Sources of Error in the Analysis of Glucosinolates by Gas Liquid Chromatography

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A study was made of the accuracy and precision of glucosinolate determination by gas liquid chromatography (GLC) after transformation of the glucosinolates to desulfoglucosinolates and silylation. The condition of the GLC column and the cleanness of the injection port were found to be crucial for quantitative determination of the glucosinolate silyl ethers. The relative stabilities of glucosinolate silyl ethers in pyridine solution remained constant over a period of weeks in air at ambient temperature.

The GLC analysis of glucosinolates is based on a simultaneous desulfation and silyl ether formation giving desulfoglucosinolate silyl ethers. Since its introduction in 1971 (1), the method has become more sensitive through the use of capillary columns (2), purification of samples by ion exchange chromatography before derivatization, and splitting off of the sulfate group with the help of the enzyme sulfatase (3–8). Despite the improvements, there are still some drawbacks. Different results are obtained when different internal standards are used, e.g. sinigrin, glucotropaeolin and trehalose (7). When two standards are present in the same sample, their ratio often fails to remain constant in all chromatograms. These results would seem to indicate that the stability of the various desulfoglucosinolate silyl ethers is not the same under the GLC conditions used. In our opinion, the assumption of equal stability is crucial to the determination of glucosinolates by the present GLC methods. In particular, a large excess of silylating agents injected to the GLC system may lead with time to contamination of the injection port with reactive decomposition products of the silylating agents leading not only to diminished accuracy of the analysis but even to completely erroneous estimation of the glucosinolate content.

In 1980 we observed that the deviation in the results for different injections of the same silylated sample solution was of the same magnitude as the deviation for different, independently prepared sample solutions of the same rapeseed sample. Both the accuracy and the precision of the analyses could only be described as very poor. The same was true of the results of the first two international collaborative glucosinolate analyses in 1974 and 1978 (9). A better precision was achieved in a later collaborative GLC analysis of six different samples of rapeseed meal and rapeseed in 1983 (10). The results of the next ring analysis, carried out in 13 laboratories, were calculated in 1985 (11). Evaluation of the determinations of glucosinolates in two different rapeseed samples and one rapeseed meal sample showed the coefficient of variation in repeatability to be 3.8%, 4.6% and 5.8% for the three samples, respectively. The coefficient of variation of the reproducibility was 15%, 16% and 9.3%. These figures refer to the total of the four most abundant glucosinolates in rapeseed (gluconapine, glucobrassicanapine, progoitrine and gluconapoleiferine) (11).

The present study was undertaken to discover the factors that diminish the accuracy and precision of glucosinolate determination by GLC. The factors examined were

- the history of the silylated sample
- the condition of the injection port
- the condition of the column.

EXPERIMENTAL PROCEDURES

Materials. Two different cultivars of turnip rape (*Brassica campestris*) were used for the glucosinolate analysis. One is a normal cultivar (EMMA) widely used in oilseed production in Finland and the other a low glucosinolate cultivar (SIGGA). Rapeseed meal from the normal cultivar was used to study the effect of column and injection port condition. Sinigrin hydrate and glucotropaeolin (CHR für Biochemie) were from Karl Roth, Karlsruhe, West Germany. Sulfatase (Type H-1) was from Sigma Chemical Co., St. Louis, Missouri. Ion exchanger (DEAE-SEPHADEX C-25) were from Pharmacia Fine Chemicals, Uppsala, Sweden. Pyridine (silylation grade) and TMCS (trimethylchlorosilane) were from Pierce Chemical Co., Rockford, Illinois, and bistrimethylsilylacetamide (BTMSA) and N-trimethylsilylimidazole (TMSI) were from Merck.

A Hewlett-Packard 5880A gas chromatograph fitted with a FID detector and silica capillary columns (30 m × 0.35 mm i.d.; Orion Analytica, Espoo, Finland) coated with OV 101 or SE-30 liquid phase were used for the GLC analyses. A Hewlett-Packard 5880A Series GC Terminal was used for peak integration. The column was programmed from 170 to 270 C at 10 C/min, with an initial period of 3 min at 170 C. The injector and detector were held at 270 C.

Sample preparation. The glucosinolate analysis by GLC was performed using the method of Heaney and Fenwick (6). This method is basically identical with that recommended as a standard method for international collaborative glucosinolate analysis (11). Only minor modifications were made: we used two standards (sinigrin and glucotropaeolin) instead of one, and a capillary column instead of a packed column. The water extract of 25 mg of rapeseed meal, together with 0.3 μmol of the added standards, was transferred to a DEAE SEPHADEX A-25 ion exchanger, which was eluted with pyridine-ethyl acetate to eliminate neutral compounds. The glucosinolates were released from the ion exchanger by using the enzyme sulfatase to split off the sulfate group, and the resulting desulfoglucosinolates were released from the column with water.

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The GLC analysis was performed after silylation of the dry desulfoglucosinolates.

The silylation was performed with a mixture of three parts of BTMSA, three parts of TMSI and two parts of TMCS. Each sample was silylated with 80 μl of this solution after the sample had been dried and dissolved in 250 μl of pyridine. A heating period of one hr at 110 C was used to persilylate the desulfoglucosinolates. Injections of 1.5 μl to the GLC were made by split injection technique.

Stability of the silylated samples. The stability of the persilylated desulfoglucosinolates was studied in three five-ml glass vials stoppered with Teflon-lined rubber caps. Each vial contained persilylated and ion-exchanged extract from 50 mg of rapeseed meal and 0.6 μmol of sinigrin and glucotropaeolin dissolved in 800 μl of pyridine and 160 μl of the silylation mixture. After one hr at 110 C, the contents of the vials were stirred at ambient temperature on a magnetic stirrer. Room atmosphere was allowed to enter one vial via a hypodermic needle through the rubber cap. Two other vials were flushed with dry argon and kept under dry argon atmosphere, by connecting an argon-filled balloon to the vials via hypodermic needles. Water was added to one of the argon vials as a 5% solution in pyridine using a hypodermic syringe. The mixture was stirred for 0.5 hr before GLC analysis.

Samples from three vials, taken with a syringe, were analyzed immediately by GLC.

Contamination of the injection port. To study the effect of a contaminated injection port, the silylation mixture with one-third (by volume) added water was injected using a 10-μl syringe. For this step the analytical column was replaced by a short 3-m SE-30 capillary column. The analytical OV-101 silica capillary column was then installed in the gas chromatograph and the condition of the injection port checked by analyzing, in the normal way, the standard silylated rapeseed meal sample with two added internal standards. The procedure was repeated until a total of 200 to 800 μl of the moist silylating mixture had been injected.

This experiment was concluded either by heating the injector overnight at 350 C, or by removing the quartz injector tube and cleaning it by rinsing with acetone, methanol and tetrahydrofuran, drying, soaking for two hr in concentrated nitric acid, rinsing with water and acetone, and drying at 150 C. The cleaned tube was silylated by immersing it in 20 ml of 2% TMCS solution in toluene, and it was rinsed with toluene and methanol and dried at 150 C.

Effect of defective columns. Column wear effects were studied using an eight-year-old SE-30 silica capillary column that had served in GLC analyses of a widely differing nature. A new OV-101 silica capillary column and a SE-30 silica capillary column that had been treated with wet ammonia gas were also used for comparison. Care was taken to use a well-cleaned injector.

The silylated mixture of the chromatographed desulfoglucosinolates of rapeseed meal, together with the added internal standards sinigrin and glucotropaeolin, was analyzed repeatedly on the three different columns. Results were calculated using the detector coefficient 1.00 and sinigrin as the quantitative internal standard.

RESULTS AND DISCUSSION

Stability of the silylated samples. The stability of the persilylated desulfoglucosinolates gluconapin, glucobrassicanapin, napoleiferin, progoitrin, sinigrin and glucotropaeolin in dry pyridine solution was found to be good at ambient temperature. The concentrations of the silyl ethers remained constant for several weeks even when air was admitted. Not unexpectedly, moisture caused the ethers to decompose, as seen in the diminished silyl ether peaks when water was added to the mixture (Fig. 1). The peaks disappeared completely

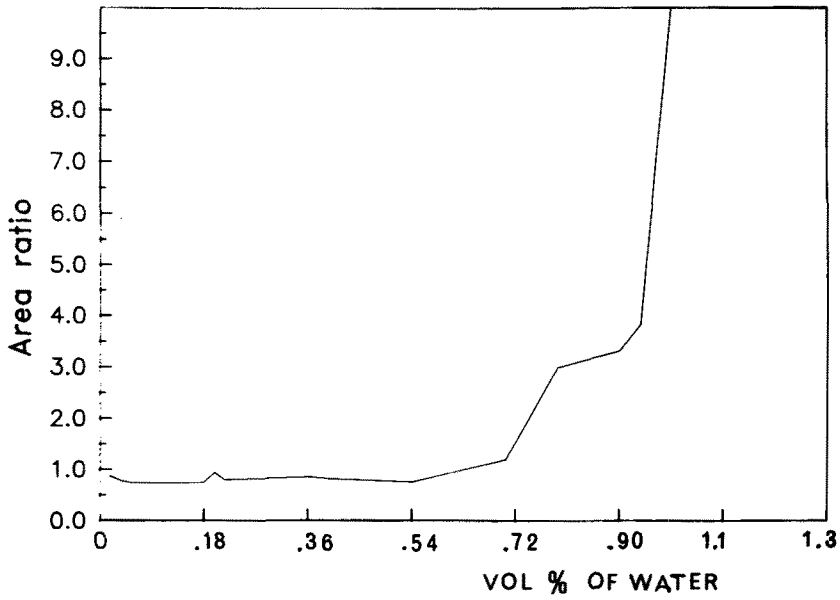

FIG. 1. Effect of water added to the silyl ether mixture of desulfoglucosinolates. Area ratio refers to that of the eluted silyl ethers of sinigrin/glucotropaeolin in GLC analysis.

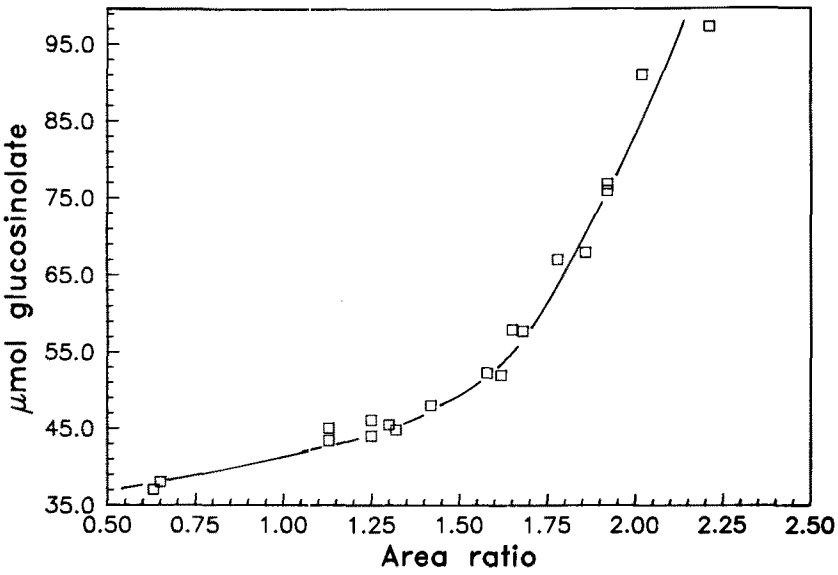

FIG. 2. Result of negligent GLC analysis of the standard rapeseed meal sample (cf. Figs. 3 and 5). Glucosinolates (total of four most abundant glucosinolates) are given as μmol/g of rapeseed meal, using sinigrin as the quantitative standard. Area ratio refers to that of the eluted silyl ethers of glucotropaeolin/sinigrin in corresponding injections. Mole ratio of glucotropaeolin/sinigrin is 1.00 in all injections.

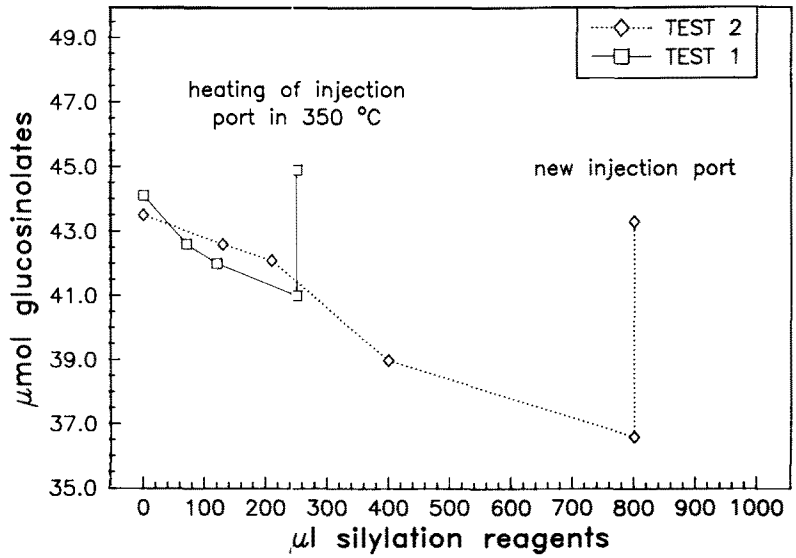

FIG. 3. Effect of injected silylation reagents on the GLC analysis of the standard rapeseed meal sample (Figs. 2 and 5). Glucosinolates (total of four most abundant glucosinolates) given as μmol/g of rapeseed meal, using sinigrin as the quantitative standard. All injections were made of the same silyl ether solution of glucosinolates.

when ca. 1% water was added. The silyl ethers of glucotropaeolin and the rapeseed glucosinolates decomposed faster than the silyl ether of sinigrin, as demonstrated in Figure 1, which shows the area ratio of internal standards (sinigrin/glucotropaeolin) in the test mixture as a function of added water. The mixture is seen to tolerate some water, but when the concentration exceeds ca. 0.5%, completely erroneous analytical results are obtained. Thus, silylated glucosinolate mixtures may be reanalyzed over a period of several weeks if special care is taken to keep the mixture dry.

Effect of a contaminated injection port and defective columns on the analysis of glucosinolates. The condition of the injection port or the column was found to affect both the accuracy and precision of the glucosinolate analysis. This is demonstrated in Figure 2, which shows the results of 19 injections of eight parallel rapeseed meal samples containing sinigrin and glucotropaeolin in 1:1 mole ratio. The sum of the four alkenyl glucosinolates in rapeseed is given as a function of the area ratio glucotropaeolin/sinigrin. The sum of the glucosinolates was calculated using sinigrin as internal

standard and a response factor of 1.00 for all glucosinolates. If the sum was calculated using glucotropaeolin as internal standard, the accuracy was improved but the precision remained poor. The 19 points in Figure 2 were obtained using two different columns in conjunction with an injection port that varied between clean and contaminated. As seen in Figure 2, anything between 36 and 97 μmol/g could be reported for the same sample. The area ratio of glucotropaeolin/sinigrin varied between 0.63 and 2.20, while the mole ratio of the two standards was 1.00. The correct area ratio of the two standards depends on the definition. If only the number of carbon atoms/molecule is considered, the correct area ratio is 1.16. This value has been used as the expected area ratio (7). However if the ratio of carbon atoms in the persilylated desulfoglucosinolates is considered, the area ratio should be 1.13. And if the contribution of the various trimethylsilyl groups is considered together with the carbon number as suggested by Verhaar et al. (12), the area ratio should be 1.155. If the internal standard ratio 1.16 was adopted for Figure 2, the corresponding correct glucosinolate content of rapeseed meal analyzed was 43.8 μmol/g. Values lower than 43.8 could reasonably be attributed to a contaminated injection port, and values higher than 43.8 μmol/g to aged columns. Obviously these two effects may be compensating to some extent, and the accuracy of the analysis may thus become acceptable if injections are repeated a sufficient number of times. But poor precision would indicate that the analytical tools were not working properly.

Figure 3 shows the effect of a contaminated injection port on the analysis of rapeseed meal glucosinolates in two different experiments. The analysis was made using a new 30-m silica capillary coated with OV-101. As seen in Figure 3, when 250 μl of the moist silylating reagent was injected, the calculated glucosinolate content decreased by 6% to 41.5 μmol/g. When 800 μl was injected, the glucosinolate content decreased by 16.5% to 37.0 μmol/g.

The situation could be corrected either by heating the injection port overnight at 350 C or by thoroughly cleaning the injector quartz tube. Figure 4 shows the same effect on the area ratio glucotropaeolin/sinigrin (mole ratio 1.00). Both Figure 3 and 4 show that silyl ethers of desulfonated glucotropaeolin and rapeseed glucosinolates are destroyed faster in a contaminated injection port than is the silyl ether of sinigrin.

The performance of the aged capillary column in glucosinolate analysis is shown in Figure 5. The gradual improvement in the performance with repeated injections of the test mixture probably was due to deactivation by the silylating agents present in the test mixture. The active sites in the aged column either adsorbed or reacted with desulfoglucosinolate silyl ethers. The performance of the aged column never equaled that of the new column.

The third column in the experiment was an old SE-30 coated silica capillary column that had been treated with moist ammonia gas. The silyl ether peaks of the desulfoglucosinolates were strongly diminished when this column was used, and the peak corresponding to sinigrin was missing completely so the results of the analysis could not be calculated in the way shown in Figure 5. Five repeated injections of the test mixture did not improve column performance sufficiently for the sinigrin peak to appear.

When a well cleaned injection port and a new OV-101 silica capillary column were used for the analysis, the mean of eight injections of one prepared silyl ether solution of the rapeseed meal was 43.7 μmol/g of glucosinolates with a coefficient of variation (CV) of 2.0%. For parallel samples the same rapeseed (eight injections) gave a mean of 44.4 μmol of glucosinolates (CV =

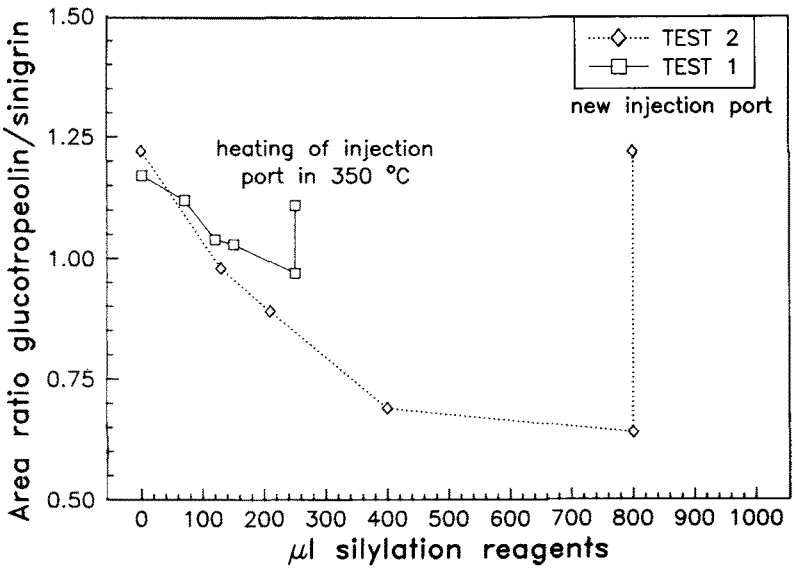

FIG. 4. Effect of injected silylation reagents on the area ratio of added internal standards glucotropaeolin/sinigrin (mole ratio 1.00) in a rapeseed meal sample. All injections were made of the same silyl ether solution of glucosinolates as was used for Fig. 3.

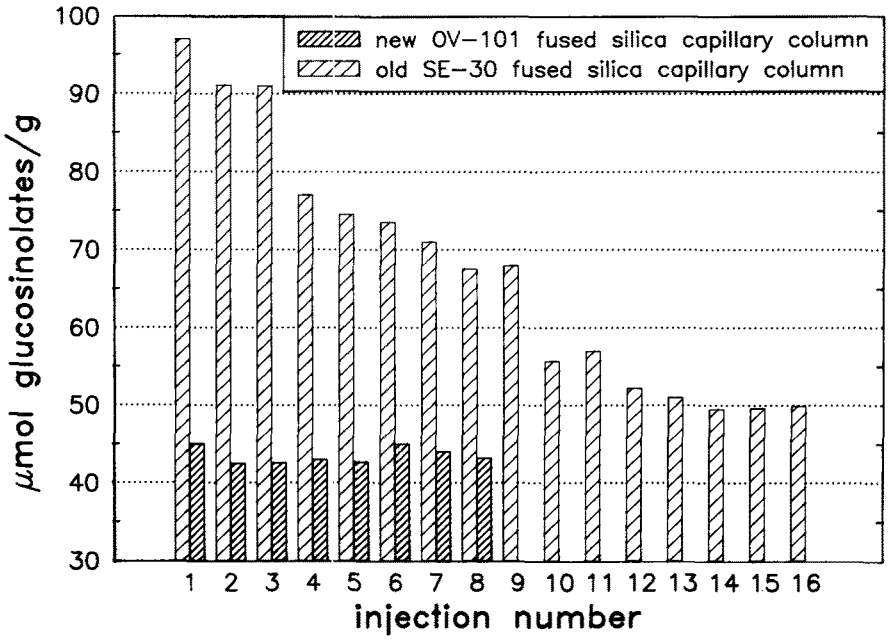

FIG. 5. Effect of column wear on the GLC analysis of the standard rapeseed meal sample (Figs. 2 and 3). Glucosinolates (total of four most abundant glucosinolates) are given as µmol/g of rapeseed meal, using sinigrin as the quantitative standard.

2.9%). Both means were close to the value of 43.7 µmol/g estimated from Figure 2.

The same value calculated from all points in Figure 2 (19 injections, 8 parallel samples) with no attention paid to the condition of the injector or the column, would be 56.6 µmol/g of glucosinolates (CV = 31.5%).

Using a well-cleaned injector and a new column to analyze four parallel samples in eight injections, 80.6 µmol/g of glucosinolates (CV = 3.4%) were found in the turnip rape sample and 13.2 µmol/g of glucosinolates (CV = 3.2%) were found in the double-low cultivar sample.

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