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Enhanced Quantitative Analytical Capability with a Nuclear Magnetic Resonance (NMR) Spectrometer by Use of an External Integrating Recorder

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ABSTRACT: A basic 60-MHz nuclear magnetic resonance (NMR) spectrometer (Varian EM360A) can be economically upgraded by external connection to an integrating chart recorder (Shimadzu C-R3A). The analog voltage signal corresponding to vertical pen position for signal peaks on the spectrometer's flatbed recorder can provide an input signal to the external recorder. Major benefits include improved objectivity in quantitation by digital electronic integration of peak areas, ability to reprocess spectra stored in memory, and improved tracing of fast signal peaks. Details of the interfacing procedure and a discussion of results are provided.

KEYWORDS: toxicology, chemical analysis, spectroscopic analysis

Historically, nuclear magnetic resonance (NMR) spectrometers have been provided with a capability for electronic integration of peak areas by means of tracing out the familiar "integral steps." Peak areas can then be determined as proportional to the heights of the integral steps corresponding to those peaks, that is, the increase in height of the integral trace's prestep baseline to the poststep baseline. NMR peak areas are, of course, of tremendous importance. Most obvious is the ability to determine relative numbers of nuclei producing a particular resonance signal, either for signal assignments of a pure compound or for analytical purposes in quantitatively determining mixture compositions. Other applications include observations of nuclear Overhauser effects and studies of relaxation phenomena. Of special importance in our laboratories were uses for mixture analyses relevant to pharmaceutical, forensic science, and toxicological studies. Forensic science applications are of special concern, for if evidence is to be truly probative, it should be demonstrably objective, quantitative, accurate, and reproducible, since evidence offered by courtroom expert witnesses can directly affect determinations of guilt or innocence.

The newer models of costly, computer-controlled high-field Fourier transform (FT) NMR spectrometers may provide a precise digital electronic integration of peak areas, but such a feature is not part of the basic and ubiquitous 60-MHz continuous wave NMR instruments. While integral step heights normally available with basic NMR spectrometers may be adequate for measurement of sharp, well-resolved peaks, we had found difficulty when integrat-

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ing broader, incompletely resolved peaks. In the latter case, inflection points in the integral traces may be poorly defined if the valley between two absorption peaks is high. In studies of optical purity determinations of drugs and analogs with chiral lanthanide shift reagents [*1*], separation of the peaks corresponding to the two enantiomers is often incomplete, making integral step height measurements increasingly subjective and dependent on operator experience. This is undesirable in forensic science evidential applications.

Electronic digital integrators and integrating recorders have been extensively applied to signals from chromatographic detectors, but we were not aware of reports of modern integrating recorders being applied to basic NMR spectrometers as an upgrading modification. Representatives of the manufacturers of our NMR and integrating recorder were similarly not aware of this application. We report here details of our experience using the Varian EM360A NMR and the Shimadzu C-R3A electronic digital integrating recorder.

Experimental Procedure

The Varian EM360A NMR spectrometer (Varian Assoc., Inc., Palo Alto, CA 94303) has circuit points available at Pins 7 and 8 of Jack J-4 at the rear of the electronic control console that we have used to provide positive and negative signals, respectively, to the external integrator. As supplied, Pins 7 and 8 are missing from the corresponding "D"-subminiature RS-232C Plug P-4. (Point 7 is identified as "Recorder Y Monitor" in Varian Accessory Interconnect Publication 276317-1, and Points 7 and 8 are labeled "Recorder Input" in Varian Electronics Console Interconnect Publication 276361 Rev. B176.) Lengths of insulated wire (15 cm) were soldered to pins (supplied by Bead Electronic Connectors, Coral Springs, FL 33075), inserted into the plug (as Pins 7 and 8) to the proper depth, and cemented with epoxy glue on the rear of the plug. The free ends of the wires were terminated with crimped-on spade lugs and attached to a terminal barrier strip that was epoxied onto the rear of the control console chassis adjacent to J-4. External integrating recorder signals were then taken from this terminal strip. Connections to the Shimadzu C-R3A Chromatopac integrator (Shimadzu Scientific Instruments, Inc., Columbia, MD 21046) were made by the standard supplied input connector cable through the appropriate (+) and (−) analog input leads.

Discussion and Results

Full scale vertical (y axis) deflection on the Varian NMR flatbed recorder is nominally 3.6 VDC as measured at our signal points, with Pin 7 going positive relative to Pin 8 for an upward peak. Signal voltage at the Pins 7 and 8 is independent of the NMR console's Base Line Position control but will vary with Spectrum Amplitude control settings. To establish a baseline level, the Shimadzu C-R3A must have an input signal between −1 and +5 mV. We observed that in the absence of a signal peak, some residual offset voltage could be noted at our signal points. For example, with minimum NMR spectrum amplitude setting (1 × 1), this offset was about 40 mV, beyond the C-R3A's acceptable range. This offset voltage was found to be critically dependent on the setting of the Fine Spectrum Amplitude control of the NMR, but essentially independent of the Coarse Spectrum Amplitude. Thus, clockwise rotation of the "fine" control (toward *increasing* amplitude) made the offset voltage progressively less positive, becoming near zero at settings ~5–6 and negative at higher settings, for example, about −60 mV at ~10. By proper setting of the "fine" control, then, the offset voltage in the absence of a signal peak could conveniently be brought into the −1- to +5-mV window required by the C-R3A.

This task is simplified by monitoring the voltage level on the 200-mV DC range of a digital multimeter (Keithley 130). Once set, actual signal voltage of real peaks can be roughly selected by the detented decade "coarse" amplitude control of the EM360A NMR, and the C-R3A "factor of two" attenuator can control integrator presentations. A maximum for the

input signal to the C-R3A of 1 V must be observed to avoid getting an "E" (error signal) in the integration. This corresponds to a peak deflection of only about 25% of full scale on the EM360A flatbed recorder; since the spectral trace is available from the C-R3A with selectable amplitude by using the integrator's independent attenuator, no problem results. Indeed, the C-R3A trace consistently proved much superior to the trace normally obtained (from the EM360A flatbed recorder with commercial disposable felt-tip pens) in terms of line density, consistency, and freedom from skipping. This was especially noticeable with sharp peaks or complex multiplets.

Note that other external computing integrators might have features that may further simplify some aspects of their use with the EM360A. One unit (Hitachi D-2000 Chromato-Integrator, EM Science, Cherry Hill, NJ 08034-0395) incorporates a liquid crystal display (LCD) display that directly shows the input signal voltage, accommodates an input signal from -10 mV to $+1$ V, and has some capability to offset a DC component in the input voltage. No separate digital multimeter would be needed here.

Increasing the NMR spectrum amplitude controls increased the signal voltage for peaks to a maximum of more than 10 V. Typically, $10 \times \sim 5$-6 or $100 \times \sim 5$-6 were used as NMR amplitude settings for normal samples to provide reasonable signal levels, signal-to-noise ratios, and offset voltages (in absence of a signal peak) with the C-R3A. Higher coarse amplitude settings resulted in high voltage fluctuations because of increased noise levels.

In adjusting the "fine" amplitude to set properly the offset voltage, higher values for the Filter time constant switch of the NMR could be selected, for example, 1, 2, or 5 s. This reduces "chatter" and provides some smoothing of noise, decreasing the fluctuations of the voltmeter reading.

Shorter time constants, about 0.05 s, can be selected after correction of the baseline offset voltage for actual spectral recording to avoid loss of signal resolution. With high Filter settings, 10 to 20 s should be allowed for settling of the offset voltage measurements. With NMR amplitude settings of about 10×5 resulting in peak heights of 2 to 7 cm on the flatbed recorder, integrator attenuator settings of 7 to 9 (2^7 to 2^9, that is, 128 to 512) gave reasonable integrator chart peak intensities.

The most common application of integrating recorders such as the one used here is most likely with chromatography detectors such as gas chromatography (GC) or liquid chromatography (LC). For well-behaved signal peaks, isothermal GC or isocratic LC (at constant mobile phase flow rates) should result in increasing component peak widths at greater retention times because of peak broadening. GC temperature programming or LC solvent composition gradient programming is commonly used to maintain reasonably consistent (narrow) peak widths in analyses of complex mixtures. Thus, either relatively constant or steadily increasing component peak widths result depending on whether programming is or is not used, respectively. In contrast, NMR signal peak widths will show no correlation to x (time) axis position in a spectrum. Sharp or broad peaks, singlets or multiplets, may occur at any chemical shift. In principle, this could pose a problem for routine NMR spectral recording with an external integrating recorder. The C-R3A, for example, ordinarily will increase its peak slope detection sensitivity parameter with increasing time to consistently detect broadening peaks (with less slope) anticipated in isothermal GC or isocratic LC runs. This "slope sensitivity doubling time" feature can be locked out for temperature programmed GC or solvent gradient programmed LC runs.

However, complex NMR spectra can pose a peak detection problem that may be difficult to solve. Selected parameter settings for slope sensitivity and peak width (the latter serving to discriminate against transient noise spikes and determining sampling interval) may result in failure to detect broad peaks or falsely identifying noise spikes as peaks, since there will be no relationship between NMR peak shape and time during the spectral scan. Appropriate settings in a spectral region of sharp peaks could be unsatisfactory in a region of broad peaks. One solution might involve time programming of these parameters with the integra-

tor, but this could prove tedious for complex spectra. Another difficulty could be posed by integration of groups of peaks, as for multiplets. These groups of peaks should appropriately have their areas combined if they represented a single kind of nucleus, rather than having the area of each peak in a multiplet (triplet, quartet, and so forth) separately tabulated. Here, too, appropriate control of the C-R3A can accommodate grouping of selected peaks, if desired. The required control settings could be easily justified for quality control work where similar samples are repeatedly analyzed.

For the specific quantitative applications with which we were most concerned (direct optical purity determinations with chiral shift reagents), only a small region of the spectrum is of analytical importance. This is also likely to be the case if nuclear Overhauser enhancements are being accurately measured or if mixtures are being assayed by specific peak area or height measurements for a selected resonance in each analyte. These are situations when the precise and objective digital integrations are readily obtainable with minimal "fussing" and resetting of controls. For optical purity measurements, peak areas should be preferable to peak heights since the two enantiomers may differ in their degrees of lanthanide-induced peak broadening. This could result from different geometries in the shift reagent-substrate complex and differing distances in the two enantiomers between corresponding nuclei and the lanthanide atom.

For low optical purity mixtures (where measured peaks are of similar intensity), most accurate results are obtainable using peak areas obtained by dropping verticals from the valley between two overlapping peaks to the baseline. For mixtures of higher optical purity, where the minor component may be a "bump" on the tail of the major component, use of the tangent skim method is more accurate. The C-R3A uses a simple straightline skim from baseline to valley or from valley to valley. Other data processors may provide a more sophisticated skim with an exponential extrapolation of the baseline that could prove more accurate. Appropriate setting of the baseline gradient (as a "Drift" parameter on the C-R3A) can impose the desired integration method, either by vertical lines from valley to baseline or by tangent skim. Although the baseline used for a specific integration method is not actually displayed or printed on the integrator plotter, peak height displays by the C-R3A (along with display of peak area measurements) permit the user to know unambiguously what baseline method is being applied. In some cases where a smaller poorly resolved shoulder follows a bigger peak, detection of the shoulder could be difficult. We found that scanning the spectrum in the reverse direction (from higher to lower field), by putting the small shoulder on the leading rather than the trailing edge of the big peak, could facilitate peak detection.

Applications of the C-R3A interfaced to the EM360A are presented in the accompanying figures. The chiral lanthanide shift reagent, tris[3-(heptafluoropropylhydroxymethylene)-*d*-camphorato]europium(III), (*1*), known as $Eu(hfc)_3$ or $Eu(hfbc)_3$, has been added to deuterochloroform ($CDCl_3$) solutions of 2,5-dimethoxy-4-ethylamphetamine, DOEt, (*2*), of known optical purity. Illustrated results do *not* represent optimum conditions for optical purity determinations of *2;* full details of this method are being published elsewhere. To provide examples of cases where *objective* reliable measurements of peak areas might prove most demanding, we have selected "difficult" conditions which would be especially problematic for quantitation by normal step integrals, involving broad, incompletely resolved resonances. Figure 1 shows the spectral expansion of the methine resonance for a sample of (−) and (+)-*2* prepared by direct weighings of the pure enantiomers in a ratio of 86.3 to 13.7. Figure 2 shows the expansion of the region for the CH_3 doublets of *2* for a (−)-*2* to (+)-*2* ratio of 33.6:66.4. Note that the doublets of the two enantiomers overlap severely, leading to the integral traces shown. Inflection points are hard to distinguish. Tables 1 and 2 summarize the results obtained from C-R3A integrations and by integral step height measurements. The latter were made by two (Table 1) or four (Table 2) independent workers. Average values and standard deviations are presented. Electronic integrations generally provide better precision, and perhaps most important, more freedom from subjective worker-to-

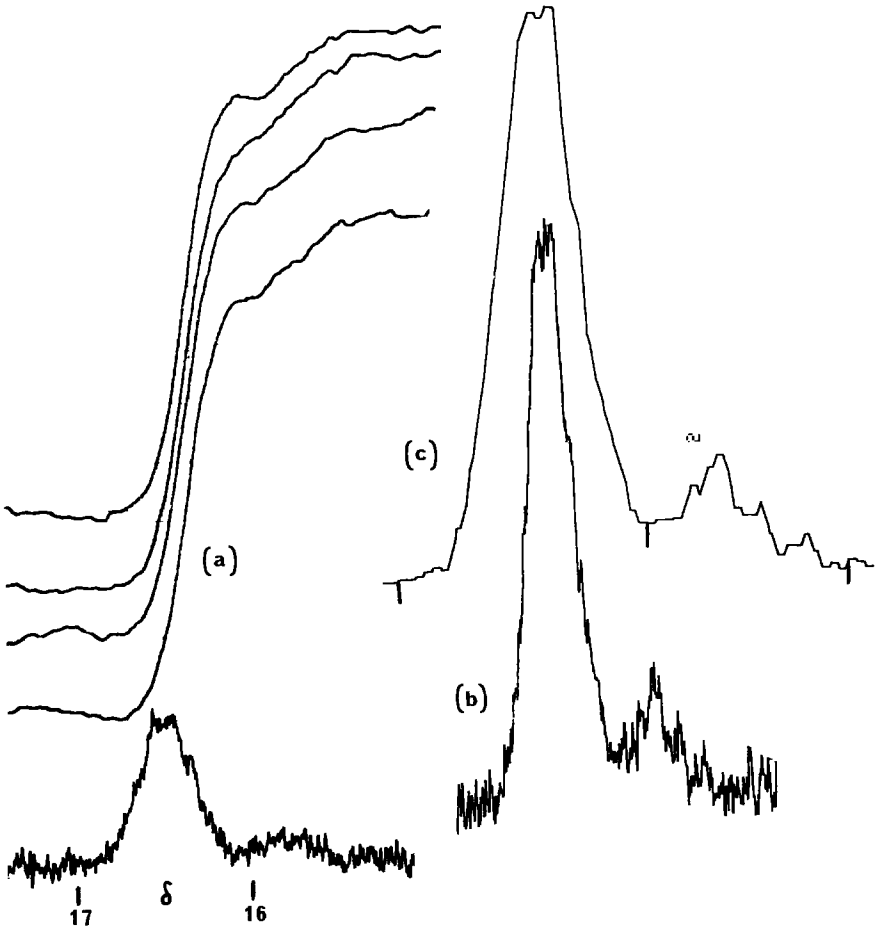

FIG. 1—*Methine resonance for a 0.217 molal solution of* 2 *in* $CDCl_3$ *at 28° with a* 1:2 *molar ratio of 1.29. Ratio of (−)-2 (downfield) to (+)-2 (upfield) is 86.3:13.7. Accompanying integral traces are shown. NMR conditions for absorption trace: 10-ppm sweep width, 0.1-s filter, 0.1-milligauss rf power, 5-min sweep time. Same conditions for integral traces, except 0.2-s filter and 1-min sweep time. Region scanned: ~δ17 to 15 ppm. C-R3A width: 10 s.* (a) *NMR recorder traces.* (b) *Representative absorption trace as recorded on C-R3A.* (c) *Reprocessed spectrogram [of trace* (b)*] from C-R3A memory. Note peak detection marks in* (c), *(emphasis added). Different chart speeds were used in* (b) *and* (c). *C-R3A traces were obtained as for integral traces in* (a), *except with 5-min sweep time. See text.*

worker variation. Interestingly, it appears that an individual may report excellent precision in measuring integral steps but worker-to-worker variations can be substantial, as seen in Table 2. This is especially undesirable in forensic science analyses.

Use of an external digital voltmeter for improved accuracy in NMR integrations had been described earlier [*2,3*]. Experimental protocol and results using the usual built-in NMR spectrometer step integrator have also been reported [*4*]. The particular virtue of using a modern computing integrator as an external upgrading accessory lies in the automatic and objective measurements for poorly separated peaks by using appropriate automatic algorithms. The accessory's cost-effectiveness is enhanced by the ability to use the integrator for more conventional applications with chromatographic detectors as well. An unexpected additional use for the C-R3A that we found was for improved accuracy in measuring spac-

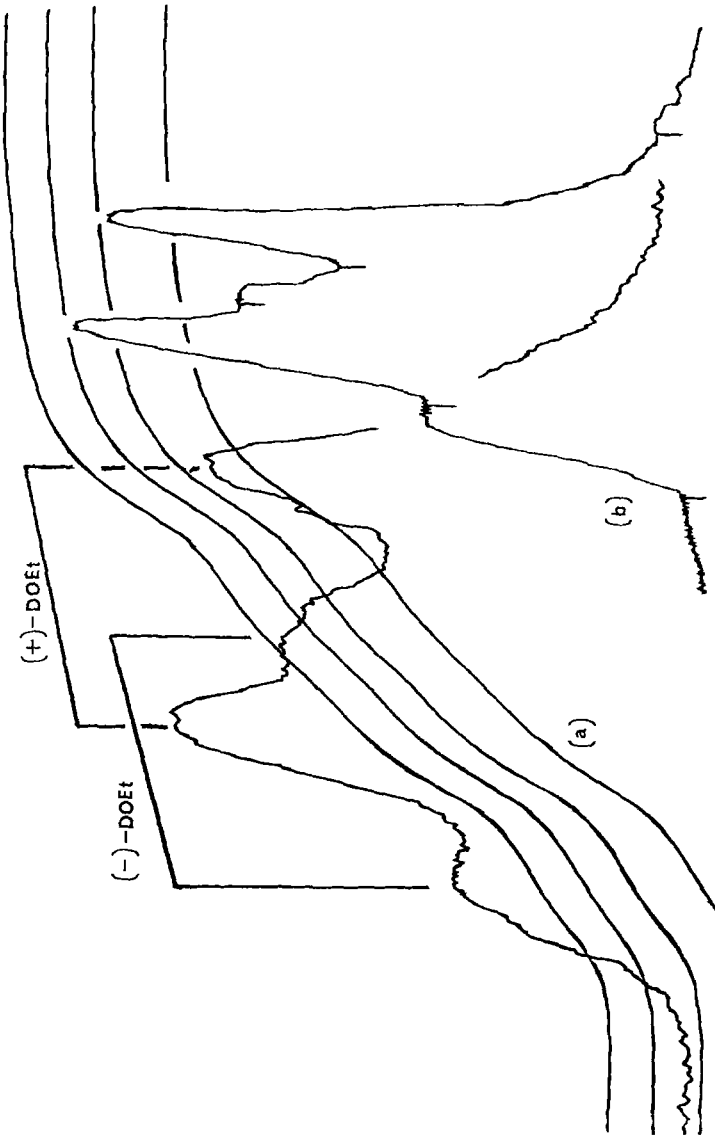

FIG. 2—CH_3-CH *resonance for a 0.277 molal solution of DOEt, 2, with a 1:2 molar ratio of 0.0175. Ratio of (−)-2 (downfield) to (+)-2 (upfield) is 33.6:66.4. NMR conditions for absorption trace as recorded on NMR in* (a)*: 0.5-ppm sweep width, 0.2-s filter, 0.1-milligauss rf power, 2-min sweep time. Same conditions were used for integral traces, except with 1-min sweep time. NMR recorder traces are shown in* (a)*. Representative absorption trace as recorded on C-R3A is shown in* (b)*: width 2 s. C-R3A traces were typically obtained as for absorption trace in* (a)*, except with 0.05-milligauss rf power. Different chart speeds were used in* (a) *and* (b)*. Note peak detection marks in* (b)*, (emphasis added). Region scanned: ~82.1 to 1.6 ppm. Indicated doublets reflect coupling constant of ~6.4 Hz. See text.*

TABLE 1—*Summary of results for CH resonance. (Actual ratio (by weight) = 86.3:13.7.)*[a]

C-R3A, %		Integral Step Heights, mm (*a*)		(*b*)	
84.28	15.72	72	13	69.8	10.9
84.66	15.34	73	12	75.2	13.7
85.47	14.53	75	14	72.0	13.8
83.72	16.28	71	11	70.5	14.2
86.58	13.42	$\bar{X}$ = 72.75	12.5	71.88	13.15
$\bar{X}$ = 84.94	15.06	SD = 1.48	1.19	2.08	1.31
SD = 0.99	0.99	(85.34)	(14.66)	(84.53)	(15.47)
		Average of (*a*) and (*b*):			
		84.94	15.06		
		SD = 0.41	0.41		

[a]C-R3A values are presented as normalized area percentages from five separate scans and calculations. Integral step height values show actual separate heights as measured independently by two different people, (*a*) and (*b*). Mean step heights and standard deviations, as well as normalized area percentages and standard deviations, are also shown.

TABLE 2—*Summary of results for CH_3 resonance. (Actual ratio (by weight) = 33.6:66.4.)*[a]

					$\bar{X}$	SD
C-R3A						
34.45	31.09	34.75	33.00	34.41	33.54	1.37
65.55	68.91	65.25	67.00	65.59	66.46	1.37
Pk. 1	Pk. 2	Pk. 3	Pk. 4	Pks. (1 + 3)	Pks. (2 + 4)	%, (−)-2:(+)-2
INTEGRAL STEP RESULTS						
(*a*) $\bar{X}$ = 21.75	48.93	24.45	56.33	46.20	105.25	30.51:69.50
SD = 0.99	2.77	2.06	1.63	2.87	2.93	1.90 1.90
(*b*) $\bar{X}$ = 24.36	46.75	30.13	49.13	54.50	95.88	36.24:63.76
SD = 0.96	1.25	0.96	1.67	1.06	0.96	0.61 0.61
(*c*) $\bar{X}$ = 20.75	41.75	31.75	58.50	52.50	100.25	34.37:65.63
SD = 1.09	0.83	1.09	0.87	0.50	1.64	0.50 0.50
(*d*) $\bar{X}$ = 25.25	42.50	27.50	37.75	52.75	80.25	39.67:60.33
SD = 1.92	1.12	1.66	1.48	0.43	2.05	0.73 0.73

[a]Peaks 1 to 4 refer to peaks going from low to high field in Fig. 2. Values (*a*) to (*d*) were obtained independently by four individuals, and represent averages of four integral scans as shown in Fig. 2*a*. Peaks 1 and 3 correspond to the minor enantiomer and Peaks 2 and 4 to the major. C-R3A data reflects normalized area percentages for sum of areas of Peaks (1 and 3) versus Peaks (2 and 4) as calculated from five separate scans. See footnote *a* for Table 1.

ings between peaks, as for coupling constant determinations. Since peak "retention times" can be assigned for the maxima of identified peaks, differences in these retention times can be converted to coupling constants (or enantiomeric shift differences or other $\Delta\nu$ values) based on NMR spectrometer sweep width and sweep time settings. The interfacing of a microcomputer to a Varian EM360 NMR has been previously described [5]. The interconnec-

tion requirements were far more complex than those described in this present work, as the modification goals were primarily to achieve computer control of the NMR, signal averaging, and so forth, and not specifically to provide improved integration capability. Most users should find it easier to match the spectrometer's output voltage to the external recorder by simple adjustment of the Spectral Amplitude controls, as reported here, than by fabrication of the interface electronics required in Ref 5.

The main point of this present work is the demonstration of an extremely convenient use of an external integrating recorder to provide cost-effectively more objective and accurate integrations using an inexpensive NMR and minimal alteration to the spectrometer. The computing integrator offers an important advantage compared to manual integral step height measurements or the external digital voltmeter method by removing the subjective aspect from determining the beginning or ending of even severely overlapped peaks. The diagram for the simple interconnection is shown in Fig. 3.

Acknowledgments

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Note

The technique described here was applied to numerous mixtures under varying conditions leading to different degrees of peak overlap. Acceptable accuracy, comparable to that reported in the above illustrative cases, was achieved in all cases. Nonetheless, we would agree with the cautionary note posed by a reviewer, who had

> reservations about the ability of this technique to provide reliable measurements of peak areas for examples such as those depicted in Figures 1 and 2. Reproducibility of measurements made on severely overlapping peaks can mask problems with the accuracy of those measurements for

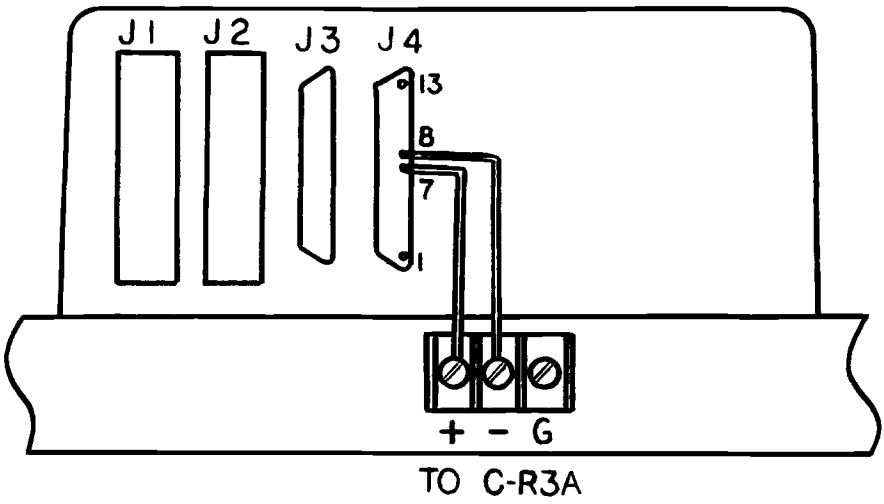

FIG. 3—*Rear of EM360A electronics control console showing location for terminal barrier strip cemented to NMR spectrometer chassis. Short lengths of wires from Pins 7 and 8 of J-4 are added as shown, through the rear of Plug P-4, to provide signal takeoff points to the external integrator.*

true individual peak intensities. On the other hand, the technique appears to be justified for situations where similar samples are repeatedly analyzed as long as the parameters are carefully chosen for integration. It should also be useful for the measurement of clearly defined peak areas.

We are grateful for this reviewer's comment and suggestion.

For details of work with *2*, see Hatzis, A. and Rothchild, R., *Journal of Pharmaceutical and Biomedical Analysis*, in press.

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