Feature

Deceit and Deception in Volatile Analysis*

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*Dedicated to the memory of Dr. Cecil B. Johnson (1937-1998).
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Introduction

Plant volatiles are low molecular weight (M_r < 250) organic molecules of long-standing scientific interest as natural pollutants, *e.g.* isoprene, 2-methylbut-3-en-2-ol, and pinenes,[1] as food flavours,[2] and as signalling molecules passing between plants and insects.[3] Modern genetic approaches have rejuvenated interest in this field of chemistry with an ongoing process of characterising the enzymes responsible for volatile production[4] and of the volatile signals that pass between plants and plants and herbivores - The Talking Trees Hypothesis.[5] There is speculation that volatiles might even provide cues by which we assess the nutritional value of our foods.[6]

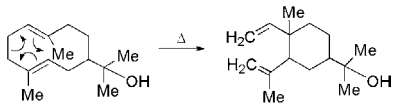
Technology for the measurement of organic volatiles, *e.g.* gas chromatography and gas chromatography mass spectrometry (GC/GCMS), is well established and continues to advance. Newer techniques, such as solid phase micro-extraction (SPME) and the ability to back flush GC columns and automatically change the GC liner to enable the analysis of very complex samples are in place. However, as new workers enter the field, old skills need to be remembered, or sometimes restored. The technology provides us with new tricks but the underlying chemistry doesn't change.

Our interest lies at the interface of chemistry with biology, with the analysis of aroma and flavour biogenesis,[7,8] and the genetic mapping and use of functional genomics to identify genes responsible for flavour production.[9] In such interdisciplinary research, inter-generational memory can be stretched and old skills may need to be rediscovered. We would, therefore, like to dedicate this reflection on GC methodology to Dr Cecil B. Johnson (deceased), GC expert, lipid chemist and long-standing member of the NZIC.

The standard technology (GC/GCMS) for measurement of organic volatiles is well established and recent advances have focused on the MS detector (Time of Flight) and automated sampling and sample preparation techniques. The GC box with its split/splitless injector is often taken for granted, but the heated injector, itself, can be the source of some interesting chemistry. The reactivity in the GC injector of thiopropanal S-oxide (EtCH=SO), and several thiosulfinates [RS(O)SR] responsible for the onion and garlic flavour of *Allium* is well known[10] to produce artefact di- and trisulfides, and thiosulfonates, and serves to illustrate that without an understanding of the chemistry of the system under consideration it is possible for the analyst to be deceived.

Deceptive Analytes and Inappropriate Methods

The possibility for self-deception was demonstrated in the analysis of products of a terpene synthase enzyme expressed in *Escherichia coli*. Solvent extraction and GCMS analysis indicated a complex profile of sesquiterpene products (Fig. 1A) when using standard splitless injection into a 240 ºC GC injection port. One of the compounds, elemol, is a known thermal rearrangement product of the sesquiterpene hedycaryol (Scheme 1),[11] and so its presence in the chromatogram was viewed with some concern. When the sample was injected directly into the start of the capillary GC column before any heat was applied (cold on-column injection), the GC showed only germacrene D (Fig. 1B). An awareness of the value of cold on-column injections was helped by our remembrance of Cecil Johnson who specialised in this technique for lipid analysis. The germacrenes are known to rearrange thermally,[12] but to our knowledge there is no previous report of germacrene D rearranging to produce the isomers of elemene, nor of their subsequent reaction with water, present in the sample, to produce elemol. This may relate to the unknown and generally overlooked state of the GC injection port. The reported thermal rearrangement products of germacrene D are the stereoisomeric β-ylangene and β-copaene, isogermacrene D, and stereoisomeric ε-muurolene and ε-amorphene (Chart 1).[13]



1, hedycaryol **2**, elemol

Scheme 1

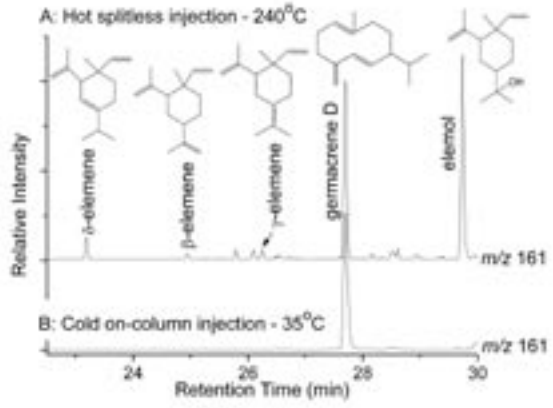
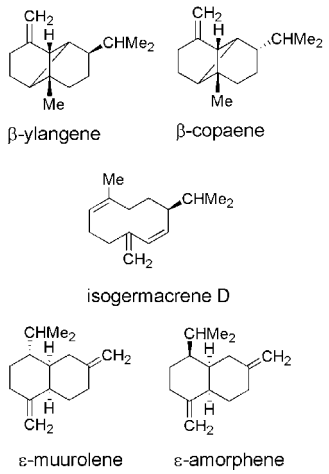


Fig. 1. Thermally labile analytes analysed by A) hot splitless GC injection, and B) cold on-column injection of a solution of germacrene D into the GC injection port

Chart 1. Thermal rearrangement products of germacrene D



The more recent innovation of SPME in volatile analysis[14] has been widely adopted. It is convenient and easy to use in the extraction of organic volatiles from both headspace and aqueous samples with minimal equipment costs. In short, volatiles are concentrated onto an adsorbent coating, which is then inserted directly into the heated injection port of the GC from where the volatiles are thermally desorbed and pass into the GC in the normal way. In Fig. 2A, the SPME-GCMS analysis of an enzyme extract incubated with farnesyl diphosphate reveals the presence of seven sesquiterpenes; a mixture of products not atypical of such enzymes based upon published work.[15] A sesquiterpene synthase from the plant *Arabidopsis thaliana*, expressed in *E. coli*, produces some eighteen sesquiterpenes, all of which are found in the wild type plant. However, in our case, GCMS analysis of a solvent rather than an SPME extract revealed that nerolidol was the sole product of the enzyme (Fig. 2B). The other six sesquiterpenes observed by SPME sampling were all artefacts, presumably formed during thermal desorption from the SPME fibre in the GC injection port. The geometrical isomerisation of the remote (6*E*) double bond to the (6*Z*) isomer shown in the formation of (3*Z*,6*Z*)- and (3*E*,6*Z*)-α-farnesenes and (*Z*)-β-farnesene (not all shown in Fig. 2) indicates the extent and reversibility of the rearrangements taking place.

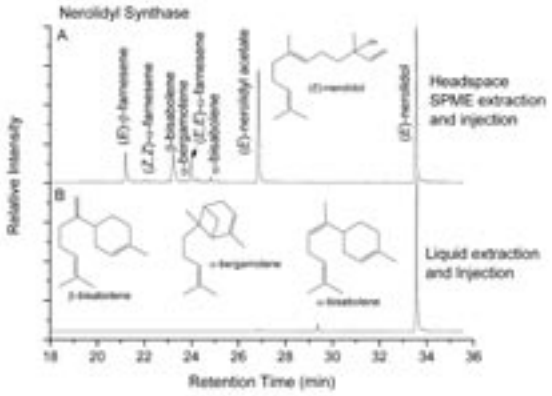


Fig. 2. GCMS following A) thermal desorption from an SPME fibre, and B) hot splitless injection of a solution of nerolidol into the GC injection port

With deceitful or *at-risk* analytes, it is not always clear where in the chain of extraction and analysis the problem lies. An example of this is the sesquiterpene zingiberene (found in ginger, *Zingiber officinale* L.), which can aromatize (-2H) in the injection port to curcumene. Fig. 3 shows that by SPME, oxidation to curcumene is extensive - only 2% of the zingiberene makes it through the system to the mass spectrometer. Based upon our experience with nerolidol, we attributed this to the SPME fibre. When a liquid injection was used zingiberene was detected at 16% but the outcome was still far from clear-cut. A liquid injection into a different GCMS system, presumably with a cleaner injection port, resulted in a chromatogram with 99% zingiberene. Problems with *at-risk* analytes can be circumvented by good GC house-keeping!

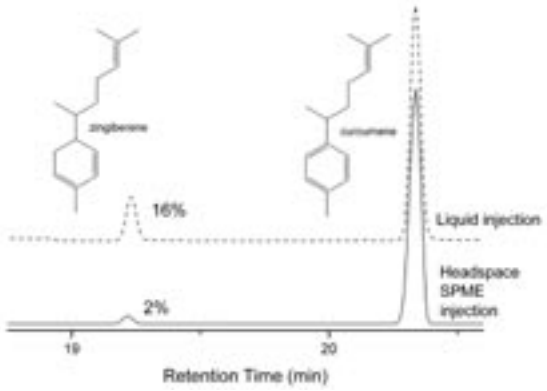


Fig. 3. Thermal lability and injection port maintenance – SPME *vs* liquid sample injection of easily oxidized zingiberene (to curcumene); injection into a totally different GCMS system provided a chromatogram with 99% zingiberene

When Good Labels Go Bad

The use of isotopically labelled compounds as internal standards is widely regarded as the gold standard for quantitative chemical and biosynthetic analysis. However, the use of such compounds is subject to the same assumptions as for any internal standard or tracer, *i.e.* identical chemical properties and consequently identical metabolism, complete and equal spatial equilibration with analyte, and non-perturbation of the system. These assumptions are known to break down in complex, heterogeneous biological systems. The commonly observed separation of deuterated and non-deuterated isotopomers in a GC capillary column demonstrates their chemical non-equivalence (Fig. 4).[16] Deuterated skatole shows reduced toxicity compared with the non-deuterated compound, and this difference has been used to infer both the mechanism of toxicity and a structure for the reactive intermediate generated *in vivo*.[17] The following examples are intended to illustrate both the usefulness and the need for care in use of isotopically labelled compounds in biological systems; they stem from our experience in this area.

α-Farnesene synthase is an enzyme identified in a number of plant species. It converts farnesyl diphosphate into the sesquiterpene, α-farnesene (Fig. 4). Expression of the apple enzyme in *E. coli*, and treatment with a number of alternative precursors, suggested that this enzyme was also able to assemble the 15C α-farnesene molecule directly from a C10 geranyl diphosphate and a C5 isoprenyl diphosphate

precursor.[9] Evidence for this novel combination of activities was provided by feeding the deuterated versions of all precursors to the purified enzyme. Analysis by headspace SPME-GCMS (Fig. 4) showed the formation of (*E*,*E*)-d_{10}-α-farnesene in addition to (*E*,*E*)-d_0-α-farnesene from the endogenous d_0-farnesyl diphosphate, which had remained bound to the protein throughout its purification. The MS fragmentation pattern indicated head-to-tail coupling of the two fragments. In this case, the two isotopomers (d_0- and d_{10}-) were completely resolved on the GC column, with the heavier deuterated isotopomer counter-intuitively eluting first. This chromatographic isotope effect is colloquially known as the reverse isotope effect.[18]

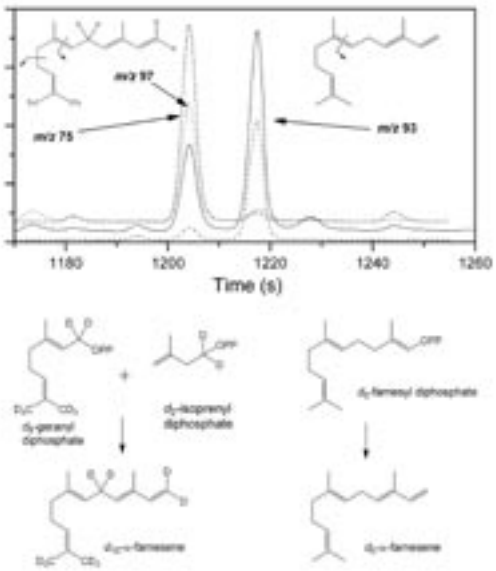


Fig. 4. Deuterium labelling of precursors to determine biosynthetic pathways; d_{10}-α-farnesene produced by incubation of a farnesene synthase gene with d_6-geranyl diphosphate, and d_2-isoprenyl diphosphate and α-farnesene produced by endogenous farnesyl diphosphate – see ref. 9.

Fruit kept in cold (or controlled-atmosphere) storage lose the ability to produce flavour volatiles resulting in a noticeable loss of flavour. In particular, apples lose their ability to produce fruity esters such as hexyl acetate and 2-methylbutyl acetate. However, not all flavour volatiles are equally suppressed and ester production can be artificially enhanced by infusing flavour precursors into the fruit tissue. This provides opportunity for identifying which enzymes in the biosynthetic pathways are being inactivated during storage.[8] Addition to the apple tissue of [6,6,6-$^2H_3$]-hexanol, a precursor for hexyl acetate, leads to the production of deuterated hexyl acetate. This suggests that the concentration of the alcohol precursor is limiting hexyl acetate production by the fruit. However, the addition of deuterated hexanol to younger, but not older, fruit also increases the production of non-deuterated hexyl acetate (Fig. 5). Presumably sufficient *total hexanol* is now available to meet the needs of other unknown biosynthetic pathways, thus allowing more non-deuterated hexanol to be converted to acetate ester.

The above example, perturbation of the biological system, was not a problem because the deuterium labelling has been used only as a tracer to test the biosynthetic capability. However, if the deuterated compound is used as

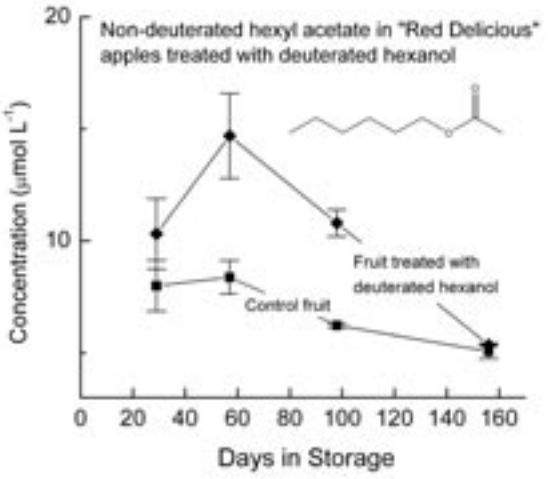


Fig. 5. Perturbation of biological systems by addition of biological precursors; elevation of endogenous hexyl acetate production in controlled-atmosphere-stored 'Red Delicious' apples treated with deuterated hexanol

a quantitative internal standard then the matter may have become more serious. In a study of sulfur esters present in kiwifruit, deuterated internal standards d_5-ethyl 2-(methylthio)acetate and ethyl 3-(d_3-methylthio)propanoate were dissolved in ethanol and then spiked into kiwifruit pulp. Upon addition of the deuterated internal standards, kiwifruit samples that did not previously contain d_0-ethyl 2-(methylthio)acetate developed levels approaching 5% of the amount of the d_5-ethyl 2-(methylthio)acetate internal standard. We assumed that this problem was caused by the presence of transesterase enzymes in the kiwifruit pulp. These would remove the d_5-ethyl moiety from the internal standard and replace it with a d_0-ethyl moiety from endogenous and added ethanol.

Adding carboxyesterase inhibitors to the fruit pulp partially clarified matters. The cysteine protease inhibitor E-64 [*trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane] that is used to block serine carboxy esterases, and 50% sodium dodecyl sulfate solutions caused 4- and 2-fold reductions, respectively, in the levels of d_0-ethyl 2-(methylthio)acetate. More of the story was revealed when deuterated standards dissolved in isopropanol rather than ethanol were added to the kiwifruit pulp. No non-deuterated esters were formed. Full clarification was reached only after the powerful esterase inhibitors paraoxon [diethyl 4-(nitrophenyl)phosphate] and DMCP (dimethyl chlorophosphate) were used in addition to isopropanol as solvent. Not only was there no formation of the d_0-ethyl 2-(methylthio)acetate, but also the peak areas of both deuterated internal standards, d_5-ethyl 2-(methylthio)acetate and ethyl 3-(d_3-methylthio)propanoate, increased 6- to 7-fold above those in the control samples. Thus, what had appeared to be the major problem, exchange of d_5- and d_0-ethyl moieties on one of the deuterated standards was, in fact, a minor problem. Moreover, it assisted in revealing the massive, and therefore much more serious, loss of both deuterated internal standards due to occult esterase enzyme activity. In previous studies of the biosynthesis of apple flavour volatiles,[19] apple tissue samples had been incubated with deuterated esters to assess transesterase

activity, and the fates of the deuterated moieties in those esters. The present case appears to be an example of re-learning something we already knew. Internal standards placed in biological systems may not be inert and can be quite misleading when used for the purposes of quantitation.

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Dates of Note

Sir *Geoffrey* ***Wilkinson***, joint recipient with Ernst Fischer of the 1973 Prize for Chemistry on sandwich compounds, was born on July 14, 1921.

Paul ***Walden***, the Latvian chemist who discovered the reversal of stereochemical configuration phenomenon in 1896 – the Walden inversion – was born on 14 July 1863.

On July 15 in 1869 margarine was patented by *Hippolyte Mège* ***Mouriés*** in France. His formula included a fatty component that mixed to a pearly luster, so he named the product after the Greek word for pearl (*margaritari*).

Sir *Frederick* ***Abel*** was the English chemist and military explosives specialist who invented cordite (1889) with Sir James Dewar; he was born on July 17, 1827. This day is also the 10th anniversary of the publication (*Science*) of the genome sequence of the bacterium that causes syphilis.

July 24 marks the 70th anniversary of the introduction of Nescafé instant coffee in Switzerland, but Dr. *Satori* ***Kato*** (Japan) presented the first instant coffee in 1881 at the Pan-American World Fair. It was patented in 1903 in the US.

Stephanie ***Kwolek***, the DuPont chemist who invented Kevlar®, was born on 31 July 1903 and *Friedrich* ***Wöhler*** on this day 103 years earlier (1800).

Aug 3 of 1926 saw installation of the first traffic lights – at Piccadilly Circus.

Aug 13 marks the 90th birthday of *Fredrick* ***Sanger*** who has twice received the Nobel Prize in Chemistry [1958: proteins especially insulin; 1980 (with Paul Berg and Walter Gilbert) for the base sequences in nucleic acids]. *Anders* ***Angstrom*** was born on this day in 1814.

Aug 14 is the 50th anniversary of the death of *Frédéric* ***Joliot*** (b.1900) who, with wife Irène (Curie) received the 1935 Nobel Prize for artificial radiation.

Linus (Carl) ***Pauling***, twice made a Nobel laureate, died on 19 Aug in 1994. He charted the chemical underpinnings of life, worked for nuclear peace, and proffered vitamin C as especially beneficial.

Sir *Hans Adolf* ***Krebs*** (of Krebs cycle fame) was born on Aug 25, 1890. In 1973 on this day the first CAT (Computer Assisted Tomography) scan was recorded.

On Sept 4 in 1888 *George* ***Eastman*** was issued the landmark US Patent 388,850 for his box camera. He registered the trademark name *Kodak* on the same day.

Felix ***Bloch***, the Swiss-born American physicist who, with E.M. Purcell, received the 1952 Nobel Prize for Physics for developing NMR, died 25 years ago – 10 Sept 1983.

Sept 20 is the 155th anniversary of the day of sale of *Elisha Graves* ***Otis***'s first safety lift equipment.

27 Sept 1910 was when US Patent 971,501 for the production of ammonia was granted to *Fritz* ***Haber*** and *Robert* ***Le Rossignol***.

Sept 30 marks the 65th birthday of German *Johann* ***Deisenhofer*** who (with Hartmut Michel and Robert Huber) received the Nobel Prize for Chemistry in 1988 for the determination of the three-dimensional structure of certain proteins that are essential to photosynthesis. It is also the 69th birthday of Nobel laureate *Jean-Marie* ***Lehn***, a recent visitor to Otago.

Oct 1, 1908 saw the the first car to be made on an assembly line. Ford's Model T sold for $US825.

October 5th marks the 50th anniversary of the death of *Earl Silas* ***Tupper***, inventor of Tupperware.

8 Oct is the 125th anniversary of the birth of *Otto Heinrich* ***Warburg*** who received the 1931 Nobel Prize in Medicine or Physiology for his discovery of the nature and mode of action of the respiratory enzyme.

October 9th marks the 90th anniversary of the birth of *Kenichi* ***Fukui*** who introduced the frontier orbital theory of reactions. He received the 1981 Nobel Prize jointly with Roald Hoffmann.