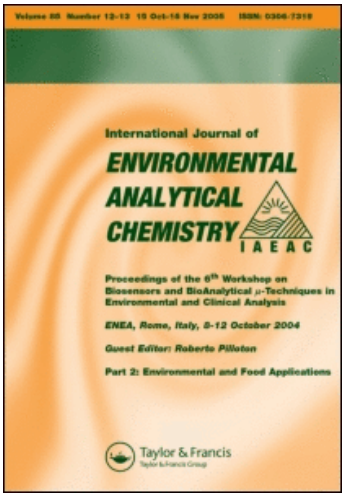

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Public concern about the impact of xenobiotics, including pesticides, on human health is greater than ever before. Pesticides constitute a potential risk to humans who are exposed to them directly and indirectly. Accordingly, the health risk assessment of pesticides is of utmost importance for the protection of human health. Risk assessment needs reliable scientific information, and one source of information is the characterisation of metabolic factors and toxicokinetics. Indeed, quantitative toxicokinetic data in humans are needed for human risk assessment to make reliable comparisons between individuals or between species. Because humans cannot be used as study subjects except in occasional circumstances, we have to rely upon *in vitro* experiments, human-derived techniques and *in vitro–in vivo* predictions. These methods combined with the advantage of novel analytical techniques (LC/TOF-MS; LC/MS-MS and LC-NMR) for metabolite identification and quantification enable the development of quantitative chemical-specific assessment factors. Here, we will briefly describe the *in vitro* techniques used to study *in vitro* metabolism and interactions, summarise the metabolic and kinetic properties of diuron as an herbicide and demonstrate how to apply current techniques to obtain relevant data for risk assessment.

Keywords: pesticides; metabolism and interactions; P450; human-derived *in vitro* techniques; risk assessment; LC-MS

1. Introduction

Hundreds of articles are available concerning the *in vitro* metabolism of pesticides utilising experimental animals, usually rodents, while only a few studies of *in vitro* human metabolism exist. However, human quantitative toxicokinetics data (e.g. metabolic clearance, metabolites and metabolic pattern and interactions) are needed to improve human risk assessment. In order to screen the metabolic factors of pesticides, we must rely upon *in vitro* approaches using human tissues and genetically engineered experimental systems. The more we know about the metabolic characteristics of pesticides *in vitro*, the more accurate will be our extrapolation of *in vitro* and animal studies to human risk assessment. Because humans cannot be used as study subjects except in occasional circumstances, predictions from human *in vitro* data must be made carefully without

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direct verification from human *in vivo* data. Fortunately, there is a lot of experience about *in vitro–in vivo* extrapolations as it has been used already for decades in the drug development process.

Xenobiotic biotransformation is the process by which lipophilic foreign compounds are detoxified through enzymatic biotransformation to hydrophilic metabolites that are eliminated directly via renal elimination or after conjugation with endogenous cofactors. These metabolic enzymes are divided into two groups, phase I and phase II enzymes.

Phase I reactions generally involve oxidation, reduction and hydrolytic reactions as well as other rarer miscellaneous reactions. These reactions are mediated primarily by the cytochrome P450 family of enzymes (CYP), but other enzymes (e.g. flavin monooxygenases, peroxidases, amine oxidases, dehydrogenases, xanthine oxidases) also catalyse the oxidation of certain functional groups [1]. CYPs are found in high concentrations in the liver, but are also present in other tissues. CYPs are classified into families and subfamilies by their sequence similarities. Humans have 18 families of CYP genes and 44 subfamilies. CYPs are important in the metabolism of various endogenous substrates as well as a wide range of xenobiotics, and they are responsible for the metabolism of the majority of drugs [2,3].

Sulfation, glucuronidation and glutathione conjugation are the most prevalent classes of phase II metabolism, which may occur directly on the parent compounds that contain appropriate structural motifs, or, as is usually the case, on functional groups added or exposed by phase I oxidation.

It is well recognised that these metabolic processes may lead to detoxification and/or activation reactions. Many compounds, such as organophosphorothioate, phosphorodithioate and phosphorothiloate insecticides, have been found to be activated. Bioactivation occurs in these compounds via CYP enzymes-mediated desulfuration to phosphate oxon metabolites.

In vitro systems have become an integral part of studying drug metabolism throughout the drug discovery process. One scheme has been originally developed for the screening of potential drugs at the preclinical screening phase. It involves liver tissue preparations of either animal or human origin for the measurement of metabolic stability, metabolite identification, metabolising enzymes recognition and enzyme inhibition [4]. However, this scheme has also been used to study the metabolism and interactions of pesticides. The use of human enzyme sources and several possible applications are reviewed by Pelkonen *et al*. [5].

2. Human-derived techniques and enzymes sources for *in vitro* metabolism studies

Human-derived techniques for measuring the metabolism of xenobiotics have been established for many different cell types and organs. For metabolism studies, the enzyme systems used at present, including subcellular fractions, recombinant enzymes, liver homogenates, liver slices and hepatocytes, clearly give reliable results, if their inherent restrictions are taken into consideration [4].

Subcellular fractions including microsomes are the most widely used *in vitro* systems in drug metabolism studies of new drug candidates [6]. Liver microsomes consist of vesicles of the hepatocyte endoplasmic reticulum and are prepared by standard differential ultracentrifugation. Liver microsomes contain almost only CYP and UGT

enzymes. Microsomes can be stored at −80°C and are commercially available. Liver preparations other than from fresh human liver can also be used (e.g., liver slices, liver cell lines, and primary hepatocytes) for the preparation of microsomes [7]. Hepatic microsomes are useful for the evaluation of metabolic stability, metabolite identification and quantification and enzyme inhibition.

Recombinant xenobiotics metabolising enzymes (CYP, UGT) are generally produced and commercially available. They can be easily applied to higher throughput screening and can be used to study the role of individual CYPs in metabolism. The disadvantage is that the effect of only one enzyme at a time can be evaluated [5,8].

Primary cultures of hepatocytes have shown good *in vitro–in vivo* correlations in the metabolic activity of a number of drugs and they allow a comprehensive view of whole cell metabolism, since drug transporters and both phase I and II enzymes are present. Currently, cultured human hepatocytes are the most recommended tools to study CYP-mediated metabolism and induction [6,9]. Hepatocytes are used to evaluate the metabolic stability of the compounds, identify the metabolising enzymes and study enzyme inhibition, much like the use of microsomes, but with the advantage of a complement of phase I and II enzymes and transporters.

Similar to whole cell systems like the hepatocytes, liver slices contain the whole complement of xenobiotic metabolising enzymes and offer a more reliable *in vitro–in vivo* correlations than subcellular systems [5,10].

Different human and animal cell lines are available (http://www.lgcpromochem-atcc.com). The most common human cell lines for biotransformation studies are probably HepG2 (Hepatocellular carcinoma), BC2 (Hepatoma) and the lung-derived line A549. Recently, a new human hepatoma cell line, HepaRG, was derived from a hepatocellular carcinoma. The HepaRG cells express a large panel of liver-specific genes including several cytochrome P450 enzymes [11].

Each of the *in vitro* techniques can provide important information on the mechanisms of xenobiotic detoxification as well as bioactivation. By using *in vitro* systems and human-derived techniques, we can carry out studies in order to answer some scientifically important questions. What is the metabolic stability of the compound? What kinds of metabolites are formed? What are the specific enzymes and isoforms involved? Is the reaction an activation or a detoxication reaction? Do induction and/or inhibition occur?

3. Analytical techniques used in metabolite identification and quantification

Determination of the structure of metabolites is most often performed either by mass spectrometry (MS) or nuclear magnetic resonance (NMR) spectrometry [12]. Mass spectrometry has been the preferred technique because of its good sensitivity. Mass spectrometry is also a very robust technique and typically only protein precipitation is needed as a sample preparation method before analysis.

Earlier separation of metabolites from incubations was performed mostly with the help of gas chromatography (GC). GC is a very good separation technique for thermally stable volatile compounds and it is easy to connect to the mass spectrometer. The most common ionisation technique used with GC is electron impact (EI) ionisation which produces a large number of fragment ions to the mass spectra. These fragmentations

give valuable qualitative information, which could be used in the identification of analytes. Because metabolites are usually polar molecules, they do not volatilise without derivatisation of analytes. Derivatisation is time consuming, complex and it generates extra costs and errors to the measurements [13].

A better separation technique for polar metabolites is high performance liquid chromatography (HPLC). During separation analytes are in the liquid phase and no derivatisation is needed. Usually a basic MS-compatible gradient HPLC method is suitable. For a long time, connection between LC and MS was not practical until the introduction of atmospheric pressure ionisation (API) techniques, such as the most commonly used electrospray ionisation (ESI). ESI is a soft ionisation process that mostly produces $[M \pm H]^{\pm}$ ions without fragmentation. A common problem for LC-MS is ion suppression. Today's higher pressure liquid chromatographs (UPLC, RRLC, etc.) and smaller particle columns have greatly improved the resolution of HPLC.

The best mass spectrometer for screening metabolites is the time-of-flight mass spectrometer (TOF-MS) because of its ability to detect all ionised compounds simultaneously without actual instrument scanning [14]. Another benefit of TOF-MS is its ability to provide accurate mass measurements (± 5 ppm), which helps in identification by providing the elemental composition of the metabolites. If the structures of possible metabolites have been anticipated based on the parent, accurate masses usually give enough information to tentatively confirm structures of metabolites. Other MS instruments used in the identification of metabolites are the triple-quadrupole mass spectrometer and quadrupole ion trap. With these instruments, identification is based on fragments of metabolite molecules produced in the collision cell with the help of a collision gas such as argon (collision-induced dissociation, CID).

Even though triple quadrupole mass spectrometers can help in structure elucidation, their best application is quantification in multiple-reaction monitoring (MRM) mode. The exceptional selectivity of MRM is based on the two-stage separation of the metabolite; first, the actual metabolite is separated from the LC flow, then its produced fragment ion. Because of the high selectivity of triple quadrupole MS perfect separation of metabolites is not needed in MRM measurements. Because metabolites usually have very similar structures they can sometimes produce the same fragments, which can cause problems if metabolites elute at the same time. Sometimes easily fragmenting conjugated metabolites can break already during ionisation, which requires high voltage and temperature to operate. This so-called in-source fragmentation can be a potential source of quantification error if analytes coelute. In any case it will decrease the sensitivity of the method. Similarities in structure can also make identification and isolation of metabolites more difficult.

To know the exact structure of a totally unknown metabolite, nuclear magnetic resonance (NMR) spectroscopy is needed. Because of the lower sensitivity of NMR, larger amounts of a pure metabolite are needed for structure determination. Fortunately recent developments in NMR probe design, electronics and software have made it possible to combine HPLC with NMR. Because of low levels of metabolites the most sensitive nuclei, like 1H, 19F and 31P, must be used. Despite this, continuous-flow measurements are not usually adequate and eluent flow must be stopped for actual NMR measurement. Nevertheless, HPLC-NMR spectroscopy has a valuable role to play in the determination of the structure of xenobiotic metabolites [12].

4. *In vitro* techniques for testing interactions

4.1 *Induction of CYP enzymes*

CYP-xenobiotic interactions involve either induction or inhibition of metabolising enzymes. Induction is defined as an increase in enzyme activity associated with an increase in intracellular enzyme concentration. Many induction studies have been conducted *in vitro* using primary human hepatocytes, human hepatoma cell lines or cell lines derived from other human tissues. Both immunoblotting and reverse transcription polymerase chain reaction (RT-PCR) techniques have been applied to examine the CYP induction of pesticides. However, problems in tissue availability, interindividual differences, reproducibility and ethical issues preclude the efficient large-scale use of human primary hepatocytes for induction screening. Therefore, the development of mechanism-based test systems for induction screening, based for example on *in vitro* pregnane X receptor/constitutive androstane receptor activation, is currently very active, and some test systems are in use as a first step for the identification of potential inducers [4].

Whereas the acute effects of exposure to high doses of pesticides are well known, the long-term effects of lower exposure levels remain controversial. The ability of chemicals to induce metabolic enzymes, including cytochrome P450 (CYP), has long been considered as one of the most sensitive biochemical cellular responses to toxic insult [15,16], since it often occurs at much lower doses of the chemical than those known to cause lethal or overtly toxic effects. One important factor regulating the expression of drug metabolising enzymes is induction by a diverse group of endogenous and exogenous substances that bind to the nuclear receptors pregnane X receptor (PXR) or constitutive androstane receptor (CAR), thereby causing significant up-regulation of gene transcription [17]. Assessment of pesticides inducibility on xenobiotics metabolising enzymes is vital for health risk assessment. Numerous pesticides are capable of inducing their own metabolism and by enzyme induction can also lead to enhanced biotransformation of other xenobiotics.

4.2 *Inhibition of CYP enzymes*

Inhibition can take place in several ways including the destruction of pre-existing enzymes, inhibition of enzyme synthesis or by complexing and thus inactivating the metabolising enzyme. The type of CYP inhibition can be either irreversible (mechanism-based inhibition) or reversible. Irreversible inhibition requires biotransformation of the inhibitor, while reversible inhibition can take place directly, without metabolism. Reversible inhibition is the most common type of enzyme inhibition and can be further divided into competitive, noncompetitive, uncompetitive, and mixed-type inhibition. The inhibition of individual CYP enzymes has been extensively reviewed by Pelkonen *et al.* [2]. The optimal model system depends on a number of factors, such as *in vivo* resemblance, expense, availability of the model and ethical consideration. The *in vitro* model is always a compromise between convenience and relevance [18].

Inhibition interactions can be determined either by single substrate assay or by cocktail assay. In the single substrate assay, traditionally CYP interaction studies are performed using specific assays for each CYP isoform. A decrease in probe metabolite formation produced by inhibition is usually analysed by UV-LC, LC-MS or fluorometry. On the other hand, the dramatically increased number of new chemicals has raised the demand for more efficient and rapid screening techniques to analyse inhibition interactions [19].

Modern mass spectrometers have a capability to analyse multiple compounds simultaneously. This has been applied to inhibition studies, and several cocktail-type N-in-one-assays have been developed for screening CYP inhibitions. The most extensive of these is the LC-MS-MS assay developed by Tolonen *et al.* [20].

5. Case study: Quantitative diuron-specific data to help in the development of diuron-specific adjustment factors for interspecies differences and interindividual variability in toxicokinetics

The 100-fold uncertainty factor (UF) is used to convert a no-observed-adverse-effect level (NOAEL) from an animal toxicity study to a safe value for human intake (ADI). The UF contains a 10-fold interspecies difference (which is subdivided into a factor of $10^{0.6}$ (4.0) for toxicokinetics and $10^{0.4}$ (2.5) for toxicodynamics) and a 10-fold interindividual variation (which is divided equally into two subfactors each of $10^{0.5}$ (3.16)) [21]. Risk assessment has to be carried out using quantitative chemical-specific data, which will define the fundamental processes influencing toxicokinetics and toxicodynamics [22]. Moreover, the International Program on Chemical Safety (IPCS) proposes the use of chemical-specific toxicological data instead of default assessment factors, whenever possible [23]. The basic idea is to provide valuable toxicokinetic data to develop one or more of these subfactors with chemical-specific factors. The assessment factor may either increase or decrease and the differences may be considerable.

Practical examples of using *in vitro* techniques to generate *in vitro* toxicokinetic data for profenofos, as an organophosphorothioate insecticide, and diuron, as one of the phenylurea herbicides, have been published [24,25]. *In vitro* biotransformations and dispositions of diuron were studied in human hepatic homogenates, different types of mammalian hepatic microsomes (LM) including human and cDNA-expressed human P450 enzymes. Human liver samples were from 10 individuals of Caucasian race including four females and six males between the ages of 21 to 62 years. Intracerebral hemorrhage was the primary cause of death. More detailed characteristics of the liver samples are presented in our previous publication [24]. Metabolites were identified by LC-TOF and quantified by triple quadrupole mass spectrometry. The only metabolic pathway detected was demethylation at the terminal nitrogen atom, and the resulting N-demethylmetabolite had a higher none target toxicity than diuron [26]. The kinetic parameters V_{max} and K_m were calculated by nonlinear regression. These values were used to calculate intrinsic clearances (V_{max}/K_m) and to compare the efficiency of catalysis between species (the clearance in each mammalian microsome was divided by the clearance in humans) and between the ten individuals. Based on efficiency of catalysis obtained from quantitative human-derived techniques, the animal to human differences in toxicokinetics was 2.3-fold. In addition, the human variability in toxicokinetics was 1.5-fold restricted to Caucasian race, as defined by Renwick and Lazarus [21] as a variation between the mean and the highest value (Figure 1).

These values are less than the default, as would be expected. Our studies, although restricted to metabolic data from human and animal liver preparations, provide valuable quantitative diuron-specific data for risk assessment, which suggest that interspecies differences and interindividual variability in toxicokinetics are within the standard applied factors for species extrapolation in toxicokinetics. These factors could not be used in risk assessment without taking into account the toxicological evaluation of the active

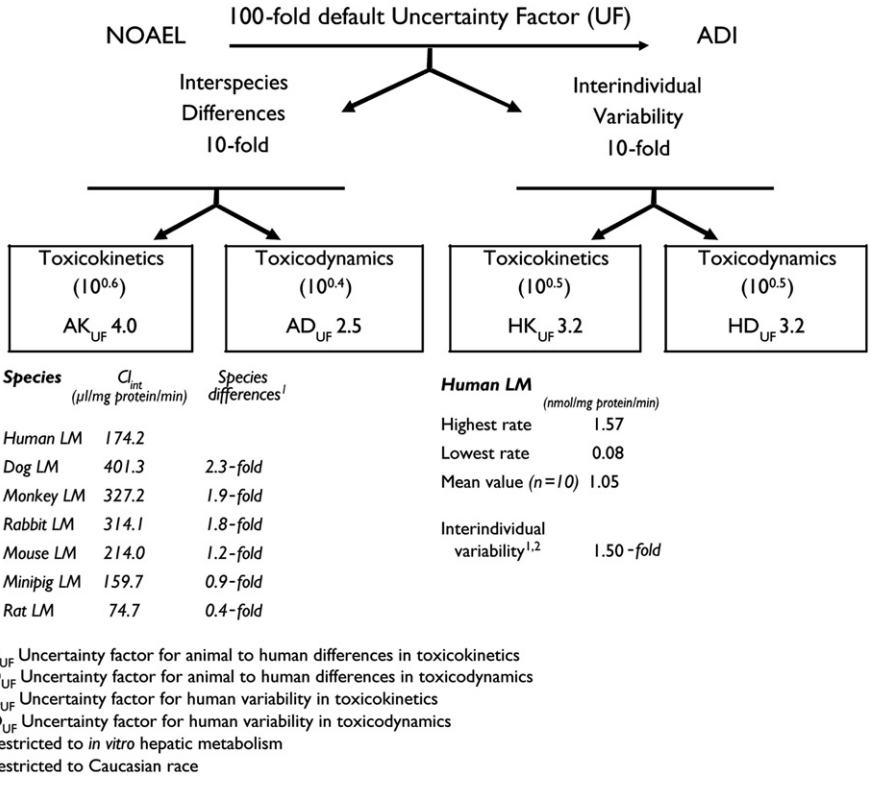

Figure 1. The subdivision of the 100-fold uncertainty factor showing the relationship between the use of uncertainty factors and proposed subdivisions based on toxicokinetics and toxicodynamics [21,23].

chemical moiety, e.g. their relative potency and elimination rates. Hence, while the data provided will be of value in risk assessment, other factors should be taken into consideration before deriving CSAFs. These results will be valuable in further defining the risks associated with exposure to diuron. It must be stressed that we measured only the hepatic metabolism of diuron, from which the above factors have been derived. On the other hand, metabolism is usually the most important factor contributing to interindividual and interspecies differences in toxicokinetics and thus, as a default, one could conclude that interspecies extrapolation with respect to diuron does not pose extraordinary uncertainties. Based on our finding, the tested animal species could be a good model for understanding and extrapolating the results of metabolism studies in a human health risk assessment.

6. Conclusions

Large numbers of enzymes are capable of metabolising pesticides into many different products. Both phase I and phase II xenobiotic metabolism pathways cannot be considered in isolation, but rather as part of a coupled, interactive system, interfacing

directly with many endogenous metabolic pathways. Human-derived techniques for measuring xenobiotics metabolism have been established for many different cell types and organs. For metabolism studies, the enzyme systems used at present clearly give reliable results, if their inherent restrictions are taken into consideration [4]. Each of the *in vitro* techniques can provide important information on the mechanisms of xenobiotic detoxification as well as bioactivation. Investigations with human-derived *in vitro* model systems to predict metabolic clearance, metabolites and metabolic pattern, metabolising enzymes and interactions are needed to improve human risk assessment. Together with the advantage of novel analytical techniques (LC/TOF-MS; LC/MS-MS and LC-NMR) for metabolite identification and quantification, the development of quantitative chemical-specific assessment factors can be achieved.

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References

[1] L.K. Low, in *Wilson and Gisvold's Textbook of Organic Medicinal and Pharmaceutical Chemistry*, edited by J.N. Delgado and W.A. Remers (Lippincott-Raven, Philadelphia, 1998), pp. 43–122.
[2] O. Pelkonen, M. Turpeinen, J. Hakkola, P. Honkakoski, J. Hukkanen, and H. Raunio, Arch Toxicol. **82**, 667 (2008).
[3] S. Rendic and F.J. Di Carlo, Drug Metab. Rev. **29**, 413 (1997).
[4] O. Pelkonen and H. Raunio, Expert Opin. Drug Metab. Toxicol. **1**, 49 (2005).
[5] O. Pelkonen, M. Turpeinen, J. Uusitalo, A. Rautio, and H. Raunio, Basic Clin. Pharmacol. Toxicol. **96**, 167 (2005).
[6] S. Ekins, B.J. Ring, J. Grace, D.J. McRobie-Belle, and S.A. Wrighton, J. Pharmacol. Toxicol. Methods **44**, 313 (2000).
[7] M.T. Skaanild and C. Friis, Pharmacol. Toxicol. **87**, 174 (2000).
[8] G.C. Moody, S.J. Griffin, A.N. Mather, D.F. Mcginnity, and R.J. Riley, Xenobiotica **29**, 53 (1999).
[9] O. Pelkonen and M. Turpeinen, Xenobiotica **37**, 1066 (2007).
[10] M.J. Gómez-Lechón, M.T. Donato, J.V. Castell, and R. Jover, Curr. Drug Metab. **5**, 443 (2004).
[11] K.P. Kanebratt and T.B. Andersson, Drug Metab. Dispos. **36**, 1444 (2008).
[12] I.D. Wilson, J.K. Nicholson, and J.C. Lindon, in *Handbook of Drug Metabolism*, edited by T.F. Woolf (Marcel Dekker, New York, 1999), pp. 523–550.
[13] T.L. Sheehan, Am. Lab. **34**, 40 (2002).
[14] S.T. Fountain, in *Mass Spectrometry in Drug Discovery*, edited by D.T. Rossi and M.W. Sinz (Marcel Dekker, New York, 2002), pp. 25–84.
[15] F.J. Gonzalez, S.Y. Liu, and M. Yano, Pharmacogenetics **3**, 51 (1993).
[16] M.S. Denison and J.P. Whitlock Jr, J. Biol. Chem. **270**, 18175 (1995).
[17] R. Wolbold, K. Klein, O. Burk, A.K. Nussler, P. Neuhaus, M. Eichelbaum, M. Schwab, and U.M. Zanger, Hepatology **38**, 978 (2003).
[18] E.F.A. Brandon, C.D. Raap, I. Meijerman, J.H. Beijnen, and J.H.M. Schellens, Toxicol. Appl. Pharmacol. **189**, 233 (2003).

[19] M. Turpeinen, J. Uusitalo, J. Jalonen, and O. Pelkonen, Eur. J. Pharm. Sci. **24**, 123 (2005).
[20] A. Tolonen, A. Petsalo, M. Turpeinen, J. Uusitalo, and O. Pelkonen, J. Mass Spec. **42**, 960 (2007).
[21] A.G. Renwick and N.R. Lazarus, Regul. Toxicol. Pharm. **27**, 3 (1998).
[22] K. Walton, J.L. Dorne, and A.G. Renwick, Food Chem. Toxicol. **39**, 667 (2001).
[23] WHO/IPCS. Chemical-specific Adjustment Factors for Interspecies Differences and Human Variability: Guidance Document for Use of Data in Dose/concentration-response Assessment (World Health Organization, Geneva, 2005).
[24] K. Abass, P. Reponen, J. Jalonen, and O. Pelkonen, Pestic. Biochem. Physiol. **87**, 238 (2007).
[25] K. Abass, P. Reponen, M. Turpeinen, J. Jalonen, and O. Pelkonen, Drug Metab. Dispos. **35**, 1634 (2007).
[26] C. Tixier, P. Bogaerts, M. Sancelme, F. Bonnemoy, L. Twagilimana, A. Cuer, J. Bohatier, and H. Veschambre, Pest. Manage. Sci. **56**, 455 (2000).