Liquid and Solid-State ^{1}H, ^{13}C and ^{11}B NMR Analysis of Magnesium Fructoborate Complex: Chemical Structure, Identification and Stability Study

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Overview of Study

Fruitex-B® (FrxB) is a patented plant mineral complex that is marketed as a nutritional supplement with potential health benefits for conditions linked to inflammation such as bone, joint and cardiovascular conditions. The original product was a calcium fructoborate complex formed by the reaction of boric acid with fructose and calcium carbonate. Recently a Magnesium salt version of the product has been produced and this study represents an investigation into the similarity between the calcium and magnesium salt versions of the product. Liquid and solid-state ^{13}C and ^{11}B NMR was utilized to establish a baseline for product quality and to establish a robust testing method for both identification and quantification of the mono-complex and di-complex present in the product, as well as free borate and free fructose that is present in the finished product. A quantitative ^{13}C NMR method was developed to quantify free fructose content in the complex. Finally, an NMR based product stability study was performed to monitor molecular level stability of the complex at temperature ranging from 35-70°C with exposure lasting from 2-18 hours.

Background

Boron is naturally occurring and essential element for plant and animal life. There are many different biological compounds that can form complexes with boron. Compounds capable of complexing with boric acid include sugar alcohols, pyranose and furanose sugars or their derivatives, organic acids. Boric acid forms esters and complexes with a wide variety of mono-, di-, and polyhydroxy compounds (Woods, 1996). One of the most stable esters of boric acid are complexes where boric acid is a bridge between two carbohydrate molecules, e.g. fructose-boron-fructose. The examination of boron complexation in plants and plant extracts by ^{11}B NMR demonstrated the majority of the boron was associated with a diester complexes of diols and hydroxycarboxylic acids in radish and apple respectively (Matsunaga & Nagata, 1995). The authors made the conclusion that fructose is the most significant boron complexing molecule. Later these hypotheses were verified (Brown & Shelp, 1997 and Hu, et al., 1997) after successful isolation and full characterization of soluble boron complexes from higher plants. Calcium fructoborate (CF) is most commonly found in fresh fruits and vegetables. As a dietary supplement it is manufacture by VDF FutureCeuticals, Inc under the commercial name Fruitex-B®(FrxB) based on the US patent 5.962.049 (Miljkovic, 1999). The characterization of this complex has been reported previously (Rotaru et al., 2010) using thermal analysis, X-rays diffraction, ICP-MS, Raman spectrometry techniques. In this study we investigate molecular composition, stability and identification of FrxB used as a dietary supplement for human nutrition (Dinca & Scorei, 2013, and Reyes-Izquierdo et al., 2012) using liquid- and solid-state ^{11}B and ^{13}C NMR.

Materials and Method

Materials

Fruitex-B ® magnesium fructobotrate (MgFrxB) was manufactured and provided by Futureceuticals, Momence IL, USA according to the Miljkovic patent (US 5, 962,049).

All NMR analysis was performed in D_2O or H_2O/D_2O. D_2O (99.9%D) was obtained from Cambridge Isotopes Laboratories, Tewksbury MA, USA.

Samples were observed directly after they were received, after they had been thermally treated in a Duratech TCON dry bath system (capable of holding temperatures to +/- 0.1 °C).

NMR Spectroscopy

Liquid-state ^{11}B, ^{13}C, and ^{1}H NMR was performed on a Varian Mercury 300MVX NMR spectrometer equipped with a 5mm Varian ATB Probe at a resonance frequencies of 96.14 MHz (^{11}B), 75.36 MHz (^{13}C) and 299.67 MHz (^{1}H), respectively. ^{11}B spectra were acquired with a 45 degree tip angle pulse width, a relaxation delays of 0.2 seconds, an acquisition time of 80 ms with 8K points acquired with a spectral width of 100 kHz, and 1024 pulses were averaged. The data was zero filled to 65K points. The ^{13}C NMR was acquired with a 30 degree tip angle pulse width, a 5 seconds relaxation delay, 0.96 second acquisition time, with 24K points acquired with a spectral width of 25 kHz, and 10-12,000 pulses were averaged. The data was zero filled to 131K points. The ^{1}H NMR spectra were obtained with a 30 degree pulse angle, a 2 second relaxation delay, a 4.448 second acquisition time, with 32K points acquired over a spectral width of 7.2 kHz, 128 pulses were averaged. The data was zero-filled to 131K points. The data was acquired in a quantitative manner with inverse gated decoupling of protons during the acquisition of the ^{11}B and ^{13}C experiments. All samples were dissolved in D_2O (Cambridge Isotope Laboratories). No pH adjustments were performed on the samples after dissolution.

Solid-State ^{13}C (50.30 MHz) and ^{11}B (64.17 MHz) NMR spectra were obtained on a Varian UnityPlus-200 NMR spectrometer equipped with a Doty Scientific 7mm Supersonic CP-MAS probe. Magic angle spinning (MAS) speeds of around 6 kHz were employed. The ^{13}C NMR data was acquired using cross polarization which prepares the magnetization on the protons initially and then transfers the spin locked magnetization to the ^{13}C nuclei. The advantage of this experiment is the fact that the experiment is performed at the spin-lattice relaxation rate (T_1) of protons in the sample which is considerably shorter than the T_1 of ^{13}C nuclei in the same sample. Thus, one obtains a significant enhancement of the ^{13}C signal from the polarization transfer and can pulse at a shorter pulse-repetition rate. The ^{13}C experiment on calcium fructoborate complex were acquired with an 8 second relaxation rate, and acquisition time of 25.6 ms, with 1K points being acquired over a spectral with of 40 kHz, and 4096 pulses were averaged. The exception to these acquisition parameters were those used for pure crystalline fructose. The ^{11}B NMR spectra were acquired with MAS and with the sample remaining static in the NMR probe. The experiments were acquired with a central transition selective pulse width, a 0.2 second relaxation time, with 1K points being acquired in an acquisition time of 10.2 ms, and with a spectral width of 100 kHz.

Structure of Fruitex-B Fructoborate Complex

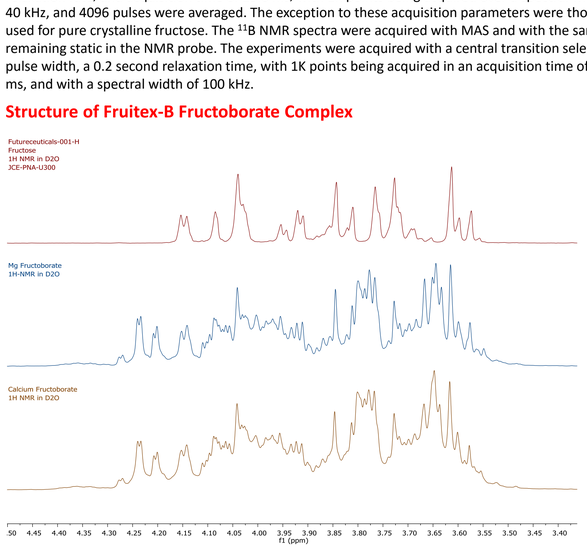


Figure 1: ^{1}H NMR spectral comparison of the proton chemistry observed in fructose, calcium fructoborate, and magnesium fructoborate.

The spectrum above shows the comparison of the ^{1}H NMR spectra of pure D-fructose, Calcium Fruitex-B fructoborate complex (CaFrxB), and Magnesium FruitexB fructoborate (MgFrxB). Free fructose is observed as well as the mono-ester/di-ester complex in the MgFrxB and CaFrxB samples, but the overall spectrum is complicated and no assignments have been made due to its complexity. However, in previous work (Edwards et al.) we have demonstrated that the ^{1}H NMR spectrum can be used to quantify the presence of CaFrxB or MgFrxB in the presence of maltodextrin or other adulterants.

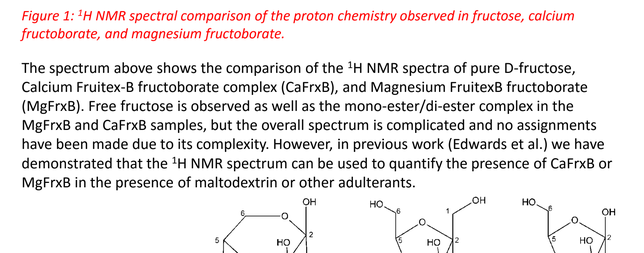


Figure 2: ^{13}C NMR spectrum of D-fructose with assignments (Consonni and Cagliani, 2008, Mazzoni et al, 1997).

Figure 2 shows the assignment of the ^{13}C spectrum of d-Fructose – the anomeric C2 carbon peaks are utilized to calculate the free fructose content in the MgFrxB product. Table I shows a typical quantitative distribution of fructopyranose/fructofuranose forms.

Table I: D-Fructose Tautomer Type ^{13}C NMR - Anomeric Region

Fructose Tautomer	Mole%
β-FP	71.1
β-FF	22.4
α-FF	6.5

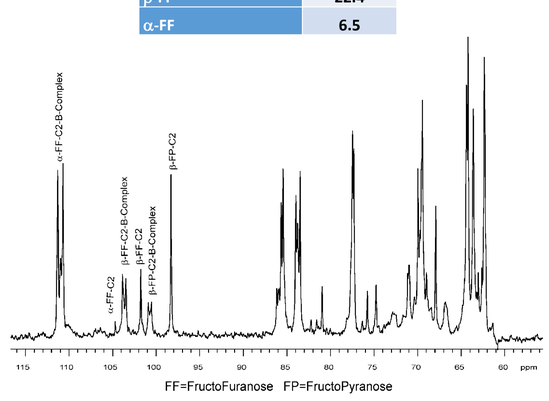


Figure 3: ^{13}C NMR of Fruitext-B Fructoborate complex with assignments

Liquid-state ^{13}C NMR and ^{11}B NMR were utilized to quantitate the optimim ratio of fructose:boric acid:magnesiumcarbonate (or magnesium hydroxide). Figure 3 shows the assignment of the ^{13}C NMR spectrum of a typical fructoborate sample. Figure 4 shows the comparison of the ^{13}C NMR spectra of D-Fructose with CaFrxB and MgFrxB. The similarity of the two fructoborate sample spectra is an indication of the very similar fructoborate complex chemistry of the magnesium and calcium forms of the fructoborate complex.

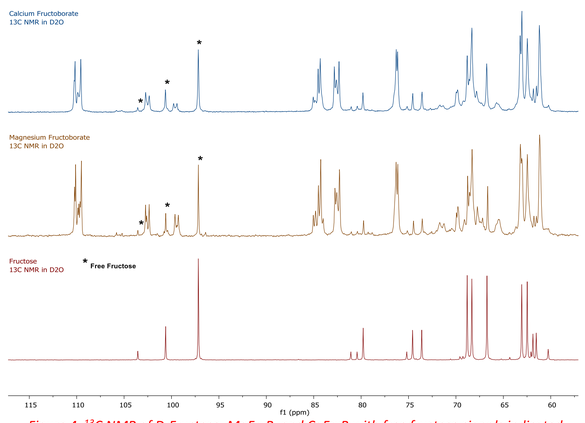


Figure 4: ^{13}C NMR of D-Fructose, MgFrxB, and CaFrxB with free fructose signals indicated.

Table II shows the free fructose content calculated from the ^{13}C NMR spectrum for each of the samples obtained by different preparation methods and with varying magnesium salt content. Figure 5 shows the ^{13}C spectra of the samples prepared with varying magnesium content

Table II: Free Fructose Content – Effect of Magnesium salt concentration

PNA ID#	Manufacturing Process and Fructose:Borate:Mg Ratio	Free Fructose (%C)
190	Mg Fructoborate Solution F:B:Mg 4:2:1	28.2
192	Mg Fructoborate Solution F:B:Mg 4:2:1.1	24.9
191	Mg Fructoborate Solution F:B:Mg 4:2:1.2	19.5
193	Mg Fructoborate Freeze Dried Powder F:B:Mg 4:2:1	29.0
194	Mg Fructoborate Freeze Dried Powder F:B:Mg 4:2:1.1	24.2
195	Mg Fructoborate Freeze Dried Powder F:B:Mg 4:2:1.2	20.8
196	Mg Fructoborate Spray Dried Powder F:B:Mg 4:2:1.2	22.2

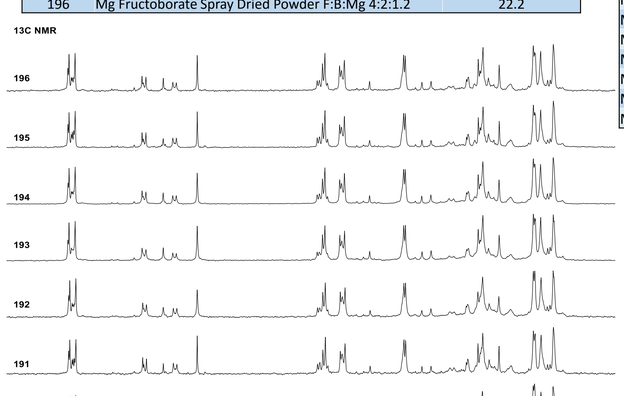


Figure 5: ^{13}C NMR spectra of MgFrxB manufactured by different methods and with varying Mg content

^{11}B NMR: Liquid-state ^{11}B NMR has been utilized often in the study of biomedical applications of boron (Bendel, 2005). In this study liquid-state ^{11}B NMR was obtained on order to observe the FrxB complex from the perspective of the boron chemistry. Previous research has identified that three basic types of boron are observed in aqueous solutions of CaFrxB and MgFrxB. Free boric acid is observed at 0 ppm, the di-ester complex (BL_2^-) is observed at -9 ppm, and the mono-ester (BL^-) complex is observed at -13 ppm (Makkee et al., 1985, Reyes-Izquierdo et al., 2012, and Smith et al., 1998). The relative molar concentrations of these three types of boron were found to be approximately 5%, 85%, and 10%, respectively. Figure 6 shows the liquid-state ^{11}B NMR spectra of boric acid and 3 batches of FrxB. Figure 7 shows the ^{11}B spectra of the same series of samples previously analyzed by ^{13}C NMR for varying manufacturing processes and with different magnesium salt content. Table III shows the ratio of free borate to the mono and diester complexes.

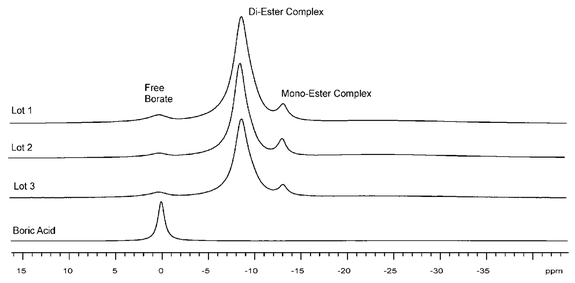


Figure 6: ^{11}B NMR of boric acid and several CaFrxB product samples

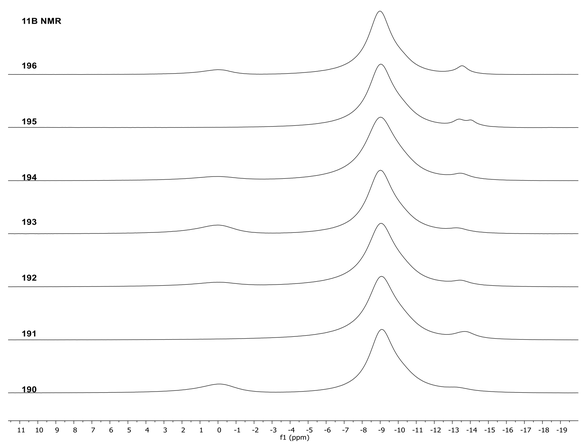


Figure 7: ^{11}B NMR of MgFrxB complex samples manufactured by different drying methods and with different magnesium ratios.

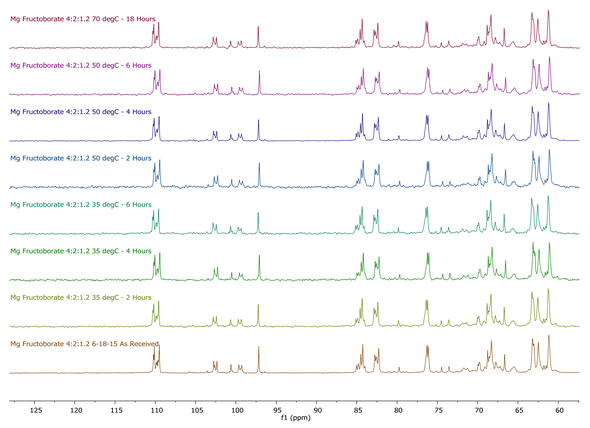
Table III: Free Fructose Content – Effect of Magnesium salt concentration

PNA ID#	Manufacturing Process and Fructose:Borate:Mg Ratio	% Free Borate	% Di-Complex	% Mono-Complex
190	Mg Fructoborate Solution F:B:Mg 4:2:1	13.8	81.1	5.1
192	Mg Fructoborate Solution F:B:Mg 4:2:1.1	8.3	86.1	5.6
191	Mg Fructoborate Solution F:B:Mg 4:2:1.2	0.0	92.6	7.4
193	Mg Fructoborate Freeze Dried Powder F:B:Mg 4:2:1	13.3	81.7	5.0
194	Mg Fructoborate Freeze Dried Powder F:B:Mg 4:2:1.1	7.1	86.9	6.0
195	Mg Fructoborate Freeze Dried Powder F:B:Mg 4:2:1.2	0.0	92.1	7.9
196	Mg Fructoborate Spray Dried Powder F:B:Mg 4:2:1.2	7.1	86.8	6.1

Thermal Stability of Magnesium Fruitex-B Complex Product

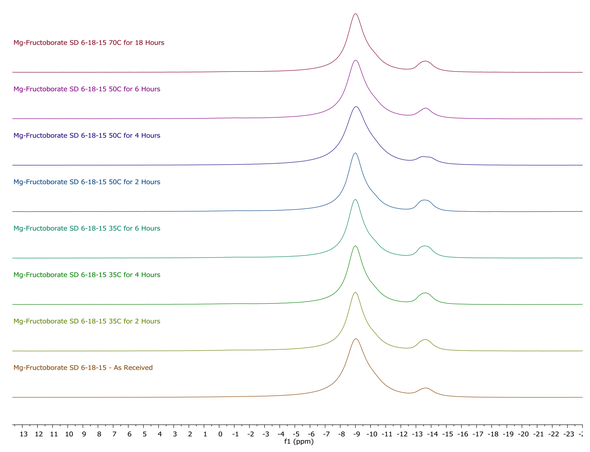
The thermal stability of the Fruitex-B product was tested by exposing the product to temperatures of 35, 50 and 70°C for between 2 and 18 hours. These figures below show the results obtained by liquid and solid-state NMR experiments. The samples showed no observable changes over the course of the stability test. Free borate, BL^- and BL^{2-} were calculated from the ^{11}B NMR and the ^{13}C NMR was utilized to calculate free fructose and the α-FF/β-ff/β-FP component complex concentrations and these values were used to assess the stability of the MgFrxB complex.

Liquid-state ^{13}C NMR spectra of heat treated MgFrxB and Calculated Free Fructose Concentrations



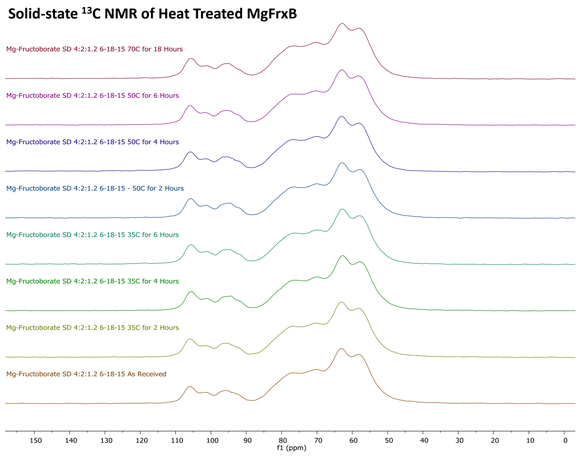
Sample and Heat Treatment	Free Fructose (%C)
Mg Fructoborate SD - F:B:Mg 4:2:1.2 6-18-15 As Received	19.12
Mg Fructoborate SD - F:B:Mg 4:2:1.2 6-18-15 - 35C for 2 Hours	19.09
Mg Fructoborate SD - F:B:Mg 4:2:1.2 6-18-15 - 35C for 4 Hours	19.35
Mg Fructoborate SD - F:B:Mg 4:2:1.2 6-18-15 - 35C for 6 Hours	19.6
Mg Fructoborate SD - F:B:Mg 4:2:1.2 6-18-15 - 50C for 2 Hours	19.67
Mg Fructoborate SD - F:B:Mg 4:2:1.2 6-18-15 - 50C for 4 Hours	19.51
Mg Fructoborate SD - F:B:Mg 4:2:1.2 6-18-15 - 50C for 6 Hours	19.01
Mg Fructoborate SD - F:B:Mg 4:2:1.2 6-18-15 - 70C for 18 Hours	18.16

Liquid-state ^{11}B NMR spectra of heat treated MgFrxB samples and table showing calculated concentrations of free borate, di-ester complex, and mono-ester complex.

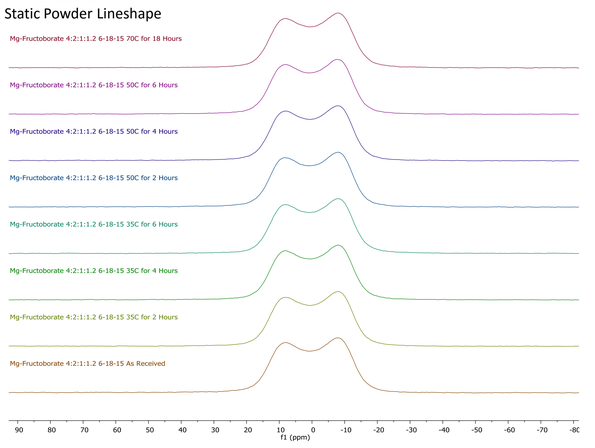


Sample and Heat Treatment	% Free Borate	% Di-Complex	% Mono-Complex
Mg Fructoborate SD - F:B:Mg 4:2:1.2 6-18-15 - As Received	0.0	90.3	9.7
Mg Fructoborate SD - F:B:Mg 4:2:1.2 6-18-15 - 35C for 2 Hours	2.1	86.4	11.5
Mg Fructoborate SD - F:B:Mg 4:2:1.2 6-18-15 - 35C for 4 Hours	2.0	86.1	11.9
Mg Fructoborate SD - F:B:Mg 4:2:1.2 6-18-15 - 35C for 6 Hours	2.4	84.9	12.7
Mg Fructoborate SD - F:B:Mg 4:2:1.2 6-18-15 - 50C for 2 Hours	2.3	85.9	11.9
Mg Fructoborate SD - F:B:Mg 4:2:1.2 6-18-15 - 50C for 4 Hours	0.0	90.2	9.8
Mg Fructoborate SD - F:B:Mg 4:2:1.2 6-18-15 - 50C for 6 Hours	1.9	87.8	10.3
Mg Fructoborate SD - F:B:Mg 4:2:1.2 6-18-15 - 70C for 18 Hours	2.1	86.0	11.9

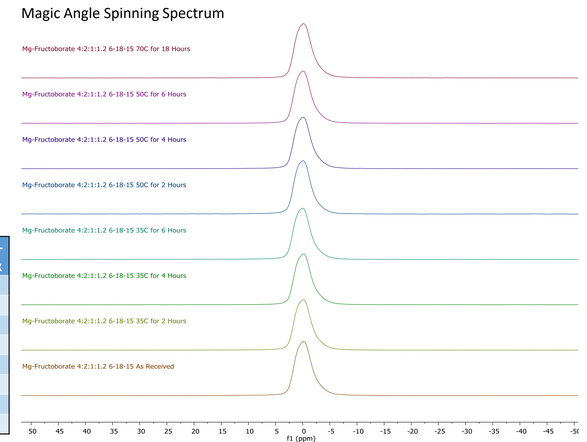
Solid-state ^{13}C NMR of Heat Treated MgFrxB



Solid-state ^{11}B NMR
Static Powder Lineshape



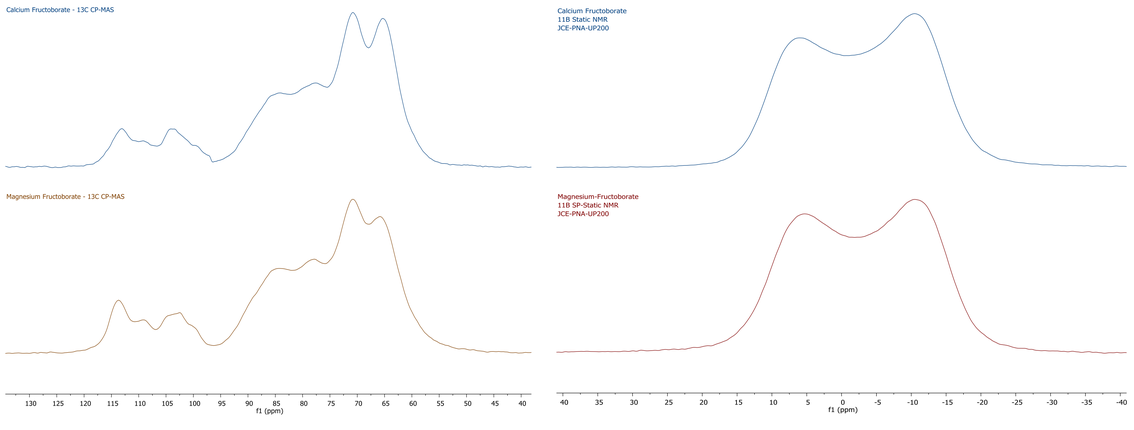
Solid-state ^{11}B NMR
Magic Angle Spinning Spectrum

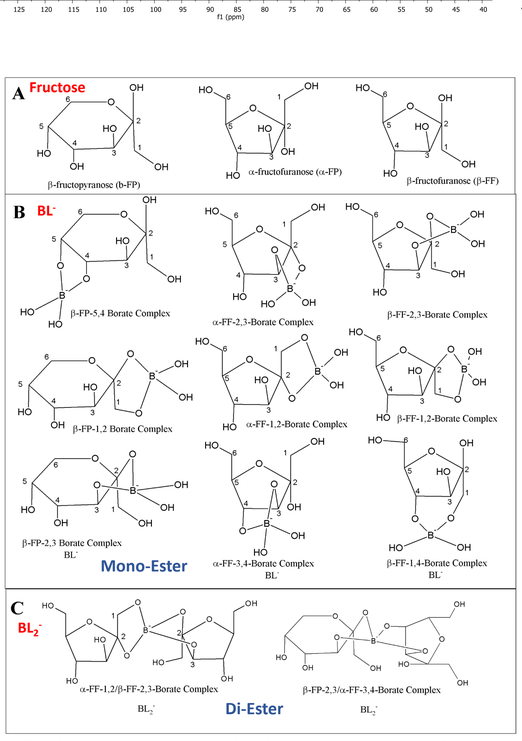


Unreacted fructose (free fructose) is observed in the complex mixture and a 3-7 ppm downfield shift of the fructose resonances is observed for the carbons coordinated to borate in the fructoborate complex. The change of relative signal intensities in the regions of the spectrum that are associated with furanose tautomers, indicates that the tautomer distribution of the complex strongly favours the reaction of borate with the fructofuranose (FF) form. The fact that the FrxB complex peaks are multi-component in all cases leads to the conclusion that the borate reacts with multiple hydroxyls with OH condensation reactions occurring predominantly on the C-1/C-2 as well as on the C-3/C-4 of the FF forms. It is expected from the mole ratios utilized in the synthesis of the FrxB complex that the complex is predominantly the di-ester form BL_2^- form (one borate coordinated to two fructose molecules) with the minor constituent being the monoester form (BL^-), as well as some free/unreacted borate.

In the stability study the MgFrxB was found to be stable with little observed change in free fructose content or the free borate/mono-ester/di-ester distribution even after 18 hours exposure to a temperature of 70°C.

Solid-State ^{11}B and ^{13}C NMR Comparison of MgFrxB and CaFrxB





The above structures represent the mixture of component fructoborate species present in the Fruitex-B product

Conclusion

Multinuclear liquid and solid-state NMR spectroscopy demonstrated the structural similarity between calcium and magnesium fructoborate complexes. NMR was also utilized to understand differences in complex chemistry with varying magnesium concentrations and the effect of these concentrations was reflected in free fructose content as well as free borate/ester ratios. The temperature stability of the MgFrxB was demonstrated with little degradation of the complex observed even after 18 hours exposure to 70°C.

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