

In vitro metabolism of tebuconazole, flurtamone, fenhexamid, metalaxyl-M and spirodiclofen in *Cannabis sativa* L. (hemp) callus cultures

Leonie Hillebrands,^{a,b}  Marc Lamshoef,^{b*} Andreas Lagojda,^b Andreas Stork^b and Oliver Kayser^{a*}



Abstract

BACKGROUND: *Cannabis sativa* L. (hemp) is a medicinal plant producing various cannabinoids. Its consumption is legalized for medical use due to the alleged positive health effects of these cannabinoids. To satisfy the demand, *C. sativa* plants are propagated in contained growth chambers. During indoor propagation, pesticides usually are used to ensure efficient production. However, pesticide registration and safe application in *C. sativa* has not been investigated in detail.

RESULTS: With this study the metabolic degradation of pesticides in recently established *C. sativa* callus cultures was examined. Tebuconazole, metalaxyl-M fenhexamid, flurtamone and spirodiclofen were applied at 10 μM for 21 days. Results were compared with metabolism data obtained from *Brassica napus* L., *Glycine max* (L.) Merr., *Zea mays* L. and *Triticum aestivum* L. callus cultures as well as in metabolism guideline studies. The successfully established *C. sativa* callus cultures were able to degrade pesticides by oxidation, demethylation, and cleavage of ester bonds in phase I, as well as glycosylation and conjugation with malonic acid in phase II and III. Initial metabolites were detected after Day (D)7 and were traced at D21.

CONCLUSION: The resulting pathways demonstrate the same main degradation strategies as crop plants. Because metabolites could be the main residue, the exposure of consumers to these residues will be of high importance. We present here an *in vitro* assay for a first estimation of pesticide metabolism in *C. sativa*.

© 2021 The Authors. *Pest Management Science* published by John Wiley & Sons Ltd on behalf of Society of Chemical Industry.

Supporting information may be found in the online version of this article.

Keywords: *Cannabis sativa* L.; *in vitro* callus cultures; hemp; metabolism; pesticide; testing for residues

1 INTRODUCTION

Since *Cannabis sativa* L. has been legalized for medical use in several countries, its demand has been rising heavily.¹ It is known that 120–220 million users consume *C. sativa* products annually. In addition to the legal uses, *C. sativa* is the most consumed illegal drug worldwide and often is produced by local and small growers.²

Δ^9 -tetrahydrocannabinol (THC) is a psychoactive cannabinoid produced by *C. sativa* which can be used to mitigate several symptoms of a broad spectrum of diseases, such as chronic pain or spasms due to multiple sclerosis.^{3,4} Nonpsychoactive cannabinoid products such as cannabidiol (CBD) are claimed to be relevant as food additives owing to alleged positive health effects. Thus, the production and consumption of THC and CBD from *C. sativa* is demanding increasing attention.^{5,6}

Different defense mechanisms allow the detoxification of absorbed xenobiotics in plants. Oxidation in phase I via insertion of a functional hydroxy moiety is conducted by the enzyme

superfamily of cytochrome P450 monooxygenases.^{7,8} Subsequently, phase I metabolites are in phase II covalently linked to hydrophilic molecules, such as glucose and malonic acid.⁹ In addition to the rare but known processes of excretion, the plant mainly compartmentalizes metabolites in vacuoles or apoplasts within phase III.^{7,10} These mechanisms describe the general detoxification process of xenobiotics in plants, yet the specific pathways in *C. sativa* are mostly unknown.

* Correspondence to: M Lamshoef, Bayer AG Division CropScience, Alfred-Nobel-Str. 50, 40789 Monheim, Germany, E-mail: marc.lamshoef@bayer.com (Lamshoef); or O Kayser, Emil-Figge-Str. 66, 44227 Dortmund, Germany. E-mail: oliver.kayser@tu-dortmund.de (Kayser)

a Faculty of Biochemical and Chemical Engineering, Chair of Technical Biochemistry, TU Dortmund University, Dortmund, Germany

b Bayer AG Division Crop Science, Monheim, Germany

During growth, harvest, storing or processing of *C. sativa* plants, contamination with hazards including bacteria, fungi, heavy metals or chemicals is possible. Contamination can trigger adverse effects, such as lung infection, allergies and asthma, as well as nervous disorders as headache, dizziness, tremor, discoordination and convulsion.^{11,12}

As a consequence of the increasing demand for cannabinoids, commercial *C. sativa* production faces new challenges. As a result of regulatory restrictions, to avoid theft and to ensure constant THC levels, *C. sativa* generally is grown indoors. Although the contained growth pattern may protect against certain pathogens and pests, there is still a risk for contamination, particularly in the case of fungi and insects.^{13,14} Several pest species of relevance to *C. sativa* have been identified: insects including Aphidoidea (Aphids) and Thysanoptera (thrips), Tetranychidae (spider mites) arachnids, and the fungal pathogens *Fusarium oxysporum*, *Botrytis cinerea* and particularly powdery mildew.^{13–15} Growers thus could have a need for insecticides and fungicides, whereas herbicides are not relevant for greenhouse cultivation.¹³ The United States Environmental Protection Agency (EPA) provides for the exemption of the application of minimal risk pesticides which are mostly botanicals, such as rosemary oil.^{16,17} The current registered pesticide products to use in hemp are available via the EPA online portal.¹⁸ Canadian regulations prescribe testing for 96 pesticide residues in *C. sativa* consumables.¹ Although the regulation of synthetic pesticides used in *C. sativa* remains unclear, it is known that in seized and legal *C. sativa* products synthetic pesticide residues are common.^{2,12,14,16,19–21}

As a result of the use of nonregistered pesticides in several countries, growers and consumers are confronted with unanswered questions around the potential presence of residues of these pesticides and their impact on consumers.¹⁶ Therefore, it would be helpful to growers and consumers alike to legalize the use of appropriate pesticides, determine the potential metabolites of these active substances, and set Good Agricultural Practice on residue levels and maximum residue limits (MRL). Previous investigations focused on the residue analysis of the active ingredients (A.I.) and disregarded potential metabolites.^{19,22–24} Therefore, we set out to determine novel technical methods to detect the pesticide residues and *C. sativa* metabolites.^{16,25}

An *in vitro* screening assay using plant cell cultures was applied to test for the metabolic degradation of pesticides. It is known that plant cell cultures of different plant species can be used to elucidate the metabolic pathway of xenobiotics in plants.^{26–29} Thus, callus cultures of *C. sativa* were incubated with five different pesticides: three fungicides (tebuconazole, metalaxyl-M and fenhexamid), one herbicide (flurtamone) and one miticide (spirodiclofen). Analyzing their metabolic degradation in *in vitro* *C. sativa* callus cultures shows the metabolic capability of *C. sativa* as well as a comparison against data from metabolism guideline studies in typical crop plants. Callus cultures of *Brassica napus* L. (*B. napus*, oilseed rape), *Glycine max* (L.) Merr. (soybean), *Zea mays* L. (maize) and *Triticum aestivum* L. (wheat) were likewise incubated with the same pesticides, in order to compare the results with *C. sativa* callus cultures.

The applied pesticides are well-known substances with a complete elucidated metabolic pathway (Supporting Information, Table S1).^{30–34} They additionally are known to be detected as residues in *C. sativa*.^{2,13,19,22–24,35} The three fungicides have different modes of action in fungal cells: Tebuconazole hinders the 14- α demethylase enzyme, consequently causing a stop of ergosterol synthesis.^{36,37} Metalaxyl-M blocks the processing of ribosomal

RNA (rRNA) by stopping the polymerase I enzyme and fenhexamid hinders the 3-keto reductase which is involved in the sterol biosynthesis in fungi.^{38,39} The herbicide flurtamone inhibits carotenoid synthesis which results in a photodegradation of chlorophyll with bleaching properties in all plants.⁴⁰ Moreover, spirodiclofen blocks the acetyl-CoA-carboxylase in fatty acid metabolism of arachnids and has an impact in all stages of their development.⁴¹

In this study five pesticides were applied to *C. sativa* callus cultures in order to investigate the qualitative and semiquantitative metabolic degradation. The selected pesticides were applied for 21 days by using an *in vitro* application protocol described previously.⁴² A simultaneously conducted application of the same pesticides in callus cultures of *B. napus*, *G. max*, *Z. mays* and *T. aestivum* enabled a comparison against data from *C. sativa* to check the similarity of the metabolic nature of possible residues. Finally, the **results were correlated with data known from regulatory guideline studies** and literature for each pesticide in crops.⁴²

2 MATERIALS AND METHODS

2.1 Plant material

Cannabis sativa L. cv. Euphoria (Snorkel Spain SLU, Barcelona, Spain) seeds were germinated and grown as a hydroculture on Perlite® (granule size 0.5–5 mm). This cultivar was chosen as it is a *C. sativa* plant with a 1:1 THC:CBD ratio which reaches medical requirements for *C. sativa*. At long-day conditions (16 h:8 h, light:dark) the temperature of the TU Technical University Dortmund growth chamber (PlantMaster®, CLF Plant Climatics GmbH, Werting, Germany) was set to 25 °C with light intensity of 110 $\mu\text{m}^{-2} \text{s}^{-1}$ and humidity at 70%. Five weeks after germination a combination of three fertilizers was used. The concentration of FloraGro, FloraMicro and FloraBloom (General Hydroponics Europe, Fleurance, France) was set to 7 mL fertilizer/10 L water (0.07% each). All studies with *C. sativa* were performed with permission no. 4586416 issued by the Federal Institute for Drug and Medical Devices (BfArM), Bonn, Germany.

2.2 Initiation of callus cultures and experimental setup of *in vitro* cultures

Petiole surface sterilization of month-old *C. sativa* plants was performed for 10 min. After washing three times under aseptic conditions, the petioles were cut into small pieces and transferred to petri dishes containing phytohormones. The Murashige & Skoog (MS) medium and all applied phytohormones were purchased at PhytoTechnology Laboratories, Shawnee, KS, USA.⁴³ Initiation and maintenance of callus cultures took place on full-strength MS medium 30 g L⁻¹ sucrose and 8 g L⁻¹ agar (both Carl Roth GmbH + Co KG, Karlsruhe, Germany) with 1 mg L⁻¹ NAA and BAP. It took place three to four weeks after dissection from the tissue. Initiation and maintenance of all crop plant callus cultures is described in Hillebrands *et al.*⁴²

Callus cultures of all plant species were grown in PlantMaster® enclosure which contains BrightBoy® climate cabinets (CLF Plant Climatics GmbH) which generate long-day conditions of 16 h:8 h, light:dark photoperiod at 25 °C, 50% humidity and 101 $\mu\text{m}^{-2} \text{s}^{-1}$ light intensity. The ≤ 6 -month-old callus cultures were initiated, maintained and applied with selected pesticides at TU Dortmund University, Germany.

2.3 Experimental setup *in vitro* cultures

Powdered tebuconazole, metalaxyl-M, flurtamone and fenhexamid had a purity >99% and were obtained from Bayer AG (Monheim am Rhein, Germany) and were dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany). Powdered spirodiclofen (purity ≤100%; Sigma-Aldrich Chemie GmbH) was dissolved in acetonitrile (ACN ≥99.9% gradient; Sigma-Aldrich Chemie GmbH). A stock solution of 10 mg mL⁻¹ for each pesticide was stored at -20 °C. At the application date [Day (D)0], a 10 μM application solution was produced by adding the stock solution into the warm and liquid nutrition media. Preliminary tests showed that 10 μM was the most reliable test concentration. In doing so even in complex biological matrices it was possible to analytically detect the metabolites. Each well of a 12-microwell plate was filled with 2.5 mL nutrition medium and 0.66 g ± 0.2 g callus fresh weight (F.W.). All pesticides migrate out of the nutrition medium into the plant callus culture by passive diffusion. The callus cultures in the microwell plates were incubated for 21 days. Fresh growth index (FGI) estimation was done with *C. sativa* callus cultures incubated for 21 days.⁴⁴ Agar without the influence of a callus culture served as a negative control and was extracted and analyzed after 21 days to estimate the abiotic degradation or hydrolysis of the pesticide in the aqueous nutrition media. A preliminary test showed that callus cultures does not excrete any pesticide metabolites into the nutrition media. The utilized biological control were callus cultures without application of pesticides.

2.4 Sample preparation

Overall, 72 callus cultures for each plant species were represented by 12 negative controls as well as callus cultures treated with pesticide (tebuconazole, flurtamone, metalaxyl-M, fenhexamid and spirodiclofen) with four replicates at three time points (D7, D14 and D21). Negative controls were 12 callus cultures without any pesticide application represented four replicates after 7, 14 or 21 days respectively. Control and sample callus cultures were treated at the same time. The entire callus from each well was carefully removed from the agar and extracted with a mixture of acetonitrile and water (4:1, v/v, 2 mL g⁻¹ F.W.). The extraction took place three times, each cycle was 20 s long at 10 000 rpm in a tissue homogenizer (Precellys Evolution®; Bertin Technologies, Montigny-le Bretonneux, France). After centrifugation and filtration of the combined extraction solution with a PTFE-Chromafil® syringe filter, the supernatant was analyzed by high-performance liquid chromatography - high resolution mass spectrometry (HPLC-HRMS).

2.5 HPLC-HRMS and -MS/MS analysis

All measurements were conducted as described in Hillebrands *et al.*⁴² Additionally, the used HPLC-HRMS method is described in the supporting information.

2.6 Data analysis

Identification took place by retention time (RT), accurate mass and fragmentation pattern of LC-HRMS/MS measurements. In a second step all MS data were verified by available regulatory guideline studies on the metabolic degradation of fenhexamid, flurtamone, metalaxyl-M, spirodiclofen and tebuconazole in crop plants.³⁰⁻³⁴ The application of a software tool (Compound Discoverer®; ThermoFisher Scientific, Bremen, Germany) gave a list of *m/z* values, but as a consequence of a considerable number of false-positive results the data had to be checked manually. Each

interesting value, described by the software, was verified concerning RT, peak form and signal heights in the chromatogram, kinetic behavior over the 21 days, and the proposed sum formula aligned with the molecular structure and formula.

A.I. intensities were analyzed statistically by an unpaired *t*-test by assessing the normal distribution at *P* > 0.05. Error bars represented the standard error (SE; SE = σ/\sqrt{n}). All detected metabolites were confirmed in the negative controls. The nonexistence of any metabolite in this control was taken to mean metabolism by the callus in the *in vitro* assay. Because of abiotic degradation processes, metabolites of the tested pesticides could be observed; however, they were neglected if their detected signal in the negative control was <20% comparing to their signal in the callus extract. As a consequence, the metabolites recorded were considered to be true plant metabolites.

3 RESULTS

3.1 Initiation of *Cannabis sativa* L. callus cultures

The vegetative growth stage of unfolded cotyledons was reached after one [Fig. 1(A)] and the totally unfolded first and second leaf pair after two four-week periods [Fig. 1(B) and (C)].⁴⁵ A few months later the *C. sativa* plant was big enough to cut several petioles and induce the callus growth. After four weeks, the callus tissue was dissected from the initial plant material [Fig. 1(D)]. Finally, incubation with 10 μM of the applied pesticides started 10–12 weeks after germination of the *C. sativa* seeds [Fig. 1(E)]. At D7, D14 and D21 four replicates of the *C. sativa* callus cultures were harvested, weighed, stored at -20 °C, stepwise extracted and analyzed by HPLC-HRMS.

3.2 Vitality check in *Cannabis sativa* L. callus cultures incubated with pesticides

The 21 days F.W. development of all *C. sativa* callus cultures is an important vitality verification. Callus cultures without any pesticide application served as a negative control and indicated the

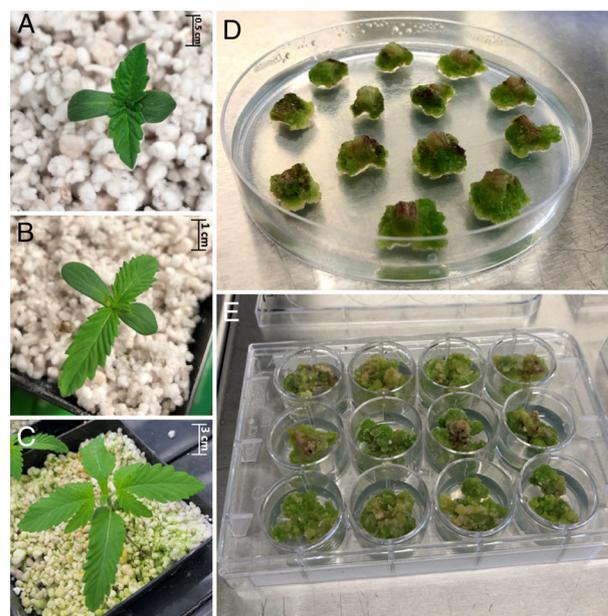


Figure 1. Development of *C. sativa* L. cv. Euphoria seedlings at D7 (A), D14 (B) and D21 (C). Callus induction (D) and incubation with pesticides in a well plate (E).

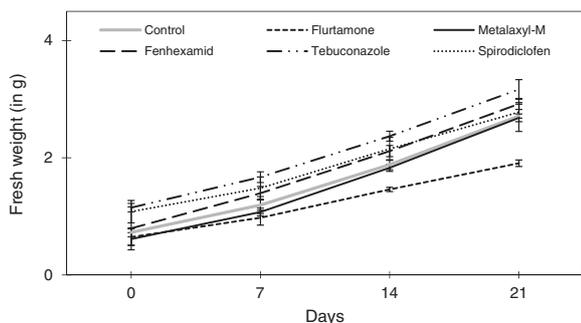


Figure 2. Fresh weight (g) of *in vitro* callus cultures of *C. sativa*: Growth curves of cultures incubated with a concentration of 10 μM flurtamone, metalaxyl-M, fenhexamid, tebuconazole and spirodiclofen. The evaluation of F.W. of *C. sativa* callus cultures without the addition of any pesticide was taken as the negative control. After D7, D14 and D21 the F.W. of each callus was determined. Each sampling point is represented by four individual callus cultures ($n = 4$; bars represent SE).

growth of unaffected callus. All incubated callus cultures showed an increase in F.W. (Fig. 2). Each value was represented by four replicates with a slight distribution of the mean variation. Starting at an average F.W. of 0.39 g (± 0.17 g), *C. sativa* callus cultures increased up to 1.01 g (± 0.47 g). The fresh growth index (FGI) showed the F.W. increase over 21 days. Callus cultures incubated without the addition of pesticide achieve a FGI of 1.65 (± 0.14 ; Table S3). This is in between the mean FGIs analyzed from the incubations with metalaxyl-M (FGI = 1.88 \pm 0.39), tebuconazole (FGI = 1.85 \pm 0.23), fenhexamid (FGI = 1.70 \pm 0.68) and spirodiclofen (FGI = 1.57 \pm 0.30) (Table S3). By contrast with the others, the incubation with herbicide flurtamone revealed a lower final F.W. of callus cultures (Fig. 2). The depicted flat growth curve was specified by an FGI of 0.96 (± 0.30) (Table S3) and the herbicidal properties (bleaching) of flurtamone in *C. sativa* callus cultures after 14 days (Fig. S1).

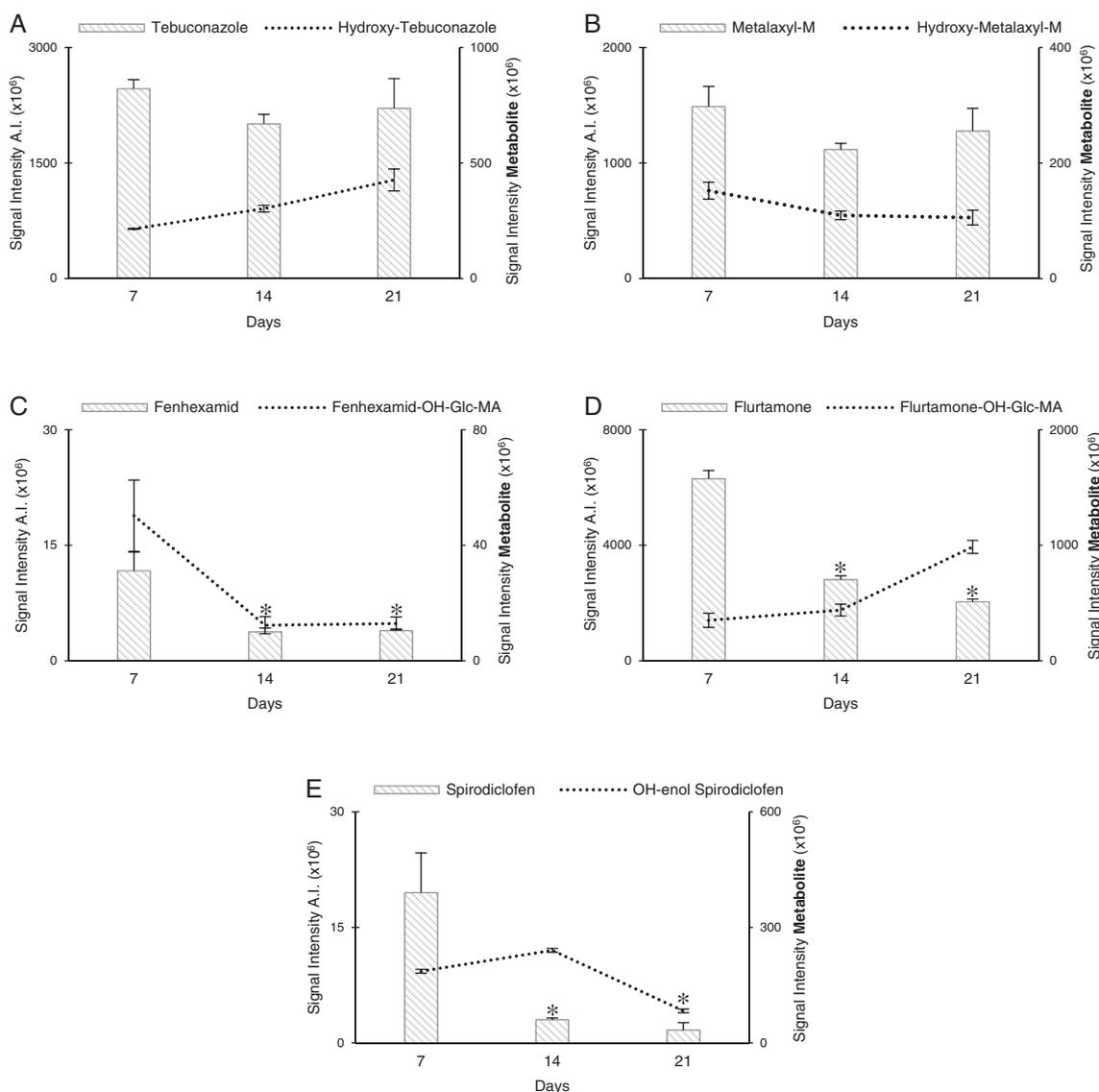


Figure 3. Degradation of pesticides and formation of their main metabolites in *C. sativa* L. callus cultures: tebuconazole (A), metalaxyl-M (B), fenhexamid (C), flurtamone (D) and spirodiclofen (E) at D7, D14 and D21. Each sampling point is represented by four individual callus cultures ($n = 4$; bars represent SE; asterisks indicate a significant difference in comparison to the D7 cultures by unpaired Student's *t*-test; $P > 0.05$). (OH-Glc-MA, glucose (Glc) and malonic acid (MA) conjugate after hydroxylation (OH); OH-enol Spirodiclofen, hydroxylated enol of spirodiclofen).

3.3 Pesticide residues occurring in *Cannabis sativa* L. callus culture extracts

The first important aspect was the uptake of each A.I. in the intracellular compartments of the callus by supposed passive diffusion. Although tebuconazole [Fig. 3(A)], metalaxyl-M [Fig. 3(B)] and flurtamone [Fig. 3(D)] showed high signal intensities, fenhexamid [Fig. 3(C)] and spirodiclofen [Fig. 3(E)] remained at low levels. The intensities of tebuconazole and metalaxyl-M continued on these high levels over the 21-day incubation period. By contrast with that, flurtamone intensity decreased within the same period [Fig. 3(D)], with a final intensity of $\approx 50\%$ of the detected intensity after the first week. In comparison, fenhexamid and spirodiclofen showed the same declining effect at a lower level. Looking at the main metabolite of the applied A.I., different kinetic behaviors were seen. The phase I metabolite hydroxy-tebuconazole increased intensity continuously over the whole time period [Fig. 3(A)]. By contrast with that, hydroxy-metalaxyl-M obtained its highest amount at D7 [Fig. 3(B)] and the hydroxy-enol of spirodiclofen at D14 [Fig. 3(E)]. The main metabolites of fenhexamid and flurtamone were phase III products whereby the A.I. was hydroxylated with additional glycosylation and conjugation of malonic acid. The malonyl-glucoside of flurtamone registered a continuous increase whereas its counterpart of fenhexamid

decreased [Fig. 3(D)]. The main metabolite of fenhexamid yielded its highest abundance after the first week and remained on a constant lower level after the second and third week [Fig. 3(C)].

The selected ion chromatograms of flurtamone [Fig. 4(A)–(C)] and tebuconazole [Fig. 5(A)–(C)] exhibited both main and some minor metabolites. At D21, flurtamone [Fig. 4(C)], showed a three-fold reduction in abundance comparing to the D7 culture [Fig. 4(A)]. However, tebuconazole underwent a slight decrease in abundance in the HPLC-HRMS measurements at D21 [(Fig. 5 (A)–(C)]. The parent A.I. of flurtamone and tebuconazole was the major residue in *C. sativa* callus cultures. For flurtamone at D7, hydroxy-flurtamone (F1) and desmethyl-flurtamone (F2) as phase I metabolites defined the first initial metabolic degradation steps [Fig. 4(A)]. At D14, these two metabolites remained the major metabolites [Fig. 4(B)]. At D21, three additional metabolites were found: F3, the hydroxy-flurtamone-glucoside, and F4 and F5, two isomers of the glucoside after malonic acid conjugation [Fig. 4(C)]. At D21, F1 and F4 were found to be the main metabolites each occurring at a similar concentrations.

Selected ion chromatograms of tebuconazole at D7, D14 and D21 outlined seven metabolites [Fig. 5(A)–(C); T1–T7]. T1–T3 were phase II and III conjugates of hydroxy-tebuconazole with glucose (T1) and additionally two isomers of the malonic acid conjugates

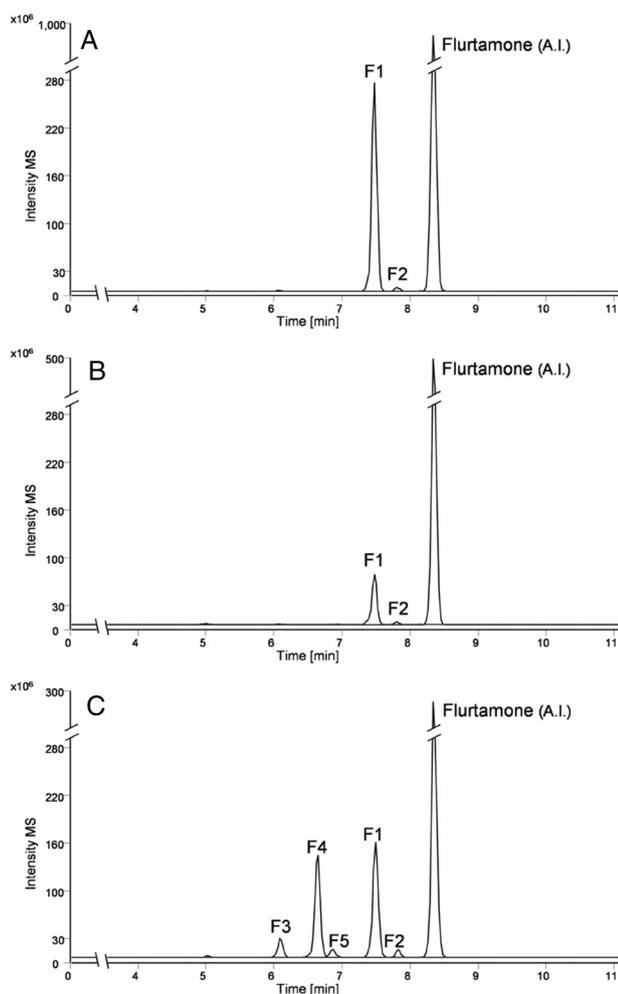


Figure 4. Selected ion chromatograms of HPLC-HRMS measurements of flurtamone in *C. sativa* L. callus culture extracts at D7 (A), D14 (B) and D21 (C).

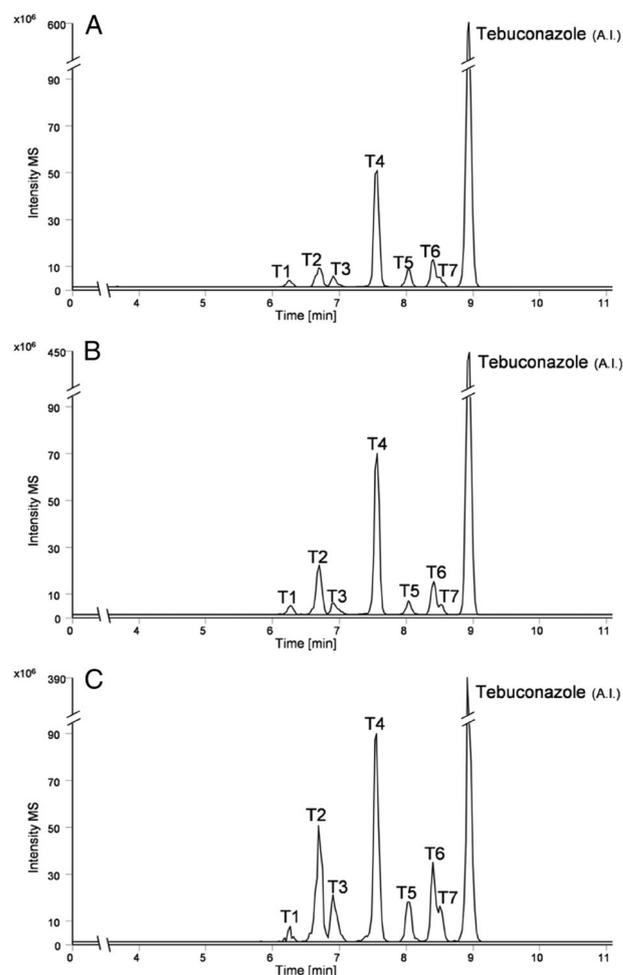


Figure 5. Selected ion chromatograms of HPLC-HRMS measurements of tebuconazole in *C. sativa* L. callus culture extracts at D7 (A), D14 (B) and D21 (C).

Table 1. Overview of detected metabolites for fenhexamid, metalaxyl-M, tebuconazole, flurtamone and spirodiclofen found in *in vitro* callus cultures incubation experiments of *C. sativa* L., *B. napus* L., *G. max* (L.) Merr., *Z. mays* L. and *T. aestivum* L. after 21 days

	Molecular formula	Mass [†]	Metabolic transformation	OSR	SOY	MAZ	WHT	HEM
Fenhexamid	C ₁₄ H ₁₇ Cl ₂ NO ₂	302.0706	(–)	✓	✓	✓	✓	✓
Hydroxy-fenhexamid (isomer 1)	C ₁₄ H ₁₇ Cl ₂ NO ₃	318.0655	Oxidation	✓	✓	✓	✓	✓
Hydroxy-fenhexamid (isomer 2)	C ₁₄ H ₁₇ Cl ₂ NO ₃	318.0655	Oxidation	✓	✓	✓	✓	✓
Hydroxy-fenhexamid (isomer 3)	C ₁₄ H ₁₇ Cl ₂ NO ₃	318.0655	Oxidation	✓	✓	✓	✓	✓
Hydroxy-fenhexamid (isomer 4)	C ₁₄ H ₁₇ Cl ₂ NO ₃	318.0655	Oxidation	✓	✓	n.d.	✓	✓
Fenhexamid-glucoside	C ₂₀ H ₂₇ Cl ₂ NO ₇	464.1233	Glycosylation	n.d.	✓	n.d.	n.d.	n.d.
Malonyl-glucoside of fenhexamid (isomer 1)	C ₂₃ H ₂₉ Cl ₂ NO ₁₀	550.1237	Glycosylation and malonic acid conjugation	n.d.	✓	n.d.	✓	✓
Malonyl-glucoside of fenhexamid (isomer 2)	C ₂₃ H ₂₉ Cl ₂ NO ₁₀	550.1237	Glycosylation and malonic acid conjugation	n.d.	n.d.	n.d.	n.d.	✓
Metalaxyl-M	C ₁₅ H ₂₁ NO ₄	280.1539	(–)	✓	✓	✓	✓	✓
Hydroxy-metalaxyl-M (isomer 1)	C ₁₅ H ₂₁ NO ₅	296.1488	Oxidation	n.d.	n.d.	n.d.	✓	n.d.
Hydroxy-metalaxyl-M (isomer 2)	C ₁₅ H ₂₁ NO ₅	296.1488	Oxidation	n.d.	✓	n.d.	✓	✓
hydroxy-metalaxyl-M (isomer 3)	C ₁₅ H ₂₁ NO ₅	296.1488	Oxidation	n.d.	✓	✓	✓	✓
Hydroxy-metalaxyl-M (isomer 4)	C ₁₅ H ₂₁ NO ₅	296.1488	Oxidation	✓	✓	✓	✓	✓
Desmethyl-metalaxyl-M (isomer 1)	C ₁₄ H ₁₉ NO ₄	266.1383	Demethylation	n.d.	✓	✓	✓	n.d.
Desmethyl-metalaxyl-M (isomer 2)	C ₁₄ H ₁₉ NO ₄	266.1383	Demethylation	✓	n.d.	✓	✓	✓
Desmethyl-metalaxyl-M (isomer 3)	C ₁₄ H ₁₉ NO ₄	266.1383	Demethylation	✓	✓	✓	✓	✓
Desmethyl-metalaxyl-M (isomer 4)	C ₁₄ H ₁₉ NO ₄	266.1383	Demethylation	✓	✓	✓	✓	✓
Desmethyl-metalaxyl-M (isomer 5)	C ₁₄ H ₁₉ NO ₄	266.1383	Demethylation	✓	✓	✓	✓	n.d.
Desethyl-metalaxyl-M	C ₁₃ H ₁₇ NO ₄	252.1227	Demethylation, demethylation	✓	✓	✓	✓	✓
Hydroxy-desmethyl-metalaxyl-M (isomer 1)	C ₁₄ H ₁₉ NO ₅	282.1333	Oxidation, demethylation	✓	n.d.	✓	✓	✓
Hydroxy-desmethyl-metalaxyl-M (isomer 2)	C ₁₄ H ₁₉ NO ₅	282.1333	Oxidation, demethylation	✓	✓	n.d.	n.d.	n.d.
Hydroxy-metalaxyl-M-glucoside (isomer 1)	C ₂₁ H ₃₁ NO ₁₀	458.2016	Oxidation, glycosylation	n.d.	✓	n.d.	✓	n.d.
Hydroxy-metalaxyl-M-glucoside (isomer 2)	C ₂₁ H ₃₁ NO ₁₀	458.2016	Oxidation, glycosylation	✓	✓	n.d.	✓	✓
Hydroxy-metalaxyl-M-glucoside (isomer 3)	C ₂₁ H ₃₁ NO ₁₀	458.2016	Oxidation, glycosylation	✓	✓	✓	✓	n.d.
Desmethyl-metalaxyl-M-glucoside	C ₂₀ H ₂₉ NO ₉	428.1912	Demethylation, glycosylation	✓	n.d.	n.d.	n.d.	n.d.
Tebuconazole	C ₁₆ H ₂₂ ClN ₃ O	308.1508	(–)	✓	✓	✓	✓	✓
Keto-tebuconazole (isomer 1)	C ₁₆ H ₂₀ ClN ₃ O ₂	322.1299	Oxidation, desaturation	n.d.	n.d.	✓	n.d.	✓
Keto-tebuconazole (isomer 2)	C ₁₆ H ₂₀ ClN ₃ O ₂	322.1299	Oxidation, desaturation	n.d.	n.d.	n.d.	n.d.	✓
Keto-tebuconazole (isomer 3)	C ₁₆ H ₂₀ ClN ₃ O ₂	322.1299	Oxidation, desaturation	✓	✓	✓	✓	✓
Hydroxy-tebuconazole (isomer 1)	C ₁₆ H ₂₂ ClN ₃ O ₂	324.1457	Oxidation	✓	✓	✓	✓	✓
Hydroxy-tebuconazole (isomer 2)	C ₁₆ H ₂₂ ClN ₃ O ₂	324.1457	Oxidation	✓	✓	✓	✓	✓
Hydroxy-tebuconazole (isomer 3)	C ₁₆ H ₂₂ ClN ₃ O ₂	324.1457	Oxidation	✓	✓	✓	✓	✓
Hydroxy-tebuconazole (isomer 4)	C ₁₆ H ₂₂ ClN ₃ O ₂	324.1457	Oxidation	✓	✓	✓	✓	✓
Dihydroxy-tebuconazole	C ₁₆ H ₂₂ ClN ₃ O ₃	340.1420	Oxidation, oxidation	n.d.	n.d.	✓	✓	n.d.
Tebuconazole-glucoside	C ₂₂ H ₃₂ ClN ₃ O ₆	470.2051	Glycosylation	✓	✓	✓	✓	n.d.
Hydroxy-tebuconazole-glucoside (isomer 1)	C ₂₂ H ₃₂ ClN ₃ O ₇	486.1998	Oxidation, glycosylation	n.d.	✓	✓	✓	✓
Hydroxy-tebuconazole-glucoside (isomer 2)	C ₂₂ H ₃₂ ClN ₃ O ₇	486.1998	Oxidation, glycosylation	✓	✓	✓	✓	✓
Hydroxy-tebuconazole-glucoside (isomer 3)	C ₂₂ H ₃₂ ClN ₃ O ₇	486.1998	Oxidation, glycosylation	n.d.	n.d.	n.d.	n.d.	✓
Malonyl-glucoside of tebuconazole (isomer 1)	C ₂₅ H ₃₄ ClN ₃ O ₁₀	572.2004	Oxidation, glycosylation and malonic acid conjugation	✓	✓	✓	✓	✓
Malonyl-glucoside of tebuconazole (isomer 2)	C ₂₅ H ₃₄ ClN ₃ O ₁₀	572.2004	Oxidation, glycosylation and malonic acid conjugation	✓	✓	✓	✓	✓

Table 1. Continued

	Molecular formula	Mass [†]	Metabolic transformation	OSR	SOY	MAZ	WHT	HEM
Malonyl-glucoside of tebuconazole (isomer 3)	C ₂₅ H ₃₄ ClN ₃ O ₁₀	572.2004	Oxidation, glycosylation and malonic acid conjugation	n.d.	n.d.	n.d.	n.d.	✓
Malonyl-glucoside of tebuconazole (isomer 4)	C ₂₅ H ₃₄ ClN ₃ O ₁₀	572.2004	Oxidation, glycosylation and malonic acid conjugation	n.d.	n.d.	n.d.	n.d.	✓
Flurtamone	C ₁₈ H ₁₄ F ₃ NO ₂	334.1056	(–)	✓	✓	✓	✓	✓
Hydroxy-flurtamone (isomer 1)	C ₁₈ H ₁₄ F ₃ NO ₃	350.1007	Oxidation	✓	✓	✓	✓	✓
Hydroxy-flurtamone (isomer 2)	C ₁₈ H ₁₄ F ₃ NO ₃	350.1007	Oxidation	✓	✓	✓	✓	✓
Hydroxy-flurtamone (isomer 3)	C ₁₈ H ₁₄ F ₃ NO ₃	350.1007	Oxidation	n.d.	✓	✓	✓	✓
Hydroxy-flurtamone-glucoside (isomer 1)	C ₂₄ H ₂₄ F ₃ NO ₈	512.1544	Oxidation, glycosylation	✓	n.d.	✓	✓	✓
Hydroxy-flurtamone-glucoside (isomer 2)	C ₂₄ H ₂₄ F ₃ NO ₈	512.1544	Oxidation, glycosylation	✓	✓	✓	✓	n.d.
Hydroxy-flurtamone-glucoside (isomer 3)	C ₂₄ H ₂₄ F ₃ NO ₈	512.1544	Oxidation, glycosylation	n.d.	n.d.	✓	✓	n.d.
Malonyl-glucoside of flurtamone (isomer 1)	C ₂₇ H ₂₆ F ₃ NO ₁₁	598.1547	Oxidation, glycosylation and malonic acid conjugation	✓	✓	✓	✓	✓
Malonyl-glucoside of flurtamone (isomer 2)	C ₂₇ H ₂₆ F ₃ NO ₁₁	598.1547	Oxidation, glycosylation and malonic acid conjugation	n.d.	n.d.	n.d.	n.d.	✓
<i>N</i> -desmethyl-flurtamone	C ₁₇ H ₁₂ F ₃ NO ₂	320.0902	Demethylation	✓	✓	✓	✓	✓
Hydroxy- <i>N</i> -desmethyl-flurtamone (isomer 1)	C ₁₇ H ₁₂ F ₃ NO ₃	336.0853	Demethylation, oxidation	✓	✓	✓	✓	✓
Hydroxy- <i>N</i> -desmethyl-flurtamone (isomer 2)	C ₁₇ H ₁₂ F ₃ NO ₃	336.0853	Demethylation, oxidation	n.d.	n.d.	n.d.	✓	n.d.
Flurtamone-trifluoromethyl- <i>N</i> -methyl-mandelamide	C ₁₀ H ₁₀ F ₃ NO ₂	234.0743	Hydrolysis and cleavage of the furan moiety, oxidation	✓	✓	✓	✓	✓
Flurtamone-3-trifluoromethyl-benzoic acid (TFMBA) [‡]	C ₈ H ₅ F ₃ O ₂	189.0166 [‡]	Hydrolysis and cleavage of the furan moiety, oxidation, dissociation dimethylamine	✓	✓	✓	✓	n.d.
Flurtamone-trifluoromethyl- <i>N</i> -methyl-phenylacetamide	C ₁₀ H ₁₀ F ₃ NO	218.0793	Hydrolysis and cleavage of the furan moiety	✓	✓	✓	✓	✓
Spirodiclofen	C ₂₁ H ₂₄ Cl ₂ O ₄	411.1130	(–)	✓	✓	✓	✓	✓
Hydroxy-spirodiclofen-enol (isomer 1) [‡]	C ₁₅ H ₁₄ Cl ₂ O ₄	327.0196 [‡]	Cleavage of ester bond, oxidation	✓	✓	✓	n.d.	✓
Hydroxy-spirodiclofen-enol (isomer 2) [‡]	C ₁₅ H ₁₄ Cl ₂ O ₄	327.0196 [‡]	Cleavage of ester bond, oxidation	✓	✓	✓	✓	✓
Hydroxy-spirodiclofen-enol (isomer 3) [‡]	C ₁₅ H ₁₄ Cl ₂ O ₄	327.0196 [‡]	Cleavage of ester bond, oxidation	✓	✓	✓	✓	✓
Spirodiclofen-enol [‡]	C ₁₅ H ₁₄ Cl ₂ O ₃	311.0247 [‡]	Cleavage of ester bond	✓	✓	✓	✓	✓
Keto-hydroxy-spirodiclofen-enol [‡]	C ₁₅ H ₁₂ Cl ₂ O ₄	325.0037 [‡]	Cleavage of ester bond, oxidation, desaturation	n.d.	✓	✓	✓	✓
Dihydroxy-spirodiclofen-enol (isomer 1) [‡]	C ₁₅ H ₁₄ Cl ₂ O ₅	343.0146 [‡]	Cleavage of ester bond, oxidation, oxidation	n.d.	✓	n.d.	n.d.	n.d.
Dihydroxy-spirodiclofen-enol (isomer 2) [‡]	C ₁₅ H ₁₄ Cl ₂ O ₅	343.0146 [‡]	Cleavage of ester bond, oxidation, oxidation	n.d.	✓	n.d.	n.d.	✓
Dihydroxy-spirodiclofen-enol (isomer 3) [‡]	C ₁₅ H ₁₄ Cl ₂ O ₅	343.0146 [‡]	Cleavage of ester bond, oxidation, oxidation	✓	✓	✓	✓	✓
Dihydroxy-spirodiclofen-enol (isomer 4) [‡]	C ₁₅ H ₁₄ Cl ₂ O ₅	343.0146 [‡]	Cleavage of ester bond, oxidation, oxidation	n.d.	n.d.	n.d.	n.d.	✓
Dihydroxy-spirodiclofen-enol (isomer 5) [‡]	C ₁₅ H ₁₄ Cl ₂ O ₅	343.0146 [‡]	Cleavage of ester bond, oxidation, oxidation	n.d.	n.d.	n.d.	n.d.	✓
Hydroxy-spirodiclofen-enol-glucoside (isomer 1) [‡]	C ₂₁ H ₂₄ Cl ₂ O ₉	489.0723 [‡]	Cleavage of ester bond, oxidation, glycosylation	✓	n.d.	✓	n.d.	n.d.

Table 1. Continued

	Molecular formula	Mass [†]	Metabolic transformation	OSR	SOY	MAZ	WHT	HEM
Hydroxy-spirodiclofen-enol-glucoside (isomer 2) [‡]	C ₂₁ H ₂₄ Cl ₂ O ₉	489.0723 [‡]	Cleavage of ester bond, oxidation, glycosylation	✓	✓	✓	✓	✓
Keto-dihydroxy-spirodiclofen-enol (isomer 1) [‡]	C ₁₅ H ₁₂ Cl ₂ O ₅	340.9991 [‡]	Cleavage of ester bond, oxidation, oxidation, desaturation	n.d.	n.d.	✓	✓	n.d.
Keto-dihydroxy-spirodiclofen-enol (isomer 1) [‡]	C ₁₅ H ₁₂ Cl ₂ O ₅	340.9991 [‡]	Cleavage of ester bond, oxidation, oxidation, desaturation	n.d.	n.d.	✓	✓	✓
Keto-dihydroxy-spirodiclofen-enol (isomer 1) [‡]	C ₁₅ H ₁₂ Cl ₂ O ₅	340.9991 [‡]	Cleavage of ester bond, oxidation, oxidation, desaturation	n.d.	n.d.	✓	✓	✓
Keto-dihydroxy-spirodiclofen-enol (isomer 1) [‡]	C ₁₅ H ₁₂ Cl ₂ O ₅	340.9991 [‡]	Cleavage of ester bond, oxidation, oxidation, desaturation	n.d.	n.d.	n.d.	n.d.	✓
2,4-dichloro-mandelic acid [‡]	C ₈ H ₆ Cl ₂ O ₃	218.9615 [‡]	Cleavage of ester bond, cleavage of acid ring structure, further degradation	✓	✓	✓	✓	✓
2,4-dichloro-mandelic acid glucoside [‡]	C ₁₄ H ₁₆ Cl ₂ O ₈	381.0146 [‡]	Cleavage of ester bond, cleavage of acid ring structure, further degradation, glycosylation	n.d.	n.d.	n.d.	✓	n.d.

OSR (oilseed rape), *B. napus* L.; SOY (soybean), *G. max* (L.) Merr.; MAZ (maize), *Z. mays* L.; WHT (wheat), *T. aestivum* L.; HEM (hemp), *C. sativa* L.; n.d., not detected; ✓, detected; (–), no metabolic transformation.
[†] Selected ion mass ([M + H]⁺).
[‡] Selected ion mass ([M–H][–]).

(T2 and T3). T4–T6 were three isomers of hydroxy-tebuconazole with M4 as the main metabolite [Fig. 5(A)–(C)]. The ratio between T4 as main metabolite and all other identified metabolites (T1–T3 and T5–T7) varied amongst the three time points. Although T4 was the major metabolite at D7 and D14, at D21 the intensity was similar to the other six metabolites, although T4 was still rising. Overall, the tebuconazole metabolites were all detectable at D7, whereas the intensities of flurtamone metabolites increased in intensity within the 21-day incubation period. Like flurtamone, fenhexamid metabolites increased within the 21-day incubation (Fig. S2). By contrast metalaxyl-M and spirodiclofen metabolites were detectable at the first sampling point (D7) (Figs S3 and S4). All flurtamone, tebuconazole, fenhexamid, metalaxyl-M and spirodiclofen metabolites could be arranged in a metabolic pathway (Figs S5–S9). All indicated molecular structures in Figs S5–S9 as described in regulatory guideline studies were identified by HPLC-HRMS and nuclear magnetic resonance (NMR) technology.

3.4 Qualitative comparison of metabolites between *Cannabis sativa* L. and crop plants

The examined A.I. were five chemical substances with known metabolic properties in crop plants as well as callus cultures of *B. napus*, *G. max*, *Z. mays* and *T. aestivum* (Table 1). The metabolic behavior of these A.I. in *C. sativa* was compared to that of the same A.I. in the crop plants. Table 1 shows the A.I. and metabolites identified for each crop plant as well as *C. sativa*. Fenhexamid exhibited five in-common, one *C. sativa*- and one crop-specific metabolite. Metalaxyl-M was degraded into 16 metabolites with

nine in-common, seven crop- and no *C. sativa*-specific metabolite. Tebuconazole exhibited 16 metabolites, ten of which were in-common between crop plants and *C. sativa*, four *C. sativa*- and two crop-specific. For flurtamone 14 metabolites were detected, nine in-common, four crop- and one *C. sativa*-specific. The miticide spirodiclofen was degraded into 11 in-common, four crop- and three *C. sativa*-specific metabolites.

Tables S4 and S5 describe the metabolic degradation in *C. sativa* and the four crop plants. The main residue of flurtamone [Fig. 4(C)], tebuconazole [Fig. 4(F)] and metalaxyl-M [Fig. S3(C)] in *C. sativa* and crop plant callus cultures was the A.I. itself. By contrast with these results, the malonyl-glucose conjugate of hydroxy-fenhexamid [Fig. S2(C); Fe2] and the desaturated spirodiclofen-enol [Figs S4(C); S5] define the main residues in *C. sativa*. Comparable results were exhibited by *B. napus*, *G. max*, *Z. mays* and *T. aestivum*, because tebuconazole, flurtamone and metalaxyl-M remained the main residue in these crops. For spirodiclofen, various phase I metabolites defined the main *in vitro* residues in all plant species (Table S4).

For all plant species incubated with an A.I., the main metabolic processes were identified (Table S5). Tebuconazole in *B. napus*, *G. max*, *Z. mays* and *C. sativa* the main metabolite was hydroxy-tebuconazole, whereas in *T. aestivum* the metabolization reached the final phase III. For all used crop plants flurtamone defined phase I metabolite as its main metabolites. *C. sativa* callus cultures metabolize flurtamone to its malonyl-flurtamone-glucoside. *B. napus* and *Z. mays* exhibited the main metabolites in the first phase of metabolic degradation of fenhexamid, whereas

G. max, *T. aestivum* and *C. sativa* metabolized to phase III conjugation product. Moreover, hydroxy-metalaxyl-M was the main metabolite in *B. napus*, *Z. mays*, *T. aestivum* and *C. sativa*. Here only *G. max* demethylated metalaxyl-M mainly. Summarizing, all plant species showed phase I metabolite of spirodiclofen as their main metabolites. In general, the additional metabolites that were detected in *C. sativa* are metabolites that represent no new metabolic pathways.

4 DISCUSSION

4.1 Vitality of *Cannabis sativa* L. callus cultures

Addition of the fungicides, tebuconazole, fenhexamid and metalaxyl-M, as well as the miticide spirodiclofen did not influence callus growth of *C. sativa* (Fig. 2 and Table S3). The observed growth curve and the occurred FGI confirmed this result. The *C. sativa* callus cultures incubated with these four A.I. and the negative controls, without the use of any A.I., showed a good vitality. As expected, flurtamone as a herbicide had a negative impact on the growth of *C. sativa* callus cultures (Figs 2 and S2). From this observation we can state that flurtamone interfered with photosynthesis by inhibiting carotenoid synthesis, even in callus cultures (Fig. S1). We suppose that chlorophyll in these callus cultures was photodegraded which had a negative effect on the callus F.W. increase compared to the controls without added pesticides. None of the applied A.I. caused a detectable increase in F.W. A narrow distribution of the mean variations indicated reliable and high-quality data concerning callus vitality.⁴⁶

4.2 *In vitro* metabolic degradation of selected pesticides in *Cannabis sativa* L.

Production of *C. sativa* plants could be improved by pesticide application, particularly insecticides and fungicides. The Canadian regulation prescribes testing for 96 pesticides which includes two of the fungicides selected in this study, metalaxyl-M and tebuconazole, as well as the miticide spirodiclofen.²⁴ The use of these A.I. have been confirmed by analysis of derived products.^{2,14,19–21} Although the authorities recommend testing for the parent A.I., we show that for the A.I.s used in these studies, in particular spirodiclofen, testing for metabolites also is important. For all investigated plant species, phase I metabolites of spirodiclofen defined the main residue found at D21 [Table 1; Fig. 3(E)]. Because in this case metabolites were the main residue, the exposure of consumers to these residues will be of high importance when compounds such as spirodiclofen are used.

The detected intensity of an A.I. was able to describe its bioavailability or enable speculations concerning its metabolic degradation rate. Although tebuconazole, metalaxyl-M and flurtamone were detected with high intensities, fenhexamid and spirodiclofen were not (Fig. 3). Several reasons could be responsible for these observations: inefficient uptake of fenhexamid and spirodiclofen, their fast metabolic degradation (e.g. prodrug), or a lack of MS sensitivity resulting from negative matrix effects.

Cannabis sativa has a high matrix load which can restrict HPLC-HRMS analysis.¹ Sample preparation or dilution of plant extracts with a high matrix load reduces chromatographic obstructions but also could decrease the qualitative detection of minor metabolites at the limit of detection. However, matrix effects or changes in matrix components within one plant species after D7, D14 and D21 generally were disregarded during the presented analytical investigations. It should be kept in mind that our model study

neglected soil or leaf uptake and translocation in the plant. Therefore, our results allow no conclusions on the absolute quantities of pesticide residues, but do constitute a first description of their metabolic capabilities in *in vitro* *C. sativa* callus cultures.

Ionization of small molecules by MS depends on different aspects: their chemical structure is decisive for their capability to become ionized by protonation or deprotonation, and simultaneously eluted matrix components can bias ion detection in the mass detector.

Therefore, a semiquantitative comparison by means of integration between A.I. and their metabolites in different plant species was not done. As a result of different matrix effects in different plant species, the ionization of the A.I. and its metabolites could vary enormously.

The uptake of an A.I. into the plant cell of a callus is dependent on different chemical and physical properties.^{47,48} For example, spirodiclofen has a water solubility of 0.05 mg L⁻¹, so in comparison to tebuconazole with 32.0 mg L⁻¹, the solubility of spirodiclofen could be lower, which could result in lower uptake by the callus.⁴⁹ The constant high intensities of tebuconazole and metalaxyl-M imply a high uptake of the A.I. in the *C. sativa* callus cultures.

The dose–response relationship of spirodiclofen is important for its miticidal activity, whereas its leaf uptake, translocation and metabolic behavior in plants is relevant for consumer risk assessment. Therefore, sometimes a fast metabolic degradation within crop plants is desirable to minimize its exposure to consumers. For spirodiclofen the successful identification of unspecified metabolites in metabolism guideline studies was possible. The desaturation of hydroxy-spirodiclofen-enol to keto-hydroxy-spirodiclofen-enol as well as the oxidation of hydroxy-spirodiclofen-enol to its dihydroxy and the glycosylation to hydroxy-spirodiclofen-enol-glucoside were not described in the metabolic guideline studies. This circumstance probably was caused by following regulatory guidelines where metabolites exceeding the defined 10% threshold of the total radioactive residue (TRR) were subject to a complete clarification of the chemical structure, whereas signals below this 10% limit were not considered further.^{50,51} However, within this study no limits were applied, so all minor metabolites were noted.

The trend of decline over time for phase I metabolites could be explained by the increase of phase II metabolite. For example, hydroxy-metalaxyl-M was further glycosylated to hydroxy-metalaxyl-M-glucoside [Fig. 3(B)]. The same effect was exhibited for spirodiclofen-enol which is further hydroxylated and conjugated by glucose. However, despite the further metabolization of hydroxy-tebuconazole to the glucose and malonic acid conjugate, its intensity increased in *C. sativa* by D21. Oxidation of tebuconazole by P450-monoxygenases probably compensated for the loss of hydroxy-tebuconazole by conjugation in *C. sativa* [Fig. 3(A)]. Nevertheless, phase II and III metabolites of tebuconazole were detectable in the extract.

The decrease of the fenhexamid malonyl-glucose conjugate was not explainable [Fig. 3(C)]. It was clearly seen in eight callus cultures at two different time points, and thus is a true observation. Different reasons could be excluded: for example, it is not a consequence of poor vitality conditions of the callus cultures because they continued to grow and gain in F.W. over the whole 21-day culture period. A further metabolization of the phase III metabolites seems illogical, so this remains a question to be followed up.

4.3 Qualitative comparison of metabolites between *Cannabis sativa* L. and crop plants

Hillebrands *et al.*⁴² showed that *in vitro* callus cultures can be used to demonstrate the metabolic degradation of selected pesticides in plants. Conducting *in vitro* experiments in *C. sativa* and crop plants offered the possibility to compare all plant species and to start to understand the xenobiotic metabolism capabilities of *C. sativa*. Table 1 gives the overview about the qualitative comparison between *C. sativa* and the crop plants: *B. napus*, *G. max*, *Z. mays* and *T. aestivum*. From the list of all metabolites it is apparent that in most cases *C. sativa* executed the same metabolic steps but often showed a greater variety in isomers of metabolites produced. For example, spirodiclofen was oxidized by crop plants into maximum three different dihydroxy-spirodiclofen-enol isomers, yet *C. sativa* produced four different isomers of the same oxidation (Table 1). After grouping of all isomers of one metabolic degradation step, the resulting pathways gave the same main degradation strategies in all of the examined plant species. Metabolic degradation of the same selected pesticides was done in another *C. sativa* hydroponic *in planta* experiment.⁵² Again it was possible to both extract and analyze known metabolites; the *in planta* *C. sativa* results support the *in vitro* results presented herein.

Metabolism guideline studies using ¹⁴C labeled pesticides, often identified the parent A.I. as the main residue.^{30–34} For example, the main residues of flurtamone in *T. aestivum* forage was the A.I. with $\leq 92\%$ of TRR.³⁴ The A.I. of tebuconazole, flurtamone and metalaxyl-M in all crop plants including *C. sativa* as well as fenhexamid in *B. napus* and *Z. mays*, defined the main residue. By contrast, for fenhexamid in *G. max*, *T. aestivum* and *C. sativa*, and for spirodiclofen in all used plant species, metabolites of the parent A.I. were detected as the major constituent in residue (Table S4). Fenhexamid was degraded by oxidation with conjugation of glucose and malonic acid, whereas spirodiclofen mainly was metabolized to phase I metabolites.

In Table S5 the majority of all detected main metabolites were phase I degradation products: oxidations, demethylations or cleavage of ester bonds. This is consistent with other reports on the metabolic degradation of xenobiotics in plant cell cultures.^{53,54}

This study demonstrated the presence of a broad spectrum of metabolites in *C. sativa* callus cultures. Especially for fenhexamid and spirodiclofen, metabolites described the main residue. Because it is known from literature that pesticide residues remain in the smoke of *C. sativa* and their concentrations can be up to ten-fold higher in *C. sativa* consumables in comparison to flowering heads as a result of processing of the raw agricultural commodities, their investigation, as well as appropriate residue and processing studies are important aspects for consumer safety.^{55,56}

Although information on the operator exposure to these chemicals can be expected to be similar to other crops grown in comparable circumstances, it will be important to investigate the potential exposure of workers who may manually harvest and process the commodities.²

5 CONCLUSIONS

The successful initiation and maintenance of *C. sativa* callus cultures enabled us to study the metabolic degradation of selected pesticides. Their degradation is comparable to their that in crop plant callus cultures and metabolism guideline data. This aspect is under-represented in most publications discussing the effect of contamination, especially pesticides, in *C. sativa* products. The presented assay is capable of qualitatively identifying plant

metabolites and characterizing their semiquantitative behavior in *C. sativa*. Because it is known that metabolites are compounds of toxicological concern, this assay is able to identify possible metabolites in a first step.

ACKNOWLEDGEMENTS

The authors would like to thank Julia Schachtsiek (TU Dortmund) for taking care of the *C. sativa* L. plants and Ricky Rohde (Bayer AG) for his support during the HPLC-HRMS measurements. They also would like to thank Elizabeth Bates (Bayer AG) for reviewing the manuscript. The studies were performed with permission no. 4586416 issued by the Federal Institute for Drug and Medical Devices (BfArM), Bonn, Germany; no more than 25 callus cultures of *C. sativa* L. were simultaneously in culture. This work is supported financially by Bayer AG, Division Crop Science, Monheim, Germany.

CONFLICT OF INTEREST

Leonie Hillebrands, Marc Lamshoef, Andreas Lagojda and Andreas Stork are employees of the Bayer AG Division Crop Science, a leading manufacturer of agricultural chemicals. Oliver Kayser declares no competing interests.

AUTHOR CONTRIBUTIONS

Leonie Hillebrands: Investigation and visualization of experimental data, Writing – Original draft. Marc Lamshoef: Conceptualization, Writing – Review & Editing, Supervision. Andreas Lagojda: Conceptualization. Andreas Stork: Conceptualization, Writing – Review & Editing. Oliver Kayser: Conceptualization, Writing – Review & Editing, Project administration.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

REFERENCES

- Craven CB, Wawryk N, Jiang P, Liu Z and Li X-F, Pesticides and trace elements in cannabis: analytical and environmental challenges and opportunities. *J Environ Sci* **85**:82–93 (2019).
- Schneider S, Bebing R and Dauberschmidt C, Detection of pesticides in seized illegal cannabis plants. *Anal Methods* **6**:515–520 (2014).
- Naef M, Curatolo M, Petersen-Felix S, Arendt-Nielsen L, Zbinden A and Brenneisen R, The analgesic effect of oral delta-9-tetrahydrocannabinol (THC), morphine, and a THC-morphine combination in healthy subjects under experimental pain conditions. *Pain* **105**:79–88 (2003).
- Rog DJ, Nurmikko TJ, Friede T and Young CA, Randomized, controlled trial of cannabis-based medicine in central pain in multiple sclerosis. *Neurology* **65**:812–819 (2005).
- Pauli CS, Conroy M, vanden Heuvel BD and Park SH, Cannabidiol drugs clinical trial outcomes and adverse effects. *Front Pharmacol* **11**:63 (2020).
- Larsen C and Shahinas J, Dosage, efficacy and safety of cannabidiol administration in adults: a systematic review of human trials. *J Clin Med Res* **12**:129–141 (2020).
- Coleman J, Blake-Kalff M and Davies E, Detoxification of xenobiotics by plants: chemical modification and vacuolar compartmentation. *Trends Plant Sci* **2**:144–151 (1997).
- Siminszky B, Plant cytochrome P450-mediated herbicide metabolism. *Phytochem Rev* **5**:445–458 (2006).
- Lamoureux GL and Rusness DG, Xenobiotic conjugation in higher plants, xenobiotic conjugation chemistry. *ACS Symp Ser* **299**:62–105 (1986).
- Korte F, Kvesitadze G, Ugrekheldidze D, Gordeziani M, Khatishashvili G, Buaдзе O *et al.*, Organic toxicants and plants. *Ecotoxicol Environ Saf* **47**:1–26 (2000).

- 11 Shaban NS, Abdou KA and Hassan NE-HY, Impact of toxic heavy metals and pesticide residues in herbal products. *Beni-Suef Univ J Appl Sci* **5**: 102–106 (2016).
- 12 Kumar N, Kulsoom M, Shukla V, Kumar D, Kumar S et al., Profiling of heavy metal and pesticide residues in medicinal plants. *Environ Sci Pollut Res* **25**:29505–29510 (2018).
- 13 Taylor A and Birkett JW, Pesticides in cannabis: a review of analytical and toxicological considerations. *Drug Test Anal* **12**:180–190 (2020).
- 14 Russo EB, Current therapeutic cannabis controversies and clinical trial design issues. *Front Pharmacol* **7**:309 (2016).
- 15 Punja ZK, Emerging diseases of Cannabis sativa and sustainable management. *Pest Manag Sci* (2021). <https://doi.org/10.1002/ps.6307>. [in press].
- 16 Stone D, Cannabis, pesticides and conflicting laws: the dilemma for legalized states and implications for public health. *Regul Toxicol Pharmacol* **69**:284–288 (2014).
- 17 McPartland JM, McKernan KJ, Chandra S, Lata H and ElSohly MA, Contaminants of concern in Cannabis: microbes, heavy metals and pesticides, in *Cannabis sativa L - Botany and Biotechnology*, Vol. 1, ed. by Chandra S, Lata H and ElSohly MA. Springer International, Berlin (2017).
- 18 United States Environmental Protection Agency (EPA), *Pesticide Products Registered for Use on Hemp*. Available: <https://www.epa.gov/pesticide-registration/pesticide-products-registered-use-hemp> [5 July 2021].
- 19 Pérez-Parada A, Alonso B, Rodríguez C, Besil N, Cesio V, Diana L et al., Evaluation of three multiresidue methods for the determination of pesticides in marijuana (*Cannabis sativa* L.) with liquid chromatography-tandem mass spectrometry. *Chromatographia* **79**:1069–1083 (2016).
- 20 Cuyper E, Vanhove W, Gotink J, Bonneure A, van Damme P and Tytgat J, The use of pesticides in Belgian illicit indoor cannabis plantations. *Forensic Sci Int* **277**:59–65 (2017).
- 21 Dryburgh LM, Bolan NS, Grof CPL, Galetti P, Schneider J, Lucas CJ et al., Cannabis contaminants: sources, distribution, human toxicity and pharmacologic effects. *Br J Clin Pharmacol* **84**:2468–2476 (2018).
- 22 Narváez Simón A, Rodríguez-Carrasco Y, Castaldo L, Izzo L and Ritienei A, Ultra-high-performance liquid chromatography coupled with quadrupole orbitrap high-resolution mass spectrometry for multi-residue analysis of mycotoxins and pesticides in botanical nutraceuticals. *Toxins* **12**:114 (2020).
- 23 Daniel D, Lopes FS and do Lago CL, A sensitive multiresidue method for the determination of pesticides in marijuana by liquid chromatography-tandem mass spectrometry. *J Chromatogr A* **1603**:231–239 (2019).
- 24 Atapattu SN and Johnson KRD, Pesticide analysis in cannabis products. *J Chromatogr A* **1612**:460656 (2020).
- 25 Wylie PL, Westland J, Wang M, Radwan MM, Majumdar CG and ElSohly MA, Screening for more than 1,000 pesticides and environmental contaminants in Cannabis by GC/Q-TOF. *Med Cannabis Cannabinoids* **3**:14–24 (2020).
- 26 Schmidt B, Thiede B and Rivero C, Metabolism of the pesticide metabolites 4-nitrophenol and 3,4-dichloroaniline in carrot (*Daucus carota*) cell suspension cultures. *Pest Sci* **40**:231–238 (1994).
- 27 Feung CS, Hamilton RH and Mumma RO, Metabolism of indole-3-acetic acid: III identification of metabolites isolated from crown gall callus tissue. *Plant Physiol* **58**:666–669 (1976).
- 28 Davidonis GH, Hamilton RH and Mumma RO, Metabolism of 2,4-dichlorophenoxyacetic acid in soybean root callus and differentiated soybean root cultures as a function of concentration and tissue age. *Plant Physiol* **62**:80–83 (1978).
- 29 Babczinski P, Plant cell cultures as a model for the biochemistry of crop selectivity. *Pest Sci* **55**:671–674 (1999).
- 30 Food and Agriculture Organization of the United Nations (FAO), Pesticide Residues in Food – 2004, Evaluations, Joint Meeting of the FAO Panel of Experts on Pesticide Residues in Food and Environment and the WHO Core Assessment Group, Rome, Italy, 20–29 September 2004. FAO, Rome (2005).
- 31 Food and Agriculture Organization of the United Nations (FAO), Pesticide Residues in Food – 2005, Evaluations, Joint Meeting of the FAO Panel of Experts on Pesticide Residues in Food and the Environment and the WHO Core Assessment Group on Pesticide Residues, Geneva, Switzerland, 20–29 September 2005. FAO, Rome (2006).
- 32 Food and Agriculture Organization of the United Nations (FAO), Pesticide Residues in Food – 1994, Evaluations, Report of the Joint Meeting of the FAO Panel of Experts on Pesticide Residues in Food and the Environment and the WHO Expert Group on Pesticide Residues, Rome, 19–28 September 1994. Food and Agriculture Organization of the United Nations (1994).
- 33 Food and Agriculture Organization of the United Nations (FAO), Pesticide Residues in Food – 2009, Evaluations, Report of the Joint Meeting of the FAO Panel of Experts on Pesticide Residues in Food and the Environment and the WHO Core Assessment Group on Pesticide Residues, Geneva, Switzerland, 16–25 September 2009. FAO, Rome (2010).
- 34 European Food Safety Authority (EFSA), Updated peer review of the pesticide risk assessment of the active substance flurtamone. *EFSA J* **15**:e04976 (2017).
- 35 Sandler LN, Beckerman JL, Whitford F and Gibson KA, Cannabis as conundrum. *Crop Prot* **117**:37–44 (2019).
- 36 Kwok IMY and Loeffler RT, The biochemical mode of action of some newer azole fungicides. *Pest Sci* **39**:1–11 (1993).
- 37 Lamb DC, Cannieux M, Warrilow AGS, Bak S, Kahn RA, Manning NJ et al., Plant sterol 14 α -demethylase affinity for azole fungicides. *Biochem Biophys Res Commun* **284**:845–849 (2001).
- 38 Krämer W and Schirmer U, *Modern Crop Protection Compounds*, 3 Volume Set. John Wiley & Sons Ltd, Chichester, UK (2007).
- 39 Debieu D, Bach J, Hugon M, Malosse C and Leroux P, The hydroxyanilide fenhexamid, a new sterol biosynthesis inhibitor fungicide efficient against the plant pathogenic fungus *Botryotinia fuckeliana* (*Botrytis cinerea*). *Pest Manag Sci* **57**:1060–1067 (2001).
- 40 Sandmann G, Ward CE, Lo WC, Nagy JO and Böger P, Bleaching herbicide flurtamone interferes with phytoene desaturase. *Plant Physiol* **94**:476–478 (1990).
- 41 Bretschneider T, Fischer R and Nauen R, Inhibitors of lipid synthesis: acetyl-CoA-carboxylase inhibitors, in *Modern Crop Protection Compounds*, ed. by Krämer W, Schirmer U, Jeschke P and Witschel M. Wiley-VCH Verlag & Co. KGaA, Weinheim, pp. 1059–1126 (2012).
- 42 Hillebrands L, Lamshoef M, Lagojda A, Stork A and Kayser O, Evaluation of callus cultures to elucidate the metabolism of tebuconazole, flurtamone, fenhexamid, and metalaxyl-M in *Brassica napus* L., *Glycine max* (L.) Merr., *Zea mays* L., and *Triticum aestivum* L. *J Agric Food Chem* **68**:14123–14134 (2020).
- 43 Murashige T and Skoog F, A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiol Plant* **15**:473–497 (1962).
- 44 Meyer-Teuter H and Reinert J, Correlation between rate of cell division and loss of embryogenesis in longterm tissue cultures. *Protoplasma* **78**:273–283 (1973).
- 45 Mediavilla V, Jonquera M, Schmid-Slembrouck I and Soldati A, Decimal code for growth stages of hemp (*Cannabis sativa* L.). *J Int Hemp Assoc* **5**:68–74 (1998).
- 46 Yeoman MM and Evans PK, Growth and differentiation of plant tissue cultures: II. Synchronous cell divisions in developing callus cultures. *Ann Bot* **31**:323–332 (1967).
- 47 Schriever C and Lamshoef M, Lipophilicity matters – a new look at experimental plant uptake data from literature. *Sci Total Environ* **713**:136667 (2020).
- 48 Briggs GG, Bromilow RH and Evans AA, Relationships between lipophilicity and root uptake and translocation of non-ionised chemicals by barley. *Pest Sci* **13**:495–504 (1982).
- 49 Lewis KA, Tzilivakis J, Warner DJ and Green A, An international database for pesticide risk assessments and management. *Hum Ecol Risk Assess* **22**:1050–1064 (2016).
- 50 OECD, Test No. 501: Metabolism in Crops, OECD Guidelines for the Testing of Chemicals, Section 5, OECD Publishing, Paris (2007). <https://doi.org/10.1787/9789264061835-en> (Accessed 3rd August 2021)
- 51 United States Environmental Protection Agency (EPA), Residue Chemistry Test Guidelines: OPPTS 860.1300: Nature of the Residue - Plants, Livestock (EPA 712-C-96-172). Available: <https://www.regulations.gov/document/EPA-HQ-OPPT-2009-0155-0006> (1996).
- 52 Hillebrands L, Lamshoef M, Lagojda A, Stork A and Kayser O, Metabolism of fenhexamid, metalaxyl-M, tebuconazole, flurtamone, and spirodiclofen in *Cannabis sativa* L. (hemp) plants. *ACS Agric Sci Technol* **1**:192–201 (2021).
- 53 Cole DJ and Loughman BC, The metabolic fate of (4-chloro-2-methylphenoxy)acetic acid in higher plants. *J Exp Bot* **34**:1299–1310 (1983).
- 54 Frear DS and Swanson HR, Metabolism of cisanilide (cis-2,5-dimethyl-1-pyrrolidinedicarboxanilide) by excised leaves and cell suspension cultures of carrot and cotton. *Pest Biochem Physiol* **5**:73–80 (1975).
- 55 Sullivan N, Elzinga S and Raber JC, Determination of pesticide residues in Cannabis smoke. *J Toxicol* **2013**:378168 (2013).
- 56 Subritzky T, Pettigrew S and Lenton S, Into the void: regulating pesticide use in Colorado's commercial cannabis markets. *Int J Drug Policy* **42**:86–96 (2017).