Practical Nuclear Magnetic Resonance Analysis of Liquid Oil in Oilseeds: I Factors Affecting Peak Width

Martin J.T. Reaney*[,a], Nancy J. Tyler[a], and Keith Brown[b]

[a]Agriculture and Agri-Food Canada, Crop Utilisation Research Section, Saskatoon, Saskatchewan, S7N 0X2, Canada, and [b]Department of Chemistry, University of Saskatchewan, Saskatoon, Saskatchewan, S7N OWO, Canada

ABSTRACT: If proton nuclear magnetic resonance (NMR) spectra of single seeds can be improved, a rapid, low-cost method of screening seeds for oil composition could be developed for use as a selection tool in plant breeding. NMR spectroscopy was performed on single seeds of borage, flax, and canola to evaluate methods for improving spectra quality (narrowing peak widths and increasing signal-to-noise ratio) to a degree necessary to measure differences among seeds in a breeding program. Immersion of seeds in a variety of solvents, including deuterated chloroform, deuterated acetone, deuterated dimethyl sulfoxide (DMSO) and completely fluorinated hydrocarbons (FC-77), narrowed peaks obtained from seeds when compared with spectra from seeds analyzed in the absence of a solvent. Deuterated chloroform and FC-77 were free of interfering solvent proton peaks while deuterated acetone and deuterated DMSO contributed interfering peaks. The spectra of dehulled seeds had narrower peak widths than did seeds with hulls. Treatments that decreased seed oil viscosity failed to substantially narrow spectral peak widths of seeds. High magnetic field strength did not improve the spectral quality of seeds, as peak widths increased with field strength. Conversely, low field strength limited resolution of oil spectra. Although the 300 MHz spectrum of vegetable oil had greater resolution (narrower peaks) than the 60 MHz spectrum, spectra of seeds produced at 60 MHz had superior resolution to 300 MHz spectra.

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KEY WORDS: Borage, *Borago*, *Brassica*, canola, flax, *Linum*, oilseed, proton NMR, single seed analysis.

Seed oil quality is an inherited trait that is reflected in the oil composition of each seed. Breeding plants to produce seeds with desirable oil composition requires the development of inexpensive and rapid screening methods. Selection of single seeds allows more rapid development of cultivars than does analysis of bulk seed samples from a single plant (1). One requirement of a successful single seed analysis method is that it must preserve viability.

Currently, most oilseed breeders working on single seeds use half-seed analysis (McGregor, D.I., personal communication). Typical half-seed analysis begins with surface sterilization of the seeds followed by germination in petri plates. One cotyledon from each germinated seed is removed. The excised tissue is solvent-extracted and the extract transmethylated or methylated to form the acyl methyl esters that are then analyzed by gas chromatography. The embryo with the other cotyledon still attached is maintained in the petri dish until results of the fatty acid composition analysis are completed. Plantlets with interesting or desirable fatty acid compositions are transferred to soil and grown to maturity.

Oil and fat in seeds exists as a liquid or liquid crystal material suspended in droplets in a solid matrix (2). In many seeds the only proton signal measurable by standard proton nuclear magnetic resonance (NMR) originates from the liquid oil (3–5). Unfortunately, solid seed materials broaden the spectral peak widths and limit the useful information derived from the NMR spectrum of a single seed. Narrow NMR peak widths are attainable in proton NMR from oilseeds using magic angle spinning (MAS; 3,4) which allows the acquisition of high-resolution spectra from nonhomogeneous samples. Unfortunately, MAS requires careful balancing of the sample and is inherently expensive. Carbon [^{13}C]-NMR (5–7) in conjunction with high-field NMR gives comparatively narrow peaks for oilseeds but it is time consuming due to the low natural abundance of [^{13}C] and high-field spectrometers are too expensive for most breeding programs. Conway and Johnson (8) identified many factors that limited the quality of proton spectra of corn embryos using a 100 MHz Fourier transform NMR. In this paper, technologies to optimize the quality of NMR spectra of individual oilseeds are described.

EXPERIMENTAL PROCEDURES

Seed samples. Samples of flax (*Linum usitatissimum* L.), a generous gift from Dr. G.G. Rowland, were taken from field experiments grown at the Kernen Crop Research Farm, Crop Development Centre, University of Saskatchewan (Saskatoon, Canada). Samples of canola (*Brassica napus* L.) were obtained from check samples from the Canola Council of Canada, while borage (*Borago officianales* L.) was obtained as a generous gift from Bioriginal Food and Science Ltd., Canada.

Reagents. Deuterated solvents and tetramethylsilane (TMS) were purchased from Aldrich Chemical Co. (Milwaukee, WI). Fluorinated carbon solvent FC-77 was a generous gift

*To whom correspondence should be addressed at Agriculture and Agri-Food Canada, Crop Utilisation Research Section, 107 Science Place, Saskatoon, Saskatchewan, S7N 0X2, Canada. E-mail: Reaneym@em.agr.ca

from 3M Industrial Products Division (St. Paul, MN). Chloroform was purchased from BDH Chemicals (Saskatoon, Canada).

NMR spectra of solvent immersed seeds. Seeds of canola and borage were fixed in position in a NMR tube between two Teflon vortex plugs (Wilmad, Buena NJ). The optimal position for obtaining the maximal signal strength was determined by lowering seeds into the NMR and recording the spectra. The best signal-to-noise ratio was obtained when the seed was centered in the receiving coil. No attempts were made to shim the NMR with the seed in the probe, as this would not be feasible in a breeding program.

Flax seeds were not easily positioned and therefore were fixed onto the end of a segment of glass capillary tubing using Lepage's wood glue. The glue was allowed to dry for more than 48 h prior to analyses. The capillary tubing extended above the top of the NMR tube and was immobilized with a wad of glass wool that tightly fit into the NMR tube. Proton NMR spectra of seed oil were obtained using a continuous wave 60 MHz NMR (Perkin-Elmer R12-B) and a pulsed 300 MHz NMR (Bruker AM-300 Spectrometer, Bruker Spectrospin, Canada) at a frequency for protons of 300.13 MHz.

The effect of immersion of seeds in various solvents on the quality of spectra at 300 MHz was examined by successively analyzing a single borage seed. Spectra of seeds were compared before and after the addition of enough solvent to the NMR tube to submerge the seed.

Dehulling of borage seed. Borage seeds were dehulled by gluing the seeds between two boards and then prying the boards apart. Approximately 50 borage seeds were fixed to a board using wood glue, and the glue was allowed to dry. Glue was applied to a second board and the board with attached seeds was placed seed face down onto the second board. The glue was allowed to dry completely. The dehulling was accomplished by first placing a spacing rod between the boards at one end to prevent the seeds from being crushed while driving a metal wedge between the boards. About 20% of the seeds treated in this way were dehulled without any apparent damage to the seed.

NMR of seeds equilibrated with TMS. Borage seeds were immersed in TMS for 2 d prior to analysis. After the 2-d treatment seeds were blotted dry, and TMS was allowed to evaporate from the seed surface for 1 min. The seed was then placed in an NMR tube and immersed in FC-77 as described above, and the spectrum was taken immediately. The seed was removed from the spectrometer and allowed to dry to a constant weight and the spectrum was recorded again.

NMR spectra of extracted oil. Oil was extracted from 0.5 kg seed samples of borage using a Komet expeller press (IGB Monforts, Monchen, Germany). The oil was centrifuged at $1,000 \times g$ to settle any fines prior to analysis. Thin-wall NMR tubes (Norell Inc., Mays Landing, NJ) were used for all samples. Spectra were acquired on samples of oil diluted to 5% w/v using $CDCl_3$/TMS (90:10) and on oil diluted with 5% TMS. Proton NMR spectra of oil were obtained at 60 and 300 MHz.

Seed survival of solvent treatment. To determine the effect of immersion in a solvent on seed viability, seeds were soaked in the various solvents for 30 min. Average exposure to solvents during analysis was less than 10 min. Only the solvents that were previously found to optimize the NMR spectra were investigated. Differences in survival were determined from the binomial probability tables for 300 events.

RESULTS AND DISCUSSION

The highest signal-to-noise ratio was obtained when the seed was centered in the receiving coil of the NMR probe. Immersion of seeds in a fluorocarbon solvent in the NMR tube greatly improved the quality of the NMR spectra compared to those obtained from nonimmersed seeds of flax, canola, and borage at 300 MHz (Fig. 1 shows data for borage). Similar data sets acquired for these seed types at 60 MHz showed a smaller beneficial effect of immersion on spectra quality (data not shown).

Since the impact of solvents on spectra quality was greatest in the 300 MHz NMR, the effect of treatments to improve spectral quality was best demonstrated at 300 MHz. Several solvents were screened for effects on the quality of the borage seed spectra obtained at 300 MHz. There were no observable differences in peak widths or intensities regardless of the nature of the solvent used (Fig. 2). However, the more polar deuterated solvents, DMSO and acetone (Figs. 2A and 2B, respectively), contributed undesirable peaks that obscured important portions of the seed oil spectrum. Conway and Johnson (8) found that performing spectra on samples covered with Freon^{TM} 113 improved the spectra although other researchers have not used this technique (3–7). Results from this study indicated that it was necessary to immerse the seeds in solvent to obtain the best possible spectra while the choice of solvent was dependent on other factors. Both deuterated chloroform and FC-77 were found to be acceptable, but the fluorinated material was less expensive and less toxic. Therefore, for subsequent analyses performed at 300 MHz all seeds were immersed in fluorocarbon FC-77.

Molecules with higher magnetic susceptibilities provide a medium that locally enhances the magnetic field strength. Oxygen, for example, due to unpaired electrons in its structure, has

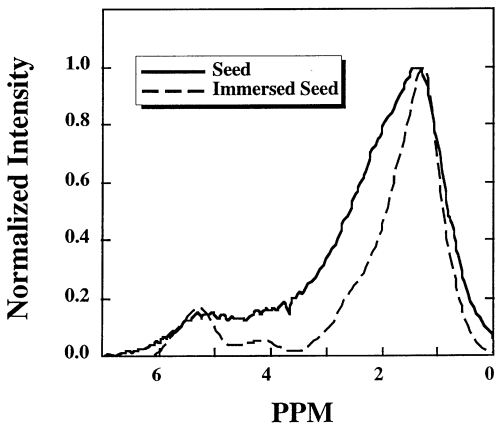

FIG. 1. Spectra of a single borage seed before and after the addition of the solvent FC-77 to the nuclear magnetic resonance (NMR) tube. Spectra were acquired at 300 MHz.

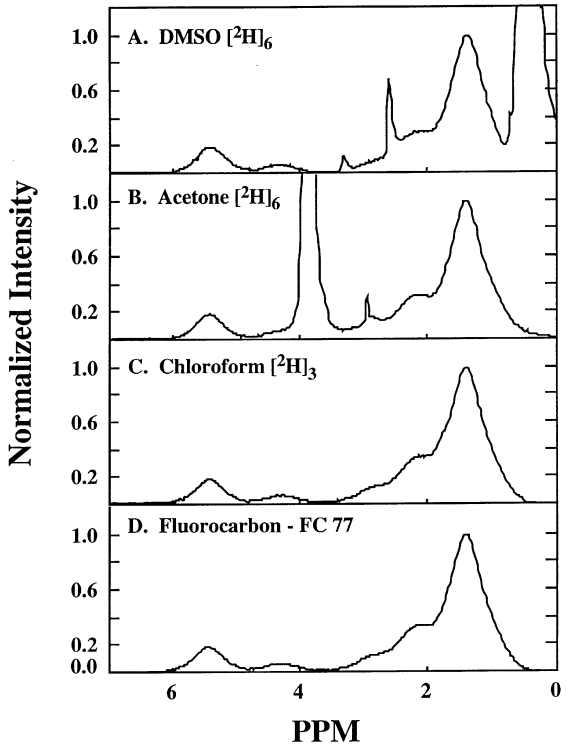

FIG. 2. Solvent effect on the *in situ* seed oil spectrum of a single borage seed analyzed by NMR at 300 MHz. All spectra were normalized to an intensity of 1.0. DMSO, dimethylsulfoxide; for other abbreviation see Figure 1.

a higher magnetic susceptibility than most of the compounds present in a seed. The presence of oxygen surrounding a seed can distort the uniform magnetic field required for NMR spectroscopy and limit the quality of the spectrum. The displacement of oxygen by the solvents described above improves the quality of the spectrum (8).

Removal of the hull material from borage (Fig. 3) narrowed the peak width of the 300-MHz spectra. Seed hull material has relatively low oil content with respect to the meats, and the hull oil often has characteristics different from the oil-bearing tissue (9). Therefore, the loss of the hull oil from the sample should not affect the NMR analytical results regarding the measurement of seed oil. Removing the hull may be a nondestructive practical method of improving the NMR signal of seeds. This may be especially important in seeds that contain an air gap between the hull and the cotyledons (e.g., borage, peanut, and sunflower) which would contribute a layer of substantially different magnetic susceptibility. Similar studies of canola and flax were not performed, as it is difficult to remove the hull of these seeds without damaging the seed meats.

Immersion of borage seed in TMS for 48 h followed by a brief drying period resulted in the appearance of a new peak in the 300 MHz NMR spectrum (Fig. 4) which is attributable to TMS. As TMS is soluble in vegetable oil it was anticipated that some or all of the TMS would dissolve in the seed oil. Integration of the area under the TMS and triglyceride peaks indicates that the concentration of TMS in the seed is 12.9% by weight of the seed oil, which substantially reduces the viscosity of

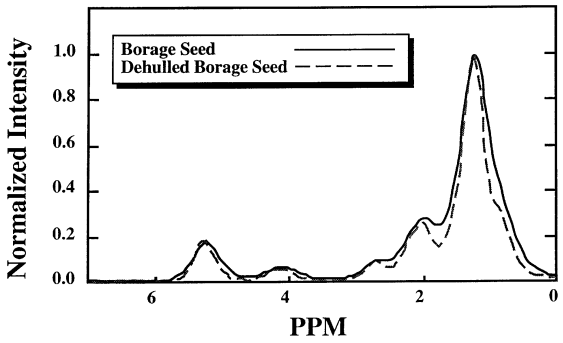

FIG. 3. Effect of removing the hull of a borage seed on the NMR oil spectrum acquired at 300 MHz. For abbreviation see Figure 1.

the oil. Comparison of the spectrum of the borage seed taken before and after TMS treatment demonstrates that the spectra of the triglyceride in the oil are comparable in peak width before and after TMS treatment. Spectra of extracted borage oil containing 5% TMS (diluted borage oil), 5% in deuterochloroform and TMS, and borage seed analyzed at 60 MHz are compared in Figure 5. From Figure 5B it is apparent that high viscosity lowers the spectral resolution of oil solutions by increasing the width of each peak in the multiplet. However, the effects of factors that cause peak broadening in seeds, such as magnetic inhomogeneity, are much larger than the effect observed for viscosity; therefore, lowering seed oil viscosity *in situ* would have little impact on improving the quality of seed spectra.

The NMR spectra of oil acquired with a 60 MHz NMR spectrometer (Fig. 5B) showed significant overlap in multiplets (e.g., at 4.2 ppm) that was not observed in spectra acquired at 300 MHz (Fig. 6B). Despite the decreased quality of oil spectra obtained at 60 MHz when compared with 300 MHz spectra, the spectra of seeds at 60 MHz were superior to those obtained at 300 MHz. Spectra of seeds acquired at 300 MHz (Fig. 6) had broader peaks and less resolution than those acquired with a 60 MHz (Fig. 5) spectrometer. Thus at 300

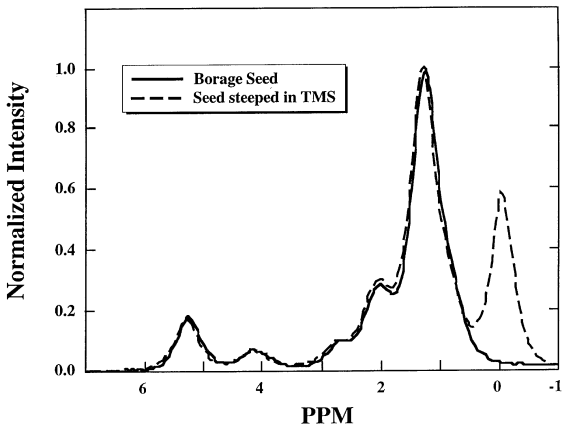

FIG. 4. The effect of pre-equilibrating a borage seed in tetramethylsilane (TMS) on the NMR spectrum acquired at 300 MHz. Both spectra were normalized to a relative intensity of 1.0. For abbreviations see Figure 1.

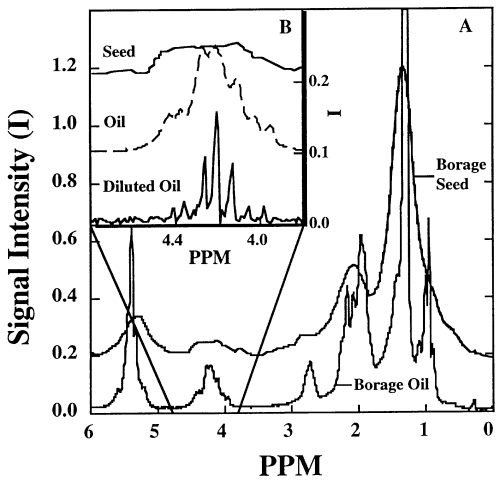

FIG. 5. (A) The NMR spectra of a borage seed immersed in FC-77 fluorocarbon (seed) and borage oil with 5% TMS (oil). (B) expansion of region indicated showing comparison with 5% borage oil in $CDCl_3$/TMS 90:10 (diluted oil). All spectra at 60 MHz. For abbreviations see Figures 1 and 4.

MHz the rank of factors contributing to spectral peak width is magnetic susceptibility >> peak multiplicity > viscosity. At 60 MHz the rank of factors contributing to peak width is magnetic susceptibility ~ peak multiplicity >> viscosity.

The observed effect of field strength on the spectra of seeds is common in other semisolid materials and is attributable to differences in magnetic susceptibility (10). For example, bubbles, fissures, and other structural irregularities which cause differences in bulk magnetic susceptibility and greatly increase peak widths as field strength increases, degrade spectra of polyethylene polymers (10). Seed structures such as cotyledons, embryos, and hulls could greatly affect the peak width if they vary in bulk magnetic susceptibility. Pockets of air that may exist among the internal seed structures could also have significant deleterious effects on spectra quality due to the high magnetic susceptibility of oxygen. Dehulled borage seeds likely displayed improved spectra compared with intact seeds due to the displacement of trapped air from between the seed coat and the cotyledons with solvent.

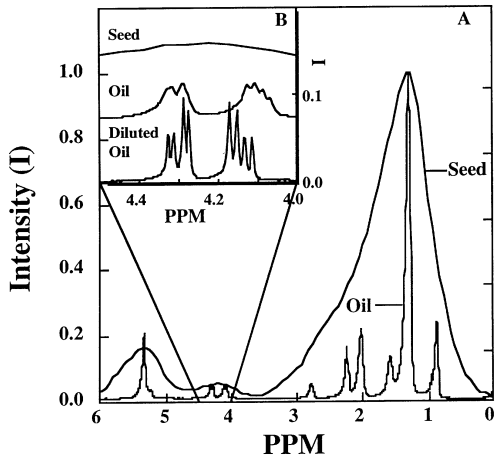

FIG. 6. (A) The NMR spectra of a borage seed immersed in FC-77 fluorocarbon (seed), borage oil with 5% TMS (oil). (B) Expansion of region indicated showing comparison with 5% cold-pressed borage oil in $CDCl_3$/TMS 90:10 (diluted oil). All spectra at 300 MHz. For abbreviations see Figures 1 and 4.

Selection of field strength may be the most important variable in selecting an NMR device for rapid analysis of seeds. Field effects broaden the spectra in higher field devices while in lower field devices peak multiplicity becomes a major factor affecting peak resolution (10). The first step in NMR analysis is optimization of the spectra, which was previously achieved by Rutar and coworkers (3,4,7). Nevertheless, for a repetitive task like seed selection in a breeding program, the quality of the spectra may be partially sacrificed if the speed of analysis can be increased. In this paper, methods to improve the quality of spectra attained from oilseeds without using time-consuming methods like [^{13}C] NMR or MAS NMR have been outlined. Immersion of the seeds in solvent greatly improved the resolution at both frequencies tested and seeds were tolerant of all solvent immersion treatments of less than 30 min. Using the methods reported, it was possible to repeatedly record the spectra of individual seeds of borage, flax, or canola in less than 1 min using a 60 MHz continuous wave NMR or a 300 MHz pulsed NMR. In the next paper of this series, the methods of analysis of lower quality spectra attained from NMR and the limitations imposed by lower quality spectra will be reported.

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