Non-invasive Localization of Thymol Accumulation in *Carum copticum* (Apiaceae) Fruits by Chemical Shift Selective Magnetic Resonance Imaging

P. V. GERSBACH[1],* and N. REDDY[2]

[1]*Centre for Biostructural and Biomolecular Research, University of Western Sydney, Richmond, NSW 2753, Australia and* [2]*School of Science, Food and Horticulture, University of Western Sydney, Kingswood, NSW, Australia*

Magnetic resonance imaging was used to localize the site of essential oil accumulation in fruit of *Carum copticum* L. (Apiaceae). A chemical shift method is described that utilized the spectral properties of the aromatic monoterpene thymol, the major component of the essential oil, to image thymol selectively. The presence of essential oil secretory structures in the fruit and an essential oil containing a high proportion of thymol were confirmed with optical microscopy and gas chromatography-mass spectrometry, respectively. Selective imaging of whole *C. copticum* fruits showed that thymol accumulation was localized to the secretory structures (canals) situated in the fruit wall. The technique was considered non-invasive as the seeds used in the imaging experiments remained intact and viable.   © 2002 Annals of Botany Company

Key words: *Carum copticum*, ajowan, nuclear magnetic resonance, magnetic resonance imaging, chemical shift imaging, histochemistry, essential oil, monoterpenoids, thymol.

INTRODUCTION

Techniques involving nuclear magnetic resonance (NMR) have been used increasingly over the past decade in the study of plants (De Graaf, 1998; Brown and Semelka, 1999). NMR imaging (MRI) is a non-invasive analytical technique, making it attractive for application to biological specimens and, as sample preparation is unnecessary, artefacts are avoided (Young, 1988; Mitchell, 1999).

MRI has been used to study several aspects of plant physiology and metabolism, including phloem and xylem water flow (Kockenberger *et al.*, 1997), disease and senescence processes (Goodman and Glidewell, 1997), accumulation of heavy metals and pesticide residues (Ratcliffe and Roscher, 1998), sucrose concentration and metabolism (Goodman *et al.*, 1993; Tse *et al.*, 1996), lipid and water distribution (Halloin *et al.*, 1993), and chemical composition (Gussoni *et al.*, 1994). In all of these publications, the non-invasive nature of the technique has been emphasized.

This paper describes the application of chemical shift selective MRI to localization of essential oil accumulation in fruits of *Carum copticum* (ajowan). The method is an example of non-invasive plant histochemistry, approaching the concept of histochemistry from a perspective quite different from conventional methods that involve cutting, fixing and staining. The application of MRI to the location of terpenoids in plants has been described in two previous reports, where it was used to image the aromatic monoterpene anethole accumulated in the fruit of *Foeniculum vulgare* (Sarafis *et al.*, 1990), and to localize terpenoids in the female cones of two Podocarpaceae species (Glidewell *et al.*, 2002).

Ajowan fruits are an important commercial product for the food/flavouring industry, and they accumulate up to 5 % essential oil in compartments referred to as canals or vittae (Bhargava and Haksar, 1959). Reports on the antimicrobial and antioxidant properties of thymol have been reviewed in a previous article (Gersbach *et al.*, 2001), and similar properties have been attributed to extracts of ajowan seeds (Mehta *et al.*, 1994; Srivastava *et al.*, 1999).

The composition of the essential oil has been analysed extensively. Up to 55 % is the aromatic monoterpene thymol, and there are significant amounts of the thymol precursors p-cymene and γ-terpinene (Balbaa *et al.*, 1973; Ashraf and Bhatty, 1975; Nagalakshmi *et al.*, 2000). The relatively high essential oil content of the ajowan fruit, with a high proportion of thymol, make it a promising subject for MRI. The aromatic ring structure of the monoterpene thymol, with its aromatic protons and their particular spectral characteristics, is a feature that is conducive to detection by NMR methods. In ajowan, the main component of the fruit is lipid in the form of reserve oil (triglyceride). This is present in greater abundance than monoterpenes, but the triglyceride and essential oil are located in separate, well-defined compartments, the seed endosperm and vittae, respectively. This, combined with the well separated NMR peaks of the two substances, suggests the choice of chemical shift selective imaging (CSSI). The principles of CSSI and its application to the analysis of two-component systems, such as water and lipid in biological specimens, have been reported in several publications (Sun *et al.*, 1994; Pope *et al.*, 1995; Gerald *et al.*, 1996; Schick *et al.*, 1997; Schick, 1998; Weis *et al.*, 1998).

* For correspondence. E-mail: p_gersbach@hotmail.com

MATERIALS AND METHODS

Volatile analysis

Mature dry mericarps of *Carum copticum* (Apiaceae) were collected from stock plants grown at the University of Western Sydney, Australia. Volatiles were sampled by solvent extraction: 250 mg of the fruit was extracted in 1·0 ml methanol for 24 h at room temperature. The extract was analysed by gas chromatography-mass spectrometry to confirm the presence of aromatic monoterpenes suitable for detection by NMR microscopy. The instrument was an HP-5890 gas chromatograph fitted with a J&W DB-1 $30·0$ m $\times$ $0·25$ mm $\times$ $1·0$ μm column, and interfaced with an HP-5971 mass selective detector. The injector temperature was 240 °C, detector temperature 260 °C and the oven temperature programme was 80–150 °C at $2·5$ °C min^{-1}, then 150–250 °C at 5 °C min^{-1}. Volatiles were identified by mass spectra and Kovats retention indices calculated from the retention times of C_8–C_{14} hydrocarbon standards under identical operating conditions.

Bright-field microscopy

Whole mericarps and transverse sections were examined and photographed using bright-field microscopy. The microscope was an Olympus SZH zoom stereomicroscope equipped with a DF-Plan $\times 1·0$ objective and $\times 2·5$ projection eyepiece. The zoom control was adjustable from $7·5$ to $\times 60·0$. The aperture was adjusted to the minimum setting. The microscope was also fitted with an Olympus C35AD-4 camera, operated by an Olympus PM-10AD5 automatic photomicrographic system attachment. Images were recorded on 100 ASA print film.

NMR spectroscopy

A high resolution ^{1}H-NMR spectrum of thymol was acquired with a Varian Unity Plus 300 MHz spectrometer, interfaced with a $7·05$ Tesla/89 mm wide-bore Oxford magnet system. The sample of pure thymol was obtained from Aldrich (Sydney, Australia), and was dissolved in CDCl$_3$ containing $0·1$ % CHCl$_3$. All chemical shifts were reported relative to TMS (TMS = 0 ppm).

MRI

Experiments were carried out to acquire NMR images of aromatic monoterpenes present in secretory structures in the ajowan seeds using a chemical shift selective method. The NMR imaging experiments were performed on the same spectrometer system as for the acquisition of the high resolution ^{1}H-NMR spectrum, which was also equipped with a Varian micro-imaging accessory. The MRI probe was a V-120 supplied by Doty Scientific Instruments (Columbia, SC, USA), containing coils for the generation of magnetic field gradients for spatial delineation.

Images of 300 μm transverse slices across ajowan schizocarps and mericarps were acquired by means of a chemically selective fast-spin-echo sequence, having a chemical shift selective 90° Gaussian shaped excitation pulse of $8·0$ ms duration and 400 Hz band-width, and a slice selective 180° Gaussian shaped re-focusing pulse of $2·0$ ms duration and 800 Hz band-width. Gradient strengths were $10·0$ G cm^{-1} for the slice selection and $30·0$ G cm^{-1} for the readout and phase encoding gradients. The spin-echo time was $12·0$ ms and the repetition time $1·0$ s.

RESULTS AND DISCUSSION

Volatile analysis and optical microscopy

The GC-MS analysis of the methanol extract showed that thymol was the major volatile component of *Carum copticum* (ajowan) fruit. The volatiles identified and their qualitative contribution to the essential oil were as follows: thymol $45·60$ %, γ-terpinene $23·79$ %, p-cymene $21·25$ %, β-pinene $3·44$ %, α-pinene $1·40$ %, carvacrol $1·20$ %, camphene $0·88$ %, β-myrcene $0·65$ % and limonene $0·51$ %. The remaining $1·28$ % contained about 15 trace compounds (<$0·10$ % each) that were not identified.

Images acquired by light microscopy confirmed the fruit of *C. copticum* to be a schizocarp with two single-seeded mericarps (Fig. 1A). The mericarps had six oil canals (vittae) which are commonly associated with essential oil accumulation in the Apiaceae (O'Brien and McCully, 1981; Sarafis *et al.*, 1990). Two canals were located in the mesocarp on the flat inner side of the mericarp where the two mericarps are joined and four were located around
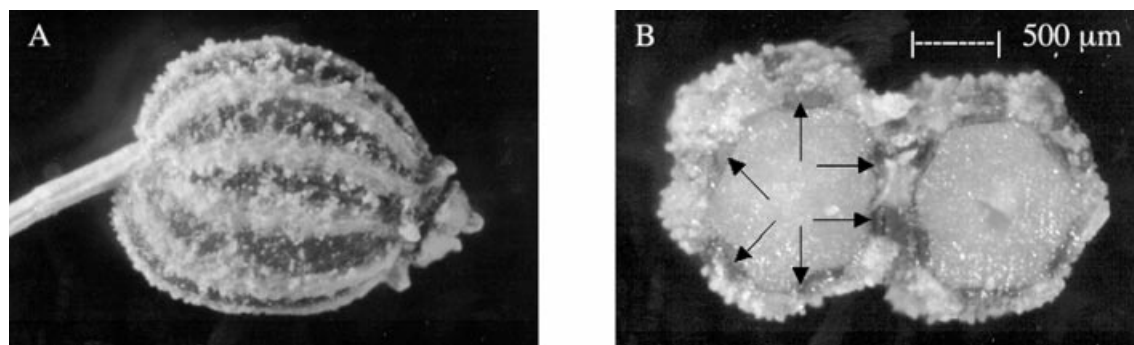

Fig. 1. A, Intact schizocarp of *Carum copticum*, $\times 150$. B, Transverse section through schizocarp showing location of oil canals (arrows) in one mericarp. There were six canals in each mericarp, situated in the fruit wall.

the convex outer surface of the mericarp in the mesocarp (Fig. 1B).

The information collected by light microscopy and GC-MS confirmed that ajowan fruits accumulated significant quantities of essential oil containing a high combined proportion of aromatic monoterpenes (thymol, carvacrol and p-cymene), and the presence of secretory structures where accumulation of the terpenoids is believed to be localized.

NMR and MRI

The high resolution NMR spectrum of thymol contained signals from the aromatic protons and the methyl and hydroxyl groups. The spectrum was used as a reference to identify signals arising from thymol in the subsequent spectrum of whole ajowan fruit. The peaks were assigned so as to correspond to the numbered groups in the structure of thymol, included with the spectrum (Fig. 2A).

The ^{1}H-NMR spectrum of the whole ajowan fruit revealed three main signals (Fig. 2B). The signal at $\delta = 1.5$ ppm contained several unresolved resonances resulting from the methyl and methylene groups of reserve oil, with a contribution from the methyl and hydroxyl groups of thymol; resonance from the aromatic protons of thymol appeared at $\delta = 6.2$ ppm, while the peak at $\delta = 5.2$ ppm has been confirmed by 2-dimensional correlated spectroscopy (2D-COSY) spectroscopy to derive from olefinic protons adjacent to methylene groups in the reserve oil (Aue *et al.*, 1976; Sarafis *et al.*, 1990).

The images in Fig. 3A and B are non-selective, and represent total lipid distribution: reserve oil in the endosperm and thymol in the vittae. The location of the vittae is evident in these images (highlighted in Fig. 3B), which can be compared with the optical microscopic image of a transverse slice through the schizocarp in Fig. 1B, where the canals are also indicated.

Peak resolution in the spectrum of ajowan fruit was adequate for selective imaging of thymol. The obvious choice for selective imaging of thymol was by excitation at $\delta = 6.2$ ppm, the peak resulting from resonance of the aromatic protons of thymol. Two chemically selective NMR images were acquired, one indicating the distribution of reserve oil or triglyceride in a single seed (Fig. 3C) and the other the distribution of thymol, corresponding to the six oil canals located in the mericarp (Fig. 3D). The latter probably included a contribution from p-cymene, which made a significant contribution to the essential oil and, having aromatic protons, will result in resonance in the range of $\delta = 6.2$ ppm. Averaging of 512 transients was required for each pixel to produce the image of thymol.

The selective image of thymol (Fig. 3D) verified that thymol accumulation and, consequently, a significant proportion of essential oil accumulation, was confined to the canals in the mesocarp of the ajowan fruit. The image corresponds with the location of the canals in the optical image (Fig. 1B): thymol in the fruit was identified by the volatile analysis and by the comparison of the high resolution NMR spectrum of thymol with the spectrum of the whole fruit. Because of the selectivity of the method used, it can be stated that the image in Fig. 3D results from thymol, and also that it indicates its spatial distribution in the fruit.

The use of CSSI for localizing chemical constituents in plant material was completely non-invasive, and this is its greatest advantage over conventional methods. Fruits used in the imaging experiments remained intact throughout, and the seeds contained in the fruits retained their viability. The seeds germinated and developed normally subsequent to the experiments. Although the images are referred to as slices, or sections, through the plant material, there was no mechanical sectioning or cutting involved in the conventional sense; the 'slice' is accomplished by means of magnetic field gradients.

CSSI is yet to be routinely used in histochemical studies involving plant material or essential oil, but, in addition to the method described here, CSSI has been successfully applied to histochemistry of plant materials, such as
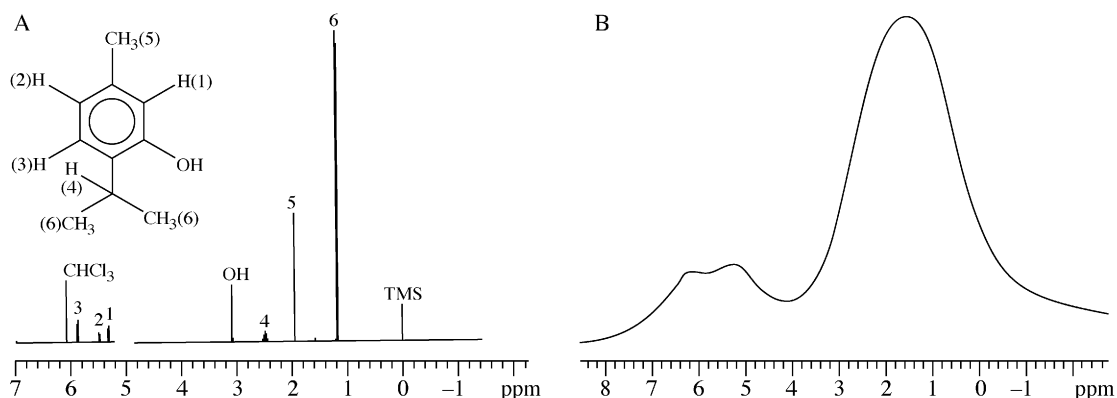

FIG. 2. A, ^{1}H-NMR spectrum of pure thymol. Assignment of peaks corresponds to the adjacent structure of thymol. CHCl$_3$ at $\delta = 6.1$ ppm is the residual peak of chloroform in the CDCl$_3$ solvent. All chemical shifts are reported relative to TMS at $\delta = 0$ ppm. B, ^{1}H-NMR spectrum of whole dried ajowan fruit. The signal at $\delta = 6.2$ ppm arises from the aromatic protons of thymol; the olefinic protons in reserve oil (triglyceride) produce the peak at $\delta = 5.2$ ppm; the broad resonance at $\delta = 1.5$ ppm contains signals from the methyl and methylene groups of reserve oil, and the hydroxyl and methyl groups of thymol.
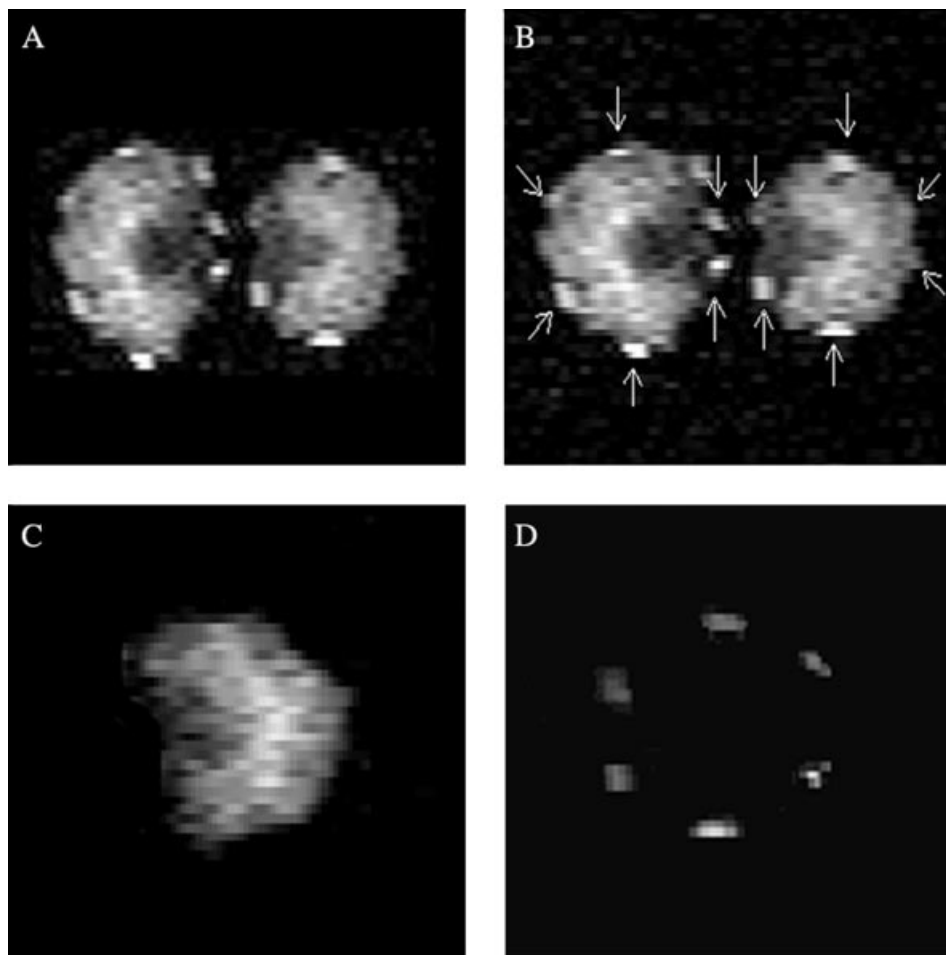
FIG. 3. NMR images of transverse slice across ajowan schizocarp (A and B) and mericarp (C and D). Spatial resolution is 25 μm × 25 μm and slice thickness is 300 μm. A, Non-selective image of overall lipid distribution, comprising reserve oil and thymol; B, same image as in A showing location of oil canals; C, chemical shift selective image (δ = 5·2 ppm) of reserve oil (triglyceride) distribution; D, chemical shift selective image (δ = 6·2 ppm) of thymol distribution. The image corresponds with the location of the canals as shown in Fig. 1B, and confirms that thymol is localized in the canals.

anethole in fennel seeds (Sarafis *et al.*, 1990). Therefore, it should be feasible to image other compounds selectively, and in different types of plant material (Stark and Bradley, 1999). In addition to ajowan and fennel, the fruits of other Apiaceae species have oil canals, for example, those of cumin contain significant amounts of the aromatic mono-terpene cuminaldehyde, making it a prospective subject for chemically selective NMR imaging.

Imaging of essential oil components in leaves or flowers should also be possible, although this would require some modification of the technique due to compositional differ-ences. In leaves, for example, which commonly have structures that accumulate essential oil, the major component, by a significant margin, is water, and the problem arises of resonances from molecules in compara-tively very low concentration being obscured by that of water.

NMR microscopy should be considered as a technique that is yet to be even moderately exploited in its application to plant histochemistry, and one that can be further

developed and improved for the non-invasive imaging and selective identification and localization of compounds in plant material.

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