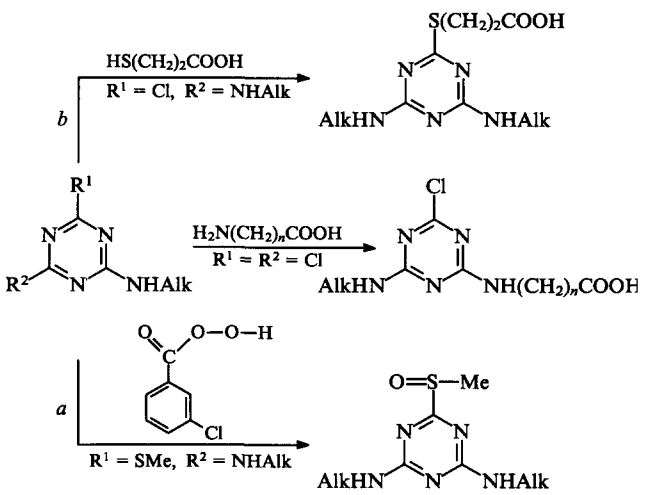
II. Principal components of the immunochemical assay methods

The immunochemical assay methods are based on the specific binding of a particular compound to the corresponding antibodies. The complex form is detected by introducing a label (enzyme, fluorescence) into one of the initial components of the reaction system. If one of the components of the system is bound chemically (covalently) or by sorption to a solid carrier in order to separate a specific complex from the components of the reaction mixture, then the methods are referred to as heterogeneous. Otherwise, if there is no need to separate the components of the immunochemical reaction, the methods are called homogeneous.

Numerous studies devoted to the development and different aspects of the optimisation of immunochemical methods for the assay of herbicides of the 1,3,5-triazine group in objects within the environment and in food products have been published in recent years. The vast majority of the investigations are devoted to methods for the solid-phase immunoenzyme assay — ELISA (enzyme-linked immunosorbent assay). In order to carry out such an assay, it is necessary to have specific antibodies, a conjugate of the substance to be determined with a protein or an enzyme, as well as a solid phase on which one of the components of the immunochemical complex is adsorbed.

The principal components in any immunochemical assay method are specific antibodies — polyclonal and monoclonal. Like all pesticides, 1,3,5-triazines are haptens, i.e. they are incapable of inducing the production of antibodies when introduced into the organism and therefore, in order to obtain antibodies, they are conjugated with proteins. In the 1,3,5-triazine molecule there are no suitable reaction groups for binding to a high-molecular-mass carrier (a protein or an enzyme), so that investigators have to resort to the modification of the herbicide. The questions concerning the selection of the correct structure of the hapten in the synthesis of the pesticide immunogens for the successful production of antibodies have been described in detail.[16] We shall consider the procedures for the modification of 1,3,5-triazines. A carboxy-group is usually introduced into the *N*-alkyl side chain or in the 2-position in the trazine ring (see the Scheme below).

Scheme



2,4-Dichloro-1,3,5-triazines are conjugated to unbranched aminoacids with a terminal amino-group (the 2,4-dichloro-derivatives can be used immediately for conjugation).[17-19] 2-Methylthio-1,3,5-triazines are modified by oxidising them to the sulfoxides by 3-chloroperbenzoic acid (pathway *a*),[18,20-22] while 2-chloro-1,3,5-triazines are conjugated with 3-thiopropionic acid (pathway *b*). The reasons for using the latter modification method are as follows: the sulfur atom is most suitable for the simulation of a chlorine atom as it contains, like chlorine, two lone pairs; the resonance interaction between the sulfur atom and the triazine ring is limited; the atomic radii of sulfur and chlorine are similar; proteins do not add to sulfur just as they do not add to oxygen or nitrogen: the nucleophilicity of the thiolate anion facilitates the substitution of chlorine; finally, all the reagents can be synthesised.[17]

Table 2 presents the structures of the modified 1,3,5-triazines which were used, after conjugation with proteins, to obtain polyclonal antisera and monoclonal antibodies. The table shows that the polyclonal antisera and the monoclonal antibodies exhibit an appreciable percentage of cross-reaction with structurally similar herbicides. Furthermore, one may note the following features: an appreciable percentage of cross-reaction with atrazine is observed for antisera against a simazine derivative (hapten **2**); the antibodies obtained against atrazine derivatives (hapten **3**) exhibit an appreciable percentage of cross-reaction with propazine. The antibodies obtained to the conjugates of 1,3,5-triazines with 3-thiopropionic acid (haptens **9** and **10**) recognise also 2-methylthio-derivatives of herbicides (for example, ametryn and prometryn). In certain cases, the specificity of the antibodies obtained was unexpected.[18,19] Both homologous and heterologous haptens may be used for the synthesis of conjugates with an enzyme and a protein and also in analysis. We recall that, if a single hapten is used to obtain the immunogen and the conjugate and the method used to achieve its addition to the high-molecular-mass carrier is the same, then such a system is referred to as homologous. If, however, the haptens are different in the two cases the system is heterologous. The structure of the hapten used for conjugation to high-molecular-mass carriers can then exert an appreciable influence on the sensitivity of the assay. As will be seen below, heterologous systems are more sensitive than homologous ones.[17,23-26]

The conjugation of the modified carboxylated 1,3,5-triazine with the amino-groups of a protein or an enzyme to form a peptide linkage is carried out by the traditional methods used in protein

Table 2. The structures of the modified 1,3,5-triazines, the methods for the conjugation of derivatives with a carboxy-group, and the characteristics of the specificity of the monoclonal antibodies and polyclonal antisera obtained.

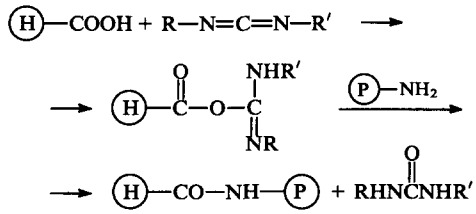
Compound	R^1	R^2	R^3	Method of conjugation with protein	Antibodies	Herbicide	Cross reaction (%)	Ref.
1	Pr^i	Cl	Cl	–	mAb	aziprotryn	100	18
						propazine	70	
					mAb	terbutylazine	100	19
						atrazine	7	
2	Et	$(CH_2)_nCOOH$ $n = 1-5$	Cl	AE	pAb	simazine	100	17, 23
						propazine	180	
						atrazine	250	
						hapten **2**	1830	
				CD (CMC)	mAb	atrazine	100	39
						propazine	136	
						cyanazine	28	
3	Pr^i	$(CH_2)_nCOOH$ $n = 1-5$	Cl	AE	pAb	atrazine	100	17, 23
						hapten **3**	700	
						propazine	260	
						simazine	12	
				AE	mAb	–	–	24
				MA	pAb	atrazine	100	28
						propazine	87	
						simazine	10	
				AE	pAb	atrazine	100	42
						propazine	90	
				CD (CMC, DCC)	pAb	atrazine	100	47
						propazine	195	
						simazine	20	
				CD (CMC)	pAb	atrazine	100	68
						propazine	116	
						prometryn	10	
				CD (CMC)	pAb	–	–	75
4	Et	$(CH_2)_2COOH$	OH	AE	pAb	hydroxysimazine	100	25
						hapten **3**	96	
						hydroxyatrazine	38	
5	Pr^i	$(CH_2)_5COOH$	OH	AE	mAb	hydroxyatrazine	100	42
						hydroxypropazine	100, 83[a]	
6	H	$(CH_2)_5COOH$	Cl	CD(CMC)	pAb	de-ethylatrazine	100	40
						deisopropylatrazine	100	
7	Pr^i	Et	SOMe	–	mAb	terbutryn	100	18
						terbutylazine	64	
				–	mAb	prometryn	100	18
						ametryn	25	
				–	pAb	atrazine	100	22
						ametryn	106	
						propazine	57	
8	Et	Bu^t	SOMe	–	pAb	terbutryn	100	21
						hydroxyterbutryn	78	
						terbutylazine	64	
9	Pr^i	Et	$S(CH_2)_2COOH$	AE	pAb	ametryn	100	17, 23
						hapten **9**	200, 670[a]	
				AE	mAb	–	–	24
				AE	pAb	–	–	72
10	Et	Et	$S(CH_2)_2COOH$	AE	pAb	simetryn	100	17, 23
						hapten **10**	1050, 157[a]	
11	Pr^i	$C(CN)Me_2$	$S(CH_2)_2COOH$	MA	pAb	cyanazine	100	29
						terbutylazine	3	
12	$(CH_2)_3COOH$	$C(CN)Me_2$	Cl	–	pAb	–	–	48, 49

Note. Notation adopted: pAb = polyclonal antisera, mAb = monoclonal antibodies, DCC = dicyclohexylcarbodiimide, CMC = 1-cyclohexyl-3-(2-*N'*-methylmorpholinoethyl)carbodiimide toluene-*p*-sulfonate, AE = activated esters method, CD = carbodiimide method, MA = mixed anhydride method.

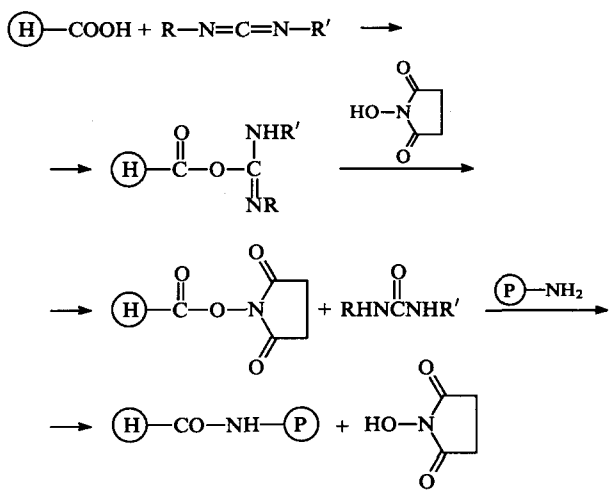
[a] The values for different antibodies or the values obtained by different methods for the determination of specificity are quoted.

chemistry. Among these we may specify the carbodiimide method, the activated esters method (the *N*-hydroxysuccinimide ester is used),[27] and the mixed anhydride method employing isobutyl chloroformate.[28,29]

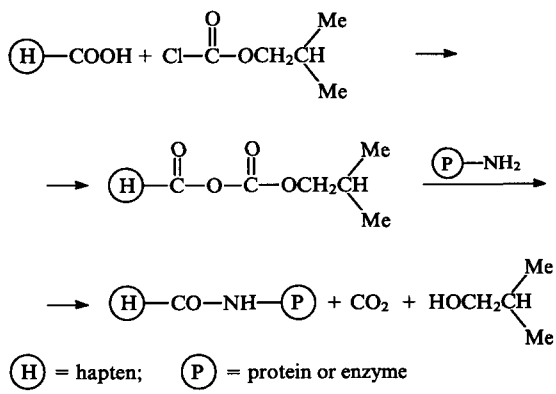
Carbodiimide method



Activated esters method



Mixed anhydride method



(H) = hapten; (P) = protein or enzyme

In most of the studies on the development of ELISA methods for 1,3,5-triazines, 96-well polystyrene trays are used. A number of investigators have also reported the employment of polystyrene test tubes,[30-37] spheres,[20-22] and magnetic particles.[29,38] The methods used to carry out the competitive enzyme-linked immunosorbent assay (formats) are also different (Fig. 1). Format I is usually employed for the screening of the antibodies obtained and is distinguished by a large number of stages. One of the modifications of format III involves preliminary adsorption of the antispecies antibodies or the protein A on the solid phase. It has been noted that an advantage of formats II and III over format I is a higher sensitivity and the fact that less time is required for the assay,[24-26,39] but they are more sensitive to the effect of the matrix and solvents.[24] The studies concerned with the comparative assessments of different forms of the ELISA method will be examined in greater detail below.

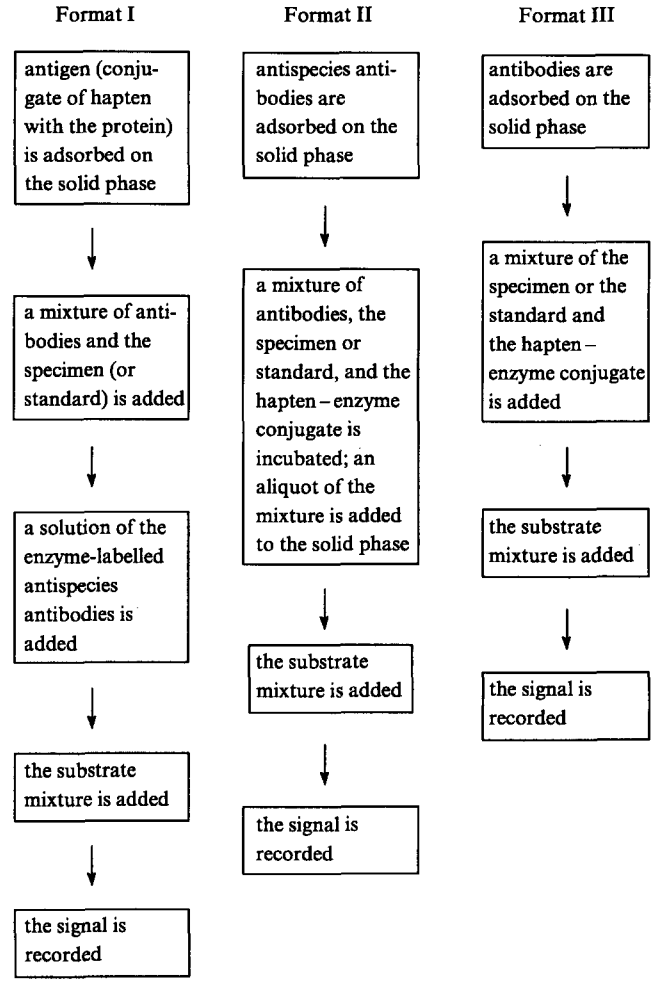


Figure 1. The ELISA formats used in the assay of haptens. After incubation, the unreacted components are washed off in each stage with a wash buffer containing clean Tween 20. Modification of format III: IIIa—antispecies antibodies are adsorbed on the solid phase in the first stage; IIIb—a protein A is adsorbed on the solid phase in the first stage.

A survey of published literature shows that 1,3,5-triazines are usually assayed by the ELISA method in water and soil although there have been reports also of other objects of study (Tables 3 and 4). Water is used for analysis without preliminary preparation, since its matrix does not as a rule affect the results of the assay. The data obtained by the ELISA and gas-chromatographic methods,[24,29,39-41] by the HPLC method,[26,30,36,37,42-44] and by gas chromatograph-linked mass spectrometry (GC/MS) [35] are satisfactorily correlated and the ELISA method does not afford any false negative results. A correlation of the results with HPLC data in the assay of liquid food products has been reported.[31] Herbicides from the soil, solid food products, and other objects are extracted with organic solvents or water. The results of such measurements are also satisfactorily correlated with physicochemical analytical data (Table 4).

III. Solid-phase immunoenzyme assay using polyclonal antisera

1. Determination of 1,3,5-triazines in objects within the environment

Among 1,3,5-triazines, most attention is devoted to atrazine, this being the most common herbicide of the given group used abroad, and its metabolites (Table 3). Assay methods developed on the basis of polyclonal antisera have been described in a series of publications.

Table 3. Characteristic of ELISA for 1,3,5-triazines.

Substance assayed	Detection limit/ng ml^{-1}	Samples	ELISA format[a]	Solid phase	Antibodies	Ref.
Atrazine	0.01	water	III	assay tray	pAb	20, 22
	0.1	"	III	spheres	pAb	20, 22
	0.05	water, soil	I, II, **IIIa**	assay tray	mAb	24
	0.5 – 10	water, soil	III	test tubes	pAb	30, 31
		food products			pAb	36, 37
	0.2 – 6.4	milk	III	"	pAb	32
	20 – 1000	soil	III	"	pAb	33, 34
	0.05	water		magnetic particles		38
	0.03	water, soil	II, III, **IIIa, IIIb**	assay tray	mAb	39
	0.05	" "	I	" "	mAb	42
	1 – 10	water, soil, algae	I	"	pAb	43
	1[b]	soil	III	" "	pAb	52
		water	**II**, IIIa	" "	mAb	55
	0.03 – 1	" "	IIIa	" "	mAb	58
	0.01 – 10	" "	I	" "	pAb	75
Deisopropylatrazine	0.01	" "	III	" "	pAb	40
De-ethylatrazine	0.01	" "	III	" "	pAb	40
	2[b]	soil	III	" "	pAb	52
Hydroxyatrazine	0.1	water, soil, manure, fungal micro-organisms	I, II, **IIIa**	" "	pAb	25
	0.1[c]	water, soil	I	" "	mAb	42
Hydroxysimazine	0.1[c]	water, soil, manure fungal micro-organisms	I, II, **IIIa**	" "	pAb	25
		soil	**II**, IIIa	" "	mAb	55
Atrazine mercaptourate	0.5	urine	IIIa	" "	mAb	57
Simazine		water, soil	I, II, **IIIa**	" "	mAb	24
		soil	**II**, IIIa	" "	mAb	55
Cyanazine	0.035	water	III	magnetic particles	pAb	29
	3.5	soil	III	" "	pAb	29
	0.5	water	I	assay tray	pAb	48, 49
	10	soil	I	" "	pAb	48, 49
Terbutryn	25 – 1500	water	III	" "	pAb	21
	0.1 – 600	" "	III	spheres	pAb	21
Atrazine, simazine, cyanazine	1[c]	" "	I, II, **IIIa**	assay tray	mAb	26

[a] The formats used for the assay are specified.
[b] The detection limit is expressed in μg kg^{-1}.
[c] The detection limit is expressed in μmol litre^{-1}.

The first studies on the ELISA of atrazine were published in 1985.[20,45] The radioimmunoassay method was used to characterise the antiserum described in a patent.[45] The conjugate of the hapten with the methyl ester of tyrosine, which was suitable for the introduction of the ^{125}I label, was synthesised by the mixed anhydride method; the atrazine detection limit was 1 ng in the sample. An ELISA with a lower atrazine detection limit of 0.1 ng ml^{-1} was developed later.[28] In assay by format I of ELISA use was made of secondary antibodies conjugated with biotin which interacted with the streptavidin – horseradish peroxidase conjugate in the following stage.

Polystyrene trays and spheres were used in the development of a homologous ELISA for atrazine.[20,22] Purification of the polyclonal antiserum by immunoaffinity chromatography made it possible to lower the detection limit by a factor of 100. It was noted that, when spheres are used as the solid phase, the assay is less sensitive (Table 3). The characteristic features of this procedure are the different ratio between the volume of the specimen and the area of the solid phase, a possible increase in sensitivity with increase in the volume of the specimen only within a limited range (a large volume of the specimen is then required for the assay), a long assay time (5 h), and difficulties in mechanisation and automation. The same procedure has been used in the development of a homologous ELISA for terbutryn.[21] The terbutryn detection limit was improved by a factor of 250 using polystyrene spheres as the solid phase.[46] A similar range of measurable concentrations was obtained using ^{14}C-labelled terbutryn in radioimmunoassay.[46]

Wittmann and Hock[47] used different carbodiimides to synthesise the immunogen: 1,3-dicyclohexylcarbodiimide (DCC), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), and CMC as well as various hapten : protein and carbodiimide : hapten ratios. A number of the immunogens proved to be insoluble (especially when EDC was used). An immunogen having the hapten : protein = 4 : 1 composition was used for immunisation in the initial stage (hapten : protein = 200 : 1 and DCC : hapten = 1 : 2), but, since the titres of the antisera were low, an immunogen with the maximum number of attached molecules (hapten : protein = 35 : 1; reactant ratios — hapten : protein = 100 : 1, and DCC : hapten = 1 : 1) was employed for subsequent immunisation. Also synthesised were homologous conjugates with enzymes such as horseradish peroxidase (by the activated esters method) and alkaline phosphatase (using CMC). The hapten : enzyme and hapten : carbodiimide ratios were also varied in the synthesis. The conjugate with peroxidase exhibited a higher sensitivity of assay and was used for subsequent

Table 4. The extractants and the procedures used in the preparation of the specimen for the assay of 1,3,5-triazines by the ELISA method.

Specimen analysed	Extractant and procedure for preparation of specimen [a]	PCM for correlation	Ref.	Specimen analysed	Extractant and procedures for preparation of specimen [a]	PCM for correlation	Ref.
Soil	water, aqueous methanol, dilution with buffer	GC	23	soil	water, tenfold dilution	–	52
	ethyl acetate; acetonitrile : water (9 : 1), evaporation reconstitution with buffer	GLC	24		ethyl acetate, evaporation and dilution with buffer	CC [b]	55
	acetonitrile, evaporation to dryness, reconstitution with buffer	–	25		water : methanol (3 : 2), dilution with buffer	GC	56
	methanol : water (3 : 1), 50-fold dilution with buffer	–	29	manure	methanol : ethyl acetate (1 : 1), evaporation, reconstitution with buffer	–	25
	aceonitrile : water (9 : 1), evaporation to dryness, reconstitution with buffer	HPLC	30	fungal microorganisms	methanol : water (1 : 10)	–	25
	water; methanol; water : methanol (3 : 2); acetonitrile, dilution with buffer	GC	34	solid food products (maize, pineapple)	acetonitrile : water (9 : 1), evaporation to dryness, reconstitution with buffer	HPLC	31
	methanol : water (4 : 1), 40-fold dilution with buffer	GLC, HPLC	42	algae	organic solvents, dilution with buffer to 1%	HPLC	43
	water, electro-ultrafiltration	GC	43	urine	chloroform, evaporation to dryness, reconstitution with buffer	–	57
	methanol : water (4 : 1), 0.5 M ammonium acetate, acetonitrile : water (9 : 1), dilution of organic-extracts with buffer	–	51				

[a] The extractants used for the assay are specified.
[b] Probably column chromatography (Ed. of Translation).

optimisation. The method developed was applied to the analysis of water, soil, and algae.[43] In the last case, an ecosystem model with algae and organisms into which specific amounts of atrazine had been introduced was employed. The authors note that the method is fast and convenient, but appreciable cross-reactivity with propazine is one of its disadvantages. Three types of ELISA, based on various combinations of two antisera obtained against immunogens of different structure, and two enzyme conjugates, were subsequently assessed.[41] In the most sensitive assay, the cross-reactivity was 195% for propazine and 20% for simazine.

The use of magnetic particles as the solid phase in the development of ELISA for atrazine has been reported.[38] The degree of cross-reaction was 171% for ametryn, 132% for propazine, and 105% for prometryn. It was shown that the presence of various salts, metal ions, or a change in pH from 2 to 12 do not affect the results of the analysis of water specimens. The authors note the advantages of the assay developed: the assay time is 45 min and there are no problems with the coverage of the solid phase by antibodies (the antibodies are attached to magnetic particles via glutaraldehyde).

Investigators of the Shell Development Company obtained a highly specific antiserum to cyanazine which was used to develop the ELISA.[48,49] Cross-reaction was demonstrated only for cyanazine metabolites. The method was used to monitor water and soil and the analytical results were well correlated with gas-chromatographic data. The ELISA for cyanazine in water and soil on magnetic particles has been developed[29] by the method of Rubio et al.[38] A high specificity of the method for cyanazine and tolerance towards the matrix as well as sensitivity to changes in pH and different components of the medium (organic solvents, salts) were noted.

In Western countries, some firms already manufacture kits of immunochemical reagents for the assay of 1,3,5-triazines,[16] for example: Res-I-Mune™, Res-I-Quant™ ("Immunosystems"); Triazin-ELISA ('Riedel-de-Haen'); RaPID Assays® ('Ohmicron'); EnviroGard™, Triazine Test Kit ('Millipore'). Studies have been published where such kits have already been applied successfully. Bushway and coworkers developed an ELISA for atrazine in water,[30,36,37] soil,[30] liquid and solid food products,[31] and milk[32] using Res-I-Mune™, which incorporates polystyrene test tubes coated with polyclonal antibodies and all the necessary reagents. In one assay, it is possible to analyse eight specimens and the assay time is 15 min. The study of a large number of water, soil, food products, and milk specimens containing both natural and introduced herbicides demonstrated the high accuracy and satisfactory reproducibility of the results. In the analysis of food products, positive results were obtained for

two specimens. However, the HPLC method demonstrated the absence of herbicides from these samples. In the analysis of milk specimens, the authors noted that unduly low results are obtained for samples with a high fat content. They put forward a hypothesis according to which atrazine dissolves partly in the fat and becomes inaccessible to the antibodies. The authors divided milk into three groups in terms of its fat content: > 3.5%, 1.5%–3.5%, and < 0.5% and proposed that the milk specimens should be diluted to give a fat level of the order of 1%, although this lowers the detection limit. The cross-reactions of polyclonal antibodies have been investigated[32] and it has been observed that ametryn, propazine and simazine give rise to the highest percentage of cross reactions. In view of the simplicity and rapidity of the proposed method, the authors suggest that it be used also for assay under field conditions. It has shown that the assay is relatively insensitive to changes in pH, temperature, and the concentrations of calcium, sodium, and nitrate ions.[50]

An attempt to apply the Res-I-Mune™ kit to the assay of atrazine in the soil has been described.[33,34] It has been reported[33] that coextracted substances interfere with the determination. When extraction with water was used, atrazine was determined at concentrations of 0.5–5 ng ml^{-1}, while in the case where methanol was used as the solvent the concentration range was 5–20 ng ml^{-1}. Various solvents for extraction have been investigated (Table 4).[34] A water–methanol mixture and methanol were selected taking into account the ability of the solvents to extract the herbicide. However, the authors abandoned the use of methanol, since it affected the results of the assay at atrazine concentrations less than 50 ng ml^{-1}. The authors note that this assay is suitable for use in fields subjected to a known chemical treatment. The same kit has been used to analyse water specimens.[35]

A commercial kit for the immunoenzyme assay of atrazine on Res-I-Quant™ 96-well trays has been described.[51] The authors tested two different methods and three solvents (Table 4) in the extraction of atrazine from the soil. The presence of organic solvents begins to affect the results of the assay at concentrations greater than 3.5%. It was shown that the best solvent as regards the percentage detection of the herbicide is acetonitrile.

The metabolites of 1,3,5-triazines have not been neglected either. A study has been devoted to the development of a homologous ELISA for deisopropyl- and de-ethyl-atrazine.[40] The calibration curves for both compounds virtually coincided. The method using a conjugate of the hapten to horseradish peroxidase proved to be more sensitive than the one employing a conjugate with alkaline phosphatase. The method developed for the assay of atrazine[43] and of its principal metabolite (de-ethyl-atrazine)[40] has been used to study the degradation of atrazine in the soil in the course of a year.[52] It was suggested that a simplified scheme be used for the extraction of the herbicide with water, which makes it possible to achieve its extraction from the soil at the level of 1/3–2/3 of the extraction with methanol. The ELISA for de-ethylatrazine has been used successfully in the analysis of water specimens.[44]

The application of ELISA for the determination of hydroxysimazine and hydroxyatrazine in various media (water, soil, and manure) and for the investigation of the degradation of atrazine by micro-organisms resistant to UV light has been demonstrated.[25] For use in the assay, haptens **2** ($n = 2$), **4**, and **5**, were conjugated to bovine serum albumin (BSA) and alkaline phosphatase by the activated esters method. The attempt to develop homologous and heterologous (conjugate based on hapten **5**) systems for hydroxysimazine was unsuccessful for all formats of ELISA. An unexpectedly high sensitivity was achieved using the enzyme conjugate based on hapten **2** ($n = 2$). The study of the antisera obtained by means of this system showed that three of the antisera recognise effectively hydroxysimazine and one recognises hydroxyatrazine. By using different modifications of format III, it was established that the direct coating of the tray with antibodies reduces the sensitivity by a factor of 2–5. The antiserum responding to both metabolites was selected to develop assay methods. It was shown that different salts and metal ions over a wide range of concentrations, pH (5–9), and matrices do not affect the results in the analysis of water specimens. It was suggested that this method be used to investigate ecosystems as regards their tolerance and ability to degrade xenobiotics.

Interesting data on two interlaboratory tests have been published.[53] For the first test, ten water specimens of different composition were sent to thirteen laboratories. Each laboratory analysed the specimens by the ELISA method, and the results were confirmed by GC/MS data. It was reported that only some laboratories were able to determine the correct amount of atrazine and none of the laboratories were able to determine correctly the total amount of 1,3,5-triazines. For the second test, six water specimens of different composition were analysed in twelve laboratories with the aid of the three ELISA procedures which were most successful in the first test. These results were also compared with gas chromatographic and GC/MS data and showed that among the three ELISA methods, one determines atrazine specifically, while the others determine the total amount of 1,3,5-triazines in the sample. Some useful comments were made: (1) it is necessary to certify immunochemical laboratories (the personnel working in these laboratories must be highly qualified); (2) it is necessary to describe the assay procedure in detail; (3) the working concen-tration range must be close to the middle of the calibration curve; (4) at least 6 standard solutions must be employed to plot the calibration curve; (5) if the matrix is unknown [?known (Ed. of Translation)], the substances analysed should be introduced into it; (6) four replicate measurements must be made for each specimen in order to avoid erroneous results.

2. The influence of the structure of the immunoreagents and of the format of the analysis on the sensitivity of the determination

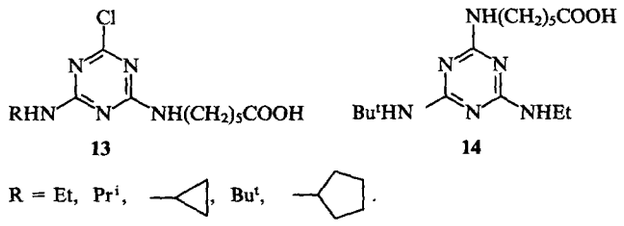
It is seen from the foregoing that homologous systems are used as a rule in the development of ELISA. Hammock's group carried out a fundamental study designed to develop and optimise the ELISA methods for herbicides of the 1,3,5-triazine class and to determine the influence of the structure of the immunoreagents on the parameters of the analysis.[17,23] Two types of atrazine and simazine derivatives were synthesised and characterised: (a) haptens **9** and **10**; (b) haptens **2** and **3** with different lengths of the spacer, ranging from $n = 1$ to $n = 5$. Conjugates of haptens **2**, **3**, **9**, and **10** as well as dichlorosimazine and dichloroatrazine with BSA, conalbumin (CONA) and tyroglobulin (TG) were synthesised. Since problems concerning the solubility of the conjugates with TG and CONA arose, those with BSA were used as antigens in the analysis.

During the screening of the antibodies, the authors[17,23] noted the following features: the maximum binding was observed for all the antibodies in homologous systems, the majority of the antibodies exhibiting appreciable binding to at least one heterologous antigen. When immunogens based on atrazine were used, the sera were obtained with higher titres than in the case when simazine derivatives were used. For the former, high titres were observed in many instances when a heterologous antigen was applied, while in the latter this happened in only one case. Next, the assay was optimised for antisera with the highest titre in relation to a large number of heterologous systems using atrazine and simazine as inhibitors. It was shown that homologous systems are least sensitive. In a study of the conjugation site, of the length of the 'foot' (the length of the alkyl chain), and of the changes in the *N*-alkyl groups, it was observed that the sensitivity depends to the greatest extent on the conjugation site, although this rule has not always been observed. For example, the highest sensitivity of the antisera obtained against the protein conjugate of hapten **5** was attained using a protein conjugate of hapten **1** (in which the 'foot' was altogether absent).

In the study of the specificity of the antisera obtained, it was noted that homologous haptens induce the greatest inhibition. The authors note that the antisera can be divided into two groups: antisera obtained against protein conjugates of haptens **9** and **10**, which recognise not only atrazine but also other 2-methylthio-1,3,5-triazines, and antisera obtained against protein conjugates of haptens **2** and **3**, which effectively recognise the 2-chloro-compounds and hardly react with the 2-methylthio-derivatives. The loss of an alkyl group on substitution of a chlorine atom (methylthio-group) by a hydroxy-group led to an appreciable decrease of recognition by all the antisera.

The influence of the matrix and solvents in the sample has been examined[17,23] in the assay of water and soil specimens. It was observed that the most sensitive systems proved least resistant to the influence of the matrix and solvents in the extract. The authors note that, in the development of any assay method, it is necessary to find a compromise solution: between the most sensitive assay and the greatest tolerance to the influence of the matrix. The authors also emphasise that, in the presence of a set of well characterised antibodies and antigens of different structure, it becomes possible to determine quantitatively structurally similar substances in specimens of complex composition.

An attempt has been made[54] to follow the influence of the structure of the enzyme conjugate on the sensitivity of the assay. Conjugates with horseradish peroxidase were synthesised by the activated esters method. The haptens employed were compounds **13** with various side groups (ethyl, isopropyl, cyclopropyl, *tert*-butyl, cyclopentyl) and 2-*tert*-butylamine-4-(1-carboxypent-5-yl)amino-6-ethylamino-1,3,5-triazine **14**.



A distinct dependence of the sensitivity of the assay on the structure of the conjugates could not be found. For the antibodies obtained against the protein conjugate of hapten **3**,[47] the sensitivity decreased in the sequence (the substituents R are indicated) isopropyl > ethyl > cyclopropyl > *tert*-butyl > cyclopentyl when conjugates based on compound **13** were used. The same antibodies were scarcely bound to the conjugates obtained from compound **14**. For the antibodies to the protein conjugate of hapten **7**,[22] the greatest sensitivity was achieved when the enzyme conjugate of the hapten with an isopropyl side group was employed, while for the remaining conjugates scarcely any binding was observed.

As can be seen from the foregoing, the use of polyclonal antisera makes it possible to attain low detection limits for 1,3,5-triazines in various objects. However, the fact that the number of antisera obtained is limited and their quality (affinity, specificity) depends on the animal immunised precludes their standardisation. The last disadvantage can be avoided by using monoclonal antibodies for the assay.

IV. Solid-phase immunoenzyme assay using monoclonal antibodies

Monoclonal antibodies have found extensive application in the assay of 1,3,5-triazines. One of their advantages is the possibility of their standardisation and production in a virtually unlimited amount. Monoclonal antibodies to atrazine and its metabolite hydroxyatrazine have been described.[42] In the homologous ELISA, format I was used and the mixture of antibodies and the specimen was subjected to preliminary incubation before adding them to the tray.

Karu et al.[24] summarised the results of studies in three laboratories on the development of ELISA methods for 1,3,5-triazines using monoclonal antibodies. Three formats were investigated—I, II, and IIIa. They all exhibited approximately the same sensitivity (conjugates with hapten **3** were used), but the application of the enzyme conjugate of hapten **2** ($n = 2$) in format IIIa increased the sensitivity of the assay by a factor of 5. The study of the specificity of the antibodies obtained showed that monoclonal antibodies bind preferentially to 1,3,5-triazines containing an isopropyl group in the 4- or 6-position and a methylthio-group in the 2-position; *N*-dealkyl and hydroxy-derivatives hardly react with the antibodies. It was shown that the results of the assay of 75 water specimens by gas chromatography for atrazine and of 48 soil specimens by gas–liquid chromatography for simazine were satisfactorily correlated with ELISA results. The method was used to determine atrazine and simazine in sewage.

A whole series of studies have been carried out in which the monoclonal antibodies proposed by Karu et al. were used.[24] Lucas et al.[55] compared the advantages of two ELISA methods for simazine and atrazine using monoclonal antibodies[24] and polyclonal antisera.[17] One of the polyclonal antisera and the monoclonal antibodies were employed against the protein conjugate of hapten **9**, while another polyclonal antiserum was used against the protein conjugate of hapten **3**. Two ELISA formats were investigated (II and IIIa) using the conjugate of hapten **3** ($n = 2$) with alkaline phosphatase for the monoclonal antibodies and the conjugate of hapten **3** ($n = 3$) for polyclonal antisera. Comparison of the antibodies showed that the polyclonal antisera were more specific to atrazine than to simazine, while the monoclonal antibodies recognised both herbicides to approximately the same extent. The monoclonal antibodies showed a greater sensitivity, a weaker background, a more intense colour of the product of the enzymic reaction, and a greater resistance to the influence of the solvent on the matrix compared with the polyclonal antisera. They were therefore selected for the assay.

In the study of different assay formats, it was observed that the use of format IIIa increases the sensitivity by a factor of 10. However, format II proved to be more resistant to the influence of the matrix and gave rise to a weaker background. For example, the presence of methanol, acetonitrile, acetone up to 10%, and tetrahydrofuran up to 2.5% did not affect the results of the assay. Format II was selected for the analysis of water and soil specimens. The assay developed subsequently[55] was used successfully in the study of the leaching of simazine.[56] Soil specimens were collected from different depths after irrigation, the highest concentration of the herbicide being observed in the upper layers of the soil (0.2 m). The application of this assay method in the study of the content of atrazine and its metabolites in urine has been reported;[57] gas chromatography, mass spectrometry, and HPLC have been used to supplement and confirm the ELISA data in qualitative and quantitative determinations of the atrazine metabolites. It was shown that the main atrazine metabolites in the urine of people working with herbicides is atrazine mercaptourate. This metabolite was found at concentrations ten times greater than atrazine or its *N*-dealkyl derivatives. Atrazine mercaptourate was isolated from urine by affinity extraction using polyclonal antisera.[17,25] After monitoring for ten days the level of atrazine mercaptourate in the urine of people working with atrazine and who have received different doses of the herbicide through the skin or via the respiratory pathways, it was suggested that ELISA be used to determine atrazine mercaptourate as a biomarker of the dose received.

The influence of the ELISA format and of the structure of the enzyme conjugate on the sensitivity of the method has been analysed in detail;[39] the optimum variant was used to analyse water and soil specimens. Enzyme conjugates with horseradish peroxidase and alkaline phosphatase were synthesised for haptens **2** and **3** ($n = 2, 5$) by the activated esters method. Four formats were investigated — II, III, IIIa, and IIIb. Different

antigen : enzyme ratios were used in the synthesis of the enzyme conjugates. It was found that the sensitivity of the assay depends little on the antigen : enzyme ratio, although an increase in the amount of the hapten entailed a proportional increase in the optical density of the product of the enzymic reaction. Comparison of the enzyme conjugates showed that the use of haptens with the longest 'foot' ($n = 5$) for conjugates with horseradish peroxidase increases the sensitivity by a factor of 3–5 compared with haptens having a short 'foot' ($n = 2$). For conjugates with alkaline phosphatase, the opposite picture was obtained. In general, the conjugates with alkaline phosphatase proved inferior to the conjugates with horseradish peroxidase, since they required a smaller dilution and a longer time for the development of the colour of the product of the enzyme reaction. Ultimately the enzyme conjugate of hapten **3** with horseradish peroxidase proved to be best. The authors[39] note that this feature, found for different enzymes, is somewhat surprising and attribute it to the different molecular masses of the enzymes and hence to different dispositions of the hapten on their surfaces and to possible steric hindrance.

Comparison of different formats of the assay showed that format II was least sensitive and economical as regards the consumption of the reagent. Formats III, IIIa, and IIIb had approximately equal sensitivities but a longer time was required for format III to develop the colour of the same intensity as for formats IIIa and IIIb. In addition, the greatest scatter of the results from tray to tray was observed for this format. As regards formats IIIa and IIIb, the former was selected for the assay as being more economical. The greatest percentages of cross-reaction were observed for atrazine (100%), propazine (147%), ametryn (70%), and simazine (32%).

The preparation of monoclonal antibodies and an extremely simple semiquantitative method for the assay of atrazine on strips have been reported.[58] The duration of the assay is 20 min. The strips are coated in succession by antispecies antibodies, monoclonal antibodies, and casein. For the assay, an aliquot of the specimen assayed and the enzyme conjugate are mixed and placed on the strips for 5 min. After washing, the colour is developed. This method makes it possible to determine visually the content of atrazine in water at a concentration in excess of 0.5 ng ml^{-1}. Monoclonal antibodies have also been used in the development of ELISA. The employment of conjugates of haptens **2** and **3** with horseradish peroxidase involved hardly any differences in their calibration curves and the conjugate of hapten **3** was used for the assay.

After the use of atrazine had been prohibited in Germany, it began to be replaced universally by terbutylazine. This is the reason why a series of studies on the development of ELISA for this herbicide have appeared recently. Giersch and Hock[18] described the synthesis of homologous conjugates of terbutylazine with alkaline phosphatase involving the use of format III. Somewhat unexpected results were obtained. For antibodies synthesised against the conjugate of hapten **7**, the greatest cross-reactivity was shown by terbutryn and prometryn (the detection limits were 0.1 and 0.3 ng ml^{-1} respectively), while the antibodies to the protein conjugate **1** (Table 2) recognised aziprotryn most effectively (detection limit 1 ng ml^{-1}). The monoclonal antibodies obtained[18] were used in numerous investigations. It has been suggested[59] that dextran be employed as the high-molecular-mass carrier. When protein conjugates and the conjugate with dextran were used in format I, it was shown that the sensitivity of the determination is approximately the same in both cases. The advantages of the conjugates with dextran were noted: resistance to denaturation and resistance to high temperatures, different pH values, and salts.

The influence of the incubation time of the enzyme conjugate on the sensitivity of ELISA has been investigated. Monoclonal antibodies,[18,42] polyclonal antisera,[22,47] and format III were used. In order to obtain antibodies to the protein conjugates of haptens **3**, a homologous enzyme conjugate was employed, whilst for the remaining antibodies use was made of the conjugate of compound **14**. Conjugates with horseradish peroxidase were obtained by the activated esters method. The incubation time of the conjugate was varied from 1 min to 40 h. For the polyclonal antibodies, the variation in the position of the central point on the calibration curve was appreciable (by five orders of magnitude). The authors explained this by the presence in the polyclonal antiserum of antibody fractions with different affinities. It was noted that such a large range of measurable concentrations permits the analysis of an undiluted specimen over a wide range of concentrations solely by varying the incubation time of the enzyme conjugate. For monoclonal antibodies, the variation was much less (by about one order of magnitude). A decrease in the incubation time led to a decrease in the optical densities of the product of the enzymic reaction. It was observed that a decrease in the assay time did not diminish the cross-reactivity. For the optimised assay of atrazine based on the polyclonal antiserum, the detection limit was 2 ng $litre^{-1}$ (incubation time 1 min), whereas for monoclonal antibodies the detection limit in the assay for terbutylazine was 30 ng $litre^{-1}$ (incubation time 7 min).

Monoclonal antibodies to the protein conjugate of hapten **1** have been obtained.[19] ELISA for terbutylazine was optimised using various formats (I, modification of I by labelling monoclonal antibodies with biotin and subsequent interaction with the streptavidin–horseradish peroxidase conjugate, IIIa). A protein conjugate of hapten **2** was used in the assay involving format I, whereas for format IIIa use was made of the enzyme conjugate of hapten **2** with an isopropyl side group. It was shown that the sensitivity increased in the following series of formats: I, modified I, IIIa.

Thus the employment of monoclonal antibodies in the assay of 1,3,5-triazines make it possible as a rule to obtain a higher sensitivity of the assay compared with polyclonal antibodies. However, it is not possible to improve radically the specificity of the assay, since monoclonal antibodies also exhibit an appreciable cross-reaction with related 1,3,5-triazines. This may be associated with only slight differences between the structures of the herbicides of the given group, which as a rule involve only the alkyl substituent in the side chain.

V. Quantitative determination of several herbicides in one sample

Owing to the appreciable cross-reaction with structurally similar 1,3,5-triazines when the sample contains several related herbicides, it is impossible to determine quantitatively just one of them. We shall consider below the results of two studies in which ways of solving this problem have been proposed.

The application of a set of characterised monoclonal antibodies for the quantitative determination of atrazine, simazine, and cyanazine in a single specimen in the analysis of waste and wash liquids has been described.[26] Five monoclonal antibodies,[24] three formats [I (with preincubation of a mixture of a solution of antibodies and the specimen or standard), II, and IIIa], and various (homologous and heterologous) structures of the ELISA antigens were investigated. From the five antibodies proposed initially, three with different cross-reactivities in relation to atrazine, simazine, and cyanazine were selected. The highest sensitivity was observed for the heterologous systems. In terms of sensitivity, format II was best, while the sensitivity for format IIIa was slightly lower but the assay time was then shorter by 2 h. Initially all the antibodies were analysed by means of format I using the protein conjugates of haptens **3** and **9** with BSA, by means of format II using the enzyme conjugate of haptens **2** ($n = 2$) and **3** ($n = 2$), and by means of format IIIa using the enzyme conjugate of hapten **2** ($n = 2$). Three ELISA methods (with different antibodies) in format IIIa were used to analyse water specimens employing a conjugate of hapten **2** ($n = 2$). An approach to a quantitative determination of several herbicides was based on the assumption that the observed response and the

response measured in the immunoassay (i.e. in ELISA) of the antibodies binding to the antigens present in the sample represents an 'overall response' to all the reacting antigens. The observed response is represented by the equation

$$\text{ELISA response} = A(X_A) + B(X_B) + C(X_C) + \ldots + Z(X_Z),$$

where the ELISA response is the amount of substance assayed determined on the basis of the calibration curve for the antigen selected as the standard, A, B, C, and Z are the concentrations of different antigens, and X_A, X_B, X_C, and X_Z are the reactivity coefficients for these antigens.

In this case, the ELISA response was expressed in micromoles of atrazine, the reactivity coefficient for which was adopted as unity. The reactivity coefficients for other herbicides were determined from the slope of the plot of the linear dependence of the ELISA response on the antigen (simazine or cyanazine) concentration. In the analysis of specimens containing three herbicides, equations were derived for each ELISA:

$$\text{ELISA response 1} = A(X_{A1}) + B(X_{B1}) + C(X_{C1}),$$

$$\text{ELISA response 2} = A(X_{A2}) + B(X_{B2}) + C(X_{C2}),$$

$$\text{ELISA response 3} = A(X_{A3}) + B(X_{B3}) + C(X_{C3}),$$

where A, B, and C are the unknown concentrations of atrazine, simazine, and cyanazine and X_{Ai}, X_{Bi}, and X_{Ci} are respectively the reactivity coefficients of atrazine, simazine, and cyanazine when different antibodies are used ($i = 1-3$).

The amount of each herbicide in the sample was determined by solving three equations with three unknowns. Analysis of water specimens showed that atrazine and the total amount of herbicides are determined with a satisfactory accuracy and reproducibility, whereas the determination of simazine and cyanazine is less accurate.

The application of two antibodies with different specificities for the detection of a known pesticide in the sample has been reported.[61] A polyclonal antiserum (the cross-reactivities with respect to atrazine and terbutylazine were 100% and 4% respectively)[47] and monoclonal antibodies (the cross-reactivities for terbutylazine and atrazine were 100% and 25% respectively)[18] were used in the analysis of water specimens. In the assay of atrazine, the cross-reactivity in relation to terbutylazine (4%) was used to calculate the concentration equivalent of terbutylazine. Similar calculations were performed in the assay of terbutylazine. Next, the ratio of the smaller and higher concentration equivalents was determined for each herbicide. If the ratio was in the range 0.4–1, it was concluded that the herbicide was present in the sample. A satisfactory correlation of the results with gas-chromatographic data was obtained. Thus it was shown that the type of herbicide employed can be determined rapidly in the sample by the ELISA method.

VI. Alternative immunoassay methods

Apart from the spectrophotometric detection of the enzymic reaction product usually employed, it is possible to detect its fluorescence or luminescence.

Two versions of the immunoassay of atrazine and simazine with enhanced luminescence have been described.[62,63] In the former case, the peroxidase-labelled hapten competed with the substance assayed for the binding centres of polyclonal antibodies adsorbed on test-tubes. The test-tubes were then washed and the amount of peroxidase remaining with the antibodies was determined using the isoluminol reagent. When assay trays were used, the range of measurable concentrations was 0.01–1 ng ml^{-1}. The concentrations of the reagents were increased compared with the usual method in order to shorten the incubation time and make the assay suitable for field conditions.

Ulrich and Niessner[64] used a fluorogenic substrate for alkaline phosphatase in the development of an immunoassay for terbutylazine with fluorescence detection of the label. For a homologous assay on monoclonal antibodies,[18] the required incubation time of the substrate mixture was 20 h. Later is was possible to shorten the incubation time to 20 min by synthesising the enzyme conjugate by means of a modified method.[65] Glyme was used as the solvent for the synthesis of the enzyme conjugate, while, instead of the traditional *N*-hydroxysuccinimide, di(*N*-succinimidyl) carbonate was employed.[66]

The study of Seare et al.[67] was devoted to the application of fluorescence immunoassay (FIA) in the quantitative determination of 1,3,5-triazines. For this purpose, the hapten was conjugated with fluorophors (dansyl chloride or fluorescein).

Recently increasing attention has been devoted to versions of the immunochemical assay methods where the procedure is simplified to the maximum extent and which are most suitable for routine analyses under field conditions. The development of a semiquantitative method for the determination of atrazine on strips was described above.[58]

It has been suggested that immunobiosensors be used to assay atrazine in drinking water.[68] The immunobiosensor consists of a piezo-crystal with a vibration frequency of 10 MHz, which is coated in succession with a protein A and polyclonal antibodies. When the immunobiosensor is bound to atrazine, the mass of the crystal and hence the frequency of its vibrations changes, the effect being proportional to the content of the pesticide in water. After the removal of the antigen from the surface, such an immunobiosensor can be used for 8–9 measurements. The linear range of the measurable concentrations is 0.03–100 μg ml^{-1}.

The first description of flow-injection immunoassay for pesticide determination in water was published in 1991.[69] A polyclonal antiserum to atrazine[47] was immobilised on immunoaffine membranes. The 'stop-go' method was used when the reagents were passed through the membrane and a fluorimeter was employed to detect the product of the peroxidase oxidation of hydroxyphenylpropionic acid. The linear range of the calibration curve was 0.02–0.3 ng ml^{-1}. The reproducibility of the results was poorer than in the corresponding immunoenzyme assay method. This might have been due to the nature of the flow–injection reaction, the nonequilibrium reaction conditions, and the immobilisation method used.

The optic fibre immunosensor has been employed to assay atrazine in water.[70] The hapten was immobilised on the surface of the optic fibre via glutaraldehyde. The immunosensor was placed in a cell containing a mixture of the specimen to be analysed and monoclonal fluorescein-labelled antibodies[18] and the fluorescence was measured. In a later study,[71] it was possible to reduce the detection limit by a factor of 100 (0.01 nM atrazine concentration) by selecting the hapten for immobilisation.

An immunochromatographic method for the assay of atrazine in which ABICAP (antibody immunocolumn for analytical processes, Abion, Germany) immunochromatographic columns with a microheterogeneous medium, containing antispecies antibodies, are used, has been developed.[72] In the assay, the reagents are passed in succession through the column in the course of 10 min; the lower limit in the detection of atrazine is 0.1 ng ml^{-1}. Various detection methods (spectrophotometric, fluorescence, and chemiluminescence) are considered in the above communication.

The present authors are conducting studies designed to develop a polarisation fluorescence immunoassay (PFIA) for herbicides of the 1,3,5-triazine group and to investigate the influence of the structure of the labelled hapten on the sensitivity of the assay. The method is homogeneous, simple, and rapid. It is based on the competition between the antigen to be determined and the antigen with a fluorescence label for the limited number of binding centres of the antibody and on the measurment of the degree of polarisation of the reaction mixture. A PFIA has been developed[73] for simazine on the basis of a polyclonal antiserum with a lower detection limit of 5 ng ml^{-1}. The influence of the structure of the labelled antigen on the sensitivity of the assay has been studied.[74] It was shown that the assay employing

heterologous labelled antigen with the shortest chemical bridge is the most sensitive. The PFIA method is less sensitive than the ELISA method, but it is simpler and rapid.

One may conclude that immunochemical methods for the assay of herbicides of the 1,3,5-triazine group are used successfully in the analysis of various specimens. The results obtained by immunochemical and physicochemical analytical methods are as a rule satisfactorily correlated. The simpler and fast immunochemical methods will apparently also soon find extensive application in our country.

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