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Extraction and Determination of Glucosinolates from Soil

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The use of glucosinolate-containing plants as soil-incorporated biofumigants for pest and disease control has raised questions regarding the fate of glucosinolates in soil; however, no method for routine analysis of glucosinolates in soil has been reported. A simple method to extract glucosinolates from soil with quantification as desulfoglucosinolates by HPLC is presented. The method involves two extractions with 70% methanol at room temperature, centrifugation, and filtration prior to the desulfation step. The desulfoglucosinolates are then quantified by HPLC using established protocols for plant tissue analysis. There were no significant interfering peaks from the soil extracts, and the method provided high extraction efficiencies (around 100%) for both aromatic (benzyl) and aliphatic (2-propenyl) glucosinolates when amended at a wide range of realistic field soil concentrations (1.6−120 nmol/g of soil). The method was equally effective in three diverse Australian soils that varied in organic matter, clay content, and pH. The method was effective in air-dried or field-moist soil, although evidence for rapid glucosinolate degradation in field-moist soil indicates that extraction of moist soils should be performed as soon as possible after sampling. The method is compatible with field soil sampling at remote sites and utilizes the same equipment and protocols already established for plant tissue analysis. Extraction of glucosinolates in the field following incorporation of Indian mustard (*Brassica juncea*) and rape (*Brassica napus*) green manure crops was also tested. Eight different glucosinolates contained in the plant tissues were identified and quantified in soil extracts at concentrations ranging from 0.11 to 21.7 nmol/g of soil.

KEYWORDS: ***Brassica*; 2-propenyl glucosinolate; benzyl glucosinolate; HPLC; biofumigation**

INTRODUCTION

Glucosinolates are a group of compounds produced by plants in the family *Brassicaceae* and a few other plant families and are responsible for the flavors in well-known crops such as mustard, radish, and cabbage (*1*). Glucosinolates consist of a β-thioglucose moiety, a sulfonated oxime moiety, and a variable side chain derived from an amino acid. More than 120 different side chains have been described (*2*), although glucosinolates are often divided into three groups according to the nature of their side chain: aliphatic, aromatic, and indolyl. The difference between the glucosinolates with regard to chemical properties and biological activity and the hydrolysis products formed is largely determined by the side-chain structure (*1*).

Interest in the fate of glucosinolates in soil derives from recent research on the use of brassicaceous plants as biofumigants (*3*). Biofumigation refers to the suppression of soil pests and diseases by the biocidal glucosinolate hydrolysis products, principally isothiocyanates, released when brassicaceous green manure crops are incorporated into the soil (*3*−*7*). When tissues of glucosinolate-containing plants are damaged, the glucosinolates come in contact with the endogenous enzyme myrosinase, which hydrolyses the glucosinolates to several biologically active compounds. Of these, isothiocyanates are generally considered the most toxic (*6*, *8*, *9*). It is generally believed that the glucosinolates will be hydrolyzed or degraded quickly in soil after incorporation, but there have been no studies to confirm this by measurement. Unhydrolyzed glucosinolates may, at least in part, account for the inability to achieve the maximum liberation of isothiocyanates during biofumigation (*6*). Measurements of glucosinolates in soil will also be important to understand their fate relative to the generation of their hydrolysis products in the soil environment. For instance, the glucosinolates are expected to easily leach from soil, in contrast to the corresponding isothiocyanates. Hitherto there have been no published methods for the extraction and quantification of glucosinolates from soil.

Glucosinolates are polar and highly water soluble compounds (*10*), but when they come into contact with the enzyme myrosinase, they hydrolyze relatively quickly especially if there

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Table 1. Properties of the Three Experimental Soils

	pH (water)	% clay	% silt	% organic carbon	CEC[a] (cmol/kg)
Endeavor	6.2	14.6	6.0	0.6	2.45
Ginninderra	7.2	15.9	23.9	0.9	6.93
Southedge	5.8	7.4	6.0	0.2	0.88

[a] CEC is the cation exchange capacity.

is water present (*11*−*13*). Myrosinase is not only produced by plants, but also by insects, fungi, and bacteria, and myrosinase activity may be found in soil (*12*, *14*−*16*).

Extraction and determination of glucosinolates from other matrixes such as plant tissues and foods have been performed for many years, but no methods are available for determination in environmental matrixes such as soils and natural waters. Soil represents a completely different matrix compared to plants and foods, and interactions with the inorganic and organic soil constituents often occur for organic compounds. In addition, coextracted compounds can interfere in the analysis, requiring cleanup and preconcentration steps prior to determination. The solvent used for extraction of glucosinolates has to inactivate the myrosinase enzyme, so that hydrolysis of the glucosinolates is inhibited during extraction (*17*, *18*). Some methods simply use boiling water (*19*, *20*) for the extraction, while others add a denaturing agent like methanol to the extraction solvent (*3*, *17*, *21*, *22*). Extraction with hot 70% methanol is commonly used (*3*, *17*, *21*, *22*) but 100% methanol has also been used (*23*). On the basis of this knowledge, it seems likely that glucosinolates could be extracted from soil with a polar solvent but that care must be taken to avoid hydrolysis.

This paper presents a method to extract and measure glucosinolates from soil. The developed extraction procedure enables the extraction to be initiated in the field. The analysis of the glucosinolates in the soil extracts follows the standard desulfation and HPLC procedure used routinely for glucosinolate analysis of plant tissue samples (*3*, *21*, *22*).

MATERIALS AND METHODS

Three Australian agricultural soils with different properties were used in the study (**Table 1**). The Ginninderra soil was sampled at the CSIRO Ginninderra Experiment Station close to Canberra ACT, Australia (149.10°E, 35.20°S) and is generally cultivated with temperate crops or pastures, predominately wheat. The Endeavor and Southedge soils were both sampled close to Mareeba in tropical north Queensland (16.59°S, 145.28°E). The Endeavor soil was sampled from a horticultural farm growing subtropical vegetables such as tomatoes, while the Southedge soil was sampled from a former tobacco experimental station. The texture, soil organic matter content, and pH of the soils varied considerably and represented the range typical of lighter textured soils used for irrigated horticulture in northern Europe, western United States, and Australia. Some preliminary experiments were carried out using the Ginninderra soil at field moisture content (15% g/g), but all soils were subsequently air-dried and sieved to 2 mm for comparative studies.

Extraction Procedure. Two pure glucosinolates, an aromatic (benzyl glucosinolate; Canola Council of Canada, ≥99% purity) and an aliphatic (2-propenyl glucosinolate; Sigma, ≥99% purity) were used in the study because both types are abundant in commonly used biofumigant crops (*3*).

The extraction experiments were carried out by applying an aqueous solution (10, 75, or 750 μL depending on the amount added) containing the pure glucosinolates at a concentration of 1.6 mM to 10 g of soil in a polypropylene 50-mL centrifuge tube. The tubes were shaken gently for 5 min. The extractions were done at ambient temperature. After a contact time of approximately 30 min, 70% methanol was applied (the amount varied depending on the experiment; see below for details), and the tubes were shaken vigorously (manually) and left to stand for 45 min. The contact time of 30 min was chosen to allow time for any chemical reactions of the glucosinolates with the soils without their microbial degradation or hydrolysis playing a significant role. The tubes were then centrifuged and the supernatant was filtered through a 0.22 μm syringe-mounted nylon filter. In experiments in which two extractions were performed, a second portion of 70% methanol was applied (the amount varied depending on the experiment; see below for details), and the tubes were shaken periodically over a 45 min period and then centrifuged. The supernatant was filtered, and the two filtrates were combined. All experimental treatments were prepared in triplicate with the exception of the first experiment, which was prepared in duplicate.

Investigations of Extraction Requirements. Three preliminary experiments were carried out to investigate the impacts of extraction number, soil type, glucosinolate type, and concentration on the glucosinolate recovery in soil. The Ginninderra soil used in these experiments was at field moisture content, while the other two soils had been air-dried.

In experiment 1, 750 μL of aqueous solution containing 1.2 μmol of 2-propenyl glucosinolate was applied to 10 g of each of the three soils (120 nmol/g of soil), which were then extracted as described above using either one extraction with 6 mL of 70% methanol or two extractions each with 5 mL of 70% methanol to investigate the effect of the number of extractions on the extraction efficiency.

In experiment 2, only the Ginninderra soil was used, but lower concentrations of glucosinolates were added. The first experiment was repeated by applying 0.12 μmol of benzyl and 2-propenyl glucosinolate separately to 10 g of soil (12 nmol/g of soil) and extracting either once with 10 mL of 70% methanol or twice with 5 mL of 70% methanol to investigate the effect of one versus two extractions.

In experiment 3, we investigated the effect of soil moisture on extraction efficiency as the Ginninderra soil used in experiments 1 and 2 was at field moisture content. In this experiment, 0.016, 0.12, and 1.2 μmol of benzyl and 2-propenyl glucosinolates were applied to 10 g of field-moist or air-dried Ginninderra soil (1.6, 12, and 120 nmol/g of soil) and subsequently extracted with 2 × 5 mL 70% methanol after 30 min.

Effect of Soil Type and Glucosinolate Concentration on Extraction Efficiency. In experiment 4, air-dried Southedge and Endeavor soils were used and 2-propenyl and benzyl glucosinolate were added using the same procedures as in experiment 3 (1.6, 12, and 120 nmol/g of soil). Controls were performed by extracting soils with no glucosinolates applied with 2 × 5 mL 70% methanol. Data for air-dried Ginninderra soil derived from experiment 3 were directly comparable with results from this experiment.

Extraction of Glucosinolates from Field Soil after *Brassica* Green Manure Incorporation. To verify the effectiveness of the method for extraction of glucosinolates applied as fresh plant material in the field, the method was applied at the Ginninderra field site to extract glucosinolates following incorporation of flowering *Brassica* crops. Indian mustard (*Brassica juncea*) and rape (*Brassica napus*) green manure crops were macerated with a mulcher at flowering, and the tissues incorporated into the top 0−10 cm of soil using a rotary hoe. Immediately after incorporation, soil samples were taken from the top 0−10 cm of the soil and visible fragments of plant material were removed. The samples were extracted by placing 20 g of soil into preweighed 50-mL plastic centrifuge tubes containing 10 mL of 70% methanol. The tubes were shaken vigorously immediately after sampling and were taken to the laboratory where they were weighed and then centrifuged for 5 min at 3000 rpm. The supernatant was filtered through a 0.22 μm syringe-driven nylon filter. Another portion of 10 mL of 70% methanol was added to the soil, the centrifuge tubes were shaken vigorously for 5 min and left for 45 min, shaking three times in that period. The centrifuge tubes were centrifuged again, the supernatants filtered, and the two filtrates combined.

HPLC Measurement of the Glucosinolates. Quantification of the glucosinolates in all soil filtrates was performed using the method described by Kirkegaard and Sarwar (*3*). Briefly, the glucosinolates in the filtrates were captured and washed on an anion-exchange resin (Sephadex A-25) and then transformed to desulfoglucosinolates using

sulfatase from Sigma (*3*). The desulfoglucosinolates were separated by gradient HPLC using a Waters HPLC system consisting of a 600E multisolvent delivery system, a 717 autosampler, and a 486 tuneable absorbance detector set to 229 nm (Waters Inc., Milford, MA) with a Maxima 820 chromatographic workstation (Dynamic Solutions, Chino, CA). A 250 mm × 4.6 mm (i.d.) Spherisorb C-18 reversed-phase column (ODS2, 5 μm from Alltech) was used for separation. The mobile phase consisted of two eluents: A, 100% MilliQ water; B, 50% acetonitrile:MilliQ water. Both eluents were filtered and degassed prior to use using vacuum. The flow rate was 1 mL min^{-1} except for a reduction to 0.97 mL min^{-1} over the initial 20 min. The program started with 99% A and 1% B for 1 min followed by a linear gradient over 20 min to 1% A and 99% B. This was held for 3 min before the program returned to 99% A and 1% B by a linear gradient for 1 min followed by 11 min equilibration. The glucosinolates were quantified using external standards prepared in 70% methanol. The concentrations of the standards were 0.0016, 0.016, 0.047, 0.091, and 0.15 mM, and the standard curves were linear over this range.

For the samples from the field, the identification of the glucosinolates was based on previous measurements of the plant material before maceration (*3*). The concentration of the glucosinolates was determined by use of an internal standard (75 μL of 16 mM 2-propenyl glucosinolate for the rape, and 75 μL of 16 mM benzyl glucosinolate for the mustard, the 75 μL was added to the 10-mL extract) and the response factors published by the European Economic Community (Commission Regulation (EEC) No 1864/90).

Statistical Analysis. The extraction efficiency was calculated as the percent of glucosinolate recovered relative to the amount of glucosinolate added to the soil. The effect of different treatments and their interactions on extraction efficiency was analyzed using ANOVA (The SAS system for Windows v8) using models appropriate for each experiment: soil type × extraction times (experiment 1), glucosinolate type × extraction times (experiment 2), soil moisture content × glucosinolate concentration × glucosinolate type (experiment 3), and soil type × glucosinolate concentration × glucosinolate type (experiment 4). Treatments were considered to be significant at $P < 0.05$. The confidence interval (95%) was used to compare the recovery efficiency for each treatment with a 100% extraction benchmark.

RESULTS AND DISCUSSION

The chromatograms of extracts from the control treatments in experiment 4 (i.e. no addition of glucosinolates) showed no detectable peaks, implying that there were no interfering compounds present in the soil extracts and that no further sample preparation was necessary. This is in contrast to extraction and measurement of other plant-produced compounds where extensive sample preparation prior to analysis is often required, e.g. in the case of the glycoside ptaquiloside produced by the bracken fern (*24*).

In experiment 1, using duplicate samples with 120 nmol/g of 2-propenyl glucosinolate added to the three soils, extraction efficiency was generally high (>95% in most cases) but was significantly improved ($P = 0.03$) when two methanol extractions were performed (data not shown). There was no difference in extraction efficiency between the soil types. Variability between duplicates (i.e. higher confidence intervals) increased in the moist Ginninderra soil compared to the two other soils, which were air-dried. In experiment 2, using both of the glucosinolates added to moist Ginninderra soil at a lower concentration (12 nmol/g of soil) and in triplicate, significantly more glucosinolate was recovered if two extractions were used ($P = 0.01$, **Figure 1**). There was no effect of glucosinolate type on extraction efficiency, which approached 90% in both cases. The somewhat lower and more variable extraction efficiency observed for the moist Ginninderra soil (compared to air-dried soils from the other sites in experiment 1) raised the possibility that the water content was influencing recovery in that soil. The

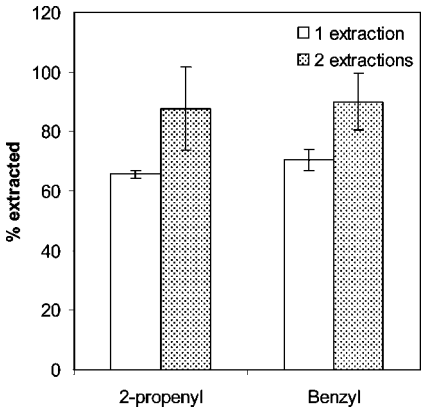

Figure 1. Extraction efficiency for two glucosinolates (2-propenyl and benzyl) in moist Ginninderra soil with either one or two methanol extractions from experiment 2; 12 nmol/g of soil of the glucosinolates was applied. ANOVA indicates no significant effect of glucosinolate type; the number of extractions was significant at $P = 0.001$. The bars show the 95% confidence interval for each treatment.

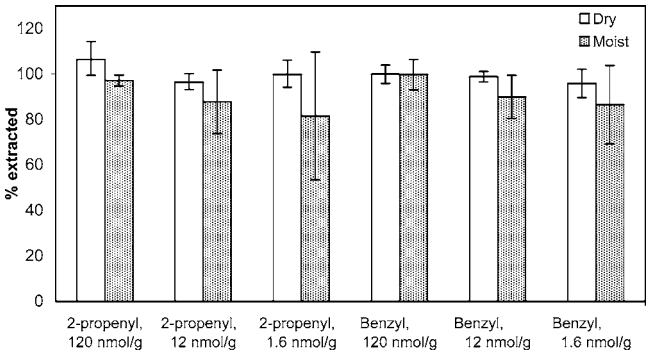

Figure 2. Extraction efficiency of 2-propenyl and benzyl glucosinolate amended at 120, 12, and 1.6 nmo/g of soil in moist and air-dry Ginninderra soil from Experiment 3. ANOVA indicates no effect of soil type or glucosinolate concentration; soil moisture was significant at $P = 0.01$. The bars show the 95% confidence interval for each treatment.

difference in extraction efficiency from air-dried and moist Ginninderra soil in experiment 3 was confirmed by the statistical analysis, which confirmed the impact of moisture content, with significantly less glucosinolate recovered from the moist soil than the dry soil ($P = 0.001$; **Figure 2**). This suggests that microbial degradation may be contributing to some loss of glucosinolates in moist soil, although considering the 95% confidence intervals shown in **Figure 2**, the overall efficiency even for moist soil remains higher than 90%.

In experiment 4, recovery efficiencies of both glucosinolates were measured for a range of concentrations added to all three air-dried soils (**Figure 3**). Between 90 and 100% of both the aromatic and aliphatic glucosinolates were recovered from all three soils when amended to soil at a range of concentrations. There was no significant difference in recovery efficiency between glucosinolate types or soil types; however, the recovery was slightly higher ($P = 0.03$) for the lowest glucosinolate concentration. Thus the method appears to be robust across these soil types, effective for both aromatic and aliphatic glucosinolates, and likely to be effective at the low concentrations anticipated in field soils. The concentrations used in this study were chosen to cover the range of concentrations anticipated in soil based on the report of Kirkegaard and Sarwar (*3*). Those authors screened a wide range of brassicaceous crops and showed that the maximum amount of glucosinolate produced

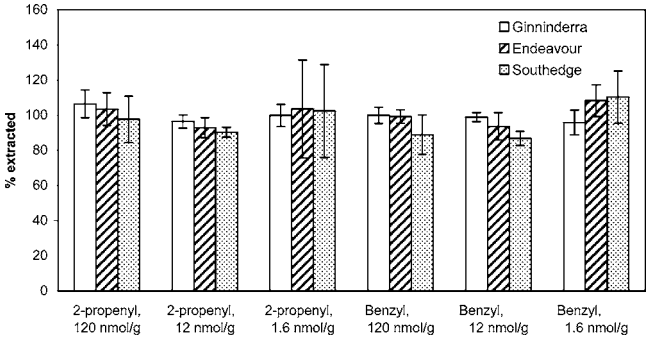

Figure 3. Extraction efficiency of 2-propenyl and benzyl glucosinolate amended at 120, 12, and 1.6 nmol/g of soil for three contrasting Australian soils. ANOVA indicates no significant effect of soil type or glucosinolate type; glucosinolate concentration was significant at $P = 0.03$. The bars show the 95% confidence interval for each treatment.

Table 2. Glucosinolate Contents (nmol/g) in Field Soil Following Incorporation of Indian Mustard (*B. juncea*) and Rape (*B. napus*) in the Field[a]

glucosinolate	rape	mustard
2-propenyl	nd	21.74(7.78)
4-methylsulfinyl	1.48(0.68)	nd
3-butenyl	4.39(0.95)	nd
4-pentenyl	1.63(0.47)	nd
3-indolylmethyl	1.50(0.79)	0.21(0.12)
4-methoxy-3-indolylmethyl	1.92(0.18)	0.43(0.07)
2-phenylethyl	0.12(0.14)	0.26(0.09)
1-methoxy-3-indolylmethyl	0.91(0.39)	0.15(0.02)

[a] Samples where taken from four plots and standard deviations are shown in parentheses. nd = not detected.

in the plant tissues was equivalent to 324 nmol/g of soil, assuming all material was incorporated into the top 15 cm of soil. Much of this is likely to be hydrolyzed during incorporation so that the range of concentrations in this study (120−1.6 nmol/g) spans the range of glucosinolates likely to be found in soil.

Glucosinolates were detected in soil extracts following incorporation of Indian mustard (*B. juncea*) and rape (*B. napus*) green manures in the field at the Ginninderra Experiment Station (**Table 2**). It should be noted that the glucosinolates detected could have been extracted from the soil or from the finely macerated plant tissue in the soil, which was impossible to completely remove from the soil. Eight of the different glucosinolates known to be contained within the plant tissue, including aliphatic (2-propenyl, 4-methylsulfinyl, 3-butenyl and 4-pentenyl), aromatic (2-phenylethyl) and indolyl (3-indolylmethyl, 4-methoxy-3-indolylmethyl, 1-methoxy-3-indolylmethyl) glucosinolates could be identified and quantified in concentrations ranging from 0.12 to 21.7 nmol/g. This represented up to 14% of the original GSL content of the plant biomass, and the fate of the glucosinolates and production of other hydrolysis compounds following incorporation in this experiment is reported in more detail elsewhere (*25*).

The method developed and tested in this study has been shown to be effective in extracting several glucosinolates from a range of light-textured Australian agricultural soils both in the laboratory and in the field. The glucosinolates may be quantified as desulfoglucosinolates using existing HPLC techniques without further sample preparation, even at low concentrations (down to 0.12 nmol/g). It is a simple but effective means of identifying and quantifying glucosinolates in soil. Two extractions with 70% methanol are necessary, and there is a tendency for decreased extraction efficiency and increased variation if moist soil is used, possibly due to microbial degradation. As a result we suggest that extractions of field-moist soil should begin as soon as possible after sampling, as delays (even within 30 min) could result in significant loss of glucosinolates. In practice this could be achieved by placing the soil samples directly into preweighed tubes containing methanol for transport to the laboratory. This is the first report of a methodology for glucosinolate extraction from soils, and further testing and refinement of the methodology is warranted in more diverse soils with higher clay or organic matter contents.

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