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CAPILLARY GC OF GLUCOSINOLATE-DERIVED HORSERADISH CONSTITUENTS

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Key Word Index—*Armoracia lapathifolia*; Cruciferae; horseradish; root; glucosinolate derivatives; capillary GC; MS.

Abstract—The technical requirements of glass capillary GC allowing the separation and identification of a large number of mustard oils and other nitrogenous components in horseradish are presented. Certain of these require support surfaces under the stationary phase which are free from basic sites. The deactivation of adsorptive sites, which is necessary in the case of a number of mustard oils, was accomplished by employing polyglycols as stationary phases. Sampling into conventional vaporizing injectors may cause strong discrimination of high boiling compounds as well as pyrolysis of labile mustard oils within the syringe needle. By employing cold on-column injection these effects could be avoided. Glucosinolates present in horseradish were identified through the corresponding mustard oils using MS. Of the glucosinolates in this species, 30 were identified including at least six compounds that have not been described before as natural products.

INTRODUCTION

A surprisingly large number of mustard oils and glucosinolates were described before efficient isolation and identification techniques were available. In the early fifties the separation efficiency was much improved when the 'ingenious tool of paper chromatography' [1] was introduced. GC was first employed by Youngs and Wetter [2] for the analysis of rape seed. The suitability of the method was restricted to compounds which were not adsorbed and highly volatile, and the limit of detection (some 100 ppm) was rather high. However, it showed that a plant species may produce as many as 10 different mustard oils. A disadvantage of GC, the low sample capacity, yielding the separated compounds only in microgram quantities, was overcome by GC–MS. Packed column GC–MS recently allowed the identification in cabbage of 17 nitrogenous constituents deriving from nine different glucosinolates [3].

The most comprehensive work on identification of mustard oils from a wide variety of plants using GC–MS was performed by Cole [4]. By comparison with horseradish (*Armoracia lapathifolia*, formerly *Cochlearia Armoracia*) this paper reported that in *Cochlearia anglica* and *C. danica* only *sec*-butylglucosinolate was found, which in *C. officinalis* was accompanied by a small amount of allylglucosinolate.

In contrast, GC analysis of horseradish (*A. lapathifolia*) has revealed the presence of six different glucosinolates: methyl-, ethyl-, *iso*-propyl-, *sec*-butyl-, allyl- and 3-butenylglucosinolate [5]. Among the volatile constituents of horseradish root, the products of butyl-, allyl, 4-pentenyl- and 2-phenylethylglucosinolate were identified using GC–MS analysis [6]. This list was later extended when GC–MS analysis led to the identification of the following compounds: *iso*-propyl-, allyl-, 3-butenyl-, 4-pentenyl-, phenyl-, 3-methylthiopropyl-, benzyl- and 2-phenylethylglucosinolate [7]. All of these identifications are correct with the exception of phenylglucosinolate which was not detectable in the glass capillary GC–MS system used in the present study.

RESULTS AND DISCUSSION

Evaluation of columns

The suitability of different glass capillaries for the analysis of mustard oils was tested with a mixture of critical mustard oils. Moreover, the columns were subjected to a standardized test procedure [8]. Provided that the capillaries do not have a strongly basic support, the functional groups typical for glucosinolate-derived products (isothiocyanates, thiocyanates and nitriles) are neither adsorbed nor degraded by any type of high quality column. Special requirements as to the properties of the columns are associated with additional functional groups: (a) some mustard oils with hydroxy groups tend to adsorb and, hence, tend to tail on insufficiently deactivated surfaces; (b) acidic compounds such as phenolic mustard oils require systems that are free of basic sites, in the presence of basic sites they may not be eluted at all; (c) the sulfinyl as well as the sulfonyl compounds require not only a perfectly deactivated surface but also the absence of basic sites and a slightly acidic capillary.

Basic mustard oils (derived from basic amino acids) have not been discovered so far. If the existence of such compounds can be neglected, the absence of basic sites and a low general adsorption are the principal properties of a column that will efficiently elute all the known mustard oils

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except some very polar species such as *p*-glucosyloxybenzyl- and 3,4-dihydroxybenzyl-*iso*-thiocyanate.

Stationary phases of dimethyldisiloxanes or slightly substituted silicones (e.g. OV-1, SE 30, SE 52, SE 54) have several advantages. They have a low bleed rate and, therefore, are suitable for GC–MS. Retention is low so that even highly boiling compounds are eluted. Since the concentrations of major and minor mustard oils differ by factors up to 1000 it was necessary to choose a film thickness of the stationary phase of 0.3–0.4 μm to ensure complete separations despite the inevitable overloading of the chromatograms. These thick stationary phases resulted in an increased retention and, thus, facilitated the separation of the most volatile compounds. Among several pretreatments of the support surface for nonpolar coatings, strong leaching of the glass (20% HCl, 160°, 18 hr) and subsequent silanization [9] turned out to be particularly suitable. A disadvantage of apolar columns is their poor durability which is caused by the tendency of polar compounds to adsorb to the support of the stationary phase, affecting the deactivation and wettability of this surface and thus leading to increased adsorption and poor separation efficiency. Moreover the nonpolar columns fail in the elution of dihydroxybenzyl-, sulfinyl- and sulfonyl-mustard oils. Since this was the case even when columns produced reasonably shaped peaks for carboxylic acids, it was concluded that adsorption is associated with an acid–base independent mechanism. Columns coated with polyglycol phases eluted these substances satisfactorily as a result of excellent shielding and blocking of active sites. Furthermore, the durability of such columns is not affected by polar compounds present in the sample. Polyglycol columns were prepared by employing the barium carbonate procedure [10]. In order to eliminate basic sites, the columns were treated with sulfuric acid dissolved in ether, resulting in the transformation of carbonate into sulfate. The criteria for selecting suitable polyglycols were good deactivation and low bleed. Deactivation was found to be similar in the case of polyethylen- and polypropylene-glycols. On a given support the bleed, which may cause severe disturbance of the MS, is strongly dependent on the column temperature. Thus, polymers with polypropylene-glycol having a relatively low polarity appeared to meet the requirements and we finally chose the well-defined and relatively pure Pluronic L 64 [11] with a temperature range of 15–240°.

Injection system

Sampling into classical vaporizing injectors was difficult, particularly in the case of sulfide-, sulfinyl- and sulfonyl-mustard oils. Standard deviations of quantified peak areas were high and the accuracy was poor. Strong discrimination of the higher boiling compounds is mainly caused by the fact that most of the sample is evaporated already in the hot syringe needle. As a consequence the volatiles are evaporated selectively whereby a large proportion of the high boiling materials remain behind in the dead volume of the needle. Furthermore, the evaporation from the hot metal surface of the syringe needle causes pyrolysis of certain mustard oils. As shown previously [12], the proportion of the sample 'shooting' out of the syringe needle in the form of small droplets (safely carrying high boiling and labile components out of the needle) is dependent on the syringe needle temperature at the moment the sample is transferred into or through it. This 'hot needle technique' was again found to give more accurate results. The sample is withdrawn into the barrel of the syringe as the needle is introduced into the injector and the plunger is pushed down rapidly, but not before a preheating period of 3–5 sec. Still another difficulty concerns the injector temperature. At high temperatures discrimination of high boiling compounds is decreased, yet the pyrolysis of labile compounds is increased. In the case of horseradish- and radish mustard oils the optimal injector temperature was *ca* 225°. Under these conditions pyrolysis and discrimination reached 15% for the most critical compounds. However, the variance of the results was still unsatisfactorily high, exceeding 20% in some cases.

A decisive improvement of the quantitative analysis was associated with the employment of an injector allowing the sample to be transferred as a liquid into the capillary [13]. Cold on-column injection avoids the discrimination upon selective evaporation out of the needle as well as pyrolysis during the sampling. Indeed, breakdown products were not observed and the standard deviations of peak areas (normalized from an internal standard) did not exceed 8% even in the case of the labile and adsorptive sulfinyl compounds.

Mustard oils present in horseradish roots

The chromatograms shown in Figs. 1 and 2 were obtained with SE 52 and with a medium polar Pluronic L64 capillary column, respectively, using a nitrogen specific alkaliflame ionization detector (AFID). Comparison with chromatograms obtained with FID demonstrate that the nitrogenous glucosinolate derivatives represent by far the most abundant volatiles of crude horseradish extracts. The strongly misshaped peak in the first third of the chromatogram of Fig. 2 represents dicyclohexylamine which is strongly adsorbed on the slightly acidic column. Peaks Nos. 9 and 10 (a sulfinyl and a sulfonyl compound) are well shaped in the chromatogram of the Pluronic capillary (Fig. 2); in contrast, these compounds are strongly absorbed on the SE 52 column (Fig. 1).

The identification of mustard oils was carried out using GC–MS with capillaries of both SE 52 and Pluronic L 64. In most cases both the corresponding isothiocyanates and the nitriles were detected, providing an opportunity for direct comparison. The R_t data therefore provided a most valuable confirmation of the MS identifications.

The 30 glucosinolates identified are listed in Table 1. This number exceeds the number of glucosinolates detected before in any one species. A close examination of the chromatograms may indicate that the number of structures present in horseradish is much larger. The structures that have not been detected before among the products of mustard oil-producing plants are marked in Table 1. It is noteworthy that indole derivatives of the glucobrassicin type appear to be absent in horseradish.

Glucosinolates are known to originate from amino acids [14, 15]. It is therefore tempting to consider the possible origins of the compounds identified. The largest group of known glucosinolates originates from methionine. In horseradish almost 70% of the total amount of identified glucosinolates (273 of 398 mg/kg fr. root) is represented by methionine-derived compounds. The parent structure, 2-methylthioethylglucosinolate, which is derived from methionine without chain elongation or alteration of the sulfide group, has not been detected before

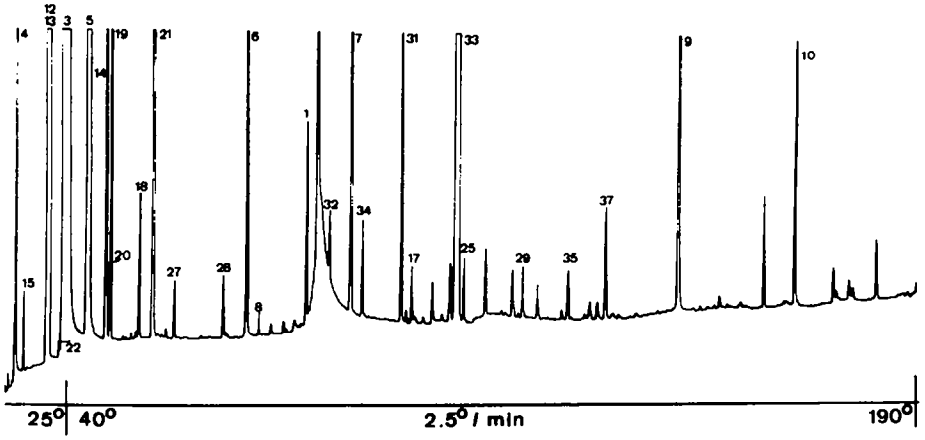

Fig. 1. Glucosinolate-derived horesradish constituents present in a crude extract, chromatographed on a SE 52 capillary column (15 m × 0.31 mm) using AFID detection. Carrier gas: 0.7 at He. The peak numbers refer to the identifications listed in Table 1.

in mustard oil-producing plants. Of the entire series of higher homologues up to the 6-methylthiohexylglucosinolate, the 3-methylthiopropylglucosinolate is the most abundant. This structure is known to represent the precursor of allylglucosinolate (Sinigrin [16]) which is by far the predominant glucosinolate in horseradish.

Of the four alkenylglucosinolates detected, the 5-hexenylglucosinolate is new in horseradish. It has been found, however, in *Wasabia japonica* [7]. These structures undoubtedly originate from the methylthioalkyl compounds through the elimination of methyl mercaptan. The vinylglucosinolate (derived from 2-methylthioethylglucosinolate) is missing in the otherwise complete co-existence of methylthioalkyl and alkenyl compounds. In all cases the quantity of the alkenylglucosinolates is much larger than the quantity of the corresponding methylthio compounds. It is interesting to note that both 3-hydroxy-4-pentenyl- and 3-hydroxy-5-methylthiopentylglucosinolate are present in horseradish, indicating that the hydroxy group is possibly introduced into the methylthio compound (or into its precursor), the 3-hydroxy-4-pentenylglucosinolate representing a derivative of this structure. Of the large number of known sulfinyl and sulfonyl structures only the derivatives of 3-methylthiopropylglucosinolate could be detected. This is probably due to the low concentrations of the other corresponding compounds. Moreover, their MS are complicated and difficult to interpret in the case of very small peaks.

Of the straight-chain isomers of alkylglucosinolates only the C_4 and C_5 structures (not the C_3 structure) were found to be present in fairly low amounts. Two small peaks yielded MS of hexyl and heptyl *iso*thiocyanate which, however, were not in agreement with published spectra of *n*-alkyl isomers [17]. They probably represent *iso*-alkyl derivatives. Valine appears to be a precursor candidate of the smaller *iso*-alkyl-homologues: *iso*-propyl-, and 3-methylpentylglucosinolate. *iso*-Leucine is a precursor candidate of *sec*-butylglucosinolate. The spectra of the C_6 and C_7 compounds neither exclude nor sufficiently confirm the identity with 4-methyl- and 5-methylhexylisothiocyanate, nor do they fit acceptably with the spectrum recently published for 4-methylpentyl-*iso*-thiocyanate [18]. Two *iso*-alkylglucosinolates of horseradish, *iso*-butyl- and 2-methylbutylglucosinolate cannot be derived from valine through chain elongation, but from leucine and *iso*-leucine, respectively.

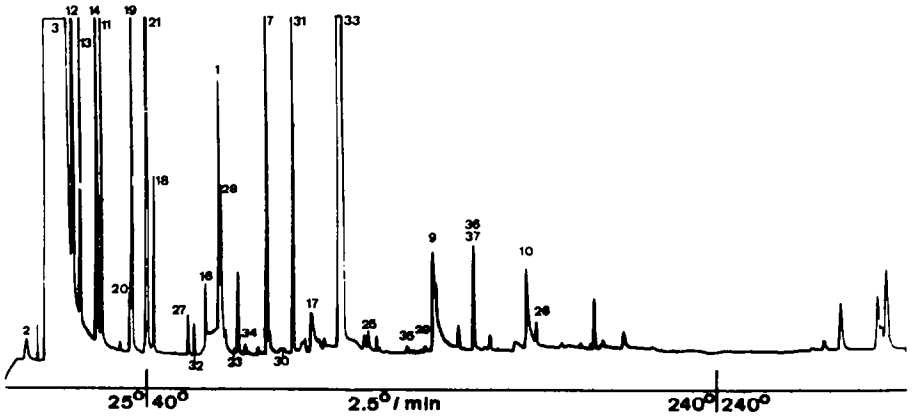

Fig. 2. Extract and conditions as indicated in Fig. 1 except for the capillary column (18 m × 0.30 mm) which was coated with Pluronic L 64. The strongly misshaped peak between peaks 1 and 32 is dicyclohexylamine.

Table 1. Glucosinolates of horseradish roots as identified by their corresponding mustard oils

Glucosinolate	Concentration μg/g fr. wt.	GC-peak No.	Type of mustard oil
C_2 2-methylthioethyl-	2	1	-NCS new
C_3 *iso*-propyl-	1	2	-NCS
allyl-	240	3	-NCS
		4	-CN
		5	-SCN
2-hydroxypropyl-	3	6	-NCS new
3-methylthiopropyl-	5	7	-NCS
		8	-CN
3-methylsulfinylpropyl-	3	9	-NCS
3-methylsulfonylpropyl-	3	10	-NCS
C_4 *n*-butyl-	5	11	-NCS
sec-butyl-	22	12	-NCS
iso-butyl-	6	13	-NCS
3-butenyl-	14	14	-NCS
		15	-CN
3-hydroxybutyl-	0.5	16	-NCS
4-methylthiobutyl-	0.5	17	-NCS
C_5 *n*-pentyl-	1	18	-NCS
3-methylbutyl-	8	19	-NCS new
2-methylbutyl-	0.5	20	-NCS
4-pentenyl-	20	21	-NCS
		22	-CN
2-hydroxypentyl-	0.1	23	-NCS new
3-hydroxy-4-pentenyl-	0.8	24	-NCS
5-methylthiopentyl-	0.2	25	-NCS
3-hydroxy-5-methylthiopentyl-	0.2	26	-NCS
C_6 *iso*-hexyl- structure unknown	0.4	27	-NCS new ?
5-hexenyl-	2	28	-NCS new in horseradish
6-methylthiohexyl-	0.1	29	-NCS
C_7 *iso*-heptyl- structure unknown	0.05	30	-NCS
benzyl-	4	31	-NCS
		32	-CN
2-phenylethyl-	55	33	-NCS
		34	-CN
3-phenylpropyl-	0.1	35	-NCS new ?
4-phenylbutyl-	0.5	36	-NCS new
methoxybenzyl-	0.5	37	-NCS

The numbers of GC peaks refer to the chromatograms shown in Figs. 1 and 2.

Phenylethylglucosinolate represents the most abundant of the five structures derived from phenylalanine. Of the series of homologues with chains elongated by 1, 2 or 3 carbon atoms, neither 3-phenylpropyl- nor 4-phenylbutylglucosinolate were known as natural compounds. 3-Phenylpropylglucosinolate was postulated to be present in horseradish as early as in 1931 [19] but its occurrence in this plant has lately been doubted [1].

The spectrum of glucosinolate derivatives identified, together with the considerable number of unidentified GC peaks suggests that horseradish (and perhaps also other mustard oil-producing plants) produces the entire theoretical complement of glucosinolates. Undoubtedly, the cells are equipped with the enzymes necessary for the transformation of the amino acids (chain elongation, hydroxylation, etc.) to yield the glucosinolate function and the various structures of the residues. It is perhaps a small group of enzymes which are unspecific enough to catalyse the reactions in a wide variety of compounds. From this, it is not to be expected that a species like horseradish would accumulate a few glucosinolates in large quantities and a large number of others in comparatively small amounts. Since glucosinolates are known to be sequestered exclusively in vacuoles [20], it may be speculated that the specificity of transport of glucosinolate precursors, e.g. at the level of thiohydroximate, into this compartment is responsible for the preferential accumulation of species-typical compounds. In conjunction with a possible product-inhibition of enzymes involved in the biosynethetic processing, preferential removal of certain compounds from the cytoplasm would lead to the phenomena observed.

EXPERIMENTAL

The following methods were employed for extracting mustard oils from horseradish roots. (i) Extraction of glucosinolates from the intact tissue in boiling H_2O [21], removal of nonpolar substances prior to the formation of mustard oils. Pieces of tissue were dropped into boiling H_2O, boiled for 5 min, and subsequently homogenized in the same H_2O using a blender. The slurry was centrifuged and the supernatant extracted $\times 3$ with hexane and then supplemented with crude myrosinase [22], 0.1% L-ascorbic acid and Na-citrate to pH 6.5. The mustard oils produced upon incubation at 30° for 2 hr were extracted with CH_2Cl_2. This extract contained the corresponding nitriles and *iso*thiocyanates of most glucosinolates in similar amounts. (ii) Alternatively, pieces of frozen root tissue were grated directly into CH_2Cl_2. After incubating the brei for 30 min at 30° the solvent was separated from the solid material which retained the aq. phase. Further purification of the extract was not necessary since it contained surprisingly little volatiles other than the mustard oils which were present almost exclusively as *iso*thiocyanates.

MS. The glass capillary was coupled directly to the ion source of a medium resolution instrument by means of a narrow platinum capillary; 70 eV.

MS of new iso*thiocyanates*. 2-Methylthioethyl-*iso*thiocyanate: *m/e* 61 (100%), 133 = M^+ (40), 99 (35), 44 (35), 72 (25), 75 (25), 45 (23). Spectrum known from ref. [17]. 2-Hydroxypropyl-*iso*thiocyanate: *m/e* 59 (100%), 99 (90), 72 (75), 45 (60), 39 (40), 41 (30), 66 (30). *n*-Pentyl-*iso*thiocyanate: *m/e* 43 (100%), 41 (70), 72 (40), 101 (35), 55 (35), 129 = M^+ (30), 114 (20), 96 (20), 59 (15). Spectrum known from ref. [17]. 3-Methylbutyl-*iso*thiocyanate: *m/e* 43 (100%), 41 (60), 114 (45), 72 (35), 55 (35), 129 = M^+ (15). Spectrum known from ref. [17]. 2-Hydroxypentyl-*iso*-thiocyanate: *m/e* 43 (100%), 59 (85), 69 (60), 127 (50), 72 (45), 41 (45), 71 (40), 99 (20). *iso*-Hexyl-*iso*thiocyanate: *m/e* 41 (100%), 43 (100), 73 (35), 128 (30), 56 (30), 110 (25), 143 = M^+ (20), 115 (15), 101 (15), 102 (15). *iso*-Heptyl-*iso*thiocyanate: *m/e* 43 (100%), 115 (55), 99 (50), 114 (25), 124 (25), 157 = M^+ (15), 72 (15), 89 (10), 71 (10), 55 (10). 3-Phenylpropyl-*iso*thiocyanate: *m/e* 91 (100%), 119 (40), 72 (35)... 105 (20), 145 (15), 117 = M^+ (10). 4-Phenylbutyl-*iso*thiocyanate: *m/e* 41 (100%), 72 (90), 91 (60), 104 (45), 191 = M^+ (40), 100 (40), 99 (40), 79 (25), 118 (20), 158 (15), 190 (15).

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