MEASURING GLUCOSINOLATES BY FLOW INJECTION ANALYSIS

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INTRODUCTION

Glucosinolates are a heterogeneous group of secondary metabolites which occur in plants, mainly in species of Brassica. They have alkenyl, aromatic or indolyl radicals attached to common beta-thioglucose and sulphonated oxim groups. Breakdown products of glucosinolates have been shown to be toxic to some animal species with effects mainly on the thyroid gland (Fenwick & Heaney, 1983) but also on other tissues, particularly in poultry (Fenwick et al, 1983). The development of varieties of oilseed rape with much reduced concentrations of glucosinolates has been pursued vigorously, and to develop such varieties plant breeders require rapid yet precise methods to enable selection of lines low in seed glucosinolate content. Glucosinolates can be measured quantitatively in several ways, for example individually by HPLC or GC or collectively by glucose or sulphate release after enzymic reaction or formation of a coloured complex with palladium. Indirect, rapid, non-destructive methods such as NIR or X-ray fluorescence are also utilised, particularly the latter since it became an official EEC rapid test.

Plant breeders have made use of most of these tests, their requirements being speed, reasonable accuracy and precision, with safety and economy also being important. We report here on the accuracy, precision and speed of the use of flow-injection analysis (FIA) compared with a microtitre plate reader method for measuring glucose released from glucosinolates in small, crushed, non-defatted samples of rapeseed. A rate of about 600 analyses per day is required to service our winter rapeseed breeding programme in the short interval between harvest and selection prior to planting.

MATERIALS AND METHODS

Tests for accuracy were made using EEC reference rapeseed sample CRM 190. The certificated value for glucosinolate content of this seed is 25.5 ± 0.9 µmol/g expressed as whole seed as received. This certificated value was determined after independent analysis by 13 European laboratories who applied a variety of methods, including HPLC, GC and glucose release (Wathelet & Wagstaffe, 1989). Measurements of precision were made using a sample of rapeseed, c.v. Samurai, grown at Cambridge in 1990.

Glucosinolates were extracted by slight modifications to the methods described by Smith et al. (1985) and by Smith and Donald (1988). Samples were rapidly logged and accurate weights recorded (200mg ± 5mg) by utilising bar-code label readers and electronic balances linked to a computer, then crushed in thick-walled, glass test tubes and four mls of water added at room temperature. After vortexing, the suspensions were heated at 70°C for 40 minutes in a water bath before cooling in running water for 10 minutes. Activated charcoal was added by dispensing 1 ml of a 100mg/ml (continuously

stirred) suspension into the sample followed by rapid mixing. After a further 10 minutes an aliquot of the extract was centrifuged for 5 minutes at 10,000 r.p.m.. The glucose concentration in the clear supernatant was measured to assess glucosinolate content in the seeds. In our screening work samples are handled in batches of 36 containing two reference samples. Endogenous glucose was measured after whole seeds were dropped into boiling water for 10 minutes before extraction as above.

The flow-injection analyser (Tecator FIAstar^{R} 5020) was used to measure glucose concentration in these extracts by glucose oxidase and peroxidase mediated oxidation of phenol and 4-aminophenazone. The reagent was prepared by mixing two stable stock solutions in the ratio of 5:1. Stock 1 consisted of 60 mg glucose oxidase (Boehringer-Mannheim, grade III), 60 mg of 4-aminophenazone (Aldrich) and 90 mg of peroxidase (Boehringer-Mannheim, grade II) in a litre of 0.16M phosphate buffer, pH 6.8. Stock 2 was a 0.15% (W/V) solution of phenol. Ten drops of a 10% Brij solution were added to each 250ml of degassed working reagent and sample wash. Standard solutions of glucose (ranging from 50-300µg/ml - equivalent to 7.4 to 44 µmol glucosinolate/g seed) were prepared in water containing 0.01% sodium azide. The analyser was used in the configuration shown in Fig. 1, and operated at 70 samples per hour. Data was collected and processed automatically and merged with sample identities and weights taken at the time of weighing (to calculate glucosinolate concentrations). This system has been used for three seasons to analyse about 100,000 samples. A team of five people were required to weigh out samples, produce extracts, feed the analyser, handle data and recycle glassware in processing 600 samples per day.

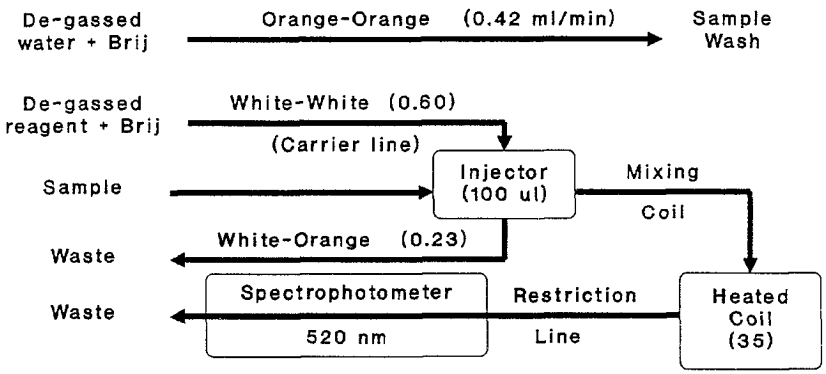

Fig. 1. Configuration of Flow Injection Analyser.

The microtitre plate system (Titertek Multiscan^{R}MCC/340) utilised the same reagents and standards, except no Brij was used. Thirty µl of extract or standard were manually pipetted into each well and 200µl of reagent was added rapidly by automatic pump. After incubation for 10 minutes at 20^{o}C colour was read at 540 nm and data collected and processed automatically. Correction was made for endogenous glucose

(about 5 µmol/g) by subtracting the value found for reference seeds extracted in boiling water. Only one well per plate was used to ascertain this value. Variations in endogenous glucose levels were not sufficient to justify determinations for each sample in a screening programme.

RESULTS AND DISCUSSION

Accuracy

Accuracy of the analyses was determined by extracting separately nine lots of the EEC reference sample on three occasions. The same extracts were analysed by FIA and platereader and the results are summarised in Table 1. These results show good agreement with the reported mean value of the European laboratories and, in view of the method-related variation reported by these laboratories (Wathelet & Wagstaffe, 1989), appear to be good for a rapid-screening system.

Table 1. Glucosinolate content of EEC reference sample (Certificated value 25.5 ± 0.9 µmol/g)

	FIA	Platereader
Mean (µmol/g)	26.32	25.21
Standard Deviation	3.093	3.159
Coefficient Variation (%)	5.87	6.26
n	27	27

In our experience accuracy of quantitative glucose release can be affected in two ways. Underestimates of glucosinolates occur if endogenous myrosinase levels are inadequate (only normally occurs if seeds are grossly overheated during drying; 60°C for 20 hours, for example, has no effect). Overestimates occur if glucose from other sources is present. This can occur at low temperature by autolysis, but does not occur at 70°C (Smith & Donald, 1988). Extracts of rapeseed contain high concentrations of sucrose (we have measured up to 17mg/ml), thus slight contamination of reagents by β-fructosidase will result in overestimates. As this (and endogenous glucose) also occurs in boiled extracts, these can be used for corrections if long incubations are used, but as β-fructosidase in our reagent acted at a slow rate (a in Fig. 2), the short dwell-time in the FIA (<1 minute) rendered interference negligible. However, this is an important point to consider when using a glucose-release method. Endogenous sugars can be removed from boiled extracts by passing them through an ion exchange resin to retain glucosinolates which are later hydrolysed by exogenous myrosinase (Heaney et al. 1988). However, this procedure is too slow for rapidly screening several hundreds of samples per day.

Precision

The standard deviation and coefficients of variation given in Table 1 combine errors of extraction and analysis. To determine repeatability of the FIA, thirty extracts were made of the Samurai reference sample and analysed twice and then pooled, mixed, re-divided and analysed 60 times (30 on each of two days). These samples were also analysed at the same time by plate-reader and the results are summarised in Table 2.

These results show that similar errors accrue using both procedures and that most of the error is in analysis rather than extraction. As only 200mg subsamples were used and several steps are involved this is rather surprising, but reassuring when using such small samples and justifies the use of such samples, necessary when screening early-generation material.

Table 2. Glucosinolate content of Samurai reference sample (µmol/g)

		FIA	Platereader
Separate measurements	Mean	13.00	13.46
	SD	1.406	1.229
	CV	10.82%	9.12%
	n	60	60
Pooled extracts	Mean	13.22	13.76
	SD	0.761	0.872
	CV	5.76	6.33
	n	60	60
% of SD due to extraction		46.0	30.8

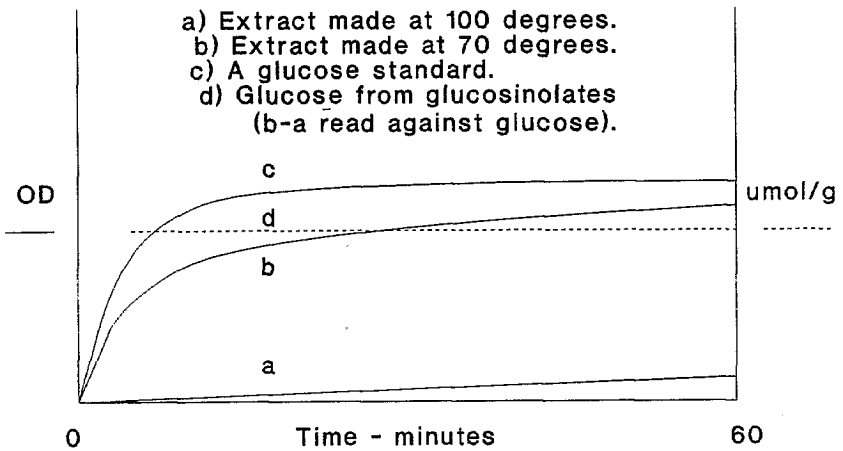

Fig 2. Schematic diagram to show effect of interference by endogenous sucrose if fructosidase is present in reagent. The rates shown were measured in microtitre plates. Reaction rates in FIA are more rapid.

Conclusion

The extraction procedure described is well suited to high-throughput batch production of extracts to feed an appropriate analysis system. The FIA, coupled to a suitable data processing system, is shown to work with acceptable accuracy and precision at a rate of 70 analyses per hour. The platereader system operates with similar accuracy and precision but requires manual pipetting of each sample and standard into microtitre plates.

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