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GAS CHROMATOGRAPHIC DETERMINATION OF INDOLEACETONITRILES IN RAPESEED AND *BRASSICA* VEGETABLES

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SUMMARY

Indole glucosinolates are susceptible to thermal decomposition with the production of indoleacetonitriles. The indoleacetonitriles are only one of several enzymatic degradation products of indole glucosinolates which have been reported to have both beneficial and harmful effects in animal and human studies. A gas chromatography (GC) method was developed for the quantification of 3-indoleacetonitrile and 4-hydroxy-3-indoleacetonitrile, which correspond to the indole glucosinolates glucobrassicin (3-indolylmethyl) and 4-hydroxyglucobrassicin (4-hydroxy-3-indolylmethyl), respectively. Following extraction with methylene chloride, trimethylsilyl derivatives of indoleacetonitriles from commercial low-glucosinolate rapeseed meal and cooked *Brassica* vegetables were effectively separated by GC with a total run time of 20 min and detected by using a flame ionization detector. Extraction of samples with methylene chloride was maximal at 15 min extraction time and sample to water ratios (w/v) of 1:2 for rapeseed meal and 1:10 for freeze-dried vegetables were shown to be effective. Derivatization of the indoleacetonitriles was effected at room temperature.

INTRODUCTION

The indole glucosinolates, glucobrassicin (3-indolylmethyl) and 4-hydroxyglucobrassicin (4-hydroxy-3-indolylmethyl), represent the major portion (40–60%) of the total glucosinolate content of low-glucosinolate rapeseed and *Brassica* vegetables[1,2]. It was shown for both of the indole glucosinolates that they are markedly susceptible to thermal degradation with thiocyanate ion (SCN) as a major thermal degradation product[1,3]. Recent studies have indicated the presence of two new thermal degradation products of indole glucosinolates in rapeseed meal[4] and *Brassica* vegetables[5]. These compounds were identified as 3-indoleacetonitrile (IAN) and 4-hydroxy-3-indoleacetonitrile (OH-IAN), which correspond to the intact indole glucosinolates glucobrassicin and 4-hydroxyglucobrassicin, respctively. A scheme showing the thermal decomposition of indole glucosinolates to SCN and indoleacetonitriles is given in Fig. 1.

Since thermally treated cruciferous crops are widely used as human foods and animal feedstuffs, there is a growing interest in the potential antinutritive properties of

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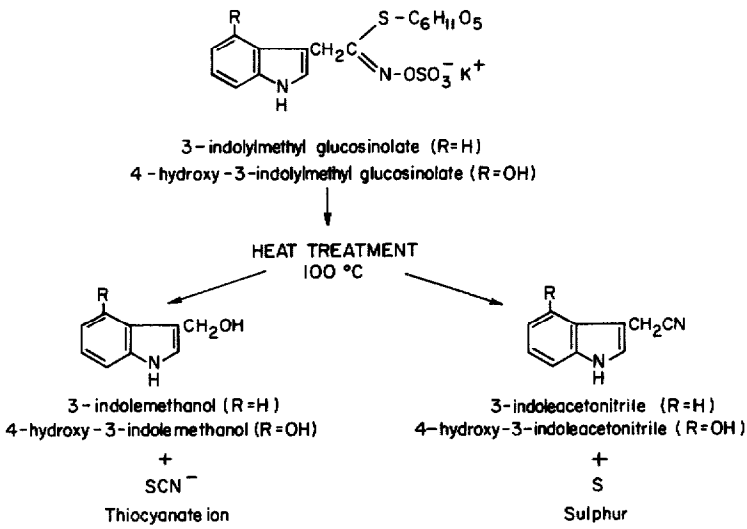

Fig. 1. Proposed pathway of the thermal degradation of indole glucosinolates.

the thermal degradation products of indole glucosinolates. In contrast to the potential antinutritive effects of *Brassica* vegetables, studies have indicated a possible beneficial effect in humans due to the fact that some indole compounds originating from the intact glucosinolates in the vegetables were shown to be inhibitors of chemically induced carcinogenesis and to stimulate the activity of key enzymes involved in cellular detoxification systems[6]. Further research in these areas is warranted and the procedure reported herein for determining indoleacetonitriles should facilitate such research.

EXPERIMENTAL

Materials

Commercial low-glucosinolate rapeseed meal samples were obtained from crushing plants located in Western Canada and the *Brassica* vegetable samples were purchased at a local supermarket. Indole-3-acetic acid, 3-indoleacetonitrile and *n*-octadecane were obtained from Sigma (St. Louis, MO, U.S.A.). Pyridine and bis(trimethylsilyl)-trifluoroacetamide (BSTFA) were purchased from Pierce (Rockford, IL, U.S.A.). Methylene chloride was from Burdick and Jackson (Muskegon, MI, U.S.A.).

Gas chromatographic analysis of indoleacetonitriles

Rapeseed meal or *Brassica* vegetable samples (0.5 g) were weighed into 125-ml erlenmeyer flasks. Distilled water was then added and the mixture was allowed to stand for 5 min to form a paste. The standard procedure involved the use of 1 and 5 ml of water for rapeseed meal and freeze-dried vegetable samples, respectively. Portions of 20 and 30 ml of methylene chloride were then added, respectively to rapeseed meal and vegetable samples, followed by 1 ml of a methylene chloride solution of internal standard (0.25 μmol ml^{-1} *n*-octadecane). The flasks were sealed and shaken in an orbit shaker for 15 min at 200 rpm. Following extraction, the methylene chloride extracts

were filtered (Whatman No. 3 filter paper) into 50-ml evaporating flasks, concentrated under vacuum to approximately 1 ml and transferred by Pasteur pipette into 4-ml silylation vials. Following drying under a stream of nitrogen the samples were derivatized at room temperature by adding 50 μl pyridine and 50 μl BSTFA to each vial which was then capped and mixed well prior to gas chromatographic (GC) analysis. The trimethylsilyl (TMS) derivatized indoleacetonitriles were separated by using a Varian Vista 6000 gas chromatograph equipped with a flame ionization detector and a Vista 402 computer. A glass column (1.8 m × 2 mm I.D.) packed with 3% OV-1 on Chromosorb W (HP) (100–120 mesh) was used with helium gas at a flow-rate of 40 ml min^{-1}. The oven temperature was programmed from 120 to 220°C at 5°C min^{-1}. Injection port and detector temperatures were 200 and 250°C, respectively.

During development of the method, sample to water ratio and extraction time were studied to effect complete extraction of indoleacetonitriles from the samples. In addition, time and temperature conditions of derivatization with BSTFA were also studied.

Determination of relative response factors

Relative response factors (RRF) were determined by using pure IAN (Sigma) and a sample of isolated OH-IAN. A known amount (1 μmol) of each compound was used with *n*-octadecane as internal standard in the determination of the response factors.

Isolation of OH-IAN was done since this compound is not commercially available. Column chromatography (CC, Sepahdex LH-20) and thin-layer chromatography (TLC, silica gel 60) as described by Slominski and Campbell[4] were used in the isolation and purification. In the quantification of OH-IAN, GC of a 80% methanol TLC eluate was conducted while using indole-3-acetic acid as a reference compound. Following derivatization with pyridine and BSTFA (1:1, v/v) for 15 min at 50°C[7] indole-3-acetic acid and OH-IAN were separated on a 3% OV-1 column with the response set at 1.00. This was based on the assumption that both compounds would respond identically in the flame ionization detector due to similar chemical structures, *i.e.*, two TMS groups attached to the molecule and the same number of carbon atoms.

Indoleacetonitrile analysis and recovery studies

Seven samples of low-glucosinolate rapeseed meal and four samples of cooked *Brassica* vegetables were analyzed for content of indoleacetonitriles by using the proposed procedure. The samples of *Brassica* vegetables included white and red cabbage, broccoli and Brussels sprouts. Prior to analysis the vegetable samples were boiled (100°C) for 40 min, freeze-dried and ground. Three recovery experiments were conducted using rapeseed meal and white cabbage with a known amount (1 μmol g^{-1}) of IAN added to each sample.

RESULTS AND DISCUSSION

Examples of gas chromatograms of trimethylsilyl derivatives of indoleacetonitriles from commercial low-glucosinolate rapeseed meal and cooked white cabbage leaves are shown in Fig. 2. Although both indoleacetonitriles were well separated in a total run time of 17 min, the analysis was continued for an additional 3 min to allow the removal of impurities from the column.

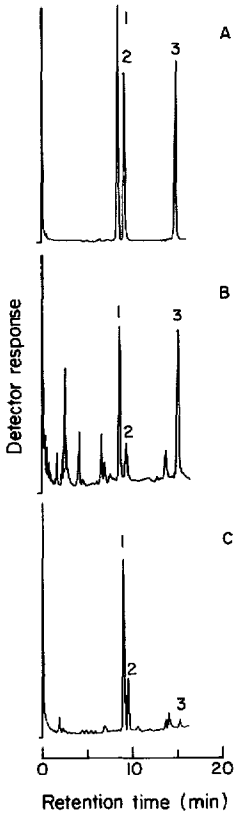

Fig. 2. Gas chromatogram of TMS derivatives of indoleacetonitriles from (A) a mixture of pure compounds, (B) low-glucosinolate rapeseed meal and (C) cooked white cabbage leaves. Peaks: 1 = *n*-octadecane (internal standard); 2 = IAN; 3 = OH-IAN.

The influence of the amount of water added to rapeseed meal samples on the extraction of indoleacetonitriles by methylene chloride is shown in Fig. 3. The data indicated that a ratio of 1:1 (w/v) was sufficient to result in maximal extraction of both IAN and OH-IAN from rapeseed meal samples. As a standard procedure, however, it is recommended that for air-dry rapeseed meal samples a ratio of sample to water of 1:2 be used. In the case of freeze-dried vegetable samples the ratio of sample to water to effectively extract the indoleacetonitriles was found to be 1:10. No additional water was required, however, for the analysis of non-freeze-dried (*i.e.* fresh) cooked or steamed samples of vegetables.

The effect of time of extraction on the recovery of indoleacetonitriles is shown in Fig. 4. Extraction was maximal within 10–15 min and consequently an extraction time of 15 min was adopted for the standard procedure. Derivatization of a methylene chloride extract of rapeseed meal under different conditions showed no significant differences with respect to the times (1–20 min) or temperatures (20 and 60°C) applied (Fig. 5). In previous work derivatization of indoleacetonitriles was carried out at 60°C for 15 min[4]. Under those conditions it was demonstrated that the nitrogen atom of the indole ring of both IAN and OH-IAN and the hydroxyl group of OH-IAN were

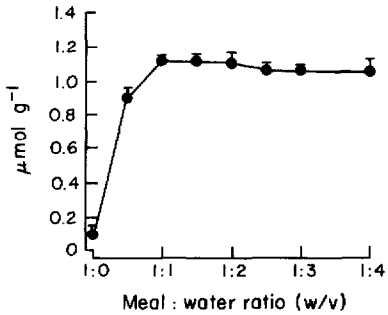

Fig. 3. Effect of different meal to water ratios on the extraction of indoleacetonitriles with methylene chloride.

effectively trimethylsilylated. The results from the current study indicated that derivatization is effective at 20°C and consequently it is recommended that derivatization be conducted at room temperature in the proposed procedure.

The RRF values determined for IAN and OH-IAN are given in Table I. The RRF values for both compounds are approximately 15% higher than values calculated based on TMS-nitrile and *n*-octadecane carbon number. This apparent discrepancy may be explained by a differential response between aromatic and aliphatic compounds in the flame ionization detector. A similar response difference between aromatic and aliphatic glucosinolates has been reported[1,8]. It should be noted that the RRF values reported herin cannot be applied arbitrarily and should be determined for specific column and chromatographic conditions.

The recoveries of IAN added to samples of rapeseed meal and white cabbage leaves are shown in Table II. Since IAN is readily soluble in methylene chloride and due to the fact that no clean-up procedure is used in the proposed method, the data demonstrating complete recovery (98–101%) are more a confirmation of the accuracy of the RRF value for IAN than recovery *per se*. Another estimate of recovery, however, can be obtained from the data presented in Fig. 3 and 4. The constant amounts of indoleacetonitriles determined in samples of rapeseed meal and cabbage leaves subjected to varying but optimum extraction conditions is an indication of adequate recovery of the nitriles from these samples.

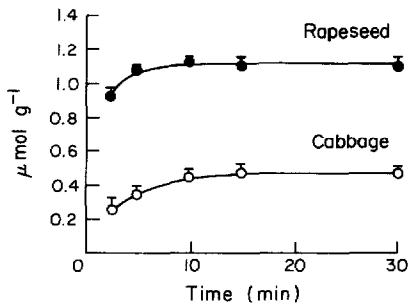

Fig. 4. Effect of varying times of extraction on the recovery of indoleacetonitriles from rapeseed meal and cooked white cabbage leaves.

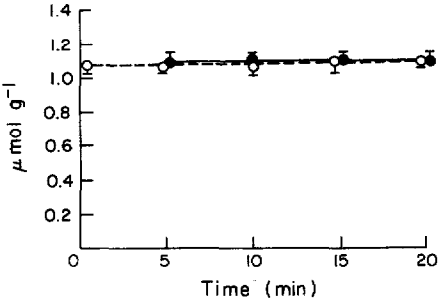

Fig. 5. Effect of different derivatization conditions on the recovery of indoleacetonitriles. (●) 60°C; (○) 20°C.

A number of samples of low-glucosinolate rapeseed meal and of cooked *Brassica* vegetables were analyzed for IAN and OH-IAN by the proposed method (Table III). Total indoleacetonitriles content averaged at 0.8 μmol g^{-1} for the seven rapeseed meal samples. The predominant indoleacetonitrile was OH-IAN which would be expected since 4-hydroxyglucobrassicin is the major intact indole glucosinolate in low-glucosinolate rapeseed meal[1]. Results have shown that in rapeseed meal approximately 5–6 μmol g^{-1} of intact indole glucosinolates are decomposed during heat treatment and that of the decomposed indoles 2.0 μmol g^{-1}, on average, appear as free SCN[1]. The results from the current study indicate that indoleacetonitriles may account for an additional 0.8 μmol g^{-1} which leaves approximately 2–3 μmol g^{-1} of decomposed indoles unaccounted for. This confirms that degradation products other than SCN or indoleacetonitriles are formed during the heat treatment of indole glucosinolates as suggested by Campbell and Slominski[3].

TABLE I

RELATIVE RESPONSE FACTORS (RRF) FOR IAN AND OH-IAN WITH *n*-OCTADECANE AS INTERNAL STANDARD (RRF = 1)

Compound	*Relative response factor*	
	Calculated	*Determined*
IAN	1.38	1.58
OH-IAN	1.12	1.36

TABLE II

PERCENT RECOVERY OF IAN ADDED TO SAMPLES OF RAPESEED MEAL AND WHITE CABBAGE LEAVES

Sample	*Recovery (%)**
Rapeseed meal	101.0 ± 1.0
White cabbage	98.2 ± 2.3

* Mean ± S.D.

TABLE III

CONTENTS OF IAN AND OH-IAN IN COMMERCIAL LOW-GLUCOSINOLATE RAPESEED MEAL AND COOKED *BRASSICA* VEGETABLES

Sample	*IAN*	*OH-IAN*	*Total*
Rapeseed meal (μmol g^{-1} air-dry weight)			
Meal 1	0.15	0.60	0.75 ± 0.02*
Meal 2	0.11	0.66	0.77 ± 0.02
Meal 3	0.24	0.68	0.92 ± 0.01
Meal 4	0.10	0.56	0.66 ± 0.03
Meal 5	0.31	0.82	1.13 ± 0.03
Meal 6	0.14	0.61	0.74 ± 0.02
Meal 7	0.09	0.64	0.73 ± 0.01
Vegetable (μmol g^{-1} dry weight)			
White cabbage	0.41	0.07	0.48 ± 0.01
Red cabbage	0.19	0.07	0.26 ± 0.03
Broccoli	0.20	0.03	0.23 ± 0.02
Brussels sprouts	2.43	0.11	2.54 ± 0.06

* Mean of duplicate determinations, ±S.D.

The amount of indoleacetonitriles in *Brassica* vegetables (Table III) was more variable than that noted for the rapeseed meal samples, which could be explained by the high variability in intact indole glucosinolate content among and within species of cruciferous vegetables[2]. In contrast to the rapeseed meal samples, IAN rather than OH-IAN was the predominant indoleacetonitrile which corresponds to the high amount of glucobrassicin in these vegetables[2]. In addition, the highest level of IAN was present in Brussels sprouts which are known to contain a relatively high amount of glucobrassicin[9]. The data presented in Table III, however, does not represent a total amount of IAN and OH-IAN produced upon the thermal decomposition of indole glucosinolates in *Brassica* vegetables since substantial leaching may occur during the cooking process[5].

ACKNOWLEDGEMENTS

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