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Short Communication

Analytical methodology for the study of endosulfan bioremediation under controlled conditions with white rot fungi

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ABSTRACT

A general procedure to study the biodegradation of endosulfan under laboratory conditions by white rot fungi isolated from native sources growing in YNB (yeast nitrogen base) media with 1% of glucose is presented. The evaluation of endosulfan biodegradation as well as endosulfan sulfate, endosulfan ether and endosulfan alcohol production throughout the whole bioremedation process was performed using an original and straightforward validated analytical procedure with recoveries between 78 and 112% at all concentration levels studied except for endosulfan sulfate at 50 mg L^{-1} that yielded 128% and RSDs < 20%. Under the developed conditions, the basidiomycete *Bjerkandera adusta* was able to degrade 83% of (alpha + beta) endosulfan after 27 days, 6 mg kg^{-1} of endosulfan diol were determined; endosulfan ether and endosulfan sulfate were produced below 1 mg kg^{-1} (LOQ, limit of quantitation).

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1. Introduction

Endosulfan (6,7,8,9,10,10-hexachloro-1,5,5a,6,9,9a-hexahydro-6,9-methano-2,4,3-benzodioxathiepin 3-oxide) is a pesticide (insecticide and acaricide) still used in agriculture in some countries but already banned in the European Union since 2006 (Legislation: 05/864/EC). In the field, technical grade endosulfan is employed, which is a mixture of two stereoisomers, α- and β-endosulfan in a 7:3 ratio. The contamination of atmosphere, soils, sediments, surface and rain waters and foodstuffs by endosulfan has been documented in several studies [1]. This chlorinated pesticide persists in the environment for very long periods, having a half-life of 60–800 days [2,3]. It undergoes bioaccumulation and biomagnification, therefore becoming toxic to non-target organisms including human beings. Its harmful impacts on aquatic fauna [4] and numerous mammalian species including human beings have been reported several times in the literature [5]. Particularly, endosulfan is extremely toxic to fish and aquatic invertebrates and it has been increasingly implicated in mammalian gonadal toxicity, genotoxicity, and neurotoxicity [6]. The persistence of endosulfan in soil and water environments has been observed by several researchers under different conditions [3,7]. Up to date, some physicochemical and biological remedial strategies which lead to degradation of endosulfan into both toxic and non-toxic metabolites, have been described in the literature [8–10].

In Uruguay, endosulfan was authorized to be applied only to soybean plantations as insecticide until 2011. Its repeated applications and misuses, resulted in the focal contamination of soil and water environments in several countries including Uruguay [11], causing deleterious effects on the environment and public health.

Bioremediation could be a solution for this complex situation because it enables the biodegradation of recalcitrant compounds that cannot be chemically degraded. White rot fungi, which occur naturally on wood, have demonstrated the ability to bioremediate contaminated sites [12]. These fungi have a considerably higher tolerance to toxic pollutants than other organisms that need to uptake the toxin in order to decompose it [12]. Oxidation of the sulfite portion of the endosulfan moiety leads to the formation of the toxic and more persistent endosulfan sulfate whereas the sulfite hydrolysis yields the nontoxic endosulfan diol. To our knowledge there is no report on the degradation of endosulfan isomers by white rot fungi. Therefore, we faced the task of evaluating the capability of native fungi, isolated from *Eucalyptus* sp., to degrade endosulfan under controlled conditions; looking for the prevention of pollution by POPs (persistent organic pollutants) of surface and groundwaters in Uruguay.

There are no general and validated analytical methods reported in the literature for the proper evaluation of endosulfan

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dissipation and metabolite production (endosulfan ether, endosulfan diol, endosulfan sulfate) by white rot fungi in laboratory bioremediation assays. Trying to fill this gap, we undertook the development of a validated analytical procedure for the analysis of endosulfan and the metabolites produced by *Bjerkandera adusta* (taken as fungal model), grown in 1% glucose yeast nitrogen base culture medium.

2. Materials and methods

2.1. Standards and reagents

Analytical grade organic solvents, pesticide residues free were purchased from Merck (Darmstadt, Germany). Pesticide standards and the internal standard were from Dr. Ehrenstorfer (Augsburg, Germany, 99%). The culture media were provided by Difco (Detroit, MI, USA). Standard bags of 177 mm × 305 mm for Stomacher apparatus were purchased from Seward (Worthing, United Kingdom). Stock solutions were prepared from the standard substances at 1000 mg L^{-1} in ethyl acetate. Working standard mixtures were prepared by appropriately diluting the stock solutions with ethyl acetate. All solutions were stored at 4 °C.

2.2. Apparatus and experimental conditions

Gas chromatographic (GC) analyses were performed using a Shimadzu GC 17A equipped with an ECD (electron capture detector) and a PTV (programmed temperature vaporizing) injector using internal standard method. All compounds were resolved on a capillary column Mega 68 (30 m, 0.32 mm ID, 0.25 μm film thickness) Mega Legnano (Italy). The experimental conditions were as follow: PTV injector temperature, 60 °C (0.3 min), then 5 °C min^{-1} to 280 °C (40 min). Oven temperature, 100 °C (3 min), 100–180 °C at 10 °C min^{-1}, 180 °C (15 min), then 180–270 °C at 5 °C min^{-1}, 270 °C (10 min). Detector temperature, 280 °C. Stomacher apparatus: Seward U.K. laboratory blender 400.

2.3. Microbiological matrix preparation for validation study

Bjerkandera adusta (CCM0379) was subcultured in Malt Extract Agar and grown for five days. It was then transferred using a 5 mm plug as an inoculum, to 52 mm Petri dishes containing 7 g of YNB (yeast nitrogen base) medium with 1% of glucose. Samples were incubated at 28 ± 2 °C for 18 days to perform the analytical method validation.

2.4. Recovery tests

For recovery studies the matrix was prepared with spiking at three levels: 1, 25 and 50 mg kg^{-1} with the pesticide under study and its metabolites. Level 1 mg kg^{-1} was prepared by adding 0.7 mL of a solution containing 10 mg L^{-1} of endosulfan α and β, endosulfan ether, endosulfan diol and endosulfan sulfate to the grown fungi in culture medium and placed in a stomacher bag (5 replicates); level 25 and 50 mg kg^{-1} were prepared by adding 1.75 and 3.5 mL, respectively, of a 100 mg L^{-1} mixture solution of the same analytes.

2.5. Extraction and clean-up methods comparison

The compared methods were: QuEChERS [13] as a dispersive acetonitrile based method, Mini-Luke [14] as a liquid–liquid acetone:dichlormethane partition method, ethyl acetate [15] as another solvent based dispersive method and the novel methodology developed using ethyl acetate and Stomacher® assistance for the extraction/homogenization described in Section 2.6.

The comparison of the extraction methods was performed with Petri dishes inoculated at 50 mg kg^{-1} of endosulfan.

2.6. Sample preparation for endosulfan and its metabolites analysis

The whole Petri dish content (culture media + fungi) was placed in a Stomacher® bag where 40 mL ethyl acetate were added; the bag is afterwards placed in a Stomacher® apparatus during 2 min for extraction/homogenization. An aliquot of 28 mL of the extract was driven to dryness with a rotary vacuum evaporator. Then, the extract was redissolved in 0.5 mL of an ethyl acetate solution with 10 mg L^{-1} chlorpyrifos methyl as internal standard (IS). Afterwards, the obtained solution was diluted to volume with ethyl acetate in a 5 mL volumetric flask obtaining a final vial concentration of 1 mg L^{-1} of the IS. These aliquots and dilutions were performed for the level 1 mg kg^{-1} and adjusted for the levels 25, 50 mg kg^{-1} in order to obtain a final vial concentration of 1 mg L^{-1}. Therefore, the final matrix coextractives' concentration varied with the dilutions made during the sample preparation. The chromatographic analysis was performed in a GC-ECD under the conditions explained in Section 2.2.

Matrix matched standards were prepared using a blank sample extracted with the same procedure as the recovery test samples, driven to dryness and redissolved in an ethyl acetate solution containing the studied analytes and the IS in an adequate concentration according to DG-SANCO [15].

2.7. Method validation

The following parameters were evaluated for the analytical method validation: linearity, recoveries, repeatability (RSDr), within-laboratory reproducibility (RSDwR), limits of quantitation (LOQs) and limits of detection LODs. All the tests were performed at three levels, five replicates in three different days. Solvent and matrix matched calibration curves were compared and matrix effects were quantified. Matrix effects were evaluated at three different amounts of matrix 0.98, 0.042, and 0.021 g mL^{-1} of extract which correspond to the dilutions needed to cover the wide range of concentrations (1–50 mg L^{-1}) necessary to assess the biodegradation of endosulfan. Percentage matrix effects were calculated as the matrix matched calibration curve slope minus the solvent calibration curve slope relative to the solvent calibration curve slope ((matrix matched calibration curve slope-solvent calibration curve slope) * 100/solvent calibration curve)

3. Theory

White rot fungi have the capability of degrading xenobiotics due to the production of extracellular laccases, oxidases and hydrolases. The degrading capability is checked in trial experiments, growing the fungi in contact with the xenobiotic for a specific period of time while monitoring endosulfan disappearance. The analytical task is to quantitatively extract the compound and its metabolites from the culture media in the presence of the fungal mycelium. In this very complex matrix, different physical and biochemical phenomena occur. Fungi grow heterogeneously in the culture media and interact with endosulfan in a physical and biochemical way, either occluding the compound in the mycelium or through the extracellular enzymes which degrade it. All these phenomena add up to make a very heterogeneous matrix with a concentration gradient of analytes within it that has to be properly homogenized for the extraction step. The vigorous sample stirring and beating supplied by the Stomacher allows an efficient matrix disruption and analyte extraction.

Table 1
Percentage recoveries (%) and Precision RSDr repeatability and RSDwR within laboratory reproducibility (relative standard deviations (%)) of endosulfan ether, endosulfan alcohol, endosulfan α, endosulfan β and endosulfan sulfate for the novel methodology using ethyl acetate and Stomacher® assistance at 1, 25 and 50 mg kg^{-1}.

	1 mg kg^{-1}			25 mg kg^{-1}			50 mg kg^{-1}		
	Recovery (%)	RSDr	RSDwR	Recovery (%)	RSDr	RSDwR	Recovery (%)	RSDr	RSDwR
Endosulfan ether	91	4	7	80	8	14	102	3	8
Endosulfan alcohol	78	4	9	103	9	10	112	2	11
Endosulfan α	94	4	7	94	8	10	103	4	7
Endosulfan β	95	3	9	101	7	7	112	2	5
Endosulfan sulfate	95	5	7	104	8	6	128	5	11

4. Results and discussion

The first trial aiming to find a good methodology to analyze endosulfan and its metabolites in the sample, was to test well-known sample preparation methodologies for pesticide multiresidue analysis: QuEChERS [13] and Mini Luke [14]. These methods were chosen because they present acceptable recoveries for a wide range of pesticides and matrices. The recoveries obtained in the experiments performed under our laboratory conditions for these methodologies were not adequate (30 and 40% recoveries with 7 and 10% RSDs for QuEChERS and Mini Luke, respectively). Then, a modification of the Ethyl Acetate methodology as reported in [15], using an orbital shaker was evaluated. Eventhough, the recoveries were better, they were not good enough to accomplish the DG-SANCO (European Commission' Directorate-General for Health and Consumers) [16] for pesticide residue analysis guidelines criteria. The problems detected were that these methods were not able to assure neither homogeneity nor representativeness. It was not possible to perform the sub sampling and sample comminution steps prior to solvent addition in order to achieve good accessibility of the solvent to the analytes because the fungi grows heterogeneously in the culture media. The enzymatic diffusion was assayed with Poly-R 480 and Remazole-blue. It was observed that the decoloration zone was bigger than the growth halo, concluding that the extracellular enzymes diffuse at a higher rate than the colony linear growth rate. Presumably, this is the case for the degradation of endosulfan as well; for this reason it is important to analyze the whole Petri dish content. The analytical methodology was developed taking into account the particular characteristics of the assayed matrix. Consequently, the new method was chosen as the recoveries were better than with the other evaluated methodologies and it does not need the salts addition step. For this reason, the time and costs of analysis are lower. Another advantage is that it allows to extract the whole biotransformation media in the Petri dish; which will be a useful tool to assess the real whole biotransformation process.

Overall good results were obtained for endosulfan and the different metabolites assayed (Table 1); recoveries were between 78 and 112% at all concentration levels studied, except for endosulfan sulfate at 50 mg L^{-1}. The method was precise: its repeatability (RSDr) was below 9% and its within-laboratory reproducibility (RSDwR) is below 14% for all analytes at all the evaluated levels. The evaluated linearity ranges were 0.125–1 mg L^{-1} with correlation coefficients >0.99 for all analytes. The determined LOQs and LODs were adequate for the purpose of the method: the quantification of endosulfan residue and its metabolites for a fungi growing in YNB media. The values for LOQs and LODs are 1 and 0.3 mg kg^{-1}, respectively.

The fungal mycelium and the growth media are responsible for marked matrix effects when the amount of matrix injected is higher, being very important for experiments when the initial endosulfan concentration was 1 mg kg^{-1} but it was very small at 50 mg kg^{-1}. In particular, at these concentrations, this effect was observed for endosulfan metabolites while it was not observed for endosulfan isomers (Table 2). The low retention time metabolites suffered negative matrix effects and the higher ones showed enhanced effects that disappeared upon matrix dilution. At 25 mg kg^{-1}, negative effects disappeared but the positive matrix effects are noticeable, according to the classification proposed [17]. Matrix effects are a combination of positive and negative contributions to analyte determination within the separation/detection system. Positive matrix effects are generally explained due to the protective effect of the matrix in the GC inlet active sites rather than on the detector response, while negative matrix effects could be caused either by reactions between the analytes and matrix components at the injection port or by co-eluting electronegative matrix compounds that capture the electrons and decrease the analyte signal, particularly aromatics and volatile sulfur derivatives. However, matrix-matched calibration curves were used for quantitation at all concentration levels because the influence of the amount of matrix on the analyte response is very heterogeneous due to the intrinsic variability of the biological system under study. The recommendation is to always quantify with matrix matched calibration curves when assessing endosulfan dissipation and its metabolites generation with a blank extract prepared in the same way as the samples (same amount of matrix injected).

Table 2
Percentage matrix effect (ME%) for endosulfan and its metabolites calculated at 1, 25 and 50 mg kg^{-1} using for the novel methodology using ethyl acetate and Stomacher® assistance with GC–ECD analysis.

	1 mg kg^{-1} ME (%)	25 mg kg^{-1} ME (%)	50 mg kg^{-1} ME (%)
Endosulfan ether	−50	0	0
Endosulfan alcohol	−25	10	−12
Endosulfan α	1.8	33	−6.5
Endosulfan β	4.3	38	−4.8
Endosulfan sulfate	49	96	−0.7

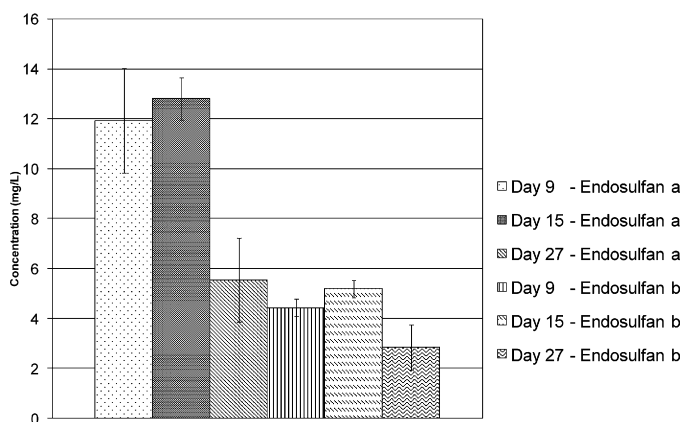

Fig. 1. Degradation of endosulfan isomers by *B. adusta* growing in Yeast Nitrogen Base + 1% glucose with 50 mg kg^{-1} of endosulfan at 28 ± 2 °C during 27 days of incubation.

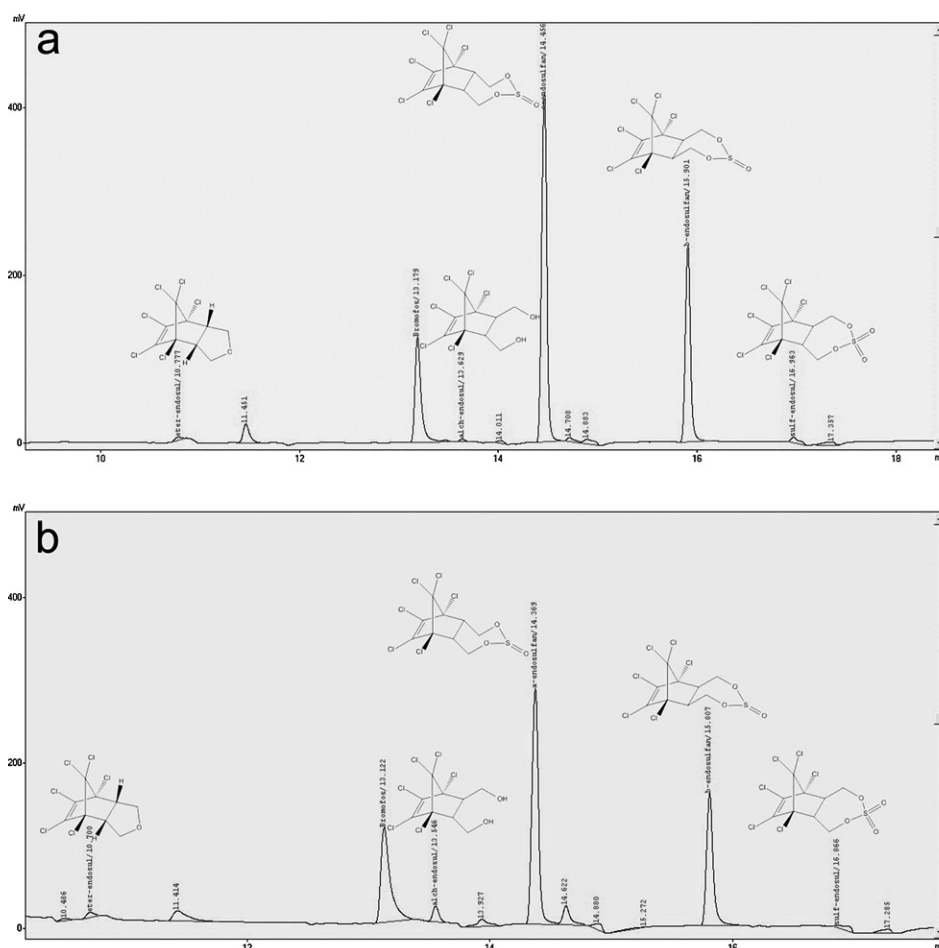

Fig. 2. Chromatograms of endosulfan degradation and metabolites production. After 9 days (part a) and after 27 days (part b) of incubation of *B. adusta* in the media yeast nitrogen base with 1% of glucose.

One of the advantages of this method is that as the sample contained in the Petri dish (live fungi + culture medium) is completely transferred to the stomacher bag and is effectively extracted, no sub sampling is necessary, therefore all problems related to representativeness are avoided. Also this characteristic improves the results in terms of repeatability and reproducibility. Another advantage of this method is that probably due to the strong media and fungal disruption provided by the stomacher extraction/homogenization, it allows better accessibility of the extraction solvent to the analytes. The method has few steps and is easy to perform in the laboratory considering the complexity of the matrix such as a microbiological organism grown in a culture media degrading a recalcitrant pesticide.

The method was further tested in real conditions using *B. adusta*, a white rot fungi (WRF) isolated from *Eucalyptus* sp. The fungi was able to degrade 83% of (alpha + beta) endosulfan under laboratory conditions (Fig. 1). The degradation of the pesticide was easily followed and endosulfan decay over time was proven.

The GC-ECD chromatograms show (Fig. 2) the profile of a typical biotransformation experiment of endosulfan by *B. adusta*. The main metabolite produced was endosulfan–diol along with lower levels of endosulfan ether and sulfate at every sampling day. These findings are very promising as the most environmentally dangerous amongst the metabolites, endosulfan sulfate, is produced at very low levels, and diminishes with time, being detected below the LOQ (1 mg kg^{-1}) at 27 days of incubation. The results suggest that the S—O bond is cleaved preferentially, accumulating little endosulfan or endosulfan sulfate, favoring the production of non sulfur containing molecules like endosulfan diol, that can presumably be easily degraded by the fungal oxidases, as no other ECD detectable compound could be observed. Moreover, the amount of endosulfan diol detected is almost the same after 9 or 27 days (6 mg kg^{-1}), reaching a stationary state, whereas the amount of (alpha + beta) endosulfan present in the transformation media diminished significantly, indicating its degradation through a hydrolysis–oxidation pathway.

5. Conclusions

This novel extraction and clean up methodology of sample preparation from a solid microbiological culture for pesticide residue analysis has shown to be fit to purpose as it allows the accurate determination of endosulfan dissipation as well as its metabolites production by GC-ECD. The procedure has been successfully applied to the evaluation of endosulfan degradation by *B. adusta*, a uruguayan white rot fungi isolated from *Eucalyptus* sp.

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