TEST ARTICLE STORAGE AND STABILITY

COMMENTS ON DRAFT NTP TECHNICAL REPORT ON THE TOXICOLOGY AND CARCINOGENESIS STUDIES OF ISOEUGENOL (CAS NO. 97-54-1) IN F344/N RATS AND B6C3F1 MICE (GAVAGE STUDIES) NTP TR 551 NIH Publication No. 08-5892

The following comments have been prepared following a review of the Draft NTP Report 2-Year Gavage Study of Isoeugenol TR155 and the Batelle Quality Assurance Statement, Bulk Chemical Limited Analysis Report, Isoeugenol (Batelle Study No G002840-NN, TherImmune No. 7244-203, pp 487 – 521).

1. Background

The formation of oxidation and polymerization products during the storage of isoeugenol is an area of investigation that AQUI-S New Zealand Ltd (AQNZ) has undertaken for the preparation of the Chemistry and Manufacturing file for AQUI-S, an aquatic anaesthetic containing 50% isoeugenol as active ingredient. This work has included an analysis of the oxidation and polymerization products of isoeugenol and the development of analytical methods to monitor the formation of isoeugenol degradation products including polymers.

1.1 Isoeugenol Polymerisation

Polymerisation of isoeugenol occurs rapidly on exposure to air and is usually noted by the development of colour from light straw to dark yellow. This can occur within a few days for a sample that is open to the air at room temperature, or over a few months for samples that have been exposed to the air while being transferred from one container to another and that have been stored at room temperatures. The polymers have not been characterized in detail but include the dimers: diisoeugenol and dehydrodiisoeugenol. The formation of vanillin, an oxidation product often accompanies the formation of polymers.

Isoeugenol usually has a 2-year shelf life if kept in dark un-opened plastic lined metal containers. The extent of polymerisation of a 5 to 7-year old sample will depend on storage conditions, including exposure to air and light. Experience at AQNZ suggests that deterioration commences as soon as a container is opened and exposed to the air. For this reason all storage containers are nitrogen purged and blanketed.

GC/MS profiles for unpolymerised (sample name: feed141207) and polymerised isoeugenol (sample name: feed71207) are attached as Figures 1 and 2 respectively. The unpolymerised sample was approximately 3 months old and had been stored in a sealed plastic lined steel drum at room temperature (15^{o}C to 20^{o}C). The polymerized sample was 2 years old and had been stored in an opaque HDPE container at room temperature.

2. NTP Analytical Method for Test Article Stability

The GC method used to determine the stability of isoeugenol, the test article, for the NTP study, is described on page H-2 of the draft report. Method B uses an initial oven temperature of 80^{o}C and ramps to 240^{o}C over 8 minutes followed by a hold period of 9 minutes. The total time is approximately 17 minutes. This method is commonly referred to in the scientific literature but it

does not indicate the presence of isoeugenol polymerization products. Therefore these materials are not normally reported by the suppliers of isoeugenol or in the literature.

AQNZ uses a modified GC method (SOP831) for which the temperature is held at 150^{o}C for 13 minutes followed by ramping to 300^{o}C over 3 minutes and then holding for a further 14 minutes. The total time is approximately 30 minutes. The higher temperature used for this method is necessary to indicate the presence of the high molecular weight polymers.

Figure 3 shows comparative traces using the NTP Method B for unpolymerised isoeugenol (Figure 3a) and polymerized isoeugenol (Figure 3b). The polymerized sample shows a small increase in peaks at 7.5 minutes and 8.5 minutes but does not indicate significant material at longer retention times. The method terminates after approximately 19 minutes. The main peaks as shown are eugenol (7.3 minutes), cis-isoeugenol (7.7 minutes) and trans-isoeugenol (8 minutes).

A comparison of the NTP Method B and the AQNZ SOP831 for polymerized isoeugenol is shown in Figure 4. An expanded scale is used to allow identification of specific peaks. Figure 4a shows the NTP Method B. Although peaks are shown at 8.6 minutes and 9.3 minutes there are no significant peaks at longer retention times. Figure 4b shows the same sample using the AQNZ SOP831 which indicates significant material at retention times between 18 and 20 minutes.

The identified compounds for the AQNZ SOP831 method are as follows:

No	Compound	Residence Time, minutes
1	4-(2-propenyl)-phenol	6.66
2	Eugenol	7.43
3	2-methoxy-4-propyl phenol	7.82
4	vanillin	8.22
5	cis-isoeugenol	9.29
6	trans-isoeugenol	11.22
7	1-(4-hydroxy-3-methoxyphenyl)-2-propanol	13.14
8	Diisoeugenol	18.56

3. NTP Study Storage Conditions

3.1 Test Article Storage

There is confusion regarding the storage conditions used for the NTP study.

The Battelle Quality Assurance Statement refers to storage of the test article in 1-L amber glass bottles, with Teflon®-lined lids, at room temperature (p491, Battelle Study No G002840-NN).

The NTP Draft report refers to storage at less than or equal to -20 ^{o}C, protected from light, in 1-L Teflon® bottles (page H-2, Isoeugenol NTP TR551).

The test article was received on May 30, 1997 and June 11, 1997. No information has been provided regarding the date of manufacture. This material was used in the 2-year gavage study commencing in April 2002, with the final dose being administered in April 2004. This means that the isoeugenol was at least 5-years and possibly more than 7-years old for the study.

Prolonged storage periods are known to result in the polymerization of isoeugenol. The storage conditions in the NTP study may have resulted in degradation by polymerization in the test article. The extent of polymerization will depend on the actual storage conditions used.

3.2 Dose Formulations Storage

The dose formulations were prepared as a mixture of isoeugenol in corn oil. The formulations were stored at room temperature in amber glass bottles with Teflon®-lined lids for up to 35 days.

Experience with the preparation of formulations of isoeugenol has found that although some materials will inhibit polymerization others can promote polymerisation. The influence of corn oil on the polymerization process is unknown.

4. Conclusions

The conclusions of this review are as follows:

(i) Isoeugenol is subject to polymerization when stored for extended periods.

(ii) The GC Analytical Method B as used by Batelle is not suitable to determine the stability of isoeugenol because it does not detect polymers.

(iii) The conditions of storage of the test article in the NTP study must be verified.

Dr D J Bell
AQUI-S New Zealand Limited
PO Box 44-269
LOWER HUTT
New Zealand
Phone: +64 4 587 0389
Fax: +64 4 587 0388

8 February 2008

Figures Attached

Figure 1 GC/MS Fresh Isoeugenol (no polymerization)
Figure 2 GC/MS Polymerised Isoeugenol
Figure 3 NTP Method B. a) Fresh Isoeugenol b) Polymerised Isoeugenol
Figure 4 Polymerised Isoeugenol. Expanded Scale a) NTP Method B. b) AQNZ SOP831

Figure 1. GC/MS Fresh Isoeugenol (no polymerisation)

Isoeugenol

File :J:\2007\Dec2007\IanSims\~~Eugenol~~\feed141207.D
Operator : IMS
Acquired : 19 Dec 2007 14:03 using AcqMethod EUGENOLHP5MS
Instrument : Agilent 5
Sample Name: feed141207
Misc Info :
Vial Number: 3

Fresh Isoeugenol
(no polymerisation)

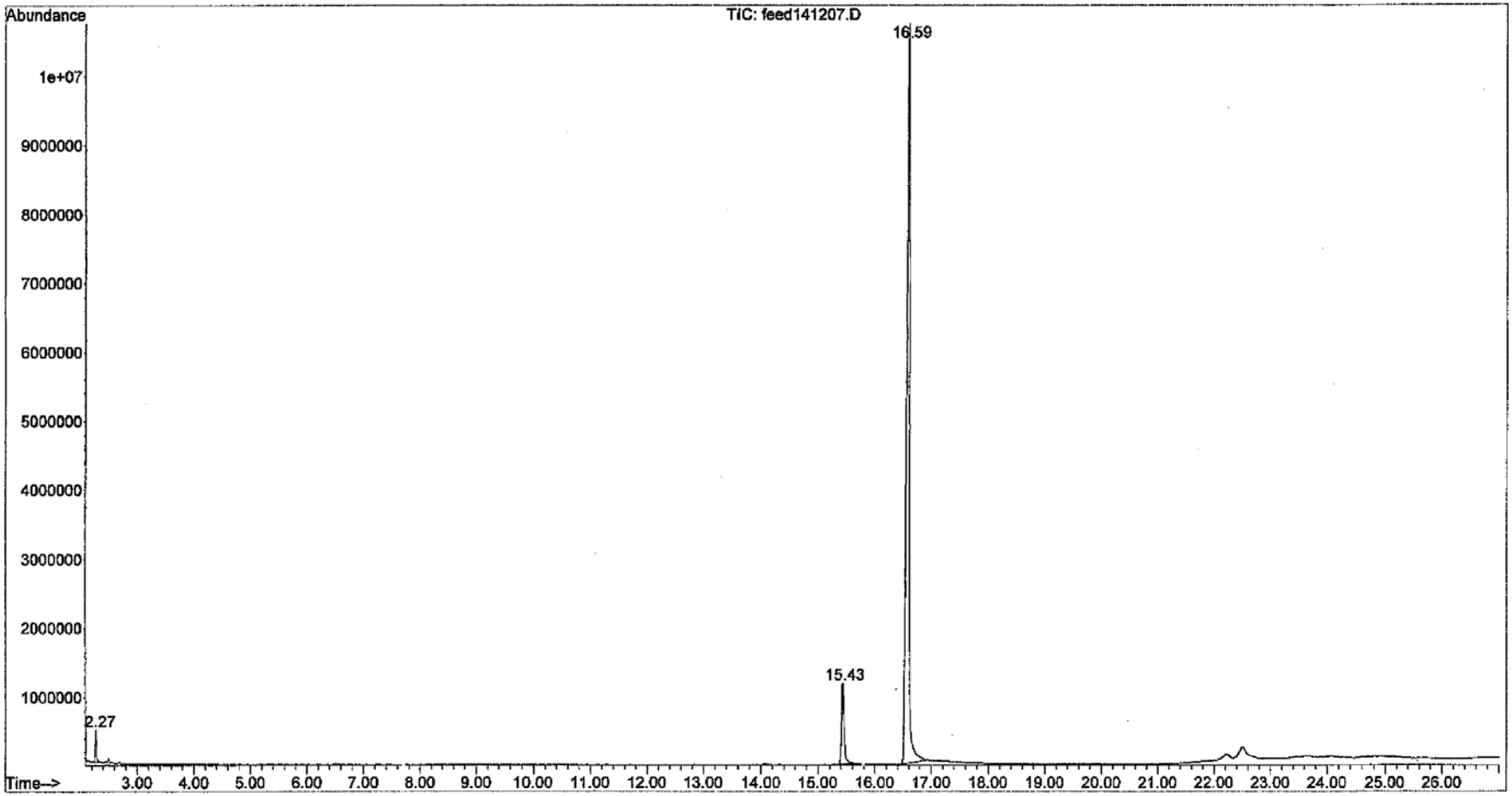
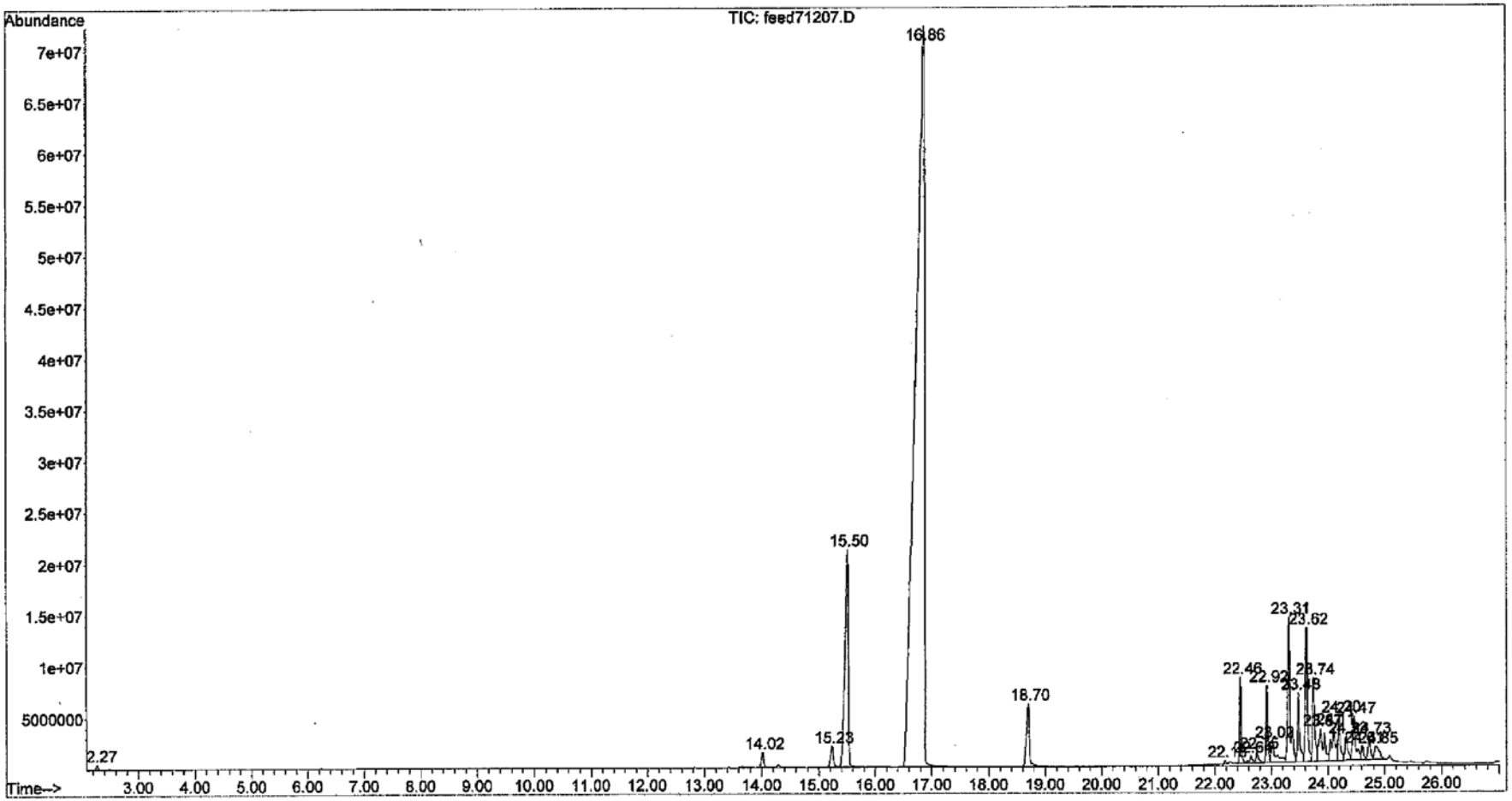


Figure 2. GC/MS Polymerised Isoeugenol

Isoeugenol

File :J:\2007\Dec2007\IanSims\[redacted]\feed71207.D
Operator : IMS
Acquired : 19 Dec 2007 13:30 using AcqMethod EUGENOLHP5MS
Instrument : Agilent 5
Sample Name: feed71207
Misc Info :
Vial Number: 2

Polymerised Isoeugenol.



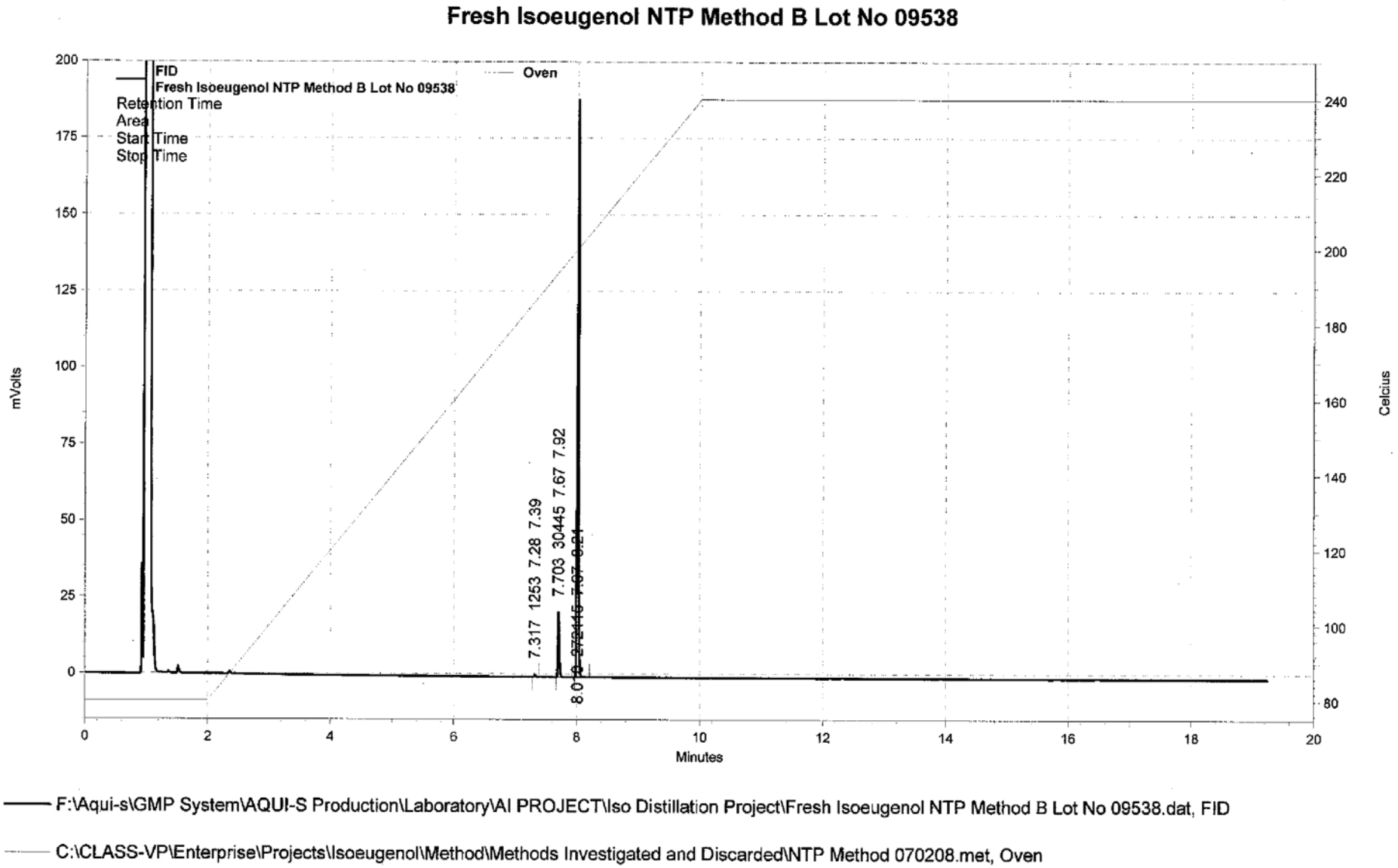


Figure 3a. NTP Method B. Fresh Isoeugenol

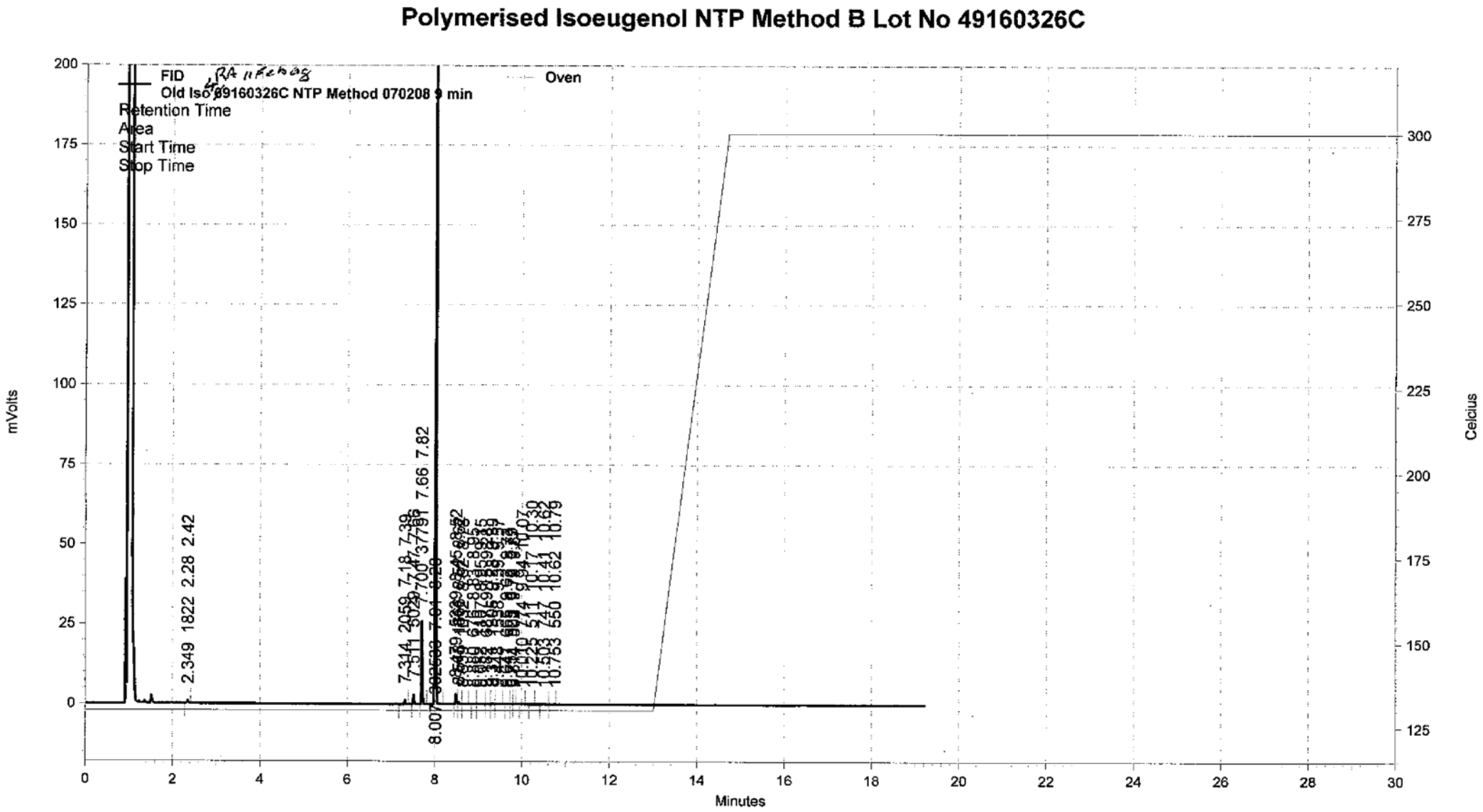


Figure 3b. NTP Method B. Polymerised Isoeugenol

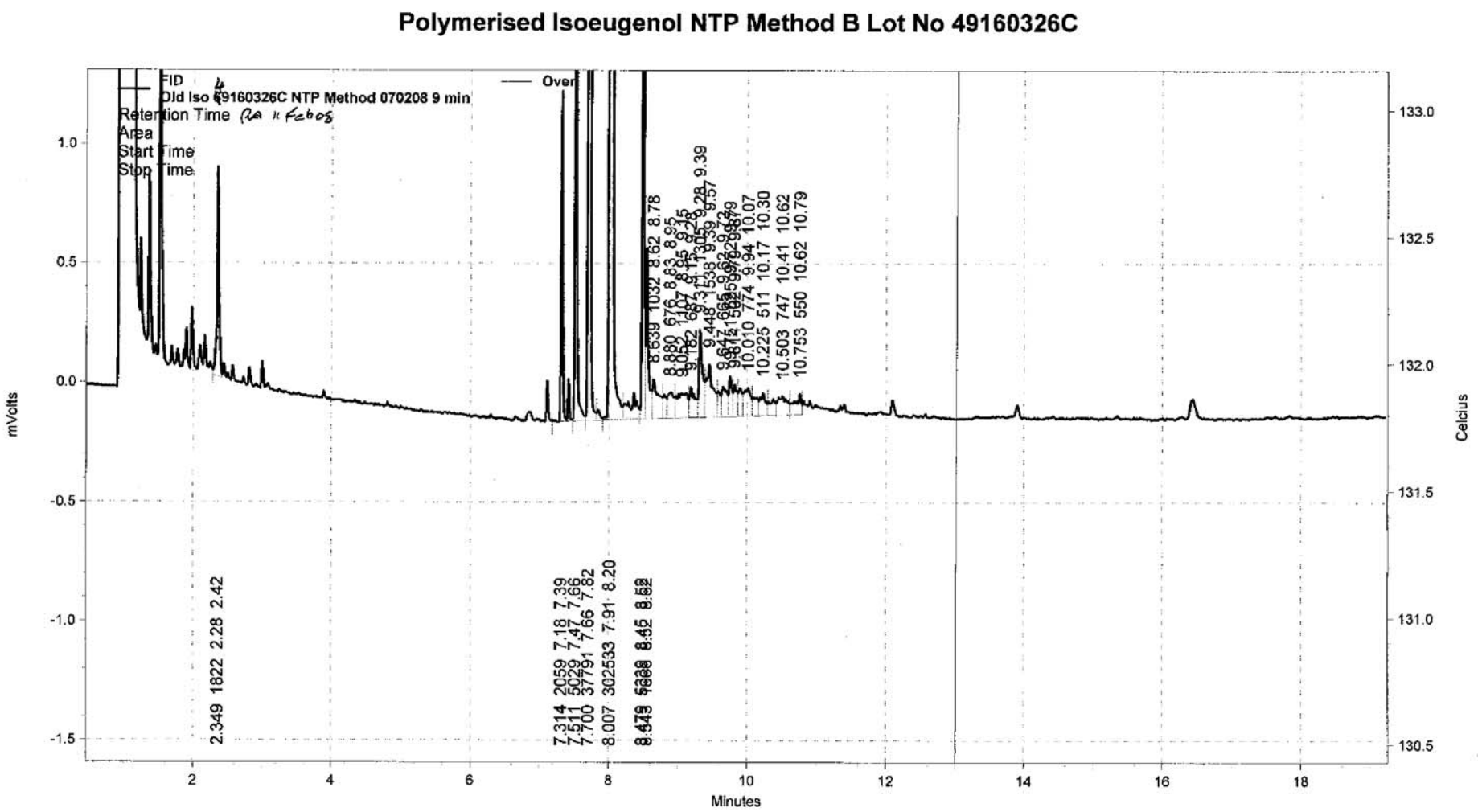


Figure 4a. Polymerised Isoeugenol. Expanded Scale. NTP Method B

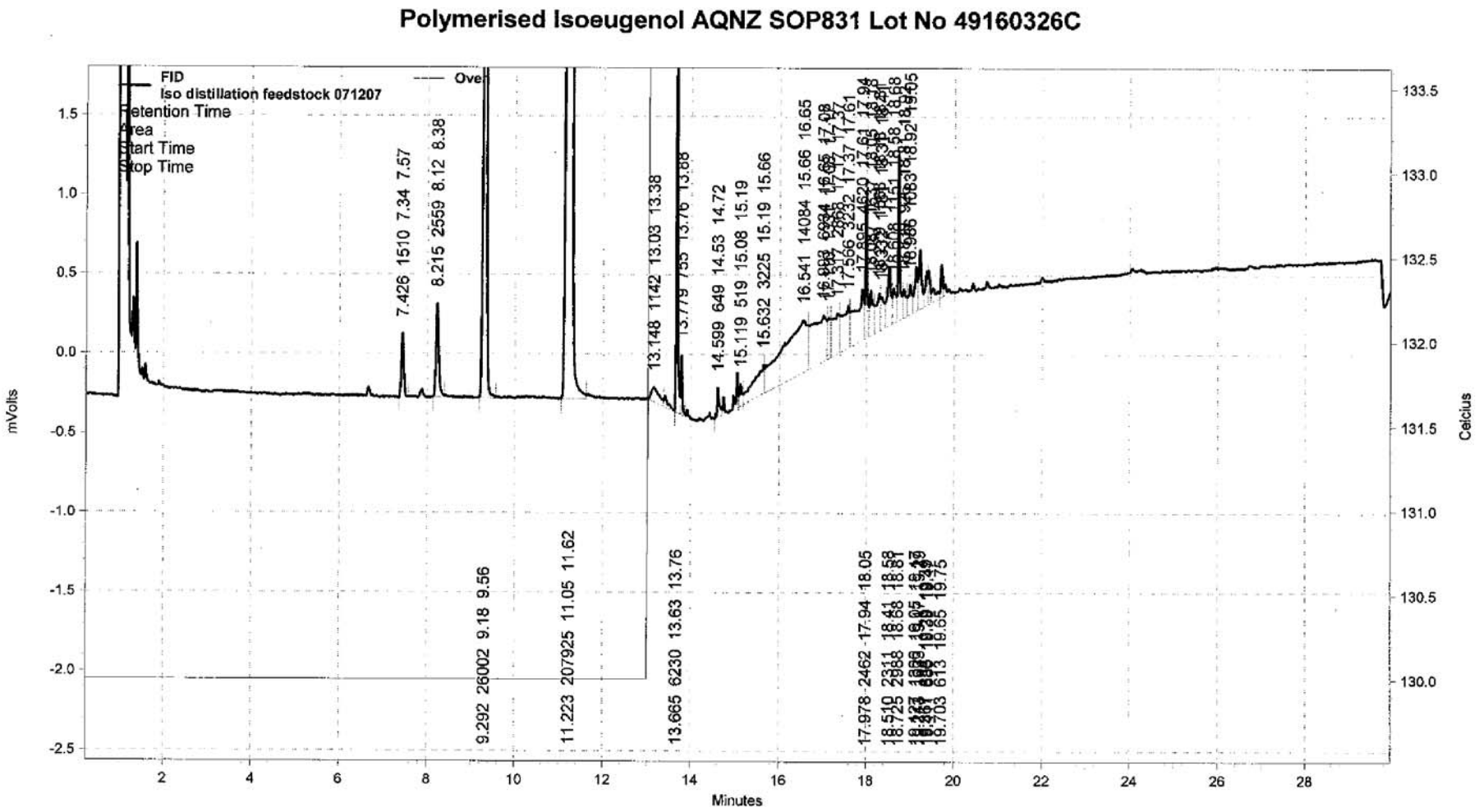


Figure 4b. Polymerised Isoeugenol. Expanded Scale. AQNZ SOP831