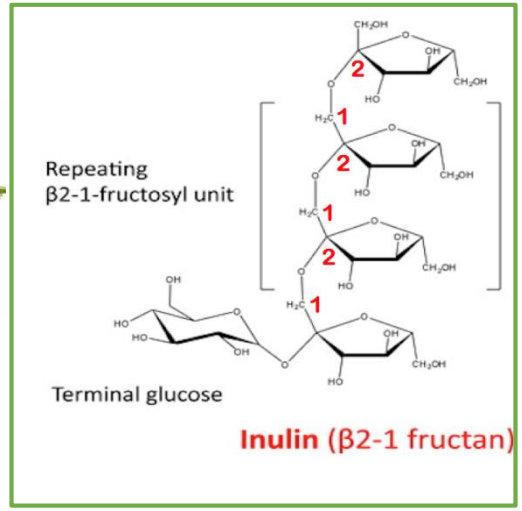
133th AOAC Annual Meeting & Exposition, Denver, 6th-12th September, 2019
Lucie Charmier– lucie@megazyme.com

Challenges in the Measurement of Fructan in Biological Materials

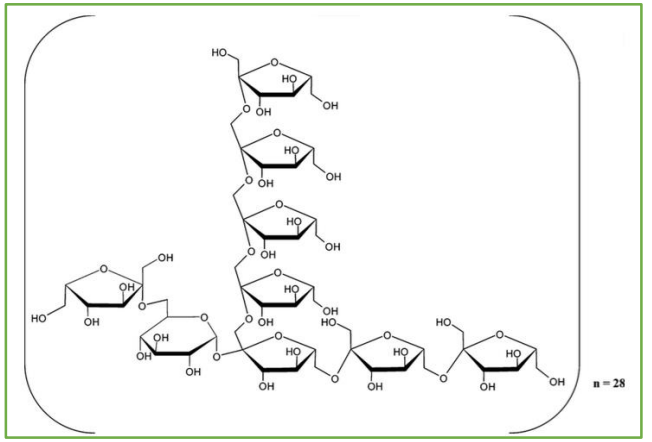
Lucie M.J Charmier and Barry V. McCleary
Megazyme

Fructan is defined as any compound where one or more fructosyl-fructose linkages constitute a majority of the linkages

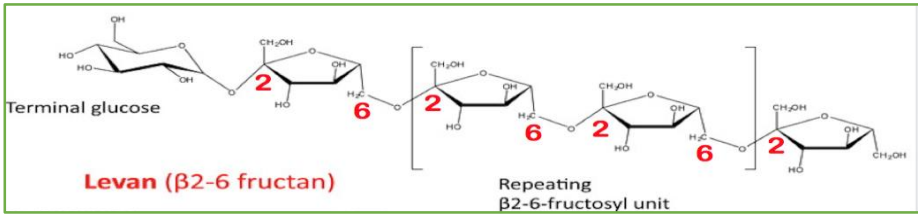
Inulin [containing exclusively the β(2-1) fructosyl-fructose linkage] e.g. chicory inulin



Branched fructan [containing both β(2-1) and β(2-6) fructosyl-fructose linkages in significant amounts]
e.g. agave, wheat and onion fructans



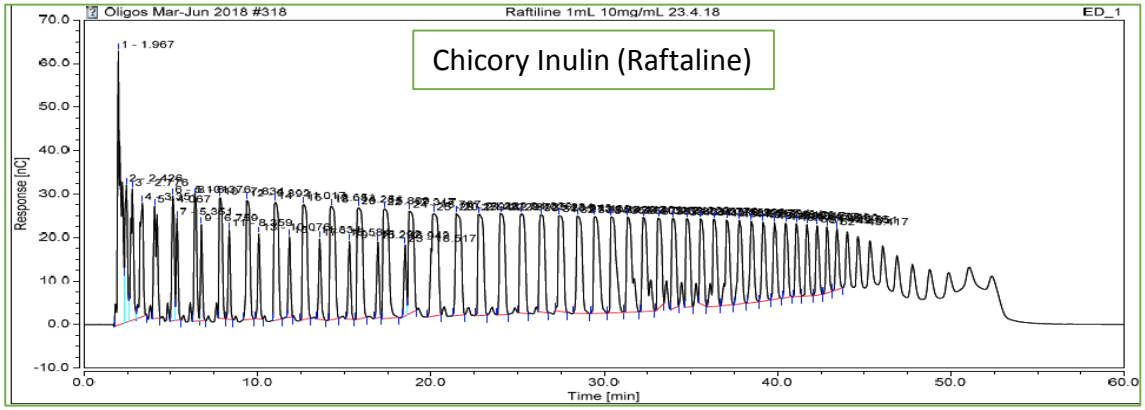
Levan [containing mostly or exclusively the β(2-6) fructosyl-fructose linkage] e.g. Timothy grass Levan



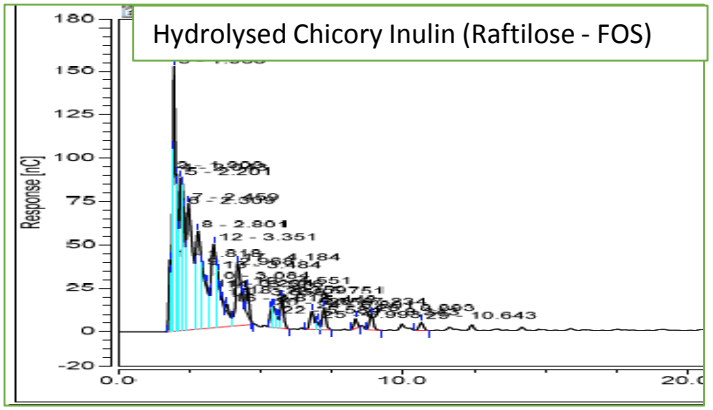
Megazyme

COMPLEXITY OF NATIVE FRUCTANS – ION CHROMATOGRAPHY

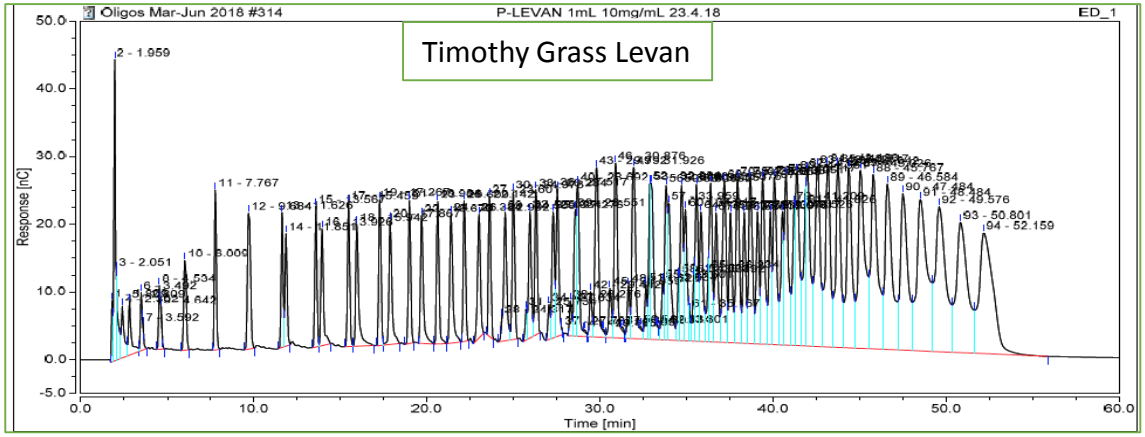
- Dionex HPAEC-PAD analysis with a 10mg/mL solution of polymer - Column PA200
- **Fructans** are polysaccharides of varying complexity and degree of polymerisation occurring in nature in various forms and shapes.



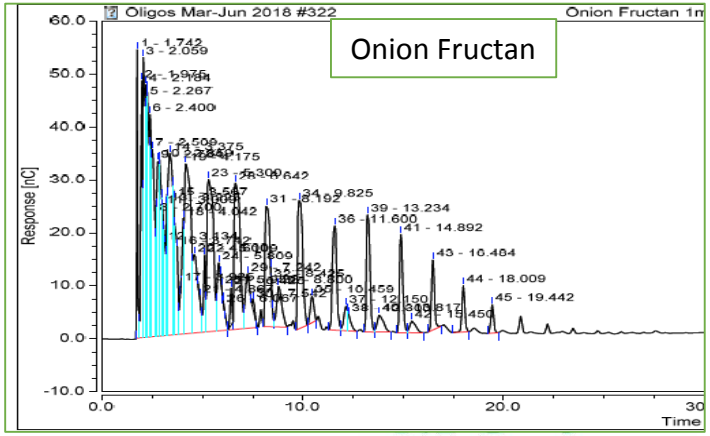
Chicory Inulin (Raftaline)



Hydrolysed Chicory Inulin (Raftilose - FOS)



Timothy Grass Levan



Onion Fructan

ENZYMATIC FRUCTAN MEASUREMENT (AOAC Method 999.03)

FRUCTAN

All enzymatic polysaccharide measurement requires selective degradation into its constituent monomers

FRUCTAN CONTENT

Megazyme

ENZYMATIC FRUCTAN MEASUREMENT (AOAC Method 999.03)

FRUCTAN

Inulin and Agave (branched fructan)
(AOAC Method 999.03)

↓ *endo*-Inulinase
+ *exo*-Inulinase

FRUCTOSE + GLUCOSE

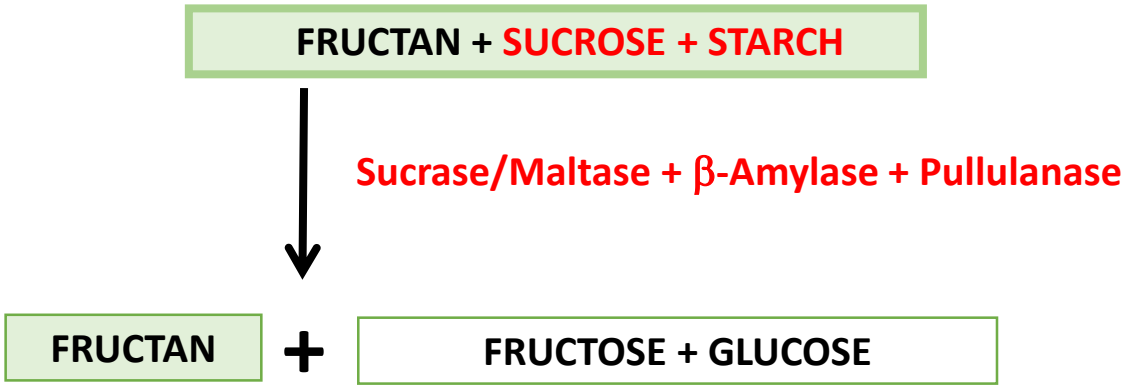
↓ PAHBAH reducing sugar determinations

FRUCTAN CONTENT

All enzymatic polysaccharide measurement requires selective degradation into its constituent monomers

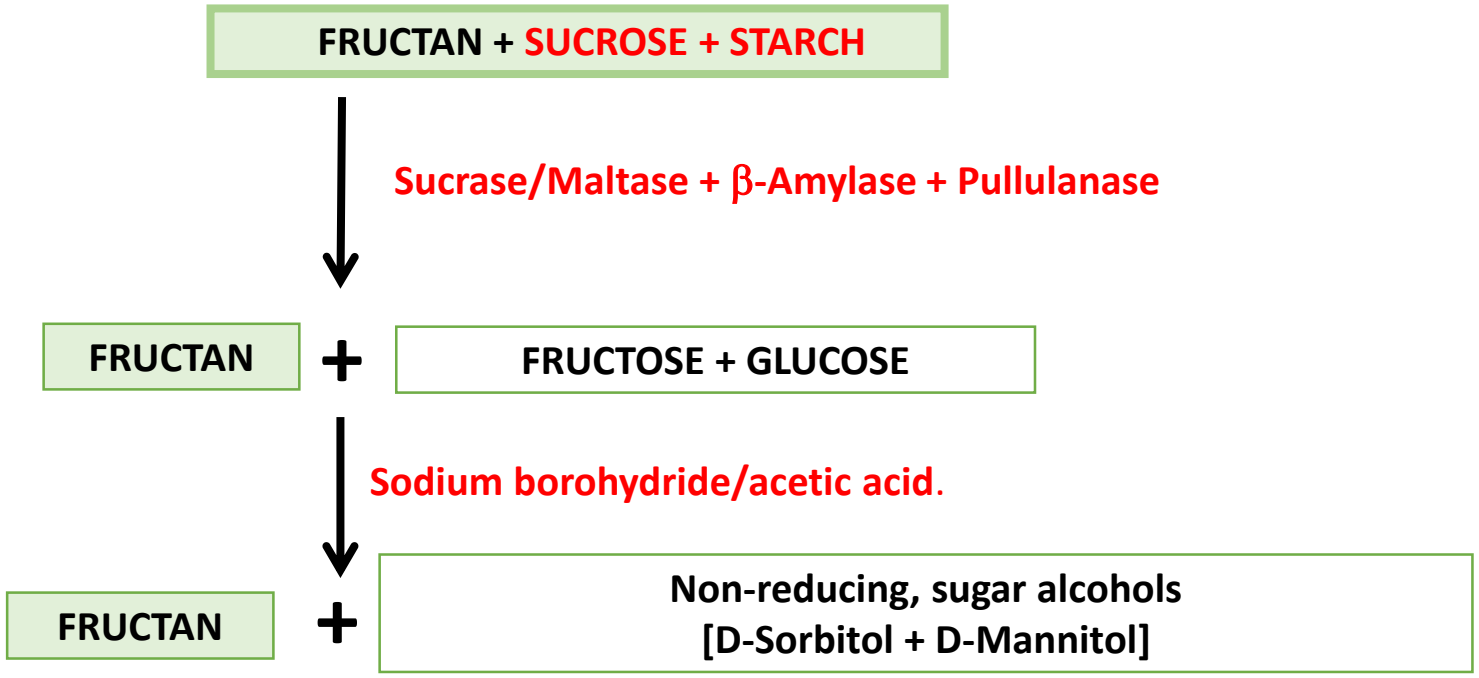
Megazyme

FRUCTAN MEASUREMENT Megazyme K-FRUC (AOAC Method 999.03)



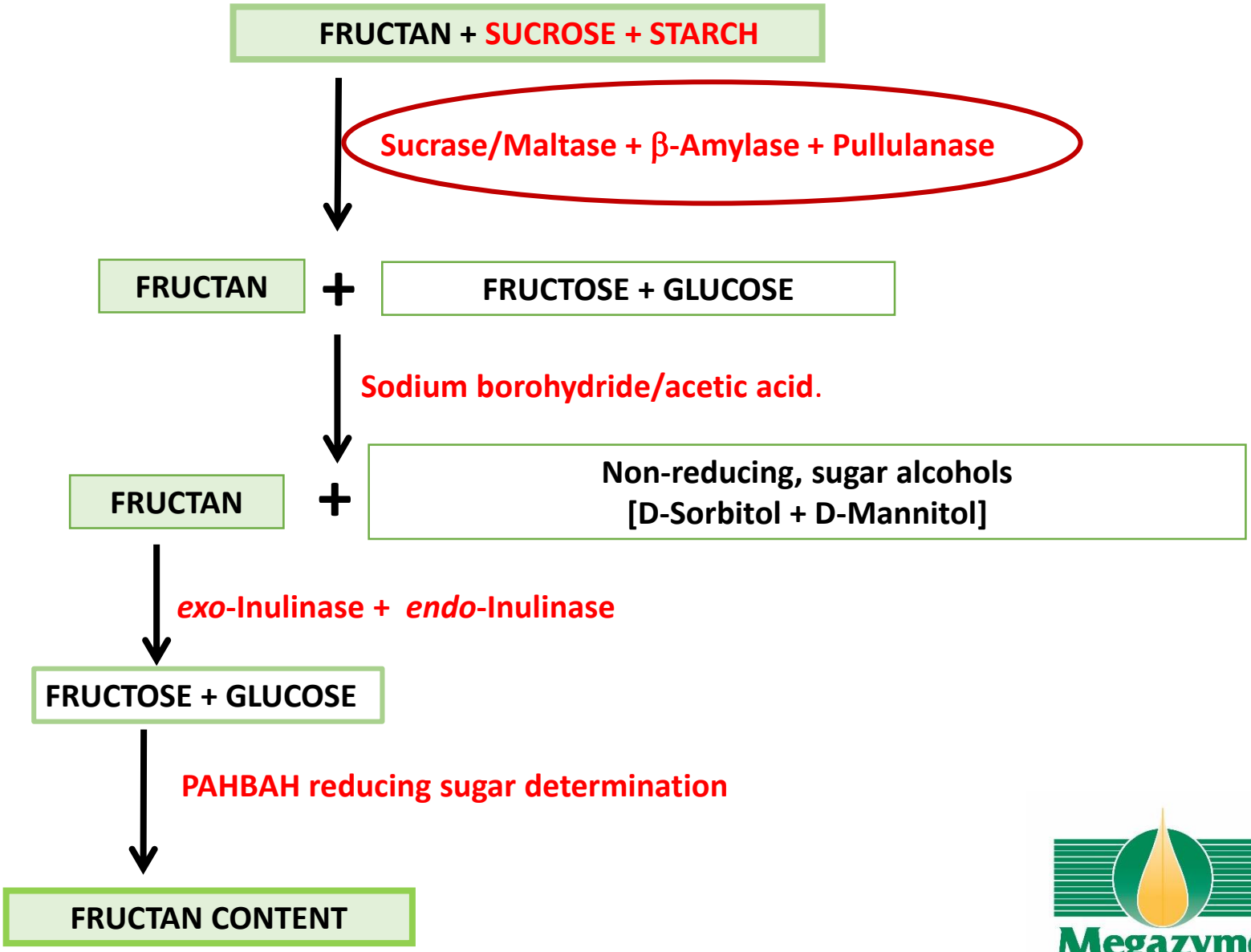
Megazyme

FRUCTAN MEASUREMENT Megazyme K-FRUC (AOAC Method 999.03)



Megazyme

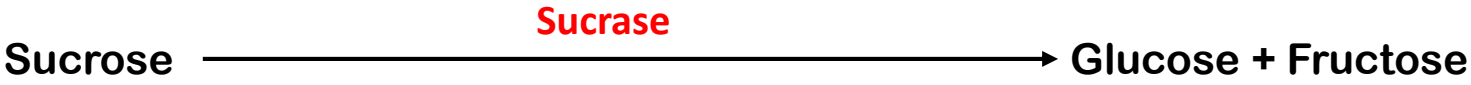
FRUCTAN MEASUREMENT Megazyme K-FRUC (AOAC Method 999.03)



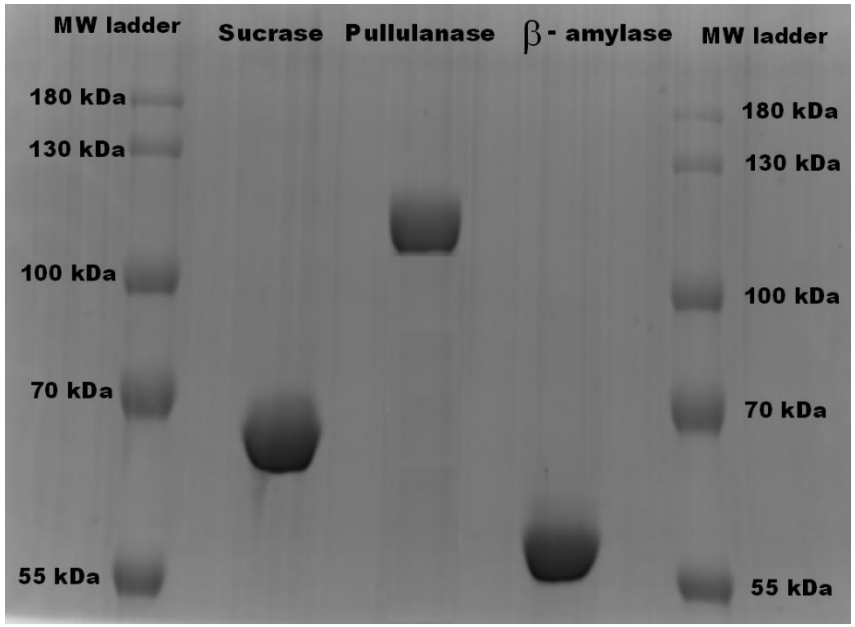
Megazyme

Requirement for Ultra-pure Enzymes for Starch & Sucrose Hydrolysis

It is important to remove interfering compounds prior quantitative fructan measurement (e.g free glucose, fructose, sucrose and other interfering compounds) as this may lead to over/under estimation of fructan content in biological materials



SDS-PAGE Electrophoresis of KC-FRUC1 Starch and Sucrose degrading enzymes



A - Sucrase/Maltase (chromatographically purified)

B - Pullulanase (affinity purified)

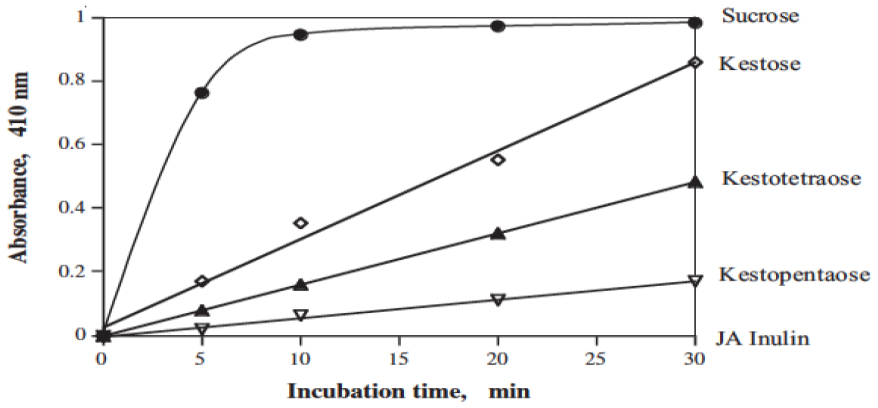
C - β-Amylase (recrystallized)

All enzymes must be devoid of action on Fructan

Megazyme

Importance of enzyme specificity in Sucrase/FOS hydrolysis

Hydrolysis by Yeast Invertase



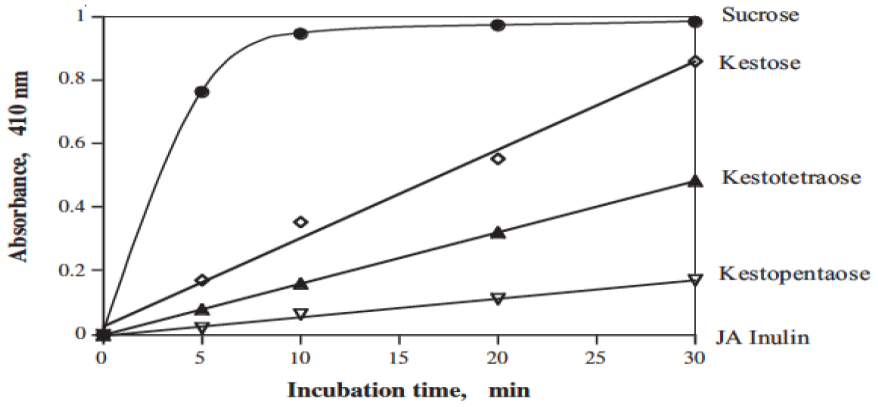
Hydrolysis conditions are essentially the same as those used in the Fructan assay procedure

- Crystalline yeast invertase hydrolyzes lower degree of polymerization FOS
- This enzyme was used in Pontis *et al.* 1966 method leading to underestimation of Fructan content (FOS)

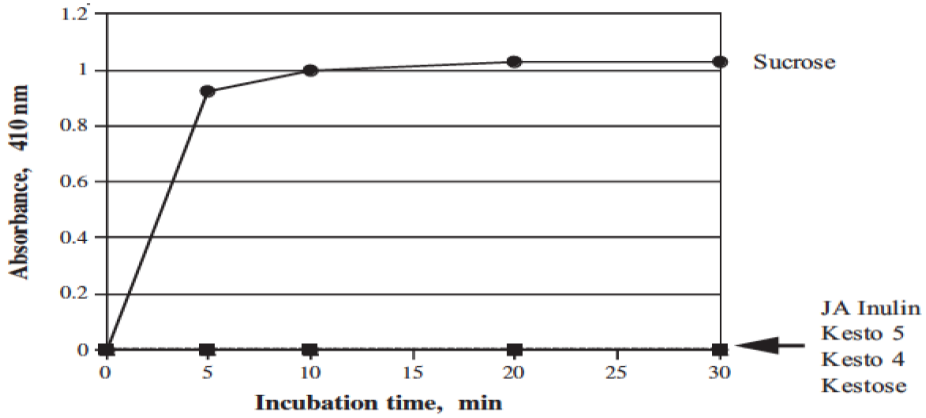
Megazyme

Importance of enzyme specificity in Sucrase/FOS hydrolysis

Hydrolysis by Yeast Invertase



Hydrolysis by K-FRUC Sucrase

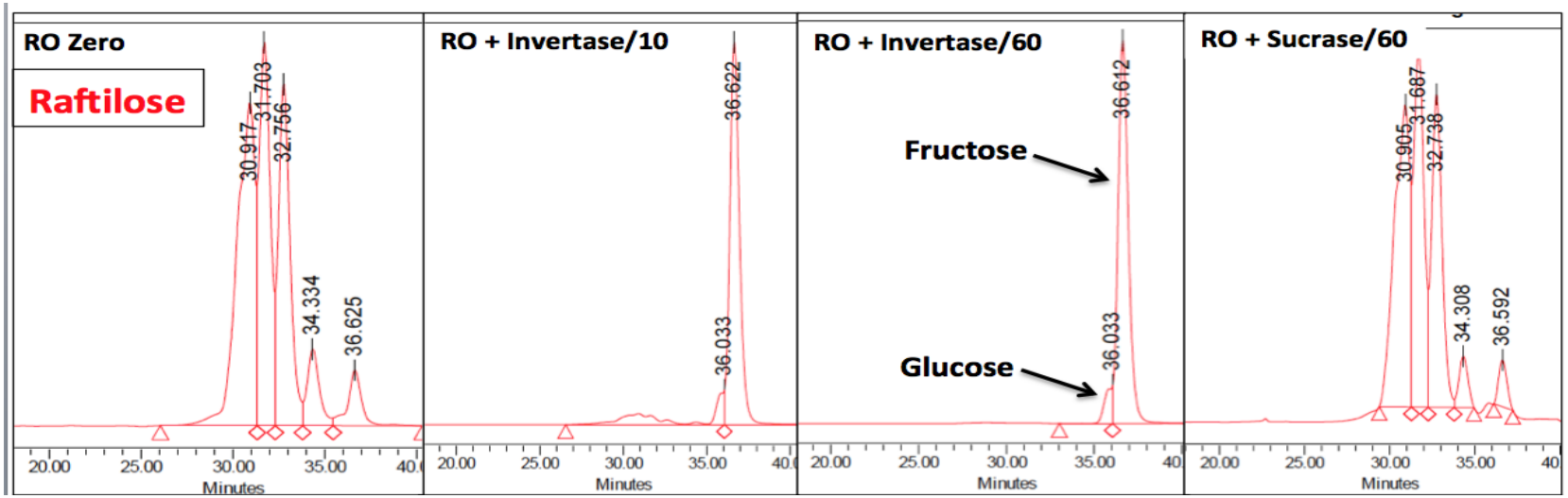


Hydrolysis conditions are essentially the same as those used in the Fructan assay procedure.

- Crystalline yeast invertase hydrolyzes lower degree of polymerization FOS
- This enzyme was used in Pontis *et al.* 1966 method leading to underestimation of Fructan content (FOS)
- AOAC method 999.03 uses a sucrase enzyme that is specific for Sucrose hydrolysis
- The sucrase has no action on lower degree of polymerization FOS such as 1-kestose and 1,1-kestotetraose

Megazyme

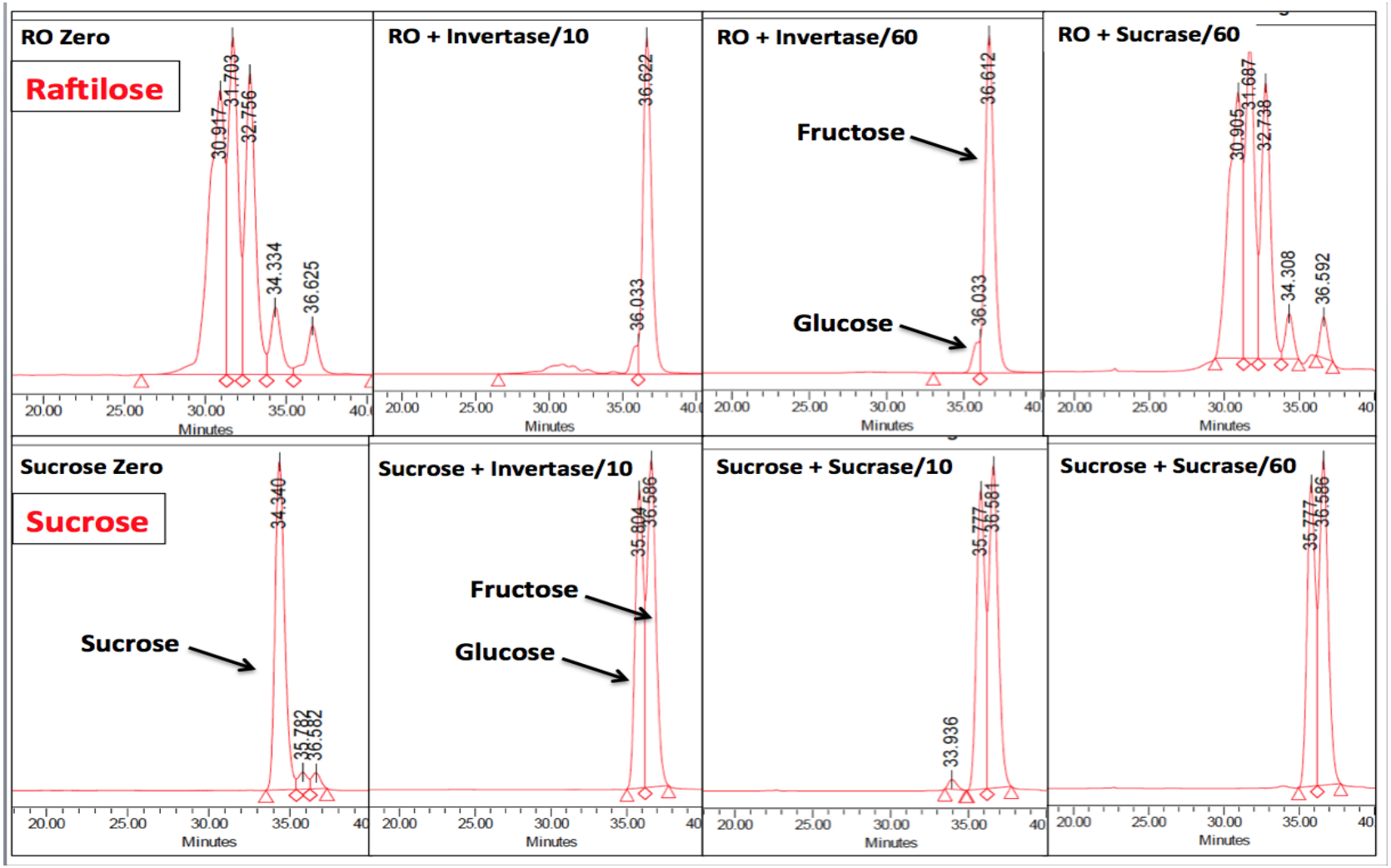
Specificity of Sucrase in Hydrolysis of Sucrose vs Non-specificity of Invertase



- Invertase hydrolyses Raftilose (FOS) and Sucrose, leading to underestimation of Fructan

Megazyme

Specificity of Sucrase in Hydrolysis of Sucrose vs Non-specificity of Invertase



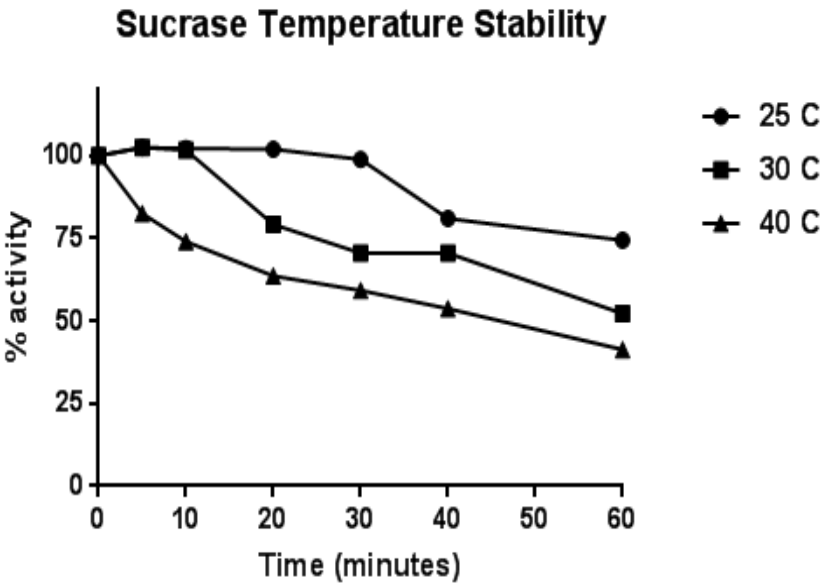
- Invertase hydrolyses Raftilose (FOS) and Sucrose, leading to underestimation of Fructan
- Sucrase is specific for Sucrose with no activity towards Raftilose (FOS)

Megazyme

Importance of Sucrase Activity and Stability on Sucrose control

- Enzyme purity and specificity is crucial for the removal of interfering compounds (e.g sucrose)
- Reaction conditions such as concentration, temperature and pH are critical for the optimal hydrolysis of these compounds

	% Sucrose remaining	
	30°C	**40°C**
No Sucrase	8.885	8.946
Sucrase A	**0.069**	2.096
Sucrase B	0.139	4.198

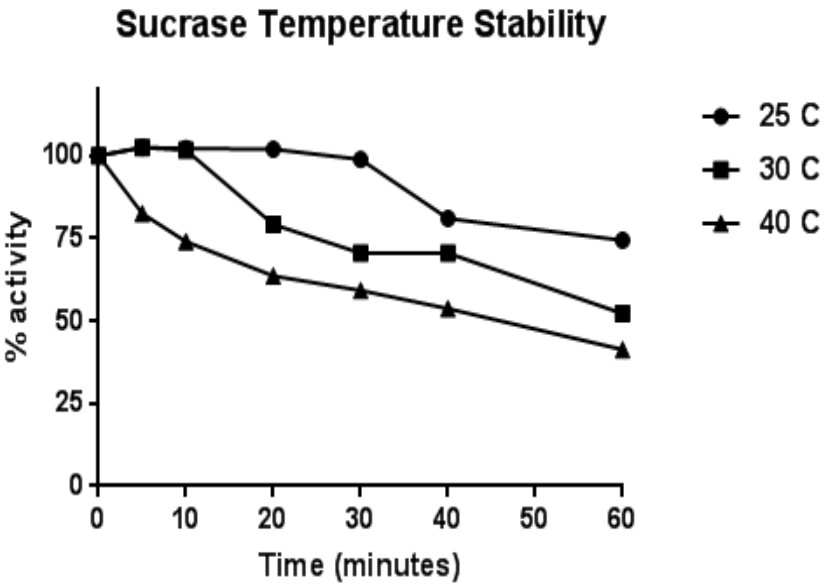


Megazyme

Importance of Sucrase Activity and Stability on Sucrose control

- Enzyme purity and specificity is crucial for the removal of interfering compounds (e.g sucrose)
- Reaction conditions such as concentration, temperature and pH are critical for the optimal hydrolysis of these compounds

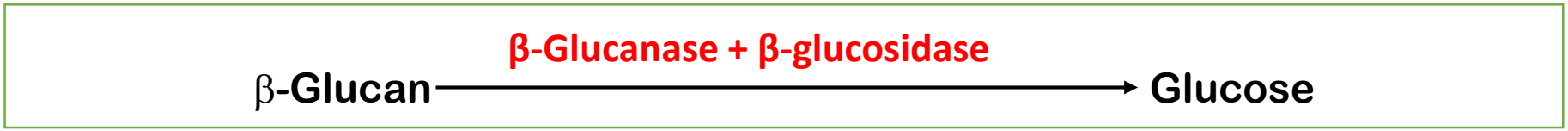
	% Sucrose remaining	
	30°C	**40°C**
No Sucrase	8.885	8.946
Sucrase A	**0.069**	2.096
Sucrase B	0.139	4.198

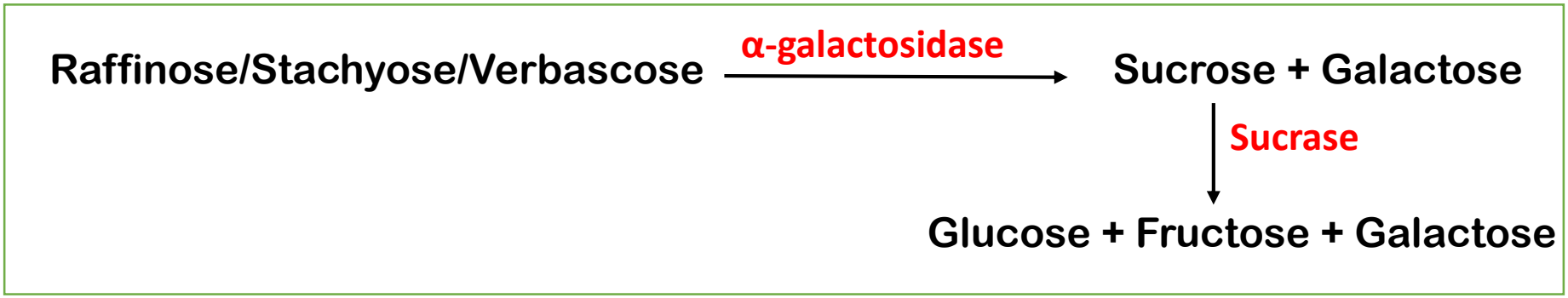


Sucrase Formulation A provided near complete hydrolysis of sucrose at 30C

Megazyme

Removal of Other Interfering Compounds





Megazyme

Removal of Other Interfering Compounds

β-Glucan —(β-Glucanase + β-glucosidase)→ Glucose

Raffinose/Stachyose/Verbascose —(α-galactosidase)→ Sucrose + Galactose

Sucrose —(Sucrase)→ Glucose + Fructose + Galactose

All enzymes must be ultra pure and devoid of action on Fructan

Megazyme

Table 2. Interference of α- and β-glucans, maltodextrins, and galactosyl-sucrose oligosaccharides on fructan determination using the Fructan Assay Kit

Sample	Sample weight analyzed, mg	Apparent "fructan" content, % (w/w; "as is")
Sucrose/cellulose control (8.9% sucrose)	400	0.13 – 0.14
Wheat starch	400	0.03 – 0.05
Maltodextrins (Matsutani Chemical Co.)	400	0.08 – 0.10
β-Glucan (Megazyme Cat. No. P-BGBM)	200	0.05 – 0.06
α-Cellulose	400	0.01 – 0.02
Raffinose (galactosyl-sucrose)	7.5	7.5 – 7.7
Raffinose pretreated with α-galactosidase[a]	7.5	0.11 – 0.13
Mung beans (containing 3.05% galactosyl-sucrose oligosaccharides)	400	2.85 – 3.05
Mung beans (3.05% galactosyl-sucrose oligosaccharides) plus α-galactosidase[a]	400	0.10 – 0.11

[a] Sample (0.2 mL) was incubated with 0.05 mL of α-galactosidase solution (20 U) in 50 mM sodium acetate buffer (pH 4.5) for 30 min at 40°C.

Megazyme

FRUCTAN MEASUREMENT (AOAC Methods 999.03 & 2018.07)

FRUCTAN

Inulin and Agave (branched fructan)
(AOAC Method 999.03)

↓ *endo*-Inulinase + *exo*-Inulinase

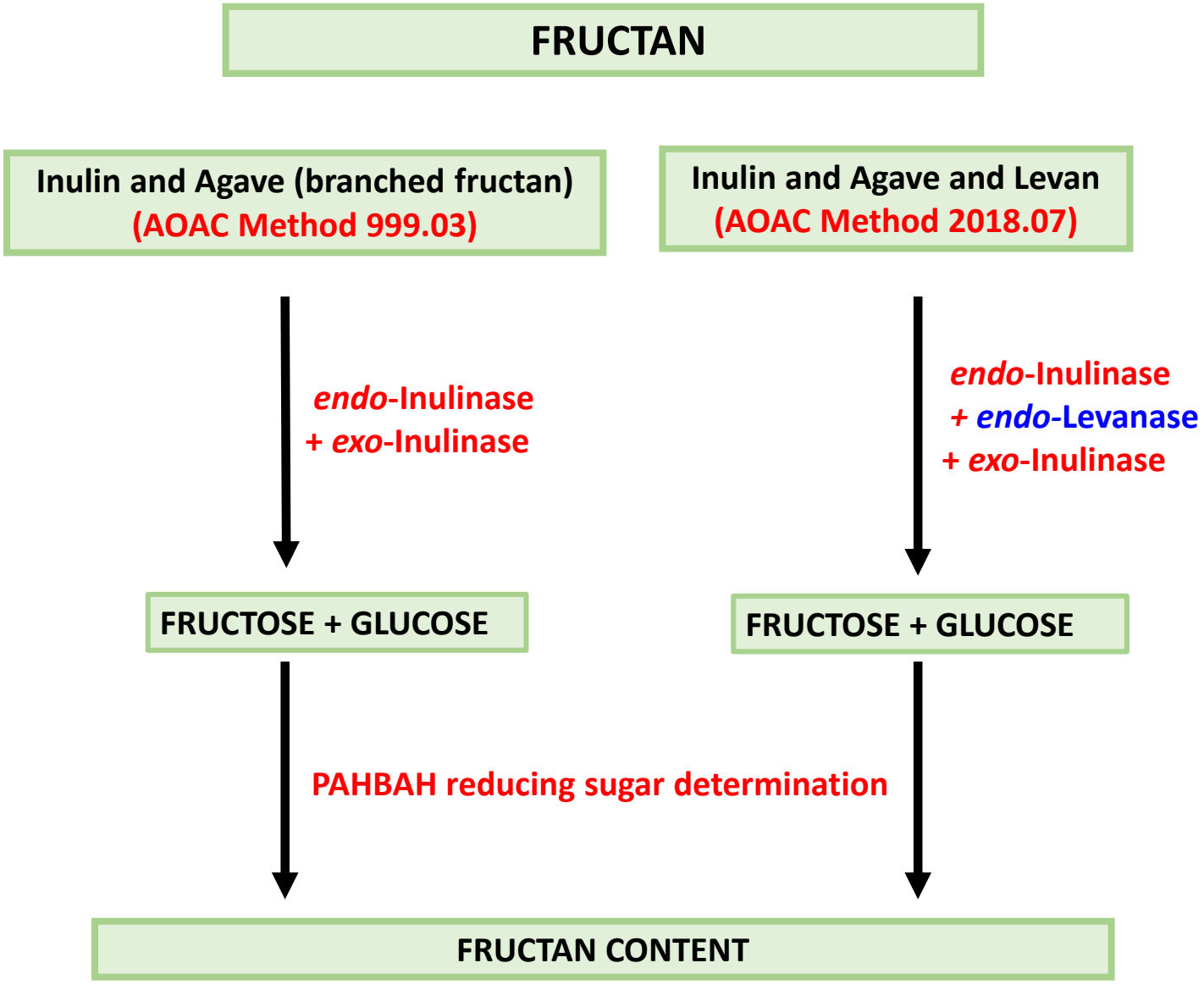
FRUCTOSE + GLUCOSE

↓ PAHBAH reducing sugar determination

FRUCTAN CONTENT

Megazyme

FRUCTAN MEASUREMENT (AOAC Methods 999.03 & 2018.07)



Megazyme

Measurement of plant levan “Phleins” through K-FRUC AOAC 999.03

Comparison of a colorimetric and a high-performance liquid chromatography method for the determination of fructan in pasture grasses

Annette C Longland,[a*] Mewa S Dhanoa[b] and Patricia A Harris[c]

J Sci Food Agric 2012; **92**: 1878–1885

Megazyme AOAC 999.03 colorimetric technique was not reliable substitute for HPLC in the quantitative measurement of Fructan content of pasture grasses

Table 5. Degree of fructan hydrolysis of grass and inulin incubated with the fructan hydrolases used in the colorimetric method for fructan determination

Grass type	Fructan hydrolysis	SD	Megazyme : HPLC[a]
Ryegrass C	0.29	0.040	0.36
Cocksfoot	0.28	0.007	0.27
Timothy hay 1	0.07	0.089	0.09
Timothy hay 2	0.58	0.121	0.52
Inulin (control)	0.91	0.004	1.03

[a] Ratio of fructan values obtained by the colorimetric method relative to those determined by HPLC.

Megazyme

Measurement of plant levan “Phleins” through K-FRUC AOAC 999.03

Comparison of a colorimetric and a high-performance liquid chromatography method for the determination of fructan in pasture grasses for horses

Annette C Longland,[a*] Mewa S Dhanoa[b] and Patricia A Harris[c]

J Sci Food Agric 2012; **92**: 1878–1885

Table 5. Degree of fructan hydrolysis of grass and inulin incubated with the fructan hydrolases used in the colorimetric method for fructan determination

Grass type	Fructan hydrolysis	SD	Megazyme : HPLC[a]
Ryegrass C	0.29	0.040	0.36
Cocksfoot	0.28	0.007	0.27
Timothy hay 1	0.07	0.089	0.09
Timothy hay 2	0.58	0.121	0.52
Inulin (control)	0.91	0.004	1.03

[a] Ratio of fructan values obtained by the colorimetric method relative to those determined by HPLC.

Megazyme AOAC 999.03 colorimetric technique was not reliable substitute for HPLC in the quantitative measurement of Fructan content of pasture grasses

METHOD DEVELOPMENT TO INCLUDE LEVAN

Megazyme

Measurement of plant Levan “PHLEINS” using K-FRUC test kit

- The K-FRUC method was first developed for the measurement of **inulin-type and branched fructans**.
- This method employs a mixture of ***exo*-inulinase** and ***endo*-inulinase**.
- ***exo*-Inulinase** hydrolyses both β(2-1) and β(2-6) fructosyl-fructose linkages in lower DP oligosaccharides, but Levan is not depolymerised by ***endo*-Inulinase**.

Megazyme

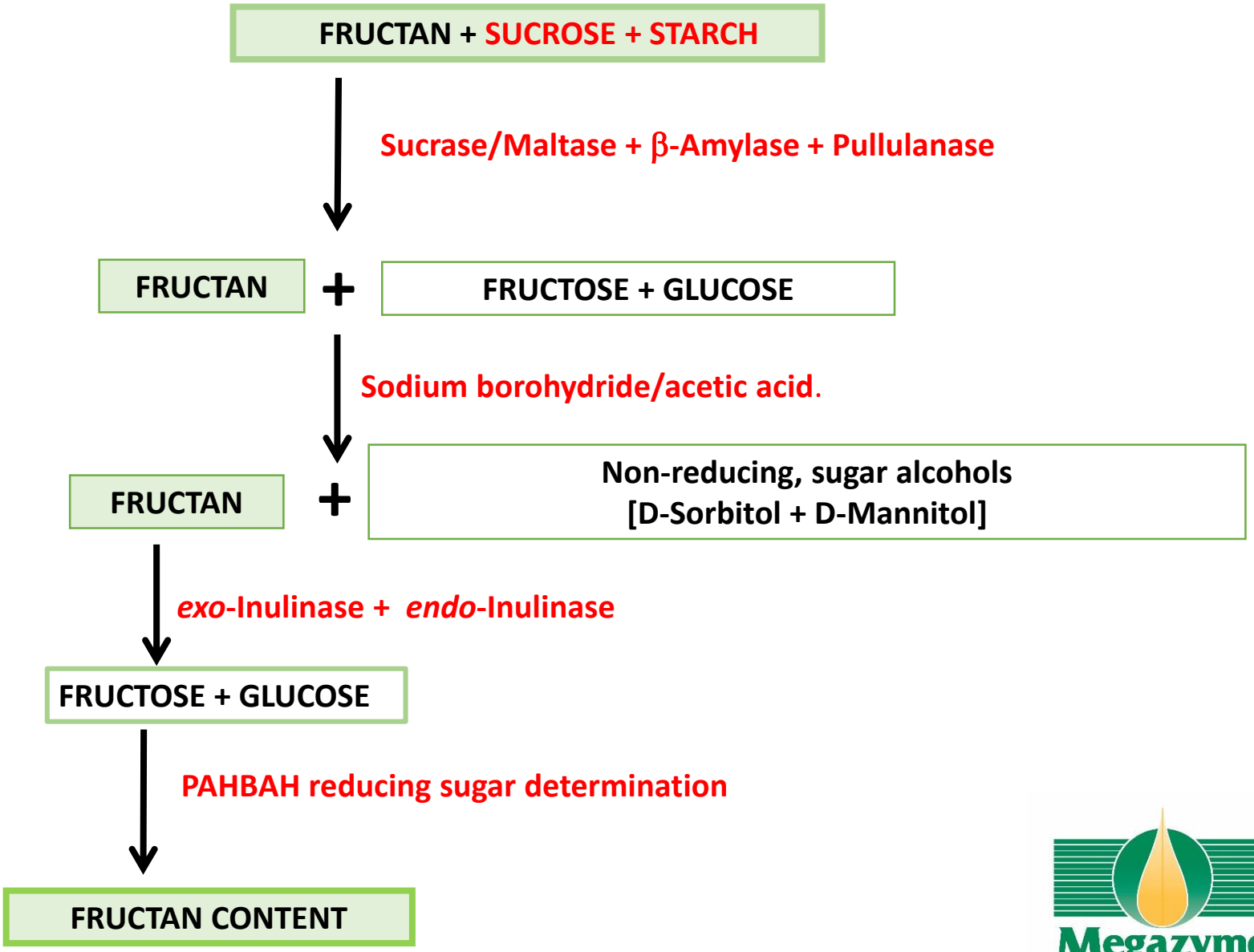
Measurement of plant Levan "PHLEINS" using K-FRUC test kit

- The K-FRUC method was first developed for the measurement of **inulin-type and branched fructans**.
- This method employs a mixture of ***exo*-Inulinase** and ***endo*-Inulinase**.
- ***exo*-Inulinase** hydrolyses both β(2-1) and β(2-6) fructosyl-fructose linkages in lower DP oligosaccharides, but Levan is not depolymerised by ***endo*-Inulinase**.

- **For quantitative measurement of Levan-type polysaccharides [(2-6) fructosyl-fructose linkages] the enzyme mixture needs to include an *endo*-Levanase.**
- **Such an enzyme has been recombinantly produced by Megazyme and is now included in the K-FRUC fructanase enzyme mixture in the K-FRUC assay kit.**
- **The enzyme mixture has been evaluated using a Levan-type polysaccharide purified from Timothy grass.**

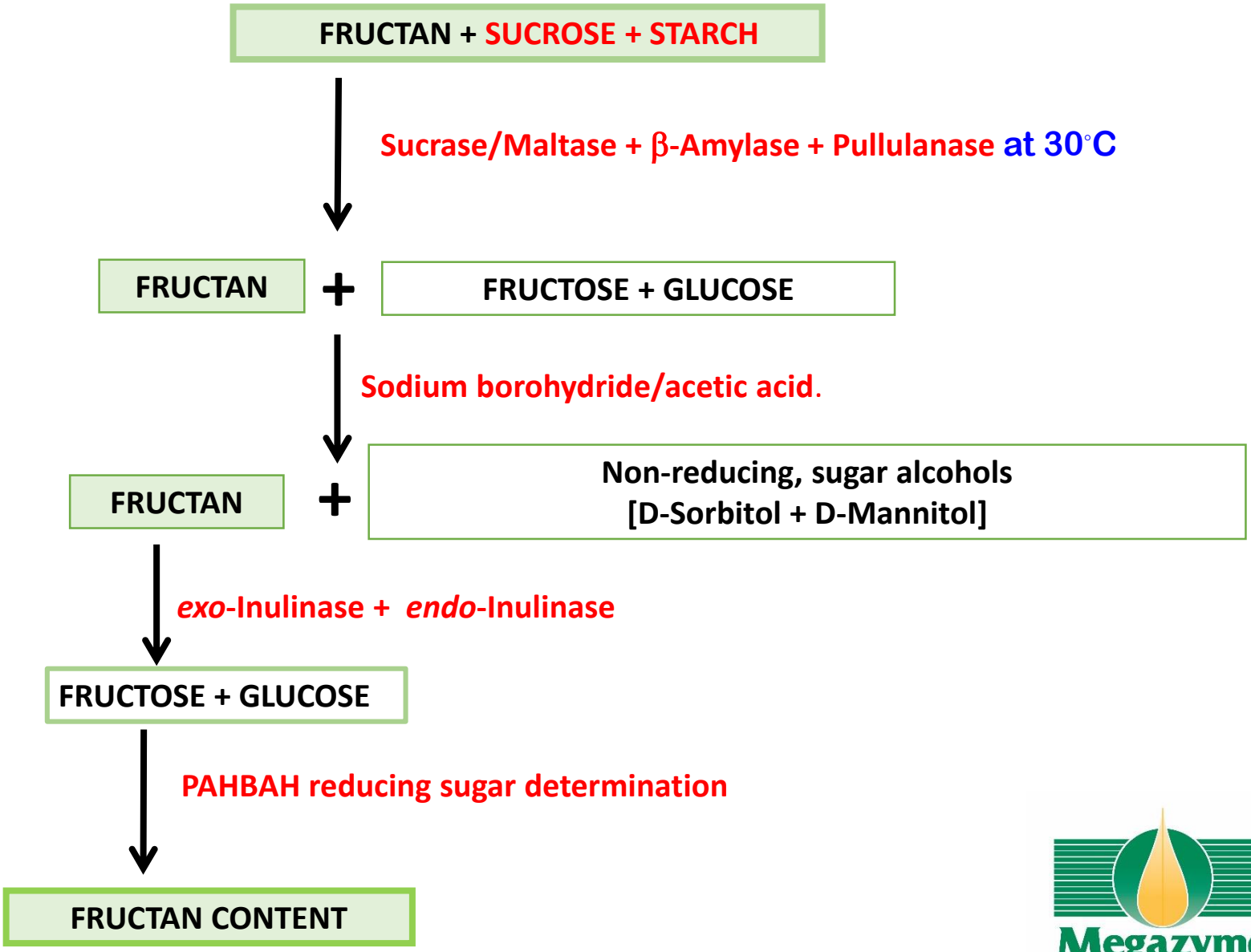
Megazyme

FRUCTAN MEASUREMENT Megazyme K-FRUC updated (AOAC Method 2018.07)



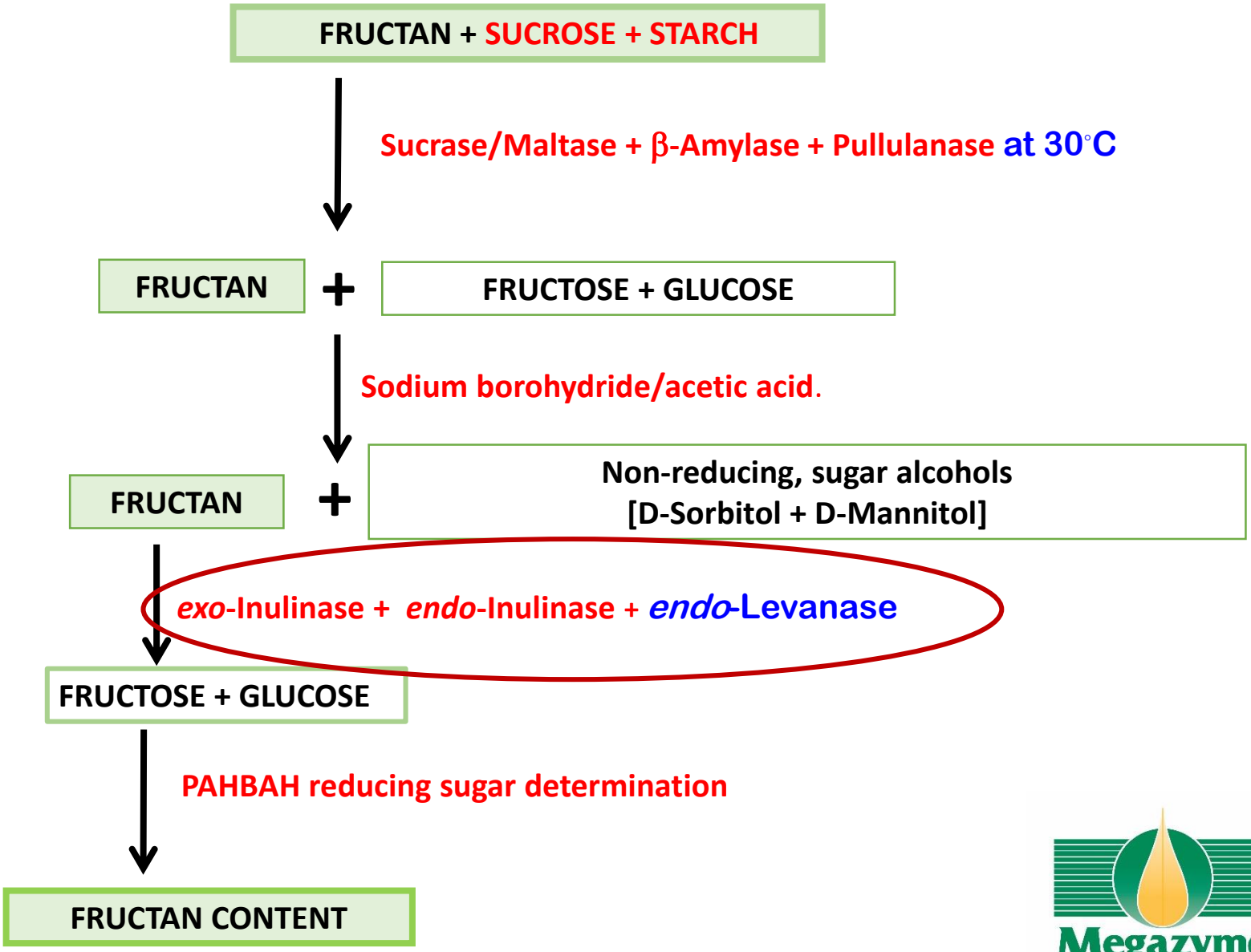
Megazyme

FRUCTAN MEASUREMENT Megazyme K-FRUC updated (AOAC Method 2018.07)



Megazyme

FRUCTAN MEASUREMENT Megazyme K-FRUC updated (AOAC Method 2018.07)



Megazyme

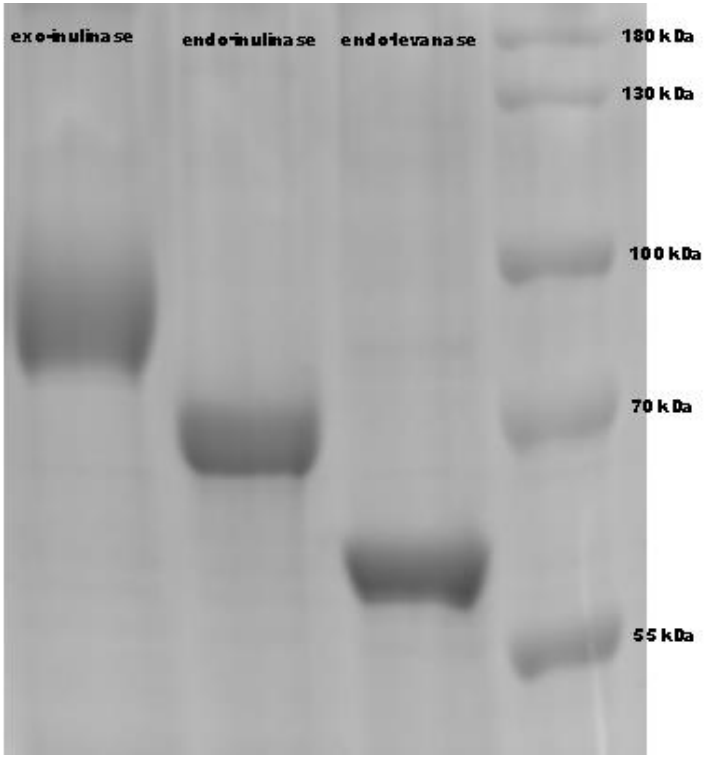
Requirement for Ultra-pure Enzymes for Fructan Hydrolysis

Initially, *exo*-inulinase and *endo*-inulinase were purified from a commercial Fructanase enzyme preparation. This material became unavailable, so the required enzymes are now produced recombinantly.

An added advantage is the ability to produce ultra-pure enzymes.

Fructan —(***exo*-inulinase + *endo*-inulinase + *endo*-levanase**)→ **Glucose + Fructose**

SDS-PAGE Electrophoresis of KC-FRUC2 Fructanase enzymes



A – *exo*-Inulinase (recombinant)

B – *endo*-Inulinase (recombinant)

C – *endo*-Levanase (recombinant)

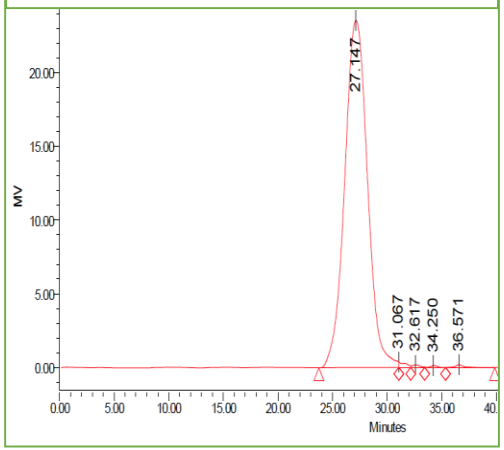
Analytical requirements for use of fructanase enzymes in the measurement of Fructans:

- **Prior removal of interfering compounds**
- **Devoid of action in any other polysaccharide that might be present in the biological sample**

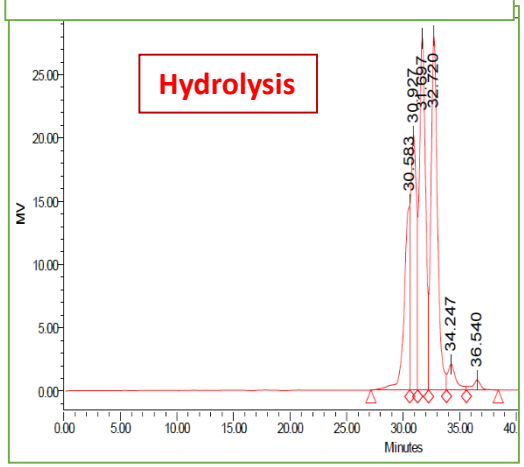
Megazyme

Specificity of *endo*-Inulinase and *endo*-Levanase in Hydrolysis of Inulin and Levan

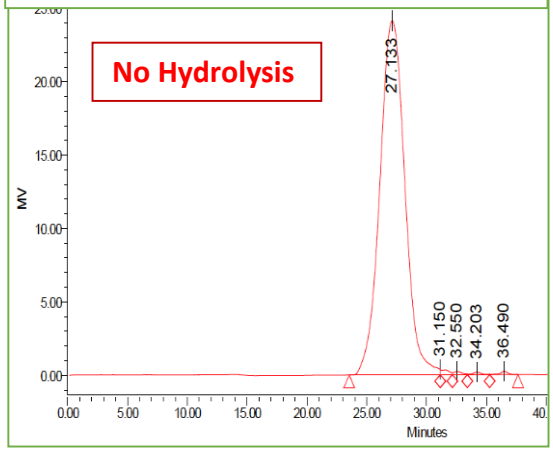
Inulin no enzyme treatment



Inulin + *endo*-Inulinase for 10 min



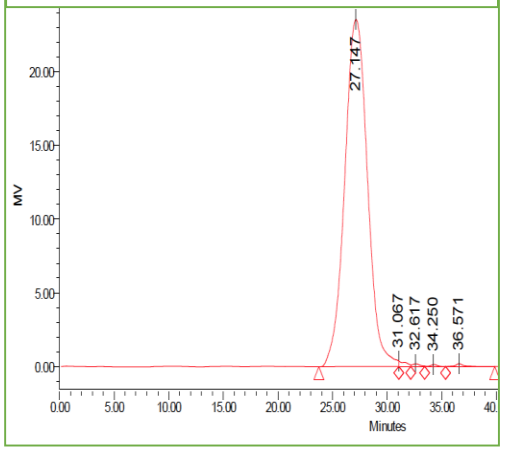
Inulin + *endo*-Levanase for 60 min



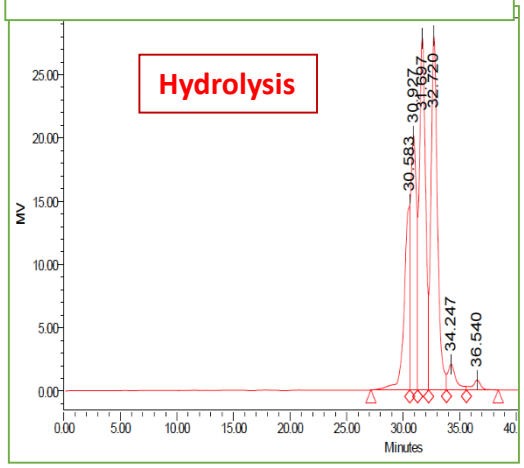
Megazyme

Specificity of *endo*-Inulinase and *endo*-Levanase in Hydrolysis of Inulin and Levan

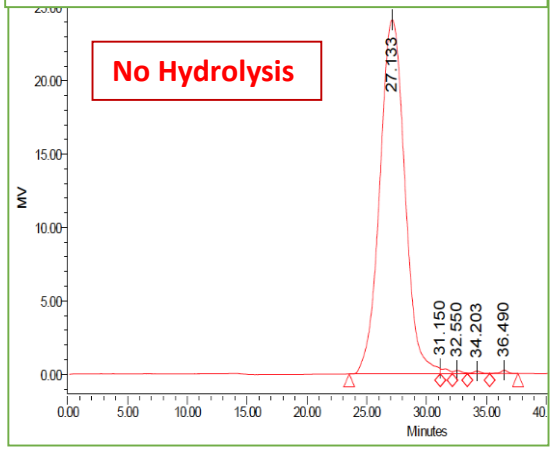
Inulin no enzyme treatment



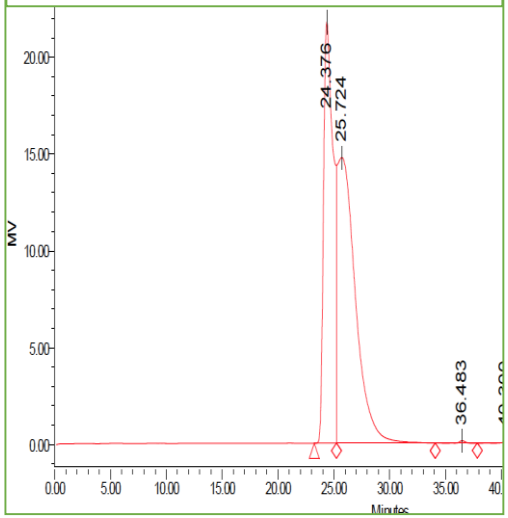
Inulin + *endo*-Inulinase for 10 min



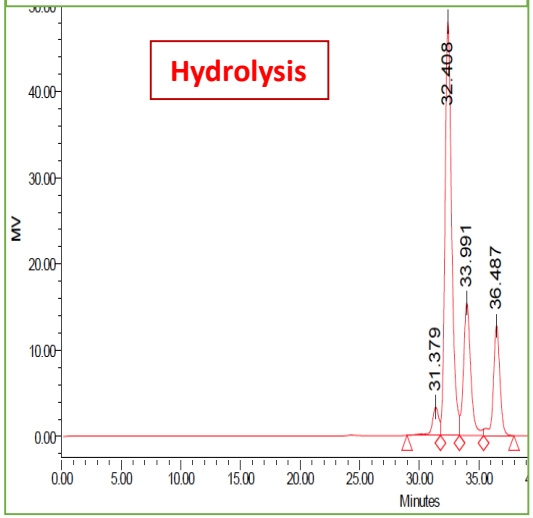
Inulin + *endo*-Levanase for 60 min



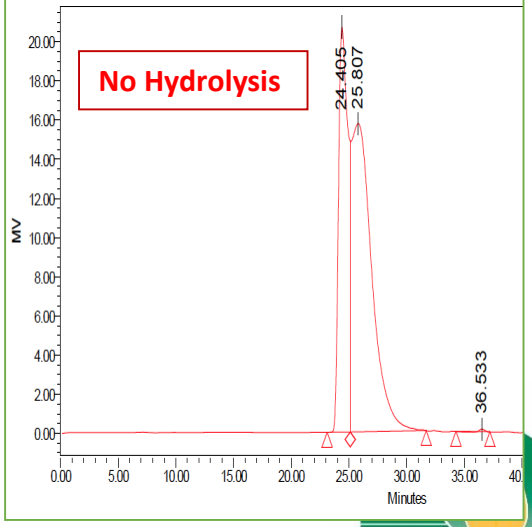
Levan no enzyme treatment



Levan + *endo*-Levanase for 10 min



Levan + *endo*-Inulinase for 60 min



Megazyme

Effect of the Incorporation of *endo*-Levanase into the K-FRUC assay method on the measurement of Levans

	Fructan content, % w/w (*as is* basis)	
Sample	***exo*-Inulinase + *endo*-Inulinase**	***exo*-Inulinase + *endo*-Inulinase + *endo*-Levanase**
Timothy grass (sample A)	4.9	**13.8**
Timothy grass (sample B)	3.2	**6.2**
Rye grass	8.9	9.9
Oaten hay	10.7	10.9
Barley MAX (grain)	12.8	12.8
Purified Levan from timothy grass	59.2	**91.2**
Purified Inulin from chicory	92.0	92.3

- ***endo*-Levanase** incorporation in K-FRUC allows quantitative measurement in pasture grasses
- K-FRUC 999.03 partially measured Levan-type Fructans prior ***endo*-Levanase** incorporation
- K-FRUC is now designed to measure all types of Fructan as Total Fructan Content, % w/w.

Megazyme

Repeatability of the Megazyme K-FRUC fructan assay procedure across a range of Fructans (Inulin, Levan and Highly Branched)

Sample	Fructan, % (w/w), dwb mean ±2 SD, (CV, %)				Interday mean, ±2 SD, (%RSDr, %)
	Day 1	Day 2	Day 3	Day 4	
Raftaline Inulin (Native chicory fructan)	99.8 ± 4.1	98.2 ± 1.2	98.3 ± 0	97.2 ± 1.5	98.4 ±2.6
	2.04	0.61	0.00	0.77	1.34
Levan (from Timothy grass)	97.5 ± 1.6	98.3 ± 2	99.2 ± 1	98.6 ± 0.9	98.4 ±1.7
	0.83	1.01	0.50	0.45	0.88
Frutafit Agave fructan	85.7 ± 3.5	86.4 ± 2	86.8 ± 3	83.2 ± 1.5	85.6 ±3.6
	2.07	1.17	1.75	0.91	2.10
Timothy grass	15.6 ± 0.2	15.9 ± 1.1	15.2 ± 1	14.9 ± 0.8	15.4 ±1
	0.51	3.58	3.20	2.84	3.37
Barley max	14.4 ± 0.2	14 ± 1.4	13.6 ± 0.5	13.7 ± 0.9	13.9 ±0.9
	0.60	4.92	1.90	3.23	3.36
Rye Grass	11 ± 0.4	10.8 ± 0.3	10.4 ± 0.6	10.4 ± 0.1	10.7 ±0.6
	1.73	1.25	2.98	0.46	2.96
Brett brother swine feed	2.01 ± 0.03	1.96 ± 0.01	1.98 ± 0.12	1.94 ± 0.1	1.97 ±0.09
	0.78	0.31	3.05	2.64	2.16
Barley Flour	1.7 ± 0	1.7 ± 0.1	1.7 ± 0.1	1.5 ± 0.1	1.6 ±0.1
	0.32	2.80	3.04	2.78	3.59
Purina GoCat dry cat food	0.98 ± 0.03	0.99 ± 0.18	1.03 ± 0	0.97 ± 0.02	0.99 ±0.09
	1.73	9.18	0.16	0.78	4.74
Top spec alfalfa	0.2 ± 0.02	0.2 ± 0.02	0.21 ± 0.01	-	0.21 ±0.02
	4.20	3.82	2.91	-	3.73

Megazyme

Repeatability of the Megazyme K-FRUC fructan assay procedure across a range of Fructans (Inulin, Levan and Highly Branched)

Sample	Fructan, % (w/w), dwb mean ±2 SD, (CV, %)				Interday mean, ±2 SD, (%RSDr, %)
	Day 1	Day 2	Day 3	Day 4	
Raftaline Inulin (Native chicory fructan)	99.8 ± 4.1	98.2 ± 1.2	98.3 ± 0	97.2 ± 1.5	98.4 ±2.6
	2.04	0.61	0.00	0.77	1.34
Levan (from Timothy grass)	97.5 ± 1.6	98.3 ± 2	99.2 ± 1	98.6 ± 0.9	98.4 ±1.7
	0.83	1.01	0.50	0.45	0.88
Frutafit Agave fructan	85.7 ± 3.5	86.4 ± 2	86.8 ± 3	83.2 ± 1.5	85.6 ±3.6
	2.07	1.17	1.75	0.91	2.10
Timothy grass	15.6 ± 0.2	15.9 ± 1.1	15.2 ± 1	14.9 ± 0.8	15.4 ±1
	0.51	3.58	3.20	2.84	3.37
Barley max	14.4 ± 0.2	14 ± 1.4	13.6 ± 0.5	13.7 ± 0.9	13.9 ±0.9
	0.60	4.92	1.90	3.23	3.36
Rye Grass	11 ± 0.4	10.8 ± 0.3	10.4 ± 0.6	10.4 ± 0.1	10.7 ±0.6
	1.73	1.25	2.98	0.46	2.96
Brett brother swine feed	2.01 ± 0.03	1.96 ± 0.01	1.98 ± 0.12	1.94 ± 0.1	1.97 ±0.09
	0.78	0.31	3.05	2.64	2.16
Barley Flour	1.7 ± 0	1.7 ± 0.1	1.7 ± 0.1	1.5 ± 0.1	1.6 ±0.1
	0.32	2.80	3.04	2.78	3.59
Purina GoCat dry cat food	0.98 ± 0.03	0.99 ± 0.18	1.03 ± 0	0.97 ± 0.02	0.99 ±0.09
	1.73	9.18	0.16	0.78	4.74
Top spec alfalfa	0.2 ± 0.02	0.2 ± 0.02	0.21 ± 0.01	-	0.21 ±0.02
	4.20	3.82	2.91	-	3.73

This level of precision indicates that the Fructan assay method AOAC 2018.07 is reliable and repeatable.

Megazyme

Single Laboratory Standard Method Performance Requirements
AOAC SMPR 2018.07

AOAC SMPR® - Standard Method Performance Requirements

	AOAC SMPR® 2018.002	Megazyme (K-FRUC)
Operating range (% w/w)	0.2 to 100	0.21 to 98.4[a]
Limit of quantitation (LOQ) (% w/w)	0.20	0.119[b]
RSD_r, % (0.2 to 1% w/w Fructan)	7	4.74
RSD_r, % (>1 to 10% w/w Fructan)	5	3.59
RSD_r, % (>10 to 100% w/w Fructan)	3	2.96
RSD_R, % (0.2 to 1% w/w Fructan)	14	8.47
RSD_R, % (>1 to 10% w/w Fructan)	10	6.36
RSD_R, % (>10 to 100% w/w Fructan)	6	5.77

[a] Precise range dictated by fructan content in samples tested.

[b] Based on replicate measurements for a sample with ~ 1% (w/w) fructan.

- Method is robust for all specified range of samples
- This method is suited to measure Total Fructan in food and feed samples.

Megazyme

Conclusions

Enzymatic methods can be quite challenging:

- Method requires ultra-pure specific enzymes to remove interfering compounds and allow the quantitative measurement of Fructan.

- Enzyme selectivity and reaction parameters such as temperature and pH are crucial to prevent under or overestimation of Fructan content .

Megazyme

Conclusions

Enzymatic methods can be quite challenging:

- Method requires ultra-pure specific enzymes to remove interfering compounds and allow the quantitative measurement of Fructan.

- Enzyme selectivity and reaction parameters such as temperature and pH are crucial to prevent under or overestimation of Fructan content .

Updated K-FRUC procedure AOAC 2018.07:

- Allows quantitative measurement of inulin-type fructans, highly branched fructans and levan-type fructans across a wide range of samples.

- Employs ultra-pure, recombinant enzymes (*exo*-inulinase, *endo*-inulinase and *endo*-levanase) and therefore is specific for Fructan measurement.

- This method is user-friendly, robust and reliable for the intended application.

Megazyme

Thank you for your attention

Acknowledgements

Artur Rogowski – Megazyme, Molecular Biology Principal Scientist

Ciara McLoughlin – Megazyme, Carbohydrate and Enzyme Research Laboratory Scientist

Megazyme

How to account for reduced FOS underestimation?

FRUCTOOLIGOSACCHARIDES OR PURE FRUCTAN

Sodium borohydride/acetic acid.

FRUCTAN + **Non-reducing, sugar alcohols [D-Sorbitol + D-Mannitol + reduced FOS]**

***exo*-Inulinase + *endo*-Inulinase + *endo*-Levanase**

FRUCTOSE + GLUCOSE

PAHBAH reducing sugar determination

FRUCTAN

Borohydride Reduced Fructan Sample

Calculate the percentage recovery of fructan in the standard procedure following borohydride reduction as:

Percentage recovery = absorbance BRF / absorbance NBRF x 100

Non-Borohydride Reduced Fructan Sample

Megazyme

Spiking Recovery Studies

Table 5. Recovery of inulin (Raftiline) and levan (Timothy grass) spiked into GoCat dry cat food and into Timothy grass

Sample	Rec. of spike, % (mg added)		Rec. parameter for AOAC, %
	Inulin	Levan	
GoCat dry cat food	99.0 (27)	99.6 (26)	95–105
	97.8 (27)	95.2 (26)	95–105
Timothy grass	95.0 (22)	102.8 (21)	95–105
	99.4 (22)	97.2 (21)	95–105
Timothy grass	99.4 (11)	101.5 (11)	95–105
	103.4 (11)	95.1 (11)	95–105

Megazyme