Journal of Chromatography, 452(1988) 61–73
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 20 656

APPLICATIONS OF GAS CHROMATOGRAPHY TO THE STUDY OF TERPENOID METABOLISM

D. MICHAEL SATTERWHITE and RODNEY B. CROTEAU*
Institute of Biological Chemistry, Washington State University, Pullman, WA 99164-6340 (U.S.A.)

SUMMARY

Studies on the enzymology and mechanism of biosynthesis of the essential oil terpenes are often hampered by the need to resolve and detect trace levels of these metabolites, an analytical requirement for which gas chromatography is ideally suited. Essential principles in the application of gas chromatography to terpenoid metabolism are described, with particular emphasis on experimental strategies employing flame ionization, mass spectrometric and thermal conductivity–radiochemical detection methods. The general approaches described can be readily adapted to studies on the origin of other volatile natural products.

INTRODUCTION

The monoterpenes and sesquiterpenes are two large and diverse families of natural compounds produced primarily by plants in which they often cooccur as complex mixtures known as essential oils[1]. Although the monoterpenes and sesquiterpenes were of key significance in the historical development of isoprenoid biogenetic theory[2], studies on the biochemistry of these volatile terpenoids were often hindered by the inability to resolve and detect trace levels of isomeric metabolites. Gas chromatography (GC), with ancillary detection systems, is ideally suited to this analytical problem, and the application of this technique to metabolic investigations is in large part responsible for recent progress in this area[3,4].

The application of gas chromatographic methods to terpene biosynthetic investigations was last reviewed in 1983[5] and since that time significant refinement in methods has been made, particularly in the use of fused-silica capillary columns and associated on column injection techniques. A number of assays for specific metabolites, based on these and related techniques, have been reported[6–11], yet the guiding principles and strategies underlying the method are rarely provided in the primary literature. We address these issues in the context of terpenoid metabolism by describing the applications of GC in conjunction with flame ionization, mass spectrometric and thermal conductivity–radiochemical detection methods. The general procedures are applicable to any volatile, thermally stable metabolites.

0021-9673/88/$03.50

EXPERIMENTAL

Enzyme systems

General procedures for isolating the enzymes of terpenoid metabolism have been described[12,13]. Descriptions of specific enzymes, and the substrates and standards required for their assay, are provided by citation in the appropriate sections of the text.

Special considerations

The extraction of proteins from terpene-producing tissues with aqueous buffer systems is always accompanied by the co-extraction of greater or lesser amounts of terpenoid metabolites which adhere to the various cellular constituents. These endogenous terpenoids can interfere with subsequent kinetic assays by acting as inhibitors or substrates of the relevant enzymes or, alternatively, by compromising quantitative analysis of the enzyme reaction by masking or diluting the product(s) of interest. Such endogenous materials can be removed by incorporating polymeric adsorbents, such as soluble or insoluble polyvinylpyrrolidone and polystyrene beads[14,15], into the extraction buffer[12,13]. For particularly oily or resinous tissue, an equal tissue weight of such polymers may be required to reduce the levels of endogenous terpenoids to negligible levels. Beaded polystyrene containing the adsorbed terpenoids is sufficiently large to remove from cell free homogenates by simple filtration through cheesecloth. Insoluble polyvinylpyrrolidone can be removed from soluble enzyme extracts by centrifugation, since this material pellets readily at 27 000 *g*. For membranous enzymes, soluble polyvinylpyrrolidone is substituted for the insoluble form of the polymer in the extraction buffer, and is then discarded in the supernatant when the membranes are collected by centrifugation. Detailed protocols for these procedures have been published[8,12,13].

Since the products of most of the metabolic reactions of interest are relatively hydrophobic, extraction of the aqueous incubation mixture with organic solvent is generally the first step of the assay. Pentane is most often chosen for this purpose because such an extract can be eluted through a short column of silica (3 cm × 0.5 cm I.D. of silica gel Type 60A-special, 100–200 mesh, Mallinckrodt 6447) to separate terpene olefins from oxygenated metabolites which remain adsorbed to the column. Subsequent extraction of the assay mixture with ether and passage of this extract through the same column allows the collection of the oxygenated products in a separate fraction. More polar co-solvents such as methanol or acetic acid may be required to elute highly oxygenated or acidic metabolites. Prior to the column chromatography step, the extract may be decolorized if needed with a minimum amount of activated charcoal and, following filtration, the extract can be back-extracted with water to remove possible additional contaminants. Drying of these organic extracts is most easily accomplished by surmounting the above silica columns with a small plug of anhydrous magnesium sulfate. Internal standards may be added at any stage of the work up; however, this is preferably done at the extraction step to prevent evaporative losses of product. In any case, the minimum volumes of high purity solvents should be employed during extraction and column elution to reduce product loss during the solvent concentration steps.

Sample concentration in preparation for chromatographic analysis is efficiently carried out by solvent evaporation under a stream of dry nitrogen or argon. Removal

of solvent under vacuum (Savant Speed-Vac) is also efficient and rapid, and permits concentration of a few milliliters of solvent to *ca.* 100 μl with 80–90% sample recovery. Samples at this volume are adequate for most applications; concentration to 10–20 μl, if required, is best performed in a microvial equipped with a Mininert valve (Wheaton 986294 or 986293 vial with Pierce 10127 valve).

Numerous derivatization procedures can be applied to the analysis of specific product types and several of these are described in the following text. In many instances it is also desirable to remove specific classes of potentially interfering compounds, and for the preparation of saturated terpenoid products a useful procedure for removing olefinic contaminants has been developed. For this application, the initial extract is treated with 0.1–0.5 mg of osmium tetroxide and a few drops of pyridine. Following agitation for one to several hours, osmate esters are decomposed with saturated sodium metabisulfite solution and the organic layer processed through the silica column as above. In this way olefinic bonds are converted to the corresponding diols, rendering these compounds water-soluble or of sufficient polarity to adhere tightly to silica.

Radio gas chromatography

Radio GC was performed on a Gow-Mac 550P programmable chromatograph equipped with a low volume 10-955 thermal conductivity detector, with gold-plated tungsten-rhenium filaments, which was coupled directly to the oxidation–reduction furnace of a Packard 894 flow-through gas proportional counter. The thermal conductivity detector has more than adequate sensitivity for this application; however, the primary rasons for this choice of detection are the non-destructive nature and the fact that the optimum gas flow for separation is perfectly compatible with the flow-through counter. Thus, direct coupling of the instruments without sample loss is possible, thereby eliminating the need for make-up gas or effluent splitting with the attendant variation in split-ratio with temperature.

The helium carrier-gas stream exiting the chromatograph detector is introduced directly into a 10 × 0.7 cm quartz tube containing copper oxide wire at 750°C (both ends plugged with quartz wool). The combustion products (*i.e.*, $^{14}CO_2$ and $^{3}H_2O$) are passed through a short capillary tube into a 15 × 0.7 cm quartz tube packed with chloroform–methanol washed, preconditioned (750°C) steel wool (000 or finer) also at 750°C. The emerging gases ($^{14}CO_2$, $^{3}H_2$) are conducted across short columns of carbon dioxide adsorbent [Mallcosorb (Mallinckrodt) is employed for $^{3}H_2$ detection to eliminate the possibility of carbon dioxide-based quenching] and magnesium perchlorate (to remove traces of water), and then mixed with the appropriate proportion (6–8% of column flow) of the propane quench gas before passage, via zero-dead-volume capillary fittings, into the 20-ml counting tube held at ~1700 volts.

Using conventional packed columns [3.7 m × 2 mm I.D. stainless steel containing, for example, 15% Silar 10C (olefins), Superox 20M or FFAP (most oxygenated terpenoids) on 80–100 mesh Chromosorb WHP] the optimum column flow is about 35 ml/min, prior to combustion, and not greater than 40 ml/min emerging from the counting tube (exhausted to a hood). With sample loads up to about 50 μg per component (with mass detection to about 1 μg), baseline resolution with peak widths (at half-height) of roughly 30 s can be expected. Under these conditions, quantitation of 2000 ± 200 dpm is easily attainable (counting efficiencies ranging to 80% for $^{3}H_2$ at

optimum flow and peak width), with reliable detection to about 600 dpm. The system is calibrated externally with [^{3}H]toluene or [^{14}C]toluene, or a similar primary standard. A System Instruments, America 7000A chromatogram processor is employed for simultaneous (dual channel) monitoring of the thermal conductivity detector and gas proportional counter outputs, and for data reduction.

Fused-silica, wall coated open tubular, capillary columns (530 μm) can be employed with the radio chromatographic system described. Resolution at optimum flow-rates (*ca.* 6 ml/min) is far superior to that of packed columns; however, sample capacity is reduced (5- to 10-fold) and counting efficiency is slightly diminished because of the greater sample dilution in the gas stream. For such applications, helium make up gas (to total 35 ml/min) is added to the thermal conductivity detector and a small quantity of hydrogen (2–4% of total flow) is added at the inlet of the reduction furnace to insure efficient purging of the $^3H_2$ formed.

Capillary gas chromatography

Capillary GC (hydrogen as carrier) was done on a Hewlett-Packard 5890 chromatograph equipped with flame ionization detector, split injection and on-column injection ports, and a 7673A autoinjector. Data were analyzed using a Hewlett-Packard 3392A recording integrator.

Combined capillary gas chromatography–mass spectrometry (GC–MS) (helium as carrier) was carried out on a Hewlett-Packard 5840A-5985B system with SIDS data system. The instrument was equipped with a dual injector system as above, and modified to allow column selection and source isolation with a Valco switching valve. The transfer line, to within 0.5 cm from the ion source, was a 30 cm × 0.1 mm I.D. deactivated fused-silica tubing.

For cold, on-column injection (splitless), a 10-cm section of 530 μm deactivated fused-silica tubing was coupled to a 1-m section of similar 250 μm tubing, and thence connected to the capillary column (30 m × 250 μm I.D., with bonded Superox or methyl silicone type phase). This system permits up to 1 μl injections using a conventional 10-μl syringe with 26-gauge needle and eliminates most problems associated with on-column injection using fused-silica needles. Removable couplings (Valco ZU.5TFS.4) permit easy removal of the deactivated inlet tubing for cleaning. For most applications, 0.5-μl injections are employed (50–100 pg sample load) and a 5-pg component can be accurately quantitated.

RESULTS AND DISCUSSION

Assays with radioisotopes

Radiochemically based enzyme assay employing coupled GC–gas proportional counting is the method of choice when maximum sensitivity is required, because this technique permits detection of a few hundred dpm in a single component diluted in the gas volume of the counting tube. Additional advantages of the method include continuous monitoring of the chromatographic separation, thus avoiding the collection of fractions, and the ease of operation when the counter is coupled, via the combustion–reduction train, directly from the outlet of a thermal conductivity detector, thus avoiding the complications of effluent splitting.

Since the enzymatic cyclization reactions appear to be the slow steps of many

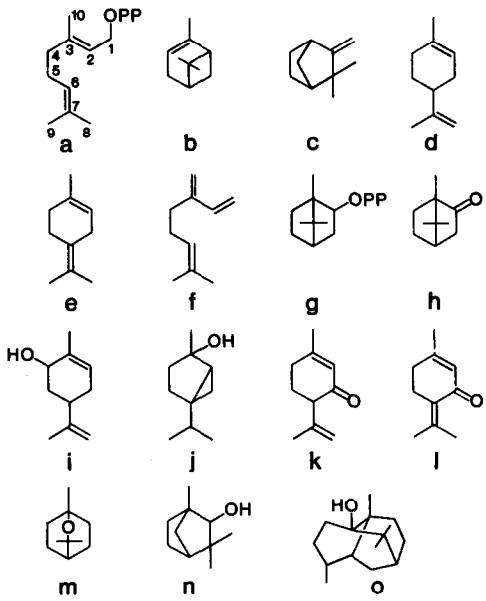

Fig. 1. Structures of (a) geranyl pyrophosphate, (b) α-pinene, (c) camphene, (d) limonene, (e) terpinolene, (f) myrcene, (g) bornyl pyrophosphate, (h) camphor, (i) carveol, (j) sabinene hydrate, (k) isopiperitenone, (l) piperitenone, (m) 1,8-cineole, (n) fenchol and (o) patchoulol.

biosynthetic sequences in the terpene series[2,3], this basic technique, involving conversion of ^{3}H- or ^{14}C-labeled immediate precursors (acyclic prenyl pyrophosphates) and radio GC analysis of the resulting products, has found wide application in the assay of these "cyclase" enzyme types. Several important considerations arise in exploiting this technique, including the fact that assays of this type, especially with relatively crude enzyme extracts, often produce complex mixtures of isomeric products, whereas the radio chromatographic separations, because of the flow requirements of the proportional counter, are largely restricted to relatively low-resolution packed columns. Most cyclic products are soluble in organic solvent and thus easily separated from the residual substrate by extraction. More importantly, radioisotopic labeling permits the addition of carrier standards (to a dilution of 4 nCi/μmol) as a precaution against loss of these volatile products in handling, and it thereby allows preliminary column or thin-layer fractionation by class (olefins, oxygenated metabolites, etc.) thereby minimizing the gas chromatographic separation requirements.

The technique has been applied to the conversion of variously labeled geranyl pyrophosphates to mixtures of cyclic monoterpene olefins (Fig. 1) by a partially purified enzyme preparation from common sage (*Salvia officinalis*). In this application the products of the cyclase assay mixture are extracted with pentane, and the pentane passed through a short column of silica to adsorb oxygenated species. The olefin fraction, following dilution with appropriate authentic standards, is concentrated and separated in a straightforward manner, yielding essentially baseline resolution of the standards and revealing which of the many potential biosynthetic products are labeled

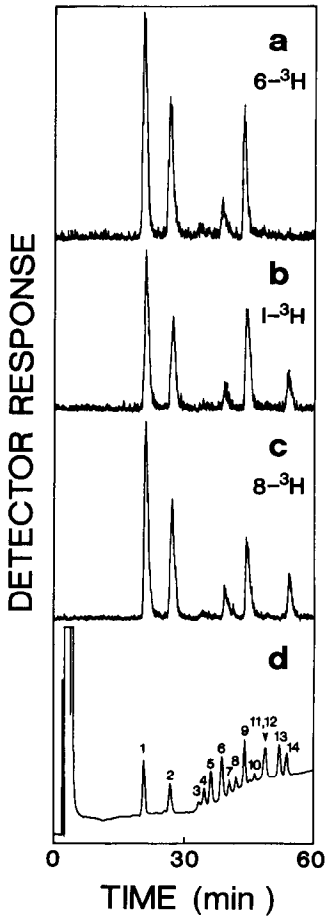

Fig. 2. Radio GC of the monoterpene olefins derived from geranyl pyrophosphate labeled with ^3H at C-6 (a), C-1 (b) and C-8 (c) by a partially purified cyclase from sage (*S. officinalis*) leaves. The smooth lower tracing (d) is the thermal conductivity detector response to a mixture of authentic α-pinene (1), camphene (2), β-pinene (3), sabinene (4), 3-carene (5), myrcene (6), α-phellandrene (7), α-terpinene (8), limonene (9), *cis*-β-ocimene (10), β-phellandrene (11), γ-terpinene (12), *p*-cymene (13), and terpinolene (14). The 3.7 m × 2 mm I.D. stainless-steel column was packed with 15% AT 1000 on 60–80 mesh Gas-Chrom Q-II and was programmed from 80°C (20 min hold) to 150°C at 4°C/min at a helium flow of 35 ml/min. The gas proportional counter was set at 1000 cpm full-scale.

or devoid of isotope (Fig. 2). Analysis of the product mixtures generated separately from 1-^3H, 6-^3H and 8-^3H labeled geranyl pyrophosphate has allowed the regiochemistry of the deprotonations leading to the various olefins, and other mechanistic features of the cyclization reaction, to be deciphered[11,16].

In some applications only a single product need be sought, thus both simplifying the chromatographic analysis and often permitting detailed probing of the enzymatic reaction. Two separable enzymes from sage (differing in molecular weight) were known to convert geranyl pyrophosphate to bornyl pyrophosphate en route to camphor (Fig. 1), a natural product of sage known to occur in the leaf oil primarily as

the (+)-isomer and thus prompting an examination of the optical purity of the two enzymatic products[17]. Incubation of the separate enzymes with [1-^{3}H]geranyl pyrophosphate gave the respective products which, following ether extracton of the reaction mixtures to remove non-polar materials, were liberated as borneol from the aqueous phases by treatment with acid phosphatase plus apyrase. Each product was then diluted with racemic carrier and the mixtures treated with excess osmium tetroxide to convert the double bonds of geraniol (liberated from the substrate by the enzymatic hydrolysis) to the corresponding diols before purification by thin-layer chromatography (TLC). Each isolated borneol was then oxidized to camphor which was converted to a mixture of diastereomeric ketals with D-(−)-2*R*,3*R*-butanediol[17,18]. Radio GC separation of the diastereoisomers gave compelling evidence that the two original enzymatically derived bornyl pyrophosphates, from which these derivatives were obtained, were in fact enantiomeric products (Fig. 3), indicating that the responsible enzymes were functionally distinct.

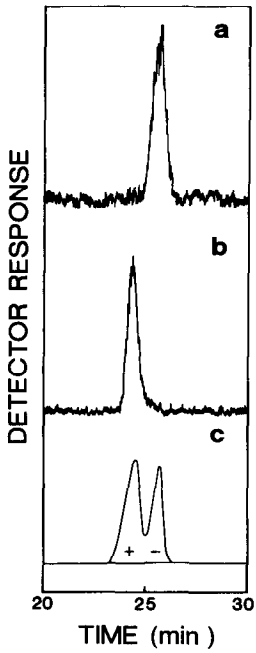

Fig. 3. Radio GC separation of the diastereomeric ketals obtained by condensation of D-(−)-(2*R*,3*R*)-butanediol with [^{3}H]camphor derived from [^{3}H]bornyl pyrophosphate generated by two enzymes from sage (*S. officinalis*). Each enzyme was incubated with [1-^{3}H]geranyl pyrophosphate, and the bornyl pyrophosphate product was enzymatically hydrolyzed to borneol, diluted with racemic standard, oxidized to camphor and converted to the ketal. Separation of products obtained from the lower-molecular-weight enzyme (a) and from the higher-molecular-weight enzyme (b) is illustrated. The smooth lower tracing (c) is the thermal conductivity detector response obtained from a mixture of the diastereomeric ketals derived from (+)- and (−)-camphor, and the order of elution was established by separate injection of each authentic diastereomer. The separation was performed on a 3.7 m × 2 mm I.D. stainless-steel column packed with 15% Silar 10C on 80–100 mesh Chromosorb WHP which was programmed from 90°C (10 min hold) to 150°C at 4°C/min at a helium flow of 35 ml/min. The radioactivity response is 3000 cpm full-scale for (b) and 1000 cpm full-scale for (a).

Although the two examples described above represent applications to the *in vitro* metabolism of terpenoids, the technique is readily adapted to *in vivo* investigations. Because of the permeability barriers encountered with intact tissue, such *in vivo* labeling studies can rarely employ advanced precursors such as geranyl pyrophosphate, and so are most often carried out with more basic precursors such as mevalonate, acetate, sucrose and even $^{14}CO_2$. This naturally leads to the labeling of a broader variety of terpenoid and non-terpenoid products, which increases the need for pre-fractionation of metabolites before radio GC analysis. Micro-scale steam distillation of the crude tissue extracts has proven to be a suitable method for isolating volatile terpenoids prior to chromatographic separation[19].

Quantitation is readily accomplished by combination of aliquot counting of the sample and integration of the radio-monitor trace, and the system is externally calibrated by injection of known quantities of [^{3}H]toluene or of a similar volatile standard.

Assays with unlabeled substrates

GC assays with unlabeled substrates are generally preferred for studies on the subsequent enzymatic conversions of the parent cyclic terpenoids since the available enzyme activities (levels) are higher for these secondary transformations[4] and the sensitivity requirements are therefore in the range of capillary chromatography using on-column injection and a flame ionization detector (unfortunately, the activity levels available are rarely in the range of spectrophotometric assays). Analysis time is comparable to that of radio chromatographic based assays (including sample handling and prefractionation); however, the major advantage of the simple mass detection technique is in avoiding the preparation of many labeled substrates which would otherwise be required (*e.g.*, in substrate specificity studies). Wide bore pre-columns coupled to standard 30-m phase-bonded capillary columns provide more than sufficient resolution, and the increased sample delivery is a requirement for on-column injection. A major consideration with this approach is the need to reduce the level of endogenous metabolites in the enzyme extract to a negligible level so as not to interfere with the analysis. Partial purification of soluble enzymes by gel permeation or ion-exchange chromatography is generally sufficient to remove small hydrophobic substances which bind to protein (and are extracted in the subsequent assay)[20], but such purification is often not possible when working with membranous enzymes where adsorption of small molecules to the hydrophobic membrane matrix is most severe[8]. This difficulty is overcome by treating the preparations with polymeric adsorbents such as polyvinyl pyrrolidone or polystyrene which are subsequently removed by filtration or centrifugation[8,13]. This procedure may need to be repeated several times to lower the background to acceptable levels, but it does permit the assay of even crude enzyme extracts.

The technique has been applied to study the hydroxylation of limonene to *cis*-carveol (en route to carvone) by microsomal membrane preparations from spearmint (*M. spicata*) leaves[21]. For this purpose, the relevant enzymes are isolated by selective extraction of the cellular contents of leaf epidermis oil glands in the presence of polystyrene beads, soluble polyvinylpyrrolidone and bovine serum albumin[13]. The former is removed by simple filtration through cheesecloth, whereas the soluble polymers are removed when the microsomal membranes are collected by high-speed

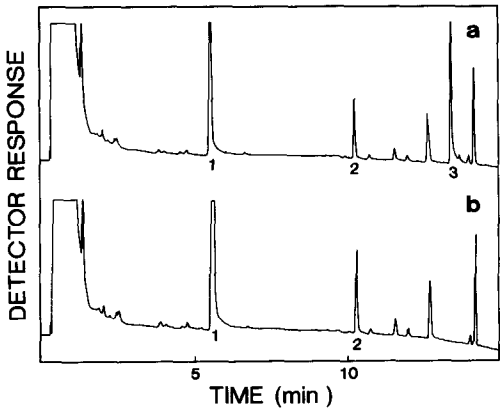

Fig. 4. Capillary gas chromatograms (flame ionization detection) of the diethyl ether-soluble products obtained from the incubation of a microsomal preparation from spearmint (*M. spicata*) leaves with 1 m*M* NADPH and 0.5 m*M* of (−)-limonene (a), and from a solvent denatured control similarly incubated (b). The peaks identified are (−)-limonene, at 200 nmol in the assay (1), camphor, 25 nmol added as internal standard (2), and the product, (−)-*cis*-carveol (3). The separation was performed on a 30 m × 0.25 mm I.D. fused-silica column coated (0.1 μm) with Superox-FA which was programmed from 70°C (5 min hold) to 220°C at 10°C/min at a hydrogen flow of 2 ml/min. Injection was on-column at 35°C.

centrifugation. Following suspension of the membrane fraction in buffer, and the addition of the substrate and appropriate cofactors, the reaction mixture is incubated in a sealed screw-capped tube (Corning 9826-13). At the appropriate time, the tube is chilled, a known amount of internal standard is added (in this case camphor), and the contents are extracted with ether, the extract concentrated and an aliquot analyzed (Fig. 4). The detection limit for the carveol product by this method is about 100 fg, which is more than adequate for kinetic evaluation of the hydroxylase system even at low substrate and cofactor levels. A very similar approach has been applied to assay numerous other enzymes of terpenoid metabolism in mint, including dehydrogenases and double bond reductases and isomerases[6–8].

In instances when quantitation is of lesser importance, and preparative scale incubations can be carried out, the method can be applied to the stereochemical analysis of biosynthetic products. Thus, to resolve the fenchol (Fig. 1) derived from geranyl pyrophosphate by a cyclase from fennel (*Foeniculum vulgare*), the pooled biosynthetic product from several large-scale incubations was (after treatment with osmium tetroxide and preliminary column fractionation on silica) converted to the corresponding isopropyl urethane[10]. This derivative was then separated on a chiral phase capillary column (XE-60-*S*-valine-*S*-α-phenylethylamide) to prove conclusively that the sole product was (−)-*endo*-fenchol (Fig. 5). Detection in this instance was by coupled GLC–MS using selected ion monitoring of the characteristic double-cleavage ion at *m/e* 80 for the isopropyl urethanes. A very similar strategy was employed to distinguish the sesquiterpene alcohol patchoulol (molecular weight, MW 222) from sesquiterpene olefins (MW 204) produced from farnesyl pyrophosphate by a crude cell free extract from *Pogostemon cablin*[9]. In this instance, the analysis by flame ionization detection was complicated by the co-extraction of interfering substances from the assay mixture (Fig. 6, inset), yet by selective-ion monitoring of the

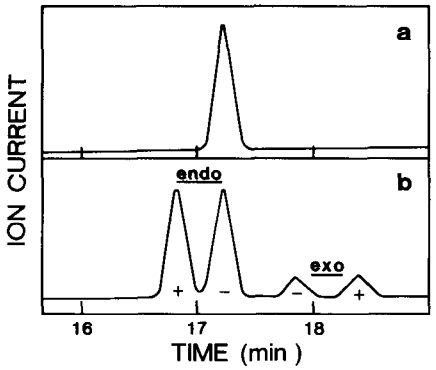

Fig. 5. Chiral phase capillary GC separation of the isopropyl urethane of fenchol derived from geranyl pyrophosphate by a cyclase from fennel (*F. vulgare*) (a). The separation of authentic standards of the isopropyl urethanes of both (+)- and (−)-isomers of both *endo*- and *exo*-fenchol are also illustrated (b). Retention times were established by separate injection of the derivative of each authentic stereoisomer. The column employed was 50 m × 0.17 mm I.D. fused silica coated with XE-60-*S*-valine-*S*-α-phenylethylamide (Chrompack) and was programmed from ambient temperature (25°C) to 160°C at 30°C/min at a helium flow of 0.5 ml/min. Injection was on-column at 25°C, and detection was by selected ion monitoring of the double-cleavage ion at *m/e* 80 for the isopropyl urethanes. Quantitation was by summation of ion current (5–10 scans) over the appropriate retention interval.

capillary-column effluent at *m/e* 204 and *m/e* 222 very simple chromatograms were produced which allowed straightforward detection of the products of interest (Fig. 6).

The higher boiling point of the sesquiterpenes and of derivatized monoterpenes allows considerable manipulation of extracts containing these compounds, yet even in

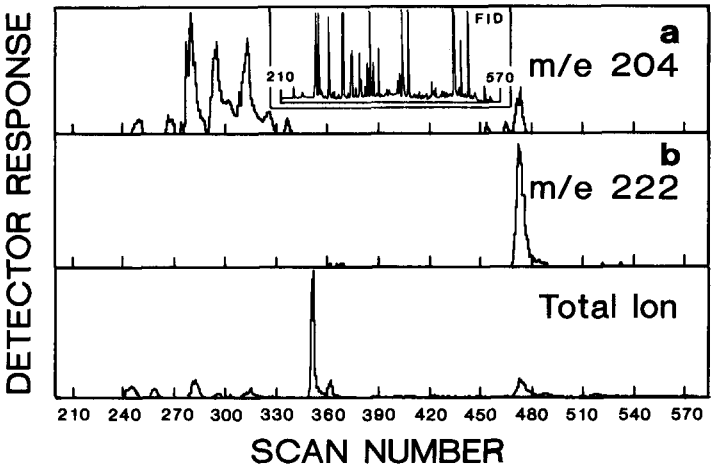

Fig. 6. Capillary GC separation of the diethyl ether-soluble products obtained from the incubation of a crude enzyme extract from patchouli (*P. cablin*) leaves with farnesyl pyrophosphate. The inset illustrates the flame ionization detector response in the relevant section of the chromatogram, the bottom trace is the total ion current, and panels (a) and (b) illustrate detection by selected ion monitoring of *m/e* 204 and 222, respectively, over the same interval. The column was the same as described under Fig. 4, and was programmed from 45°C (5 min hold) to 220°C at 10°C/min at a helium flow of 2 ml/min. Injection was on-column at 45°C.

these cases solvent volumes should be kept to a minimum to avoid evaporative losses of product during the required concentration steps. Quantitative analysis by this general method dictates the accurate addition of a suitable (non-interfering) internal standard as early as possible in the assay procedure to compensate for handling losses. For most applications the internal reference standard should be of comparable boiling point and polarity to the product(s) of interest, and bear a response factor near unity. In addition to the normal boiled enzyme or solvent denatured controls run with assays of this type, additional controls should include incubations of enzyme without substrate and of substrate without enzyme to confirm the absence of interfering materials in the chromatographic analysis.

Assays with stable isotopes

MS detection and product analysis is invaluable for mechanistic studies using stable isotopes. General considerations for this type of experiment are essentially the same as for assays with unlabeled substrates, and particular care must be taken to avoid interferences which will result in apparent isotopic dilution of the ions to be monitored. Preliminary experiments with unlabeled compounds will usually reveal such potential problems which can often be overcome by simple prefractionation or derivatization of the product of interest; however, any manipulation of this type will lead to at least some product loss.

In examining the source of the hydroxyl oxygen atom of *cis*-sabinene hydrate (Fig. 1) produced by a soluble cyclase form majoram (*Majorana hortensis*)[22] crude enzyme preparations adjusted to suitable assay conditions and containing the substrate geranyl pyrophosphate were frozen and lyophilized, and the dry powder then dissolved in $H_2{}^{18}O$ or $H_2{}^{16}O$ prior to the assay. The reaction product formed was extracted into ether along with a variety of coproducts and then separated directly by on column injection capillary GLC–MS while monitoring specific ions in the range of the possible parents (*i.e.*, *m/e* 154–157). The observed isotopic enrichment of the sabinene hydrate, evidenced by the complete shift of the parent ion from *m/e* 154 (in $H_2{}^{16}O$) to *m/e* 156 (in $H_2{}^{18}O$) clearly indicated that water was the exclusive source of the oxygen atom incorporated in the cyclization[22]. Related experiments have confirmed that the hydroxyl oxygen of the aforementioned (−)-*endo*-fenchol cyclization product is similarly derived by water trapping of a cationic reaction intermediate[23,24]. However, studies with [1-^{18}O]geranyl pyrophosphate as a substrate demonstrated that the exclusive source of the ester oxygen of bornyl pyrophosphate formed in this enzymatic cyclization was the corresponding ester oxygen of the acyclic precursor, thus providing strong evidence for ion-pairing in this unusual reaction[25,26]. In this instance, the product was examined by GLC–MS analysis of the corresponding bornyl benzoate, since preparation of the higher molecular weight derivative minimized evaporative losses and gave a superior parent ion.

Attempts to demonstrate directly the intramolecular hydrogen transfer thought to be involved in the isomerization of isopiperitenone to piperitenone (Fig. 1) by an enzyme from peppermint (*Mentha piperita*) were thwarted by very low product yields as the result of a pronounced isotope effect when the specifically deuterated substrate was employed[6]. However, indirect evidence for such an internal transfer was obtained when the enzymatic isomerization was examined in ^2H_2O, since analysis of the resulting piperitenone by GLC–MS (monitoring a series of $[P+n]^+$ ions) revealed that

the product contained no detectable deuterium[6]. A recent examination of the cyclization of geranyl pyrophosphate to 1,8-cineole (Fig. 1) by an enzyme from *S. officinalis* has confirmed that one hydrogen atom from water is acquired in this product during the course of the reaction[27]. In this case, the lyophilized enzyme preparation was incubated with substrate in either H_2O or ^2H_2O, and the resulting products extracted into pentane and adsorbed to a small silica column. Following rinsing of the column with pentane to remove interfering olefins, each cyclic ether was eluted in a minimum volume of 10% ether in pentane, then separated from oxygenated coproducts and analyzed by combined GLC–MS. Monitoring the column at the elution volume of 1,8-cineole revealed a shift of one a.m.u. in the parent ion from *m/e* 154 (in H_2O) to *m/e* 155 (in ^2H_2O).

As with assays employing unlabeled substrates, experiments with stable isotopes can be calibrated by the use of internal standards. However, since the response to normal and isotopically enriched materials can differ markedly, by either single ion or total ion-current monitoring, it is often simpler to avoid this complication by external calibration with authentic labeled material.

CONCLUSION

The application of GC, along with ancillary prefractionation and detection methods, to the study of terpenoid metabolism has been illustrated by way of several diverse examples. Without these chromatographic assay methods, studies on this class of natural products would be severely limited since few other techniques offer the combination of sensitivity and high resolution required. The general approach appears to be underutilized in other areas of study where the experimental constraints are similar, as for example in the biosynthesis of flavor substances and pheromones. It is hoped that this article will stimulate applications of this powerful method to research on the origin of these and other naturally occurring metabolites.

ACKNOWLEDGEMENTS

This investigation was supported by grants from the U.S. National Institutes of Health, National Science Foundation, and Department of Energy, and by Project 0268 from the Research Center, College of Agriculture and Home Economics, Washington State University, Pullman, WA 99164, U.S.A. We thank Nancy Madsen for typing the manuscript.

REFERENCES

1 S. R. Srinivas, *Atlas of Essential Oils*, ANADAMS Consulting Service, New York, 1986.
2 L. Ruzicka, *Pure Appl. Chem.*, 6 (1963) 493.
3 R. Croteau, in L. E. Craker and J. E. Simon (Editors), *Herbs, Spices and Medicinal Plants, Recent Advances in Botany, Horticulture and Pharmacology*, Vol. 1, Oryx Press, Phoenix, AZ, 1986, p. 81.
4 R. Croteau, *Chem. Rev.*, 87 (1987) 929.
5 R. Croteau and R. C. Ronald, in E. Heftmann (Editor), *Chromatography: Fundamentals and Applications of Chromatographic and Electrophoretic Methods, Part B: Applications*, Elsevier, Amsterdam, 1983, p. 147.
6 R. B. Kjonaas, K. V. Venkatachalam and R. Croteau, *Arch. Biochem. Biophys.*, 238 (1985) 49.
7 R. Croteau and K. V. Venkatachalam, *Arch. Biochem. Biophys.*, 249 (1986) 306.

8 F. Karp, J. L. Harris and R. Croteau, *Arch. Biochem. Biophys.*, 256 (1987) 179.
9 R. Croteau, S. L. Munck, C. C. Akoh, H. J. Fisk and D. M. Satterwhite, *Arch. Biochem. Biophys.*, 256 (1987) 56.
10 D. M. Satterwhite, C. J. Wheeler and R. Croteau, *J. Biol. Chem.*, 260 (1985) 13901.
11 R. Croteau, C. J. Wheeler, D. E. Cane, R. Ebert and H.-J. Ha, *Biochemistry*, 26 (1987) 5383.
12 R. Croteau and D. E. Cane, *Methods Enzymol.*, 110 (1985) 383.
13 J. Gershenzon, M. A. Duffy, F. Karp and R. Croteau, *Anal. Biochem.*, 163 (1987) 159.
14 W. D. Loomis, *Methods Enzymol.*, 31 (1974) 528.
15 W. D. Loomis, J. D. Lile, R. P. Sandstrom and A. J. Burbott, *Phytochemistry*, 18 (1979) 1049.
16 R. M. Coates, J. F. Denissen, R. B. Croteau and C. J. Wheeler, *J. Am. Chem. Soc.*, 109 (1987) 4399.
17 R. Croteau, D. M. Satterwhite, D. E. Cane and C. C. Chang, *J. Biol. Chem.*, 261 (1986) 13438.
18 D. M. Satterwhite and R. Croteau, *J. Chromatogr.*, 407 (1987) 243.
19 R. Croteau and F. Karp, *Arch. Biochem. Biophys.*, 176 (1976) 734.
20 M. A. Johnson and R. Croteau, *Arch. Biochem. Biophys.*, 235 (1984) 254.
21 D. M. Satterwhite, K. Wagschal and R. Croteau, in preparation.
22 T. W. Hallahan and R. Croteau, *Arch. Biochem. Biophys.*, 264 (1988) 618.
23 R. Croteau, J. Shaskus, D. E. Cane, A. Saito and C. Chang, *J. Am. Chem. Soc.*, 106 (1984) 1142.
24 D. M. Satterwhite and R. Croteau, *Arch. Biochem. Biophys.*, submitted for publication.
25 D. E. Cane, A. Saito, R. Croteau, J. Shaskus and M. Felton, *J. Am. Chem. Soc.*, 104 (1982) 5831.
26 R. Croteau, J. J. Shaskus, B. Renstrøm, N. M. Felton, D. E. Cane, A. Saito and C. Chang, *Biochemistry*, 24 (1985) 7077.
27 R. Croteau and D. M. Satterwhite, in preparation.