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Review

Isolation, Determination and Sorption Modelling of Xenobiotics in Plant Materials

M. Michel[1], B. Buszewski[2]*

[1]Pesticide Residue Laboratory, Plant Protection Institute, 20 Węgorka St., 60-318 Poznań, Poland
[2]Chair of Environmental Chemistry and Bioanalytics, Faculty of Chemistry,
Nicolaus Copernicus University, 7 Gagarin St., 87-100 Toruń, Poland

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Abstract

Crop models use mathematical equations to simulate the physical and chemical processes that generally control the uptake, translocation, and sorption of xenobiotics in all part of plants. Each compartment is anatomically characterized and described by a series of mathematical equations.

Sample preparation, such as liquid extraction methods and solid-phase-based methods are presented. Analysis of xenobiotics are generally carried out by gas chromatography (GC) or liquid chromatography (LC) coupled to different detectors, especially to mass spectrometers (MSs) and hyphenated techniques that have become extremely developed in recent years. As an example the wheat plant, as a model to describe xenobiotic uptake by roots and sorption of xenobiotic in grain, is applied.

Keywords: pesticide residue, food, modeling, sample preparation, separation techniques

Introduction

Chemical substances, natural and xenobiotic – not native to living organisms, are distributed in the environment between compartments such as soil, water, air and biota. The anatomical and physiological features that enable plants to accumulate nutrients, water, and CO_2 (carbon dioxide) also make them vulnerable to contamination by these chemicals from those compartments [1]. Understanding the potential for toxic chemical uptake, accumulation, degradation, and loss by plants is an important health and ecological issue. Via the food chain, the accumulation of chemicals in plants endangers wild and domestic animals as well as humans. Understanding the uptake of chemicals is a complex problem illustrated by the large number of chemicals that must be considered, the large number and diversity of plants, and the complex interactions between soil/water/roots and air/leaves. Plant protection and along with it agrochemical operations are applied not only to agricultural, but also medicinal plants.

Scientific investigation has made these problems possible to understand, describe, and estimate the principal fate of chemicals in water, soil, and atmosphere, whereas despite their importance, the role of plants in the behavior of chemicals, sometimes biologically active substances, is much less understood.

The various processes that function simultaneously, the movement and behavior of xenobiotics within plants, are highly complex. Computer simulation models can be useful for better understanding the interplay between the numerous processes that determine xenobiotic allocation in a plant following uptake from soil and air.

Currently, a variety of models are available for predicting the uptake, translocation, and elimination of organic contaminants by plants [2-4]. The major concept adopted in these models is the partition–based theory [5]. These models range from simple deterministic risk assessment screening tools to more complex models that consider physical,

*e-mail: bbusz@chem.uni.torun.pl

chemical, and biological processes in a mechanistic manner. These models differ in terms of their scope, methodological approach, and complexity [6, 7]. The selection of an appropriate plant uptake model will therefore depend on the requirements of the assessment, the nature of the environmental media, and the duration of the source term.

Among these models, the more complex ones require extensive plant physiological data, which are difficult or expensive to obtain. There is a need for a model which can generate reasonably accurate predictions of the bioaccumulation of organic chemicals by plants, but which do not require extensive plant physiological data. For the plant uptake model EU authorities recently notified the usage of the PLANTX model developed by Trapp and Matthies [8] in the European Union System for Evaluation of Substances (EUSES). This model has successfully exhibited the most probable distribution of a xenobiotic pesticide in crops. In our investigation we have improved this model and a novel one was developed which aims at satisfying this need by accounting for chemical migration from soil into four plant compartments, namely root, stem, leaf and seed. The model is applicable to the agricultural plant wheat. Our aim is to develop the seed model too, which accounts for pesticide uptake with a new parameter expressing the pesticide loss by adsorption onto inner surface of xylem and will elucidate residual profiles of xenobiotic in seed.

In addition, the experiments provide new ideas for description of sorption and migration and improvements in methods of separation science, such as methods extraction and determination not only of complex agricultural and medicinal plant matrices, but also many chemical mechanism in the environment.

Mechanism of Uptake by Vegetation

There are several pathways through which xenobiotics enter vegetation. The chemical may enter the plant by partitioning from contaminated soil to the roots and may also enter vegetation from the atmosphere by gas-phase and particle-phase deposition onto the waxy cuticle of the leaves or by uptake through the stomata and then be translocated in the plant [9]. Fig. 1 presents anatomy for monocot and dicot plants.

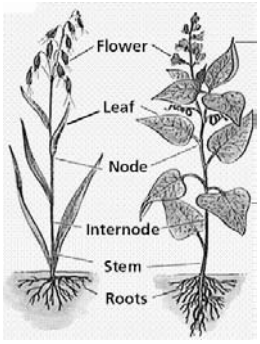

Fig. 1. Comparison of monocot (left, oat) and dicot (right, bean) anatomy [45].

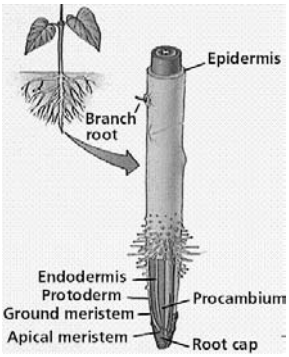

Fig. 2. View of the structure of the root and root meristem [45].

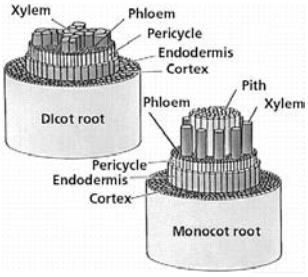

Fig. 3. Diagram illustrating the tissue layers and their organization within monocot and dicot roots [45].

Roots

Long-distance transport of xenobiotics within the plant occurs in its vascular tissues. Figs. 2 and 3 present structure and organization of tissue layers in monocot and dicot roots. The xylem is involved in the rapid movement of water and ions from the roots to the shoots. Movement within this system is normally in an upward direction, to the stems and leaves with the transpiration stream. The direction of movement in the phloem is more complex. This tissue distributes food produced by mature leaves (sources) to roots and rapidly growing regions of the shoot (sinks). Thus, movement is away from mature leaves toward the roots, but also toward the young, growing leaves as well as flowers, fruits or seeds if they are developing.

Phospholipid Bilayer – the Basic Structural Unit of Membranes

Membranes are vital because they separate the cell from the outside world. They also separate compartments inside the cell to protect important processes and events. Membranes are selective barriers and function as transport systems.

Despite the variable compositions of biological membranes, the basic structural unit of virtually all biomembranes is the phospholipid bilayer. The view of membrane model is presented in Fig. 4. This bilayer is a sheet-like structure composed of two layers of phospholipid mole-

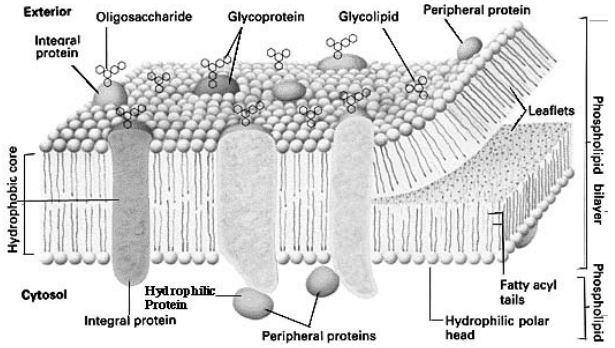

Fig. 4. View of membrane model [46].

cules whose polar head groups face the surrounding water and whose fatty acyl chains form a continuos hydrophobic interior. Each phospholipid layer in this lamellar structure is called a leaflet [10].

Knowledge of construction, architecture, composition and properties of natural membrane inspired scientists to search for new solutions and possible advantages in separation sciences. High performance liquid chromatography is the most frequently used separation technique. This method gives possibility of investigation between complicated interactions of analytes – the stationary phase which mimics biological membranes. Investigation of models imitating natural membranes gives huge advantages, especially in range of the possibility of examining xenobiotics – membranes in plant protection, toxicology, pharmacology without interference in natural systems.

Xenobiotic partitioning into biomembrane lipid bilayers is essential for diffusion of xenobiotics across the membranes, which is a major pathway for xenobiotic adsorption. In partitioning studies, stationary phases with chemically bound individuals entering in composition of biological membranes (phospholipids, glicolipids, steroids – cholesterol in animal tissue and sitosterol, campesterol or stigmasterol in plant) have been used as membrane models. Chromatographic systems have been designed to facilitate the analysis. Compared with the phospholipid bilayers, an immobilized artificial membrane (IAM), chemically immobilized alkylamide chains and immobilized molecule of cholesterol – such stationary phases are the most popular and closely resemble biological membrane. Pidgeon described [11, 12] a new packing generation of IAM for biochemical separations. IAMs contain different types of phospholipid monolayers covalently bound to the silica particles and this material appears to provide a more reliable and convenient model of natural membranes.

Utilization of lipids as a new generation of stationary phases in HPLC and related techniques such as solid-phase extraction (SPE), capillary zone electrophoresis (CZE) or/and capillary electrochromatography (CEC) offers the possibility for prediction of diffusion and transport processes of xenobiotics across the phospholipid bilayer barrier and the possibility for bonding with the surface of the membrane. There are possible chemical structures of the stationary phase materials in Fig. 5.

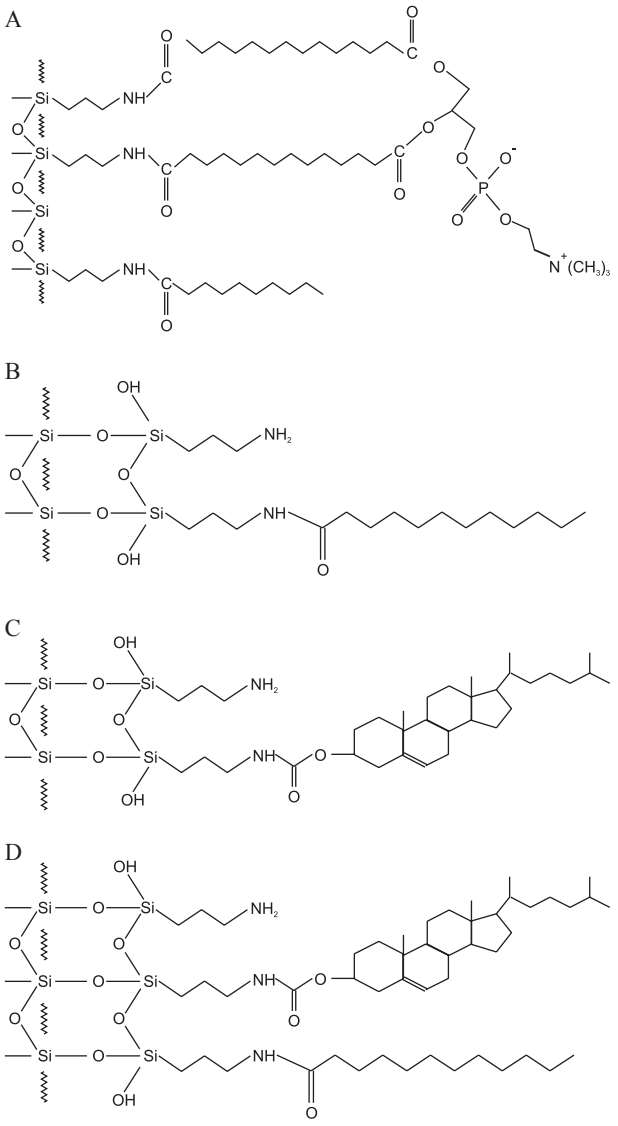

Fig. 5. Possible chemical structures of the stationary phase materials: (A) IAM, (B) AP, (C) CHOL, (D) MIX CHOL/AP [13].

Packings proposed by Buszewski et al. [13, 14] have properties resembling those of natural systems. Near the residual silanols and non-blocked -NH_2 groups, these phases contain N-acylamino groups built into the hydrophobic chain, also cholesterol bonded to a silica matrix. These materials were used to separate basic xenobiotics, antibiotics, amines, proteins, and hydrocarbons with diverse stereogeometries.

Leaves

Leaves of all plants are protected by the cuticle. It is the non-living outermost covering of high plants, and the initial barrier to the movement of foliar applied systemic chemicals. A diagram of the leaf structure is presented in Fig. 6. The cuticle consists of a surface coating of epicuticular wax underlayed by a mixed sub-

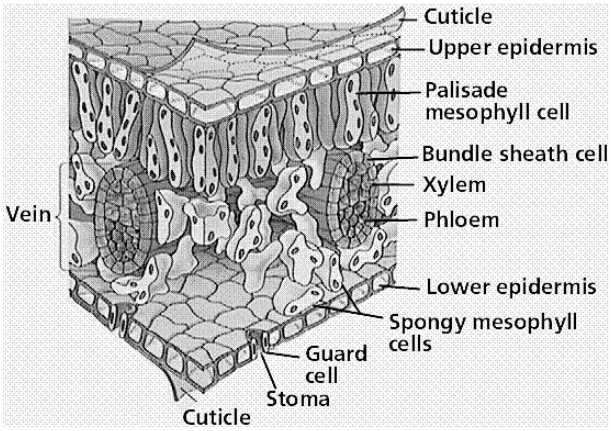

Fig. 6. Diagram of leaf structure.

strate of cutin and wax. The wax portion of the cuticle is lipophilic and helps reduce water loss from the leaf. Cutin is a hydrophilic substance providing the foundation for the cuticle. Under the cuticle rests the cell wall, a mixture of cellulose, chemicellulose and other hydrophilic substances. The final barrier is the cell membrane (plasma membrane), a lipophilic structure that controls movement of materials in and out of plant cells. Xenobiotic absorption in leaves is driven by the concentration gradient between the leaf surface and leaf interior. In order to be absorbed into the leaf, the xenobiotic must be able to move through both lipophilic and hydrophilic substances.

Theory – Mathematical Formalism

Factors Affecting Chemical Uptake and Distribution

These factors [15] within plants have been shown to depend on:

- physical-chemical properties of the compound such as molecular weight, water solubility, vapor pressure, octanol-water partition coefficient, and Henry's law constant;
- environmental characteristics such as temperature, wind speed, and organic, water and mineral content of soil;
- the techniques used for the application of the active ingredient and formulants – the form and distribution of the spray deposit, and variations in spray parameters such as dose, droplet size and number, concentration and application volume;
- plant characteristics (anatomical, physiological and biochemical) such as the nature and dimensions of roots, stem, fruits or seeds and foliage, xylem / phloem connections, membrane permeability, chemical composition of plant tissue, especially lipid, wax and water contents and metabolic capability, apoplast, symplast and vascular sap pH.

Mathematical Overview

In the model the following processes must be considered [8]:

- diffusive exchange in soil water and air pores to roots
- transfer into roots with transpiration stream
- translocation into stem and leaves via the transpiration stream
- partition into the stem
- transport into fruits via the assimilation stream
- diffusive exchange between air and leaves via stomata and cuticle
- metabolism
- dilution by growth.

There are many mathematical relationships developed for the model to describe xenobiotic movement into different compartments and across barriers based upon equation and they are in the basically approximate form which are given above [16-22].

Partitioning

Xenobiotic partition between liquids and the solid materials in the cell walls and membranes is in much the same manner as between two liquid phases such as *n*-octanol and water. Liquids contact plant tissues directly from irrigation, in soil, and from within the plant in the xylem and phloem sap and intercellular solution. This partitioning is described by the formula:

$$K_{pw}=C_p/C_w=(W_p+l_pK_{ow}^{\ b})\times\rho_p/\rho_w \quad (1)$$

K_{pw} - equilibrium partition coefficient between plant tissue and water;
C_p - equilibrium concentration in plant tissue (kg/m^3);
C_w - concentration in surrounding water or other solution (kg/m^3);
W_p - water fraction of the plant (ww., wet weight);
l_p - lipid fraction (ww., wet weight);
K_{ow} - partition coefficient between *n*-octanol and water;
b - exponent to correct differences between plant lipids and n-octanol
0.77 for macerated roots; 0.75 for cut pieces of roots and stems
0.95 for macerated shoots
0.97 for isolated cuticles;
ρ - density.

Uptake and Transport Kinetics

Xenobiotics in plants follow the same laws as other substances. Specific carriers for active uptake and translocation are likely only for naturally occurring (or similar) compounds. Usually, the uptake of xenobiotics is therefore passive, facilitated either by diffusion or by co-transport on flowing media.

Diffusive Uptake Into Roots

Chemical uptake from the external (soil) solution into the plant is driven by two forces: diffusion and mass flow. Diffusion occurs in air- and water-filled soil pores. A solution for Fick's law of diffusion to a cylindrical surface is given. Applying this to the transfer root-soil solution yields the formula:

$$N_{dr}=(K_{aw}\times D_{a,eff}+D_{w,eff})\times(C_w-C_r/K_{rw})\times 2\times L\times\pi/[ln(R_2/R_1)] \quad (2)$$

N_{dr} - sum of the diffusive flux of chemical to the roots in air- and water-filled pores (kg/s);
K_{aw} - partition coefficient of air to water (the dimensionless Henry's law constant);
K_{rw} - partitioning coefficient between roots and water;
$D_{a,eff}$ - effective diffusion coefficient in air-filled soil pores (m^2/s);
$D_{w,eff}$ - effective diffusion coefficient in water-filled pores (m^2/s);
C_w - concentration in the external (soil) solution (kg/m^3);
C_r - concentration in roots (kg/m^3);
L - total length of roots (m);
R_1 - radius of roots;
$R_2 - R_1$ - diffusion length;
R_2 - radius of a deficiency zone surrounding roots.

The radius is difficult to estimate, and default values R_2 and R_1 are used.

Uptake and Transport with the Transpiration Stream

Mass flow of solute with the transpiration stream N_t (kg/s) depends on the flow of transpiration water Q_w (m^3/s)

$$N_t=Q_w\times C_w \quad (3)$$

To enter the xylem, the xenobiotic must pass the symplast of the endodermis. Xenobiotic entry has been shown to correlate to its lipophilicity. The concentration ratio between the transpiration stream in the xylem and external solution is expressed as TSCF (transpiration stream concentration factor). Briggs et al. [23] found for barley plants:

$$\text{TSCF} = 0.784 \times \exp[-(\log K_{ow} - 1.78)^2 / 2.44 \quad (4)$$

The equation describes an optimum curve with a maximum TSCF of 0.784 at $\log K_{ow} = 1.78$. The concentration of a xenobiotic in the xylem solution C_{xy} (kg/m^3) is then

$$C_{xy} = \text{TSCF} \times C_w \quad (5)$$

and the translocation with the transpiration stream in the xylem to the stem, N_{tst} (kg/s), is

$$N_{tst} = Q_w \times C_{xy} = Q_w \times \text{TSCF} \times C_w \quad (6)$$

The fraction of the xenobiotic that enters the plant with the transpiration stream but is reflected at the endodermis remains in the roots:

$$N_{tr} = N_t - N_{tst} = Q_w \times (1 - \text{TSCF}) \times C_w \quad (7)$$

N_{tr} - mass flow of xenobiotic (kg/s) with the transpiration water remaining in the roots.

The following simplifying assumptions are made for the mass balance of the portion of chemical being translocated within the xylem into stem and leaves:

– When entering the stem, the transpiration stream concentration C_{xy} (kg/m^3) is determined by the concentration in soil water C_w and the TSCF as described above (Eqn. 5).
– When leaving the stem, the concentration in the transpiration stream C_{xy} (kg/m^3) is in equilibrium with the concentration in the stem C_{st} (kg/m^3), and C_{xy} is then

$$C_{xy} = C_{st} / K_{stxy} \quad (8)$$

K_{stxy} is the partition coefficient between stem and xylem sap (Eqn. 1). The flux into the stem, N_{tst}, is described above. The flux out of the stem, N_{tl}, and into the leaves is

$$N_{tl} = Q_w \times C_{st} / K_{stxy} \quad (9)$$

and is a loss from the stem. This description of the xylem transport and partitioning processes gives similar results to the formerly used concept of calculating explicitly the diffusion from the xylem sap into the stem, but it is advantageous because it uses fewer parameters (i.e., no xylem and stem radii).

Transport with the Assimilation Stream

The only considered transport in the phloem is the flux from leaves to stem and then into seeds. For the calculation of the phloem flux, similar assumptions are made as for the xylem flux:

In the leaf, the phloem sap is in equilibrium with the leaves, and the flux is from leaves into the stem:

$$N_{pst} = Q_p \times C_l / K_{lw} \quad (10)$$

N_{pst} - flux of the xenobiotic within the phloem from the leaves to the stem (kg/s);
Q_p - flow of the assimilation stream (m^3/s);
C_l - concentration in the leaves (kg/m^3);
K_{lw} - partition coefficient between leaves and water in the assimilation stream (Eqn. 1).

In the stem, the concentration of the xenobiotic in the phloem sap is equilibrated with the stem and comes to the same concentration as within the xylem sap (when leaving the stem).

The flux of xenobiotic from stem to seed, N_{pf}, within the phloem (kg/s) is

$$N_{pf} = Q_p \times C_{st} / K_{stxy} \quad (11)$$

and is treated as a loss from the stem.

Exchange: Leaves to Atmosphere

Substances other than water also volatilize from leaves. When this process is slower than the vaporization of water, an accumulation in the leaves can occur. The process is diffusive and reversible. The total conductance of exchange, g_{total} (m/s), is composed of parallel conductances of stomata, g_s, and cuticle, g_c, and atmospheric conductance, g_a, in series to them:

$$1 / g_{total} = 1 / g_a + 1 / (g_c + g_s) \quad (12)$$

Methods to calculate these parameters have been described in literature. The flux between leaves and atmosphere, N_{la} (kg/s), can occur in both directions, depending on the concentration gradient:

$$N_{la} = A \times g_{total} \times (C_a - K_{aw} \times C_l / K_{lw}) = A \times g_{total} \times (C_a - C_l K_{la}) \quad (13)$$

A - leaf area (m^2);
C_a - concentration of xenobiotic in air (kg/m^3);
K_{la} - partition coefficient between leaves and air, calculated from the ratio K_{lw} / K_{aw}.

The calculation method needs only a few input parameters (humidity, temperature, transpiration, leaf area, molecular weight, K_{ow}, K_{aw}, and air concentration).

Metabolism

Plants are known to be very reactive environments for xenobiotic chemical metabolism, although not much is known about rates. After uptake of substances, transformation reaction may occur (oxidation, reduction, and hydrolysis), followed by conjugation reactions and the building as bound residues, such as via glucoside complex. Conjugates are often deposited in vacuoles and cell walls (bound residues). Because metabolite character cannot yet be predicted, metabolite fate is not addressed in this model. Metabolism rates are required as input and must be determined outside the model. First-order reaction is assumed with rate constants λ (1/s):

$$\delta C / \delta t = - \lambda \times C \quad (14)$$

and the half-life $t_{1/2}$ (s) is

$$t_{1/2} = ln\ 2 / \lambda \quad (15)$$

The model calculates the amount of metabolites formed, and in the case of ^{14}C experiments, the simulated metabolite concentration may be added to the concentration of the parent compound for a comparison to the experimental results. Because of the inability to predict the chemical characteristics of the metabolic products, the present model assumes the metabolites to be immobile and to stay in the plant organ where they were formed.

Equation System of the Model

The individual processes are combined to give the model for the calculation of uptake, translocation, accumulation, metabolic conversion, and volatilization of xenobiotics in plants. Four compartments are considered, namely roots, stem, leaves, and seeds. Reactions and fluxes within each compartment are assumed to be homogeneously mixed. Below are the equations describing the mass balance of a chemical in the plant are given (Eqn. 16-20).

Roots

mass change = ± diffusion from / to soil (Eqn. 2)
+ mass flow within the transpiration stream (Eqn. 7)
– metabolism (Eqn. 14)

$$V_r \times \delta C_r\, \delta t = (K_{aw} \times D_{a,eff} + D_{w,eff}) \times (C_w - C_r / K_{rw}) \times 2 \times L \times \pi / [\ln(R_2 / R_1)] + Q_w \times (1 - TSCF) \times C_w - \lambda_r \times V_r \times C_r \quad (16)$$

Stem

mass change = + mass flow with the transpiration stream from soil (Eqn. 6)
– mass flow within the transpiration stream to leaves (Eqn. 9)
+ mass flow within the phloem from leaves (Eqn. 10)
– mass flow within the phloem to seeds (Eqn. 11)
– metabolism (Eqn. 14)

$$V_{st} \times \delta C_{st} / \delta t = Q_w \times (C_w \times TSCF - C_{st} / K_{stxy}) + Q_p \times \times (C_l / K_{lw} - C_{st} / K_{stxy}) - \lambda_{st} \times V_{st} \times C_{st} \quad (17)$$

Leaves

mass change = + mass flow within the transpiration water from stem (Eqn. 9)
± diffusive flux from / to air (Eqn. 13)
– mass flux within the phloem into the stem (Eqn. 10)
– metabolism (Eqn. 14)

$$V_l \times \delta C_l / \delta t = Q_w \times C_{st} / K_{stxy} + A \times g_{total} \times (C_a - C_l / K_{la}) - Q_p \times C_l / K_{lw} - \lambda_l \times V_l \times C_l \quad (18)$$

Seeds

mass change = + mass flow within the phloem from stem (Eqn. 11)
– metabolism (Eqn. 14)

$$V_f \times \delta C_f / \delta t = Q_p \times C_{st} / K_{stxy} - \lambda_f \times V_f \times C_f \quad (19)$$

Growth

The growth of plants has a diluting effect. From the law of mass conversation it follows that

$$C_0 \times V_0 = C_t \times V_t \quad (20)$$

C_0 and V_0 – concentration and volume at time zero;
C_t and V_t – concentration and volume at time *t*.

Modern Hyphenated Analytical Techniques for Plant Analysis

A lot of pesticides as xenobiotics are applied widely to protect plants from disease, weeds and insect damage. The development of a complete analytical method includes a number of steps involving sample storage, sample preparation, separation and isolation of analytes, their identification and, finally, their quantification. Particular attention has to be paid to the critical steps of sample preparation and isolation of analytes, because they may be possible sources of imprecision. But attention also has to be given to the method of separation, the stationary and mobile phases, and the detection method chosen.

Sample Preparation

The principal objectives of sample preparation for chromatographic and electrophoretic analysis are dissolution of the analytes in a suitable solvent and removal of as many interfering compounds as possible from the solution [24-26]. Plant sample preparation techniques can be categorized into mechanical, digestive or extractive instruments. Plant tissue samples, although solid, should be considered highly aqueous in nature, a characteristic that can be exploited to rupture cells within the tissue matrix. Overview of the sample preparation techniques in plant analysis are presented in Fig. 7.

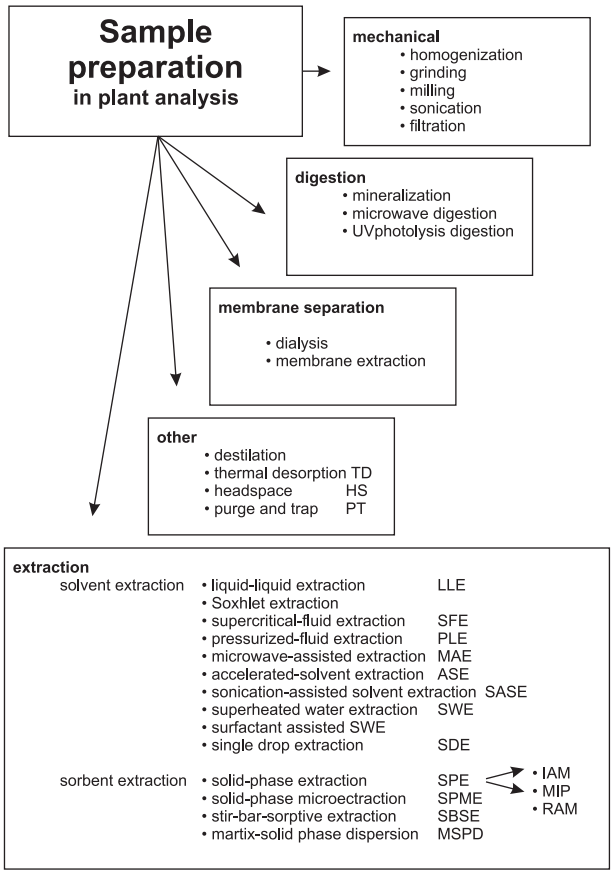

Fig. 7. Overview of sample preparation techniques in plant analysis.

Extraction of Plant Constituents

The extraction methods and strategies used depend on whether the aim of the extraction is analytical or preparative and whether the plants to be extracted contain known compounds where thermal stability may be important. Even with the same technique of extraction for different active compounds in different plant materials, different operating conditions, such as solvent use, temperature and more may be required. The nature of both plant materials and the bioactive components should be considered in order to achieve good extraction efficiency. Lipophilicity or hydrophilicity affects the solubility of a xenobiotic in the extracting solvent and, conversely, polarity of a solvent also has an impact on extraction efficiency.

The other problem for the selection of methods of extraction is that when most active compounds are present during the uptake by plants, significant analyte–matrix interaction will be present, hence spiking of the target compounds into the plant matrix will not mimic the real environment and could be difficult to overcome and predict. Depending on how the method is validated, it may be possible to have a method with high recovery but lacking accuracy.

The extraction and recovery of a solute from a plant matrix can be regarded as a five-stage process:

i) the desorption of the compound from the active sites of the matrix;
ii) diffusion into the matrix itself;
iii) solubilization of the analyte in the extractant;
iv) diffusion of the compound in the extractant;
v) collection of the extracted solutes.

In order to obtain quantitative and reproducible recoveries, careful control and optimization strategy of each step are required and will strongly depend on the nature of the matrix to be extracted; in particular, the collection of the extract needs to be carefully controlled as it is often neglected when compared to the extraction step.

The traditional extraction techniques for plant matrices include the well known liquid-liquid extraction (LLE), Soxhlet extraction, sonication, and blending. Even though efficient extractions may be achieved using these simple

techniques, they present major drawbacks, namely long extraction times (especially for Soxhlet), high solvent consumption and low temperatures, and have yet to be readily automated. In addition, the final extracts usually require subsequent concentration and clean-up prior to the analysis step, as well as filtration in the case of sonication and blending methods, which may lead to losses or contamination.

Mechanical techniques might do little to disrupt cellular structure and extract analytes from non-vascularized or low-water-content plant tissues. Extreme measures such as digestion with strong acid (mineralization) are used routinely. Alternatively, analysts can use microwaves to digest such samples.

The selective nature of membranes has made them a unique alternative to solvent extraction for sample clean up, especially if coupled with chromatographic techniques. The relative sizes of different molecules largely determines the permeation selectivity of a membrane in the absence of strong specific interactions. The main advantages over solvent extraction are the use of high ratio between surface area and volume, the lack of emulsions and no phase separation step.

During the last decade, new techniques have emerged that will supersede traditional techniques. These modern techniques include solid-phase microextraction (SPME), supercritical-fluid extraction (SFE), pressurized-liquid extraction (PLE), microwave-assisted extraction (MAE), accelerated-solvent extraction (ASE), and others. A comparison of different analytical extraction methods for plant material are presented in Table 1. More detailed descriptions of the basic principles of these modern sample preparation techniques for the extraction of plant materials in general are available in number of excellent review articles recently appearing in the literature [27-29].

Current methodology involves recently determined miniaturized techniques, such as headspace solid-phase microextraction (HP-SPME), liquid-phase microextraction (LPME) or matrix-solid phase dispersion (MSPD). MSPD that homogenized plant matrices with water–different sorbent and eluting with appropriate solvents is used to extract target compounds. Compared with classical methods, the MSPD procedure is simple and less labor intensive, and it does not require preparation and maintenance of equipment. Although these techniques seem to provide good results, we should be cautious because there are still too few reports to establish their usefulness, criticize them or compare them with other techniques.

Purification of Plant Constituents

After solid–liquid extraction, the next step in sample preparation is the purification of the raw extract, which is a crucial for plant matrices. During the extraction step many interfering (mainly organic) components are co-extracted from plant samples together with target analytes. The aim of the clean-up stage is to remove these substances that can interfere with the identification and the quantitation of target analytes.

Traditional liquid–liquid partitioning clean-up has clearly been displaced from analytical procedures. Solid-

Table 1. A brief summary of the experimental conditions for various methods of extraction (NA – not applied).

Extraction method	Common solvents used	Temperature (°C)	Pressure applied	Time required	Volume of solvent required (mL)
Soxhlet extraction	Methanol, ethanol, or mixture of alcohol and water	Depending on solvent used	NA	3–18 h	150–200
Sonication	Methanol, ethanol, or mixture of alcohol and water	Can be heated	NA	1 h	50–100
Solid-phase microextraction (SPME)	NA	40-100	atmospheric	20-60 min	NA
Supercritical fluid extraction (SFE)	Carbon dioxide or carbon dioxide with modifiers, such as methanol	40–100	250–450 atm	30–100 min	NA
Microwave assisted extraction (MAE)	Methanol, ethanol, or mixture of alcohol and water	80–150	Depending on if it is closed or opened vessel extraction	10–40 min	20–50
Accelerated solvent extraction, static (ASE)	Methanol	80–200	100 bar	20–40 min	20–40
Pressurized liquid extraction, dynamic (PLE)	Methanol	80–200	10–20 bar	20–40 min	20–30
Superheated water extraction (SWE)	Water or water with 10–30% ethanol	80–300	10–50 bar	40–50 min	40–45
Surfactant assisted SWE	Water with surfactants, such as Triton X100 or SDS	80–200	10–20 bar	40–50 min	40–45

Table 2. Comparative study of SPE clean-up techniques.

SPE technique	Characteristics	Advantages	Disadvantages
Polar-SPE (silica, alumina, Florisil)	Applicable to organic extracts	Good clean-up for most apolar pesticides, such as organochlorine and some organophosphorus compounds	Not suitable for clean-up of compounds covering a wide polarity range
C18-SPE	Applicable to aqueous extracts	On-line coupling with LC; Analyte enrichment; Allows retention of a wide variety of analytes with different polarities; Low cost and low organic solvent consumption; Simple to use; Well-known technology; Wide variety of formats for consumables	Tendency to plug; Low pH of the aqueous solution required to retain acidic herbicides; Modified silicas do not resist extreme pH; Partial removal of co-extracted compounds; It is difficult to co-extract compounds with polarities that are too different
Graphitized black carbon (GBC)	Applicable to aqueous extracts	Analyte enrichment; Retains acidic herbicides at any pH	Not suitable for on-line application because it is not pressure resistant; Partial removal of co-extracted compounds
Ion-exchange column	Applicable to aqueous extracts	Ionic analytes	Recoveries influenced by the extract characteristics
SPME Carbowax-divinylbenzene (CW-DVB)	Applicable to aqueous extracts	Elimination of organic solvents; Automation of the process with possibility of coupling on-line with GC and LC	There is no knowledge of the effects that the matrix has on the process
MI-SPE	Works best with organic extracts	Highly selective for individual compounds or compound class; Stable at extreme pHs	Custom-made product developed for each analyte; Little usable for enrichment of many different compounds or unknowns

phase extraction (SPE), introduced in the 1970s, is still the dominant method for purification of plant extracts. A large number of sorbents are used to isolate organic compounds from the extracted solutions, including alumina, Florisil, ion-exchange resins, silica gel, many silica-based sorbents (e.g., octadecyl-, octyl-, phenyl-, and diol-bonded silica) and graphitized black carbon (GBC). Table 2 compares different SPE procedures. SPE lends itself particularly well to automation and is especially helpful when large numbers of samples have to be routinely purified for chromatographic analysis [30]. Pre-packed SPE cartridges may be used in one of two modes, such that the interfering matrix compounds of a sample are retained on the cartridge while the components of interest are eluted or *vice versa*.

Immunoaffinity extraction (IAE) and molecularly imprinted polymer (MIP)-based extraction are applied to SPE and SPME methods as specific, efficient sample preparation techniques [31]. These techniques, which are based on adsorption or partitioning of analytes, are responsible for removing the majority of the biological material of interest from the sample matrix prior to analysis.

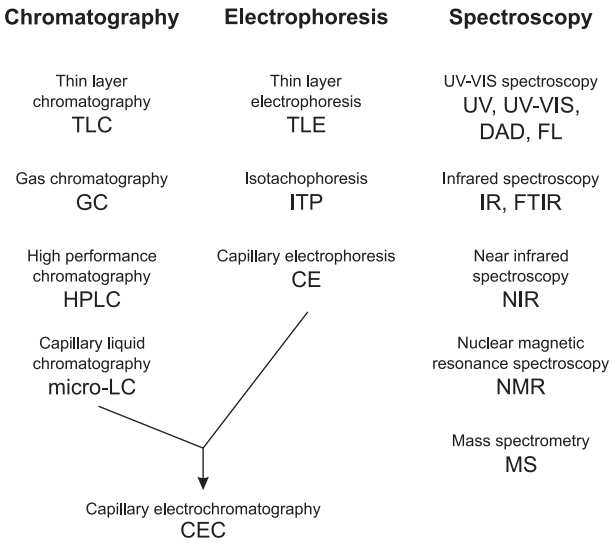

Fig. 8. Overview of analytical techniques used in phytomics.

Separation Methods

Today, analytical chemistry offers the opportunity to investigate the separation methods of plant matrices in different ways (Fig. 8) using [32]:

- chromatographic techniques, such as thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC) or gas chromatography (GC);
- electrophoretic techniques, such as thin-layer electrophoresis (TLE), isotachophoresis (ITP) or capillary electrophoresis (CE);
- combined methods, such as capillary electrochromatography (CEC);
- spectroscopic methods, such as mass spectrometry (MS), nuclear magnetic resonance (NMR), ultraviolet (UV), visible (VIS), infrared (IR) or near-infrared (NIR).

Table 3. Comparative study of different analytical techniques to determine pesticide residues.

Technique	Advantages	Disadvantages	Solutions
GC	High resolving power and ability to resolve individual analytes; High sensitivity and good selectivity with element-selective detectors	Inadequate for polar, thermo-labile and low volatility compounds; High consumption of expensive, high-purity gases	Derivatization (currently used for only glyphosate and some metabolites)
GC/MS	High resolving power and ability to resolve individual analytes; High sensitivity and selectivity; Existence of mass spectrum libraries for screening unknown samples	Inadequate for polar, thermo-labile and low volatility compounds; High consumption of expensive, high-purity gases	Derivatization (currently used for only glyphosate and some metabolites)
LC-UV	Application to virtually any organic solute, regardless of its volatility or thermal stability; Compositions of both mobile and stationary phase are variable; Can be automated and miniaturized (microchip technology); Low price, simplicity, robustness and large linear range	Insufficient separation efficiency and selectivity; Large amounts of expensive, toxic, organic solvent used as mobile phase; Lack of matrix interferences	Development of more efficient and selective materials for clean-up and separation (immunosorbents, MIPs and restricted access materials)
LC-Fluorescence	High separation efficiency	Few compounds are fluorescent	Derivatization (reported to determine only N-methylcarbamates using o-phthaldehyde and mercaptoethanol)
LC–MS	Application to virtually any organic solute regardless to its volatility or thermal stability; Compositions of both mobile and stationary phase are variable; Can be automated and miniaturized (microchip technology)	Strongly affected by matrix interferences (ion enhancement and, most often, ion suppression can be expected); Identification difficult using interfaces that provides soft ionization; Lack of spectral libraries	Development of good separations and sample clean-up; Use of isotopically labeled standard; Tandem MS (MS/MS)

Determination

GC and HPLC methods have been published for the determination of different classes of pesticides in plant origin samples. Table 3 summarizes their advantages and disadvantages [32].

Gas Chromatography

Pesticide residues have often been analyzed by GC with nitrogen–phosphorus detection (NPD) or electron-capture detection (ECD). Moreover, GC/MS has been used in multi-residue methods for pesticide analysis. GC is very useful for simultaneous determination of several pesticides at trace levels, and, in general, higher sensitivity can be obtained using GC rather than HPLC.

Comprehensive two-dimensional gas chromatography (GC × GC) is currently receiving widespread attention for the analysis of complex samples [33.34]. This is due to the fact that in GC × GC very high peak capacities can be obtained. Peak capacity is an important measure of separation, and in comprehensive two-dimensional GC, interesting separations are obtained not only under orthogonal, but also under non-orthogonal conditions – i.e., with polar × less/non-polar column combinations. The heart of the GC × GC process is the presence of a modulator between the two dimensions. The function of the modulator is to trap, refocus and then release continuous fractions of the primary column effluent onto a shorter "fast" column [35].

High Performance Liquid Chromatography

HPLC is ideally suited for the analysis of polar compounds. As most researchers would agree, HPLC is perhaps the most popular and reliable system among all chromatographic separation techniques for the separation of plant matrices. The versatility of HPLC is also aided by the different separation modes and types of detection methods, among which is the diode array detector (DAD) coupled with mass spectrometer (MS).

UV–VIS spectrophotometry has long been used for quantitation of organic compounds that absorb light in the ultraviolet and visible region. The application of UV–VIS detector in separation technologies, particularly the combination of DAD and HPLC, has gone beyond quantitation by light absorbance. HPLC–DAD has played important roles in the identification of analytes. In HPLC–DAD the spectral information of known standards can be obtained online and saved as a library database. The UV–VIS spectral data of all eluting peaks of a sample can be scanned, stored and later retrieved for comparison with

the library data. A match of both UV–VIS spectrum and retention time can lead to highly positive identification of an active compound.

As a detector, DAD is also capable of simultaneously detecting and recording chromatograms at different wavelengths. This feature significantly enhances the performance of the separation system, particularly when different groups of compounds are mixed in one sample. When proper wavelengths are chosen, e.g. at maximum absorptions, all groups of compounds can be detected with the highest sensitivity. An appropriate selection of the detection wavelength can also make possible the quantification of an unresolved or poorly resolved peak. DAD can also be used to examine the purity of a peak. In-depth discussion of HPLC–DAD and its use in identification of active compounds are beyond the scope of this paper, and there are several excellent reviews recently published if readers want to obtain further information.

There are many excellent recent reviews on the application of LC–MS in quantitative and qualitative analyses of pesticides in plant matrices [36, 37].

Capillary Electrophoresis

Capillary electrophoresis (CE) proved to be a powerful alternative to HPLC in the analysis of polar and thermally labile compounds. Reviews on the analysis of active compounds in complex matrix by CE are well reported [24.27]. Many publications show that all aspects of CE, such as capillary zone electrophoresis (CZE), micellarelectrokinetic capillary chromatography (MEKC) and capillary isoelectric focusing (cIEF), have been used for the separation of analytes from plant. The separation in CZE is based on the differences in the electrophoretic mobilities, resulting in different velocities of migration of ionic species in the electrophoretic buffer in the capillary. For MEKC, the main separation mechanism is based on solute partitioning between the micellar phase and the solution phase. Factors that are known to affect separation in CZE and MEKC include pH of running buffer, ionic strength, applied voltage and concentration and type of micelle added.

Hyphenation Procedures

The use of chromatographic separation with mass spectrometry for chemical characterization and composition analysis of plants has been growing rapidly in recent years. Reviews on the use of mass spectrometry and high-performance liquid chromatography mass spectrometry (HPLC/MS) on plants had been reported [38, 39].

The use of hyphenated techniques, such as high resolution gas chromatography mass spectrometry (HRGC/MS), high performance liquid chromatography/mass spectrometry (HPLC/MS), liquid chromatography tandem mass spectrometry (HPLC/MS/MS) and tandem mass spectrometry (MS/MS) to perform on line composition and structural analyses provide rich information that is unsurpassed by other techniques.

The use of HRGC/MS, remains the method of choice for the analysis of volatile and semi-volatile components, such as essential oil and others in plant matrices.

For the analysis of components present in plant matrices, HPLC/MS has been playing an increasingly significant role as the technique is capable of characterizing compounds that are thermally labile, ranging from small polar molecules to macromolecule, such as not only pesticides, but also peptides/proteins, carbohydrates and nucleic acids. The most common mode of ionization in HPLC/MS included electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI). Mass analyzers, such as single quadruple, triple quadruple, ion-trap, time-of-flight, quadruple time-of-flight (Q-TOF) and others, are used.

With tandem mass spectrometry, additional structural information can be obtained for the xenobiotic and natural products in complex matrices such as crude plant extracts. They represent a strategic element to avoid finding known constituents and to target the isolation of almost all bioactive compounds. These methods also give a unique possibility to study unstable compounds which rapidly degrade or which are not separable at a preparative level. However, methods using HPLC/MS is still limited to conditions that are suitable for MS operations. There are restrictions on pH, solvent choice, solvent additives and flow rate for HPLC in order to achieve optimal sensitivity.

LC-hyphenated techniques are also extremely useful for metabolomic studies. Indeed the use of LC-MS, in particular, in conjunction with efficient comparison algorithms, permit the precise detection of small metabolic modifications that occur upon stress induction in plants [27]. Fig. 9 shows schematic representation of the experimental setup used for LC-hyphenated analyses in phytochemistry [39].

New Approaches to Microseparation Systems

Whilst reversed stationary phases are the most commonly used materials, the development of new stationary

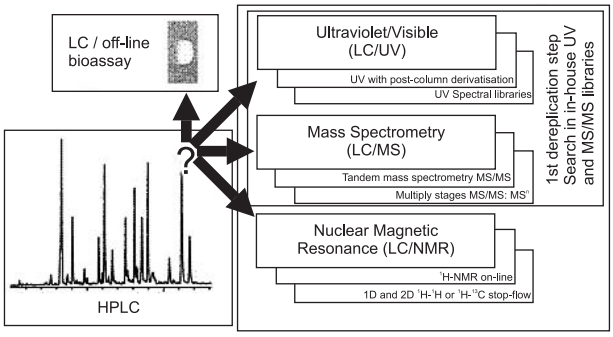

Fig. 9. Use of LC-hyphenated techniques in phytochemistry as strategic analytical tool [39].

phases for higher efficiency and selectivity to analytes in solid-phase extraction (SPE) as well as for analytical use in HPLC, μLC or CEC is of great interest in this field. The trend towards miniaturized separation systems is a target not only in proteomics and genomics, but also in phytochemistry, or phytomics, as we call it. Microseparation systems, such as μLC, offer a useful tool for fast, economic analysis, because of the very small inner diameter (i.d.) of separation columns (70 mm x 0.2 mm i.d.), low flow rates (2-2.5 μL/min) and low injection volumes (0.5 μL) [32].

Application of Model Prediction and Hyphenated Techniques – Uptake of Xenobiotics by Wheat Plants from Hydroponic Solution

Wheat was sown to cultivation soil and grown in a greenhouse and later outdoors. Plants at an appropriate growth stage of seed bearing were carefully taken out from the cultivation soil, and their roots were thoroughly washed with running tap water prior to being used in the experiment. A 168-h exposure chamber experiment measuring the uptake of carbendazim into wheat roots, stems, leaves and seeds from exposure via a hydroponic solution was conducted. This experiment was selected as it contains data on how plant concentrations change over time and it provides information on plant characteristics. The roots of plants were completely dipped into the solution. Direct chemical transfer from the solution to the air compartment was prevented and covered in this experiment by the use of an aluminum foil. Chemical concentrations were measured only in the plant seeds and chaff [40-42].

One of the chemicals used was carbendazim, systemic fungicide, methyl benzimidazole carbamate (MBC) with protective and curative action [43]. Benomyl and thiophanate-methyl are closely related fungicides and function by the generation of carbendazim. It acts by inhibiting development of the germ tubes, the formation of appressoria, and growth of mycelia. They are registered for use in various crops, for example in cereals (barley, wheat, winter rye), fruit (pome, stone, citrus, currants, strawberries, bananas, pineapples, mangoes, avocados, papaws, etc.) and stored fruit, vines, hops, vegetables (Brussels sprouts, beans, lettuce, tomatoes, cucumbers, pepper, celery, onions, cabbage, potatoes), ornamentals, coffee, cotton, rice, flax, beet, sugar cane, peanuts, rape seed, cucurbits, rubber, tobacco, turf, mushrooms, and other crops.

Carbendazim disrupts the production of sperm and damages testicular development in adult rats, probably partly through disrupting the assembling of cells in tissues which is the same way as carbendazim works as a fungicide. In addition, carbendazim is also a teratogen--damaging development of mammals in the womb. Experiments have shown that exposure of developing rats in the womb leads to deformities such as lack of eyes and hydrocephalus ("water on the brain"). It has been firmly established that direct binding of carbendazim to tubulin is required for the toxic effects of the methylbenzimidazoles. Through this mechanism carbendazim and its precursors provoke toxic effects on reproduction and induce numerical changes in chromosomes (aneuploidy) in mammalian cells *in vitro* and in bone marrow and male and female germ cells of rodents dosed *in vivo*.

The Specialized Experts [44] recommended in *Preliminary opinion of the Scientific Committee on Plants regarding the evaluation of benomyl, carbendazim and thiophanate-methyl in the context of Council Directive 91/414/EEC concerning the placing of plant protection products on the market*, that carbendazim should be classified as a Category 2 mutagen and a Category 2 reproductive toxicants. Opinion was adopted by the Scientific Committee on Plants on 7 March 2001.

Model of Wheat Plant

The model developed here is primarily designed to be fitted to experimental data in which an established plant (wheat) is exposed to a chemical (carbendazim) introduced into the hydroponic solution, and concentrations in plant tissues are monitored over a period of days. Validation by successful fitting and prediction can lead to credible expressions for partitioning, transport, and transformation processes and to confirmation of the required number and configuration of compartments. The plant model is constructed as illustrated schematically in Fig. 10.

The figure also represents a simplification of the pathways through which xenobiotics enter vegetation and the factors controlling mechanisms of chemical and physical

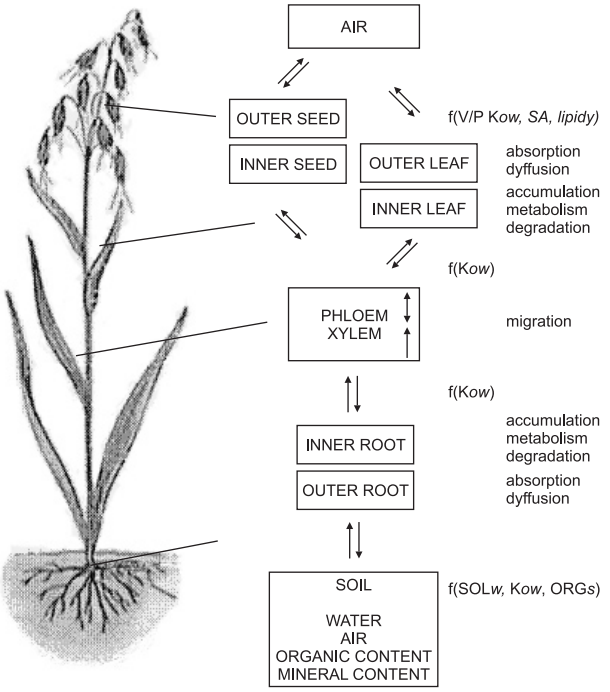

Fig. 10. Simplified illustration of plant model.

processes. In reality, vast differences exist between different plant species (about 250,000 higher plants) and more than 1,000 environmentally suspect chemicals. It cannot be expected that the approach will always work correctly. But hopefully, the equation can give some advice about the main processes. These pathways have been investigated using controlled laboratory exposure experiments.

Two bulk compartments, air and soil, surround the plant, which consists of four parts: root, stem, foliage and seed. The air compartment consists of two subcompartments of pure air and aerosol particles, and the soil consists of four subcompartments of air, water, organic and mineral matter.

Processes considered are the following: absorption, diffusion, migration, translocation to shoots, deposition on leaves, accumulation, metabolism and degradation processes, dilution by exponential growth. The main factors that affect the residual amount in plants are defined and presented in Table 4.

Perspectives

Further comparative studies of this sort need to be undertaken in order to clarify the organic chemical uptake, translocation, and elimination behavior of plant species and to enable authoritative conclusions to be reached over model theory and performance. High-quality independent data sets are still required, particularly for exposure durations equivalent to entire growing seasons. A broader range of plant species needs to be evaluated, and the results from these species must be incorporated into model theory. Important agricultural crops justify particular concerns. The models and experimental data sets should focus especially on the edible portions of the plants, as these are the most significant for risk assessment purposes. This will mean more detailed consideration of the plant fruit compartment and the reformulation of existing models. Quality information relating to model input parameters is another priority area. Model results can only be as accurate as the information from which they are derived.

Table 4. Parameters of plant model.

Parameter	Symbol	Unit
water solubility	Sol_W	L/kg
octanol-water partitioning coefficient	K_{OW}	-
octanol-air partitioning coefficient	K_{OA}	-
Henry's law constant	H	Pa×kg/mol
vapor-particle partitioning	V/P	-
organic concentration of the soil	Org_S	kg/kg
plant surface area	SA	m^2
plant lipid concentration	lipids	kg/kg

Conclusion

The theoretical framework presented in this paper provides the basic foundation for the development of a computer simulation model describing whole plant xenobiotic transport and allocation following root uptake. The individual mathematical relationships described will be linked in a multicompartment model and plant physiological processes, xenobiotic physiochemical parameters, and relevant environmental parameters can reasonably predict xenobiotic absorption, whole plant allocation, and metabolism following application.

Overall, the concept of utilizing computer simulation modeling to predict xenobiotic behavior following root uptake may prove useful in the rational chemical design of pesticides for enhanced uptake and improved whole plant distribution and effectiveness, and also in understanding these processes involved, maximizing and predicting the efficacy of applied xenobiotics and minimizing the hazard to human health and ecosystems.

The complexity of the processes involved, the high variability in plant morphology and physiology, and the potentially high number of chemicals with a wide range of properties are the reasons for the highly interdisciplinary nature of this research. It becomes inspiration for the scientists searching for new solutions, and a possible advantage in separation sciences. Modern highly sophisticated analytical methods are the most important to describe and understand biological processes: sample preparation and separation techniques, especially chromatographic techniques. This method makes possible investigations between complicated interaction analytes – the stationary phase, which mimics biological systems and could be applied in plant protection, toxicology, pharmacology without interference in natural systems. A universal model has been suggested which links the physico-chemical parameters describing the xenobiotics with the anatomical, physiological, and biochemical properties of the plant.

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