Journal Article

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Determination of the degree of polymerisation of fructans from ryegrass and chicory using MALDI-TOF Mass Spectrometry and Gel Permeation Chromatography coupled to multiangle laser light scattering

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Highlights

- Rye-grass and chicory fructans have been characterised by MALDI-TOF and GPC/MALS
- Complementary molecular mass data was obtained using the two techniques
- Rye-grass fructans have potential application in food and personal care products.

Key words

MALDI-TOF MS, Gel Permeation Chromatography, molecular mass distribution, inulin, fructans

Abstract

This study is concerned with the determination of the degree of polymerisation (DP) of fructans from chicory and rye-grass (*Lolium perenne* L.) using Gel Permeation Chromatography coupled to multiangle laser light scattering and refractive index detectors (GPC / MALLS) and Matrix-Assisted Laser Desorption/Ionisation Time of Flight Mass Spectrometry (MALDI-TOF MS). The results show that fructans isolated from ryegrass have a DP in the range 2 - >100 and the commercially produced fructans derived from chicory have a DP in range 2 - 61. It has been demonstrated that MALDI-TOF MS is particularly effective at determining the DP of low molar mass material but is less effective for detecting the presence of high DP molecules. On the other hand GPC / MALLS is able to provide a much broader range of DP values although it is less sensitive at very low DP. It has been shown that the two techniques give complementary information thus providing a more accurate estimate of the overall DP of the fructan molecules.

1. Introduction

Inulin is a storage polysaccharide and is found in a large number of plants including chicory, garlic, leek, banana and Jerusalem artichoke. The main industrial source is chicory roots, with production centred on mainland Europe around Belgium and the Netherlands, with over 350,000 tonnes produced annually (Franck, 2006; Meyer, & Blaauwhoed, 2009). Inulin is a fructan and consists of linear chains of between 2-60 β-(2,1) fructose units with a glucose unit attached at the reducing end. It is finding increasing application in food products because of its ability to form gels (Bot, Erle, Vreeker, & Agterof, 2004; Glibowski, 2010) and the fact that it is classed as a dietary fibre (Meyer, Bayarri, Tarrega, & Costell, 2011; Ritsema & Smeekens, 2003; Gibson, Beatty, Wang, & Cummings, 1995). Inulin has also been used in pharma applications as an encapsulant for active ingredients and in other drug delivery pathways and is said to have preventative effects against a range of illnesses (Barclay, Ginic-Markovic, Cooper, & Petrovsky, 2010).

Perennial ryegrass, *Lolium perenne L.*, is a common agricultural pasture grass in Europe and is also rich in fructans. It is becoming increasingly attractive as a biorefinery feedstock (Charlton, Elias, Fish, Fowler, & Gallagher, 2009; Kromus et al., 2004) but has potential application in the food and related industries. The fructans present in rye-grass, which can constitute up to 40% of the total mass, differ slightly to fructans from chicory in that the fructose chains contain some branching (Van Loo, Coussement, De Leenheer, Hoebregs, & Smits, 1995). The solubility and gel properties of inulin are very dependent on the degree of polymerisation (DP) and it has been shown that solubility decreases and gel strength increases with increasing DP (Franck, 2006). In view of the importance of the molecular mass on the fructan functional properties this study sets out to determine the molecular mass distribution of fructans from chicory and ryegrass using both Gel Permeation Chromatography (GPC) equipped with multiangle laser light scattering and refractive index detectors (MALLS) and Matrix-Assisted Laser Desorption/Ionisation Time of Flight Mass Spectrometry (MALDI-TOF MS).

2. Materials and Methods

2.1 Materials

Fructan samples were obtained from ryegrass using an extraction process developed in-house at IBERS (Bryant et al., 2014) which involved membrane filtration and purification by precipitation using ethanol. The samples are coded AB1, AB2 and AB4. Commercial fructan samples derived from chicory (inulin) were obtained from BENEO-Bio Based Chemicals, Belgium. These were coded N25 (DP >23), N10 (DP ≈13) and H25P (DP 2-8) where the manufacturer quoted DP values are provided in brackets. Deionised water was used for sample preparation. Raffinose, dextran GPC standards (1kDa, 5kDa and 12kDa) and 2,5-dihydroxybenzoic acid (DHB) and 3-aminoquinoline (3AQ) matrix materials were purchased from Sigma-Aldrich. Pullulan standards (5kDa and 11kDa) were sourced from Polymer Standards Service GmbH. All other reagents were supplied by Fisher Scientific.

2.2 Gel Permeation Chromatography-Multiangle Laser Light Scattering

The molecular mass distribution of the chicory and ryegrass fructan samples and the polymer standards were determined by GPC-MALLS using a GE Healthcare Superose 12 GL column with 0.1M NaCl as eluent. Detection was made with Wyatt DAWN DSP light scattering and Wyatt Optilab DSP refractive index detectors connected in series. Samples (1% fructan concentration dissolved in 0.1M NaCl with mixing at room temperature) were introduced at 0.5 ml/min via a Rheodyne injection valve with a 200 μl injection loop after passing through a 0.45 μm syringe filter. This concentration was chosen to ensure as high a signal to noise ratio as possible when using the refractive index and light scattering detectors simultaneously. The molecular mass was determined using Astra for Windows 4.90.08 QELSS 2.XX. The Debye model was used for all evaluation analyses. A value of 0.131 was used for the refractive index increment (dn/dc) (Verraest, Peters, Batelaan, & Vanbekkum, 1995).

2.3 Matrix-Assisted Laser Desorption/Ionisation Time of Flight Mass Spectrometry

The DP for the fructan samples was obtained using an Applied Biosystems Voyager DE-PRO MALDI-TOF mass spectrometer. Two different matrix preparations were evaluated and sample preparation involved the dissolution of 12 mg of sample with 1 ml of matrix solution, namely, either 2,5-dihydroxybenzoic acid or 3-aminoquinoline (10 gL^{-1}) in 50% acetonitrile-deionised water solution. Following mixing this was then further diluted 1:9, analyte to matrix solution. Samples were introduced into a stainless steel 100-well plate via the dried drop method. Analysis was completed in linear positive mode with a nitrogen laser.

Ion acceleration was set at 15 kV, laser intensity and shots were varied and repeated multiple times in order to obtain clear spectra.

3. Results and Discussion

3.1 Determination of molecular mass and molecular mass distribution using GPC-MALLS

The GPC refractive index (RI) elution profiles for ryegrass fructan can be seen in Figure 1a, those for chicory inulin in Figure 1b, and those for fructose, sucrose and raffinose in Figure 1c. It is shown in the RI profiles presented that the ryegrass samples tested elute at lower elution volumes than those of the commercial chicory samples suggesting that they have a hogher molar mass. By comparison of Figures 1b and 1c it can be seen that the N10 and H25P samples contain a molecules which elute at elution volumes similar to mono-, di- and trisaccharides. The average molecular mass values were determined from the light scattering and refractive index signals using the instrument Astra software and the results are presented in Table 1. The high percentage errors for the N10 and H25P are a consequence of their low molecular mass and this is discussed below.

The RI and Mw elution profiles for the chicory inulin samples are shown in Figure 2 and it is noted that the Mw values at elution times > 32 mins become more erratic. This is as a consequence of the fact that the light scattering signal is very weak due to the low molecular mass (and in certain cases low concentration) of the fructan molecules eluting. Increasing the overall sample concentration will increase the light scattering intensity but the refractive index signal can then become saturated. This means that low molecular mass molecules particularly for the N10 and H25P samples, are, therefore, not taken into account when the average Mw and Mn values are determined using the instrument software. In order to overcome this problem and obtain a more realistic profile for the DP of molecules eluting at elution times > 32 mins, a calibration curve was obtained using pullulan and dextran standards together with sucrose and raffinose and the elution profiles and calibration curve are presented in Figures 3a and 3b respectively. The overall results are presented in the form of a histogram in Figure 4 which was created by plotting the relative intensity for each DP mass unit from the GPC RI intensity at particular elution times.

3.2 Determination of DP using MALDI-TOF MS

Initial experiments were undertaken to compare the ability of DHB and 3AQ to ionise the fructans and the spectra for the N10 sample are presented in Figure 5. Both sets of spectra show peaks with a mass separation of 162 which corresponds to one fructose unit but it was concluded that DHB performed better than 3AQ as evidenced by a greater number of higher molecular mass peaks. The mass spectra for each of the other fructan samples which were determined using DHB as the matrix material are shown in Figure 6. This differs from a report by Borromei et al (2009) who looked at MALDI matrices for other commercial inulins and suggests 3AQ to be better than DHB for larger DP samples. Our experience from this work is that more reproducible and better quality spectra could be produced by a DHB matrix. The choice of matrix can vary between experiments, with different groups preferring others. A brief discussion of DHB and 3AQ matrix spectra can be found in work by Wang, Spoorns, & Low (1999), although they in fact preferred another matrix over the two (2',4',6'-Trihydroxyacetophenone).

In contrast to the situation with GPC, there is good resolution of the samples at low DP but the resolution of higher DP molecules becomes poorer due to a reducing signal to noise ratio as the amount of material of that size present decreases. Similar observations were noted in previous work with other plant material, such as agave (Lopez et al., 2003) and asparagus fructans (Suzuki et al., 2011). The molecular mass profiles for the samples obtained using MALDI-TOF MS are superimposed on the GPC data in Figure 4 and the range of DP values for each of the samples obtained by the two techniques are presented in Table 2. The results together highlight the variation between the two techniques. The MALDI-TOF MS data show an upper DP limit of 41, *cf.* >100 with GPC, but similar patterns with the ryegrass fructans larger than all of the three commercial chicory fructan samples.

The DP values obtained by GPC are of the same order as values reported for chicory inulin, (*Cichorium intybus*) which has DP up to 60, and inulin from Jerusalem artichoke (*Helianthus tuberosus*) with DP up to 50 (Meyer, & Blaauwhoed, 2009). Previous studies on *L. perenne* varieties have obtained fructan DP values up to around 35 (Turner et al., 2002; Pavis et al., 2001) and 49 (Harrison et al., 2009). Harrison and co-workers managed to obtain data up to DP 100 using a high resolution LC-MS system, with *L. perenne* L. Var. Extreme (Harrison et al., 2011).

It is evident that MALDI-TOF MS gives more reliable data for lower DP values than GPC as has been reported by Hsu et al (2007) for other polysaccharides, for example pullulan and dextran. The lower values obtained by MALDI-TOF may in part be associated with the ease of desorption/ionisation of lower molecular weight material during MALDI-TOF MS measurements which hinders desorption/ionisation of larger material. In addition, fragmentation can occur in the case of the larger material leading to an increased signal occurring for low molecular mass material (Hsu et al., 2007; Stahl, Linos, Karas, Hillenkamp, & Steup, 1997).

4. Conclusions

The DP of fructan samples derived from chicory and rye-grass have been determined using MALDI-TOF and GPC / MALLS. While MALDI-TOF is able to determine the presence of low molecular mass species it is less effective in identifying the presence of high molecular mass species. For GPC / MALLS the situation is the opposite. However, molecular mass values at low DP can be obtained by GPC by the use of standards. The combination of GPC/MALLS and MALDI-TOF gives complementary information and a more accurate determination of the overall molecular mass distribution.

Acknowledgements

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Fig.1. GPC-RI elution profiles as a function of elution time for: (a) ryegrass fructan, (b) chicory inulin & (c) fructose, sucrose and raffinose.

Fig. 2. GPC RI and Mw elution profiles for the commercial chicory inulin samples.

Fig. 3a. GPC RI profiles for the mono- oligo- and poly- saccharides. 1- Fructose, 2 – Sucrose, 3- Raffinose, 4 – 1 kDa Dextran, 5 – 5kDa Dextran, 6 – 5 kDa Pullulan, 7 – 12 kDa Dextran, 8 –11 kDa Pullulan.

Fig. 3b. Calibration curve obtained from the pullulan and dextran elution profiles.

Fig. 4. Histograms qualitatively comparing DP distribution of chicory and ryegrass fructans obtained using GPC and MALDI-TOF.

Fig. 5. Mass spectra obtained by MALDI-TOF for chicory inulin N10 comparing two different matrices with annotated DP values: (a) DHB & (b) 3AQ.

Fig. 6. Mass Spectra obtained by MALDI-TOF for chicory and ryegrass fructan samples using a DHB matrix

List of Tables

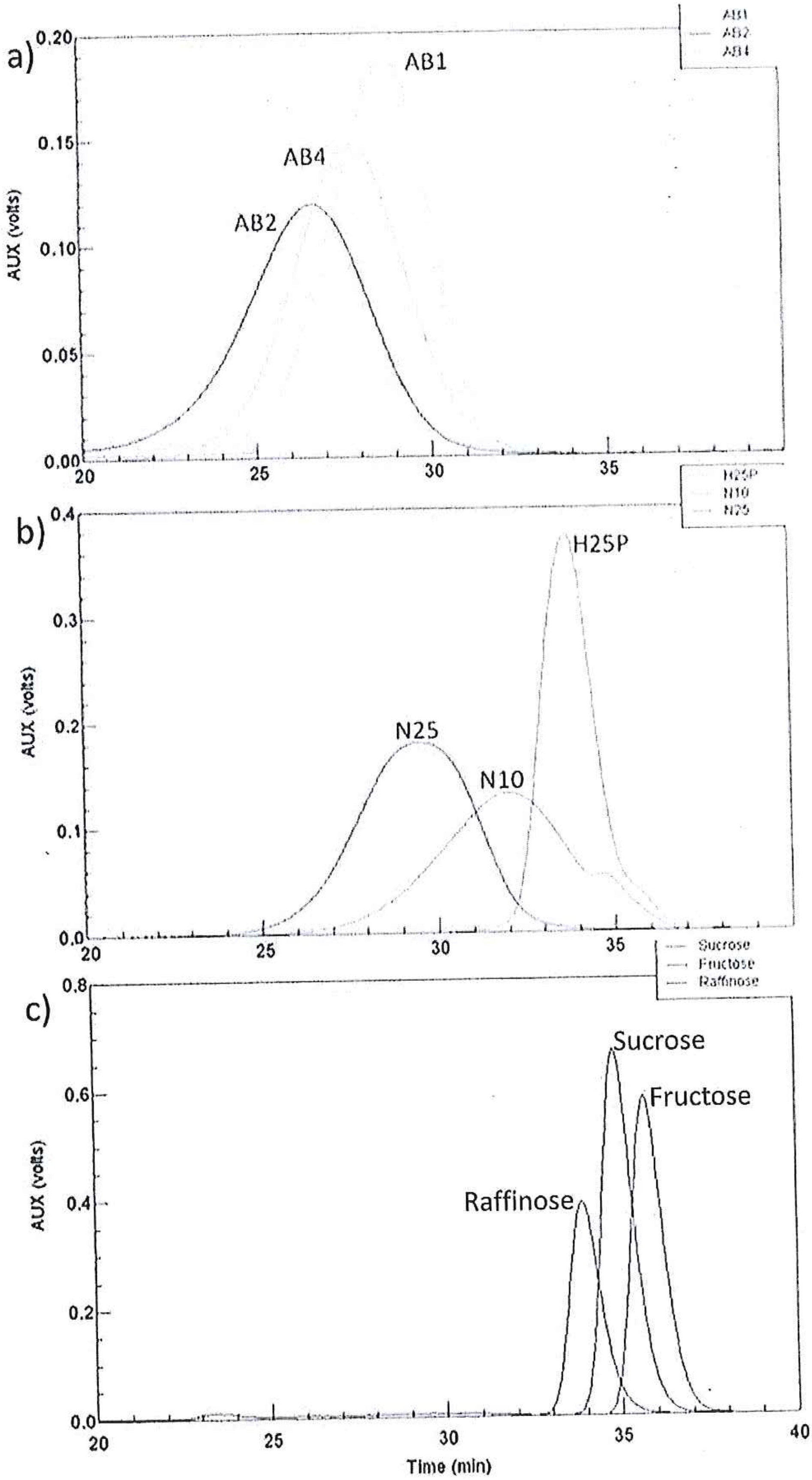
Table 1: Molecular mass values and polydispersity of chicory and ryegrass fructans

Sample	Mw (g/mol)	Mn (g/mol)	Mw/Mn
AB2	26,000 (14%)	15,000 (8%)	1.71 (17%)
AB4	19,000 (9%)	15,000 (3%)	1.28 (10%)
AB1	10,000 (10%)	8800 (8%)	1.14 (13%)
N25	8000 (10%)	6300 (5%)	1.27 (12%)
N10	2500 (16%)	1500 (24%)	1.71 (31%)
H25P	1400 (29%)	1000 (25%)	1.42 (34%)

Table 2: DP range of the fructan samples by MALDI-TOF MS and GPC.

Sample	DP (MALDI-TOF)	DP (GPC)
H25P	2 - 19	2 - 9
N10	2 - 24	2 - 56
N25	2 - 40	2 - 61
AB1	2 - 42	2 - >100
AB2	2 - 41	2 - >100
AB4	2 - 41	2 - >100

306



a)
AB1
AB2
AB4
AUX (volts)
0.20
0.15
0.10
0.05
0.00
20
25
30
35
b)
H25P
N10
N25
AUX (volts)
0.4
0.3
0.2
0.1
0.0
c)
Sucrose
Fructose
Raffinose
AUX (volts)
0.8
0.6
0.4
0.2
0.0
40
Time (min)

307 **Fig. 1.**

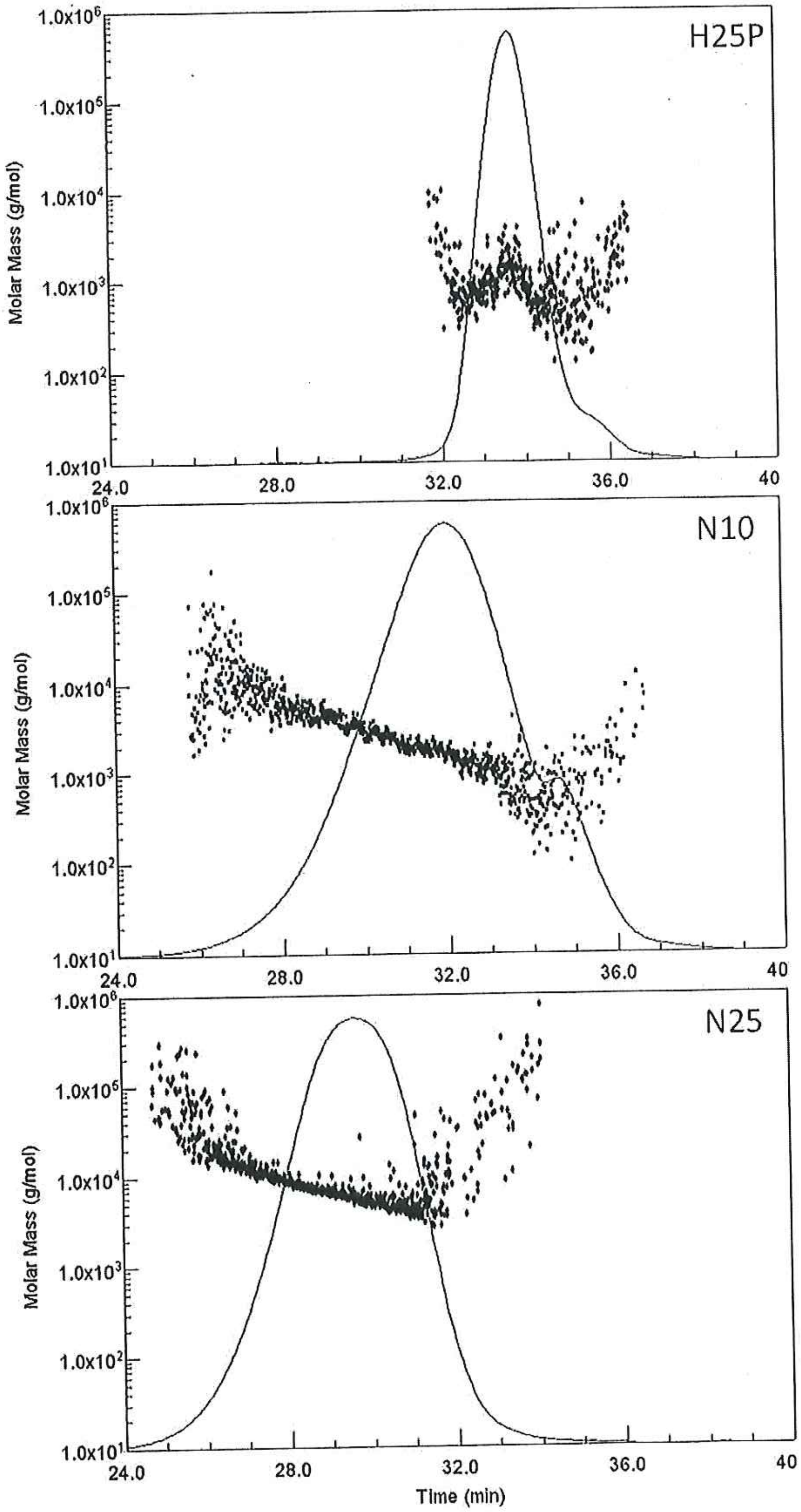

H25P
N10
N25
Molar Mass (g/mol)
Molar Mass (g/mol)
Molar Mass (g/mol)
1.0x10^6
1.0x10^5
1.0x10^4
1.0x10^3
1.0x10^2
1.0x10^1
24.0
28.0
32.0
36.0
40
Time (min)

Fig. 2.

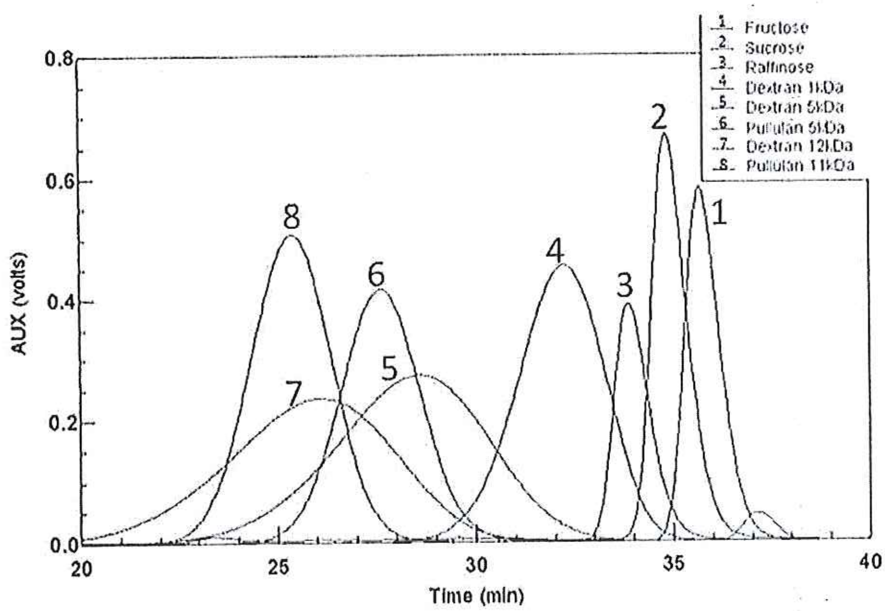

1 Fructose
2 Sucrose
3 Raffinose
4 Dextran 1kDa
5 Dextran 5kDa
6 Pullulan 5kDa
7 Dextran 12kDa
8 Pullulan 11kDa
AUX (volts)
Time (min)

Fig. 3a.

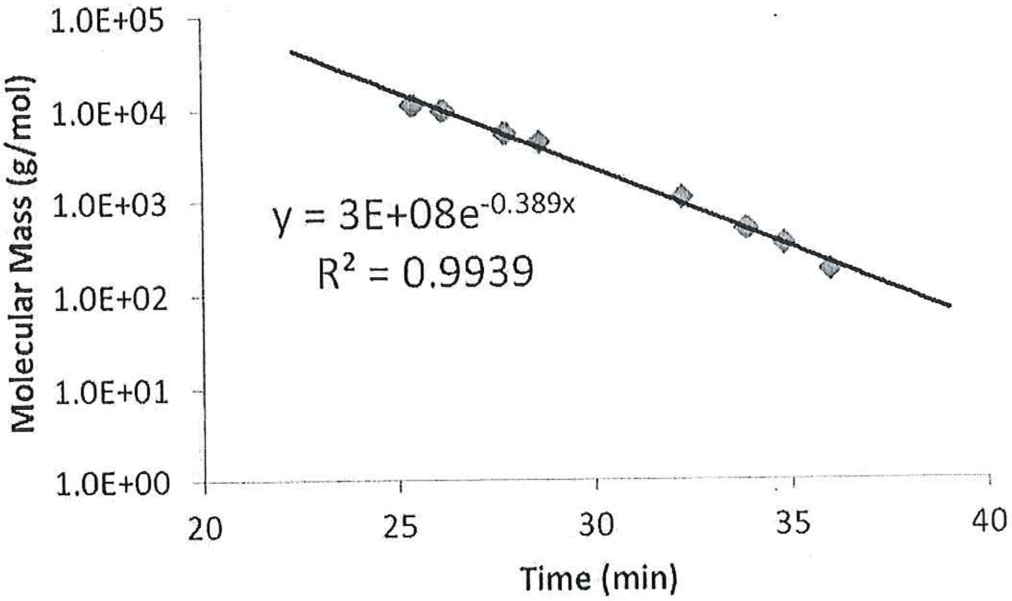
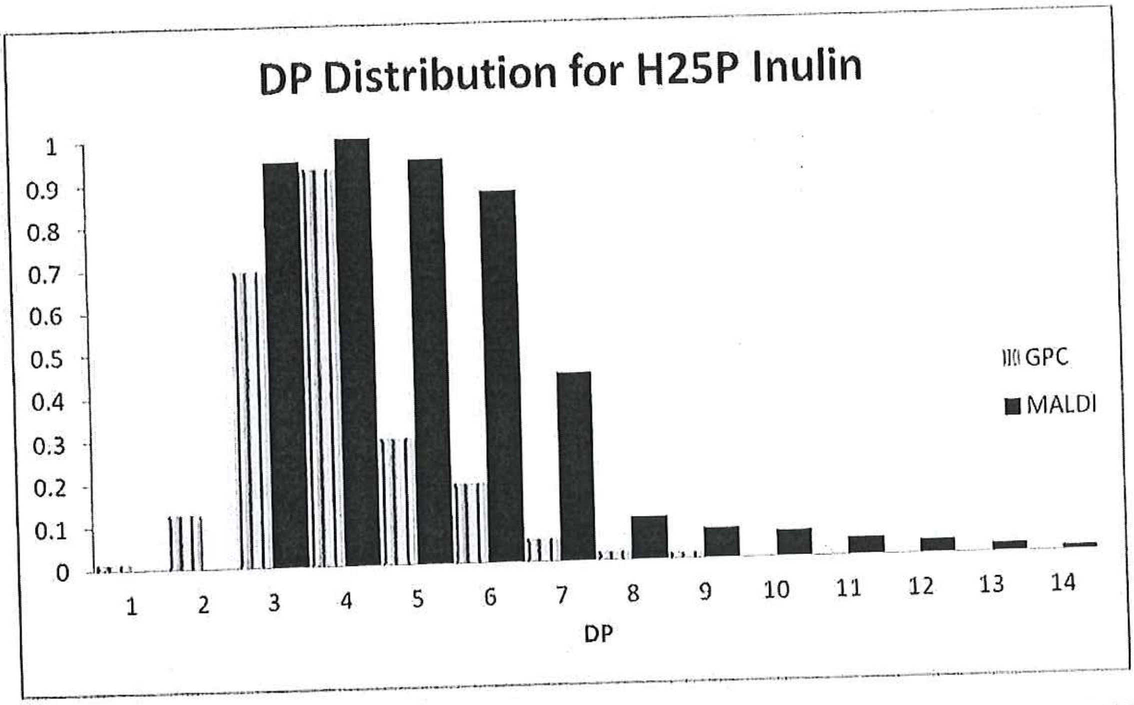
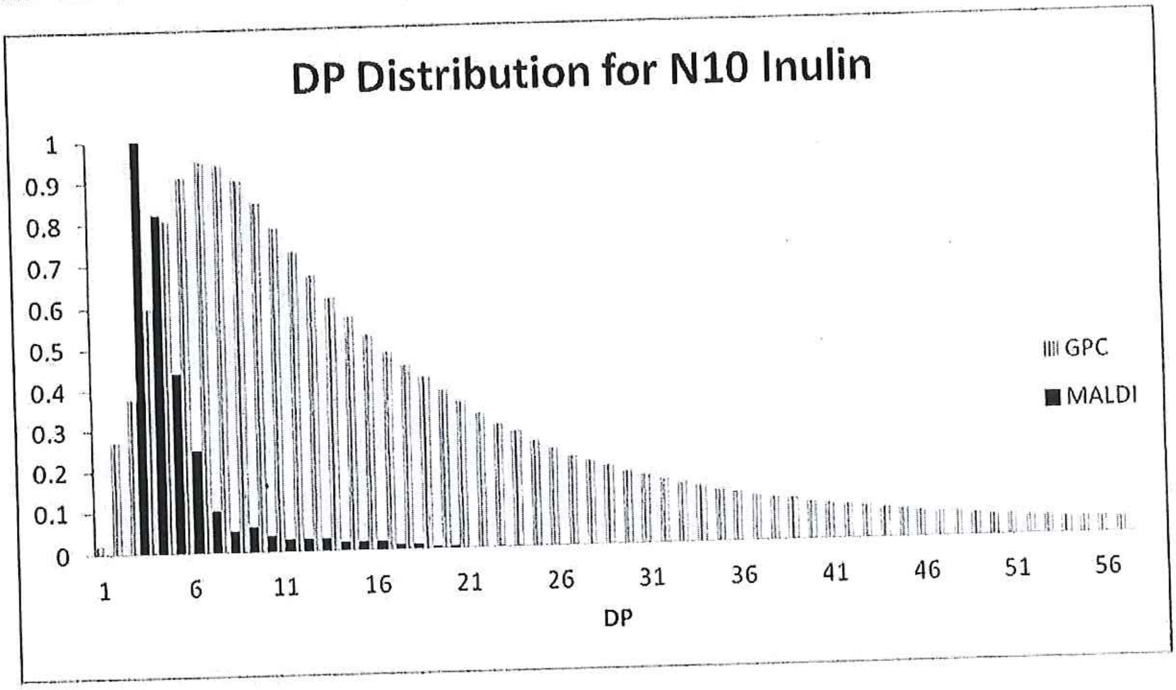

Molecular Mass (g/mol)
Time (min)
y = 3E+08e^-0.389x
R² = 0.9939

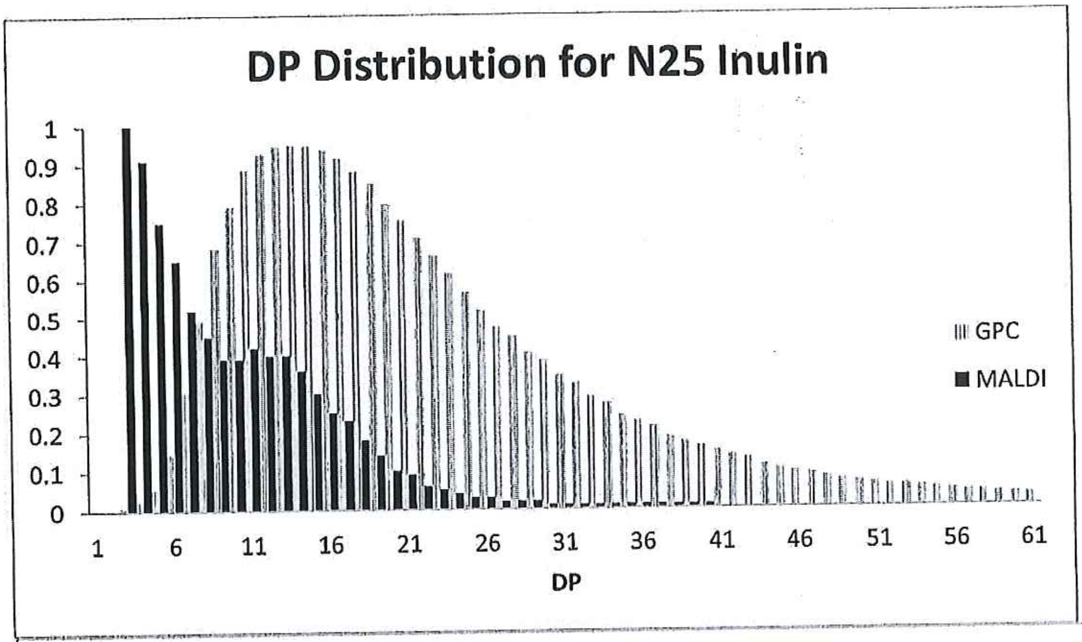
Fig. 3b.

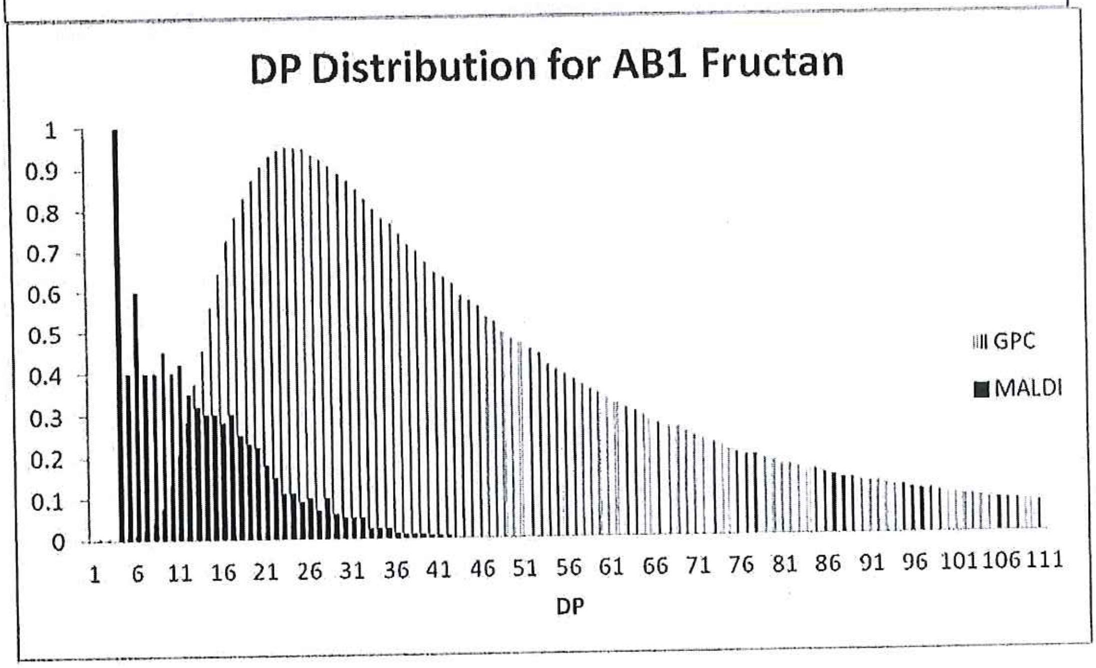


DP Distribution for H25P Inulin
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14
DP
GPC
MALDI



DP Distribution for N10 Inulin
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
1 6 11 16 21 26 31 36 41 46 51 56
DP
GPC
MALDI


DP Distribution for N25 Inulin
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
1
6
11
16
21
26
31
36
41
46
51
56
61
DP
GPC
MALDI


DP Distribution for AB1 Fructan
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
1
6
11
16
21
26
31
36
41
46
51
56
61
66
71
76
81
86
91
96
101
106
111
DP
GPC
MALDI

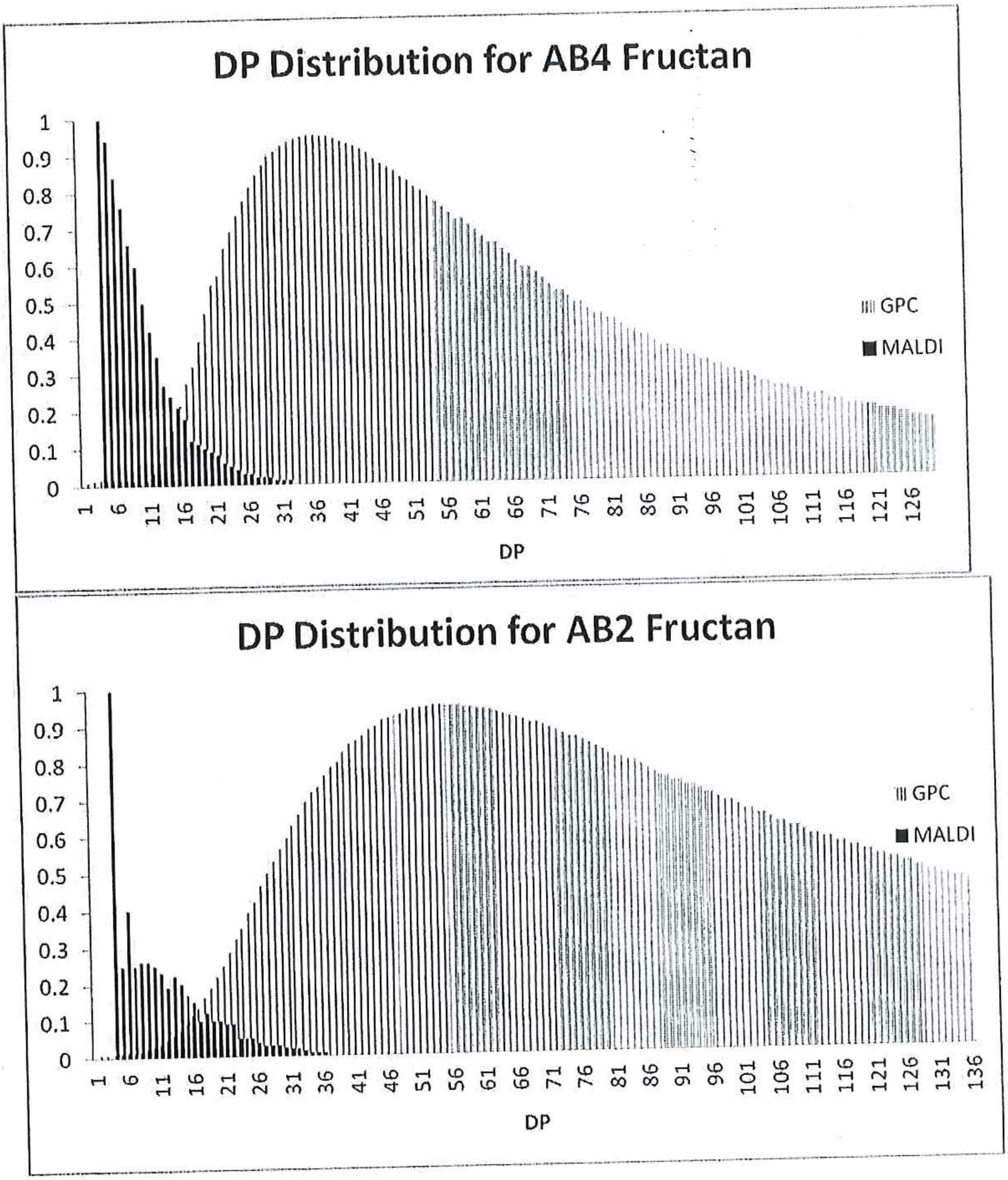

DP Distribution for AB4 Fructan
GPC
MALDI
DP
DP Distribution for AB2 Fructan
GPC
MALDI
DP

Fig. 4.

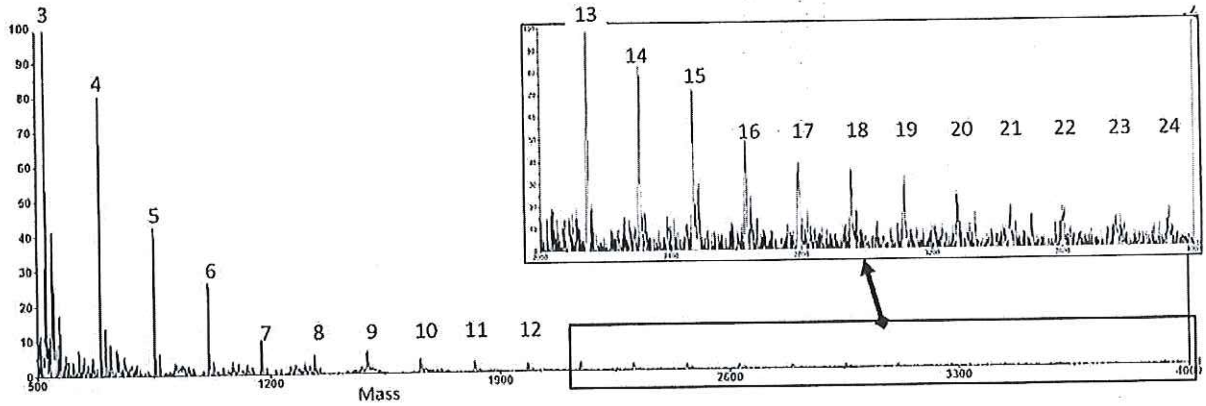

3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
100
90
80
70
60
50
40
30
20
10
0
500
1200
1900
2600
3300
Mass

Fig. 5a.

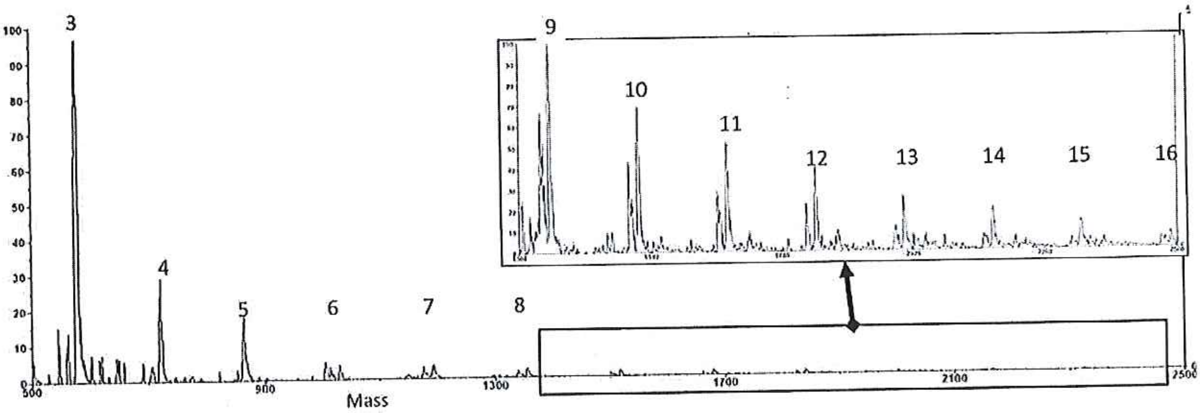
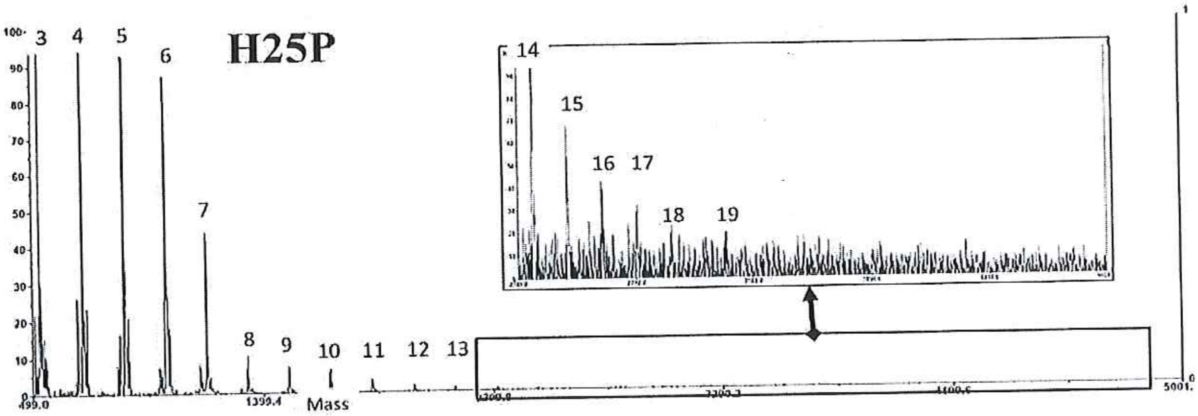
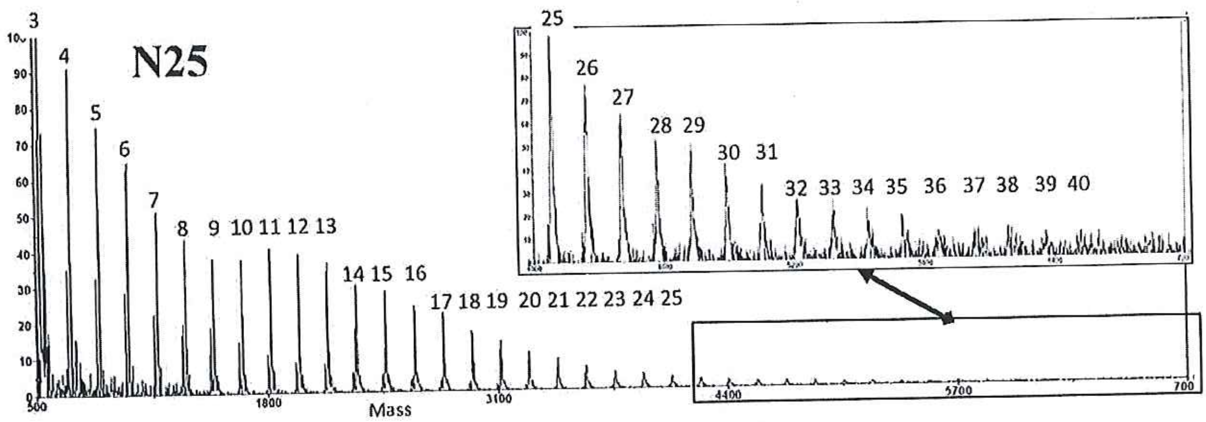
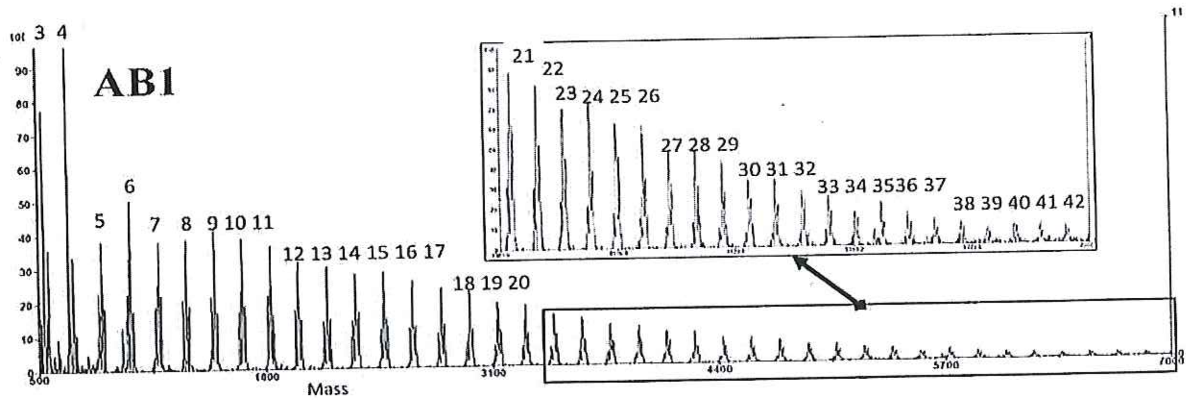
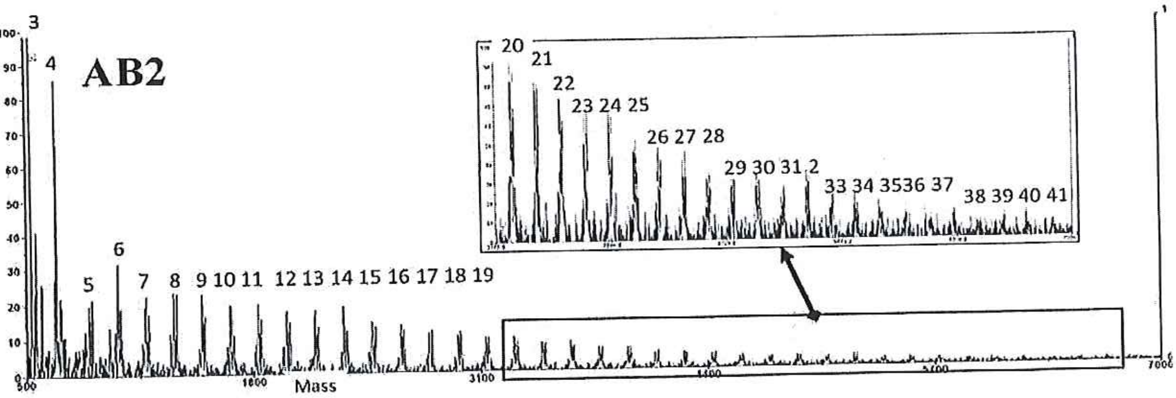

3
4
5
6
7
8
9
10
11
12
13
14
15
16
100
90
80
70
60
50
40
30
20
10
0
500
900
1300
1700
2100
2500
Mass

Fig. 5b.


H25P
3 4 5 6 7 8 9 10 11 12 13
14 15 16 17 18 19
Mass


N25
3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25
25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40
500
1800
Mass
3100
4400
5700
700


AB1
3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
21 22 23 24 25 26 27 28 29 30 31 32 33 34 3536 37 38 39 40 41 42
Mass
3100
4400
5700
7000


AB2
3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
20 21 22 23 24 25 26 27 28 29 30 31 2 33 34 3536 37 38 39 40 41
1800
Mass
3100
4400
5700
7000

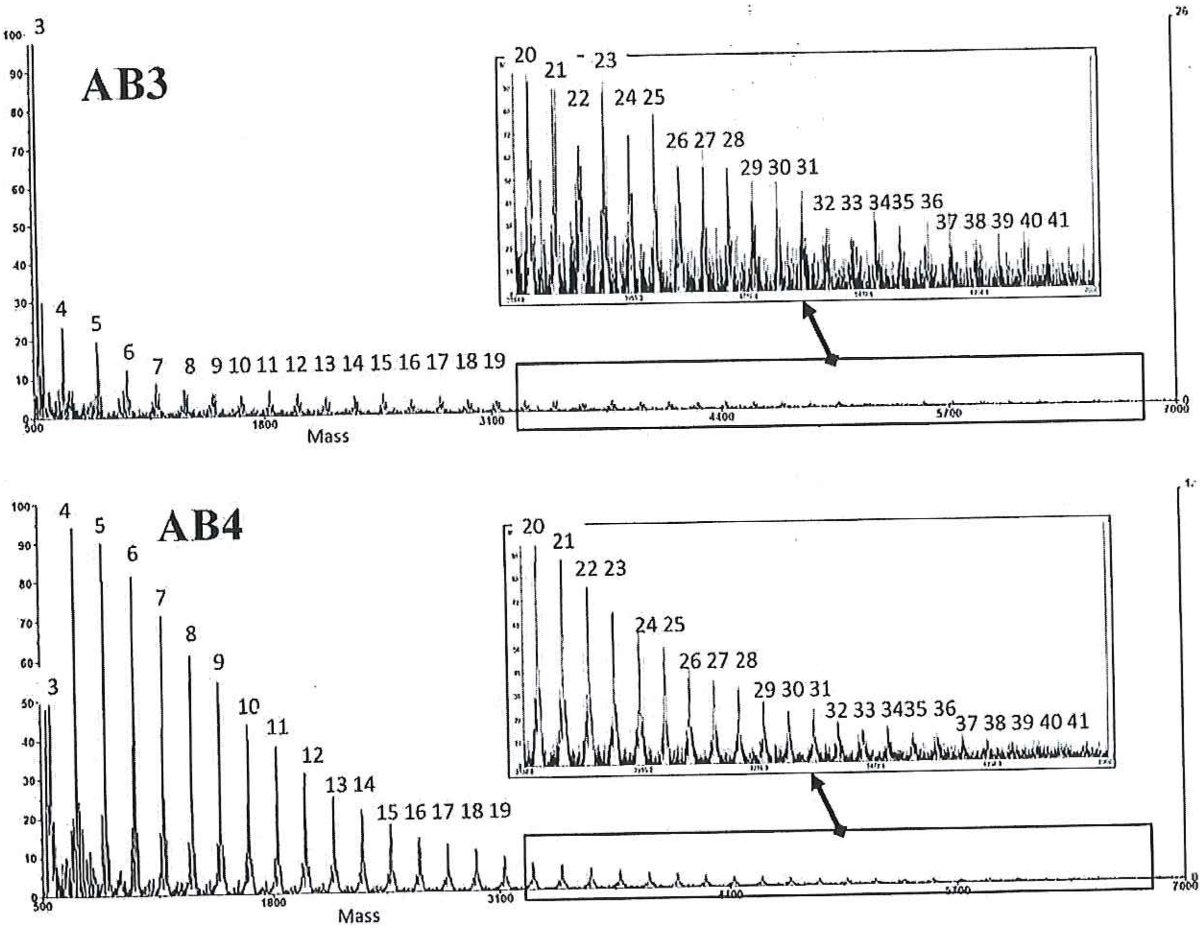

AB3
3
4
5
6
7 8 9 10 11 12 13 14 15 16 17 18 19
20 21 22 23 24 25
26 27 28
29 30 31
32 33 3435 36
37 38 39 40 41
1800
3100
4400
5700
7000
Mass
AB4
4
5
6
7
8
9
3
10
11
12
13 14
15 16 17 18 19
20 21 22 23
24 25
26 27 28
29 30 31
32 33 3435 36
37 38 39 40 41
1800
3100
4400
5700
7000
Mass

Fig. 6.