

A precision apparatus, with solid phase micro-extraction monitoring capability, for incorporation studies of gaseous precursors into insect-derived metabolites

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Dedicated to Professor R. Rickards on the occasion of his 70th birthday

Abstract

An apparatus is described that facilitates the determination of incorporation levels of isotope-labelled, gaseous precursors into volatile insect-derived metabolites. Atmospheres of varying gas compositions can be generated by evacuation of a working chamber followed by admission of the required levels of component gases, using a precision, digitised pressure read-out system. Insects such as fruit-flies are located initially in a small introduction chamber, from which migration can occur downwards into the working chamber. The level of incorporation of labelled precursors is continuously assayed by the Solid Phase Micro Extraction (SPME) technique and GC-MS analyses. Experiments with both *Bactrocera* species (fruit-flies) and a parasitoid wasp, *Megarhyssa nortoni nortoni* (Cresson) and ¹⁸O-labelled dioxygen illustrate the utility of this system. The isotope effects of ¹⁸O on the carbon-13 NMR spectra of 1,7-dioxaspiro[5,5]undecane are also described.

Keywords: Insect chamber, incorporation studies, metabolites, spiroacetals, ¹⁸O-labelling, isotope effects, carbon-13 NMR spectra, carbon monoxide.

Introduction

In recent publications,¹⁻⁴ we have disclosed details of the likely pathways utilised by certain *Bactrocera* fruit-fly species⁵ for the biosynthesis of spiroacetals,⁶ such as **1**, **2** and **3** and related oxygenated compounds such as **4** and **5**. A general paradigm² was developed which involved

mono-oxygenase mediated hydroxylation of an intermediate alkyltetrahydropyranol, eg. **6** (or biological equivalent) in the penultimate step.

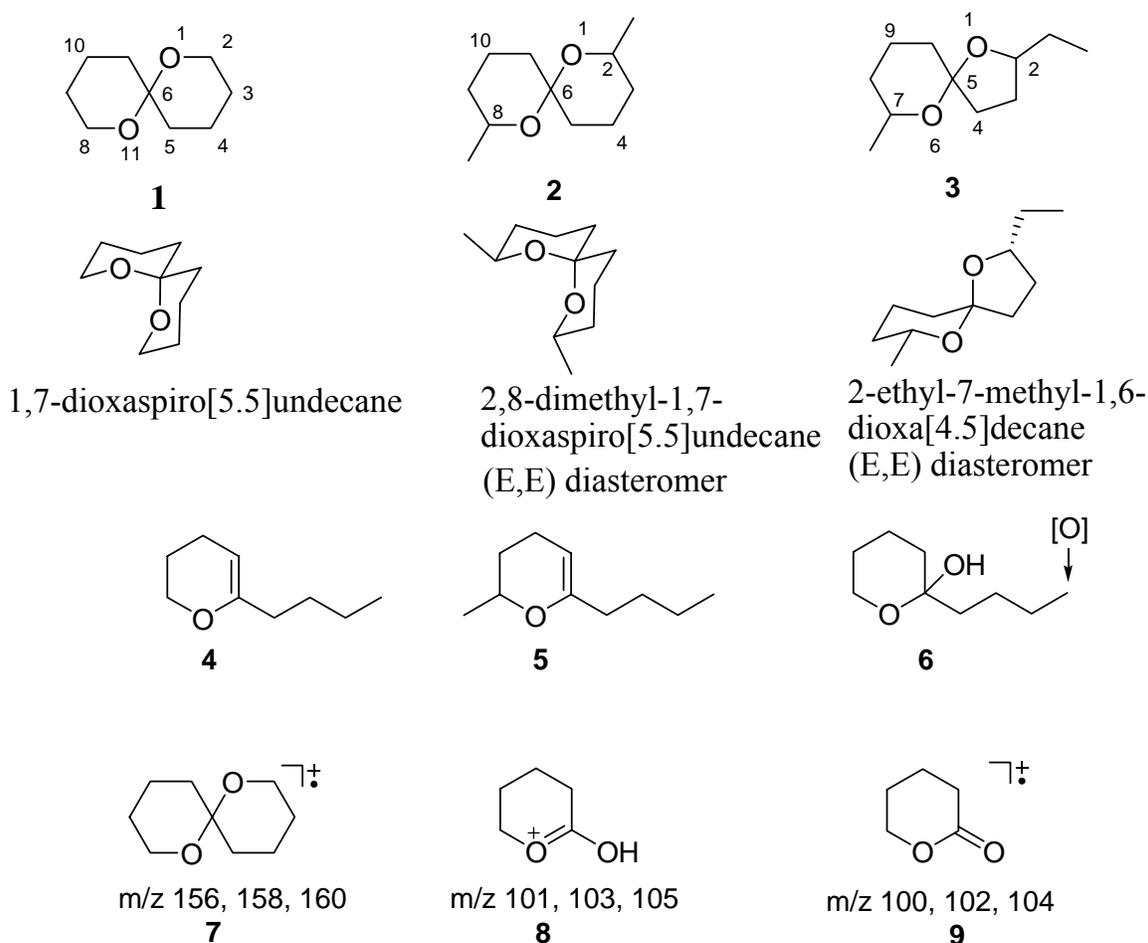


Figure 1.

In relation to this hypothesis, it was essential to demonstrate that oxygen atoms from [$^{18}\text{O}_2$]-dioxygen were incorporated into the spiroacetals, given the accepted mode of hydroxylation of saturated C-H bonds by mono-oxygenases of the cytochrome P450 family.⁷ Indeed, patterns of [^{18}O]-oxygen incorporation from both dioxygen and water into the spiroacetals from *B. oleae*, *B. cacuminata*, *B. cucumis* and *B. tryoni* strongly support this general proposal.²⁻⁴ This verification required an apparatus that would allow insect species to be exposed to an atmosphere enriched in [$^{18}\text{O}_2$]-dioxygen, and furthermore be amenable to continuous monitoring, preferably by the Solid Phase Micro-Extraction (SPME) technique. In this way, observations could be made over time without disruption to the experiment. The apparatus developed by us was characterised by ease of assembly and disassembly, precision control of initial gas compositions, and overall ease of operation. Continuous monitoring of metabolite

production and isotope incorporation was straightforward. In this report, we wish to describe the design and operation of the apparatus, and illustrate its utility.

Discussion

The essential components of the apparatus are shown in Figure 2, and consist of a detachable insect chamber, with a side-arm for connection to a vacuum system and gas inlets. Fine needle-valve systems permit controlled introduction of gases such as O₂, N₂, air or CO, and a digitised pressure monitoring system was employed. This latter unit utilises an MKS Baratron pressure gauge, a capacitance based technology to provide highly accurate and reliable low differential pressure measurement. (<http://www.mksinst.com>). The actual apparatus employed is shown in Figure 3.

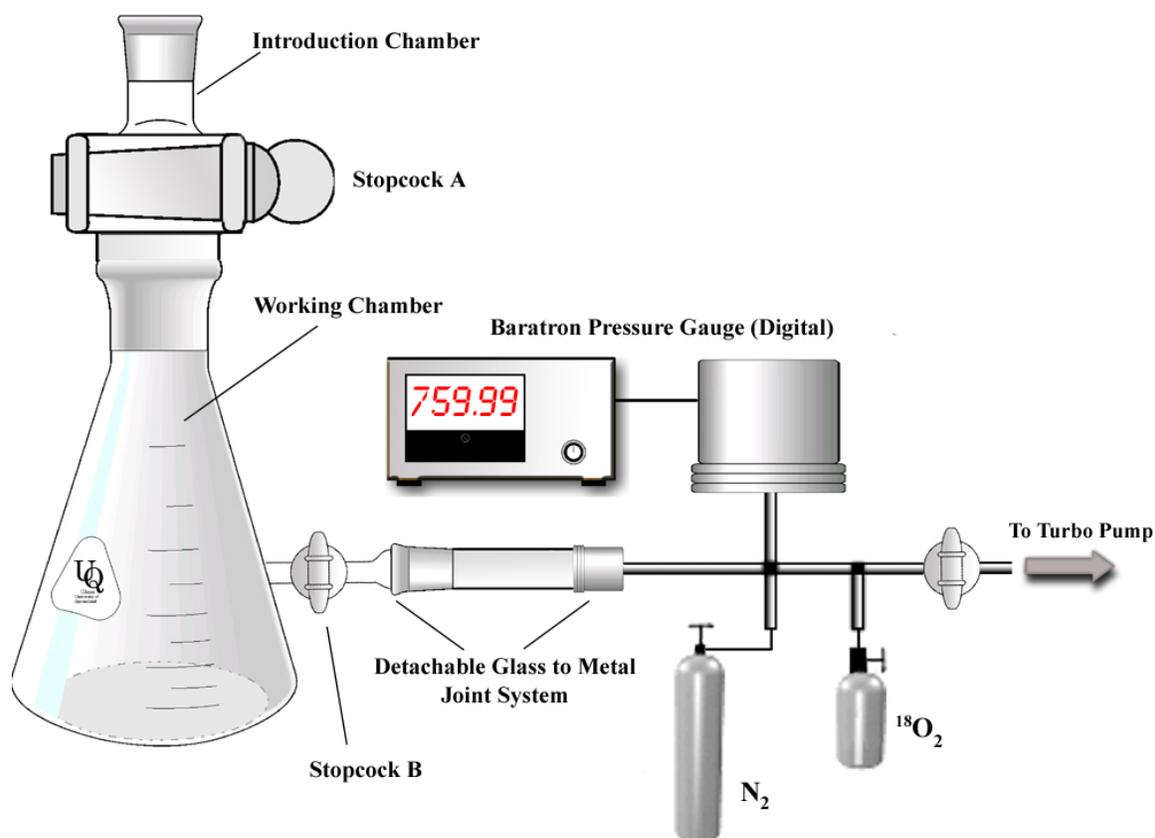


Figure 2. (NOT TO SCALE)

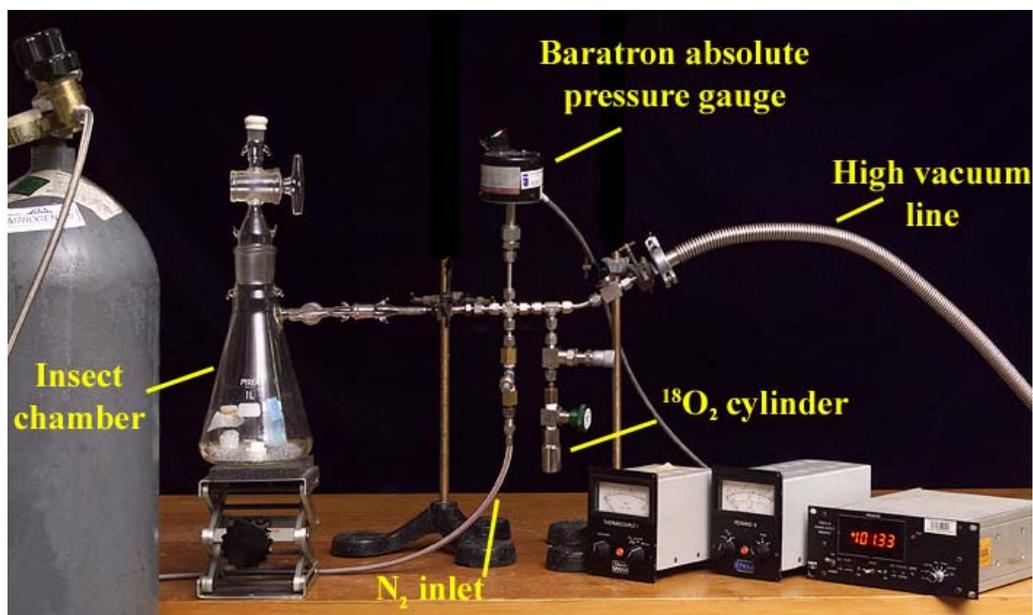


Figure 3.

For monitoring purposes a Supelco SPME unit⁹ (Figure 4) was inserted through the self-sealing waxed, white suba seal, with the needle then positioned for sampling through the open stopcock A. This is made clear in Figure 5.

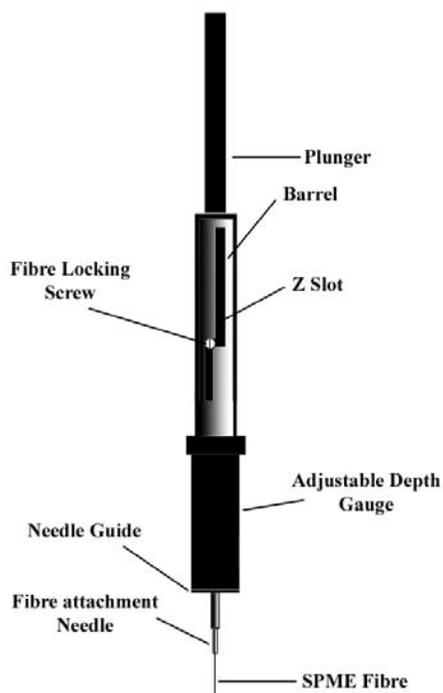


Figure 4.

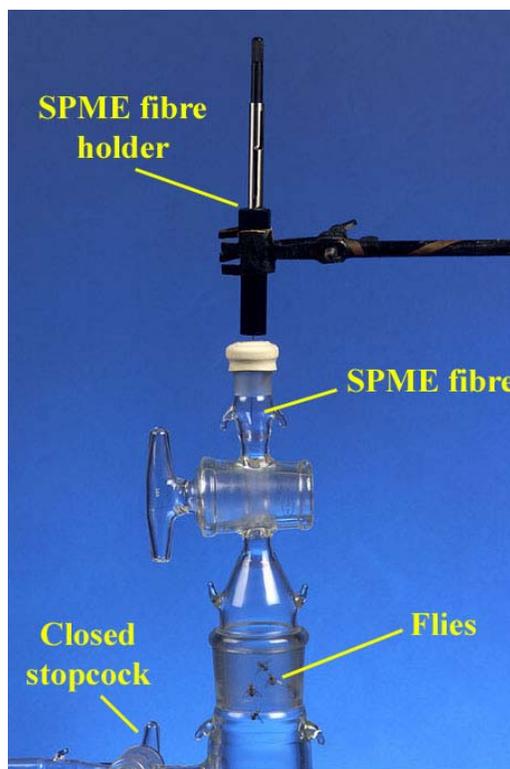


Figure 5.

Experiments have been conducted with several *Bactrocera* species⁵ (fruit-flies) and the parasitoid wasp, *Megarhyssa nortoni nortoni*.⁸ These insects differ considerably in size necessitating different procedures for introduction of the insects, and preparation of the chamber.



Figure 6. *Bactrocera tryoni* (left) and *Megarhyssa nortoni nortoni* (right)

With respect to the experiments with fruit-flies, the following procedure was adopted. With stopcock A closed, the introductory chamber (ca. 4 mL), was isolated from the working chamber (ca. 350 mL, see Figure 2). This latter chamber - a modified conical flask, with a side-arm, stopcock and male joint - was evacuated to 0.5 mm with a turbo pump (Figure 2) and then 0.2 atm of [¹⁸O₂]-dioxygen⁹ from a mini-cylinder (at ca. 44 atm, 12 mL volume), was admitted, followed by 0.8 atm of dinitrogen. At this stage, stopcock B (side arm) was closed and the glass working chamber, now a closed system, could be detached. The flies (5-10) were sedated by cooling and transferred to the introduction chamber, which was then sealed from the general atmosphere and opened (stopcock A) to the working chamber. Opening of stopcock A (which had a 10 mm orifice) allowed the flies to migrate downwards into the main chamber (now with an [¹⁸O₂]-enriched atmosphere). A dry Wettex strip was placed in the chamber before evacuation and gas admissions, and then dosed with a 10% sugar solution from a long-needle syringe through the septum. In early experiments, cubes of sugar and water (as ice for the evacuation procedure) were provided. The SPME fibre⁹ and holder (see Figures 4 and 5) could be inserted through the white suba-seal and the fibre guided towards the stopcock orifice, and thereby accessible to gases and insect-generated volatiles present in the chamber. Levels of [¹⁸O] incorporation into metabolites were monitored by direct GC-MS analysis of the SPME fibre. This method permitted monitoring on a regular basis and revealed a progressive dilution of the initial endogenous metabolites with [¹⁸O] isotopomers generated in the [¹⁸O₂]-dioxygen enriched atmosphere. Complementary labelling experiments were also easily conducted in this chamber under a normal atmosphere in the presence of [¹⁸O]-H₂O (~ 20% [¹⁸O]-enrichment).⁹ The results and interpretations of experiments conducted in this way have been described in several reports,¹⁻⁴ and are not repeated here.

In the case of spiroacetal **1**, the major pheromonal component from both *B. oleae* and *B. cacuminata*,⁵ it was established that both oxygen atoms originated from dioxygen.³ Although we had demonstrated previously^{1,2} that a dihydropyran (or its hemiketal) already containing one oxygen atom, was processed, by further oxidation, to form **1**, the question nevertheless, remained as to whether **both** oxygen atoms could be introduced into **1** from the **same** molecule of dioxygen. This proposal was easily investigated with our apparatus. Precise quantities of [¹⁸O₂]-dioxygen and regular air (with [¹⁶O₂]-dioxygen) could be admitted, and the distribution of oxygen atoms in **1** determined by GCMS analyses. Use of an atmosphere consisting of 10% [¹⁸O₂]-dioxygen and 90% instrument grade air (~18.8% [¹⁶O₂]-dioxygen final conc.) negated this proposal as now explained. This atmosphere would contain ¹⁶O:¹⁸O in the ratio of 65.3:34.7. Ten male *B. cacuminata* were placed in the introduction chamber after the controlled gas admissions, and then released into the working chamber by opening stopcock A. The system was examined by SPME-GCMS over three days.

In the mass spectra of **1**, significant ions **7**, **8**, and **9** (Figure 1) contain two oxygen atoms and in the **absence** of endogenous **1**, the distribution of [¹⁶O] and [¹⁸O] atoms calculated for independent sequential incorporation of the two oxygen atoms would be 3.6:3.8:1 for ¹⁶O-¹⁶O, ¹⁶O-¹⁸O and ¹⁸O-¹⁸O. Note that the ¹⁶O-¹⁸O arrangement in **1** has a statistical advantage of 2. Because of the persistence of some endogenous **1**, the data for ¹⁶O-¹⁸O and ¹⁸O-¹⁸O containing ions is more meaningful. For **8** this ratio is 4:1 and for **9** is 5.5:1, in satisfactory agreement with the calculated ratio of 3.8:1. Another demonstration of this is the significant ion at m/z 158, corresponding to M⁺ with one [¹⁶O] and one [¹⁸O] atom (C₉H₁₆¹⁶O¹⁸O).¹⁰ For coordinated introduction of two oxygen atoms from a single dioxygen molecule, M⁺ at m/z 156 and 160 should be present, but the ion at m/z 158 should be insignificant (this assumes there is no facile mechanism for oxygen-isotope scrambling between different dioxygen molecules, as seems reasonable).¹¹

The above analysis is supported by the 187.5 MHz ¹³C NMR spectrum of the gland secretion extracted directly into CDCl₃ (Figure 7). In addition to the resonance of the spirocarbon (C6) at 95.006ppm, (corresponding to the presence of two [¹⁶O] atoms in **1**) a higher field resonance at 94.980ppm ($\Delta = -0.026$ ppm) is present, with this isotope effect being appropriate^{3(a)} for C6 flanked by one [¹⁶O] and one [¹⁸O] atom. Under the conditions used for generating **1**, this arrangement should be ca. 3.8 times that incorporating two [¹⁸O] atoms, but about equally abundant (3.6:3.8) with the species incorporating two [¹⁶O] atoms, if no endogenous material remained. The doubly [¹⁸O] labelled species was not clearly identifiable in this ¹³C NMR spectrum (Figure 7), but it was when the atmosphere contained exclusively [¹⁸O₂]-dioxygen, as is evident in Figure 8. That the duplication of most signals in Figure 8 is due to (upfield) isotope shifts when [¹⁸O] replaces [¹⁶O] is confirmed specifically for the resonance of C2/C8 (at ca. 60 ppm) by the 2D-spectrum shown in Figure 9.

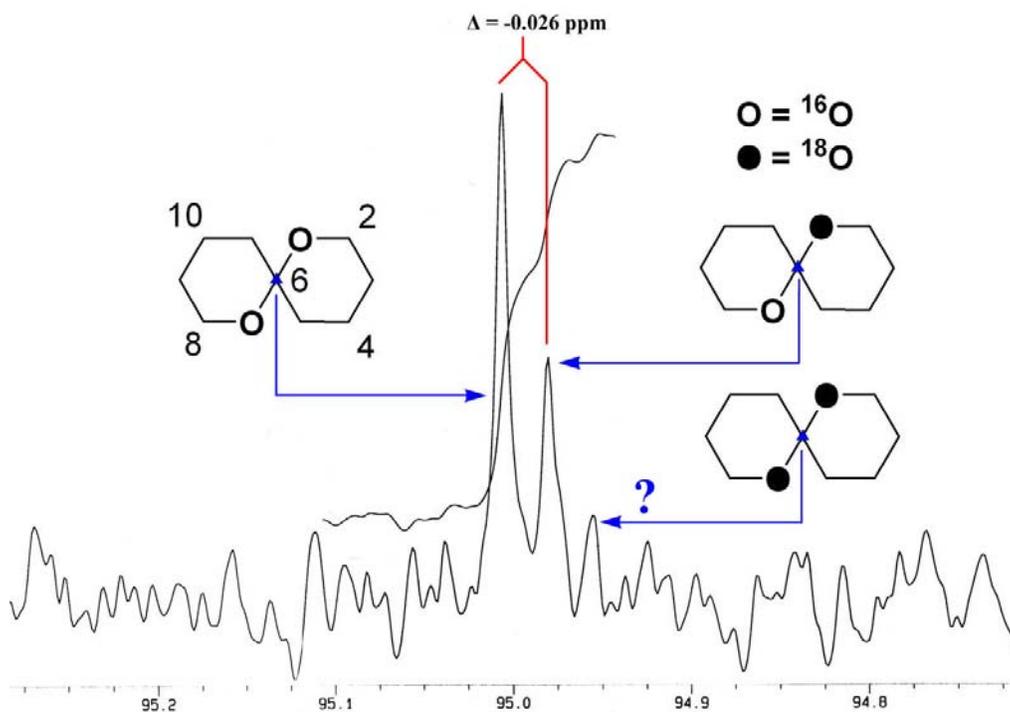


Figure 7.

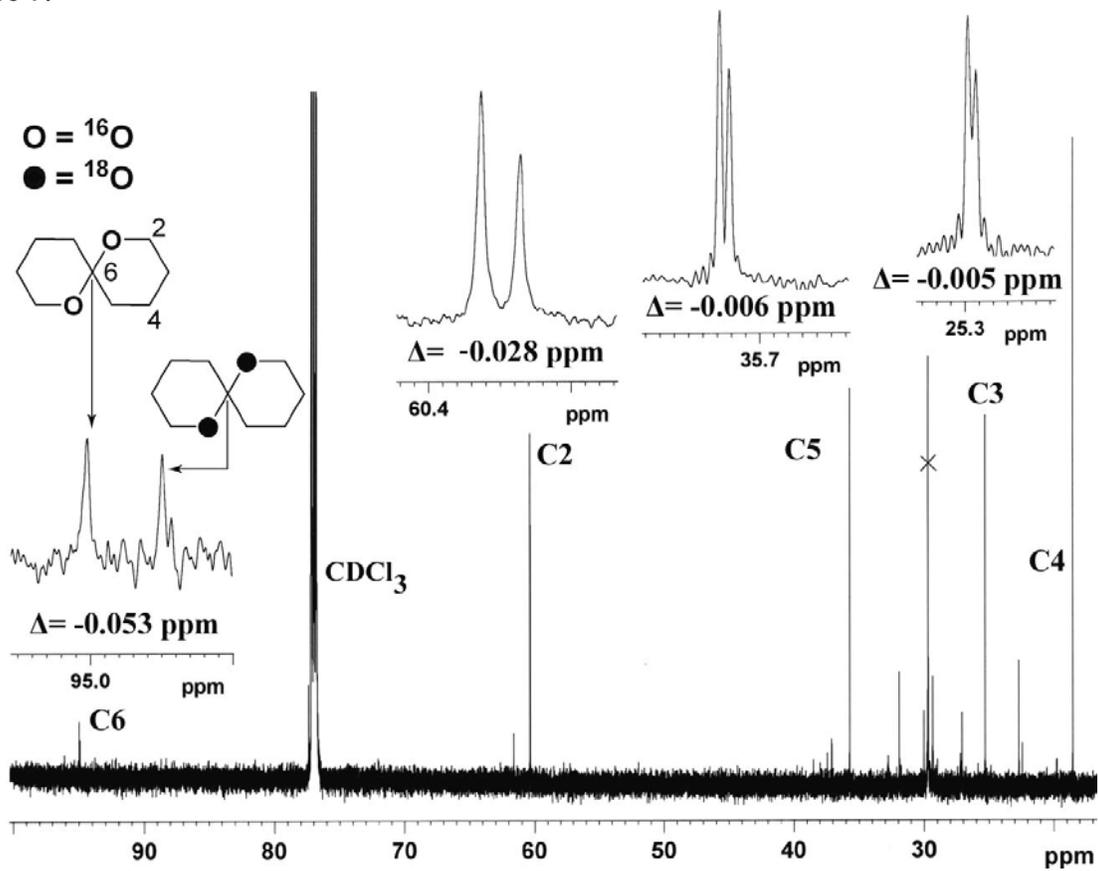


Figure 8.

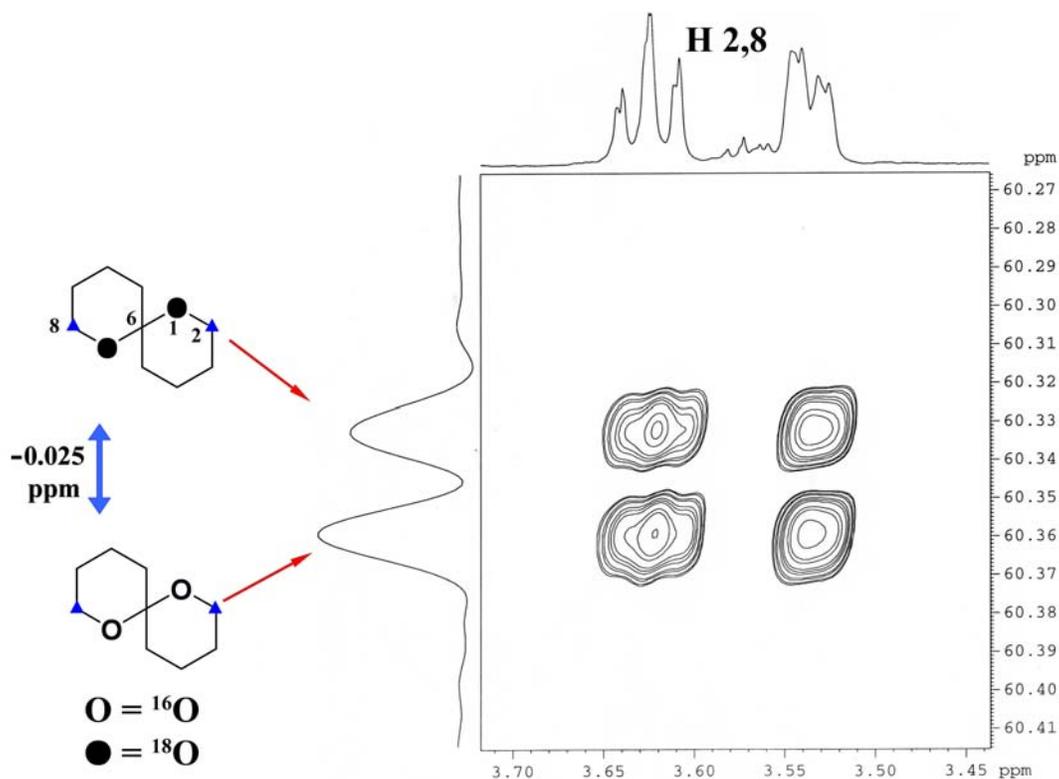


Figure 9.

This apparatus for evaluating incorporation from gaseous precursors was also employed with the wasp species, *Megarhyssa nortoni nortoni* (Cresson), a parasitoid introduced into Australia as a natural enemy of the wood wasp, *Sirex nortilis*. These parasitoid wasps are much larger than fruit-flies (7 mm) and body length can range from 15 mm to 45 mm, with ovipositor up to an additional 90 mm.⁸ These features, together with the less rigid body and appendages, render impossible the migration of a wasp from the introductory chamber through the 10 mm bore stopcock A, into the working chamber. Consequently, the operating procedure was adapted in that a single female wasp was placed directly into the larger working chamber, and a gas exchange procedure was undertaken that would not ‘pressure-stress’ the insect. The flask was evacuated to 373 mmHg (0.5 atm) and then filled with N₂ to 746 mm (now ca. 90% N₂-10% O₂). Again, evacuation to 373 mm was followed by admission of N₂ to 600 mm and then [¹⁸O₂] to 746 mm. The final situation corresponds to 24% dioxygen (of which 80% was [¹⁸O₂]-dioxygen) and 76% N₂. The incorporation of [¹⁸O] atoms into volatile metabolites could then be monitored by SPME in the normal way, and has confirmed important monooxygenase activity in this wasp species. The results of these newer investigations will be reported in due course.

Controlled admission of CO at various pressures was also performed for experiments designed to induce suppression of cytochrome P450 activity,⁷ postulated to be responsible for the oxygenation events on the pathways to the various spiroacetals. The above cases demonstrate the

value of precision admission of various gases for the conduct of experiments of this type and the continuous sampling capability of the SPME technique.

Acknowledgments

The authors are grateful to the Australian Research Council (ARC) for support of this work, to Stiftelsen Bengt Lundqvist Minne for financial support to Dr. Fredrik Rahm, to Dr. Charlma Phillips (ForestrySA, Mount Gambier SA) for the specimens of *Megarhyssa nortoni nortoni*, and Dr. Annice Lloyd (DPI, Indooroopilly, Qld) for supply of *Bactrocera* fruit-fly species. Fabrication of the glassware was performed by Mr. Bob Pryke and Mr. Ian Crighton of the University Glassblowing Services.

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9. Dioxygen [¹⁸O₂] of > 99% atom % [¹⁸O] was obtained from Isotech., Miamisburg, Ohio. [¹⁸O] enriched H₂O (20 atom % [¹⁸O]) was manufactured by Yeda R & D Co. Ltd, Israel. A Carboxen PDMS fibre (Supelco) was employed.
10. Mass spectra of spiroacetal **1** generated in both normal air and an [¹⁸O₂]-dioxygen atmosphere are reproduced in ref. 3(b).
11. For some discussion of oxygen-atom exchange between dioxygen and water see footnote 13 in Vanderwel, D.; Oehlschlager, A.C. *J. Am. Chem. Soc.* **1992**, 114, 5081.