Glucosinolate Content and Isothiocyanate Evolution − Two Measures of the Biofumigation Potential of Plants

Ben Warton,* John N. Matthiessen, and Mark A. Shackleton

CSIRO Entomology, Private Bag 5, Wembley, Western Australia 6913, Australia

A total of 570 lyophilised *Brassica* root and shoot tissue samples were hydrolyzed, and the liberated isothiocyanates (ITCs) were analyzed by gas chromatography−flame photometric detection (GC−FPD). Glucosinolates (GSLs) were extracted from samples of the same tissues and analyzed by high-performance liquid chromatography (HPLC). The concentrations of six GSLs/ITCs (2-propenyl, 3-butyl, 4-pentenyl, benzyl, 4-methylthiobutyl, and 2-phenylethyl) as determined by the two techniques were compared. In 79% of the samples, the concentration of GSLs in the tissues was greater than that of the ITCs released on hydrolysis. Several possible reasons for the difference are proposed, including the effect of tissue storage time, hydrolysis of GSLs may be less efficient than the GSL extraction procedure, or some of the ITCs formed reacted with plant proteins and amino acids in the sample and were therefore not detected in the extract. GSL concentration in plant tissues is used to estimate the biofumigation potential of the plant tissue, whereas the actual biofumigation effect is thought to be due to the ITCs formed by hydrolysis of the plant-based GSLs. The variation between ITC and GSL values therefore has implications for the assessment of the biofumigation potential of the plant tissue.

Keywords: *Isothiocyanates; glucosinolates; hydrolysis; biofumigation; brassicas; GC; HPLC*

INTRODUCTION

Glucosinolates (GSLs) are a class of naturally occurring anionic compounds found in plants (*1*), usually as the potassium or sodium salt. They consist of a β-D-thioglucose moiety, a sulfate attached through a C═N bond, and a side chain that distinguishes one GSL from another (*2*). GSLs are hydrolyzed by enzymes of the family myrosinase (β-thioglucoside glucohydrolase; EC 3.2.3.1) in the presence of water, to yield glucose and an unstable aglucone, which spontaneously undergoes a Lossen rearrangement to form an isothiocyanate (ITC) as the major product (*2*). Myrosinase is stored in specialized myrosin cells (*3, 4*). These cells do not contain GSLs, which are located separately within a variety of plant cells. GSLs and myrosinase come into intimate contact when the plant tissue is damaged by mechanical stress such as crushing, or during pathogen attack, with the resultant formation of ITCs.

GSLs have been the subject of much recent attention because of their involvement in the process termed "biofumigation" (*5*). In biofumigation, plant-based GSLs are hydrolyzed in field soil to form toxic products including ITCs, thiocyanates, nitriles, oxazolidinethiones, and ionic thiocyanate (*2*). These GSL degradation products may exert a suppressive or control effect on a wide range of soil-borne plant pathogens including wheat take-all fungus (*5*), root-knot nematode (*6, 7*), *Rhizoctonia solani* (*8*), and *Fusarium oxysporum* (*9*). A comprehensive review of allelochemical effects of glucosinolate degradation products can be found in Brown and Morra (*2*).

* To whom correspondence should be addressed. Telephone: +61 8 9333 6638. Fax: +61 8 9333 6646. E-mail: b.warton@ccmar.csiro.au.

Hydrolysis of GSL-containing plant tissue, or pure GSLs isolated from plant tissues, can be acheved in the laboratory using pure myrosinase isolated from *Brassicas* such as white mustard (*Sinapis alba*) (eg. (*10*)), so that reaction conditions can be well controlled. This hydrolysis reaction has also been performed using endogenous myrosinase present in lyophilized, ground, and rehydrated *Brassica* plant tissue (*11, 12*). In the former study, seed meal was wetted and incubated for 10−15 min followed by extraction using organic solvents (*11*).

In the present study, a modified GSL hydrolysis procedure is reported in which the hydrolysis and solvent-extraction steps are combined. After a simple cleanup procedure, the hydrolysis products in the organic phase were qualitatively and quantitatively determined by gas chromatography with a flame photometric detector (GC−FPD). Molar concentrations of ITCs liberated from the original plant tissues were calculated, and results were compared to GSL concentrations determined by high-performance liquid chromatographic (HPLC) analysis of desulfoglucosinolates extracted from the same plant tissues, a method previously used to assess the biofumigation potential of *Brassica* plants (*13*). The potential of these two procedures as indicators of the biofumigation potential of plant tissues is discussed.

MATERIALS AND METHODS

Reagents and Solvents. Propenyl GSL, sephadex, sulfatase, and methyl ITC were purchased from Sigma-Aldrich (Castle Hill, Australia), and benzyl GSL was purchased from Merck (Kilsyth, Australia). All reagents and solvents were used as purchased.

GSL and ITC Comparisons. A total of 570 root and shoot tissue samples were taken from *Brassica* plants grown at

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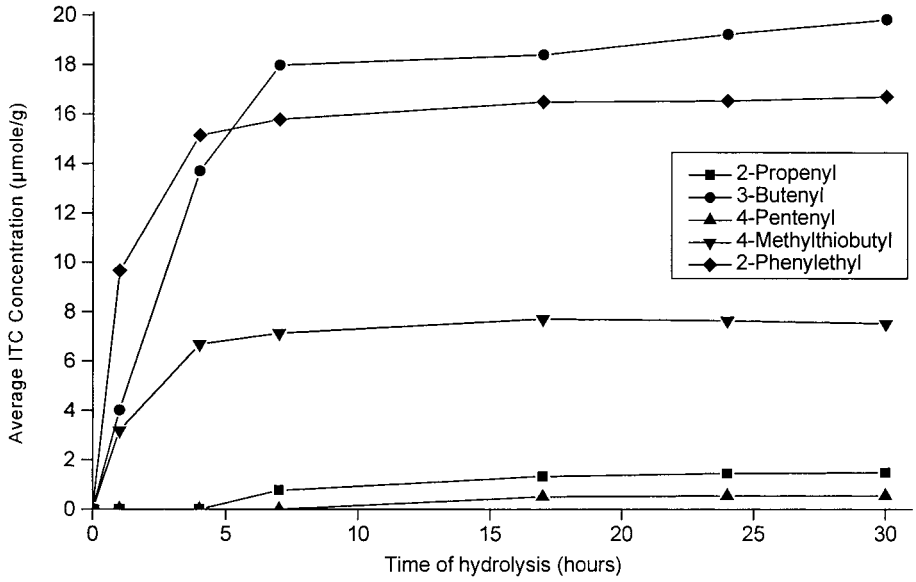

Figure 1. Concentrations versus time for five ITCs produced by the hydrolysis of a freeze-dried *Brassica* tissue sample.

different locations in Australia and New Zealand. The samples were freeze-dried and ground (Wiley mill with 1 mm screen), and stored (15 °C) for different periods. The majority of samples were taken from mature plants as they began to flower. The GSL content of, and ITC quantity liberated from, these samples were measured according to the procedures outlined below, and then compared. Analyses were not replicated. Theoretically, one mole of GSL has the capacity to produce one mole of ITC under optimal conditions for the conversion of GSLs to ITCs.

Extraction and Analysis of Glucosinolates from *Brassica* Tissue Samples. Glucosinolates were extracted and analyzed according to the procedure of Kirkegaard and Sarwar (*13*). Briefly, freeze-dried and ground *Brassica* root and shoot material (300 mg), HPLC-grade methanol (10 mL, 70%, preheated to 70 °C) and an internal standard (15 μL, 16 mM, propenyl glucosinolate or benzyl glucosinolate, as appropriate) were added to 50-mL centrifuge vials. The vials were sealed without delay, hand shaken, and stood in a water bath at 70 °C for 20 min. The samples were then agitated briefly, cooled, and centrifuged at 3500 rpm for 6 min.

The supernatant (3 mL) was carefully applied to a 0.5-cm plug of A-25 sephadex in 10-mL poly-prep chromatography columns, and the effluent was discarded. The sephadex was washed with milli-Q purified water (1 mL) followed by sodium acetate (1 mL, 0.02M), and the combined effluents were discarded. Prepared sulfatase (75 μL) was added to each of the columns, which were then capped and allowed to stand overnight. Subsequently, milli-Q water (1 mL) was applied to each column, and the effluent was collected in 1-mL HPLC vials that were frozen until analysis.

Analysis for glucosinolates was performed using HPLC according to the procedure given in Kirkegaard and Sarwar (*13*) and references therein.

Hydrolysis of *Brassica* Tissue Samples and ITC Analysis. Ethyl acetate (8 mL), methyl isothiocyanate (MITC, 2 mL, 100 mg L^{-1}) in ethyl acetate (normalization standard), and deionized water (10 mL) were added to freeze-dried *Brassica* root and shoot tissue (200 mg) in 100-mL Erlenmeyer flasks. The flasks were sealed without delay and placed on an orbital shaking table operating at 150 rpm. Samples were shaken for 24 h (except for the timed hydrolysis experiment), removed, and allowed to settle. Aliquots (1 mL) of the upper organic layer were drawn off, then dried and filtered through a plug of anhydrous magnesium sulfate (approx 4 cm) in a Pasteur pipet for analysis by GC.

To determine the optimal time for hydrolysis of GSLs to liberate ITCs, a *Brassica* plant tissue sample was selected that contained measurable quantities of five ITC-producing GSLs: 2-propenyl GSL, 3-butyl GSL, 4-pentenyl GSL, 4-methylthiobutyl GSL, and 2-phenylethyl GSL (*Brassica napus* root tissue). Samples of this plant tissue were shaken with water for a range of time periods, and the amount of ITC produced was measured, according to the experimental procedure detailed above.

Isothiocyanates were analyzed using a Hewlett-Packard 6890 GC equipped with an FPD in sulfur mode (394 nm). A 30 m × 0.32 mm i.d. wall-coated open tubular fused silica capillary column coated with a 0.25 μm methylsilicone stationary phase (HP-1, Hewlett-Packard) was used at an oven temperature of 50 °C. Helium was used as the carrier gas at a linear velocity of 19 cm s^{-1}. The GC oven was programmed from 50 to 220 °C at a rate of 8 °C min^{-1} with a 1 min initial hold time at 50 °C. Samples for analysis were injected splitless using a HP 7683 auto sampler.

RESULTS AND DISCUSSION

Effect of Hydrolysis Time on ITC Release from *Brassica* Tissue. A total of eighteen GSLs were identified, eleven of which form ITCs on hydrolysis. Figure 1 shows ITC concentration vs shaking (hydrolysis) time for five homologues. Formation was rapid for the first 6 h for 3-butenyl ITC, 4-methylthiobutyl ITC, and 2-phenylethyl ITC, with little or no further ITC formation after this time. For 2-propenyl ITC and 4-pentenyl ITC, formation was rapid between 5 and 17 h, with a further small increase to 24 h. For all of the ITCs, formation was not significant after 24 h, and on this basis 24 h was chosen as a suitable time for hydrolysis of plant tissue to release ITCs.

Effect of Tissue Storage Time on ITC Release. GSL and ITC analyses were not performed at the same time for most of the samples − GSL analyses were performed first, and the samples were freeze-dried and placed in storage prior to ITC analysis. The time interval between analyses ranged from a few days up to six years. ITC vs GSL concentrations, grouped by the time interval between the analyses, were plotted to determine whether the time of storage had an effect on the GSL concentration, as has previously been reported (*14, 15*), and hence on the capacity of the tissue to liberate ITCs on hydrolysis (Figure 2).

As Figure 2 shows, the slopes of the trendlines decrease with increasing storage time. This suggests

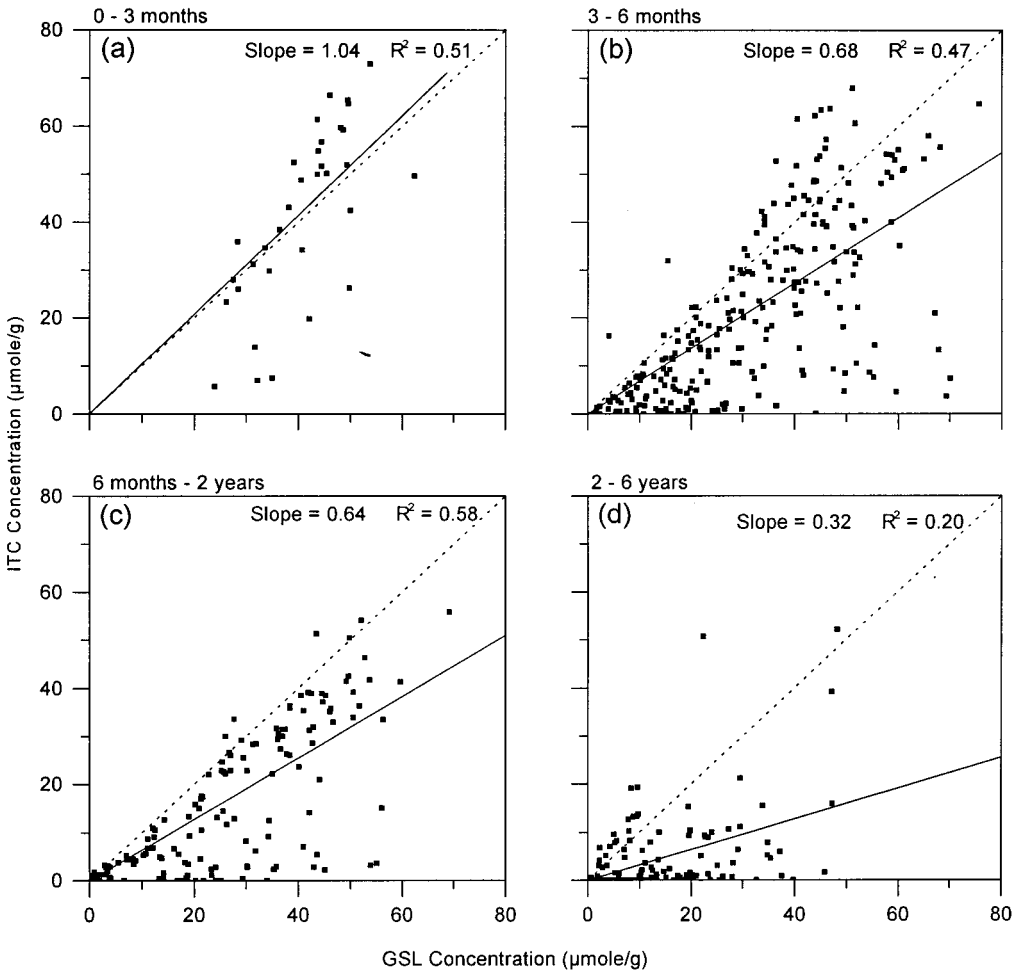

Figure 2. Sums of molar concentrations of ITCs produced by hydrolysis of a *Brassica* meal versus sums of molar concentrations of GSLs present in the meal for four time intervals between GSL and ITC analyses. Also shown are the $x = y$ line (dashed), the trendline (solid) and slope, and the R^2 values.

that since the time of the GSL analysis, the concentration of GSLs in the tissue had been decreasing during storage, resulting in lower concentrations of ITCs formed upon hydrolysis of the plant tissue. To investigate other causes of variation between GSL and ITC concentrations, samples that had been stored for longer than two years (Figure 2d) were removed from the sample set. In these samples, the effect of storage time was greatest and may therefore mask other causes of variation.

Factors Influencing GSL Abundance and ITC Quantities Liberated. Plots of the abundances of ITCs liberated by hydrolysis of plant tissue samples and the GSLs extracted from the same tissues for six GSL/ITC homologues are shown in Figure 3. Several points are apparent from inspection of Figure 3. First, the position of the trendlines indicates that, in most cases, the molar amount of ITC produced by hydrolysis is lower than the amount of the corresponding GSL present in the plant tissues (theoretically, one mole of GSL will produce one mole of ITC). There are several possible reasons for this, including incomplete hydrolysis of GSLs, decomposition, volatilization, or reaction of the produced ITCs and the formation of non-ITC hydrolysis products. Continuation of the hydrolysis procedure for extended periods (to 96 h) resulted in no decrease of ITC concentration, suggesting that decomposition was not occurring (data not shown). Opening of the sealed hydrolysis vessel during the procedure also did not affect the ITC concentration (data not shown), suggesting that quantities of ITCs present in the flask headspace were not significant. Analysis by GC−MS of randomly selected hydrolyzed samples revealed that significant quantities of organic non-ITC hydrolysis products were not produced in the procedure used.

It is possible that the lower molar amount of ITC produced relative to GSL is due to incomplete hydrolysis, and was unaffected by extending the time period of hydrolysis. In plant tissue, GSLs and myrosinase are stored in separate intact cells (*2*), and come into contact when cells are damaged to effect the hydrolysis reaction. The shaking method used in this study is fairly gentle and may not be causing all of the GSL present in the plant tissues to come into contact with myrosinase in order to be hydrolyzed. GSLs are reported to occur in cell vacuoles, and they are probably distributed in a variety of cell types (*16*), so it is possible that some GSLs are in cells that are not damaged by the hydrolysis procedure used here, but are recovered in the GSL extraction procedure used.

Another possible reason for the lower molar amount of ITC produced relative to GSL lies in their reactivity with proteins and amino acids. ITCs interact irreversibly with sulfhydryl groups, disulfide bonds, and amines (*2*), and may react with such functional groups present in the proteins of the same plants from which the ITCs themselves are produced.

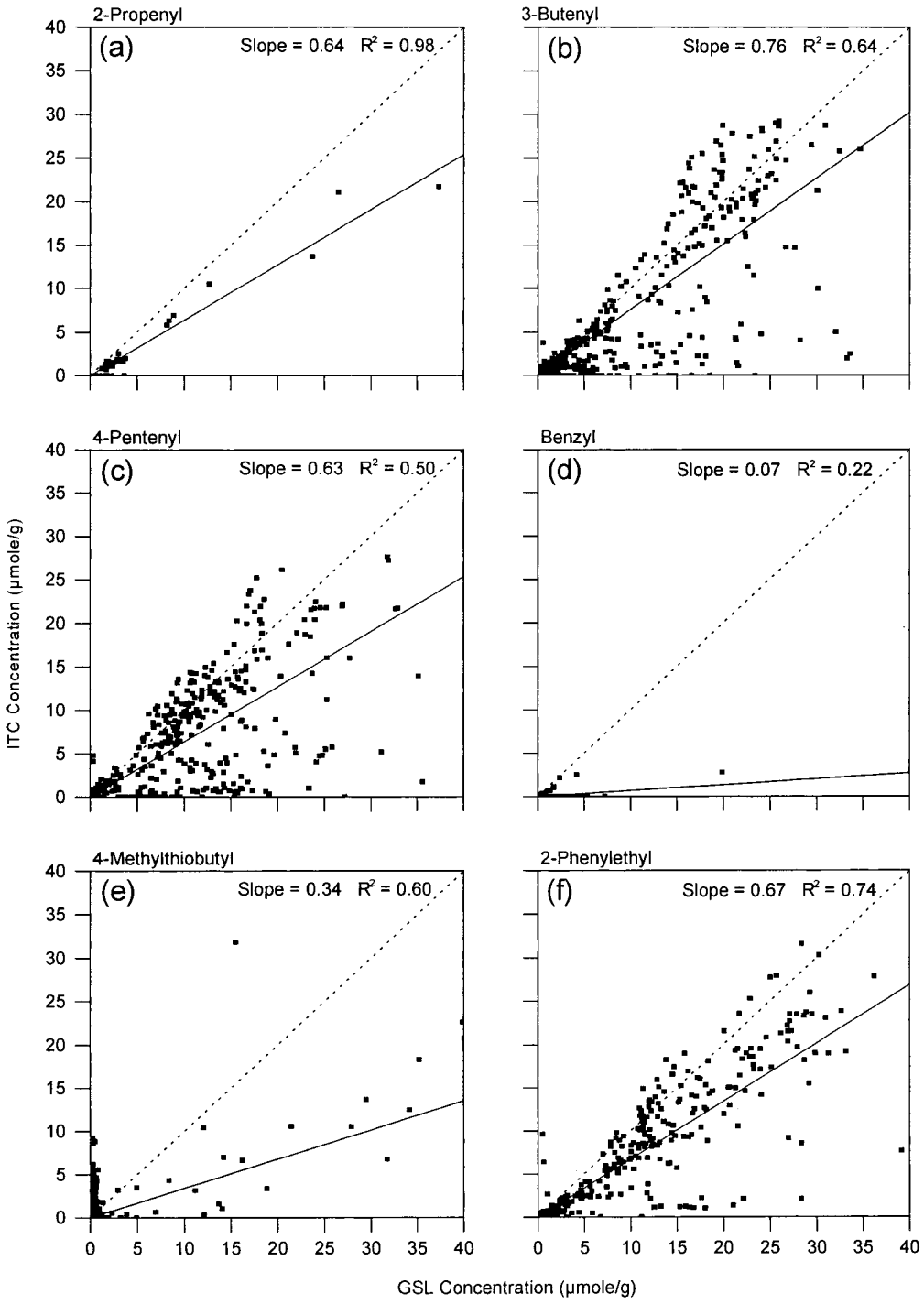

Figure 3. Molar concentrations of ITCs produced by hydrolysis of a *Brassica* meal versus molar concentrations of GSLs present in the meal for six ITC/GSL homologues. Also shown are the $x = y$ line (dashed), the trendline (solid) and slope, and the R^2 values.

A second point clearly shown in Figure 3 is that the trendlines (i.e., the ratios of ITCs to GSLs) are different for different homologues. The slope of the trendline for 3-butenyl of 0.76 was the highest of the six homologues, indicating that the biofumigation potential of this compound as determined by GSL quantification most closely matches the actual amount of toxic ITC produced by hydrolysis of the GSL. The slopes for 2-propenyl, 4-pentenyl, and 2-phenylethyl were all slightly lower (in the range 0.63−0.67) suggesting a lower correlation between biofumigation potential measured by GSL concentration and toxic ITC production. The slope for 4-methylthiobutyl (0.34) suggests that the actual biofumigant effect of this compound in soil would be much lower than that predicted from the GSL concentration in plant tissue. Only 26 of the 570 samples yielded benzyl on hydrolysis, so this homologue is not considered further.

There are several possible explanations for the apparent differences in the efficiency of hydrolysis for different GSL homologues. ITCs in different parts of plants may be exposed to different amounts of proteins and/or amino acids, with which they may react. There may be differences in the activity of myrosinase, the enzyme responsible for hydrolyzing GSLs to ITCs, toward different GSLs. Different GSLs may form dif-

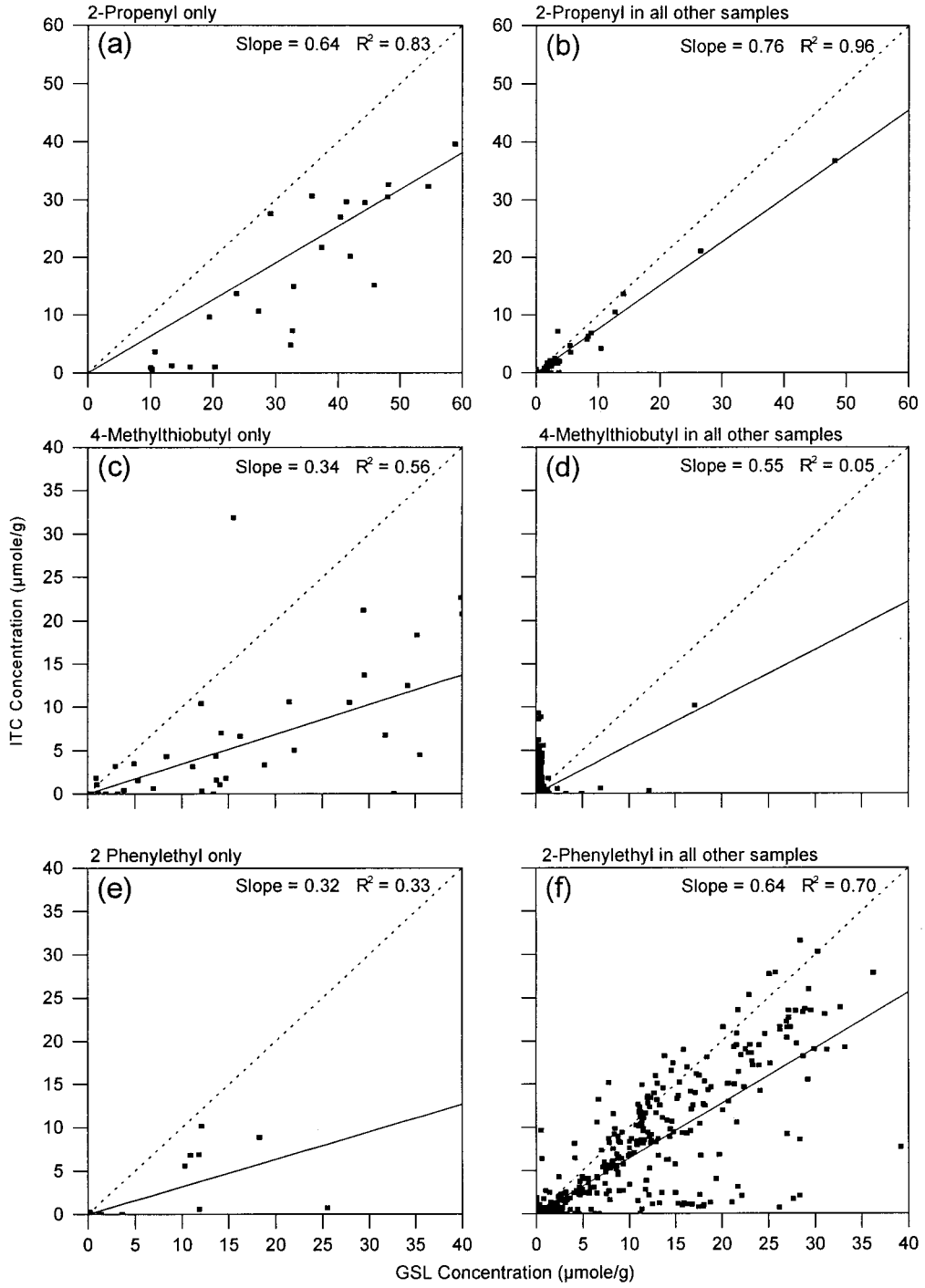

Figure 4. Molar concentrations of ITCs produced by hydrolysis of a *Brassica* meal versus molar concentrations of GSLs present in the meal for three ITC/GSL homologues, differentiated into samples containing > 95% of a single GSL and samples containing mixtures of GSLs. Also shown are the $x = y$ line (dashed), the trendline (solid) and slope, and the R^2 values.

ferent amounts of other hydrolysis products, including nitriles and thiocyanates, as well as isothiocyanates. Further research is necessary to identify the causes of the difference in efficiency of ITC release between different GSLs.

Another point apparent from inspection of Figure 3 is the variability in the relationship between GSL and ITC values. Although there are relatively few samples in which the ITC value is higher than the GSL value, a considerably larger number exhibit significantly lower (a factor of 5 or greater) ITC values than GSL values. Several factors were examined to determine whether they had an impact on the variation between GSL and ITC values (aside from tissue storage time as discussed earlier): GSL/ITC composition of the tissue (single component vs multicomponent), tissue type (root vs shoot), and growing season (spring vs autumn). No significant GSL/ITC variability was observed for growing season; however, the absolute amounts of GSLs/ITCs in spring crops were approximately double those in autumn crops.

Comparison of root vs shoot tissues revealed an apparently lower efficiency of formation of ITCs from GSLs in shoot tissue than from GSLs in root tissue for the 3-butenyl and 4-pentenyl homologues. This may be due to reasons discussed earlier, including GSLs being

present in different cell types in roots and shoots, or the presence of higher levels of proteins or amino acids in shoot than in root material that react with the liberated ITCs.

To compare GSL/ITC values for a given compound in single component and multicomponent tissue samples, samples were identified in which a single compound comprised greater than 95% of the GSL/ITC concentration. Three of the six compounds (2-propenyl, 4-methylthiobutyl, and 2-phenylethyl GSL) accounted for greater than 95% of the total GSLs present in some (approximately 5%) of the tissue samples examined. ITC vs GSL concentrations for these tissue samples, and for the same compound in tissues containing a mixture of GSLs/ITCs, for each of these three compounds were plotted (Figure 4). For all three homologues, the slope of the trendline for the homologue in samples containing only (i.e., > 95%) that homologue was lower than the slope of the trendline for the homologue in samples containing more than one homologue (2-propenyl, 0.64 vs 0.76, respectively; 4-methylthiobutyl, 0.34 vs 0.55; 2-phenylethyl, 0.32 vs 0.64). This suggests that the hydrolysis is less efficient in samples with only one GSL homologue, regardless of what the actual homologue is. However, because of the small number of samples containing only one homologue, it is unclear whether this observation represents a real trend (Figure 4).

Differences between the molar amounts of ITCs released by hydrolysis and the molar amounts of GSLs present in the plant tissue may reflect factors inherent to some plants that restrict the availability of some GSLs for hydrolysis. This may partly explain some of the anecdotal reports from growers of inconsistencies in the actual biofumigation effect of some plants as observed in the field, although variation in the level of tissue disruption in the soil incorporation process may also be a significant factor.

The variability between concentrations of GSLs in tissues and ITCs liberated from the same tissues has implications for the assessment of the biofumigation potential of *Brassica* tissues. To date, the biofumigation potential of *Brassica* has been determined by quantitative analysis of the GSL concentrations in the plant tissue (*13*, *17*). However, there are numerous methods for analyzing the individual GSL concentrations in plant tissue (*18*) and these may have varying degrees of efficiency of extraction of GSLs from the plant tssue.

If hydrolysis of GSLs in any given *Brassica* species is less 'efficient' than others (i.e., the percentage of GSL present that is converted to ITC is lower), then the actual biofumigation action of the plant may be lower than that predicted on the basis of GSL abundance alone. Further investigation is required to identify the reasons for this variability.

The hydrolysis procedure described here is a simple and rapid method of degrading GSLs in plant tissue to liberate ITCs. Quantitation of the liberated ITCs provides an additional method of assessing the biofumigant capability of plants, aside from the more commonly used method of determining the GSLs present in the plant tissue. Analysis of biofumigant plant tissue by using both techniques employed here may provide a more accurate method of assessing biofumigation potential than by using GSL analysis alone. It should be noted, however, that this study utilized only freeze-dried plant material, as many of the samples were grown in different parts of Australia and New Zealand for a variety of purposes and fresh samples could not be obtained in many cases. This study has not attempted to compare hydrolysis of freeze-dried and fresh tissue samples, an important factor in assessing actual biofumigation potential.

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