Protoplasma (1990) 159: 70–73

PROTOPLASMA

Non-invasive histochemistry of plant materials by magnetic resonance microscopy

Rapid communication

V. Sarafis[1], **H. Rumpel**[2], **J. Pope**[3,*] and **W. Kuhn**[2]

[1] University of Western Sydney, Hawkesbury, Richmond, N.S.W., [2] Hauptabteilung Medizintechnik, Fraunhofer-Institut für zerstörungsfreie Prüfverfahren, St. Ingbert, and [3] Department of Physics, University of New South Wales, Kensington, N.S.W.

Received October 15, 1990
Accepted October 30, 1990

Summary. We have combined nuclear magnetic resonance (NMR) imaging on the microscopic scale with chemical shift selection to demonstrate the application of magnetic resonance imaging (MRI) to plant histochemistry. As an example of the method we have obtained separate images of the distribution of reserve oil and anethole in dried fennel mericarps. The technique can be employed to separately image the distribution of aromatics, carbohydrates, oils, water and possibly fatty acids in suitable plant materials.

Keywords: Histochemistry; Nuclear magnetic resonance; Non-invasive techniques; Magnetic resonance imaging.

Abbreviations: NMR nuclear magnetic resonance; MRI magnetic resonance imaging; COSY correlation spectroscopy; TMS tetramethylsilane.

Introduction

The non-invasive nature of nuclear magnetic resonance microimaging (Aguyao et al. 1986, Eccles and Callaghan 1986, Harrison et al. 1988, Kuhn 1990, Walter et al. 1989) combined with the chemical specificity of the NMR method, suggests its application to histochemistry. Spatial resolution down to 10 μm is currently achievable in NMR microscopy (Walter et al. 1989), while a number of methods for achieving selectivity on the basis of chemical shift have been described (Dixon 1984, Rosen et al. 1984, Bottomley et al. 1984, Hall et al. 1984, Joseph 1985, Haase et al. 1985, Dumoulin 1985, Hennig and Friedburg 1986). Plant materials such as fruit and seeds are particularly suited to such investigations since they may contain a variety of chemical constituents in well defined compartments. NMR spectroscopy can be used to identify the major constituents and chemical shift imaging to spatially localize them. Taking the dried umbelliferious fruit of fennel as an example, we have applied the methodology of chemical shift selective imaging to demonstrate, for the first time, non-invasive histochemical localization of some of the main chemical constituents.

Chemical shift imaging has previously been used to discriminate between fat and aqueous phases in clinical whole-body MR systems. We show here that NMR microscopy at high field strength can exploit the particular spectral characteristics of plant materials to achieve non-invasive histochemistry. The immobility and stability of such materials also facilitates the use of signal averaging to achieve an adequate signal to noise ratio in the resulting images, even for chemical constituents whose spectral contributions are relatively weak.

Materials and methods

The experiments presented here were performed on a Bruker MSL 400 spectrometer, equipped with a 9.4 Tesla, 89 mm-bore magnet and microimaging accessory. The probe-head contained coils for the generation of constant magnetic field gradients for spatial delineation. Gradient strengths of up to 55 G/cm could be switched within less than 100 μs. A 10 mm homebuilt solenoid was tuned to 400 MHz for radio-frequency excitation and reception of ^{1}H-NMR signals. The images were taken by means of the conventional "spin-

* Correspondence and reprints: Department of Physics, University of New South Wales, P.O. Box 1, Kensington, N.S.W. 2033, Australia.

warp" spin-echo method (Morris 1986) modified to provide a chemical shift selective 90° excitation pulse with slice selection on the 180° re-focussing pulse.

Sweet fennel mericarps (*Foeniculum vulgare* Mill. var. dulce) were selected for imaging. The fruit of fennel is a schizocarp which splits into two one-seeded mericarps upon drying at maturity. Fennel mericarps contain reserve fat in the endosperm of the seed as well as essential oil canals (vittae) in the adherent fruit wall. There are four outer vittae in the mesocarp located in the grooves between the ribs on the convex outer side and two larger vittae in the mesocarp on the flat inner side. The endosperm contains fat droplets, aleurone grains and oxalate crystals in its cells (Gassner et al. 1989, Melchior, and Kastner 1974). The essential oil of the sweet fennel contains up to 60% 1-methoxy-4-(-1-propenyl)-benzene (transisomer), i.e., transanethole (Wagner 1989).

Results and discussion

The ^{1}H-NMR spectrum of the whole dried fennel readily reveals four main signals of approximately 300 Hz halfwidth each (Fig. 1 a). For assignment a better resolved spectrum (Fig. 1 b) was achieved by pulverizing the fennel seed and applying the magic angle spinning technique (Yannoni 1982). This allowed resolution of the methyl and methylene resonances in the range between 0.9–2.3 ppm and also of the aromatic and olefinic protons of anethole. The spectrum of pure trans anethole (Aldrich) is shown in Fig. 1 c as a reference. The signals in Fig. 1 a, are related to the main components, reserve oil and anethole, as follows. The signal at $\delta = 1.3$ ppm is due to the methylene groups of reserve oil, overlapped (partially resolved on the low field shoulder) by the styrene-sided methyl group of anethole. The methoxyl group of anethole appears at $\delta = 3.7$ ppm, while the signals of the aromatic protons and of the olefinic protons overlap at $\delta = 6.7$ ppm. The peak at $\delta = 5.4$ ppm is confirmed by 2D COSY spectroscopy (Aue et al. 1976) to derive from olefinic protons adjacent to methylene groups in the reserve oil. All chemical shifts are relative to TMS.

The separation of the peaks in the spectrum of the whole fennel enabled imaging of the individual components to be carried out. Figure 2 a shows the result of imaging the total lipid component by selective excitation at $\delta = 1.3$ ppm. The image represents lipid present in both endosperm and vittae. It may include a contribution due to the styrene-sided methyl group of anethole. By comparison, Fig. 2 b, obtained by selective excitation at $\delta = 6.7$ ppm, shows transanethole to be confined to the vittae. It should be emphasized that imaging of these transverse sections did not involve mechanical sectioning. Thus in chemical shift NMR microscopy we have a powerful tool for investigating the histochemistry of plant material. Difficulty exists in specimen preparation of dry mericarps for study by optical microscopy in that fixation, embedding and sectioning in such material introduces artefacts (O'Brien and McCully 1981). Lipid is difficult to preserve in situ if its dimensions are large as in the vittae. The histochemical demonstration of anethole as described in this paper can be extended to other compounds in plant material with freedom from the artefacts associated with the classical methods of fixation, embedding, cutting, and staining.

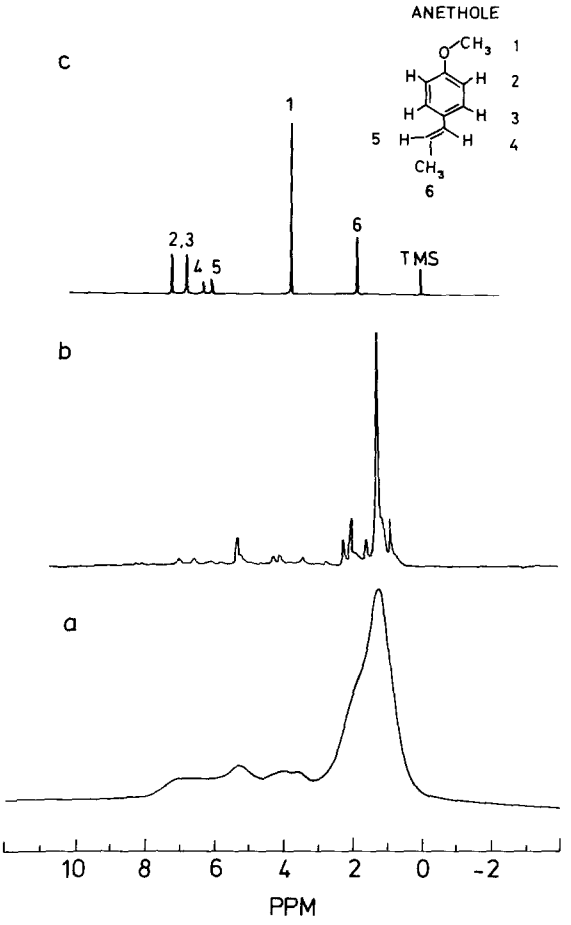

Fig. 1. ^{1}H NMR spectra of **a** whole dried fennel fruit, **b** pulverized dry fennel fruits, and **c** pure transanethole. The assignment of peaks in **a** is follows: The signal at $\delta = 6.7$ ppm arises from the aromatic and olefinic protons of anethole. The methoxy group of anethole gives rise to the signal at $\delta = 3.7$ ppm, while the methyl group contributes to the unresolved (low field) shoulder at $\delta = 1.4$ ppm. The methyl and methylene groups of reserve oil are contained in the broad resonance at 1.3 ppm, while the olefinic protons in the reserve oil give the peak at $\delta = 5.4$ ppm. The resonances are better resolved in the spectrum of **b**. Resolution enhancement was achieved by 2000 Hz magic angle spinning. The sample for **c** was obtained from Aldrich

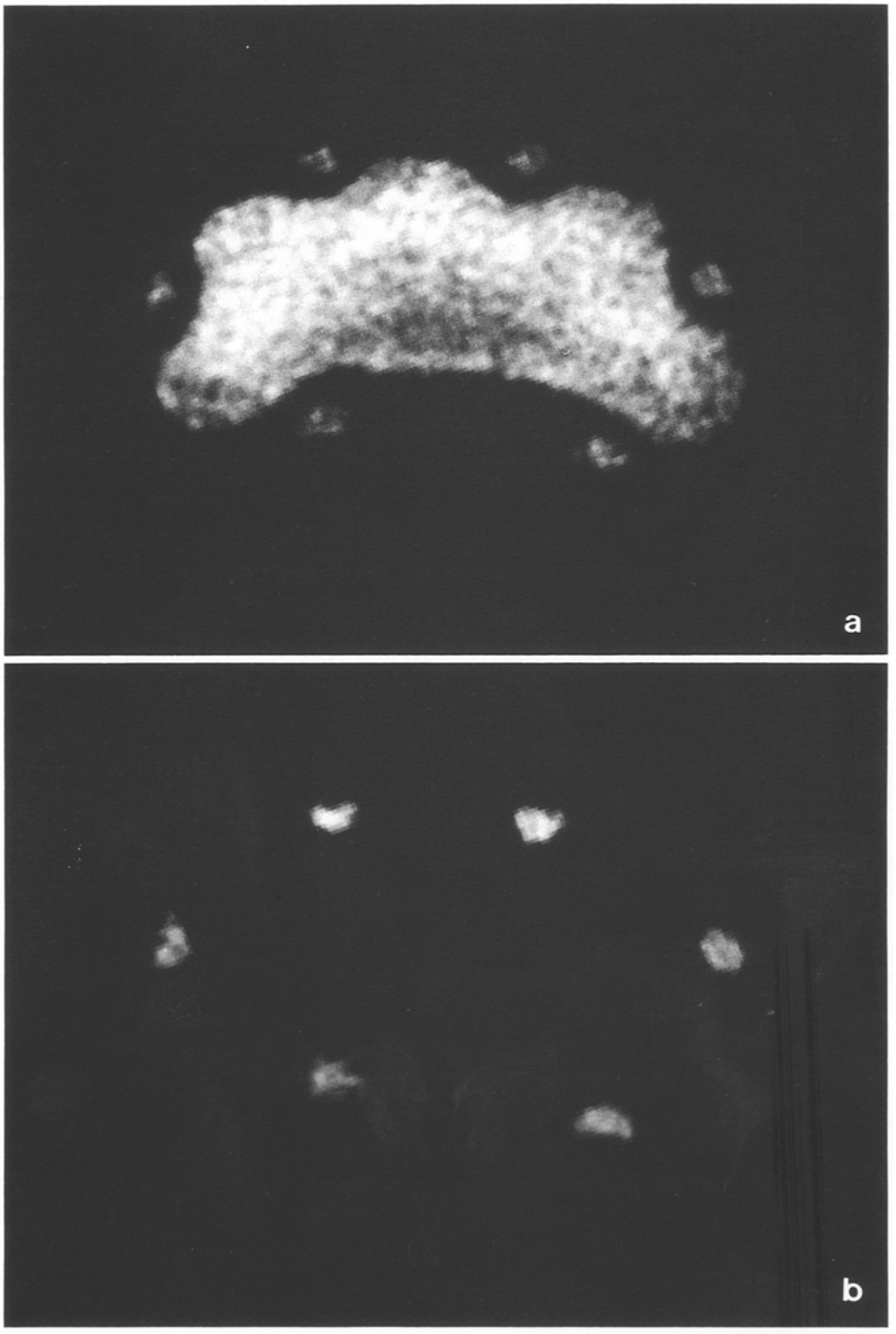

Fig. 2. NMR chemical shift images of a transverse slice across a fennel mericarp. Spatial resolution is 15 μm × 15 μm and slice thickness 260 μm. **a** Lipid distribution. A contribution due to the styrene-sided methyl group of anethole may be included. Selective excitation was performed at $\delta = 1.3$ ppm, 64 data acquisitions were averaged. **b** Distribution of anethole obtained by selective excitation at $\delta = 6.7$ ppm. 256 data acquisitions were averaged. The images were taken by means of a 90°-τ-180°-imaging sequence using a chemical shift selection 90° gaussian shaped pulse of 2.56 ms duration and 600 Hz bandwidth and a slice selective 180° hermite shaped pulse of 2 ms duration and 1200 Hz bandwidth. The gradient strengths used were 10 G/cm for the slice selection and 30 G/cm for the read and phase gradients. The spin echo time TE was 11.4 ms, the repetition time TR was 1 s

We believe that chemical shift selective NMR microscopy, as demonstrated here, may have wide application in biology, agriculture and food science. Other possibilities in plant sciences include ^{13}C imaging (Sillerud et al. 1988) of plant materials grown in a $^{13}CO_2$ enriched atmosphere (as the substrate for photosynthesis), studies of oil accumulation in seeds and its utilization during germination, and sugar distribution and storage during ripening of fruits. In the future such methods may be combined with flow imaging (Jenner et al. 1988) techniques to visualize the transportation pathways of specific chemical constituents.

References

Aguyao JB, Blackband SJ, Schoeniger J, Mattingly MA, Hintermann M (1986) Nuclear magnetic resonance imaging of a single cell. Nature 322: 190–191

Aue WP, Bartholdi E, Ernst RR (1976) Two dimensional spectroscopy: application to nuclear magnetic resonance. J Chem Phys 64: 2229–2246

Bottomley PA, Foster TH, Leue WM (1984) In vivo NMR chemical shift imaging by selective irradiation. Proc Natl Acad Sci USA 81: 6856–6860

Dixon WT (1984) Simple proton spectroscopic imaging. Radiology 153: 189–194

Dumoulin C (1985) A method for chemical shift selective imaging. Magn Reson Med 2: 583–585

Eccles CD, Callaghan PT (1986) High resolution imaging. The NMR microscope. J Magn Reson 68: 393–398

Gassner G, Hohmann B, Deutschmann E (1989) Mikroskopische Untersuchung pflanzlicher Lebensmittel, 5th edn. G Fischer, Stuttgart

Haase A, Frahm J, Hanicke W, Matthaei D (1985) ^1H NMR chemical shift selective imaging. Phys Med Biol 30: 341–344

Hall LD, Sukumar S, Talaga SL (1984) Chemical shift resolved tomography using frequency selective excitation and suppression of specific resonance. J Magn Reson 56: 275–278

Harrison LG, Luck SD, Munasinghe BDJP, Hall LD (1988) Magnetic resonance imaging approaching microscopic scale: maturation stages of *Acetebularia mediterranea* reproductive caps. J Cell Sci 91: 379–388

Hennig J, Friedburg H (1986) Fat and water separation of 0.23 T using simultaneous shift selective imaging. Magn Reson Med 3: 844–848

Jenner CF, Xia Y, Eccles CD, Callaghan PT (1988) Circulation of water within wheat grain revealed by NMR micro-imaging. Nature 336: 399–402

Joseph PM (1985) A spin echo chemical shift MR imaging techniques. J Comput Assist Tomogr 9: 651–658

Kuhn W (1990) NMR microscopy, fundamentals, limits and possible applications. Angew Chem (english edition) 29: 1–19

Melchior H, Kastner H (1974) Gewürze. Paul Parey, Berlin

Morris PC (1986) NMR in medicine and biology. Clarendon Press, Oxford

O'Brien TP, McCully ME (1981) The study of plant structure: principles and selected methods. Termacarphi, Melbourne

Rosen BR, Wedeen VJ, Brady TJ (1984) Selective saturation NMR imaging. J Comput Assist Tomogr 8: 813–818

Sillerud LO, van Hulsteyn DB, Griffey RH (1988) [^{13}C] polarization transfer proton NMR imaging of a sodium [^{13}C] formate phantom at 4.7 Tesla. J Magn Reson 76: 380

Wagner H (1988) Drogen und ihre Inhaltsstoffe, 4th edn. G Fischer, Stuttgart [Stahl E, Deutschmann F, Hohmann B, Reinhard E, Sprecker E, Wagner H, Pharmazeutische Biologie, part 2]

Walter L, Balling A, Zimmermann U, Haase A, Kuhn W (1989) NMR imaging of leaves of *Mesembryanthemum crystallinum* L. plants grown at high salinity. Planta 178: 524–530

Yannoni CS (1982) High resolution NMR in solids: the CPMAS experiment. Accounts Chem Res 15: 201–208

Verleger: Springer-Verlag KG, Mölkerbastei 5, A-1010 Wien. – Herausgeber: Prof. D. J. Morré, Arthur G. Hansen Life Sciences Research Building, Purdue University, West Lafayette, IN 47907, U.S.A. – Redaktion: Mölkerbastei 5, A-1010 Wien. – Hersteller: Adolf Holzhausens Nachfolger, Kandlgasse 19-21, A-1070 Wien. – Verlagsort: Wien. – Herstellungsort: Wien. – Printed in Austria.