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REVIEW
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ANALYTICAL TOXICOLOGY: FROM ENVIRONMENTAL MONITORING TO RESIDUE ANALYSIS

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Analytical toxicology is more than analytical chemistry. Although it does imply much analytical work, it should not stop with a statement about the more or less accurate level of a certain chemical in some biological or ecological matrix. The toxicologist performing analytical toxicology has to make clear which conclusions can be drawn from his/her analysis, and maybe even more important, which conclusions cannot be drawn. Generally, the analytical toxicologist has to find the balance between a very reliable analysis (which is often quite laborious and expensive) and the time and money available for performing the analysis; often the two demands are contradictory. In future, particular attention has to be paid to the quality assurance of toxicological analyses, to a more international collaboration, and to the development of new analytical approaches.

Key words:
biomonitoring, feed additives, food chain, veterinary drugs

The ideal toxicologist requires a knowledge of chemistry, biology, biochemistry, physiology, pharmacology, clinical medicine, ecology, agricultural practices, and industrial practices. Nowadays it is very hard to find individual scientists having the breadth and the depth of knowledge necessary to cover all aspects of the subject, so today also the toxicologist has become a specialist. However, much like medical doctors toxicologists are firstly seen as generalists, and not as specialists. All toxicologists experience questions, not only from the general public but also from colleagues in related fields of science, about totally different fields of toxicology than the one in which they happen to be specialised. But this is of course one of the charms of being a toxicologist.

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In all toxicological problems analytical toxicology has to produce the solid data. Thus analytical toxicology has the task to detect and identify minute traces of (natural or synthetic) toxicants in the most bizarre variety of matrices: surface waters and soil, fruits and vegetables, or animal organs, sometimes in an extreme state of decomposition. Since mankind has roughly 80,000 chemicals in commercial use (Table 1), this is a huge task.

Table 1 *Estimation of the number of chemicals in use**

Number of chemicals	5,000,000
Chemicals in commerce	80,000
Industrial chemicals	72,000
Pesticides	600**
Food additives	8,700
Cosmetic ingredients	7,500
Human pharamaceuticals	3,300
Annual number of new chemicals	2,000***

* Estimated by the US-EPA, 1995; communicated by H. Koëter at the annual meeting of the Netherlands Society of Toxicology, 11 January 1996.
** 600 active compounds, 2.100 products.
*** 1.000 of which in the USA.

Chemical analysis is the basis. Since generally the quantities to be determined are at the ppm or ppb level or even lower, it is always necessary to start with an extraction and clean-up procedure which removes the bulk of the matrix. Once a "clean" extract is obtained, it is possible to apply modern techniques for separation of the particular compound from co-extracted chemicals in which one is not interested. Chromatography, gas and/or liquid, is generally the first choice. Only after the compound to be measured is separated from other ("disturbing") constituents, it is actually possible to detect the compound by e.g. atomic absorption spectrometry, mass spectrometry, or whatever.

It should be stressed that analytical toxicology can only answer to problems dealing with a well-defined toxicant. One has to know what compound should be analysed before the analysis can be performed. Hence in case of problems potentially caused by some unknown compound, as is seen particularly in forensic and related sciences, the first question to be answered is "Is it possible that some chemical is involved in this problem, and if so, what are the most likely candidates?". Exactly this is why analytical toxicology is more than just analytical chemistry: it is the task of the analytical toxicologist to decide upon the question what chemicals should be analysed. In answering this primary question he (or she) must take into consideration that (I) many biological effects may have their origin in some kind of interaction with some (or many) chemicals, but also other causes are possible and often quite likely, (II) in many cases the answer has to be given in a very short period of time, and (III) generally chemical analyses are quite costly. In addition the analytical toxicologist is frequently asked to assist

in resolving structure and identity problems, as in research on metabolism of xenobiotics where metabolites have to be identified, or in problems of environmental decomposition. Next to mass spectrometry analytical toxicology applies powerful tools like NMR and IR to solve these problems.

The essential interaction between the analytical toxicologist and other scientists involved in a particular problem can be nicely illustrated with a small example from own experience. Early 1988 we were consulted in a case of acute paralysis in suckling and weaner piglets on a pig breeder farm. At autopsy the pathologist observed characteristic lesions in the central nervous systems of these piglets, indicative of a selenium poisoning. Since selenium poisoning in pigs is very rare in The Netherlands we were reluctant in going into a large number of laborious analyses, but after bacteriological and virological investigations proved to be negative, chemical analysis was done. Indeed selenium was present in significantly enhanced levels (Table 2), and selenium poisoning was proven. The cause of this incident appeared to be a batch of feed containing an extremely large amount of selenium due to human failure (1).

Table 2 *Levels of selenium determined by chemical analysis proving selenium poisoning in piglets*

Selenium in piglets	Liver *	Kidney *	Blood **
17 days after initial paralysis	11.7 – 84.6	5.9 – 16.3	0.77 – 2.43
35 days after initial paralysis	4.2 – 11.4	7.1 – 7.7	0.21 – 0.52
Normal values	0.7 – 1.8	5.0 – 11.5	0.1 – 0.3

* Range of selenium levels, in mg per kg dry material.
** Range of selenium levels, in mg per liter.

Analytical toxicology thus has a link with most if not all branches of toxicology: environmental and biomonitoring, forensic toxicology, drug development, molecular and biochemical toxicology, occupational toxicology, clinical toxicology, and residue toxicology, to name but a few.

BIOMONITORING

Biomonitoring, the regularly repeated analysis of certain chemicals in (tissues and/or organs of) particular animal or plant species, is generally conducted to monitor the status of that species, or to take advantage of biomagnification effects to monitor more generally an ecological system. Thus, ideally biomonitoring should give early warning of environmental deterioration or impending catastrophes.

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Heavy metals and persistent organic chemicals are among the ecotoxicants for which an early indication of increasing levels are needed.

Recently we published two reports in which the barn owl (*Tyto alba guttata*) and the buzzard (*Buteo buteo*) were studied for their suitability as biomonitors (2, 3). Both bird species are widespread among habitats, are essentially territorial and non-migratory, are at the top of a food chain, occur in numbers allowing sufficient sampling, and have sufficient body mass to allow accurate analysis. The choice for birds as the objects of study was a pragmatic one, since the large number of bird-watchers in The Netherlands guaranteed an easy way of collecting large and representative samples of birds found dead.

The barn owl has a small food spectrum: in The Netherlands it consists for over 60% of the common vole (*Microtus arvalis*) and the common shrew (*Sorex araneus/coronatus*). The population density cycle of the vole has a period of 3-4 years between very high (climax) and very low (crash); in climax years the barn owl diet may consist of 70-100% voles, while in crash years the main source of food is the shrew. This dietary fluctuation has an important impact on the transfer of heavy metals to barn owls. Shrews are carnivores, feeding mainly on earthworms, insects, larvae and spiders, and particularly earthworms are known to accumulate vast amounts of cadmium and lead. In contrast, voles are herbivores feeding mainly on grasses which hardly accumulate heavy metals. Moreover, a barn owl needs at least two shrews to get the amount of food comparable to one vole. An overview is presented in Table 3.

Table 3 *Lead and cadmium in liver and kidney of barn owls, shrews and voles*

	Lead*		Cadmium*	
	Liver	Kidney	Liver	Kidney
Barn owls	< 0.64	0.94	0.55	1.09
Shrews (polluted area)**	15.9	269	200	151
Shrews (control site)**	2.2	18.2	26	29
Voles (polluted area)**	5.1	15.8	3.8	2.0
Voles (control site)**	2.8	5.9	0.12	0.19

* Median values, in mg per kg dry material.
** Data from ref. 4 and 5

The results indicate that all birds with high loads of both lead and cadmium had been found in areas contaminated with these metals, but a lot of questions still have to be solved. For example it is very intriguing how the barn owl copes with a large intake of heavy metals, particularly when its nutrient reserves are low: tissue levels of cadmium and lead of shrews from contaminated areas are hazardous to mammals (4, 5).

In contrast to the barn owl the buzzard has a broad food spectrum: small mammals, birds, reptiles and amphibians, worms and insects, and baits. This makes it a suitable instrument for investigating trends in the bioavailability of

ecocontaminants in time and space over relatively large areas, and gross effects in food webs (Table 4).

Table 4 *Heavy metals in buzzards*

	Cadmium	Copper	Lead	Manganese	Iron
Liver	1.2	15.9	1.9	11.4	2287
Kidney	5.2	14.5	1.9	5.5	819
Tibia	< 0.03	0.8	4.7	2.3	79

Median values, in mg per kg dry material.

For this type of studies analytical toxicology has the task to analyse the heavy metals in various organs, but is also involved in the scientific discussion about the meaning of the measured parameters. It appeared for example that in general the metal load of an organ (that is, the total amount of metal in the organ) correlated much better with body condition than the concentration of metal in that organ.

DRUG TOXICITY

Carbadox (methyl-3-[2-quinoxalinylmethylene]carbazate-N1,N4-dioxide; Mecadox) and related quinoxaline-type veterinary drugs were introduced in the seventies as growth promoting feed additives in pig husbandry. Carbadox has also antimicrobial activity and was used for this reason in medicated feed for the treatment and prophylaxis of postweaning diarrhoea and swine dysentery. Field observations however suggested some unwanted side-effects. The normal dosage as feed additive is 50 ppm in feed, but for therapeutic and prophylactic purposes often 100–150 ppm was applied.

Experiments showed toxic effects when carbadox was given at levels above 50 ppm: a decrease in food intake and weight gain, dry faeces, decreased abdominal volume, long, withered hair and restless behaviour. Pathological examinations revealed a picture of severe dehydration and growth retardation, extensive microscopic observations showed lesions in the cortex of the adrenal glands. Together these findings suggested some interaction of carbadox with mineralocorticoid activity. Indeed it was shown that carbadox administration at levels of 100 ppm and higher in the feed resulted in a significant decrease of plasma aldosterone levels, accompanied by a decrease of plasma sodium and an increase of plasma potassium (Figure 1), indicating an interference of carbadox with aldosterone biosynthesis or release in the zona glomerulosa of the adrenals (6). In further *in vitro* research using freshly isolated porcine adrenocortical cells we were able to confirm the inhibition of aldosterone release by carbadox (7).

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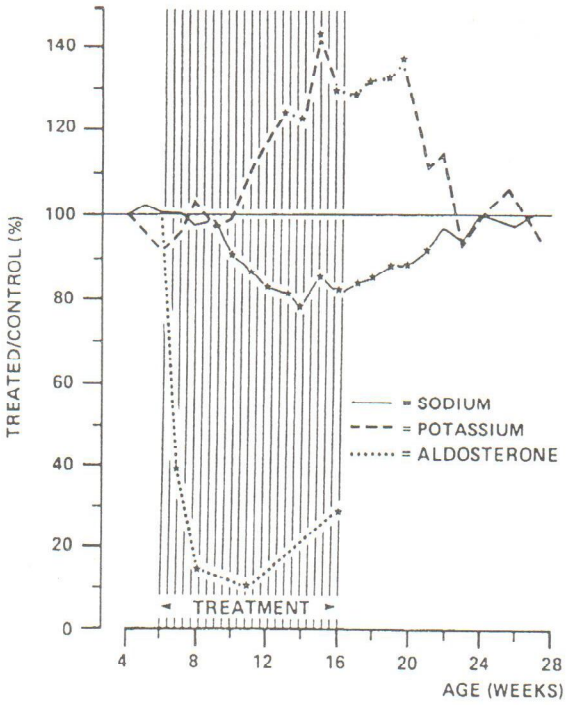


Values given are the values relative to the average values measured simultaneously in untreated animals. Significant differences between the groups (150 ppm versus 0 ppm) are indicated with an asterisk (Wilcoxon's test, $P<0.05$). The number of pigs per group varied with time due to mortality and post-mortem examination: from 1–5 weeks n=13, from 6–10 weeks n=10, from 11–15 weeks n=8, and from 16–21 weeks n=6.

100% = 136±4 mM Na^+, 6.1±0.8 mM K^+ and 1.2±0.2 nM aldosterone (mean SEM).

Figure 1 *Effect of the administration of 150 ppm (mg per kg feed) carbadox on the plasma levels of aldosterone, sodium and potassium in pigs*

Research of this type requires involvement of analytical toxicology with regard to the determinations of the various compounds involved, like aldosterone, sodium, potassium, and carbadox in plasma, tissues and organs, but also of carbadox in feed (controls had to be absolutely free of quinoxaline-type compounds). Thus the starting point of these studies was the development of an analytical method allowing accurate determination of carbadox in biological samples as well as in feed samples, which in turn required careful laboratory management because levels in feed are orders of magnitude higher than levels in organs and plasma of experimental animals, presenting great risks of cross-over contamination in the laboratory.

RESIDUE ANALYSIS

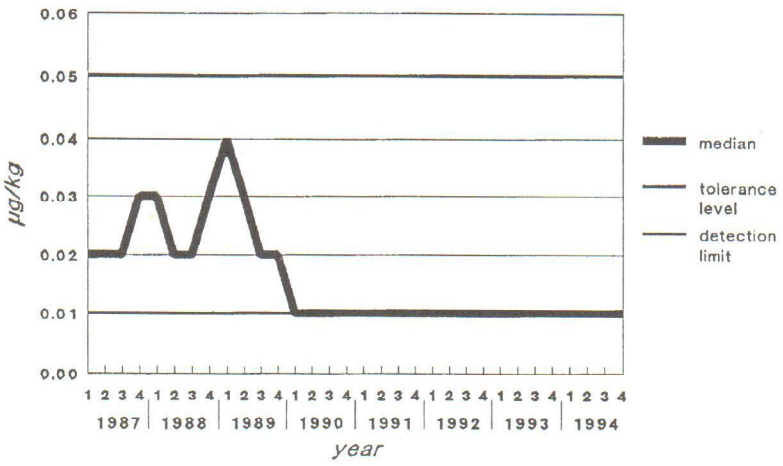
Residue toxicology and residue analysis in food production are of paramount importance in public health. Firstly, extensive toxicity testing is needed for any

agrochemical or veterinary drug before it can be allowed on the market, secondly the food producer and the government both have a responsibility with respect to the risk assessment of residues of such compounds. During the past decades maximum residue levels (MRLs) have been established for numerous compounds and products, and producers and governmental institutes have developed extensive monitoring programmes to control the quality of food products.

Table 5 *Control of illegal growth promoters according to EU-rules*

HORMONES (synthetic)	Estrogens	Diethylstilbestrol Hexestrol Dienestrol Ethinylestradiol Zeranol
	Androgens	17ß-Nortestosterone Chlorotestosterone acetaat Methyltestosterone Trenbolon
	Gestagens	Chlormadinon acetate Medroxyprogesterone acetate Melengestrol acetate Megestrol acetare
HORMONES (natural)	Estrogens Androgens	17ß-Estradiol 17ß-Testosterone Nortestosterone
BETA-AGONISTS		Clenbuterol Salbutamol Cimaterol Terbutaline Mabuterol Mepenterol Bromobuterol

With respect to controlling the use of growth promoters like b-agonists and (naturally-occurring and synthetic) steroid hormones, RIKILT-DLO is one of the two National Reference Laboratories in The Netherlands on behalf of the European Union, and the institute thus fulfils a number of statutory duties and service tasks. Samples are taken by the General Inspection Service of the Ministry of Agriculture, Nature Management and Fisheries at farmers and at cattle markets, and shipped to our institute. We have to provide an answer whether or not growth promoters can be detected, and we have to provide that answer within 48 hours (Table 5). The analyses have to fulfil rigid quality criteria as developed by the European Union. Generally we start by screening the samples using immunochemical methods, applying gas- or liquid chromatography followed by mass spectrometry to confirm the positive ones, thus presenting unequivocal evidence of identity and quantity of illegal compounds present (8-10).

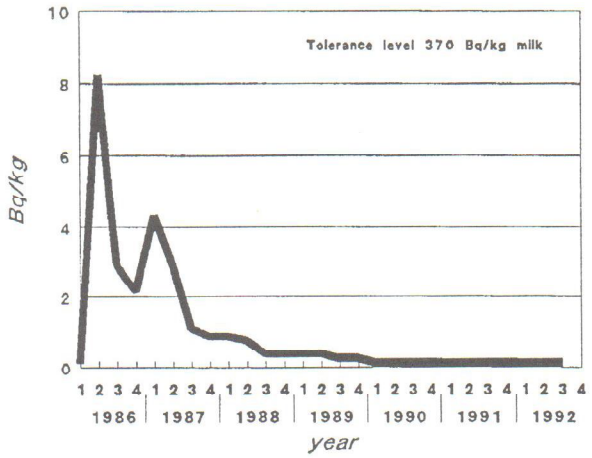


Source: Netherlands Controlling Authority for Milk and Milk Products

Figure 2 *Aflatoxin M1 in milk (mg per kg milk) during the period 1987–1994, showing that after the introduction in 1988 of a rigid quality control of feed stuff, in which the tolerance level was set at 0.05 mg per kg, the levels decreased significantly to the level of detection (0.01 mg per kg)*

The use of assays based on specific antibodies against these compounds allows for a rapid screening of many samples (several hundreds per day), thus selecting the positive ones without knowing – in that stage – the exact identity of the compounds present. We are also working to develop other principles of detecting residues and contaminants based on their biological effect (11). From an analytical-toxicological point of view this is very advantageous, because one does not immediately have to go into complicated and laborious chemical-analytical methods to identify the compounds involved.

The Dutch Quality Programme for Agricultural Products, executed by RIKILT-DLO, is a national cooperative programme of the agricultural industry and the government, and processes the results of monitoring programmes that determine the presence or absence of residues and contaminants in agricultural products (12–14). Annually it processes about 200,000 measurements, which makes its database one of the largess of its kind. A few examples will illustrate some of its activities. Figure 2 illustrates the aflatoxin M1 levels in milk during the past years. Aflatoxin B1 is a mycotoxin which can develop in feed stuff, particularly when humidity is high. Although metabolised by dairy cattle, some 2% is transferred to milk in the form of aflatoxin M1. The introduction of a rigid control for the aflatoxin contents of imported feed stuff resulted in a virtual absence of this mycotoxin in milk. Figure 3 presents radioactivity in milk by caesium-134 and caesium-137, clearly showing what happened during and after the Chernobyl disaster in April 1986.



Source: Netherlands Controlling Authority for Milk and Milk Products

Figure 3 *Caesium-134 + caesium-137 (Bq per kg milk) during the period 1986–1992. After the peak in 1986, caused by the Chernobyl disaster in April, the levels steadily decreased to virtual absence in 1990*

Producing and continuously updating a database like this requires a firm control with respect to the data produced by the various monitoring programmes, including a thorough statistical evaluation. Data retrieval to answer specific questions is possible, but also this requires specific knowledge of all analytical and statistical aspects of food chemistry and toxicology, since quite often the questions put forward are rather simple, but the answers inevitably might be quite complicated.

CONCLUDING REMARKS

The issues addressed above are no more than just a few aspects of analytical toxicology and related fields of science, and are meant to illustrate the broadness and depth of analytical toxicology without trying to cover the whole field. Clearly analytical toxicology is and will be challenged to intensify control procedures in the broadest sense of the word, performing these procedures efficiently, and guaranteeing their quality. This in turn will force European countries to intensify collaboration, to develop new analytical approaches and methods, preferably on a real-time basis, to validate these methods and to implement them into a uniform European system.

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Sažetak

ANALITIČKA TOKSIKOLOGIJA: OD NADZIRANJA PRISUTNOSTI KEMIJSKIH SUPSTANCIJA U OKOLIŠU DO MJERENJA PREOSTALIH KOLIČINA

Analitička toksikologija nije samo analitička kemija. Iako podrazumijeva mnogo analitičkog rada, ne bi se smjela ograničiti samo na utvrđivanje više ili manje točne koncentracije nekog kemijskog spoja u biološkom ili ekološkom sustavu. Na osnovi analize koju je načinio toksikolog analitičar mora izvesti odgovarajuće zaključke i, što je vjerojatno još važnije, utvrditi koji se zaključci ne mogu postaviti. To se može ilustrirati trima primjerima. Prvi je primjer u području biološkog nadziranja. Ispitivanja na pticama-grabežljivicama (sovi i škanjcu), kao mogućim modelima za biološki nadzor kvalitete okoliša, pokazali su da su različite koncentracije teških metala u organima u svezi s njihovim posebnostima u načinima hranjenja. Drugi primjer odnosi se na otkrivanje razloga neočekivanih popratnih štetnih učinaka ljekovitih spojeva u svakodnevnoj uporabi. Tako je pri dodavanju karbadoksa (kinoksalinskog spoja) krmivu u slučaju proljeva u mladih svinja uloga toksikologa analitičara bila utvrditi da su dodavane doze bile značajno više od propisanih i uzrokom ozbiljnih poremećaja funkcije hormona aldosterona sa smrtnim ishodom. U potonjem slučaju analitički toksikolog morao je biti u stanju izmjeriti različite vrste spojeva (aldosteron, natrij, kalij te karbadoks u plazmi, tkivima i u krmivu) i dobivene nalaze protumačiti na odgovarajući način. I treći primjer, nakon uporabe i prisutnosti svih mogućih vrsta kemijskih spojeva u poljoprivrednoj proizvodnji, nužne su sveobuhvatne analize preostalih količina. Općenito uzevši, toksikolog analitičar mora pronaći ravnotežu između veoma pouzdane analitičke metode (što je i naporno i skupo) i vremena i novca kojima raspolaže za izvođenje analitičkog mjerenja; dva često kontradiktorna zahtjeva. U budućnosti bi posebnu pozornost trebalo posvetiti osiguravanju kvalitete toksikoloških analiza, međunarodnoj suradnji i razvoju novih analitičkih pristupa.

Ključne riječi:
biološki monitoring, lanac prehrane, lijekovi - dodaci stočnoj hrani

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