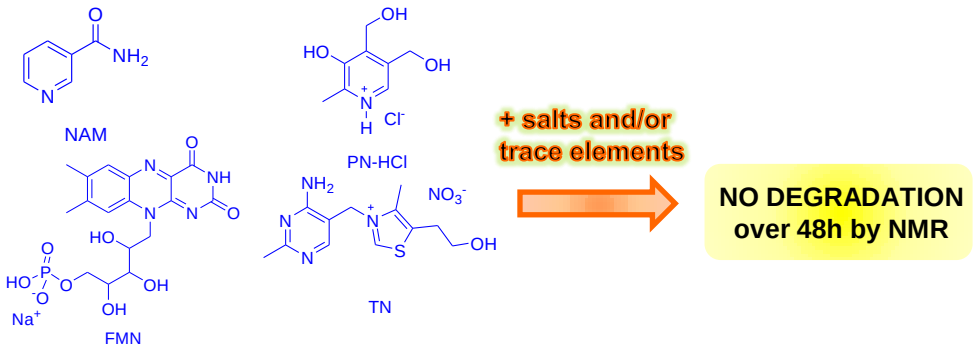
***Graphical Abstract**



***Highlights (for review)**

- We applied NMR spectroscopy in the study of stability of hydrophilic vitamins.
- Stability of vitamins was investigated in the presence of salts and trace elements.
- No degradative effects were observed in presence of salts and trace elements.
- The stability of vitamins solutions was assessed at least for 48 h.

# Stability of hydrophilic vitamins mixtures in the presence of electrolytes and trace elements for parenteral nutrition: a nuclear magnetic resonance spectroscopy investigation

Gloria Uccello-Barretta[a,*], Federica Balzano[a], Federica Aiello[a], Niccolò Falugiani[a] and Ielizza Desideri[b]

[a]*Dipartimento di Chimica e Chimica Industriale, Università degli Studi di Pisa, via G. Moruzzi 3, 56124 Pisa, Italy*

[b]*U.O. Farmaceutica – Gestione del farmaco, Azienda Ospedaliero Universitaria Pisana, via Roma 67, 56126 Pisa, Italy*

Correspondance to:

Prof. G. Uccello-Barretta, Dipartimento di Chimica e Chimica Industriale, Università di Pisa, Via G. Moruzzi 3, 56124 Pisa, Italy. Tel: +39 050 2219232; fax: +39 050 2219260. E-mail address: *gloria.uccello.barretta@unipi.it*

**Non-standard abbreviations**

AIO: all-in-one

DOSY: Diffusion Ordered SpectroscopY

FDP: fructose 1,6-diphosphate

FMN: riboflavin-5'-phosphate

NAM: nicotinamide

PN-HCl: pyridoxine hydrochloride

TN: thiamine nitrate

TPN: total parenteral nutrition

**Abstract**

In Total Parenteral Nutrition (TPN), especially in the case of preterm infants, simultaneous administration of vitamins and trace elements is still a problematic issue: guidelines put in evidence the lack of specific documentation. In this work NMR spectroscopy was applied to the study of vitamins (pyridoxine hydrochloride, thiamine nitrate, riboflavin-5'-phosphate and nicotinamide) stability in presence of salts and trace elements. Vitamins in $D_2O$ were first analyzed by $^1H$ NMR spectroscopy in absence of salts and trace elements; changes in chemical shifts or in diffusion coefficients, measured by NMR DOSY technique, were analyzed. The effects of salts and trace elements on single vitamins and on their admixtures were then investigated by performing quantitative analyses during 48 h. Selected vitamins are subject to intermolecular interactions. No degradative effects were observed in presence of salts and trace elements. Only riboflavin-5'-phosphate is subject to precipitation in presence of divalent cations; however, at low concentration and in presence of other vitamins this effect was not observed. Solutions analyzed, in the condition of this study, are stable for at least 48 h and vitamins and trace elements can be administered together in TPN.

**KEYWORDS:** Nuclear Magnetic Resonance; Total Parenteral Nutrition; Hydrophilic Vitamins; Trace Elements; Electrolytes

## 1. Introduction

Total Parenteral Nutrition (TPN) is a needful feeding mode in the case of very low birth-weight premature infants [1-3]. All-in-one (AIO) admixtures are nowadays considered the best infusion system for the administration of TPN [4,5]: all substrates are admixed in a single container and simultaneously administered through one intravenous line. This method, besides reduction in costs and practical advantages for clinicians and nursing staff, allows to reduce the number of manipulation and so contamination risk, and the fact that only one intravenous line is required leads to reduced risk of infection and makes this method particularly safe especially for premature infants [4,5].

However, problems of compatibility and stability can occur when so different compounds are mixed together [6-16], in particular in AIO admixtures addressed to premature infants, considering the low final volume that can be administered [6]. Vitamins are considered the least stable components of the admixture; it is generally recommended not to add vitamins and trace elements to the same admixture [5]. Oxidation of ascorbic acid, the least stable among the water-soluble vitamins, has been widely investigated and the role of catalyst of some bivalent ions, especially copper, has been established [12,13,16]. Ascorbic acid is also involved in reduction of selenite ion to elemental selenium that could precipitate [16]. However, guidelines highlight the lack of specific documentation [5] on the compatibility of trace elements with other vitamins and suggest the administration of vitamins and trace elements on alternated 12 h every day [11] or by two separated intravenous applications [5]. Nevertheless, especially for neonates, the need of a continuous administration of all the nutrients, and the complexity to use two intravenous lines are also claimed: a general accepted compromise is to add vitamins and trace elements to the admixture immediately before the administration to minimize interactions and eventual degradation [10]. In any case, vitamins stability should be proved at least during infusion period; furthermore, a higher stability would allow the preparation of complete AIO admixtures in hospital pharmacies [4].

Considering the clinical relevance of this issue, Nuclear Magnetic Resonance (NMR) spectroscopy was applied to the study of vitamins stability in presence of salts and trace elements up to 48 h, in experimental conditions mimicking pharmaceutical formulations, as a non-invasive analytical procedure. The investigation focused on water-soluble vitamins, in particular pyridoxine hydrochloride (PN-HCl), thiamine nitrate (TN), riboflavin-5'-phosphate (FMN) and nicotinamide (NAM) (Figure 1). Among the several NMR parameters, translational diffusion coefficients were exploited, which are remarkably responsive to phenomena of intermolecular aggregation, together with chemical shifts as local parameters, well reflecting structural changes due to eventual degradation processes.

**FIGURE 1**

## 2. Material and Methods

### *2.1. Materials*

Nicotinamide, pyridoxine hydrochloride, thiamine nitrate, riboflavin-5'-phosphate, zinc chloride, copper chloride, manganese chloride tetrahydrated and phosphate buffer were purchased from Sigma Aldrich (St. Louis, USA). Deuterated solvents, deuterium oxide and dimethyl sulfoxide, were purchased from Deutero GmbH (Kastellaun, Germany). Calcium gluconate (Polichimica, Bologna, Italy), magnesium sulfate (Fagron, Bologna, Italy), Esafosfina® (Biomedica Foscama, Ferentino (FR), Italy) and Peditrace® (Fresenius Kabi, Isola della Scala (VR), Italy) were commercially available.

## *2.2. NMR measurements*

NMR measurements were performed on a Varian Inova spectrometer (Agilent Technologies, Santa Clara, USA) operating at 600 MHz for $^{1}H$ nuclei. The temperature was controlled to ±0.1 °C. DOSY (Diffusion Ordered SpectroscopY) experiments were carried out by using a stimulated echo sequence with self-compensating gradient schemes, a spectral width of 8000 Hz and 64 K data points. Gradient strength was varied in 20 steps (16 transients each), while values of the diffusion delay and the gradient pulse duration were optimized to obtain an approximately 90–95% decrease in the resonance intensity at the largest gradient amplitude. The baselines of all arrayed spectra were corrected prior to processing the data. The data were processed with the DOSY macro (involving the determination of the resonance heights of all the signals above a pre-established threshold and the fitting of the decay curve for each resonance to a Gaussian function) to obtain pseudo two dimensional spectra with NMR chemical shifts along one axis and calculated diffusion coefficients along the other.

### *2.2.1. $^{1}H$ NMR characterization of vitamins.*

Nicotinamide (15 mM, 600 MHz, $D_2O$, 25 °C) δ (ppm): 8.79 (1H, $H_a$, dd, $J_{a-b}$ = 2.2, Hz, $J_{a-c}$ = 0.8 Hz), 8.57 (1H, $H_d$, dd, $J_{d-c}$ = 5.0 Hz, $J_{d-b}$ = 1.7 Hz), 8.10 (1H, $H_b$, dt, $J_{b-c}$ = 8.0 Hz, $J_{b-a}$ = 2.2. Hz, $J_{b-d}$ = 1.7 Hz), 7.45 (1H, $H_c$, ddd, $J_{c-b}$ = 8.0 Hz, $J_{c-d}$ = 5.0 Hz, $J_{c-a}$ = 0.8 Hz).

Pyridoxine hydrochloride (15 mM, 600 MHz, $D_2O$, 25 °C) δ (ppm): 8.01 (1H, $H_a$, s), 4.87 (2H, $H_c$, s), 4.67 (2H, $H_b$, s), 2.50 (3H, $H_d$, s).

Thiamine nitrate (15 mM, 600 MHz, $D_2O$, 25 °C) δ (ppm): 7.91 (1H, $H_a$, s), 5.31 (2H, $H_c$, s), 3.74 (2H, $H_f$, t, $J_{f-e}$ = 6.0 Hz), 3.04 (2H, $H_e$, t, $J_{e-f}$ = 6.0 Hz), 2.42 (3H, $H_d$, s), 2.35 (3H, $H_b$, s).

Thiamine nitrate (15 mM, 600 MHz, DMSO, 25 °C) δ (ppm): 9.46 (1H, $H_g$, s), 8.06 (1H, $H_a$, s), 7.13 (2H, $NH_2$, br s), 5.33 (2H, $H_c$, s), 5.23 (1H, OH, t, $J_{OH-f}$ = 4.9 Hz ), 3.65 (2H, $H_f$, dt, $J_{f-e}$ = 5.5 Hz, $J_{f-OH}$ = 4.9 Hz), 3.04 (2H, $H_e$, t, $J_{e-f}$ = 5.5 Hz), 2.51 (3H, $H_d$, s), 2.37 (3H, $H_b$, s).

Riboflavin-5'-phosphate (15 mM, 600 MHz, $D_2O$, 25 °C) δ (ppm): 7.65 (1H, $H_a$, s), 7.50 (1H, $H_b$, s), 4.92 (1, $H_{e/e'}$, dd, $J_{e-e'}$ ($J_{e'-e}$) = 14.8 Hz, $J_{e-f}$ ($J_{e'-f}$) = 7.4 Hz), 4.57 (1H, $H_{e'/e}$, dd, $J_{e'-e}$ ($J_{e-e'}$) = 14.8 Hz, $J_{e'-f}$ ($J_{e-f}$) = 2.3 Hz), 4.25 (1H, $H_f$, ddd, $J_{f-e}$ ($J_{f-e'}$) = 7.4, $J_{f-g}$ = 4.9 Hz, $J_{f-e'}$ ($J_{f-e}$) = 2.3 Hz), 3.98 (1H, $H_{i/i'}$, m), 3.93 (1H, $H_{i'/i}$, m), 3.92 (1H, $H_h$, m), 3.87 (1H, $H_g$, dd, $J_{g-h}$ = 7.0 Hz, $J_{g-f}$ = 4.9 Hz), 2.38 (3H, $H_c$, s), 2.25 (3H, $H_d$, s).

## 3. Results and Discussion

Pure vitamins (Figure 1) were first analyzed by $^1$H NMR without salts and trace elements in $D_2O$ solutions, i.e. in the administration conditions, and compared to binary, ternary and quaternary solutions (15 mM) in order to investigate if any interaction takes place when they are mixed together.

Changes in chemical shifts can be caused by proton transfer phenomena, which can be evaluated by comparison with buffered solutions (pH 7.4, phosphate buffer). Alternatively, intermolecular interactions between vitamins could occur, as well as self-aggregation phenomena which are well reflected in diffusion coefficient (D) changes.

Translational diffusion coefficient, measured by using the NMR DOSY technique [17-19], is a size-dependent parameter, directly related to hydrodynamic radius $r_H$ (eq. 1):

$$D = \frac{kT}{6\pi\eta r_H} \quad (1)$$

where $k$ is the Boltzmann constant, $T$ the absolute temperature, and $\eta$ the solution viscosity. In the fast exchange conditions of equilibrium between free and bound species (eq. 2)

$$A + B \rightleftarrows AB \quad (2)$$

observed parameter ($D_{obs}$) is the weighted average of its value in bound ($D_b$) and free ($D_f$) states (eq. 3)

$$D_{obs} = x_b D_b + (1 - x_b) D_f \quad (3)$$

where $x_b$ is the molar fraction of bound species. Thus, in the analysis of multicomponent solutions, a decrease of diffusion coefficient of one component is indicative of an aggregation phenomenon.

Only negligible chemical shifts variations of each vitamin were measured in the binary mixtures with the exception of mixtures containing PN-HCl, where relevant low-frequencies shifts up to -0.30 ppm of pyridoxine protons were detected, in particular in the presence of thiamine and for the nucleus in ortho to the positively charged nitrogen atom (Table 1, Figure 1). However, co-presence of vitamins in binary, ternary and quaternary mixtures leaves nearly unchanged the diffusion coefficient of each component, allowing us to rule out the occurrence of significant intermolecular interactions. No such chemical shifts changes were detected in buffered solution, pointing out the role of proton exchange phenomena rather than intermolecular association processes.

**TABLE 1**

The effect of salts and trace elements on single vitamins and on their admixtures was then evaluated. A standard solution used in TPN for premature infants includes three salts, fructose 1,6-diphosphate (Esafosfina®, FDP), calcium gluconate and magnesium sulfate, whose concentrations are remarkably higher than those of the vitamins studied (Table 2). Concentrations of trace elements, added through a formulation denominated Peditrace®, are, instead, comparable with those of vitamins or extremely lower (Table 2). Therefore, among trace elements selected salts were tested.

**TABLE 2**

For each vitamin, three samples containing one of the main salts plus one containing all the three salts were prepared. Concentration ratios were modified with respect to operative ones in order to maintain vitamin concentration 15 mM; according to the solubility of the salts, salt/vitamin ratios in these samples were 1.5 for calcium gluconate and 6 for magnesium sulfate and FDP.

Among the vitamins, PN-HCl and FMN are particularly sensitive to salts presence. In particular, PN-HCl (Table 3) undergoes significant variations of chemical shifts in presence of calcium gluconate and FDP, with the same pattern already observed in the binary mixtures with thiamine or riboflavin-5'-phosphate (Table 1). Above said effect suggests an ionic interaction between gluconate ion or phosphate group with the positively charged site of the vitamin. In the quaternary mixture, where low-frequency shifts lie between those caused by gluconate and phosphate (Table 3), also a decrease of the diffusion coefficient of PN-HCl till to 4.5 x $10^{-10}$ $m^2s^{-1}$ was detected, which could be attributed to self-aggregation processes of the vitamin, promoted by the ionic strength increase. The self-aggregating propensity of the vitamin is demonstrated by the diffusion coefficient responsiveness to concentration gradients. PN-HCl diffusion coefficient decreases from 6.7 x $10^{-10}$ $m^2s^{-1}$ at 0.24 mM till to 5.4 x $10^{-10}$ $m^2s^{-1}$ at 15 mM, thus reflecting the increase of the molar fraction of self-aggregated vitamin.

**TABLE 3**

The preparation of the samples for analogous tests on FMN revealed phenomena of precipitation due only to divalent cations. No precipitation was observed in presence of sodium fructose 1,6-diphosphate. In this case titrations of FMN with magnesium sulfate, calcium gluconate, and selected salts of Peditrace® (zinc chloride and copper chloride) were performed. For FMN 15 mM, in the presence of one equivalent of magnesium sulfate, calcium gluconate, zinc chloride and copper chloride, a 70%, 80%, 85% and 90% of precipitation was respectively detected by NMR (Figure 2).

**FIGURE 2**

A minor precipitation was detected at lower concentrations of FMN (40% at 1 mM in the presence of one equivalent of zinc chloride), with a further precipitation of 5% of FMN at the operative 1:4 vitamin/salt ratio. For FMN 0.1 mM (nearer to operative conditions of parenteral nutrition) Peditrace® was employed in the operative ratio. Quantitative analysis shows no precipitation of FMN within an hour from sample preparation, however, after 6 and 24 h a 5% decrease in areas of signals was detected.

198 Interestingly, no precipitation of FMN was observed in a quaternary mixture containing the
199 vitamins at components ratios employed in the commercially available Soluvit® solution neither in
200 1:1 nor in 1:4 ratio with zinc chloride.
201 For FMN 0.1 mM in a simulated Soluvit® mixture, the addition of Peditrace® did not cause any
202 precipitation after 24 h and 48 h from preparation. No further changes in shape, position or areas of
203 signals were observed, according to lack of degradative phenomena.
204 In order to gain more insight into the origin of this phenomenon, the diffusion coefficient of each
205 vitamin was measured in $D_2O$ in comparison with the quaternary mixture (Table 4). Pyridoxine and
206 thiamine diffusion coefficients did not undergo significant changes in the quaternary mixture, in
207 spite of the very high molar excess of nicotinamide (about 30 to 1 for TN and FMN and 15 to 1 for
208 PN-HCl). On the contrary, FMN diffusion coefficient showed a relevant increase from the averaged
209 value of $3.5 \times 10^{-10}$ $m^2s^{-1}$ for pure FMN, till to $4.9 \times 10^{-10}$ $m^2s^{-1}$ in the quaternary mixture. Veselkov
210 et al. [20] already demonstrated by $^1H$ NMR experiments that nicotinamide inhibits the self-
211 aggregating propensity of FMN especially at NAM/FMN molar ratios greater than 30, that is in
212 keeping with the increase of the diffusion coefficient of FMN in the quaternary mixture. As a matter
213 of fact, the decrease of molar fractions of self-aggregating species of FMN in favor of FMN/NAM
214 heterocomplexes is expected to produce a decrease of molecular sizes and, hence, an increase of the
215 diffusion coefficient (eq. 1). The low effect of above said intermolecular interaction on the diffusion
216 coefficient of NAM is due to the high molar excess of NAM in the mixtures. It is noteworthy that
217 the same effect was detected in unbuffered and buffered solutions.
218
219 **4. Conclusions**
220 In conclusion, NMR spectroscopy has a relevant potential in the study of pharmaceutical
221 formulations, guaranteeing the required specialist knowledge in the pharmaceutical practice. It
222 provides characterization tools that are complementary to chromatographic methods [21-23],
223 highlighting interaction phenomena, in addition to eventual degradative processes. As a matter of

fact, we demonstrated that salts and trace elements used in parenteral nutrition do not provoke degradation of the vitamins studied and are stable for at least 48 h, in favor of the simultaneous administration of vitamins and trace elements in Total Parenteral Nutrition. Among the four vitamins, only riboflavin-5'-phosphate is subject to precipitation in presence of divalent cations, but the formation of heteroassociation complexes with nicotinamide minimizes precipitation, thus guaranteeing the integrity of the formulation during the time.

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287 **TABLES**

**Table 1** - Variations of chemical shift ($\Delta\delta = \delta_{mix} - \delta_{free}$, ppm, 600 MHz, $D_2O$, 25°C) of protons of PN-HCl (15 mM) and diffusion coefficient (D, x $10^{10}$ $m^2s^{-1}$) in equimolar binary, ternary and quaternary mixtures with NAM, TN and FMN.

| mixture | Δδ | | | | D |
|---|---|---|---|---|---|
| | $H_a$ | $H_b$ | $H_c$ | $H_d$ | PN-HCl |
| PN-HCl | | | | | 5.4 |
| PN-HCl/TN | - 0.23 | - 0.03 | - 0.09 | - 0.09 | 5.3 |
| PN-HCl/FMN | - 0.12 | - 0.03 | - 0.07 | - 0.06 | 5.1 |
| PN-HCl/NAM | - 0.06 | - 0.02 | - 0.03 | - 0.03 | 5.4 |
| PN-HCl/TN/FMN | - 0.30 | - 0.06 | - 0.13 | - 0.12 | 5.1 |
| PN-HCl/FMN/NAM | - 0.15 | - 0.04 | - 0.08 | - 0.07 | 5.0 |
| PN-HCl/TN/NAM/ | - 0.24 | - 0.04 | - 0.10 | - 0.09 | 5.7 |
| PN-HCl/NAM/TN/FMN | - 0.30 | - 0.07 | - 0.14 | - 0.13 | 5.1 |

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**Table 2** - Concentration of selected vitamins, salts and trace elements in a standard solution for pediatric PN.

| Component | Concentration (mM) |
|---|---|
| Nicotinamide | 0.27 |
| Pyridoxine | 0.02 |
| Riboflavin-5'-phosphate | 9 x $10^{-3}$ |
| Thiamine | 8 x $10^{-3}$ |
| Calcium gluconate | 12.5 |
| Magnesium sulfate | 1.25 |
| Esafosfina® | 2.71 |
| $ZnCl_2$ | 0.03 |
| $CuCl_2$ | 2.6 x $10^{-3}$ |
| $MnCl_2$ | 0.15 x $10^{-3}$ |
| $Na_2SeO_3$ | 0.2 x $10^{-3}$ |
| NaF | 0.025 |
| KI | 0.07 x $10^{-3}$ |

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**Table 3** - Variations of chemical shift ($\Delta\delta$, = $\delta_{obs} - \delta_{free}$, ppm, 600 MHz, $D_2O$, 25°C) of protons of PN-HCl (15 mM) and diffusion coefficient (D, x $10^{10}$ $m^2s^{-1}$) in solutions with calcium gluconate (Ca), Esafosfina® (FDP) and magnesium sulfate (Mg).

| | Δδ | | | | D |
|---|---|---|---|---|---|
| mixture | $H_a$ | $H_b$ | $H_c$ | $H_d$ | PN-HCl |
| PN-HCl | | | | | 5.4 |
| PN-HCl/Ca | - 0.12 | - 0.02 | - 0.05 | - 0.04 | 5.4 |
| PN-HCl/FDP | - 0.40 | - 0.06 | - 0.16 | - 0.15 | 5.5 |
| PN-HCl/Mg | - 0.01 | - 0.02 | - 0.03 | - 0.01 | 5.1 |
| PN-HCl/Ca/Mg/FDP | - 0.29 | - 0.06 | - 0.13 | - 0.12 | 4.5 |

290

**Table 4** - Diffusion coefficients (D, x $10^{10}$ $m^2s^{-1}$) of NAM (3.34 mM), PN-HCl (0.24 mM), FMN (0.10 mM) and TN (0.095 mM) free ($D_f$) and in mixture ($D_{mix}$).

| Vitamin | $D_f$ | $D_{mix}$ |
|---|---|---|
| NAM | 7.5 | 7.8 |
| PN-HCl | 6.7 | 6.3 |
| FMN | 3.5 | 4.9 |
| TN | 5.1 | 5.1 |

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## FIGURES LEGEND

**Figure 1** - Structure of vitamins with numbering scheme for NMR analysis.

**Figure 2 -** $^{1}$H NMR (600 MHz, $D_2O$, 15 mM, 25 °C) spectral regions of aromatic protons of: a) FMN; b) FMN/$MgSO_4$ 1:1; c) FMN/calcium gluconate 1:1; d) FMN/$ZnCl_2$ 1:1; e) FMN/$CuCl_2$ 1:1.

**Figure 1**

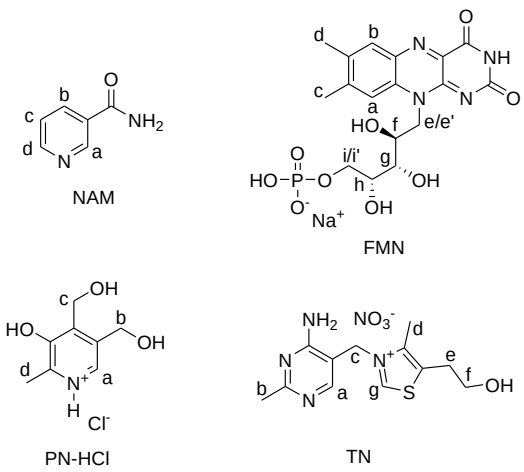


Figure 1.

**Figure 2**

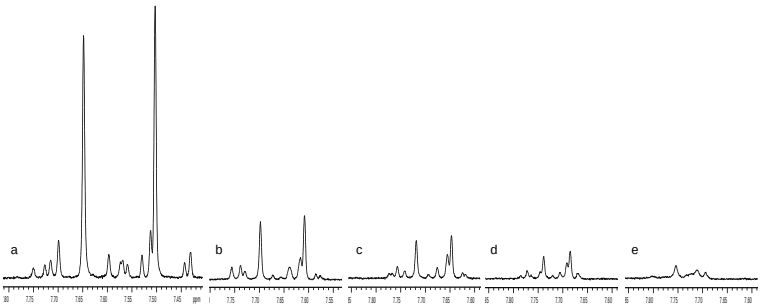


Figure 2.