Gas Chromatography of Allelochemicals Produced during Glucosinolate Degradation in Soil

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Glucosinolate degradation products from *Brassica* spp. may control soil-borne plant pests; however, a comprehensive method for the analysis of the responsible glucosinolate-derived allelochemicals in soils is lacking. Splitless injection and a 5% phenyl-substituted methylpolysiloxane fused silica capillary column in a gas–liquid chromatograph equipped with a flame ionization detector were used to separate various isothiocyanates, nitriles, and oxazolidinethione in less than 22 min. A linear relationship between detector response for isothiocyanates and molecular weight was observed, allowing estimation of isothiocyanate concentrations for which standards are not readily available. Detection limits for seven of nine compounds were less than 0.1 μg/mL for 1 μL injections. The efficacy of various solvents for extraction was examined, as was the effect of salt and extraction time. Quantities of isothiocyanate and nitrile in CH_2Cl_2 extracts of five soils exceeded 94% of the added spikes, and oxazolidinethione recovery exceeded 84%. Maximum extraction of glucosinolate degradation products from soil amended with crushed seed meal of *Brassica napus* was obtained in 15 min when 2 mL of 0.20 M $CaCl_2$ was included with the CH_2Cl_2 extractant.

Keywords: *Glucosinolates; isothiocyanates; allelochemicals; Brassica spp.*

INTRODUCTION

Glucosinolates are organic anions containing β-D-thioglucose and sulfonated oxime moieties. Nearly 100 glucosinolates, differentiated by the specific aliphatic or aromatic side group, have been isolated or identified from degradative products (Chew, 1988). Produced exclusively in dicotyledonous plants, highest concentrations of glucosinolates are found in plants from the families Resedaceae, Capparidaceae, and Brassicaceae (Fenwick et al., 1983). Enzymatic degradation of glucosinolates by thioglucoside glucohydrolase (EC 3.2.3.1) results in the formation of biologically active products. These allelochemicals have the potential to suppress a wide variety of soil-borne plant pests and thus reduce the need for application of synthetic organic pesticides. Analysis of glucosinolate degradation products in soil is necessary to evaluate the mechanisms by which glucosinolate-containing *Brassica* spp. participate in biological pest control strategies.

Packed column gas chromatography (GC) of isothiocyanates produced in plant tissue extracts as a result of glucosinolate degradation was initiated by Youngs and Wetter (1967). Improvements and modifications of this method have facilitated the analysis of additional glucosinolate degradation products (Daxenbichler et al., 1970; Daun and Hougen, 1977; Daxenbichler and VanEtten, 1977). Similar GC methods continue to be used (Cole, 1976; Louda and Rodman, 1983; Itoh et al., 1984; Al-Shehbaz and Al-Shammary, 1987; Finnigan et al., 1989). These methods were developed primarily as indirect measures of glucosinolate content in *Brassica* tissues. Because methodology for direct quantitation of glucosinolates is now possible, much of the impetus for further development of methods to quantify degradation products has been eliminated. A comprehensive method to measure the numerous glucosinolate degradation products, such as isothiocyanate (ITC), nitriles, and 5-vinyl-2-oxazolidinethione (OZT), is lacking.

Previous soil analysis has been limited mainly to methyl ITC (not common in natural sources) because of its direct application in soil fumigants or indirect application through a methyl dithiocarbamate precursor. In addition, capillary GC was used to quantify benzyl ITC in root exudates from papaya (Tang and Takenaka, 1983) and allyl ITC extracted from a simplified solid matrix (Choesin and Boerner, 1991) as well as soil (Williams et al., 1993). Our goal was to develop an improved and comprehensive method for capillary GC of glucosinolate degradation products in soils amended with *Brassica* tissues.

MATERIALS AND METHODS

Soils. Prior to the addition of any amendments, soils (Table 1) were air-dried, crushed to pass through a 2-mm sieve, and characterized according to methods previously described (Brown and Morra, 1993). Particle size for Avonville and Trout Creek soils was determined using the pipet method (Gee and Bauder, 1986).

Instrumentation. Sample analysis was conducted using a Hewlett-Packard 5890 Series II gas chromatograph, He as a carrier gas, flame ionization detector (FID), and flame photometric detector (FPD) having a sulfur filter. Columns (30 m × 0.32 mm; J&W Scientific, Folsom, CA) included a DB-Wax [poly(ethylene glycol); 0.25 μm film], DB-1701 (14% cyanopropylphenyl-substituted methylpolysiloxane; 1.0 μm film), DB-5 (5% phenyl-substituted methylpolysiloxane; 0.25 μm film), and DB-5MS (5% phenyl; 0.50 μm film). A similar GC system with a DB-5MS column (0.33 μm film), coupled to a Hewlett-Packard 5989A quadrupole mass detector, was used for identification of the compounds. Parameters for electron impact sample ionization were as follows: interface temperature, 280 °C; repeller, 7 V; emission, 300 V; electron energy, 70 eV; source temperature, 200 °C.

Product Identification. Identification was based on retention time, presence of sulfur as measured by GC-FPD, prediction from glucosinolate precursors present in the plant tissues (Brown et al., 1991), and mass spectral data. Mass spectral data were in general agreement with previously published information (G1035A Wiley Database, Hewlett-

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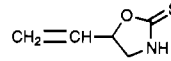
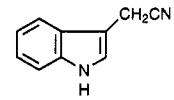
Table 1. Characteristics of Soils Used in Extraction Experiments

soil								
series	subgroup	pH	organic C, g/kg	total C, g/kg	total N, g/kg	clay, g/kg	sand, g/kg	water at −0.033 MPa, %
Portneuf sil	Durixerollic Calciorthid	7.55	11.6	15.6	1.7	157	48	27.5
Latahco sil	Argiaquic Xeric Argialboll	6.10	41.0	41.0	3.9	159	122	39.2
Andisol sil[a]	Typic Vitricryand	6.25	15.0	15.0	1.8	117	190	58.0
Avonville sil	Andic Xerumbrept	5.65	39.0	39.0	3.9	96	265	46.8
Trout Creek cl[a]	Andic Paleoboralf	5.90	2.2	2.2	0.3	698	35	36.2

[a] Not an official series name.

Table 2. Potential Glucosinolate Degradation Products, or Analogues, Examined in Study

name	abbrev	compd	source
allyl cyanide	ACN	$CH_2{=}CHCH_2CN$	Aldrich, Milwaukee, WI
allyl isothiocyanate	AITC	$CH_2{=}CHCH_2N{=}C{=}S$	Aldrich
benzyl isothiocyanate	BITC	$C_6H_5CH_2N{=}C{=}S$	Aldrich
3-butenyl isothiocyanate	3BuITC	$CH_2{=}CHCH_2CH_2N{=}C{=}S$	plant tissue
5-vinyl-2-oxazolidinethione (goitrin)	OZT		Lancaser Synthesis, Winham, NH
indole-3-acetonitrile	IAN		Fluka, Ronkonkoma, NY
methyl isothiocyanate	MITC	$CH_3N{=}C{=}S$	Aldrich
4-pentenyl isothiocyanate	4PtITC	$CH_2{=}CH(CH_2)_3N{=}C{=}S$	plant tissue
phenethyl isothiocyanate	PeITC	$C_6H_5CH_2CH_2N{=}C{=}S$	Aldrich
phenyl isothiocyanate	PITC	$C_6H_5N{=}C{=}S$	Baker, Phillipsburg, NJ
propyl isothiocyanate	PrITC	$CH_3CH_2CH_2N{=}C{=}S$	Aldrich

Packard ChemStation Library; Kjaer et al., 1963) except for the presence of an additional peak at *m/z* 99 in what we list as 4PtITC (Table 2). Loss of $CH_2{=}CH_2$ (M − 28) is not readily explained and may represent an impurity.

Analytical Standards. Commercially available compounds that are known glucosinolate degradation products, or are analogues of the products, were selected (Table 2). Mixed solutions in CH_2Cl_2 were used to construct standard curves for nine compounds. Two replicates for each of two dilution series from a single stock solution were analyzed, giving four measurements at each concentration. The entire procedure was repeated with a new stock solution such that a total of eight injections was used to calculate a mean for each concentration of the respective analyte. Peak response relative to PITC (internal standard) was calculated as the ratio of slopes between each standard and PITC. Standard curves were also constructed from solutions prepared in ether/acetone.

Extractions from Plant Tissue. Solvents screened as potential extractants were hexane, hexane/ether (1:1 v/v), ether, ether/acetone (94:6 v/v), acetone, ethanol, carbon tetrachloride, chloroform, and methylene chloride. Defatted seed meal of *Brassica napus* (*Bn*M) (0.5 g) was wetted with 1.0 mL of H_2O and incubated for 10−15 min to permit glucosinolate hydrolysis. Two additional milliliters of a 0.005 or 0.01 M $CaCl_2$ solution was added to the meal, and degradation products were extracted by shaking with 10 mL of reagent grade solvent for 2 min. The organic phase was removed using a glass syringe and dried with 0.4 g of Na_2SO_4 for at least 1 h before it was filtered through 0.45 μm syringe filters (FP-450, Supor-450, or GN-6 depending on solvent; Gelman, Ann Arbor, MI).

Extractions from Soil. Five gram soil samples in 40 mL Teflon centrifuge tubes were used to determine extraction efficiency of glucosinolate degradation products added to soil in CH_2Cl_2 or produced *in situ* by rapeseed tissues. Sample extracts were dried and filtered as described earlier.

For extraction efficiency of compounds added as soil spikes, enough water was added to obtain soil water suctions of 0.033 MPa, and the centrifuge tubes were capped. After at least 1 h of equilibration, soils were amended with 1 mL of a 1500 μM spike solution for each compound, followed in 1−2 min by 2 mL of a 0.02 M salt solution. Nine milliliters of additional CH_2Cl_2 was added to the sample, followed by shaking for approximately 10 min and centrifugation for 8 min at 650*g*. Four replicates from each of five soils were extracted. The effect of salt on CH_2Cl_2 extraction efficiency of spiked Portneuf and Latahco soils was determined by adding 2.0 mL of 0.01, 0.05, and 0.20 M KCl, $CaCl_2$, and Na_2SO_4, 1−2 min following the spike amendment.

Latahco soil was also amended with 0.5 g of *Bn*M to determine relative extraction efficiencies of glucosinolate degradation compounds produced *in situ*. Water content was equivalent to a suction of 0.033 MPa, with an additional 0.5 mL to compensate for water sorption of the *Bn*M. Moist *Bn*M-amended soil was incubated for 20 min, followed by a 2.0 mL addition of 0.011, 0.056, 0.225, or 0.563 M $CaCl_2$ (adjusted from 0.01, 0.05, 0.20, and 0.50 M to compensate for the additional 0.5 mL of water in the sample), and extracted using shake times of 2, 15, and 30 min.

Except as has been otherwise noted, replicates were performed in triplicate. In some cases, similar sets of experiments were also performed for other solvents as are described for CH_2Cl_2, most frequently using ether or ether/acetone as the extractant. Ether was used as the extractant for GC−MS work.

RESULTS AND DISCUSSION

Chromatographic Conditions. Of the columns tested, the 5% phenyl-substituted methylpolysiloxane (0.5 μM film) provided the best combination of resolution and run time for all of the products tested (Figure 1). The same phase with a thinner film would be acceptable for many applications, but the early eluting ACN peak is not adequately resolved from the solvent. The DB-1701 required longer run times and resulted in peak broadening. We have previously used the poly(ethylene glycol) column for AITC determination (Williams et al., 1993); however, OZT was retained on the column during run times exceeding 40 min.

Allyl cyanide was found to be the earliest eluting peak. No glucosinolate degradation products would be expected to elute earlier since all corresponding products (e.g. 3-butenenitrile) are larger and have higher boiling points. Of the peaks identified as glucosinolate degradation compounds, IAN was eluted last and had a retention time of about 19 min (Figure 1).

GC operating parameters were originally tested in a split mode (10:1), but it was difficult to separate ACN

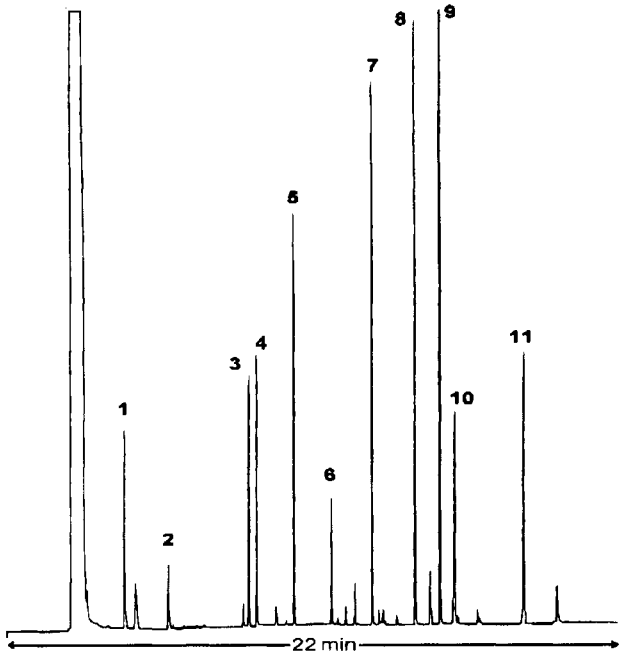


Figure 1. Gas chromatogram of a soil extract containing (1) ACN, (2) MITC, (3) AITC, (4) PrITC, (5) 3BuITC, (6) 4PtITC, (7) PITC, (8) BITC, (9) PeITC, (10) OZT, and (11)-IAN.

from the solvent peak. By changing to the splitless mode, sensitivity was improved and ACN was easily separated from the solvent peak due to the solvent effect and reconcentration at the head of the column (Grob and Grob, 1974). The following conditions were used with the FID: injector, 200 °C; detector, 260 °C; purge time after injection, 0.3 min; initial oven temperature, 35 °C for 3 min, increased at 12 °C/min to 96 °C followed by an increase of 18 °C/min to 240 °C for 6 min. Total run time including cool down and equilibrium was approximately 33 min, about half the time required for a single run of GC methods using two packed columns (Daxenbichler and VanEtten, 1977). A maximum oven temperature of 210 °C (held for 10 min) and detector temperature of 220 °C were used with the FPD.

Quantitation of Glucosinolate Degradation Products. Standard curves for six isothiocyanates, two nitriles, and OZT were linear in all cases with R^2 values greater than or equal to 0.98. Standard curves prepared in ether/acetone were essentially the same as those prepared in CH_2Cl_2 except for OZT, which had a lower response in ether/acetone. Indole-3-methanol, which can be produced along with SCN^- from indole 3-glucosinolate, was also tested but found to be unstable. Of the isothiocyanates used as standards, three occur commonly from *Brassica* spp.: AITC, BITC, and PeITC, whereas MITC, PrITC, and PITC are analogues of the naturally occurring products. We adopted an approach similar to that of Daxenbichler and VanEtten (1977) in calculating detector response factors relative to a single compound but substituted PITC for methyl palmitate. This allows quantities of other compounds to be determined from a standard curve using only PITC or by using PITC as an internal standard. The ratios of each standard relative to PITC and response factors are shown in Table 3.

FID response for the six ITC, as measured by the slopes of the standard curves, was proportional to the molecular weight of the ITC, indicating a consistent ionization pattern (Figure 2). Other compounds, such as nitriles and OZT, did not fit the same pattern as ITC. We used the linear relationship between detector response and molecular weight to predict quantities of naturally occurring ITC for which standards are not readily available (3BuITC and 4PtITC) (Table 3), instead of substituting the response of the nearest eluting peak, as is commonly practiced (Daxenbichler and VanEtten, 1977).

The detection limit, defined as 2–3 times baseline noise, for a 1 μL injection of all compounds was ≤0.1 μg/mL. Detection limits for MITC and OZT were the highest, whereas other standard compounds were detected at lower concentrations.

Extractant Selection. A variety of extractants have been used to obtain ITC or other glucosinolate degradation products from various matrices. Extraction from

Table 3. FID Response of Selected Compounds Relative to PITC

compd	slope ratio[a]	peak area conversion factor[a]	compd	slope ratio[a]	peak area conversion factor[b]
ACN	0.508	1.968	PeITC	1.304	0.767
MITC	0.192	5.221	OZT	0.474	2.111
AITC	0.499	2.004	IAN	1.146	0.873
PrITC	0.519	1.926	3BuITC	0.690[c]	1.450
PITC	1.000	1.000	4PtITC	0.867[c]	1.153
BITC	1.149	0.870			

[a] m_1/m_2, where m_2 is the slope of PITC standard curve and m_1 is the slope of the given compound. [b] Factor = $(m_1/m_2)^{-1}$. Peak area of the compound at concentration x multiplied by the factor equals the peak area of PITC at concentration x. [c] Calculated value using relationship of detector response and molecular weight reported in Figure 2.

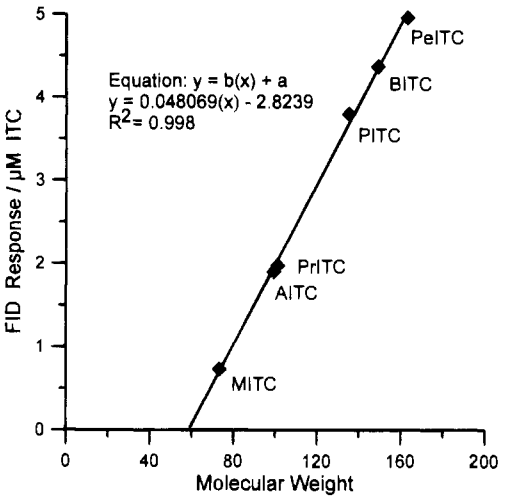


Figure 2. Relationship of FID detector response to the molecular weight of six isothiocyanates.

Table 4. Relative Effectiveness of Extracting Solvents for Glucosinolate Degradation Products from Defatted Seed Meal of *B. napus*

solvent	maximum extracted, %			
	3BuITC	4PtITC	PeITC	OZT
hexane	96	71	85	5
hexane/ether (1:1)	96	74	70	43
ether	93	77	100	87
ether/acetone (47:3)	87	68	85	100
acetone	74	61		
ethanol	80	55		
CH_2Cl_2	59	52	75	96
$CHCl_3$	75	65[a]	95	78
CCl_4	100	100	90	5

[a] Extractions were compared to the response of 4PtITC in chloroform and normalized by assigning largest extracted amount as 100%.

Table 5. Percent Recovery in CH_2Cl_2 Extracts of Soil Samples Spiked with 300 nmol of Compound per Gram of Soil[a]

		soil				
compd	no soil	Avonville	Latahco	Portneuf	Trout Creek	UVA
ACN	96.1 (3.0)[b]	97.1 (8.0)	97.2 (9.5)	96.0 (7.9)	97.5 (3.4)	99.4 (7.1)
MITC	96.9 (3.0)	98.5 (8.0)	97.5 (9.4)	96.7 (7.8)	110.4 (4.8)	102.0 (7.3)
AITC	96.7 (2.8)	97.4 (8.3)	97.9 (9.9)	96.6 (7.9)	97.9 (3.7)	98.9 (6.7)
PrITC	96.6 (2.8)	97.4 (8.4)	97.9 (10.1)	96.9 (7.7)	98.5 (4.2)	98.9 (6.8)
PITC	96.0 (2.8)	96.9 (8.8)	97.7 (10.2)	96.5 (7.8)	97.9 (4.6)	98.0 (6.5)
BITC	96.0 (3.0)	97.1 (9.3)	98.0 (10.7)	97.0 (7.6)	98.9 (4.7)	98.4 (6.4)
PeITC	96.1 (3.1)	97.1 (9.3)	98.2 (10.4)	97.2 (7.5)	98.6 (4.9)	98.0 (7.0)
OZT	90.0 (4.1)	86.3 (9.5)	84.9 (7.7)	87.9 (7.3)	90.0 (4.6)	87.8 (6.4)
IAN	93.7 (4.7)	94.2 (10.8)	94.7 (10.0)	96.5 (6.6)	99.9 (6.8)	98.6 (7.1)

[a] Extractant included 0.02 M $CaCl_2$. [b] Standard deviation in parentheses.

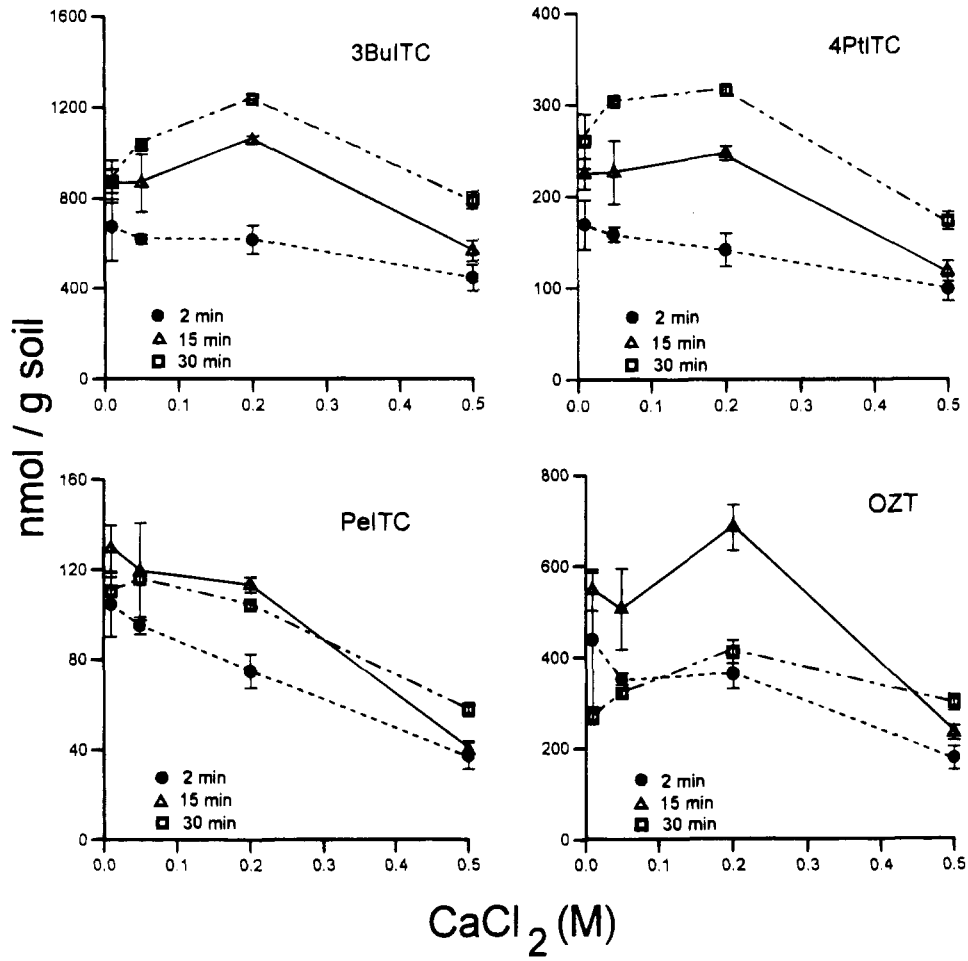


Figure 3. Effect of time and salt concentration on extracted quantities of four glucosinolate degradation compounds produced in rapeseed meal-amended soil. Error bars ±1 standard deviation.

simple systems such as acetone rinses of a resin trap containing BITC (Tang and Takenaka, 1983) and $CHCl_3$ extracts of OZT and nitriles from aqueous solutions (MacLeod and Rossiter, 1986) have been reported. In a more complicated matrix such as marine sponge tissue, organic thiocyanate and ITC were extracted with a hexane/ethyl acetate solvent mixture (He et al., 1992). In soils, carbon tetrachloride (Ashley and Leigh, 1963), 95% ether/ethanol (Sirons, 1973), ethyl acetate (Smelt et al., 1989), and isooctane/aqueous NaCl (Hogendoorn et al., 1992) have been used to extract MITC. For plant tissue extracts, CH_2Cl_2 as used by Youngs and Wetter (1967) in the first reported GC method is the most common extractant, followed by ether (Cole, 1976). Other solvent systems have also been used (Itoh et al., 1984; Appelqvist and Josefsson, 1967).

We screened solvents by comparing relative extraction efficiencies of four compounds from *Bn*M (Table 4). Soil extraction was also performed to check for potential interferences. Most solvents were unsuitable for at least one of a variety of reasons. Acetone and ethanol extracted numerous interfering substances. In addition, several glucosinolate degradation products were unstable in ethanol, a phenomenon observed also for methanol (Mullin, 1978). Nonpolar solvents such as CCl_4 and hexane (Table 4) did not extract OZT, although relative recoveries for ITC were very high, particularly for CCl_4. Chloroform has a solvent/water partition coefficient for OZT of 3:1 (de Brabander and Verbeke, 1982) and was considered as a possible extractant. However, all solvents tested except ether and CH_2Cl_2 had solvent peaks that interfered with early eluting compounds such as ACN.

Initial extractions were performed with ether or ether/acetone. However, ITC and IAN in standard solutions measured in capped vials 8 and 21 days after prepara-

Table 6. Variation in Extractable Product from *B. napus* Meal-Amended Latahco Soil Using Different Incubation and Extraction Times

incubation time, min	extraction time, min	nmol/g of soil			
		3BuITC	4PtITC	PeITC	OZT
20	2	616 (63)[a]	141 (18)	75 (8)	365 (33)
50	2	920 (44)	197 (10)	70 (3)	370 (9)
20	30	1235 (3)	316 (5)	104 (1)	413 (26)

[a] Standard deviation in parentheses.

tion became more concentrated as a result of ether evaporation. Concentrations of OZT decreased in relation to ITC during the same storage time, indicating compound instability. Because sample storage prior to GC analysis is often necessary, all further studies were conducted with CH_2Cl_2 only.

Optimization of Soil Extraction Procedures. Slopes of curves plotted from standards after storage at −10 °C for 14 days in CH_2Cl_2 showed no significant change from curves plotted prior to storage (F test, $p \geq 0.96$ for all nine compounds). Recoveries from soils spiked with 300 nmol/g were generally near 100% except for OZT, which ranged between 85 and 90% (Table 5).

Although CH_2Cl_2 was the most acceptable solvent for chromatographic purposes and appeared to be effective for extraction of spiked soils, it was one of the least effective with respect to extraction efficiency of ITC from plant tissue (Table 4). Salts were therefore included during CH_2Cl_2 extraction to improve extraction efficiency. The effects of salt concentrations or salt types on extraction from spiked soils were not significant at $p = 0.05$ (data not shown). However, trends suggested an extraction efficiency pattern for salt type of Na_2SO_4 > $CaCl_2$ > KCl, and for salt concentration a pattern of 0.20 > 0.05 > 0.01 M.

In *Bn*M-amended Latahco soil, both salt concentration and extraction time had an effect on extractable product. The same four major degradation products were measured from soil as were found from *Bn*M tissue alone (Figure 3; Table 4). Maximum amounts of 3BuITC and 4PtITC were extracted in CH_2Cl_2 at a time of 30 min using 0.20 M $CaCl_2$. Maximum concentrations for PeITC and OZT occurred at 15 min, using 0.01 and 0.20 M $CaCl_2$, respectively (Figure 3). Salt concentrations of 0.50 M resulted in minimum quantities of product for every extraction time except for OZT at 30 min.

An average total extraction value for each time and salt concentration was calculated. Optimum extraction, as measured by the sum total concentration of all four compounds, occurred at a time of 15 min and a $CaCl_2$ concentration of 0.20 M (525 nmol/g of soil). This was followed closely by 517 nmol/g of soil at 30 min and 0.20 M $CaCl_2$. All other extraction time and $CaCl_2$ concentration combinations resulted in less than 445 nmol of extracted material/g of soil.

Extraction time had the largest impact on the amount of product measured. Time is an important factor, particularly in plant tissue-amended soils, because formation of the products is not instantaneous. Also, the disappearance of some products in soil such as ITC can be fairly rapid (Choesin and Beorner, 1991; Brown et al., 1991; Williams et al., 1993) and may occur through a variety of physical and chemical mechanisms. Thus, measurement of glucosinolate degradation products in soil quantifies only what is present at the time of measurement. Some increases in amounts of degradation product observed with time were likely caused by the formation of more product through continuation of the enzymatic reaction during the extended extraction period. When incubation time was increased from 20 to 50 min (to allow for more product formation) but extraction time was kept constant, more 3BuITC and 4PtITC products were extracted from the longer incubation time, indicating increased product formation (Table 6). However, when extraction time was increased to 30 min but incubation time was kept constant (20 min), an even more dramatic increase in extraction was observed for all compounds (row 3), including nonvolatile OZT. This indicates that enzymatic hydrolysis and some product formation may occur but that longer extraction times also resulted in greater extraction efficiency.

A method that seeks to extract and quantify a broad range of compounds will necessarily result in some compromise. Additionally, the amended soil system is a dynamic one in which both the quantity of glucosinolate degradation products and the extraction efficiency are variable with time. Because of this, determination of absolute extraction efficiencies of compounds from *in situ* tissue-amended soils is difficult. However, extraction for 15 min with CH_2Cl_2 and 0.20 M $CaCl_2$ allows a wide variety of allelochemicals to be quantified using GC-FID in less than 22 min. This method has wide applicability with respect to better characterization of allelochemicals produced during glucosinolate degradation in soil.

ABBREVIATIONS USED

GC, gas chromatograph, ITC, isothiocyanate, AITC, allyl ITC; BITC, benzyl ITC; 3BuITC, 3-butenyl ITC; MITC, methyl ITC; 4PtITC, 4-pentenyl ITC; PITC, phenyl ITC; PeITC, phenethyl ITC; PrITC, propyl ITC; OZT, oxazolidinethione; ACN, allyl cyanide; IAN, indoleacetonitrile; *Bn*M, *Brassica napus* meal; FID, flame ionization detector; FPD, flame photometric detector.

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