PLATFORM SESSION 4C

ADVANCES IN RESIDUE ANALYSIS, METABOLISM AND TOXICOLOGY

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Emerging technologies in the analytical laboratory

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ABSTRACT

New technologies are continuing to change the way we do business today at an increasingly rapid rate. This is also true in the chemical, life and agricultural sciences. We are surrounded by examples of the results of these new technologies which manifest themselves in the analytical laboratory, such as (1) Higher productivity; e.g., faster turn-around times (TAT) of data and decision making; (2) Better data quality; e.g., higher sensitivity analytical methods, new method validation processes, and the ability to measure new molecules in more complex matrices with higher chemical specificity; (3) Lower fixed and variable costs; e.g., through mobile and automated instrumentation, migration to lower labor cost analyses, and computer-based intelligence; and (4) New measurement capabilities and performance.

INTRODUCTION

In the April issues of *Analytical Chemistry*, in the years shown in Table 1, papers were published that ranged from the determination of DDT in milk by volumetric titration at the parts-per-thousand (ppt) method detection limit (MDL) where the linear dynamic range (LDR) was ~ 20:1 in 1952 to the development of the Electrolytic Conductivity Detector (ELCD) which reduced the MDL to parts-per-million (ppm) and increased the LDR to 200:1 in 1972 to the advent of the micro-ECD in 1992 and subsequently to HPLC-MS which demonstrated MDL's at the parts-per-trillion (pptr) MDL with a LDR which is 5000 times greater than the 1952 method for the same analyte.

Table 1: 50 years of pesticide analysis (DDT)

Year	Technology	MDL	LDR
1952	Titration of DDT/Milk	ppt	20:1
1972	ELCD of DDT/Water	ppm	200:1
1992	μ-ECD of DDT/Soil	ppb	100,000:1
2002	HPLC-NCI/MS of DDT	pptr	100,000:1

DISCUSSION

A perspective on the impact of technology on the operation of analytical laboratories

The typical processes of the operational aspects of analytical testing, as practiced by most analytical laboratories today is entirely serial, from the development of the project plan, through funding, sampling, laboratory measurements, and onto the client(s). Similarly, they will use the results to make a decision, take an action, and/or demonstrate compliance with a regulatory requirement. Then the results are archived.

The problem with this process is that everything happens sequentially, and requires a long time to complete; esp., if you have to backup and rework one or more of the previous steps. Then the process becomes inefficient.

There is an increasing trend to migrating the business of testing today to a parallel process. This can be largely enabled by client server networks, with high speed communication links, and a change in behavior, thinking, and work-place practices. Thus all of the operations that were serial before now operate simultaneously in real-time.

This facilitates:

1. Near real-time operations and decision making,
2. Multi-dimensional, interactive decision making on work in progress, and
3. Enables a global "reach-back" capability to provide other analytical domains to be applied to the problem.

This is what Bill Gates meant when he said that computer technology is enabling us to "move at the speed of business."

Computer technologies have facilitated most of the new technologies during the last 20 years. This is illustrated by the sequential developments in microprocessors during the past 20 years, both in terms of performance and in the price/performance ratio. The last ten years have seen the advent of new hybrid processing technologies which significantly change the processes of micro-processors. These have included the Reduced Instruction Set Computing (RISC) and Explicidly Parallel Integrated Computing (EPIC).

These technologies have dramatically changed the way we work in the laboratory. For example, we have seen the speed of GC-MS (gas chromatography mass spectrometry) spectal processing and library identification improve by a factor of 60 fold between 1990 and 2003. New computing technologies will change this processing time again by another factor of 60 during the next decade as non-silicon computing technologies become available.

Another example of operational changes in laboratories today is in the productivity gains of new laboratory designs. The design of a modern environmental contract laboratory would include:

1. Ten metals instruments; from AA's (atomic adsorption spectrometry) to multiple ICP-MS's (inductively coupled plasma mass spectrometry).

2. A wet chemistry section with turbidity, pH, CN FIA (carbon/nitrogen flow injection analysis) and IC (ion chromatography) for anions and cations, etc.,

3. The pesticides area; with more than 30 fully automated dual column GC-ECD (gas chromatography with electron capture detection), GC-MSD, (gas chromatography with mass spectrometry detection) and HPLC (high performance chromatography) instruments, and

4. A semi-volatiles (S-VOC) lab with 42 GC-MSD's, 10 HPLC's, and 6 HPLC-MSD's

The chemists all work in a "central control room." In this area, chemists manage their own instrument areas completely remotely with one to two computers per person. All of the instruments are computer automated and thus can be operated without the chemist being in the laboratory. The central "control tower" also includes the laboratory manager, QA manager, shared computer peripherals, and a large central work area for interactive discussions, data interpretation and decision making.

This laboratory design was created to be a:

1. laboratory without walls,

2. laboratory without benches, and

3. laboratory without chemists.

The productivity of this laboratory is exceptional because the 106 instruments and associated samples are managed and operated by only ten people, including the lab manager and the QA manager!

Overlaying the operation of the modern analytical laboratory today are the laboratory performance and data quality requirements. Method validation, instrument qualification, laboratory accreditation and compliance, data quality, and international quality guidelines are now an integral part of laboratory operations that are meant to increase laboratory performance. Their implementation is best accomplished by integration so that they readily demonstrate compliance in a cost effective manner.

The impact of silicon-based technologies on chemical measurements

Silicon (Si) is pervasive in the analytical laboratory today (Table 2). Examples include chromatographic columns, separation media, sources and analyzers for mass spectrometers, computer systems, and hundreds of other examples. Si-based GC columns, for example continue to increase in separation efficiency because of the ability to decrease the inside diameter (to 0.05 mm) and decrease the stationary phase film thickness by selective chemical bonding to this uniquely structured surface.

Table 2: Si based technologies

Today:	CE columns, HPLC columns, GC columns,MS-analyzers, CPUs
Tomorrow:	Sample preparation • µLLE • InkJet technology • Channel plates
Tomorrow:	Analysis • µFabrication • IIP • Infomatics • Virtual instrumentation

Many new advances in this technology are currently under development which will facilitate breakthrough capabilities in the areas of micro– and nano–volume sample preparation techniques. Similarly, digital sample introduction techniques will appear in chromatography and spectroscopy based on ink jet technology, enabling the detection of attomolar concentrations. Si-based microchannel plates are already capable of migrating analytical methods from 96-well plates to plates with well volumes of 3 fL and a density of 10(7) wells/cm^2.

The next generation of analytical techniques will use technologies like micro-fabrication. Examples include the "Lab on a Chip" which is already capable of multi-channel, parallel processing of DNA and RNA samples, based on high resolution capillary electrophoresis. Other examples include entire instruments on multi-layer integrated circuit chips.

IIP is integrated information processing (IIP) on revolutionary new Si-based computer architectures which are designed by high speed specialty computing applications, such as informatics.

Virtual instrumentation is a manifestation of IIP which facilitates the remote control of measurement systems, their configuration, and measurement schema. This has significant implications for making measurements in field crops, oil exploration, counter terrorism, remote environmental sites and for globally distributed studies.

The era of nano-technologies is most promising because of the enhancements in analytical measurement performance. Examples include the analysis of neurotransmitters at zeptomole levels by capillary electrophoresis columns with 620 nm inside diameters. As the volumetric capacity decreases, the sample size decreases, and therefore detector sensitivity must increase substantially. In the example cited here, two-photon excited fluorescence was used with a laser power of 10 (12) W/cm^2. This results in the measurement detection limit of only 13,000 molecules.

Microfludics today is used for dilution, mixing, extraction, sampling, injection, on-line derivitization reactions, separations, kinetics studies, real-time binary gradient elution generation, and fast separations. These capabilities were recently demonstrated by the separation and analysis of amines in single vesicles which have a nominal volume of 65 aL to 4 fL (Zare, *et. al.*, 1998).

Other embodiments of microfluidics include a microchip-based nano-ESI interface into a TOF (Time of flight)-MS. This development results in very fast and sensitive mass spectral analysis of biological materials. Smith *et. al.* (1999) developed a microfabricated, dual-microdialysis HPLC-MS ESI (electrospray ionization) interface for the analysis of E. Coli cell lysates. This analysis classically requires days of sample preparation and fractionation and a high resolution mass spectrometer. Their micro-fluidic system reduced the analysis time to <15 minutes and gave mass accuracy, selectivity and structural identification on a bench-top mass spectrometer using MS-MS.

Mass spectrometry continues to develop through advances in nanotechnology. This is illustrated by the analysis of pesticides using HPLC-TOF/MS to achieve a mean mass measurement error of 2 ppm for a mixture of pesticides in food and agricultural samples.

An immunoassay-based biosensor system on fiber probes illustrates ultra-high selectivity for the analysis of antigens or airborne pathogens. These are microfabricated devices which makes them very portable. One recent application is the analysis in unpiloted aircraft under remote control to screen for counter bio-terrorism surveillance.

SUMMARY

All of the micro-miniaturization technologies discussed here represent significant advances in the science of chemical and biological measurement and characterization. Changes in analytical method detection limits (MDL's) as published in the reviewed scientific literature, during the last four decades have demonstrated the following:

(a) the MDL's have nominally increased three orders of magnitude every decade,

(b) there is a large range in MDL's in each decade because of the differences in measurement techniques and the sample matrices, and

(c) the MDL's improved continuously in a linear progression in each decade. The new technologies illustrated above have every promise of continuing these trends into the 21st century.

The realization of these technologies in the laboratory will follow the technology adoption life cycle, described by Gordon Moore in "Crossing the Chasm." This model says that new technologies will be adopted at different rates, by different people, in different fields of science and technology. Some technologies will never "cross the chasm". Other technologies will be applied and put into practice, but over a period of time which has a Gaussian distribution of acceptance.

Therefore, it is interesting and important to follow new technologies and to envision them as solutions to new and even more challenging problems in the future.

REFERENCES

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Uncertainty of sample processing of tomato and olive samples

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ABSTRACT

This paper discusses the importance and presents a methodology of estimating the uncertainty of sample processing. It contains the results of testing the efficiency of sample processing for olives and tomato samples using two laboratory devices and two processing conditions.

INTRODUCTION

The preparation of the analytical sample from which the analytical portion is withdrawn may consist of two distinct procedures. According to the definitions introduced by Hill and Reynolds (1999), sample preparation is the procedure used, if required, to convert the laboratory sample into the analytical sample by removal of parts (soil, stones, etc) not to be analysed. Sample processing is the procedure (e.g. cutting, grinding, mixing) used to make the analytical sample acceptably homogeneous with respect to the analyte distribution, prior to removal of the analytical portion. Sample preparation and processing shall be carried out according to the aim of the analysis. For instance, to provide data for the estimation of maximum residue limits, Codex Alimentarius Commission specifies the portion of commodities to which Maximum Residue Limits (MRLs) apply.

The method of sample preparation may be the source of substantial systematic and random errors, which cannot be estimated. To overcome this problem each laboratory must strictly follow the appropriate instructions, preferably given in the form of Standard Operating Procedures (SOPs). In the past, the effect of sample processing on the variability of the results and stability of residues gained very little attention of analysts (Ambrus, 1999). Analysts took for granted that a test portion of chopped and minced sample was sufficiently homogeneous for the purpose of analysis. Although the sample processing procedure looks simple, some preliminary results (Ambrus *et al.*, 1996) indicated that the uncertainty of sample processing can be as large as 57% and 88% (if representative test portions can be prepared at all for that small quantities) when a test portion of 5 g and 2 g of apple is analysed for pesticide residues, respectively. Since the current trend is to analyze 5-10 g analytical portions to save money and time, and to reduce waste material, the study of the efficiency of sample processing is very important.

In accordance with the new requirement of ISO 17025 Standard, and the recently approved Codex Standard (Codex, 2003) testing the efficiency (uncertainty) of sample processing shall be included in the general requirements of method validation and internal quality control of a

laboratory. However, presently there is no internationally agreed procedure for testing the efficiency of sample processing. The contact with plant fluids and enzymes during intensive cutting, mincing and grinding of sample materials may affect the stability of the analytes, which can be another source of uncertainty and bias in the measurements. Very little information is available on the effect of sample processing on the stability of the analytes present. The preliminary results indicate that some residues may substantially or completely decompose during sample processing (Fussel *et al.*, 2002; El-Bidaoui *et al.*, 2000; Hill *et al.*, 2000). The objective of the work was to study the efficiency of sample processing for tomato and olive samples using two different processing devices.

MATERIALS AND METHODS

Reagents and apparatus

Standard of chlorpyrifos-ethyl (purity >98%) was obtained from Dr Eherenstorfer. ^{14}C-Chlorpyrifos was obtained from Dow chemicals. All other solvents and chemicals used were of analytical grade from Merck. Liquid scintillation counting was carried out on a Beckmann counter, model LS 6000TA (LSC). Two different types of food processors -a "Foss Tecator" and a common kitchen aid "Braun Combimax 700", and two different processing conditions at ambient temperature and with the addition of dry-ice were tested during the study.

Methodology

To imitate the worst scenario regarding the initial inhomogeneity of residues in the sampled units, a relatively small portion of the surface of selected tomatoes (1.5 kg) was treated with a mixture of ^{14}C-labelled, and unlabelled chlorpyrifos (Maestroni *et al.*, 2000a). Since field treated crops have more uniform surface residues than the surface treatment on a small portion of the whole surface, we can assume that, during the analysis of field treated samples, the efficiency of sample processing will be equal to or better than that determined in the study. The sample was minced in a food processor, and from the thoroughly homogenised material five replicate 150 g and 15 g analytical portions (AP) were randomly withdrawn and extracted. Five replicate aliquots of each extract were analysed by liquid scintillation counting.

Principle of the methodology

Wallace and Kratochvil (1987) elaborated a method for the estimation of sampling uncertainty and studied the sampling component by adopting the concepts of sampling constants developed by Ingamells (1973). The methodology to determine the efficiency and the uncertainty of sample processing, presented in this paper, is taken from Ambrus *et al* (1996), who applied the sampling constants concept for the analysis of pesticide residues, to calculate the size of analytical portion that will hold the variability of sample processing to a specified level.

Ingamells defined a sampling constant (K_S) as the weight of a single increment that must be withdrawn from a well-mixed material to hold the relative sampling uncertainty to 1% with 68% level of confidence. K_S can be determined from the relation:

$$K_S = W \times CV_{SP}^2 \qquad \text{eq. (1)}$$

where
W = weight of a single increment (in our case it is the analytical portion)
CV^2_{SP}= uncertainty of sample processing.

According to Wallace and Kratochvil (1987), it is possible to determine with a high level of confidence whether a material is well-mixed by analyzing two sets of increments of widely differing weight ($W_{Lg}/W_{Sm} \geq 10$), where W_{Lg} is the large portion size and W_{Sm} is the small portion size. If the matrix is well mixed then:

$$K_{S(Sm)} = K_{S(Lg)} \qquad \text{eq. (2)}$$

Since the average residue concentration of the small and the large analytical portion is the same (R), the CV^2 (S^2/R^2) can be substituted with S^2 or in terms of variance (V). Rearranging the equation we obtain:

$$V_{SP\,Lg} = V_{SP\,Sm} \times \frac{W_{Sm}}{W_{Lg}} \qquad \text{eq. (3)}$$

Since the estimation of the variances based on small number of samples is not precise, we have to apply two-tail F test to decide if the two sides of equation (3) are significantly different or not. If the difference is not significant the processed analytical sample can be considered "well-mixed". The F-test should be applied at 90% or lower level of confidence since the consequences might be severe if one wrongly decides that a sample is well-mixed. The K_S can be calculated from the W_{Lg} and CV_{SPLg} (which is more precise than an estimate based on small sample increments). The K_S value (eq. 1) can then be used to calculate the uncertainty of sample processing when a different analytical portion size is taken. Since the S_{SP} can be determined only together with the random error of the analysis, the first step is to separate the analytical contributions ($S_{Analysis}$ and S_{Total}) and the effect of sample processing (S_{SP}), through equation 4:

$$S^2_{SP} = S^2_T - S^2_A \qquad \text{eq. (4)}$$

In practice, S^2_A is obtained from analysis of replicate aliquots of extracts of several analytical portions.

Method of analysis

Pre-trials were carried out to optimise the sample processing procedure by visually examining the homogeneity of the chopped material. 5 g of homogenate were withdrawn directly from the chopper, diluted in 1 l water, and mixed. The suspension was filtered through filter paper. If pieces of the processed material (e.g. peel) larger than 2-3 mm were present then the time of homogenisation was extended to get a more homogeneous material. Pre-trials were also carried out to gain experience with handling dry-ice and optimise the sample processing

procedure in the presence of dry-ice. The ratio between the total amount of dry-ice and the matrix was found to be between (1:1) up to (1.5:1).

Tomato:
In each trial 1.5 kg tomatoes were surface treated with a solution containing labelled and unlabelled pesticide to have about 0.2 mg/kg fortification level, and 1.5 *10^6 dpm/ml activity. The matrix was homogenised in a processing device, and randomly 5 times 15 g portion, 5 times 150 g portions, and 5 times 5 g portions were withdrawn and extracted with ethyl-acetate. $NaHCO_3$ was added to the sample (0.166 salt: matrix ratio), followed by ethyl acetate (1:2 sample: solvent ratio), and sodium sulphate in 1:1 w/w ratio to the sample. Extraction was carried out with ultra-turrax®, and 5 replicate measurements of 2 ml extract for each analytical portion were done by liquid scintillation counting.

Olives:
In each trial 1.5 kg de-stoned olives were surface treated with a solution containing labelled and unlabelled pesticide to have 0.2 mg/kg fortification level, and 1.5*10^6 dpm/ml activity. The matrix was homogenised in a processing device, and randomly 5 times 15 g portion and 5 times 150 g portions were withdrawn and extracted with ultra-turrax® with n-hexane. Liquid/liquid partitioning was carried out with acetonitrile, and 5 replicate measurement of 2 ml acetonitrile extract from each analytical portion were done by liquid scintillation counting.

RESULTS AND DISCUSSION

Objective of the pre-trials was the establishment of the optimal processing time as a compromise between the time required to get peels smaller than 2-3 mm, and the minimum contact time between matrix components (enzymes, organic acids, etc.) and pesticide residues.
The optimal processing time strongly depends on the matrix, and the processing equipment. Processing times of 4 minutes and 2 minutes were required for "Ccombimax 700" and "Tecator" in case of tomato samples, respectively.

Olive samples were much more difficult to homogenize with Combimax. Its processing efficiency was best at six minutes, which represented the minimum time to get the smallest possible peels size, though much larger than 2-3 mm. Olive peels were not further reduced in size by longer processing, therefore 6 minutes time was considered as a good compromise. Tecator had a better performance for the processing of olive samples, which were already well homogenised after 1 minute processing. A sampling constant value for olives was estimated in only in 3 trials out of 7. Olives were processed using "combimax" at ambient temperature, and using "tecator" at both ambient and in the presence of dry-ice. The results are presented in table 1.

In all of the experiments, a CV_A of analysis less than 2% was obtained, which shows that handling of the analytical portions, including extraction and liquid scintillation counting, was properly carried out. In two trials, OTA 1 and OTDI 1, it was not possible to estimate any sample processing error, due to the fact that $CV_{Analysis}$ and CV_{Total} were not statistically different, and therefore the two components could not be separated according to equation (4).

In other words, the processing error was negligible compared to the total error. This result showed that "Tecator" was very efficient for the processing of olives samples. This is further justified by observing the low sampling constant values (KS=0.1-0.3 kg) estimated for "tecator" at ambient conditions. The trials carried out with "combimax" showed large variability as to the residue content at 150 and 15 g AP, in accordance with the observation of the peels that showed large peels coexisting with smaller ones after 6 minutes of processing. In other words "combimax" could not prepare well mixed olive matrix.

Table 1: results for olive samples

Trial ID	AP size	Ave Recovery	CV_A	CV_T	$F_{calc}= V_T/V_A$ ($F_{0.05,24,20}$=2.08)	$V_{SP+extr}$	$F_{calc}= 10*V_{SP}150 /V_{SP}15$ ($F_{0.1,4,4}$)=6.39	KS_{150-15}
OLCMA1	150 g	62.2	1.76	4.15	5.58	0.00055	4.87	2.12
	15 g	71.7	0.90	4.76	28.19	0.00112		
OLCMA2	150 g	67.8	0.98	7.55	59.62	0.00258	24.15	nwm
	15 g	56.2	1.37	5.97	19.06	0.00107		
OTA1	150 g	77.6	0.80	0.91	1.30	0.00001	nsd	wm
	15 g	78.7	1.08	1.59	2.18	0.00008		
OTA2	150 g	73.7	0.91	1.64	3.23	0.00010	3.54	0.28
	15 g	74.3	0.67	2.36	12.63	0.00028		
OTA3	150 g	75.4	0.78	1.14	2.10	0.00004	1.03	0.10
	15 g	76.2	0.86	2.75	10.28	0.00040		
OTDI1	150 g	76.7	0.77	0.81	1.12	0.00000	nsd	wm
	15 g	77.3	0.87	1.18	1.86	0.00004		
OTDI2	150 g	74.9	1.02	2.47	5.85	0.00028	71.47	nwm
	15 g	76.1	0.58	1.01	3.05	0.00004		

OLCMA1-3= olives combimax ambient trial 1-3
OTA1-3= olives tecator ambient trial 1-3
OTDI1-3= olives tecator dry-ice trial 1-3
nsd=not significantly different
nwm= not well mixed

Results of processing of tomato samples are presented in table 2. Average recovery was about 80 % for all analytical portion sizes, with the exception of two trials, were the recoveries were substantially higher than the average. In most of the experiment a CV_A of analysis less than 2% was obtained, exception made for trials TTA3 and TTDI1 for analysis of 5 g analytical portion.

Table 2: Results of tomato samples

Trial ID	AP size	Average Recovery	CV_A	CV_{total}	$F_{calc}= V_T/V_A$ ($F_{0.05,24,20}$=2.08)	$V_{SP+extr}$	$F_{calc}= 10*V_{SP}150 /V_{SP}15$ $F_{tab\ (0.1,4,4)}$=6.39	KS_{150-15}	Well mixed status at 5 g
TCMA1	150 g	77.8	1.55	3.83	6.13	0.00075	3.08	1.85	
	15 g	76.8	1.48	6.57	19.81				
	5 g	77.3	0.81	7.28	81.07				
TCMA2	150 g	93.5	0.86	3.15	13.48	0.00080	2.98	1.37	X
	15 g	95.9	1.27	5.55	19.22				
	5 g	99.9	2.08	8.68	17.42				

Table 2: Results of tomato samples (cont.)

Trial ID	AP size	Average Recovery	CV_A	CV_{total}	$F_{calc}=V_T/V_A$ ($F_{0.05,24,20}=2.08$)	$V_{SP+extr}$	$F_{calc}=10*V_{SP}150/V_{SP}15$ $F_{tab\,(0.1,4,4)}=6.39$	KS_{150-15}	Well mixed status at 5 g
TCMA3	150 g	78.4	1.04	1.56	2.24	0.00008	2.89	0.20	X
	15 g	79.3	1.09	6.25	32.63				
	5 g	79.5	0.94	15.66	277.67				
TCMA4	150 g	82.7	0.69	3.05	19.47	0.00060	2.37	1.33	X
	15 g	80.8	0.64	6.29	97.35				
	5 g	84.1	1.39	9.55	47.02				
TCMA5	150 g	74.3	0.94	3.67	15.39	0.00070	0.06	1.89	
	15 g	80.5	0.66	1.98	8.75				
	5 g	80.0	0.72	6.80	88.38				
TTA1	150 g	99.6	0.69	9.11	173.27	0.00819	97.44	nwm	
	15 g	129.6	0.60	2.31	15.12				
	5 g	136.4	0.67	11.36	290.34				
TTA2	150 g	73.7	0.73	1.43	3.84	0.00008	1.05	0.23	
	15 g	86.4	0.51	3.43	44.52				
	5 g	85.3	0.85	2.25	6.97				
TTA3	150 g	76.1	0.75	4.04	29.22	0.00091	26.17	nwm	
	15 g	74.2	0.66	2.60	15.48				
	5 g	79.0	3.23	5.96	3.41				
TTA4	150 g	79.9	0.95	2.53	7.05	0.00035	8.68	nwm	
	15 g	75.8	0.80	2.77	11.92				
	5 g	82.1	0.88	5.34	36.58				
TTA5	150 g	82.8	0.81	2.29	7.92	0.00031	6.48	0.69*	
	15 g	82.8	1.01	2.85	7.98				
	5 g	89.9	0.53	3.91	55.20				
TTDI1	150 g	78.1	0.86	1.10	1.63	0.00003	1.02	0.07	
	15 g	73.9	1.09	2.56	5.47				
	5 g	78.3	5.23	7.45	2.03				
TTDI2	150 g	83.1	0.93	1.92	4.33	0.00020	1.43	0.43	X
	15 g	80.0	1.50	6.81	20.63				
	5 g	86.2	0.61	8.87	212.29				
TTDI3	150 g	75.2	0.70	4.22	36.28	0.00098	18.90	2.60	
	15 g	77.3	0.60	3.01	25.24				
	5 g	78.2	1.01	7.58	56.24				
TTDI4	150 g	77.8	0.60	1.90	10.13	0.00020	3.23	0.49	X
	15 g	80.3	1.27	3.32	6.80				
	5 g	82.4	0.91	3.86	18.00				
TTDI5	150 g	82.4	0.91	1.88	4.25	0.00018	3.67	0.41	X
	15 g	79.2	0.81	2.94	13.25				
	5 g	80.6	0.71	9.99	198.67				

TCMA1-5=tomato combimax ambient trial 1-5
TTA1-5=tomato tecator ambient trial 1-5
TTDI1-5= tomato tecator dry-ice trial 1-5
nwm= not well mixed
*probability=0.097 instead of 0.1

Below 15 g analytical portion the sample processing procedure is very uncertain, however in 3 trials out of 5 tomato matrix was well mixed at the 5 g analytical portion level, using "tecator" in the presence of dry-ice and "combimax" at ambient temperature (table 2). Table 3 shows the ranges of KS for tomato samples, calculated as follows:

The sample processing variances categorised by matrix and processing device, obtained from "successful" repeated trials were tested with Cochran test (α=0.05) for the presence of outliers. Then the VSp were pooled and a confidence interval based on the Chi-square distribution ($\chi 2$) at 95% probability was obtained for the average variance.

$$\nu S_{SP}^2/\chi^2_{0.025} \leq \sigma_{SP}^2 \leq \nu S_{SP}^2/\chi^2_{0.975} \qquad \text{eq.(5)}$$

The true variance of sample processing (σ_{SP}^2) has a 95% probability of being within the calculated range. The estimated V_{SP} has 4 degrees of freedom (ν) because it is derived from the analysis of 5 replicate test portions. The pooled variance has $n*\nu = n*4$ degrees of freedom. Equation (5) can be used to calculate the expectable minimum and maximum values of Ks from the typical variance (S_{SP}^2) for each matrix and each chopping devise used. The typical sampling constant ($K_{S\,TYP}$) values are calculated from

$$K_{S\,TYP} = W * CV_{SP}^2 \qquad \text{eq.(6)}$$

where CV_{SP} is calculated from the pooled variances of sample processing as described above, and W is the weight of the large analytical portion.

Table 3: KS ranges for tomato samples

	Combimax Ambient	Tecator Ambient	Tecator Dry-ice
typical Ks	1.21	0.49	0.44
min	0.67	0.22	0.23
max	2.81	1.78	1.20

An immediate distinction can be made between the efficiency of the two processing devices: "combimax" has lower efficiency compared to "tecator", however it produced well mixed samples in 4 out of 5 cases, while "tecator" at ambient temperature gave well mixed status only in 2 trials out of 5. The laboratory has to think in terms of compromises between the costs of equipment, the purpose of analysis, and the tolerated uncertainties. Taking the upper confidence limit of sample processing, and 30 g, 15 g and 5 g analytical portion, we obtain the uncertainties of sample processing ($CV_{sp}=\sqrt{(Ks/M)}$) as shown in table 4. If the laboratory is willing to accept for tomato samples an uncertainty of sample processing <10%, then "combimax" would be a suitable processing device, provided that a minimum of 30 g are taken as analytical portion, and analysed.

Table 4: uncertainties of sample processing (%) calculated from the upper Ks confidence limit

	Combimax Ambient 150-5 g	Tecator Ambient 150-15 g	Tecator Dry-ice 150-5 g
5 g	23.7	18.9	15.5
15 g	13.7	10.9	8.9
30 g	9.7	7.7	6.3

These results show that using the above-mentioned processing devices uncertainty of sample processing up to the values in table 4 can be expected, depending on which analytical portion size is going to be analysed. In general 25-30 g analytical portion size is recommended (Figure 1), as the analysis of 5-10 g leads to high sample processing uncertainty, and above 40-50 g doesn't generate any significant improvement in terms of uncertainty, on the contrary it increases the cost of analysis in terms of solvent consumption.

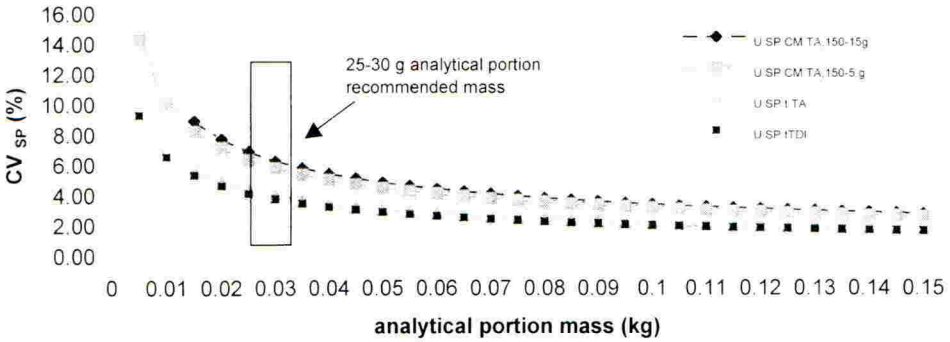

Figure 1: The estimated uncertainty of sample processing for tomato samples processed in "Tecator" and "Combimax" as a function of the analytical portion size.

Previous results (Maestroni *et al.*, 2000b) showed that K_S range for tomato samples processed in a commercial food processor, was 11-44 kg, for which up to 38% and 94% uncertainty of sample processing when processing 30 g and 5 g AP respectively could be expected. Therefore the importance of checking the efficiency of the processing devices in each laboratory, as the consequences in terms of uncertainties can be severe and unexpected (94% uncertainty deriving only from the processing step). With both commodities, olives and tomato, we conducted some trials where we carried out the processing in the presence of dry ice. Based on results from previous studies (Maestroni *et al.* 2000a), a drastic increase in the efficiency of processing was expected. However in the case of tomato samples, processed in a "tecator", only a slight improvement from the processing at ambient condition was observed. According to table 4, KS_{typ} improved only from 0.49 to 0.44 kg when processing tomatoes in the presence of dry-ice. This is another confirmation of the efficiency of the "tecator" processing device that already at ambient conditions very effectively fragments the peels of the tomatoes. Final confirmation on the best processing procedure (including type of device) to use should come

from the evaluation of the stability of residues, as it was shown (El-Bidaoui *et al.*, 2000) that processing in the presence of dry-ice maintains stable several pesticides that under ambient processing condition would degrade. In one trial for olive samples, the addition of dry-ice in the "tecator" improved the efficiency of sample processing to such an extent that its value was negligible compared to the total variance, as V_A and V_T could not be separated according to equation (4) as they were not significantly different.

CONCLUSIONS

Although the sample processing procedure looks simple, it is not always easy to obtain homogeneous samples. This shows the importance of regularly checking the homogeneity of samples as an internal quality control measure. Moreover, it emphasizes the importance of having proper skills and training to perform even easy operations such as sample processing. The present study was carried out with a radio-labelled compound so that precise (typical relative standard deviation $\leq 2\%$) and quick direct determinations of the analyte in the extract could be taken, and thus eliminating the effects of the rest of the analytical procedure. However the procedure can be carried out with normal pesticide mixtures. In this case easily and reproducibly detectable stable compounds (i.e. Chlorpyrifos, Lindane, pyrethroids) should be used for determining the K_S value for a given sample processing method.

For the estimation of the uncertainty of the residue data, the uncertainties of sampling, sample processing, and analysis have to be taken into consideration. According to Ambrus (2003) there is little point in trying to decrease the uncertainty of sample processing from 10% to 5%, as it decreases the combined uncertainty only with one percent if the sampling uncertainty is also considered. Taking into account the technical difficulties of obtaining $CV_{SP} \leq 5\%$ for many sample types and the possible consequences of the extended and intensive homogenisation process on the stability of residues, one may conclude that a CV_{SP} of $\leq 10\%$ would be an optimal target value in pesticide residue analysis.

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Relevant metabolites in soil, water, plants and animals

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ABSTRACT
Metabolites formed from crop protection products are increasingly being included in dietary and environmental risk assessments. However, the amount of separate testing and the degree of complexity of the risk assessment required to demonstrate safety is dependent on a wide variety of factors. This paper addresses some of the factors which determine whether a metabolite is relevant or not and therefore the degree to which risk assessments need to be conducted.

INTRODUCTION

Metabolites of crop protection products are formed in soil, water, plants and animals following the ingestion of plant parts or directly if the product is applied directly to the animal. Metabolites may be subsequently moved from their place of formation to another environmental compartment, for example from soil to water or from soil to primary and rotated crops. Thus an assessment of the environmental and dietary consequences of the presence of a metabolite may be necessary. The difficulty comes in deciding what type of an assessment is necessary and what new data needs to be obtained that is not already available from studies with the parent compound to complete this assessment. At one end of the spectrum a very comprehensive series of studies could be required (almost the same number as an active ingredient) and at the other end of the spectrum no testing may be needed. Thus the level of assessment required depends upon a number of factors which include the structure of the metabolite, where it is found to be present, in which quantities and for how long and if it is transferred to another environmental or dietary compartment. This paper gives an overview of the issues relating to the decision-making processes to determine if a metabolite is considered relevant and if it is relevant, the type of environmental and dietary risk assessments which are conducted.

METABOLISM STUDIES

The metabolic transformations of all xenobiotic products are investigated in each compartment that they may reach. Metabolism studies are conducted in soil, water, plants (including rotated crops) and animals. Radiolabelled atoms, for example [^{14}C, ^{3}H, ^{33}P], are incorporated into the molecule by radiosynthetic routes often in multiple locations so that the transformation and ultimate fate of each of the ring moieties of the molecule can be determined. The objective of these studies is to determine firstly the rate of transformation, secondly what is the identity of the transformation products (metabolites) and thirdly what is the rate of transformation of these metabolites. Sometimes it is not possible to determine the answers to these questions in one study and further studies with the metabolites are sometimes needed. These further studies may be conducted with radiolabelled metabolites. Soil micro-organisms can use xenobiotic compounds as a source of energy or for their carbon or other nutrient content, compounds can also be metabolised coincidentally (co-metabolism). Thus atoms or fragments can be

incorporated into microbes and become identical to the natural compounds found in soil. In many cases the ultimate fate of the xenobiotic is mineralisation to naturally occurring metabolites such as carbon dioxide and water. Many organic metabolites such as benzoic acid also occur naturally in soils.

BIOLOGICAL ACTIVITY

One of the key properties of a metabolite is whether is has biological activity against plants or harmful organisms. In many cases it is known from structure activity relationships which are the key functional groups or moieties known to be essential for providing the biological activity. For some target enzyme sites, a biochemical test may have been developed that determines the strength of inhibition and provides an I_{50} value which can then be compared with the parent molecule. In other cases this information is not known because the target enzyme or site of action is not yet determined. Also the influence of the loss/addition of certain functional groups around the molecule on the biological activity may not be known. Clearly for some compounds (pro-pesticides) the metabolite may be partly, if not completely responsible, for the majority of the biological control. The degree of control and the number of species controlled is clearly dependent upon the product. Some products have a very broad biological spectrum, whilst others are designed to be deliberately narrow. The same is true for metabolites.

If the biological activity of a metabolite is not known, then it may need to be determined. In general, the biological activity will need to be determined for metabolites found in water at significant levels and for soil metabolites if they are persistent and have the potential to leach or run-off to surface water. The degree of biological activity in comparison to the parent compound is determined by screening both parent compound and metabolite against the same weeds or organisms that the parent compound has shown activity and at the same field application rate. The molecular mass of the metabolite needs to be taken into account as given by the formula:

$$\text{Rate}_{\text{metabolite}} = \frac{M}{A} \times \text{Rate}_{\text{a.s.}}$$

Where : $\text{Rate}_{\text{metabolite}}$ = application rate at which metabolite should be tested in screen kg/ha
$\text{Rate}_{\text{a.s.}}$ = use rate of active substance kg/ha
M = molar mass of metabolite
A = molar mass of active substance

In conducting this experiment, no account is taken of the percentage formation of the metabolite. This can be taken into account during the risk assessment stage in which actual concentrations are estimated by modelling or determined in studies. In general a plant protection product needs to achieve at least an 80% level of control against an organism to pass the biological efficacy criteria which make it an efficacious product. However a metabolite may not be as effective as an active substance although still have some biological potential. Biological tests are notoriously difficult to interpret with accuracy especially at low percentage control rates (ca 20 – 30%) hence a 50% value is used to determine if the metabolite has comparable biological activity (EC Guidance Document, 25th February 2003).

METABOLITES FOUND IN SOIL

Metabolites found in laboratory metabolism studies in soil which account for greater than 10% of the active substance applied (EC Directive 91/414, 1995) will need to have their structure identified. These metabolites are then assessed for persistence under laboratory and/or field conditions. Metabolites are then considered on a case by case basis, those that are persistent (DT_{90} > 100 days) or have the potential to accumulate in soil, have likely or measured biological activity and have a high Log Kow (>2) will be examined for their ecotoxicological consequences. For terrestrial organisms studies conducted can be earthworm (acute and/or chronic) beneficial insects (Typhlodromus and Aphidius) and possibly Collembola. In addition the potential effects on the carbon and nitrogen cycles in soil are studied in a soil respiration (a general measure of soil microbial functions) and a nitrogen transformation (organic N $\rightarrow$ NH_4 $\rightarrow$ NO_2 $\rightarrow$ NO_3) test which investigates effects on specific species. A higher tier leaf litterbag test also may be performed to determine if there is an influence on organic matter decomposition.

In addition an assessment is made for the protection of ground water by determination of the half-life and the K_{oc} values and using computer modelling according to FOCUS, 2000 recommendations. Further higher tier studies such as field leaching studies may be conducted to determine the extent of exposure if the metabolite is biologically, ecotoxicologically or toxicologically relevant. It is in the area of groundwater that over the last 5 to 8 years there has been the most intensive debate cumulating in the European Commission's Guidance Document, rev 10 in February 2003. This guidance document outlines a tiered approach to testing. Parent compounds are subject to a maximum concentration of 0.1 µg/L in drinking water, which is a non-scientifically based trigger. Metabolites are allowed to exceed this value if they are shown to be non-relevant. In the first tier to determination of non-relevance, hazard based testing is performed which includes biological screening, genotox screening and toxicity screening. If the metabolite passes all these stringent hazard criteria and is below the threshold of concern of 0.75 µg/L (Monro et al. 1996 and Monro et al. 1999), then it is registerable. This threshold of concern value is based upon an assumed consumption of 2 litres of water per day and equates to 1.5 µg/person/day or 0.02 µg/kg body weight/day. If the concentrations are above this, then further assessment is required, which includes all possible sources in the diet.

It is clear that an extremely high standard is demanded of the agrochemical industry with margins of safety in this area being excessively high.

METABOLITES IN WATER

Major metabolites that are formed in hydrolysis aqueous photolysis and in water/sediment studies, in addition those metabolites, which have the potential to move from soil into surface water, have to be assessed for their potential environmental ecotoxicological impact and also dietary risk assessment. In the first instance metabolites may already have been assessed in ecotoxicological studies if they are formed by hydrolysis and this can be determined if metabolite analytical data exists from these studies. Similarly, photoproducts present in algae or Lemna tests may be available from tests with the active substance. Exposure assessments in surface water are required for major metabolites unless they are natural compounds such as CO_2 or inorganic compounds (except heavy metals) or of short chain length. The exposure in surface water accounts for three routes of entry: spray drift, sub-surface drainflow and surface

run-off into three types of water body: a ditch, a stream and a pond (FOCUS 2001). Exposure is determined in both the surface water and sediment in ten realistic worst case European scenarios which cover climatic, soil, topographic, water bodies and agronomic variations. The principle of further testing is to take the most sensitive species to determine if the metabolite is relevant with the addition of further taxonomic groups if the exposure is greater in a given compartment, e.g. sediment. If the toxicity of the parent compound to certain organisms is appreciably less than to others (*ca* 100×) there is no need to generate data on the toxicity of the metabolite to the less sensitive groups. However if the toxicity of the metabolite to any taxonomic group is greater than the parent compound then the toxicity to all groups is determined. Typical tests would include Daphnia Magna, algae and a fish in addition Lemna and Chironomus may be included. Chronic testing is conducted if the metabolite is persistent and if in acute tests the metabolite has similar toxicity to the parent compound. Thus metabolites are expected to pass the Toxicity Exposure Ratio (TER) by comparing the Predicted Environmental Concentration (PEC) with the ecotoxicity values with the same margin of safety as with active substances.

METABOLITES IN ANIMALS

Metabolism studies are conducted in rats as a surrogate mammal and may also be conducted in birds (chicken) and ruminants (goat or cow). The objectives are to determine the distribution of the active substance within the excreta and that, if any, which remains in tissues. The quantity and nature of the metabolites present and the adsorption, distribution metabolism and elimination (ADME) is determined. Information on the metabolism can aid the understanding for the basis of the toxicological properties. The metabolites found in the rat are taken as being an important basis for the likely metabolites that will be found in man with the addition of a series of safety factors.

METABOLITES IN PLANTS

The metabolites present in plants are determined in studies, which quantify and identify metabolites in plant parts at a range of growth stages with the harvest stage being the most important. Studies are performed on the key target crops and if there are a range of crops then in general a minimum of three crop groups are investigated. If the metabolism in the three crop groups is similar then it can be taken that metabolites will be the same within all the crops in those groups. For metabolites found in plants or water that can enter the food chain and have not been observed in animals then separate studies, often with the radiolabelled metabolite, are conducted.

In addition to primary crop studies, metabolism in the following crop after uptake from the soil is investigated. Plant parts which are fed directly to humans need to have metabolites, which occur above 0.01 mg/kg quantified and identified. Whereas for plant parts which are fed to animals the trigger is 0.05 mg/kg.

Once the metabolite's structure is known, it can be investigated by computer modelling to determine if it contains any functional groups that give structural alerts for mutagenicity, carcinogenicity or is a potential teratogen. If the metabolite has been observed in the rat then it will have been included by default in toxicological testing (PSD Guidance for Registrants,

2002). If not then toxicology studies would typically address genotoxicity (e.g. Ames test, gene mutation and chromosome aberration). In addition depending upon the toxicological end point of the active substance acute, chronic or reproductive toxicity testing is conducted (PSD, 2002).

However following a study by Gold et al. 1989, it has been shown that there is a "threshold of concern" below which even for known carcinogens there is an acceptable degree of risk. The dietary risk assessment then takes into account the lowest toxicological end point plus at least a 100 fold safety factor (x 10 for species variation, x 10 for extrapolation to man).

A dietary risk assessment is then performed taking into account all sources of dietary contribution. This includes drinking water, crops, processed foods and foods of animal origin (e.g. milk, meat and eggs). This is performed at the European level with a European diet and also can be conducted at the country level to take account of dietary variations.

SUMMARY

Metabolites found in soil, water, plants and animals are assessed for their environmental and dietary safety in a stepwise tiered approach.

For some metabolites, such as carbon dioxide, water and other relatively simple molecules, no assessment is necessary, as the properties of these compounds are already known. For other molecules sufficient information may be available in the literature and only a few additional studies are necessary to demonstrate that there are no concerns. For non-common metabolites between plants and animals and between water and animals, separate testing to determine the toxicological end point is conducted. Often of primary importance to an environmental risk assessment is the biological activity of the metabolite. Since, if the metabolite has biologically activity then it may have an environmental consequence. In general it has been shown (Sinclair and Boxall, 2002) that metabolites have significantly less effects than parent compounds often by a factor of more than 100. Conversely metabolites are very unlikely to exhibit toxicity greater than a 10-fold factor above an active substance.

Clearly relevant metabolites should be included in residue data gathering methods and subjected to either an environmental and/or dietary risk assessment. However, for the many compounds, the metabolites formed can be shown to be of no concern, often being identical to compounds found to occur naturally or for which sufficient data already exists and therefore no further account is needed. Thus once the actual likely quantity of the residue has been determined and the dietary risk assessment conducted then a decision can be made if it is necessary to include the metabolite in the enforcement method. In general the metabolite should only be included where it is needed to ensure consumer safety and thus justifies the additional complexity of the method and the additional cost of conducting the method in residue monitoring programs. In marginal cases the use of a multiplication (safety) factor can be included to avoid the need to be included in the enforcement method but to provide for an additional degree of safety.

Environmental and dietary assessments should be conducted to the principles of 91/414 and be on a risk based approach. If hazard based criteria are introduced to enable a tiered approach to testing, then there should always be the possibility to progress to a risk assessment. The

0.1 µg/l cut-off criteria for active substances and relevant metabolites in drinking water has no scientific basis, for non-relevant metabolites a risk assessment allows for 10 µg/L or more.

The degree in depth investigation to identify and quantify metabolites of plant protection products and to assess their environmental and dietary consequence is probably the most sophisticated assessment in any part of the chemical industry and provides consumers and the environment with an extremely high level of protection.

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Safety of genetically modified crops for food and animal feed

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ABSTRACT

The cultivation of genetically modified crops is increasing worldwide. The majority of this is herbicide tolerant and insect resistant crops. The pre-market safety assessment of genetically modified crops for food and feed purposes is based on an internationally acknowledged consensus approach. The central principle is that of substantial equivalence, in others words the comparative safety assessment, in which differences between a genetically modified crop and an appropriate counterpart are identified and further focused on in the safety assessment. Issues that are commonly addressed during the assessment include the molecular characteristics of the genetic modification, potential allergenicity, toxicity, gene transfer, unintended effects, nutritional value, and post market surveillance. While pesticide metabolism may be relevant for genetically modified herbicide tolerant crops, its safety aspects are addressed during separate pesticide registration procedures. The altered pesticide use on genetically modified crops likely affects the impact of pesticides on environment and health. Advanced methodologies for safety testing for genetically modified crops are currently developed that will add to the current methodologies for testing of future crops that will have undergone more complicated modifications.

INTRODUCTION

Since the large-scale introduction of commercial genetically modified crops in 1996, the cultivation of these crops has increased, amounting to a global 58.7 million hectares of cultivated land in 2002, predominantly in the United States of America, Argentina, Canada, China, and South Africa. The major genetically modified crops are soybeans, maize, canola, and cotton. Currently inside the EU, genetically modified maize is cultivated to a limited extent in Spain (James, 2002). Similar to the differences in adoption, EU consumers perceive genetically modified crops and foods less positively than American consumers.

The most important traits in genetically modified crops are herbicide tolerance and insect resistance, both serving agronomic purposes. Tolerance to a specific broad spectrum herbicide, such as glyphosate and glufosinate, allows for over-the-top application of the pertinent herbicide and may therefore substitute for directed applications or mechanical weed control. Insect resistance has been introduced into crops by genetic modification with insecticidal "Bt" proteins from *Bacillus thuringiensis* (itself used for decades as a biological pesticide and currently still in use). Both the herbicide tolerant and insect resistance traits likely affect the use of pesticides on these crops. Moreover, the novel proteins that have been introduced may be considered pesticides themselves in some cases. The safety of pesticides is evaluated prior to marketing, just as that of genetically modified crops *per se*, although the regulatory procedures may differ. In fact, the various national legislations on genetically modified crops

may differ among nations. In spite of these legislative and procedural differences, the regulatory pre-market safety assessment of these crops is based on an internationally acknowledged consensus approach. Besides food and feed safety, the environmental safety of genetically modified crops is also a point of consideration. An analysis of the potential environmental impact of genetically modified crops is needed with regard to, for example, altered pesticide use. The issues related to the safety of genetically modified crops are discussed below. For more details, the reader is referred to a recent review on this topic (Kuiper *et al.*, 2001).

INTERNATIONAL CONSENSUS

Prior to their market introduction, genetically modified crops have to be evaluated for their safety as required by law in the EU member states and many non-EU nations. This may include, depending on the scope of the application, human food and animal feed purposes of the genetically modified crop. Currently, this safety evaluation is carried out according to an internationally acknowledged consensus approach developed by international organisations like the International Food Biotechnology Council (IFBC), joint Food and Agriculture Organisation and World Health Organisation (FAO/WHO), International Life Sciences Institute (ILSI), and the Organisation for Economic Co-operation and Development (OECD). The efforts of the particular institutions are reviewed in more detail by Kuiper *et al.* (2001).

The principle of substantial equivalence has a central role in the safety evaluation of genetically modified organisms. It entails the comparison of a genetically modified product with a conventional counterpart, while differences that are thus identified warrant further investigation to verify their relevance for the safety of the tested product. Contrary to the views expressed by some of its critics (*e.g.* Millstone *et al.*, 1999), substantial equivalence is not an end point, but rather a starting point of the safety assessment. Interestingly, Kok & Kuiper (2003) recently proposed to substitute the designation "substantial equivalence" with "comparative safety evaluation" to avoid misconceptions about this important principle. In the following, we will use the proposed new designation. In the case of genetically modified crops, this comparison might focus on the phenotypic and compositional characteristics of the biotech crop and, for example, a near-isogenic line of the same crop that has not been genetically modified. Compositional parameters typically include the relevant macro- and micro-nutrients, anti-nutrients, and toxins for a given crop. So far, data have been collected for crops that have been altered with comparatively simple modifications, *e.g.* insecticidal proteins, for which the approach has worked well. Scenarios can be envisioned, though, where future crops with more complicated modifications that substantially alter the plant, *e.g.* the introduction of novel metabolic pathways, are notified for market-approval. In such cases, an appropriate comparator for the genetically modified crop may not be available, while other issues, such as the potential for unintended effects, may need close scrutiny. This is similar to the introduction of novel foods, *e.g.* exotic fruits or nuts, without a history of significant local use or any traditional food to compare with.

Since the formulation of the principle of comparative safety assessment by OECD in 1993, it has been endorsed and further refined. An important step in the ongoing international harmonisation is the guidelines on the safety assessment of foods derived from genetically modified plants and microorganisms. Also, in a broader sense, the principles for risk analysis (including communication, measures etc.) of genetically modified foods that were recently

issued by the FAO/WHO's Codex alimentarius commission, which sets international standards for food safety (Codex alimentarius, 2003). In addition, the OECD's Task Force on the Safety of Novel Foods and Feed develops consensus documents on key compositional parameters that need to be considered when carrying out the comparative safety assessment. At present, consensus documents on soybean, canola, sugar beet, potatoes, maize, and wheat are available, while those on rice, sunflower, cotton, forage legumes, barley, and tomato are being developed (OECD, 2003).

ILSI's International Food Biotechnology Committee (IFBiC) has developed an Internet-accessible database (www.cropcomposition.org), which provides data on the composition of field-tested conventional and genetically modified crops from recent years. These data may serve as a background range of compositional values in the comparative safety assessment, facilitating the interpretation of any detected changes in composition. In addition, ILSI is developing guidelines for the nutritional assessment of genetically modified animal feeds as well as the safety and nutritional assessment of nutritionally enhanced, biotechnology derived foods and feeds.

ISSUES IN SAFETY ASSESSMENT

As discussed above, the comparative safety assessment approach is the central principle for the assessment of genetically modified products. For crops, this generally entails a comparison of the phenotype and composition, as well as a characterisation of the inserted DNA and expressed products. Depending on the differences thus found, it is decided which further test are needed to establish crop safety. Items that are commonly addressed during the safety assessment include molecular characterisation (description of the genetic modification) and the potential for allergenicity, toxicity, gene transfer, and unintended effects, as well as nutritional value, post-market monitoring, and pesticide residues.

Allergenicity

As stated above, all known food allergens are proteins. Some of these allergens are food crops, including legumes and nuts. If used as host for the genetic modification, such crops should therefore be checked for any changes of the intrinsic allergenicity. In addition, any novel protein introduced into a crop should be assessed for its potential allergenicity. For this assessment, the FAO/WHO Codex alimentarius commission's guidelines recommend an integrated, stepwise, case-by-case approach (Codex alimentarius, 2003).

Based upon the knowledge about the allergenicity of the source of the introduced DNA, it is decided upon which tests are further required. In case the gene donor is allergenic, the genetically modified product should be screened for reactions with antisera of patients that are allergic to the donor. In case the gene donor has no history of allergenicity, such as soil bacteria, the serum screen should be performed with antisera directed against allergens that are broadly related to the gene donor. In addition, identical sequences of relevant size that are shared by the transgenic protein and allergenic proteins should be searched for with the aid of computer programs and protein sequence databases. The digestibility of the novel protein in simulated stomach fluid *in vitro* should also be established, given that stable proteins are more likely to come into contact with the gut-associated immune system and may thereby elicit allergic reactions. Additional tools may be considered, such as experimental animals. While

the experimental use of such animal models has been described, their validation would facilitate routine use. The outcome of the integrated approach is an indication of the likelihood that the new product is an allergen.

Toxicity

New gene products that have been introduced by genetic modification should be evaluated for their toxicity (*e.g.* SSC, 2003). For example, purified transgenic proteins are commonly tested in laboratory animals, such as in acute and subchronic oral toxicity studies. In acute studies, a single high dose of the test product is administered by oral gavage to animals. In the following 14 days, observations on the body weight, morbidity, and mortality of the animals are made and a gross pathology (organs etc.) performed at sacrifice. In subchronic toxicity studies, animals will receive the test product daily, for example through their feed. Compared to the acute studies, the tested parameters additionally include clinical chemistry, urinalysis, organ weights, and histopathology.

In addition to animal studies, the primary structure of novel proteins can be compared to those of toxic proteins by computer assisted sequence similarity searches. The *in vitro* digestibility of the new protein may also indicate its potential to sustain digestion and reach target sites for toxic action.

Whole food testing should be decided upon when uncertainty remains over the equivalence of a genetically modified product, for example when the comparative safety assessment is limited by lack of an appropriate non-modified comparator, such as with profound modifications. The subchronic 90-days oral toxicity test in rodents is most commonly performed and further tests be decided upon pending the outcome. Whole food testing has some limitations due to i) the limited range of dosings that can be tested; ii) impalatability; iii) bulkiness; or iv) nutritional imbalance of the product.

Currently, within the EU-sponsored project SAFOTEST, methods are developed that supplement the animal toxicity tests of genetically modified crops and that are expected to increase their sensitivity. Among others, the use of gene expression micro-arrays is studied to detect pre-clinical toxic effects.

Gene transfer

Antibiotic resistance marker genes receive special attention within this topic. Such genes are commonly employed as facilitators of the selection of genetically modified plant cells/tissues after genetic modification (*e.g.* by *Agrobacterium* or by bombardment with accelerated, DNA-coated particles). A point of concern is that the antibiotic resistance gene, after transfer to a pathogenic micro-organism within the gastro-intestinal tract, may impair antibiotic treatment of infections with this pathogen. Besides the remote possibility that gene-transfer and expression may occur, it is taken into account whether a natural background level of resistance exists and whether the antibiotic is clinically relevant. Based upon these considerations, the presence of the kanamycin resistance gene *nptII* in genetically modified crops, for example, has been found to pose no safety concern. In addition, it should be noted that also alternative marker genes need to be evaluated for their safety.

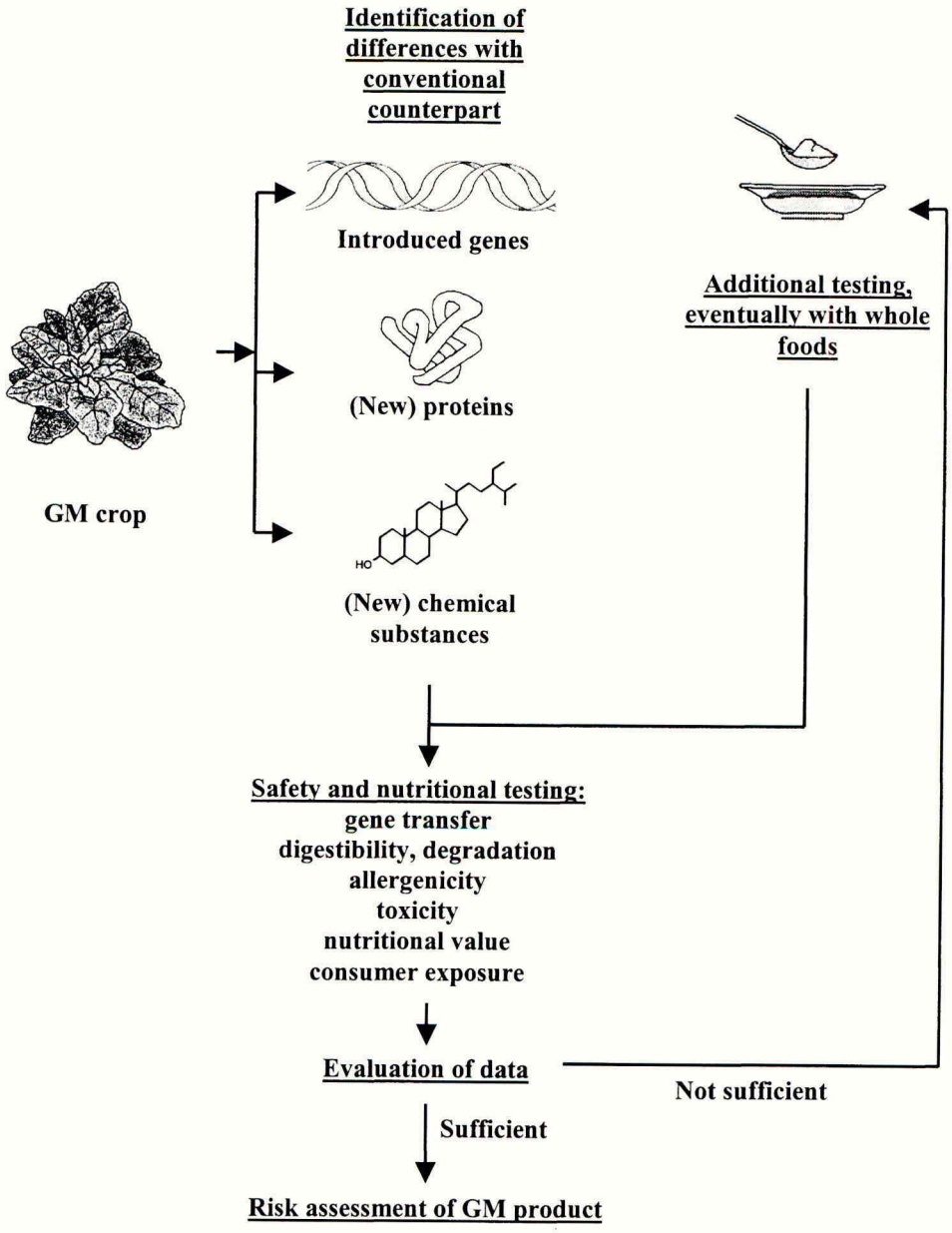

Figure 1. Risk assessment of genetically modified food crops

Unintended effects

Besides the intended effects of the genetic modification, such as the introduction of an insecticidal protein, it can be envisioned that additional unintended effects may occur due to the genetic modification. For instance, if a newly introduced gene has been inserted into the

DNA of an intrinsic gene, the latter may have lost its function. In another scenario, an introduced enzyme may generate metabolites that are further processed by intrinsic enzymes, whose action has not been anticipated. Such unintended effects can be revealed by phenotypic observations or changes in the compositional parameters. It should be noted that unintended effects are not specifically linked to modern biotechnology, as they are known to occur during conventional plant breeding as well.

It can be anticipated that in future, for genetically modified crops with more complicated genetic alterations the likelihood for unintended effects increases, *e.g.* by the introduction of novel metabolic pathways. In theory, effects may occur that do not affect the selected compounds that are measured during the commonly employed targeted analysis of genetically modified crops. In the advent of the future biotech products, the EU-sponsored project GMOCARE develops advanced analytical "profiling" techniques that supplement the current targeted techniques. With the profiling methods, holistic outputs are generated, such as peaks in a chromatogram or spectroscopic spectrum, for which the identity need not be known exactly beforehand (reviewed by Kuiper *et al.*, 2003). After differences in the outputs for the genetically modified product and its comparator have been identified, these are further examined.

Nutritional value

Besides the animal tests for toxicity, nutritional tests are performed in target animals, preferably in rapidly growing young animals which will react to any nutritional perturbance that the genetic modification may have caused. Usually, performance is measured, such as body weight increase or milk/egg production. Commonly employed animal models are the rapidly growing broiler chick and the lactating dairy cow.

Post market surveillance

Currently there are no post market surveillance programmes for genetically modified foods. Such surveillance should be a supplementary tool, rather than a substitute, for the pre-market safety assessment. It should be stressed that the pre-market assessment should exclude the possibility of adverse effects as much as possible and in case questions remain, the product should not be marketed. Post market surveillance would serve the purpose of detecting unanticipated health effects of the genetically modified food. Recently, the British Food Standards Agency studied the feasibility of post market surveillance of genetically modified foods. The agency recommended that food purchase data of local citizens collected through household surveys be linked to health survey data for the same individuals (FSA, 2003).

Pesticide residues

As discussed above, some genetic modifications are targeted at tolerance of otherwise lethal herbicides applied to the crop. It can be hypothesised that the metabolism of the herbicide may differ from that in conventional plants and also that the quantities of the particular herbicide applied to the crop may increase. For example, it has been observed that residues of the glyphosate metabolite AMPA are increased in genetically modified glyphosate-tolerant maize compared to non-modified maize (JMPR, 1997). Such issues are commonly addressed during the registration procedure for the specific purpose of the herbicide, which is separate from the safety evaluation of the genetically modified crop itself, at least in the EU.

Interestingly, the broad-spectrum herbicides (glyphosate, glufosinate), to which some biotech crops have been rendered tolerant, are currently also used on food crops, not for weed control, but as "desiccants" applied shortly before harvest to facilitate the latter. This accounts for the fact that, for example, the highest contribution to the estimated intake of glufosinate by EU citizens comes from potatoes, on which this herbicide is applied as a "leaf killer" (JMPR, 1999).

Genetically modified crops may also indirectly affect human and animal health, through a change in pesticide residues in surface and drinking water. Wauchope *et al.* (2001), for instance, predicted that pesticide residue patterns in surface water from three American watershed areas would display an environmentally favourable change upon adoption of herbicide-tolerant maize in these areas. Currently, a project of the International Union for Pure and Applied Chemistry (IUPAC), seeks to assess the environmental impact of altered pesticide use on genetically modified crops (IUPAC, 2003). For this purpose, data will be collected on the observed changes in active ingredients and application rates of pesticides applied to genetically modified crops. The impact associated with the altered use will be quantatively estimated by the use of environmental impact indicators for calculations. It is realised that, besides effects in terms of residues and toxicity, also other environmental parameters may play a role, such as biodiversity. The output will be used to create a tool for risk benefit analysis of genetically modified crop cultivation.

DISCUSSION

The safety assessment strategy described above can be considered robust and adequate for the current generation genetically modified crops. It is anticipated that future novel crops will have undergone more complicated modifications, which may increase the likelihood of unintended effects. To this end, the EU-sponsored thematic network ENTRANSFOOD (www.entransfood.nl) has brought together various EU-projects on advanced methods for safety testing of genetically modified food, such as the GMOCARE and SAFOTEST projects discussed above. These methods are a useful addition to the existing methodologies. In addition, there has been interaction with other stakeholders as well. ENTRANSFOOD was concluded recently and reviews of the pertinent areas of research are to be published shortly.

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