THE SYNTHESIS OF CONDENSED TANNINS

by

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This thesis is presented for the award of
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The thesis submitted is based upon the
Candidates own work.

Declan Moran

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I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of Masters degree is entirely my own work and has not been taken from the work of others save and to the extent that such work has been cited and acknowledges within the text of my work.

Signed: Declan Moran **Date:** August 94

Candidate

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ABSTRACT:

The Synthesis of Condensed Tannins:- Declan Moran.

A literature review with 110 references is presented which examines the history of the chemistry of condensed tannins and the development of this chemistry in parallel with the development of modern analytical techniques. The role which these compounds play in the wine and brewing industries is discussed. An examination of the different synthetic routes applied to the isolation of condensed tannins, most notable procyanidin B3, is presented. Also investigations into the modes of action for the formation of tannins in wine and beers is discussed with the implications behind their presence explained.

An experimental section that examines two different synthetic routes for the formation of procyanidin B3 from the starting materials (+)-taxifolin and (+)- catechin is presented and the advantages and disadvantages outlined. New analytical methods for the monitoring of these reactions are presented along with the NMR spectra of recovered products which are explained and compared to data generated from literature sources.

A method for the acetylation of the reaction products, thus stabilising them is presented along with the HPLC and NMR details explaining the results obtained. Finally two separate attempts at synthesising procyanidin B3 via enzymatic oxidation are presented and discussed, and the two methods are evaluated.

ACKNOWLEDGEMENTS:

My sincere thanks are due to my supervisor Dr. Paraic James for his continuous guidance and encouragement and also to my employers Bristol-Myers-Squibb Swords Laboratories for their generous financial support.

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THE SYNTHESIS OF CONDENSED TANNINS

INTRODUCTION:

The study of condensed tannins is among the oldest forms of chemistry known. The origin of the word tannin is derived from the practice for which these compounds were used in the leather industry. This industry has made use of tannins for centuries. Tanning is a process whereby dried animal skins are formed into leather by its contact with a profusion of tannins. In the Northern latitudes the most common tannin used was oak bark. The reason for this was the large acreage of oaks that were a common feature of all northern European countries in the past. This process had been carried out for generations but the science behind the expansion of leather chemistry was born out of the Industrial Revolution. For the first time leather was being produced for more than personal use and the age of mass production had arrived

During the evolution of this chemistry there have been many pioneers who have gained a knowledge of the complex chemical processes involved in tanning. The attainment of this knowledge came about very much on a trial and error basis. Consequently, there have been many definitions which have tried to encapsulate the true essence of what defines a tannin. A general description used defines a tannin as everything extracted from a plant that gives a blue colour on exposure to ferric chloride. Haslam in his book on Plant Polyphenols [1] prefers the definition coined by Bate-Smith and Swain [2]. They proposed that tannins were "water soluble phenolic compounds having molecular weights between 500 and 3,000 units and that besides giving the usual phenolic reactions, they have special properties, such as, the ability to precipitate alkaloids, gelatin and other proteins."

In overall terms there are two separate areas that can be discussed in relation to tannin chemistry. The first has the group of polyesters based upon gallic acid and its derivaties, known as hydrolysable tannins and the second contains the group of proanthocyanidins, known as condensed tannins. It is the latter with which this work is concerned. Before delving into the field of condensed tannin chemistry, it is of some importance to outline many of the uses to which these compounds have been applied since the decline of the leather industry in the Twenthieth Century.

The initial spark of interest into these compounds stemmed from the discovery that they were involved in the defence mechanisms of many plants. The astringent nature of these materials caused an unpalatable taste in the mouths of would be predators thereby insuring the plants survival. The reason behind this was identified as being due to the complexation of proanthocyanidins with macromolecular proteins such as glycoproteins [3]. Allied to this discovery was the realisation that these compounds also demonstrated anti-microbial and anti-viral properties in plants. Because these compounds are found in the human diet this was an important discovery. The basis of many oriental herbal remedies has been shown to be due to the presence of proanthocyanidins. For example these compounds have demonstrated a positive effect against *streptococcus mutans*, the primary causative agent of plaque and dental cavaties, by limiting the adhesion of the microbe on the smooth dental surface [4]. As a result many commercially available mouthwashes contain proanthocyanidins.

The anti-fungal properties of these compounds have been known since the turn of the Century when Knudson demonstrated that very few fungi could survive in the presence of 2%v/v tannin solutions [5]. Studies into the anti-viral capacity of these compounds have also been undertaken. Cadman reasoned that polyphenols complexed with the virus thus making it non-infective [6]. All of these properties have lead to the increase of research into these compounds over the past three decades. The Food and Beverage Industry by extension, have a major interest in the area of proanthocyanidins. Since these compounds are found in numerous plant types, they form part of the human food chain and are thus important in this Industry. Polyphenols contribute to the taste and flavour of foods and their presence or absence must be controlled. Too much of these compounds and the food developes an astringent taste. Astringency is defined as "a feeling of constriction, dryness, roughness, along with a sense of stiffness in the movement of the tongue with some loss of taste [7].

Within the Food Industry there has been a trend away from synthetic colourants due to possible toxic effects associated with these chemicals. Anthocyanins are compounds that are known to possess colouring capability and also may be of pharmaceutical importance. The stability of these compounds is based upon several factors: pH, temperature, partial oxygen pressure, types of co-product present, light radiation and glycosidation, as well as the nature of the heterocyclic rings. The development of commercial processes to isolate and purify these compounds is of great importance. Pifferi *et al* have outlined an optimised procedure which utilises polyvinylpyrrolidone (PVP) and alumina [8]. This group investigated several classes of anthocyanins and found that their method was very effective in separating the various compounds. The alumina was found to impose a buffering action on the compounds due to the presence of both acidic and basic sites thus allowing separation.

Since anthocyanins are used as colourants, methods for their identification and characterisation are needed if the strict rules applying to food additives are to be adhered to. In the United States beverages are only allowed to contain grape skin extracts while non-beverages are only allowed grape colour extract. With this in mind regulatory bodies routinely analyse commercial products and adulteration with banned substances can be identified. The standard way to measure these compounds was based upon their tinctorial strength (a measure of colour efficiency), however, this can be misleading as optical density and colour rank are poorly correlated due to the fact that optical density does not deal with band widths as does optical perception.

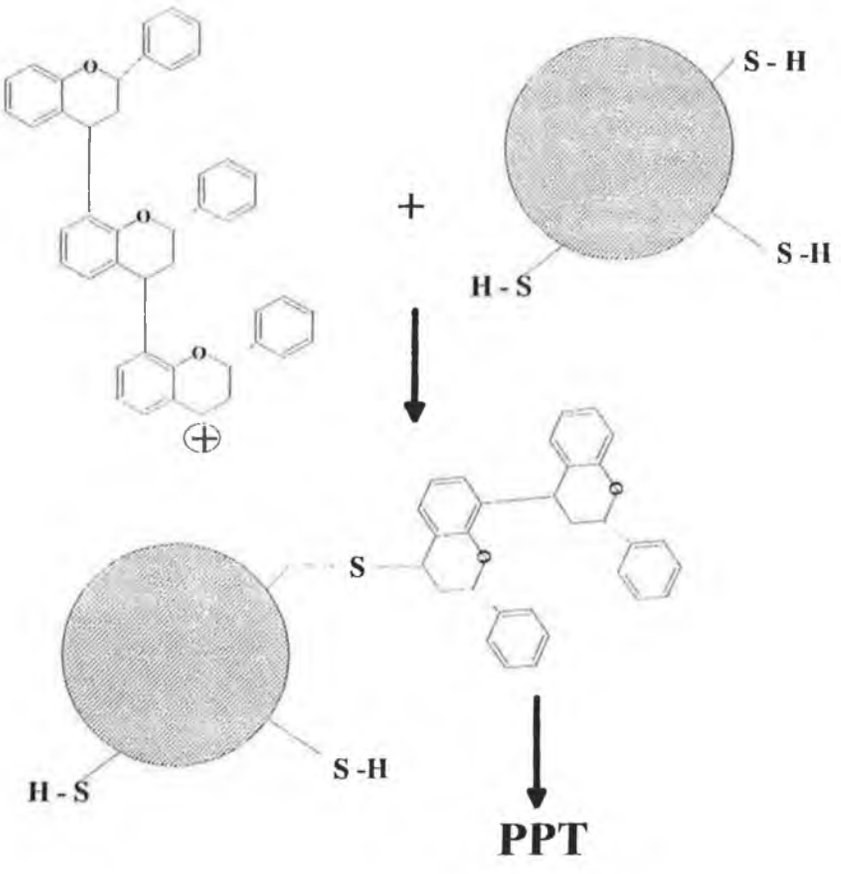
Wrolstad *et al* have proposed the use of high performance liquid chromatography (HPLC), allied to photodiode array detection (PDA) to characterise anthocyanins [9]. They subjected a whole series of compounds to analysis and from results obtained, observed that the method proved to be useful as the retention characteristics of compounds yielded data on the sugar moieties bound and on the nature of the anthocyanin. The same group further developed their technique to build a library of spectra for standards mainly characterising acidic polyphenols such as: delphinidin, cyanidin, petinidin, pelargonidin, peonidin and malvidin [10]

As well as foods, anthocyanidins contribute to the overall flavour of beverages, most notably teas, wines and beers. In wine production, particularly for whites the key aim is to limit the extraction of polyphenolic material from the grape so that oxidation of these compounds may be controlled. For red wines this trend is reversed and the extraction of polyphenols is encouraged. The presence of such compounds and their subsequent mode of action is not yet fully understood but major strides into the understanding of these processes have been made more recently.

Beers and lagers contain small concentrations of procyanidins. These are all derived from the malt (or barley) used in the manufacture of beer. Their presence in beer is a two edged sword, on the plus side, they contribute to the flavour of the beer, while on the minus side they have been implicated in the astringency of beer and also in the formation of non biological hazes that shorten the shelf life of the beer. This is a universal problem for brewers and although not fully understood, it is known to be caused by the association of the procyanidins and other polyphenols with proteins and polypeptides, it is not known however if specific proteins are involved.

During the cooling of a beer chill hazes are formed which are not permanent as these re-dissolve with heating. For the commercial production of beer, chilling is necessary and the haze is an unwelcome problem that must be removed. Currently one way to remove this haze is to immerse nylon strands into the beer which cause the polymeric material to physically adhere to it thus removing the precipitates. Over a period of time permanent hazes appear in beers which limit its shelf life. The reason for this has been proposed to be due to slow acid catalysed (pH 4.0) bond breaking and re-forming reactions characteristic of proanthocyanidins [11]. Under the weakly acidic conditions that prevail in beers, decomposition of the proanthocyanidin within the pre-formed protein-polyphenol complex is thought to occur and this generates a flavan-3-ol carbocation which is a strong nucleophilic acceptor open to attack and polymerasition. It has been suggested, however, that polymersition is not the cause of haze formation but rather that the reactive carbocation is captured by nucleophilic thiol groups of proteins. Although these adducts are acid labile, they are more stable than the proanthocyanidins. Therefore, the protein surface is more hydrophobic in nature ultimately leading to haze formation.

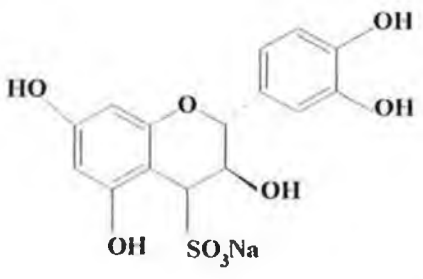
REACTION SCHEME#1: PROPOSED HAZE FORMATION MECHANISM.



Some of the other areas that make use of condensed tannins include the Oil Industry and the manufacture of natural adhesives. It has been found that sulphonation of tannins makes them more soluble and reduces viscosity. This has been applied to tannins used as lubricants in oil well drilling and adhesive applications. The reason for this is not yet fully understood. Yeap Foo *et al* established that the process only occurred to a small degree [12].

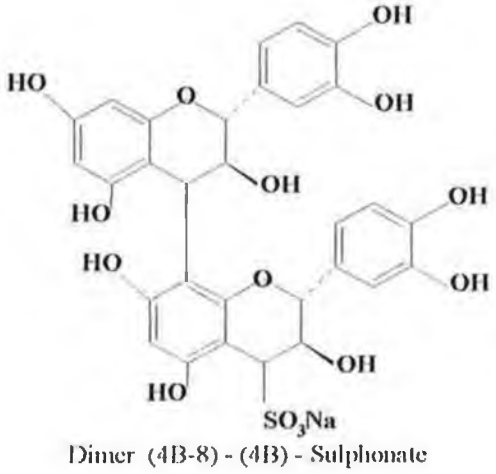
Treatment of tannins with sodium hydrogen sulphite lead to the isolation of sodium epicatechin-(4B)-sulphonate (Fig 1) and a dimer sodium epicatechin -(4B-8)-epicatechin -(4B)-sulphonate (Fig 2) in 20% and 6% yield respectively. Results showed that cleavage of the interflavanyl bond by the sulphite increased solubility. This reaction should be controlled for leather tanning as dimers and trimers exhibit poor tanning capabilities. Sulphonation of adhesive tannins also requires an increase in cross-linking agents to create cured resins.

Fig. 1



Sodium epicatechin (4B) sulphonate

Fig 2 :



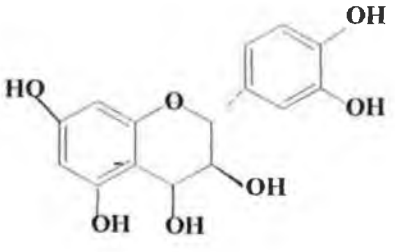
Dimer (4B-8) - (4B) - Sulphonate

Finally it is worth noting that this review is in no way a complete account of all the areas of research into this field carried out of the past three decades. Many references detail the extraction, isolation and characterisation of tannins from plant sources. To detail each of these would be a mjor undertaking well beyond the scope of this review, however, the topics discussed shall be directly related to the synthesis of condensed tannins from natural precursors, their characterisation by chromatographic and spectroscopic means with in some cases direct comparison to their natural analogues. Finally, studies that have involved model solutions that in some way mimic the processes that occur in nature shall also be outlined in some detail.

THE SYNTHESIS AND CHARACTERISATION OF PROCYANIDINS

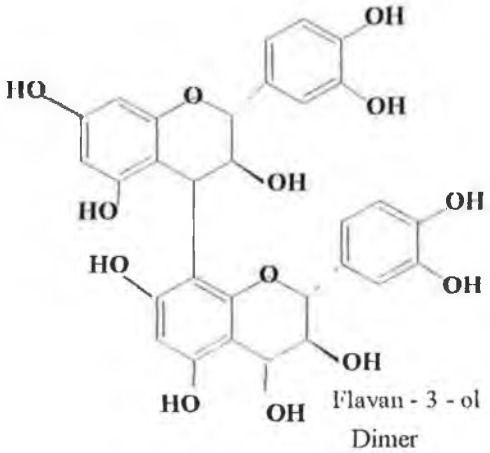
Over the past three decades there have been many groups involved in the study of procyanidins. The earliest classifications of these compounds were attributed to work carried out in the first two decades of this century. Leucoanthocyanidin was first discovered as far back as 1915 [13]. Studies by several different workers showed that these classes of compounds were confined mainly to wooded plants (i.e. bark plant types) [14,15]. Chemical studies have shown that there are two main types of compounds in this class. The first type are flavan-3,4-diols and the second flavan-3-ol dimers and higher oligomers. (fig.3/4):

Fig. 3



Flavan - 3,4 - diol

Fig. 4



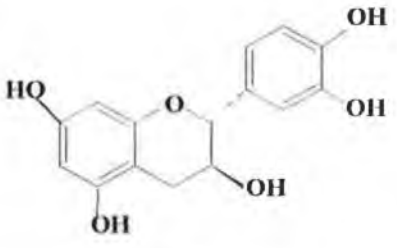
Flavan - 3 - ol Dimer

Initial analysis of the procyanidin dimers of B type (B1 to B4) was carried out on their peracetate derivatives, as these compounds were more stable than the free hydroxyl form. Haslam *et al* postulated that if biosynthesis studies were to be undertaken than analysis of the free form would be necessary [16]. However, ^{1}H Nuclear Magnetic Resonance (NMR) temperature studies on these free phenolic compounds showed that the dimers B1 to B4 exhibited conformational isomerism making chemical shift assignation difficult due to broad resonance signals.

For the procyanidin dimers B3 and B4 satisfactory analysis was possible if a few assumptions were made. Firstly the lower terminal unit's heterocyclic ring was assumed to adopt a "skew boat" conformation, with the aryl group at C-2 in a quasi-axial position. Secondly, based upon the splitting of the methylene group of procyanidin B3, as well as the signal for H-4, it was assumed that rotation about the 6'-4 or 8'-4 interflavanyl linkage was restricted rather than having heterocyclic ring flipping. However, due to the conformational isomerism a third factor is involved, namely, that the upper half of the molecule should have 2,3-trans configuration and the C-3 hydroxy group be in a quasi-equatorial position.

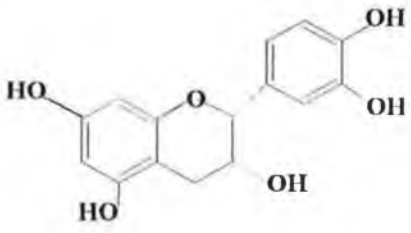
Degradation studies were carried out to confirm the structures that had been proposed using ethanolic-HCl at 60°C. In all cases the dimers yielded cyanidin and for B2 and B4 epicatechin, while for B1 and B3 (+)-catechin (Fig.5/6).

Fig. 5



(+) - Catechin

Fig. 6

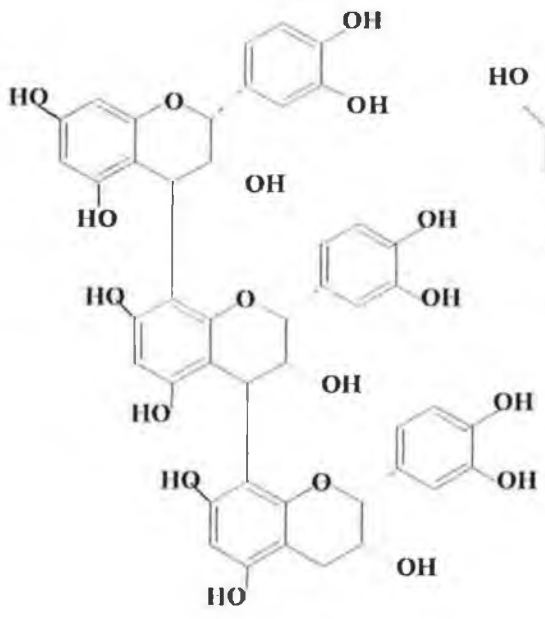


(-) - Epicatechin

The group suspected that the interflavanyl linkage was 4-8' in procyanidins. This was confirmed by the synthesis of diacetyl octamethyl-procyanidin. This was a simple cleavage reaction that served to confirm the link. Arising from this work, studies of the dimers B5 and B7 and the trimers C1 and C2 were carried out. The study of these trimers was of significance because it suggested that the controlling factor in the

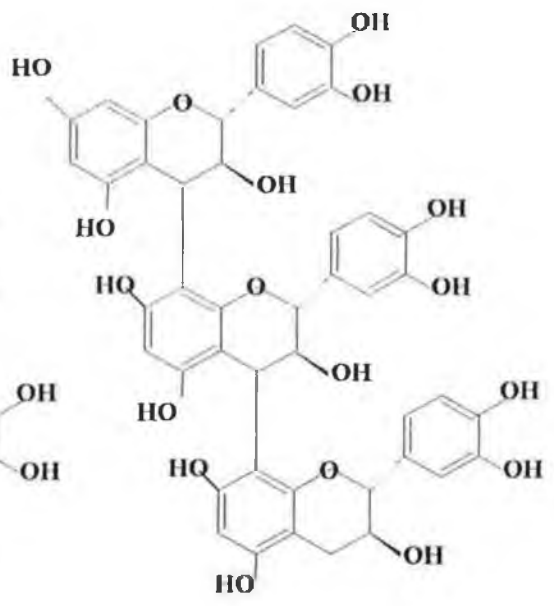
formation of higher polymeric procyanidins was a chemical rather than an enzymic process. (Fig.7/8)

Fig .7



Procyanidin C1

Fig .8

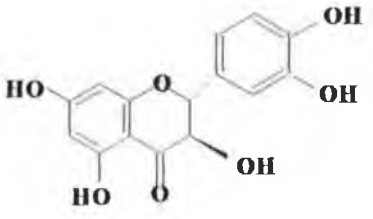


Procyanidin C2

In order to improve the study of these compounds a method for the determination of absolute configurations was needed. With the aid of ^{1}H NMR and ^{13}C NMR and also mass spectroscopy the absolute configuration should be possible to be assigned. Roux *et al* used the fact that the condensation of flavan-3-ols at the C-4 position was stereoselective when linked to resorcinol or phloroglucinol groups, holding partial or total retention of 2,3-trans isomers or inversion of 2,3-cis isomers, to develop a direct method of configurational analysis.[17]. Here multiple Cotton effects formed from aryl chromophores at C-4 dominated the circular dichroism spectra (c.d.) of the cis and trans isomers. This allowed determination of the absolute configuration of this reaction centre.

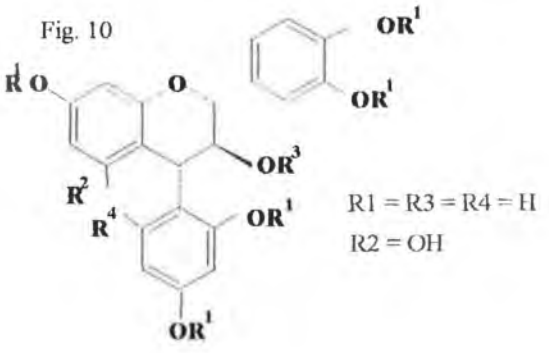
Up until the 1980's much of the isolation and characterisation of procyanidins had been carried out on compounds extracted from various plant sources. However there was no universal method of synthesis for these compounds until Roux *et al* presented their findings [18]. This group generated C-4 carbocations from flavan-3,4 diols and reacted these with strongly neuleophilic flavan-3-ol biflavanoids as well as resorcinol and phloroglucinol. The c.d spectra allowed unambigous identification of chiral centres and this lead to a general chiroptical rule that has been confirmed by other workers [19]. The reduction of (+)-taxifolin with sodium borohydride, when coupled to phloroglucinol and resorcinol gave 3.4-trans (4.8% yield) and 3,4-cis compounds (4% yield) respectively. The carbocation formed under mildly acidic conditions was found to be stereo specifically captured by phloroglucinol and resorcinol to form 2,3-cis and 3,4-trans aryl flavan-3-ols analogues containing inversion of configuration. (Fig.9/10).

Fig. 9

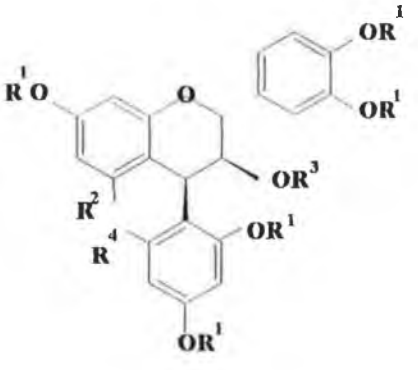


(+) - Taxifolin

Fig. 10



3,4 -Trans Analogue

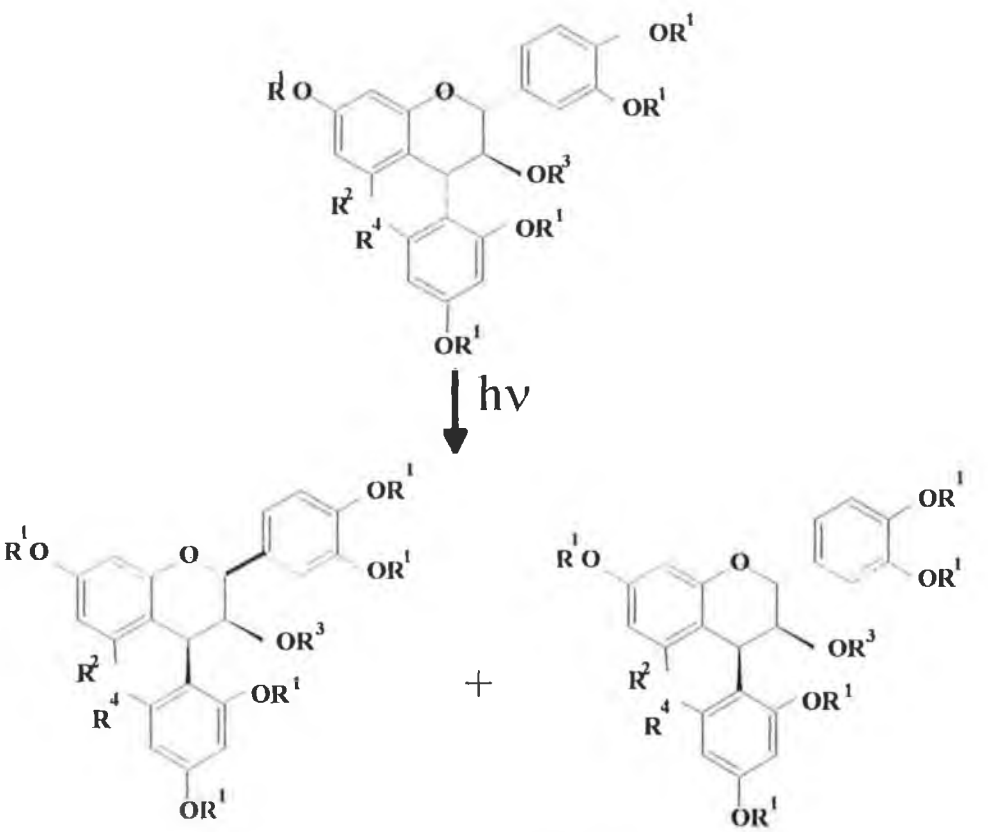


3,4 - Cis analogue

It was also observed that there was little competition from self condensation.

The same group extended their findings to the study of the first synthesised 2,3-cis-3,4-trans aryl flavan-3-ol by photolytic rearrangement. They proposed a reaction mechanism for this rearrangement [20]:

REACTION SCHEME#2: PHOTOLYTIC REARRANGEMENT MECHANISM.

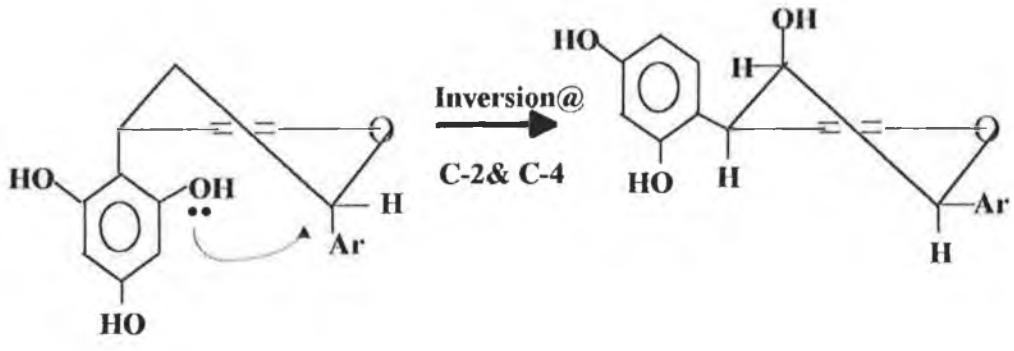


R1 = R3 = H ; R2 = R4 = OH

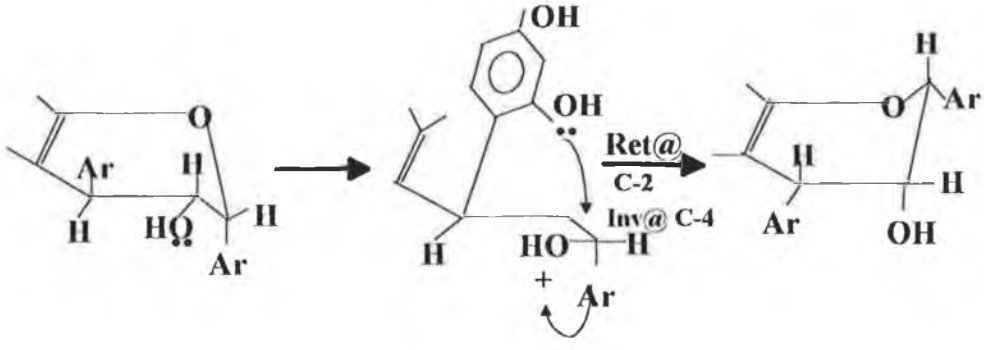
It was thought that these mechanisms needed formal hetrocyclic cleavage of the hetrocyclic ether bond with simultaneous intramolecular recyclisation via a zwitterion by neuleophlic attack of the phloroglucinol's hydroxyl group. It was also observed that in some cases inversion around the C-3-C-4 bond occurred, while in others just inversion about the 3-4 bond was observed.

The 2-OH of the phloroglucinol D ring was in close proximity to the C-2-O bond and these would be anti-oriented in an inverted C-2-C-3 semi-chair configuration. This lead to S_{N2} cleavage of the heterocyclic ether and resulted in inversion at C-2. The half chair configuration lead to the anti-configurational spacing of the 2,3-cis-3,4-trans isomer. The cleavage of the O-C-2 bond was observed to be anchimerically assisted by the lone pair electrons of the 3-OH group of the phloroglucinol ring, which formed an oxiran susceptible to nucleophilic attack by hydroxyl groups and thereby leaving the configuration at the C-2 group. Thus under photolysis, rearrangement must occur to form the more stable group.

REACTION SCHEME #3:INVERSION/RETENTION OF CONFIGURATION DURING PHOTOLYSIS



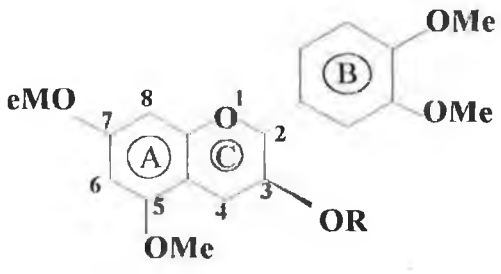
(1) Inversion gives the 2,3-trans-3,4-trans isomer



(2) Retention @ C-2,inversion @ C-4, gives 2,3-Cis-3,4-trans isomer

The interflavanyl linkages were still not fully characterised by ^{1}H NMR, as this relied upon deshielding experiments utilising methoxy derivatives, plus solvent exchange. Roux *et al* proposed a model that could be used to explain the linkages. The model was split into three distinct rings. (Fig.11) [21].

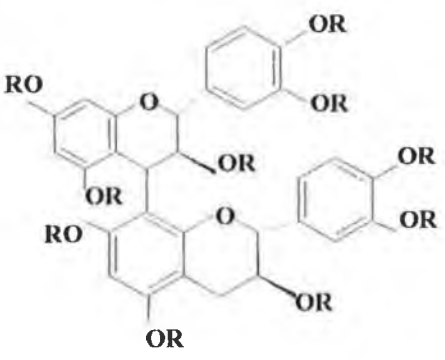
Fig .11



They used the substituted biflavanoids with 6' and 8' linkages to determine the absolute chemical shifts for the A ring protons H-6 and H-8. The methylether derivatives were directly reacted with pyridinium hydrobromide perbromide. This was applied mostly to the 8' substituted compounds as this site was more nucleophilic and less sterically hindered than the 6'. The method of formation was found to follow the trend 8'-bromo followed by 6', 8' di-bromo and finally 2', 6', 8' tri-bromo derivatives. The direct bromination of the 6' site was not possible and this was thought to be due to steric hinderence. It was felt that the 6' bromo compound might be generated by partial debromination of the 6', 8' dibromo product. The 6' bromo compound was generated in a 74% yield using n-butyl lithium at -20°C giving the first synthesis of the 6 substituted catechin species. This served as the key to the synthesis of other 6 substituted species.

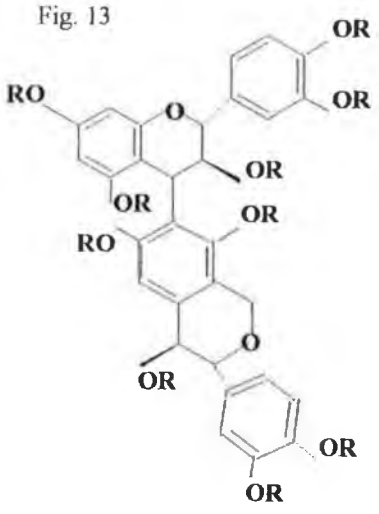
Roux *et al* used their knowledge to apply a direct biomimetic approach to the synthesis of [4,6 and 4,8] linked biflavanoids previously isolated from commercially important barks. [22]. They reacted (+)-mollisacacidin with (+)-catechin under mildly acidic conditions at ambient temperature. Three biflavanoids were identified each comprised of (-)-fisetinidol upper units and (+)-catechin lower units. The [4,8] all trans isomer predominated with a 28% yield. (Fig.12). Next was the [4,6] all trans isomer in a 5.8% yield. (Fig.13) and [4,8] 2,3-trans-3,4-cis-2', 3'-trans isomers in a 16.5% yield, not easily separated. (Fig. 14).

Fig . 12



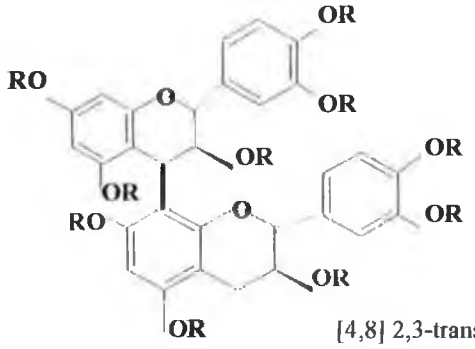
[4,8] All trans Isomer

Fig. 13



[4,6] All trans isomer

Fig. 14

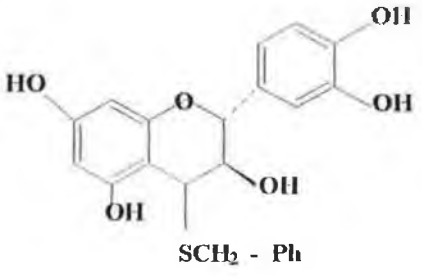


R = H

[4,8] 2,3-trans-3,4-Cis-2',3-trans Isomer

Other workers were also studying plant extracts to try and establish and confirm the linkage patterns in procyanidins. Hemingway *et al* decided to focus on procyanidin trimers, as there were several combinations of configuration possible due to the fact that these compounds were made up of more than one procyanidin group, each having three assymetric centres and a terminal flavan-3-ol unit possessing two chiral centres[23]. As well as these parameters the trimers also had either [4,6] or [4,8] interflavanyl linkages. They made use of column chromatography to fractionate their extracts and isolate the desired compounds. Once catogorised into their constituent groups the compounds were separated from each other by HPLC. Molecular weight determinations were made using gel permeation chromatography (GPC). Acid catalysed degradation involving toluene and thiol yielded 4-benzyl thioepicatechin. (Fig. 15) and (+)- catechin, implying that the trimers were composed of procyanidin units of 2,3-cis configuration with (+)-catechin as the flavan-3-ol terminal unit.

Fig.15



Studies involving partial degradation gave procyanidin dimers and benzyl sulphide procyanidins. The presence of procyanidin B1 was indicative of [4,8'] linked compounds while B7 indicated [4,6'] compounds. Desulphurisation followed by reaction with Raney nickel served to confirm this. This group also proposed a system for naming the compounds based upon oligosaccharide classification rather than using rigid IUPAC conventions. The IUPAC method used flavan as the basic ring system and

(RS) nomenclature to define absolute configuration. This was found to be misleading and difficult to apply to trimers and higher polymers.

Certain groups have concentrated their studies on the application of NMR to procyanidins. The aim of this work was to identify key chemical shifts which helped to define absolute configuration. Porter *et al* studied procyanidins of 2,3-cis conformation using ^{13}C NMR and found certain interesting features [24]. Firstly these compounds were free from conformational isomerism at 30°C allowing interpretation of their spectra. The group found that quaternary carbons attached to oxygen in the A ring could not be distinguished from one another but were found in the region 154-159 ppm while those of the pyrocatechol B ring were found near 145 ppm. They assigned shifts to the C-6 and C-8 ring carbons, the C-8 being more shielded. The C-3 and C-5 carbons were broad signals with twice the linewidth of any other signal making them easily distinguishable. The group made use of proton de-coupling to assign the signals for the pyrocatechol B ring.

TABLE#1: ^{13}C SHIFTS OF STRUCTURAL SIGNIFICANCE.

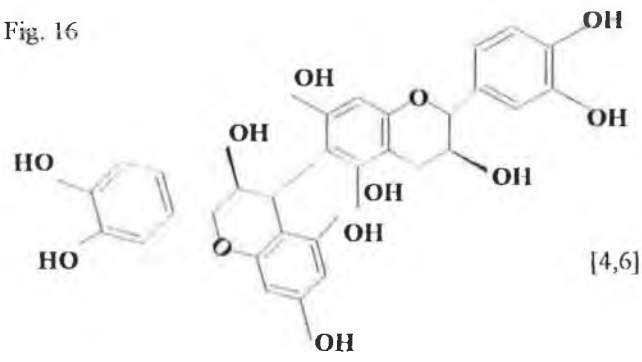
Sample Compound	Unit *	C-2	C-3	C-4	C-6	C-8
Procyanidin B1	T	76.4	72.6	36.6	96.1	95.6
	B	81.7	67.8	28.0	97.0	108.1
Procyanidin B2	T	76.6	72.8	36.7	96.5	96.0
	B	79.1	66.2	29.1	97.4	107.7
Procyanidin B5	T	76.6	72.1	37.0	96.9	96.0
	B	78.9	66.6	28.9	108.6	96.9
Procyanidin B7	T	76.7	72.0	37.0	96.4	95.9
	B	82.0	67.9	28.7	108.4	96.7

The shifts they obtained were found to be in keeping with those predicted from the monomer units. Meta-meta coupling distinguished C-6 and C-8 resonances of upper units from the lower units.

Apart from the extraction of these compounds from various plant sources, synthesis had been limited to the coupling of 4-benzyl thio - flavan 3-ols to (+)- catechin or epicatechin. However, this synthetic work overlooked the high reactivity of leucocyanidins predicted from their structure, based on the effective delocalisation of the phloroglucinol type A rings for 4-carbenium ions generated under suitable conditions. One reaction carried out was the reduction of (+) - taxifolin by sodium borohydride in ethanol under nitrogen and its subsequent condensation with (+)- catechin in 1:1 molar ratio. Delcour *et al* found 2.6% of unreacted (+)-taxifolin remained with 42% of unreacted catechin and that the products formed were two biflavanoids, two triflavanoids and a higher oligomer assumed to be a tetramer. [25]. The [4,8] all trans-bi-(+)-catechin was found in 17.5% yield. This has a spin system Amx superimposed on Abxy of the catechin moiety which was indicative of 2,3-trans-3,4-trans orientation. This was confirmed by analysis of the c.d spectra. The 6-$H_{(D)}$ of the lower unit had a shift (δ 6.16 ppm) that was used to define the 8-substitution of the phloroglucinol unit of (+)- catechin . Other parameters defined for the peracetate derivatives were shown to be directly comparable to those parameters obtained from the natural compound procyanidin B3. The [4,6] all trans biflavanoid was found in approximately 2% yield and study of its octamethyl ether derivative shown that the upfield 8-$H_{(D)}$ (δ6.28ppms) determined the position of bonding at C-6 and that the shift difference between 2-$H_{(F)}$ and 3-$H_{(F)}$($\Delta\delta$0.36) was different to the [4,8] compound and could therefore be used as a method of differentiation.

This was found to be effective even for [4,8]:[4,6] linked trimers. Other shift data determined the all trans configuration, while c.d spectra confirmed the interflavanyl linkage. (Fig.16).

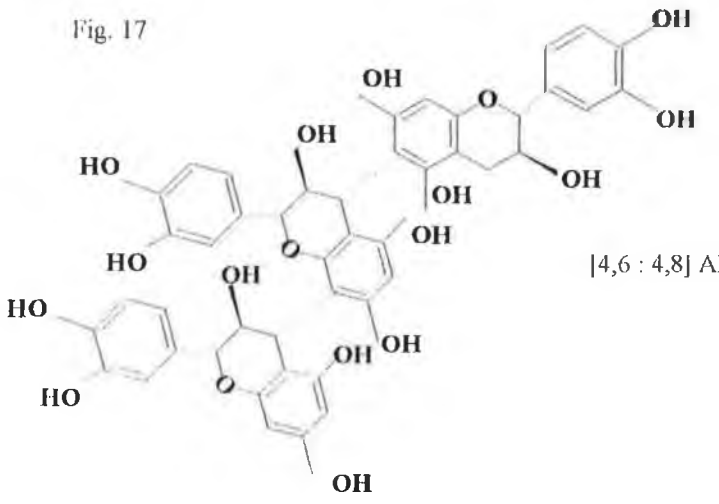
Fig. 16



[4,6] All trans Isomer

From parameters defined for the dimers, it was possible to assign the structures of the trimers. These were an all trans [4,8]:[4,8] isomer and a [4,8]:[4,6] analogue in the ratio of 12:1 respectively and their concentrations approximated those of the biflavanoids. For the [4,8]:[4,8] product the ^{1}H NMR spectra showed meta coupled doublets from the A ring (δ 6.11 and δ 6.03ppm) and two overlapping singlets 6-$H_{(D)}$ (δ 6.06ppm) and 6-$H_{(G)}$. These are indicative of of the successive [4,8] coupling of both upper units. The residual chemical shift difference (Δδ 0.23) also was indicative of this type of configuration (Fig. 8). Finally the [4,8]:[4,6] trimer was differentiated by the deshielding of one of it's singlets 8-$H_{(D)}$ (δ6.06- δ6.23ppm) and one of the two aromatic singlets relative to one another 6-$H_{(D)}$ (δ 6.07ppm). This showed linkages at 6 and 8 positions respectively. The 2-$H_{(I)}$ and 3-$H_{(I)}$ shift difference (Δδ0.33) was the same as in the biflavanoid. Again coupling constants suggested an all trans configuration (Fig.17).

Fig. 17



[4,6 : 4,8] All trans Isomer

Another group studying procyanidins from the conformational aspect presented results that served to confirm the findings of Delcour *et al.* Yeap Foo *et al* found the conformational isomeric effects in procyanidins interesting. They observed that the barrier to rotation about the interflavanyl bond was lower in those compounds where the flavan -3-ol unit was in pseudoaxial rather than pseudoequatorial coordination. Most natural compounds isolated had a configuration of 2R while those synthesised showed 2S configuration. From analysis of synthesised compounds this group confirmed that these compounds adopted a sofa-type conformation. [26].

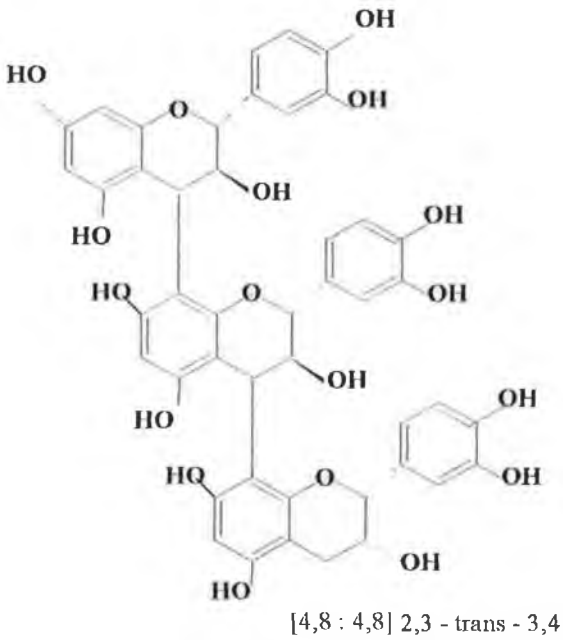
The same group have also used their findings to synthesise the first branched procyanidin trimer [27]. The acid catalysed condensation of (+)-mollisacacidin with (+)-catechin yielded the first branched catechin trimer. The reason for its formation is due to the higher activity of the phloroglucinol group in relation to the resorcinol group. Thus during the condensation the interaction of the two molecules favoured condensation to the catechin A ring over the resorcinol group. The suggested mechanism for formation was thought to be via a quinone methide intermediate as this route was thought to be more effective than the acid catalysis route.

Thus by treating the quinone methide generated from (4B) -epicatechin phenyl sulphide and epicatechin - (4B,6)-(+)-catechin,the first branched trimer was synthesised.

The notion of quinone methide intermediates had already been discussed by Hemingway *et al* [28]. While synthesis of procyanidins by acid catalysed condensations had been documented it had only been supposed that synthesis using quinone methide intermediates was possible. Thus under basic conditions (pH 9.0) Hemingway *et al* reacted (4B)-epicatechin phenyl sulphide in sodium hydrogen carbonate. They found evidence of two dimers after just 30 minutes. Therefore it was noted that phenolic anions reacted faster with quinone methides than the acid catalysed carbocations.

As can be seen most of the synthesis work has concentrated on oligomers with either cis or trans configuration. However, in nature a few examples of compounds possessing mixed stereochemistry have been reported. Delcour *et al* have already explained the reasons behind the predominance of trans configured compounds [29]. They subsequently went on to condense (+)-leucocyanidin and (-)-epicatechin. Several compounds resulted from this reaction. First were the positional isomers [4,8] and [4,6] 2,3-trans 3,4-trans 2',3'-cis-(+)-catechin-(-)-epicatechin (procyanidins B4, B8). Next a novel [4,8] 2,3-trans 3,4-cis-2',3' -cis-diastereoisomer was identified, along with a novel trimer [4,8:4,8] 2,3-trans 3,4-trans: 2,3-trans 3,4-trans:2,3-cis-bi-(+)-catechin-(-)-epicatechin. (Fig. 18).

Fig .18



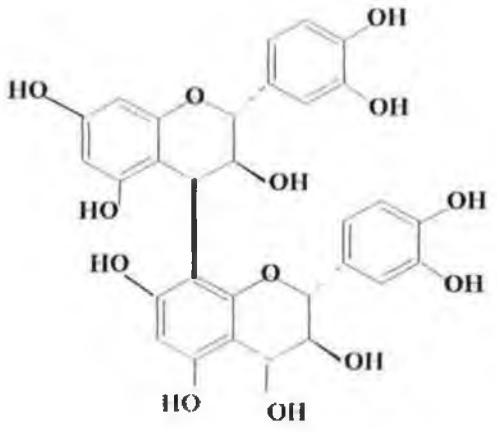
[4,8 : 4,8] 2,3 - trans - 3,4 - trans : 2,3 - trans - 3,4 - trans - bi -(+) - catechin - 2,3 - cis - epicatechin

Finally the C-4 epimer of this trimer and an all [4,8] all trans tri (+)-catechin-(-)-epicatechin tetramer were identified. The group concluded that the formation of compounds of mixed chemistry was similar to those formed having (+)-catechin as the nucleophile, the biggest difference being the formation of 2,3-trans 3,4-cis upper units. Exactly why this occurred was not fully understood.

Kolodziej *et al* provided the first concrete evidence of this type of natural compound.[30] They examined ethyl acetate extracts of tormentil rhizomes. As expected they found procyanidin B3 and its [4,6] analogue B6 but they also found the previously undiscovered (4B-8) cis analogue (Fig.19).Using ^{1}H NMR spectroscopy these compounds could be distinguished due to coupling constants ($J_{3,4}$) and the chemical shifts for two protons in the lower flavanyl units (δ 4.94, 5.0 4,4.38 ppm) for the B3 and B6 cis analogues respectively.

The natural abundance of these compounds was found to be similar to that of the synthesised compounds namely B3: B6:cis = 8:1:1 .

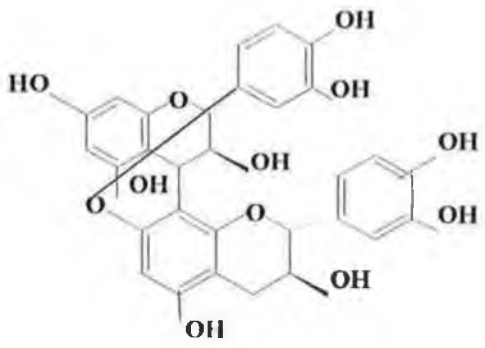
Fig 19 :



[4,8] 2,3 trans 3,4 cis bi (+) catechin

As well as the investigations into the synthesis of these procyanidin compounds, major improvements into their analysis have occured. Karchesy *et al* used fast atom bombardment mass spectroscopy (FAB-MS) to help sequence procyanidins dimers and trimers [31]. This technique proved very useful for the ability to be able to distinguish between linear and branched trimers. The types of compounds that were studied included procyanidin B dimers (B1/B7), the trimer procyanidin C1, and the alternative dimer procyanidin A1
(fig.20)

Fig. 20



Procyanidin A1

The results of this work showed FAB-MS to be an excellent technique for sequencing procyanidins. For these studies all the compounds had previously been purified chromatographically, but it was felt that the technique could be applied to sampleswithout prior purification.

PROCYANIDINS IN THE BREWING INDUSTRY

As we have already seen the industrial application of procyanidin chemistry has been the driving force behind much of the research carried out over the past three decades. No where has this been more evident than in the Brewing Industry. The processes behind haze formation in beer production has already been discussed and the role of procyanidins clearly established. Barley contains all of the procyanidins found in brewing. Outtrup *et al* were among the first workers to characterise the individual procyanidins present in barley [32]. They identified three types of procyanidin and one prodelphinidin. Reverse phase HPLC was employed to isolate and purify these compounds and their characterisation was carried out by 270MHz ^{1}H NMR of their acetylated derivatives.

The ^{1}H NMR spectra were made up of a large number of overlapping lines which were assigned to partly overlapping small spin systems. Since protons from the individual rings were found not to interfere, it was possible to interpret one dimensional spectra under standard conditions. As with the synthesised compounds some chemical characteristics were defined. Firstly all of the products yielded (+)-catechin after mild acid catalysis. Secondly, after stronger acid hydrolysis the order of elution for these compounds was established:

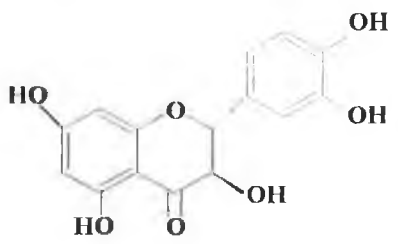
D=Dimer	**T=Trimer**
D1=Delphinidin	T1=Delphinidin
D2=Cyanidin	T2=Delphinidin +Cyanidin
	T3=Cyanidin + Delphinidin
	T4=Cyanidin

^{1}H NMR analysis showed broad signals interferring with the sharp aromatic regions and this was due to the presence of phenolic OH groups in the free forms of the compounds. Interference between the pyran ring and the aliphatic signals was also observed and finally as was found by other workers conformational isomerism was observed. The compounds in their free form were observed to be sensitive to oxidation and hence were deemed to be unstable. The bottom half of the molecule was found to be (+)-catechin while the top half was either (+)-catechin or (+)-gallocatechin.

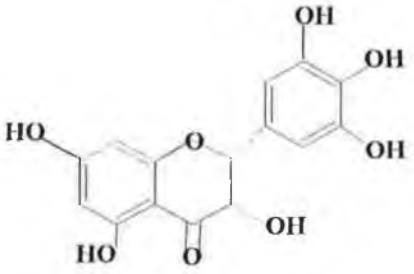
If further work investigating the possible reactions of these materials was to be undertaken, then their synthesis would be essential as extraction from plant materials was both inefficient and extremely tedious. Fonknechten *et al* employed the fairly readily available monomers of (+)-taxifolin (dihydroquercetin) and (+)-catechin as the precursors of these synthetic reactions [33]. They proposed the use of a method which essentially was an optimised procedure for a reaction first described by Delcour *et al* [25]. They suggested that dimers could be isolated in a 50% yield using this method, an improvement on the 20% yield suggested by Delcour. The isolated compounds were analysed and the results were compared to those for natural compounds and were found to be in close agreement.

So far all of the synthetic work had mainly focused on the procyanidins with little attention being paid to the prodelphinidins. Work was presented at the Phytochemical Society of Europe International Symposium (1984) which looked at the biochemistry of plant phenolics and at which it was stated that dihydroflavanol was necessary for the synthesis of prodelphinidin [34]. Based upon these findings Delcour *et al* proposed a method for the direct synthesis of prodelphinidin using (+)-catechin and (+)-dihydromyricetin as precursors (Fig.26).

Fig. 26



(A) (2R,3R) (+) Dihydroquercetin



(B) (2R,3R) (+) Dihydromyrcetin

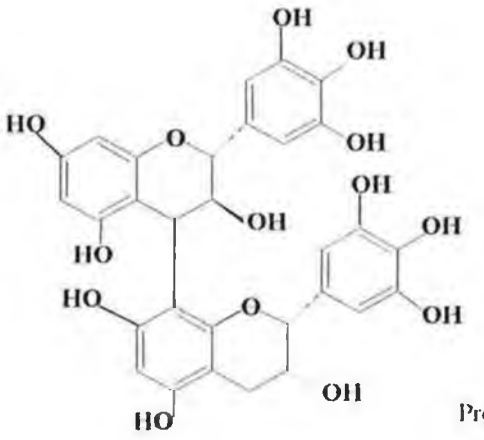
The reaction of these compounds yielded pyrogallol rings in the upper units and analysis by ^{1}H NMR of the acid derivatives of these compounds generated some of the following data [35]:

TABLE#2: 360MHz ^{1}H NMR OF PRODELPHINIDIN B3 DECACETATE

Proton No.	Chem Shifts (ppm)	Signal Type	Splitting J(Hz)
5-$H_{(E)}$	7.12	doublet	8.40
2X Ar-$H_{(B)}$	6.96	singlet	-----
2-$H_{(E)}$	6.91	doublet	2.00
6-$H_{(E)}$	6.71	doub,doub	ΣJ10.40
6-$H_{(D)}$	6.65	singlet	-----
8-$H_{(A)}$6-$H_{(A)}$	6.50	triplet	2.60
3-$H_{(c)}$	5.61	triplet	ΣJ19.50
2-$H_{(F)}$3-$H_{(F)}$	5.08-4.98	multiplet	-----
2-$H_{(C)}$	4.78	doublet	10.00
4-$H_{(C)}$	4.50	doublet	9.50
4-$Heq_{(F)}$	2.92-2.84	multiplet	-----
4-$H_{(F)}$	2.71-2.61	multiplet	-----
OAc	2.35,2.29-2.24(X6)	11 X S	-----
OAc	1.98,1.95,1.70(X2)	11 X S	-----

The above data showed that the acetate was free of conformational isomerism and the coupling constants specified all trans chemistry, which were very close to the values for procyanidin B3. The chemical shift of the residual D ring proton (δ 6.65ppms) served to confirm(4B-8) -prodelphinidin B3 linkage. (Fig. 27).

Fig. 27



Prodelphinidin B3

The same group also identified trimers with both (4B-8 and 4B-6) linked units which they identified as procyanidin C2, prodelphinidin C2 and a trimer comprised of (4,8:4,8)-(+)-gallocatechin-(+)-catechin-(+)-catechin, as well as (4,8:4,8)-(+)-catechin-(+)-gallocatechin-(+)-catechin.

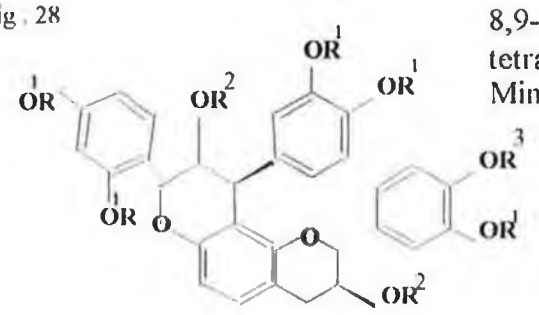
OTHER AREAS OF INTEREST IN PROCYANIDIN CHEMISTRY.

Mattice *et al* have applied fluoresence decay spectroscopy to try and establish interflavanyl linkages.[36]. Since ^{1}H NMR could not easily distinguish between the two conformational isomers observed in these compounds it was felt that time resolved fluoresence spectroscopy would be more capable of determining each of the isomers relative population. Measurements for the monomers were made in both water and dioxane while dioxane only was used for the dimers (due to poor solubility characteristics in water). Excitation of epicatechin (4B-8)-catechin and eipcatechin (4B-6)-catechin was performed at 272; 280 and 292nm giving an emission band between 310 and 321nm. These wavelengths were used as the fluoresence quantum yield was independent of wavelength or solvent effects.

The time resolved emission for monomers was in accordance to a mono-exponential function and it was found that the fluoresence lifetime of the monomer was solvent sensitive. For the dimers the decay values of their acetate derivatives showed best approximation to bi-exponential functions. For comparison the dimer procyanidin A1 was analysed as this was known not to exhibit rotation about the interflavanyl bond. This compound exhibited a mono-exponential function giving credence to the fact that bi-exponential functionality was due to rotational isomerism. The group concluded that if this was responsible for heterogenicity in fluoresence decay, than pre-exponential factors should reflect the relative populations of the major and minor rotamer. These factors were found to be dependent upon molar extinction co-efficient, the fluoresence emisson spectrum, the radiative lifetime and the concentration of the fluorescent species.

Ferreira *et al* investigated the fact that procyanidins that were extracted, or reacted, at alkaline pH's showed an increased acidity and lower reactivity towards aldehydes than if a neutral solvent system was used. They supposed that catechinic acid type rearrangement products were present. None of these types of compounds had previously been classified. They utilisied the methodology that had been developed to characterise phlobatannins on procyanidin B3, in order to obtain these compounds. They showed that over a period of 1½ hours procyanidin B3 completely converted to a mixture of oligomeric procyanidins (30%) and that following column chromatography four compounds (70%) were identified [37]. The compounds included (+)-catechin (9%) and three C ring modified compounds. These compounds were characterised as the 8,9-cis 9,10-trans-tetrahydropyrano [2,3h] chromene (Fig.28) and 2,3-cis 3,4-trans-4 aryl-2-flavanyl benzopyrans (Fig.29).

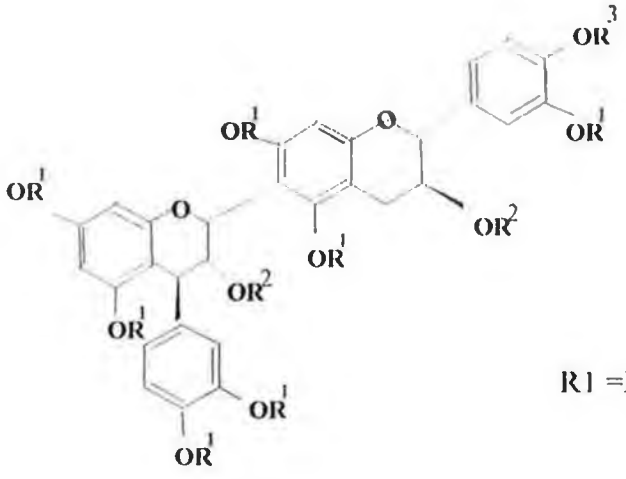
Fig . 28



8,9-cis-9,10 Trans
tetrahydropyrano-[2,3h] chromene
Minor product

R1 =R2 =R3 = H or Ac

Fig . 29



R1 =R2 =R3 = H or Ac

2,3-cis 3,4- Trans-4-aryl-2-flavanyl benzopyranes

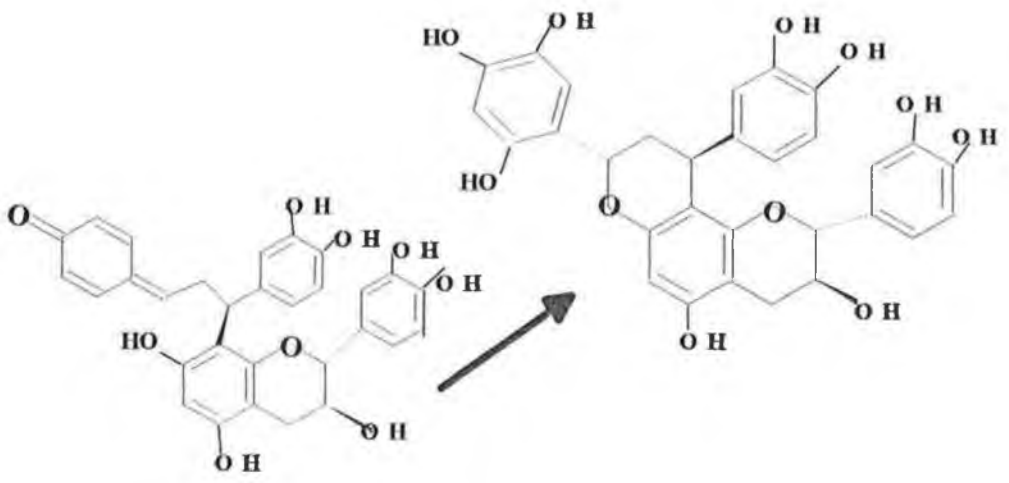
Nuclear Overhauser Effect difference spectroscopy (n.O.e diff) was used to confirm the cis-trans configurations of the C rings with associations of 8-$H_{(C)}$ and 2-$H_{(B)}$ and 6-$H_{(B)}$ for Fig. 24 and 2-$H_{(C)}$ with 2-$H_{(B)}$ and 6-$H_{(B)}$ for Fig. 25. The spectral data for the C ring isomeric products was summarised as follows:

TABLE#3: C RING ISOMER SPECTRAL DATA.

Proton No.	Chem Shifts (ppm)	Signal Type	Splitting J(Hz)
2-$H_{(B)}$	6.82	doublet	-----
5-$H_{(E)}$	6.74	doublet	-----
5-$H_{(B)}$	6.72	doublet	
6-$H_{(B)}$	6.67	doub,doub	-----
6-$H_{(E)}$	6.65	doub,doub	-----
2-$H_{(E)}$	6.64	doublet	-----
8-$H_{(D)}$6-$H_{(D)}$	6.25	singlet	-----
6-$H_{(A)}$ 3-$H_{(A)}$	6.03	singlet	-----
8-$H_{(C)}$2-$H_{(C)}$	5.58	broad singlet	-----
3-$H_{(F)}$	5.33	multiplet	-----
9-$H_{(C)}$3-$H_{(C)}$	5.20	doub,doub	-----
2-$H_{(F)}$	4.83	doublet	-----
10-$H_{(C)}$ 4-$H_{(C)}$	4.28	doublet	
4-H_{ax}	2.83	doub,doub	
4-$H_{(F)}$	2.67	multiplet	-----
OMe	3.83; 3.82; 3.78; 3.77; 3.76; 3.75; 3.60	All singlets	
OAc	1.97; 1.91	2 X S	-----

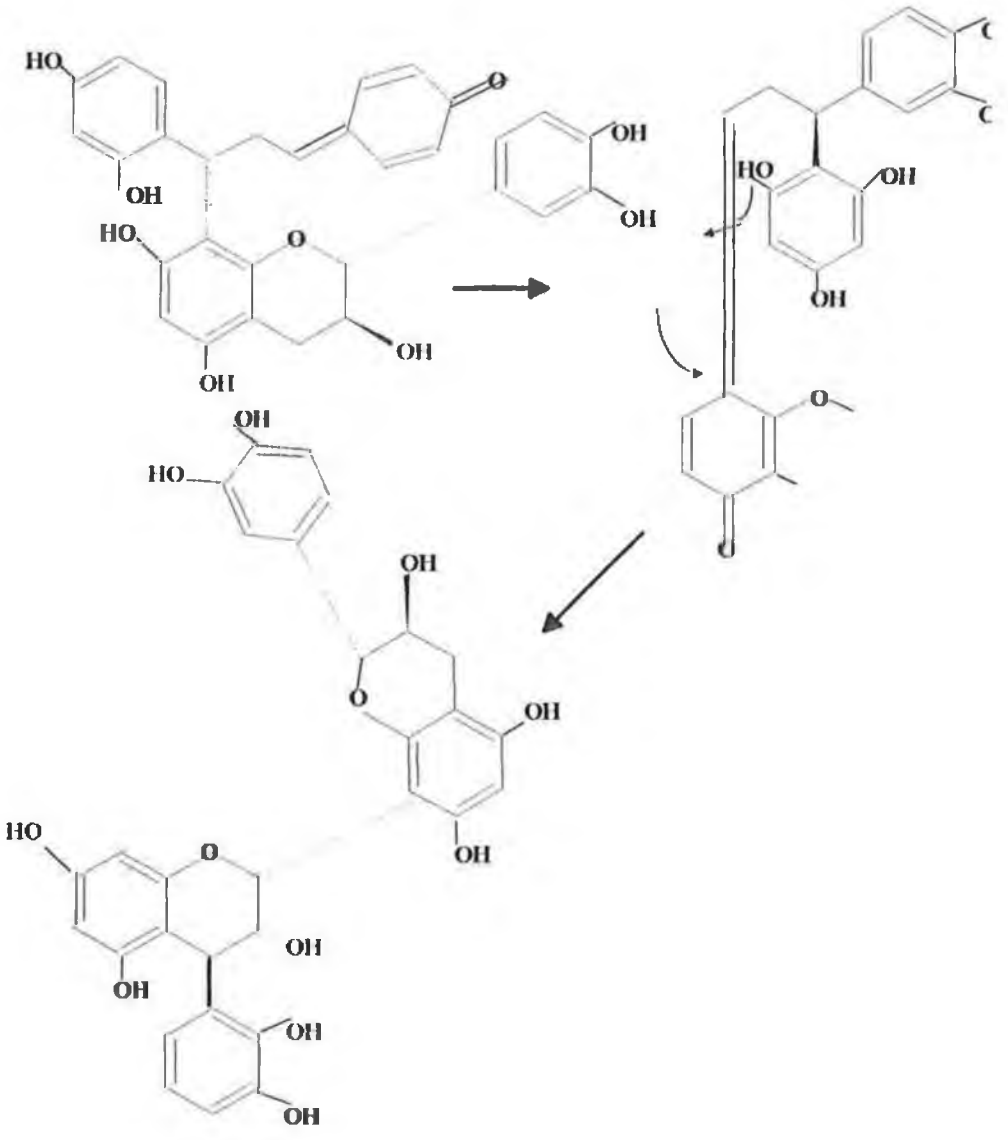
As had been previously observed it was felt that procyanidin B3 would react via its quinone methide intermediate under basic conditions. The mechanism for this reaction involves the migration of the (+)-catechin moiety, helped by the electron releasing phloroglucinol unit at C-4 to reface and C-2. This is followed by the subsequent pyran recyclisation through 7-$OH_{(D)}$ with reface of the quinone methide.

REACTION SCHEME#5: REACTION OF PROCYANIDIN B3 UNDER ALKALINE CONDITIONS.



This reaction lead to the inversion of absolute configuration at C-9/C-3 in procyanidin. The 4-ayrl-2-flavanyl benzopyranes were formed due to the enhanced migratory capacity of the phloroglucinol unit from C-4 to reface at C-2. Recyclisation at 2-$OH_{(A)}$ and the reface of the quinone methide forms the product:

REACTION SCHEME#6: FORMATION OF 4-ARYL-2-FLAVANYL BENZOPYRANES.



The group of Ariga *et al* investigated procyanidins B1 and B3 from a new stand point [38]. They wanted to study the antioxidant properties that these compounds may possess. It was felt that these compounds as well as increasing the shelf life of foods might also prevent lipid peroxidation in humans. Very little work had been done to investigate these compounds as food additives. This was partially due to the difficulties involved in their

isolation and purification. The first step in their analysis was to prepare authentic samples. The compounds were extracted from legume seeds and were characterised by ultra violet (UV), infra red (IR) and mass spec (MS). The compounds that were prepared showed the biggest differences in their ^{1}H NMR spectra. Tables outlining these differences were presented as follows:

TABLE#4: ^{1}H NMR DATA FOR PROCYANIDIN B1.

Proton No.	Chem Shifts (ppm)	Signal Type	Splitting J(Hz)
6 X $H_{(Ar)}$	7.10-6.50	multiplet	-----
8-H	6.02	doublet	-----
2-$H_{(E)}$	6.91	doublet	2.00
6-H 6'-H	5.95	doublet	-----
2-H	5.09	singlet	-----
2'-H	4.79	doublet	8.00
4-$H_{(Ar)}$	4.69	doublet	2.00
3'-H	4.05	multiplet	-----
3-H	3.98	multiplet	-----
3,3'-OH	3.62-3.54	multiplet	-----
4'-H	3.10-2.40	doub,doub	-----

These chemical shifts and coupling compounds were found to be identical to those values obtained by other workers [39]. The table for procyanidin B3 was also presented:

TABLE#5: ^{1}H NMR DATA FOR PROCYANIDIN B3.

Proton No.	Chem Shifts (ppm)	Signal Type	Splitting J(Hz)
8 X $H_{(Ar)}$	8.50-7.20	multiplet	-----
9X $H_{(Ar)}$	7.10-5.80	multiplet	-----
2-H	4.73	doublet	8.00
2'-H	4.55	doublet	3.50
3-H	4.54-4.26	singlet	-----
4-H	4.32	doublet	9.50
3'-H	4.08	multiplet	-----
3,3'-OH	3.83-3.72	multiplet	-----
4'-H		doub.doub	

This data was found to agree with the values obtained by other workers [6].

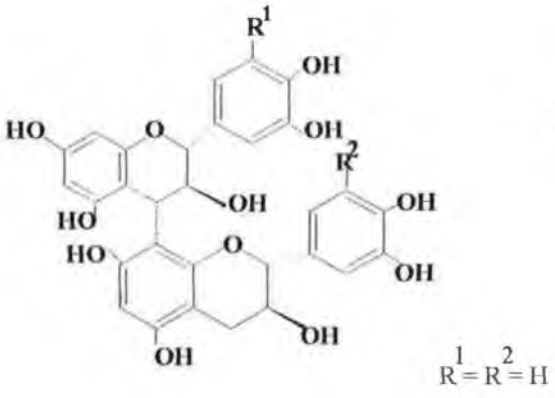
Both of these compounds were selected to be tested in model oxidative studies. This involved testing their ability to bleach ß-carotene in the presence of linoleic acid. This bleaching was due to oxidation. α-Tocopherol is a known anti oxidant and was used as a control by which the effectiveness of the procyanidins was compared. Since these compounds have been shown to be good hydrogen donors the same group set about determining how effective they might be as radical inhibitors [40]. This is important as many reports have suggested that lipid peroxidation may be linked to cancers [41,42].

The techniques developed by Yamamoto *et al*[42] as well as measuring oxygen uptake were found to be good quantitative measures for the determination of the level of lipid peroxidation, rather than the more usual substrate level determinations. The procyanidin B dimers were shown to be effective radical scavengers against peroxyl radicals and that each dimer had the ability to trap 8 peroxyl radicals. A general mechanism for phenolic antioxidants was presented and its mode of action discussed with particular reference to procyanidin B1 and B3. Studies showed the mode of action for scavenging for B3 to be relatively slow. Procyanidin B3 was found not to be effective against all types of compounds.

THE SYNTHESIS AND CHARACTERISATION OF OTHER PROANTHOCYANIDINS

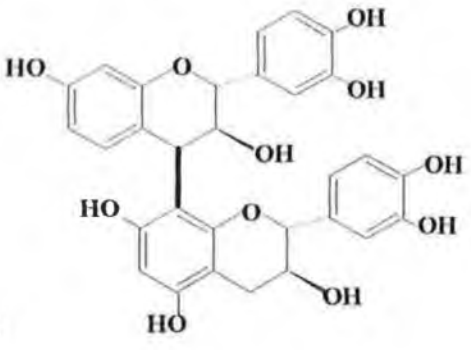
As well as the work involved in the characterisation of procyanidins that has already been discussed, it is worth mentioning some of the studies involving other related proanthocyanidins such as fisetinidins and robinetinidins. These compounds were first isolated in the 1960's by Drewes *et al*, who characterised them, in their derivative form, once extracted from black wattle bark [43,44]. For many years the absolute configurations of these compounds were open to question. Roux *et al* applied the techniques developed in the study of procyanidins to finally determine the configurations of these compounds [45]. The condensation of (+)-mollasacacidin and (+)-catechin yielded 3 dimers in 28, 16 and 5.5% yields respectively (Fig. 29-31).

Fig 29



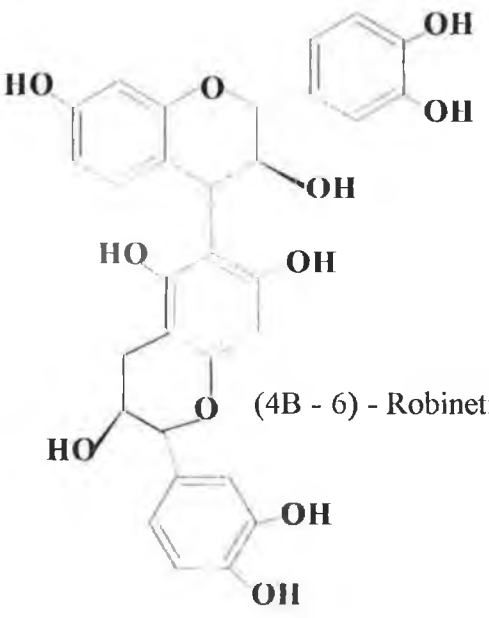
(-)-fisetinidol - (+) - Catechin

Fig 30



(-)- Robinetinidol - (+) - Catechin

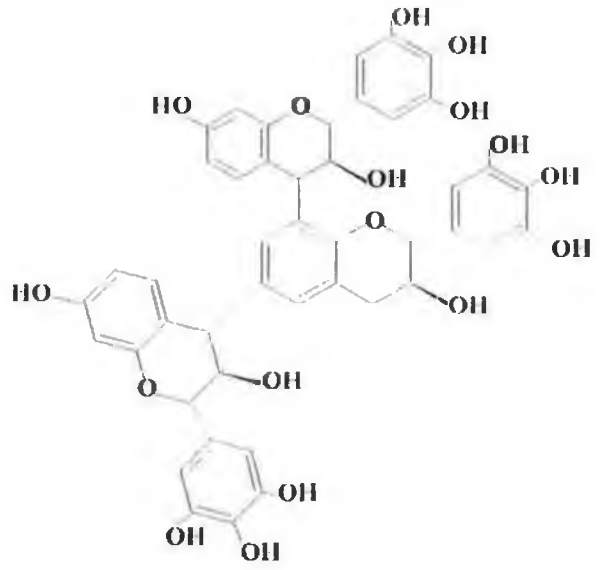
Fig 31



(4B - 6) - Robinetinidol - (+) Catechin

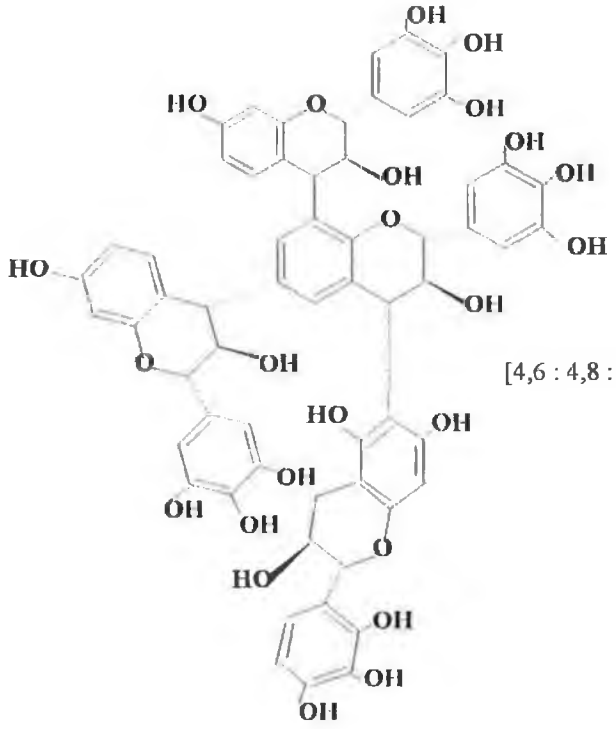
By comparison of these synthetic compounds with those isolated naturally it was concluded that in nature 2,3-trans 3,4-cis diastereoisomers predominated, while synthetically the all trans isomer was dominant. Roux *et al* by extension found that trimers existed in extracts of black wattle bark [46]. These compounds were found to be angular [4,6:4,8] prorobinetinidin triflavanoids and their absolute configurations were established (Fig. 32). The same group were also interested in tetrameric compounds, which they felt should also be present. In order to confirm this they exclusively reacted the resorcinol flavanyl units. The reaction of (+)-mollisacacidin and (-)-fisetinidol is a stereochemically selective reaction giving a good yield. This reaction yielded the expected all trans biflavanoid and its cis analogue, a new linear triflavanoid and the first branched tetraflavanoid to be synthesised [47]. ^{1}H NMR showed the tetraflavanoid to be linked to the triflavanoid, having [4,6:4,8:4,6] linkages (Fig. 33).

Fig 32



[4,6 : 4,8] All Trans bi - (-) Robinetinidol - (+) - Gallocatechin

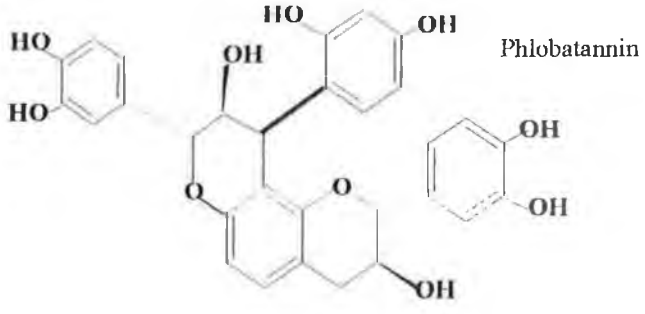
Fig 33



[4,6 : 4,8 : 4,6] - Tetramer

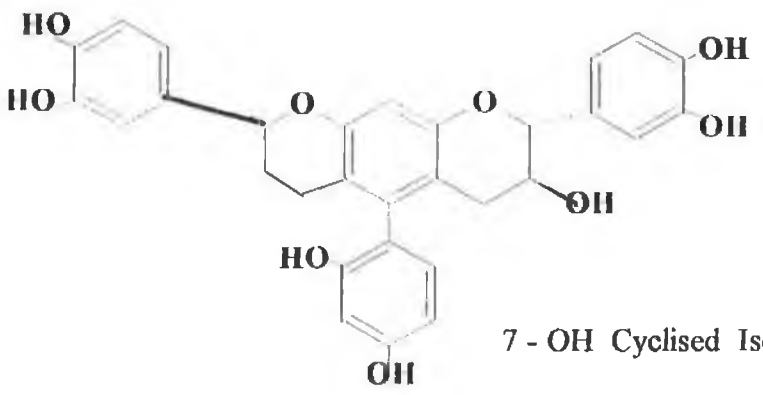
The isolation and identification of naturally occurring ring isomerised profisetinidins prompted research into their synthesis [48]. The reason for this interest was due to the usefulness as cold set adhesives that these compounds possess. Roux *et al* realised these compounds had the ability to behave as adhesives through the liberation of the reactive nucleophilic resorcinol units.[49]. This was possible if epimerisation at C-2 of (+)-catechin via the intermediate fusion of the heterocyclic ring took place [50]. Under basic conditions Roux *et al* found 5 stereospecific isomers from the (-)-fisetinidol-(+)-catechin precursor. The first was the expected phlobatannin, a product of the C ring isomerisation of the parent compound (Fig. 34). The second was its C-2 (F ring) epimer, where (+)-catechin has been converted into (+)-epicatechin. The third and fourth were positional isomers of the first and second, where (-)-fisetinidol was positioned at the C-6 position of the (+)-catechin reacting through its 5-OH group. The fifth was a structural isomer showing an alternative mode of cyclisation with the 7-OH group (Fig 35).

Fig 34:



Phlobatannin

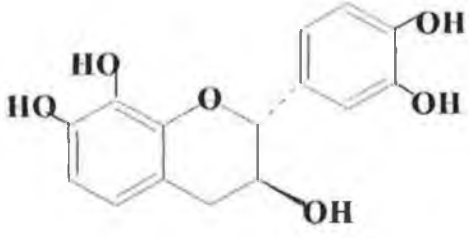
Fig 35



7 - OH Cyclised Isomer

Roux and other co-workers also investigated oxidative coupling involving flavan-3-ol units.[51]. Coupling involving flavones and flavanones was well documented [52] but, coupling involving flanan-3-ols was not common. All known reactions of this type have involved 2'-8 linkages of (+)-catechin via the respective B and A ring yielding biphenyl dehydrodicatechins [53]. The dehydrodicatechins were prepared using enzymatic oxidation or extracted from black teas after fermentative peroxidation [54]. Weinges *et al* supposed that (+)-mesquitol (Fig. 36) would more readily undergo oxidative coupling than catechin due to its more active A ring system.

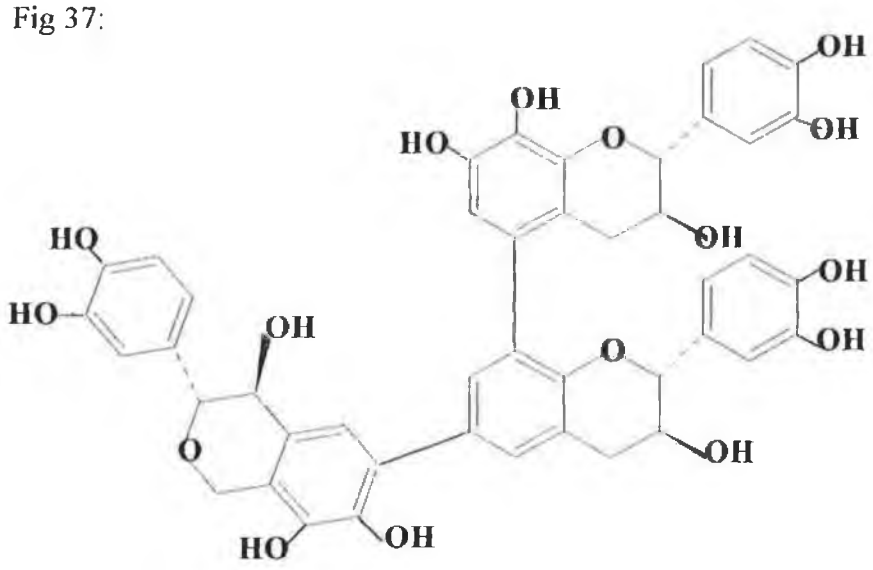
Fig 36:



(+) - Mesquitol

Synthesis of this compound with (+)-catechin lead to the isolation of a [5,6] and [5,8] dimer and a [5,6:5,8]bis-(+)-mesquitol-(+)-catechin trimer (Fig. 37). This indicated an alternative form of synthesis to those already mentioned using oxidative phenol coupling.

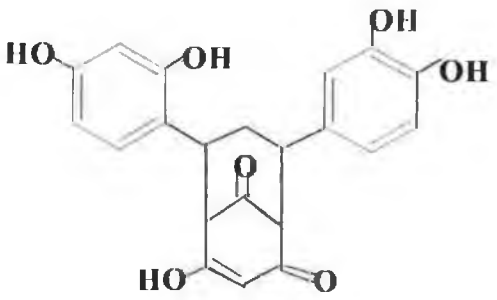
Fig 37:



[5,6 : 5,8] - bis - (+) - Mesiquitol - (+) - Catechin

Other workers were also interested in the base catalysis reactions of proanthocyanins. Laks *et al*[55] set about verifying the findings of Sears *et al*[56] who claimed that base reactions lead to an intra rearrangement of (+)-catechin yielding catechinic acid and isocatechinic acid. This acid was shown to be an enolic form of (+)-catechin-(+)-phloroglucinol (Fig.38)

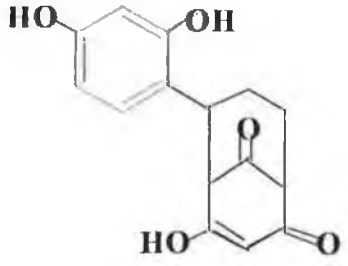
Fig 38 :



6 - (3,4 - dihydroxyphenyl) - 7 - hydroxy - bicyclo[3.3.1] - nonane - 2,4,9 - trione

Since this occurred for (+)-catechin it was postulated that it may occur in higher oligomeric compounds. Polymeric proanthocyanins were reacted with phloroglucinol at pH 12.0 at 23°C and 50°C. It was found that both the interflavanyl bond and the pyrane ether underwent rapid cleavage and that the liberated lower catechin unit was further cleaved at the pyran ring

Fig 39 :



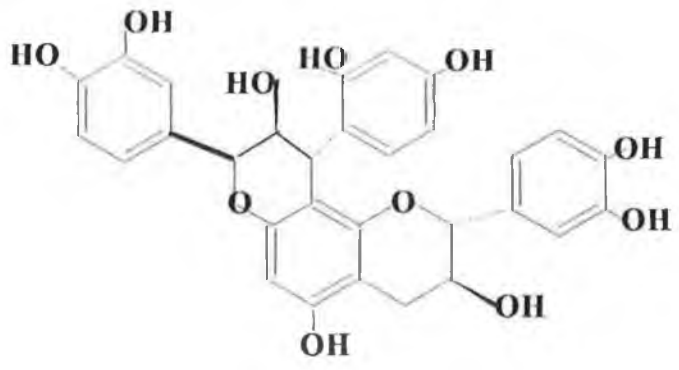
Catechinic acid

to form a reactive quinone methide that intramolecularly rearranged to form catechinic acid (Fig.39). The adduct formed from this reaction was not isolated.

Ferreira *et al* were interested in the base catalysed reaction of phlobatannins due to their more soluble characteristics [57]. The need to dissolve these compounds has already been outlined [58,59] and is very important for their practical handling in industrial situations.

The base catalysed reaction of (-)-fisetinidol [4ß-8]-(+)-catechin gave four products. These were the 8,9-cis 9,10-trans tetrahydro-2H,8H-pyrano [2,3h] chromene (Fig.40) and its all trans analogue as well as the isomeric pairs of these compounds. This group went on to detail the absolute configuration for these compounds and also presented information on the [4ß-6] analogues. Ferreira *et al* also determined the structures of base catalysed compounds of (-)-fisetinidol-(+)-catechin that had 2,3-trans 3,4-cis flavan-3-ol constitutent units [60]. Finally this group also published structures for the first profisetinidins and proguibourtinidins based on C-8 substituted (-)-fisetinidol units and their C8 ralated isomeric compounds [61].

Fig 40 :



8,9 - cis - 9,10 - trans tetrahydro - 2H,8H pyrano [2,3h] chromene

Haslam *et al* [63] have studied the role of proanthocyanidins in relation to the pigmentation of flowering plants. These compounds have been shown to be stable under acidic conditions since they can exist as flavylium ions. However, when the pH is increased deprotonation is apparent and the anhydrobase and its anion result. This is represented by a colour change from red to blue .

The proanthocyanidin on its own cannot effect this colour change and this leads to the theory of co-pigmentation, where the presence of polypeptides and polysaccharides is implicated in this reaction [63]. Furthermore, this group ascertained some other key parameters in plant colouration [64]. The variables involved were proposed to include: Anthocyanin-co-pigment type, pH, temperature, concentration and the type of metal salt present. Many potential co-pigments have been identified from flavanoyl and hydroxy cinnamyl esters [65,66,67], but no detailed quantitative analysis was presented. To this end Haslam *et al* used vegetable tannins, caffeine, theophyline, adenosine tri-phosphate (ATP), deoxyribose nucleic acid (DNA) and ribose nucleic acid (RNA) and these were associated with malvin chloride and cyanin chloride. The bathochromic shifts were established for these compounds. The phenomenum of anthocyanin-co -pigmentation may be best explained in terms of similar interactions that are forced in an aqueous media by hydrophobic effects. Therefore, in a natural context phenolic esters may be seen as electron rich systems capable of association with the electron deficient flavylium cation.

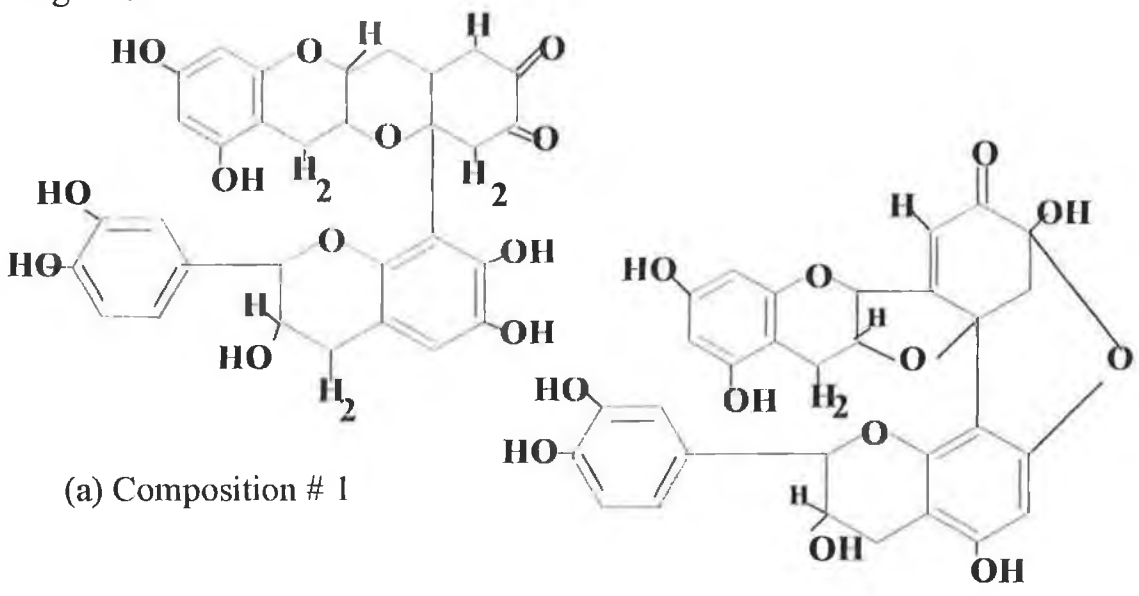
Finally, many workers have proposed that the type of binding involved between proanthocyanins and proteins is in fact hydrogen bonding [68,69,70,71,72]. Artz *et al* used the interaction between synthetic proanthocyanidin dimers and trimers and bovine serum albumin (BSA) to confirm this fact [73]. Their studies, however, also demonstrated that hydrophobic associations were also responsible for some binding in many cases.

POLYPHENOL REACTIONS IN MODEL AND NATURAL SOLUTIONS

In nature the types of reactions that have been dicussed tend to take part in areas of plants that are low in enzyme activity. There is, however, a second mechanism in nature by which proanthocyanins react. The action of oxidases generate polymeric materials that differ from each other by virtue of the position and number of hydroxyl groups on the molecule, upon which the oxidase may act. The resultant products are assumed to be of two basic structural types. The first are the group A procyanidins which have the empirical formula $C_{30}H_{24}O_{12}$ and the second, the group B procyanidins with the formula $C_{30}H_{26}O_{12}$. The enzymatic dehydrogenation of catechin is an important reaction in nature by virtue of its contribution to such processes as colour formation and flavours.

Weinges *et al* used oxidative enzymes (peroxidase, laccase, tyrosinase) to study the products formed using catechin as the precursor [74]. They found that in aqueous systems the variation and concentration and duration of exposure of catechin to the enzyme directly determined the composition of the product formed. For example, if after two days the reaction was quenched the resulting product was found to be 8-hydroxy-catechin, while after 10 to 14 days a yellow precipitate formed which when isolated and purified was assumed to be dehydrodicatechin A (Fig. 41). The same group fully assigned the configuration of this compound using further spectroscopic techniques to study the bromoheptamethyl ether derivatives [75]. The final proof was provided by Van Soest who confirmed the structure by X-ray analysis of the bromo derivative [76].

Fig 41 :



(a) Composition # 1

The effect of enzymes in natural products is dependent upon several parameters. The enzymic browning of grapes is a well studied example of these parameters [77,78], where a limiting factor in browning is said to be the oxidisable substrate. Phenolic compounds such as the flavans have been found by many workers to be important substrates in enzymatic oxidation due to their capacity to be broken down by phenol oxidases [79,80,81]. Since these compounds have been shown to effect wine during its production much interest into the nature of these reactions has been expressed. Oszmianski *et al* studied the effects of phenol oxidases on 7 compounds and monitored the reactions by HPLC [82]. Results showed that degradation of all compounds occurred but there was, however, no evidence of new peaks detected by HPLC. This was in keeping with the findings of other groups [83]. Allied to the disappearance of the input materials, was the presence of considerable browning.

The breakdown rates were observed to be different for different substrates. To some extent this could be explained by competitive reactions or, alternatively, a synergistic effect may help cause dedgradation and this has been reported in studies involving beers [84]. This was thought to occur from coupled chemical oxidation with quinones formed from enzymatic reactions. If this is the case then enzyme oxidation may not, in fact, be the limiting factor in browning, the enzyme being exhausted early on leaving any further browning to occur by non-enzymatic means.

The total content of phenols in a wine is a rough measure of the capacity that wine has for oxygen uptake, its ability to withstand oxidation and its capacity to change when exposed to oxygen. Even though the browning process is thought to be non-enzymic it is certain that enzymes have a part to play in oxidation. A good review of this area was presented by Singleton who pointed out some of the practical implications for polyphenols with specific reference to wine [85]. All of the points put forward in this review were further upheld by the work of Cheynier *et al* who studied the effects of grape polyphenol oxidase on several pheolic compounds in model solutions [86]. They concluded that oxidative polymerisation leading to the formation of brown pigments depended upon the nature and relevative concentration of the phenolic compounds present.

The same group also studied the effects of trans caftaric acid and 2-S-glutathionyl caftaric acid in model solutions. They found that the rates of oxygen uptake very much depended upon the ratios and concentrations of the substrates present and concluded that this may account for the different browning potential among various grape varieties [87]. Furthermore, this group developed a derivatisation technique involving benzene sulphinilic acid in order to study the quinone formation via enzymatic reactions [88].

Finally, they studied oxidation kinetics in enzymatic reactions and found that procyanidins were not directly susceptible to enzymatic oxidation but they are oxidised by the O-quinones generated by caffeoyltartaric acid. They demonstrated that the coupled oxidation of procyanidin products regenerated caffeoyltartaric acid from its quinone [89].

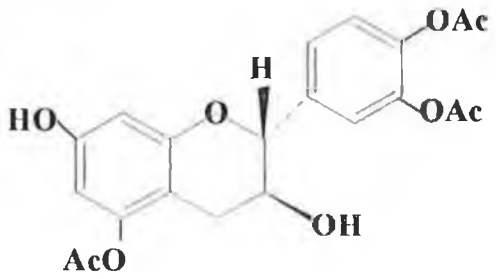
Oszmianski *et al* further investigated enzyme oxidised products of catechin and chlorogenic acid at various pH's [90]. They made use of HPLC-PDA detection to study the formation of dimers and higher oligmers and the co-polymers formed from a mixture of these compounds. Based on earlier work [91,92] the group proposed that one of the dimers produced was procyanidin B3 although no comfirmation of this was offered. The same group extended their studies to encompass the effect of oxidation of phloroetin glucoside on chlorogenic acid and catechin in model solutions [93]. Previous work had shown that phloroetin glucoside reacted very slowly to oxidation on its own but in a mixture containing epicatechin a synergestic effect was observed [94]. This was confirmed when it was shown that the rate of oxidation increased significently after addition of catechin or chlorogenic acid to a phloroetin solution.

Having established that browning processes in fruit and beverages is both enzymic and non-enzymic its practical implications must be considered. The browning of fruit tissue during or after harvesting is a major factor leading to a loss in quality and yield [95]. Therefore, for fruit processing the control of this problem has always been a difficult problem to overcome [96]. Many chemicals have been proposed as inhibitors to browning, halide compounds and aromatic carboxylic acids are known to inhibit polyphenol oxidases [97], while compounds from ascorbic acid and dextrin derivatives have also been shown to be effective [98].

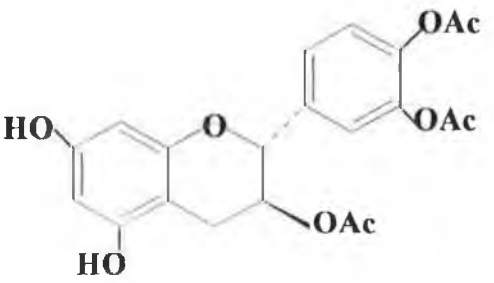
Sulphites have also been shown to be effective although their popularity is somewhat curtailed due to the possibility of toxic side effects [99,100]. Methods for the use of stabilised ascorbic acid or cyclodextrins have been proposed [101]. Very few studies into the use of thiol compounds, such as cysteine, have been undertaken although their usefullness has been long established [102]. The mode of action of cysteine was proposed as being via the formation of colourless addition complexes with the forming O-quinones [103]. Very few of these complexes have been structurally identified [104,105], and as a result Richard *et al* developed a rapid method for the preparation and purification of cysteine adduct complexes for different phenols [106]. The formation of such complexes was monitored by HPLC and their structures were identified by ^{1}H COSY NMR. For catechins it was shown that two conjugates appeared, one at the 2' position of the B ring and the other at the 5' position of the B ring.

Finally, a recent reference has cited the notion of using enzymes to regio-protect and de-protect catechin, as a means of being able to select the site of attack in further reactions [107]. Catechin and its derivatives have already been demonstrated to have important medically applications, epatoprotective [108], anti-cholesteremic [109] and anti-neoplastic [110]. They have also been used as artificial sweetners [111] and as a natural base for cosmetic products. During reactions it was noted that the B ring remained free from attack, therefore, in order to preferentially protect this group it was necessary to perform a hydrolysis rather than an esterification. Catechin however was shown to have poor solulility in aqueous solutions so a bio-catalysed alcoholysis was developed using 1-butanol in THF and an immobilised enzyme. From this two partially acetylated compounds were isolated (Fig. 42).

Fig 42 :



(i) 3,3',4',5 - O - triacetyl Catechin



(ii) 3,3',4' triacetyl Catechin

CONCLUSION:

From all of the work that has been examined in this review, a few striking features dominate. Firstly some of the earliest references date back as far as the turn of the century, indicating the importance with which this chemistry was viewed from an industrial standpoint. Secondly much of the synthetic work was derived from a need to be able to assign the absolute configurations of those compounds that were isolated from natural sources. Thirdly and perhaps most significantly a glance at the references serves to show the relatively few workers involved in this area. This would appear to be a good indicator as to the difficulty involved in the isolation and purification of such compounds.

Finally as was stated in the introduction to this publication, the body of work presented in no way is an accurate review of the entire area of proanthocyanidin chemistry. Rather the work has mainly concentrated on the synthesis of these compounds, leaving the large area of extractions from natural sources untouched to a greater extent. With the growing pressures involved in the use of more natural food additives, allied to the industrial applications, it would appear that the analysis of these compounds should continue to flourish in the future.

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Experimental section:

(1) Introduction:

(2) Reagents and Chemicals:

(3) Apparatus:

- (a) HPLC system
- (b) Freeze drying Apparatus
- (c) NMR Spectrometer
- (d) Mass Spectrometer

(4) Experimental Procedures:

- (a) Condensation Synthesis (Delcour *et al*) [1]
- (b) Condensation Synthesis (Fonknechten *et al*) [2]
- (c) Acetylation of Condensation Products [3]
- (d) Enzymatic Oxidation of (+)-Catechin (Oszmianski *et al*) [4]
- (e) Enzymatic Oxidation of (+)-Catechin (Weignes *et al*) [5]

(5) Results and Discussion:

- (a) Initial Familiarisation Experiments
  - (i) Column Chromatography Vs Cellulose Plates
  - (ii) HPLC Monitoring
- (b) Fonknechten *et al* Synthesis - Acetylation Reactions
- (c) Delcour *et al* Synthesis - Acetylation Reactions
- (d) Synthesis of Free Hydroxy Compounds
- (e) Enzymatic Oxidation Syntheses

(6) Conclusion

(1) INTRODUCTION:

The aim of the work undertaken in this project was to try and synthesise the condensed tannin dimer known as Procyanidin B3. The idea behind this was to supply Guinness Group Research with authentic standards for use with HPLC. In order to achieve this goal an investigation of synthetic procedures outlined in the literature was initiated. Most synthetic routes involved the reduction of (+)-taxifolin followed by the condensation of the reduced intermediate with (+)-catechin. Since the (+)-taxifolin could only be obtained from commercial sources having been extracted from various plant sources it was correspondingly expensive. Due to this expense, two factors predominated any investigations into condensed tannin chemistry. Firstly, all experiments had to be carried out on a small scale (largest input of (+)-taxifolin = 500mgs.). The second factor was to try and realise the maximum yield of the condensation products for the inputs used. For these reasons it was imperative that an efficient method of purification be developed.

One important factor that emerged from the literature review was the fact that procyanidin B3 in its free form was a fairly labile material. For this reason it was decided to employ two strategies during the synthesis of this dimer. Firstly, in order to establish the worth of each of the methods examined the products were isolated in their more stable peracetate derivative form. Secondly, in order to characterise the compound in its free form any product produced was subjected to lyopholisation in order to perserve it. Finally, none of the work could have been undertaken without suitable methods of characterisation for the products. For this 400mHz ^{1}H NMR was employed and spectra generated compared to those persented in literature studies. It was also noteworthy that this project involved

substantial input from analytical chemistry as HPLC methods had to be developed that allowed for in process assessment of the procedures. These analyses were scaled up to enable selective purification of compounds by semi-preparative HPLC.

(2) CHEMICALS & REAGENTS:

All (+)-taxifolin was purchased from Koch Light Limited and Extrasynthese Limited and was donated for research by Guinness Group Research. The (+)-catechin and sodium borohydride were purchased from Aldrich Chemical Co. Limited. The enzymes, tyrosinase and horse radish peroxidase were purchased from Sigma Chemical Co. Limited. All solvents used were HPLC grade and were supplied by Labscan Limited. All other reagents and solvents were ex Dublin City University Chemical stores.

(3) APPARATUS:

(i) High Performance Liquid Chromatograph (Semi-preparative)

(a) Solvent Delivery Pump; Waters 510 Dual Head Recriprocating Pump, capable of delivering a flow of 19.9mls/min.

(b) Injection Port; Rheodyne 7125 manual injector with 20µl fixed loop (analytical), 1ml fixed loop (semi-preparative).

(c) Detector; Shimadzu SPD-6A variable wavelength detector (190-700nm Range) used in ultraviolet at 220 & 280nm.

(d) Integrator; Waters 746 Integrator (128K Memory)

(e) Fraction Collector; LKB Bromma 2000 Automatic Fractionaction Unit (200 x 15ml tube collection facility).

HPLC Columns:

(a) Analytical columns used;

(i) Waters Radial Compression Module (RCM) 10cm x 8mm μBondapak C18 reverse phase packing, 15μm.

(ii) Chromex Nucleosil C18 25cm x 4.6mm, 5μm.

(iii) μBondapak C18 30cm x 3.9mm, 10μm.

(b) Semi-Preparative Columns used;

(i) Waters RCM Cartridge System 20cm x 25mm μBondapak C18, 15μm packing.

Freeze Drying Unit:

(i) Labconco 4.5lt. benchtop lyopholiser with Edwards Two Stage Vacuum Pump (<100mBar capability).

Nuclear Magnetic Resonance Spectrometer:

(i) Bruker AC-400- 400mHz FT-Pulse NMR.

Mass Spectrometer:

(i) Stand Alone Quadropole MS with electron ionisation probe (<1000 amu)

(4) EXPERIMENTAL PROCEDURES:

The following section outlines experimental procedures involved in the synthesis of procyanidin B3. In the interests of simplicity each procedure was examined separately with all values for the condensation reactions listed relative to a 100mg input of (+)-taxifolin. For the enzyme reactions concentrations were more appropriate for their discussion.

(i) Synthesis of procyanidin B3 [Delcour Method] from (+)-taxifolin and (+)-catechin in a 1:5 molar ratio [1]

Approximately 500mgs of (+)-catechin was dissolved in 10mls of ethanol containing approximately 100mgs of (+)-taxifolin in solution.
Approximately 80mgs of sodium borohydride ($NaBH_4$) was added to 2.5mls of ethanol (forms a suspension rather than solution). This was added to the ethanol solution in a round bottom flask dropwise over 10 mins. under nitrogen. Once added, 12.5mls of water was used to dilute the solution and the pH was adjusted to 5.0 using 0.15M acetic acid. The reaction was allowed to stand at ambient temperature for 1 hour with constant agitation. The reaction solution was diluted to 40mls with water and the resulting phenolic compounds were extracted with 6 x volumes of ethyl acetate.
This was evaporated to dryness by rotary evaporation having first been dried over magnesium sulphate. The dried products were then either freeze dried or further reacted to form their peracetate derivatives (cf later section). The products were then subjected to purification by several methods.
Note 1: HPLC was used for inprocess monitoring of the reactions.

Note 2: All weights and volumes were adjusted for larger scale preparations where appropriate.

(ii) Synthesis of Procyanidin B3[Fonknechten Method] using a 1:2 molar ratio of (+)-taxifolin to (+)-catechin and optimised conditions [2].

Approximately 100mgs of (+)-taxifolin was added to 5mls of water at 60°C in a round bottom flask. Approximately 50mgs of solid $NaBH_4$ was added to this slowly to avoid vigorous evolution of hydrogen. Once added the flask was cooled to ambient temperature and allowed stand for 15 mins. with constant agitation. The flask was cooled to between -6°C and -10°C with the aid of an acetone ice bath. Approximately 50mgs of (+)-catechin was dissolved in 5mls of methanol and was added to the cooling flask before the aqueous solution froze. Once the temperature had been achieved the solution was allowed stand for 20 to 30 mins. with constant agitation. The reaction was quenched using 0.3mls. of 37% HCl solution. The resulting products were extracted using 6 x volumes of ethyl acetate and isolated as outlined in the previous procedure.

Note 1: HPLC was used to monitor the inprocess reactions.

Note 2: All weights and volumes were adjusted for larger scale experiments where appropriate.

(iii) Acetylation of reaction products using a 1:1 ratio of pyridine and acetic anhydride [3]

Following rotary evoporation of ethyl acetate the resulting residue was re-dissolved in 2mls of pyridine and 2mls of acetic anhydride was added. The flask was allowed to stand overnight at ambient with constant agitation. The acetylated products were recovered following their precipitation on ice. The precipitated products were continually washed

with cool water until no further smell of pyridine was detected. All products were dried under vacuum and subjected to further analysis.

(iv) Enzymatic Oxidation of (+)-catechin using tyrosinase (mushroom polyphenol oxidase) [4]

The following procedure details the work carried out for the attempted synthesis of procyanidin B3 by enzymatic synthesis.

(i) Preparation of reagent solutions.

(a) 0.02M Acetic Acid

A stock solution was prepared by accurately weighing 1.20gms of acetic acid into a 1L volumetric flask which was diluted to volume with distilled water. The pH of this solution was adjusted to 3.5 or 6.5 accordingly using 1.0N NaOH solution. This formed a preparative solution for all other solutions used.

(b) 2.0mM Catechin Solution

Approximately 58 to 60mgs of (+)-catechin was weighed into a 100ml volumetric flask. This was diluted to volume with the 0.02M stock solution.

(c) 0.5mgs/ml Tyrosinase Solution

Approximately 5.0mgs of Tyrosinase was weighed into a 10ml volumetric flask and was diluted to volume with stock solution. The activity of the enzyme was assigned at 2100 units/mg of solid so the final activity of the enzyme was approximately 1050 units.

(ii) 19.5mls of 2.0mM (+)-catechin solution was added to a 100ml round bottom flask. 0.5mls of 0.5mgs/ml tyrosinase solution was added to this at ambient. The reaction was allowed to proceed overnight with constant agitation. The reaction was quenched with an equal volume of 50:50 CH_3CN:(3% v/v)HCl solution. The aqueous solution was subjected to 6 x volume extractions of ethyl acetate and the resulting evaporated residue was treated in a similar manner to that already described.

(v) Formation of dehydrodicatechin A by enzymatic oxidation using horse radish peroxidase. [5].

Approximately 500mgs of (+)-catechin was dissolved in the minimum quantity of acetone required to fully dissolve it,in a 100ml round bottom flask. 20mls of water was added at ambient with constant agitation. 100mgs of horse radish peroxidase was dissolved in2.0 mls of 0.05M sodium citrate buffer (Sorenson buffer), pH5.60,and was added to the catechin solution. 1.0ml of 0.3%v/v hydrogen peroxide was added dropwise. The reaction was allowed to stand at ambient for 14 days with daily additions of the enzyme and hydrogen peroxide in the prescribed concentrations. A yellow precipitate was observed and the solution was gravity filtered using Whatman No1 filterpaper. The wet product was placed into a conical flask and redissolved in acetone. This was repeatedly refluxed in the presence of activated charcoal, in order to remove the dark colour,and finally water was added to the hot filtrate and left for12-24 hours .

Note 1: HPLC was used to monitor the reaction throughout

(5) RESULTS & DISCUSSION:

(i) Initial Familiarisation Experiments:

The preliminary experiments that were completed in this field of study were done so in order to address some of the problems likely to be encountered. The first problem to be solved was the determination as to whether or not the reaction was proceeding as described in the literature. To solve this an effective method of in process determination had to be developed. Most of the references examined alluded to the use of HPLC as the method of analysis of choice. It was clear that gradient elution was used and since this facility was not available an isocratic assay that incorporated the mobile phases used in the gradient assay was developed. The first attempt at synthesis was on a100 mg input of (+)-taxifolin scale,and followed the procedure described by Fonknechten *et al*, which claimed a yield of 50% for procyanidin B3. This reaction served to validate the synthesis as well as the HPLC assay developed. Complete development of the analytical assay was hampered due to the fact that only two of the starting materials were available as HPLC markers, leaving the unequivocal assignation of the retention time for procyanidin B3 open to question. The synthesis directly followed the procedure as laid down and sampling for in process determinations was performed on a 30 min. interval scale. The analytical conditions were outlined as follows:

ANALYTICAL HPLC CONDITIONS:

Column: Chromex Nucleosil C18 25cm X 4.6mmid 10μm packing.

Mobile Phase: 90 : 10 (10%v/v)Acetic Acid : CH_3CN.

Flow Rate: 1.0 mls/min.

Detection: λ = 280 nm.

Retention Times	Time(Mins.)
(+)-Catechin	4.90
(+)-Taxifolin	16.00

Table #1: In Process Determination of Initial Synthesis reaction

Sample/Ret time	Uk1 3.33mins	Uk2 3.61mins	Uk3 3.91mins	Catechin 4.81mins	Uk4 5.44mins	Taxifolin 15.81min
T= zero	3.20	15.94	21.53	30.12	4.86	24.31
T= 30min	8.44	n/d	33.04	20.08	6.18	32.22
T= 60min	5.68	n/d	32.24	19.00	6.59	34.47
T= 90min	6.54	n/d	34.77	18.06	6.02	33.99
T=120min	1.56	n/d	42.61	20.17	6.58	29.06

Uk = Unknown n/d = not detected

From this experiment it was possible to make a few observations. Firstly it was apparent that a reaction was taking place. This was demonstrated by the disappearance of the peak with the retention time of 3.61 mins.and the consequent growth of the peak at 3.91 mins. Also the peak for catechin at 4.81 mins. was seen to decrease. Secondly it was noticed that the retention times of all the peaks were far too early and that baseline resolution had not been achieved in all cases, leading to inaccurate % impurity index(II) determinations.

It was felt therefore that an alternative assay was needed, since several peaks of interest were evident. Also it was necessary to investigate a successful method for isolating and purifying the desired compounds. A second synthesis on the same scale was started and once again monitored by HPLC with alterations having been made to the mobile phase conditions in an effort to solve some of the problems highlighted. Once the reaction had been completed and the resultant products were isolated, a method of purification had to be established. Guinness Group Research supplied a method based upon preparative scale thin layer chromatography. The method utilised cellulose plates and the mobile phase was made up of 2 :1 :1 secButanol : acetic acid : water. Once run the plates were developed with a spray solution comprised of a 0.3%v/v solution of 4-dimethylaminocinnamaldehyde in 3:1 methanol : HCl(conc). Cathecin was reported to yield a green colour, while procyanidin B3 appeared as royal blue.

The assay conditions remained the same for the in process HPLC work with the exception being the mobile phase. The ratios of it's components were altered to 92 :8 (10%v/v)Acetic acid : CH_3CN, in order to delay the retention of all compounds and therefore aid in separaton.

Table #2: In Process Analysis using Alternative Assay Conditions

sample/Ret Time	Uk1 4.24	Uk2 4.87	Uk3 5.25	Uk4 6.17	Catechin 6.80	taxifolin 23.50
T= 0	n/d	n/d	n/d	n/d	0.84	99.16
T=15min	5.07	7.70	n/d	86.00	n/d	0.75
T= 0 Post catechin Add'n	0.38	3.08	n/d	54.67	41.45	n/d
T=0 Post Acid Add'n	4.95	n/d	59.92	n/d	35.13	n/d
T=45min Post acid	7.37	n/d	63.81	n/d	28.82	n/d
T=75min Post Acid	6.30	n/d	67.14	n/d	26.55	n/d
T=100min Post Acid	5.71	n/d	69.06	n/d	25.22	n/d

Again even with the alterations that had been made to the assay the peaks of interest still eluted too early and were not fully baseline resolved. The recovered products were dissolved in ethanol and spotted onto a cellulose plate and this was developed. Two distinct bands were noticed and the characteristic green and royal blue colours appeared. Both bands were scraped from the plate and the recovered products were dissolved in ethyl acetate. This was removed by rotary evaporation and the collected compounds were analysed by HPLC:

Table #3: Isolated Product Reaction Profiles

Sample/ RetTime	Uk1 3.55	Uk2 4.08	Uk3 5.16	Catech 6.55	Uk4 7.57	Uk5 9.77	Uk6 12.68	Uk7 21.93
Rxn Pdt	0.43	2.77	47.06	35.35	9.47	0.80	2.19	1.28
1st band	0.30	2.01	39.44	30.02	11.81	5.32	4.83	n/d

From the data generated in this experiment, two points emerged. First of all the separation on the cellulose plates did not appear to be sufficient to allow purification of the procyanidin B3. Secondly there appeared to be several components present in the isolated material making identification of the B3 difficult. As a result of these findings a third experiment was undertaken, this time with double the input of (+)-taxifolin. The idea was to try and develop a column separation that would enable the fractionation of the components in a mobile phase that would be easy to remove. The product demonstrated a severe reluctance to run on a silica column under any mobile phase conditions, with dragging a common feature to most runs tried. Analysis of the recovered fractions showed silica not to be suitable for chromatographic separation of polyphenols. Cellulose powder was used as the column stationary phase and a similiar series of results were obtained. A review of the literature showed that the best results were achieved using sephadex LH-20 as the stationary phase but this was not a realistic consideration in this case due to the expense involved in the running of sephadex columns. An alternative was provided in the form of semi-preparative HPLC. Although at first it would appear that this option was also an expensive one, the flexibility involved with several different purifications was enough to justify the initial setup costs involved.

INVESTIGATION INTO SCALING FACTORS INVOLVED IN SEMI -PREPARATIVE HPLC PURIFICATIONS.

In order to determine some of the key parameters involved in semi-preparative separations some of the basic factors had to be established. Firstly it was important to ensure that both the analytical and larger semi-preparative columns were made of the same packing material, in order to directly scale the inprocess assay to semi-preparative levels. Sample loading was the first key parameter that had to be established since correct application of the sample led to the higher return of purified material. This factor was established by first of all loading the maximum quantity of product onto the analytical column without overloading the column excessively. Once this figure was calculated it could be used to determine the best loading to be placed onto the larger column. The following equation was used to determine the loading factor for the semi-preparative column:

$$Load_{(prep)} = Load_{(scaling)} \times \frac{(D1)^2 \times L1}{(D2)^2 \times L2}$$ Where,

D1 = Internal diameter of semi-preparative column.

L1 = Length of semi preparative column.

D2 = Internal diameter of scaling column.

L2 = Length of scaling column.

For (+)-catechin this factor was calculated as:

$$Load_{(prep)} = 20mgs/ml \times \frac{(2.5cm)^2 \times 10cm}{(0.8cm)^2 \times 10cm} = 195.20 \text{ mgs/ml}$$

This represents a scale factor of 10 fold.

The second parameter that was important for the scale up was the increase in flow that was needed to retain the retention times of the peaks of interest. The following equation was used to predict this increase:

$$Q_{(prep)} = Q_{(scaling)} \times \frac{(D1)^2}{(D2)^2} \quad \text{where,}$$

Q = Flow(mls/min) and D = diameters as before.

The values for Catechin were predicted as follows:

$$Q(prep) = *0.88 \text{ mls/min} \times \frac{(2.50cm)^2}{(0.80cm)^2} = 8.59 \text{ mls/min}$$

* The reason for the unusual flow is due to the pump head size on the semi -preparative pump. The flow on the dial read 0.40mls/min but this has to be multiplied by a factor of 2.2 to calculate the actual flow.

Once these parameters were established a 500mg input scale synthesis was undertaken to provide sufficient crude material for semi- preparative isolation. The previous assays had shown that the peaks of interest eluted too early and it was therefore decided to use 10% v/v acetic acid as the mobile phase. This was a highly unusual mobile phase and needed alteration before it could be used. The stationery phase of C18 HPLC column is stable between a pH range of 2.0-8.0 and for this reason the acetic acid solution had to be adjusted to a pH greater than 2.0. One good reason for using this mobile phase was the ease with which the products could be extracted into an organic solvent. The in-process analysis for this experiment was summarised as follows:

Table #4: In Process Analysis of 500mg Input Material

Sample/ Ret Time	System 3.61	Uk1 4.10	Uk2 4.56	Uk3 5.80	Catechin 6.20	Uk4 7.29
T0 $NaBH_4$ Add'n	n/d	n/d	n/d	99.08	0.91	n/d
T0 Cat Add'n	0.37	n/d	n/d	77.21	22.42	n/d
T=20 post Cat Add'n	0.69	3.73	61.80	n/d	32.50	1.29

Note 1: Very poor chromatography with no baseline resolution.

Note 2: The in-process analysis was run using 92:8 10% v/v acetic acid: CH_3CN.

The next problem addressed was the type of solvent that should be used to dissolve the compound. The use of the above mobile phase was limited as the product was not fully soluble. The compound was readily soluble in methanol but injecting neat methanol using a rheodyne injector has been known to cause peak broadening making its use non-viable. The semi -preparative assay conditions are summarised as follows:

Column: μBondapak C18 10cm x 25mm id RCM cartridge.

Mobile Phase: 10% v/v acetic acid solution.

Flow Rate: 5mls per min.

Detection: λ = 280nm.

Retention Times:	Time (mins.) Based on analytical column
B3 Suspect:	7.14
Catechin:	13.56

Table # 5: In-process Analysis prior to purification.

Sample/ Ret Time	Uk1	Uk2	Uk3	Catechin
Product	1.22	58.30	7.42	33.05

The product was made up to a final concentration of 50 mgs/ml as it was felt that a concentration below the optimum loading would give baseline resolution for separation purposes. Five 1ml injections were passed down the column and fractions collected manually into test tubes. Analysis of these fractions by analytical HPLC showed appreciable improvement in the % II of the B3 suspect. Due to this a more rigid collection routine was established, whereby the peak of interest was shaved to give far more fractions of less volume. Once completed the purified fractions were analysed by HPLC (App. A Chrom#1). The isolated product was found to have a % HI purity of 87.02% with 10.75% of an unknown that eluted after the B3 suspect and before catechin. No catechin was detected, however, spiking of the sample showed catechin to co-elute or elute close to the unknown peak. The reason for the presence of this unknown/ catechin may have been due to one of three reasons. Firstly, the procyanidin B3 may not have been stable in this particular mobile phase and may have degraded to the later eluting peak. Secondly, the separation on the column was insufficient to yield material of the required purity for further NMR studies. Finally, the isolated product may also break down during the organic extraction procedure used to isolate it.

Even though the isolated material was only assumed to have a purity approaching 87.0% it was decided to analyse it by 400mHz NMR. Before any determinations of the product were carried out it was decided to fully characterise (+)-catechin by NMR as some of the spectral information obtained by this may be common to the product isolated. The authentic (+)-catechin standard was examined using ^{1}H NMR, ^{13}C NMR, 2-D COSY

and C-H correlation studies. This data was invaluable as catechin formed the monomeric backbone of the desired dimer and other higher oliogmer compounds.

Characterisation of (+)-catechin by 400mHz NMR:

Table # 6: (i) ^{1}H NMR spectrum (App. B NMR# 1)

Proton No:	Chemical Shift (ppms)	Signal Type	Splitting (J Hz)
3 X H aromatic	6.70- 6.60	doub,doub	ΣJ = 12.07
1 X H	5.85	singlet	---
1 X H	5.76	singlet	---
1 X H	4.50	doublet	6.54
1 X H	3.95	multiplet	---
1 X H	2.70 - 2.60	doub,doub	ΣJ = 10.93
1 X H	2.25	multiplet	---

From the integration nine protons were distinguished for (+)-catechin.

Table # 7: (ii) ^{13}C NMR spectrum (App. B NMR#2)

Sample/δppm	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12	C13	C14	C15
Catechin	28.4	68.7	82.8	95.5	96.3	100.8	115.2	116	120	132.1	146.1	146.1	156.8	157.5	157.7

From the chemical shift values obtained 15 carbons were found in (+)-catechin having the following arrangement:

(a) 7 Carbons with no H attached (Aromatic)

(b) 5 Carbons with H attached (Aromatic)

(c) 3 Aliphatic Carbons

(iii) 2-D COSY NMR spectrum (App.B NMR#3)

The advantage of this spectrum was that a two dimensional plot of the compound was obtained which aided in the assignation of which protons were coupled to each other. The first plot is a contour layout of the spectrum along the diagnal of identical axes. The second plot spatially related components along either side of the diagnol and once these components could be linked by a square or a series of squares the components were said to be related. The related components were summarised as follows:

Table #8: Chemical Shifts of Related Peaks

Chemical Shift(ppms)	1st Peak	2nd Peak	3rd Peak
2.50	2.80	4.15	---
2.80	2.50	4.15	---
4.15	2.50	2.80	4.75
4.75	4.75	---	---
6.05	---	---	---
6.85	---	---	---

(iv) C-H correlation spectrum (App.B NMR#4)

(a) Dept 45 for projection unto C-H correlation.

A specialised spectrum called a Dept 45 involves scanning for ^{13}C carbon with hydrogens attached which form positive peaks if they are even and positive peaks if they are odd (Positive = Vertical). Quaternary carbons are excluded as are solvent peaks. From this spectrum it was apparent that there were 8 carbons with hydrogens attached and that they have the following chemical shifts:

Table # 9: Dept 45 ^{13}C Chemical Shifts for C-H Projection.

Sample/δppms	C1	C2	C3	C4	C5	C6	C7	C8
Catechin	28.4	68.7	82.8	95.5	96.3	100.8	115.2	116

(b) ^{1}H NMR spectrum for projection unto C-H correlation.

Table # 10: ^{1}H NMR data for Projection Spectrum

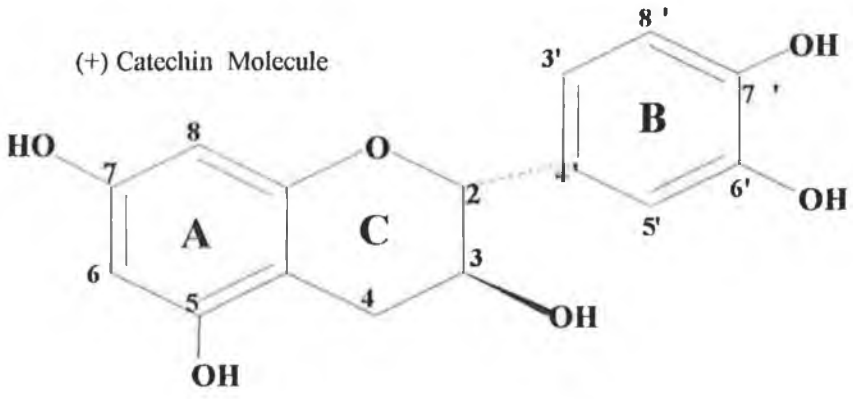
Chemical Shift (ppms)	Signal Type	Splitting (Hz)
6.71	singlet	---
6.65 - 6.58	doub,doub	ΣJ = 12.00
5.80	singlet	---
5.73	singlet	---
4.50 - 4.35	doublet	5.68
3.88 - 3.82	multiplet	---
2.75 - 2.69	doub,doub	ΣJ = 10.10
2.40 - 2.35	multiplet	---

(c) C-H correlation data.

Table # 11:C-H Correlation NMR data for Projection Spectrum

Carbon No/δppms	$^1H_1$(δppms)	$^1H_2$(δppms)
C1 28.4	2.40 - 2.35	2.75 - 2.69
C2 68.7	3.88 - 3.82	----
C3 82.8	4.50 - 4.35	----
C4 95.5	5.73	----
C5 96.3	5.80	----
C6 100.3	6.65 - 6.58	----
C7 115.2	6.65 - 6.58	----
C8 116.0	6.71	----

Since the structure for (+)-catechin was already known these spectra served to confirm its assigned structure as follows:



By contrast the ^1H NMR spectrum for the suspect procyanidin B3 product was very poor. There were a large number of interferring peaks that further questioned the purity of the isolated material (App.B NMR # 5).

Two courses of action were adopted following this result. The first was to try and alter the mobile phase for the analytical HPLC assay thereby allowing improved analysis involving photodiode array detection. The second was to submit a fraction of the sample to Guinness Group Research for analysis using a HPLC assay for which the retention time for B3 was already assigned.

Investigation into alternative mobile phase composition.

Since the purity of the isolated compound was questionable a range of alternative mobile phases were examined in order to try and afford a more accurate purity determination. The mobile phase compositions examined were summarised as follows:

(i) 85:15 10% v/v acetic acid: CH_3CN

(ii) 85:15 H_2O: CH_3CN

(iii) 85:15 0.5% v/v H_3PO4: CH_3CN

(iv) 85:15 O.5% v/v H_3PO4: CH_3CN (pH adjusted to 2.0)

(v) 80:20 CH_3CN: H2O (pH<3.0)

(vi) 95:5 10% v/v acetic acid: CH_3CN

The first of these when run gave a retention time for (+)-catechin of 8.81 mins. However, the peak obtained was very broad and showed signs of tailing. The second mobile phase showed both fronting and tailing characteristics and as a result was discounted. The third also gave a broad peak. The adjustment of pH for this mobile phase gave a well defined peak with a retention time of 10.6 mins. at a flow rate of 1.0 ml. per min. Finally in order to investigate whether any late eluting compounds were present the mobile phase was altered to (v) above with catechin eluting at 3.30 mins. There was however no evidence of any late eluting products which further served to confirm that procyanidin B3 had an earlier

retention time than catechin. Finally, the mobile phase (vi) was used to examine the two compounds. Although this did not afford the best separation between the two compounds it did demonstrate the sharpest peaks. Catechin had a retention time of 4.65 mins while the procyanidin B3 suspect eluted at 4.10 mins.

Photodiode Array Detection (PDA):

PDA detection was used to try and establish the integrity of the suspect compound. The mobile phase used was as (vi) above but the flow rate was lowered to 0.5 mls. per min. This gave (+)-catechin a retention time of 11.56 mins. The contour plot for (+)-catechin showed a maximum between 280-284 nm indicating that the choice of wavelength used was appropriate. Analysis of (+)-catechin yielded a single peak. (App.AChrom#2). The retention time for the suspect compound was found to be 8.75 mins, however, detailed analysis of this compound was not possible due to insufficient sample quantity.

Guinness Group Research Purity Determination:

As a result of the poor NMR spectrum obtained it was decided to consult the Analytical Department of Guinness Group Research with the aim of analysing the isolated fraction on a system upon which the retention time of procyanidin B3 was known. They examined the compound using the following HPLC conditions:

Column: Reverse Phase C18 25cm x 4.6 mm id, 5μm packing.
Mobile Phase: 80:20 0.25% v/v H_3PO4: methanol.
Flow Rate: 1.0 ml per min.
Detection: λ = 220nm.

Retention Times:	Time (Mins.)
Unknown#1	5.92
Procyanidin B3	7.75
(+)-Catechin	9.50
Unknown#2	12.70

Analysis by this Group showed that the product had a % HI of only 66.0% with 14% of unknown #1, 18% catechin and 2% of unknown #2. It would appear that the use of a lower wavelength was useful for observing peaks other than B3 and (+)-catechin. (App.A Chrom#3)

Confirmation of the products lower purity prompted a few changes in the method of analysis. It was felt that this was the key area since purification of the compound was only feasible by semi- preparative HPLC. The radial compression module had a 15µm packing and this was claimed to be as effecient as a 10µm packing in a stainless steel column. A direct comparison between the cartridge system and the more conventional steel column showed much better chromatography for the latter and thus the cartridge system was discarded for analytical determinations.

Having defined many of the parameters involved in both analytical and semi-preparative work a 500mg input of (+)-taxifolin was undertaken following the method of Fonknechten *et al* and the final recovered weight was calculated at 680mgs.

Theoretical Maximum Recovery (100% conversion + 100% recovery)

$$\frac{500 \text{ mgs. (+)-taxifolin} \times 579.3 \text{mol wt. B3}}{302.4 \text{mol wt. (+)-taxifolin}} = 957.8 \text{mgs.}$$

Assume only 50% yield implies theoretical max. = 478.9mgs.

Total recovered compound = 680mgs.

% B3 (by HPLC) = 33.45 %= $\frac{680.00\text{mgs} \times 33.45\%}{100\%}$ = 227.50mgs

Therefore % recovery of B3 = $\frac{227.50\text{mgs}}{478.90\text{mgs}}$ x 100% = 47.50%

In theory therefore the implied 50% yield attributed to this synthesis held.

Table #12: HPLC Profile of isolated product

Sample/Ret Time	uk1 5.01	uk2 6.23	uk3 7.19	uk4 8.20	uk5 9.86	uk6 11.01	Catn 14.40	uk7 16.03	uk8 18.24	uk9 23.52
Product	0.98	1.53	1.01	33.4	3.72	11.08	33.68	11.62	0.31	0.83

The isolated product was split into two fractions and two attempts at its purification by semi-preparative HPLC were made. The first used a concentration of 10mgs/ml and the time window between 17.0 and 18.6mins. for the fraction collection. The isolated material only had a % HI of 67.3% with the other unknown impurities making up the difference. The second attempt used a more concentrated solution at 12mgs/ml. This lead to the recovery of a peak with a retention time of 11.5mins with a % HI purity greater than 90% for the collected fractions. However, when this was analysed by 400mHz NMR it was apparent that the material was impure and that a large quantity of catechin was still present.

Even though the recovered product was not pure there were some distinguishable features in the ^{1}H NMR spectrum. The presence of aromatic hydrogens at a chemical shift greater than 8.0ppms was unexpected and suggested that the peak collected was not that for procyanidin B3.

In literature [6] it was stated that procyanidin B3 in its free form was a labile material and this meant that the isolated product was probably susceptible to degradation during isolation. Since no firm evidence for the production of procyanidin B3 had been obtained using the synthesis conditions as laid down by Fonknechten *et al* it was decided to regress to the original (and more rigorous) synthetic procedure described by Delcour *et al*.

Synthesis of Procyanidin B3 from (+)-taxifolin and (+)-catechin in a 1:5 molar ratio.

Following the procedure detailed in the experimental section a reaction using 200mgs input of (+)-taxifolin was undertaken. The more stringent conditions involved in this synthesis included the use of ambient temperature and a nitrogen atmosphere, in order to try and control the initial reduction and subsequent condensation reaction. The reaction appeared to have proceeded as described with all steps being monitored by HPLC. The in-process details were summarised as follows:

Table #13: In-process analysis.

Sample/Ret Time	uk1 8.90	uk2 10.80	uk3 14.00	Catechin 16.10	Taxifolin 75.00
T0 pre$NaBH_4$	n/d	n/d	n/d	99.24	n/a*
T0post$NaBH_4$	0.26	1.24	12.80	81.50	n/a
T0 post pH	0.11	n/d	12.16	87.08	n/d**
T=1Hr hold	0.12	1.35	13.74	84.04	n/d
T=2Hr hold	0.10	1.29	13.08	83.10	n/d

* N/A = Not Analysed ; N/D = Not Detected

From the above analysis it was observed that the procyanidin B3 had formed in a 13% yield. This was less than the proposed 20% yield claimed but this may be explained due to the small scale of this experiment. Once the reaction was complete the products were extracted in the same fashion as the previous experiments, using ethyl acetate. The isolated product had the following reaction profile;

Table# 14: Isolated Product Reaction Profile.

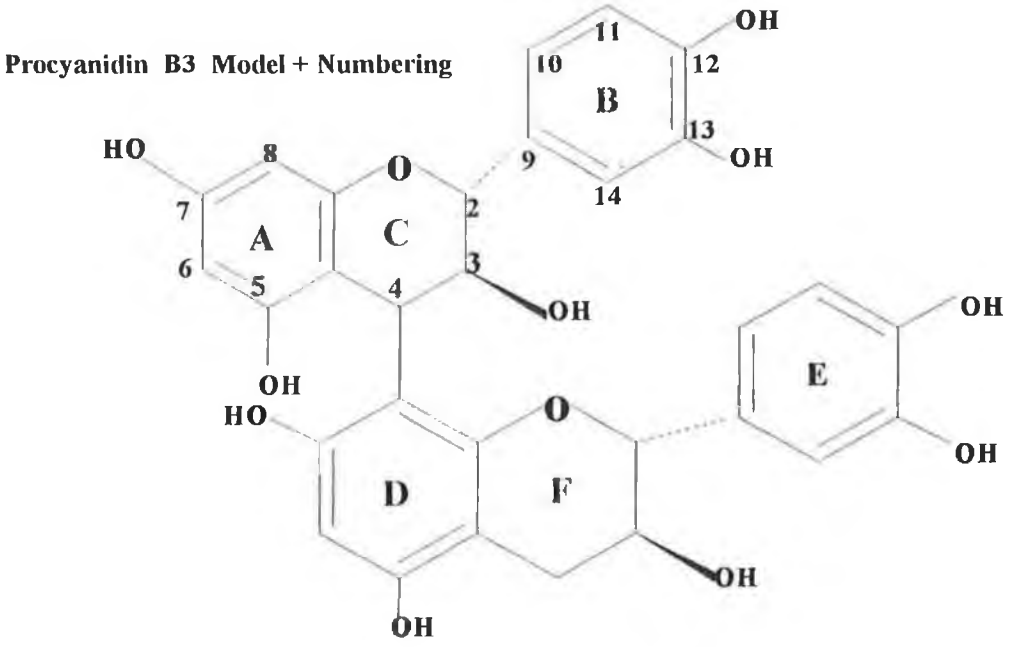
Sample/Ret Time	uk1 3.25	uk2 5.02	uk3 8.24	uk4 9.97	uk5 10.71	uk6 13.30	Cat 14.40	uk7 15.90	uk8 17.80	uk9 19.50
Product	0.18	0.14	21.4	1.37	0.94	0.10	70.27	4.45	0.58	0.33

This material was purified using semi-preparative HPLC and the peak of interest was fractionated into forty tubes. Analysis of the purified fractions showed that the first 9 fractions contained the desired compound with a %HI purity of 95.0% or better (App.A Chrom.#4). The combined fractions were extracted using ethyl acetate in the usual manner. The resulting residue was analysed by 400mHz NMR. The spectrum showed interference from ethyl acetate in an important part of the spectrum. The spectrum showed resonances, previously observed in literature assignations of procyanidin B3. One observation that was noteworthy was the fact that the spectrum exhibited signs of resonance broadening and this was consistent with spectra reported in literature reviews and which were attributed to conformational isomerisation. Some of the key features of this spectrum were compared to literature assignations:

Table# 14: Spectral details of Isolated Product (Delcour Methodology).

Proton Type	Chemical Shift (ppms)	Signal Type	Splitting (Hz)
6 X Ar-$H_{[B/Ering]}$	7.00 - 6.56	multiplet	----
6-$H_{[D]}$	6.16	singlet	----
8-$H_{[A]}$; 6-$H_{[A]}$	6.05	singlet	----
3-$H_{[C]}$	5.84	doub,doub	ΣJ = 18.84

The following diagram affords an assignment of the peaks defined by the above table:



Table# 15: Spectral details of Isolated product:

Proton Type	Chemical Shift (ppms)	Signal Type	Splitting (Hz)
	7.20	singlet	----
6 X Ar-$H_{[B/Ering]}$	7.00 - 6.65	multiplet	----
6-$H_{[A]}$; 8-$H_{[A]}$	6.50	doublet	2.57
4-$H_{[C]}$	6.29	doublet	7.70
	6.20	singlet	----
	6.05	singlet	----
	6.02	singlet	----
	5.95	singlet	----
	5.85	doub,doub	$\Sigma J = 17.78$
	5.65	singlet	----

Even though the recovered product showed evidence for the presence of procyanidin B3 it was not of sufficient quality to allow a complete identification. Also, there was insufficient quantities to allow for a second purification. A separate attempt at isolation was undertaken using the remaining quantity of this material. This used a more concentrated input which it was felt might lead to a greater yield of purified procyanidin B3. All the conditions were kept the same for both attempts and once again only the material from the first 9 fractions was found to be of sufficient purity to attempt isolation. A rough attempt at the determination of the concentration of the product not extracted by the ethyl acetate showed that almost 20% of the recovered material failed to be extracted and hence was lost. The material was analysed by 400mHz NMR but once again was not pure enough to allow identification.

One final experiment was undertaken and again an attempt at purification using semi-preparative HPLC undertaken. The concentration of the solution loaded onto the column was 100.00 mgs/ml (approximating optimum loading). Once isolated the product was freeze dried and analysed by HPLC. This showed at least 16% (+)-catechin was still present and this was not unexpected as catechin was present at five times the concentration of the (+)-taxifolin starting material. Also it was felt that at higher loadings loss of resolution would lead to catechin contamination. The resulting product was purified a second time and although HPLC analysis of the collected fractions appeared pure catechin was observed to be present upon isolation. The main problem with this second purification was the low yield of the recovered material. By contrast the volume of the collected fractions remain the same making qualitative analysis by HPLC impossible. Analysis of the isolated product showed catechin to be present at 30% II. This was not thought to be from impure isolation but rather from the de-composition of the product to yield (+)-catechin.

It was felt that this may have occurred during the final extraction into ethyl acetate (App.A Chrom#5). This called into question the stability of procyanidin B3 in its free form and lead to analysis of this compound in its acetylated derivative form.

Acetylation of Condensation Products:

Most analytical studies carried out on condensed tannins were done so using acetylated or methylated derivatives of these compounds as this greatly aided their stability. The lack of success in isolating procyanidin B3 in its free form prompted the use of such derivatisation techniques. In order to employ this procedure some issues again had to be resolved, most notably, the development of a suitable HPLC assay that would allow separation of the acetylated compounds. In order to prepare a peak marker for assay development a repeat of the Delcour *et al* synthesis was undertaken with the difference that once extracted from ethyl acetate the products were re-dissolved in a 1:1 equivalent of pyridine:acetic anhydride. This was allowed to stand at ambient temperature overnight with constant agitation. The products were recovered by their precipitation onto ice and the recovered material was repeatedly washed with chilled water until no smell of pyridine was apparent.

Several different mobile phase compositions were tried in which both methanol and acetonitrile were used in conjunction with water until a suitable assay was developed. The column used was that used in the previous experiments but the mobile phase was altered to 50:50CH_3CN:H_20 (ApH = 3.0). This gave retention times for the acetylated products that approximated those of the products in their free form in the previous assay. All other parameters remained unchanged. One major advantage to this assay was the lack of buffers or organic modifiers which therefore allowed the assay be used directly for semi-preparative HPLC work.

The composition of the acetylated compounds showed two main peaks, thought to be (+)-catechin and the procyanidin B3 suspect. In order to confirm the order of elution it was first necessary to acetylate (+)-catechin as a peak marker. This also proved useful for analysis by 400mHz NMR and mass spectroscopy as the resonances and ions of this compound would most likely be found in the spectra generated for the product. The HPLC profile for acetylated catechin was summarised as follows:

Table #16: HPLC Profile for Acetylated Catechin

Sample/Ret Time	uk1 7.06	uk2 7.99	uk3 8.34	uk4 8.71	Catn 13.40	uk5 19.12	uk6 21.87
Cat-oAc	0.15	0.41	0.27	0.38	98.40	0.10	0.13

The mass spectrum (App.2 spec #1) for acetylated Catechin gave a molecular ion (M/e) of 500, consistent with the mass expected for acetylated catechin. The ^{1}H NMR for the compound was summarised as follows: (App.B NMR#6)

Table #17: 400mHz NMR Spectral Details:

Proton Type	Chemical Shift (ppms)	Signal Type	Splitting (Hz)
3 X Ar-$H_{[B]}$	7.30 - 7.15	multiplet	----
1 X Ar-$H_{[A]}$	6.65	singlet	----
1 X Ar-$H_{[A]}$	6.55	singlet	----
2-$H_{[C]}$	5.25	multiplet	----
3-$H_{[C]}$	5.15	doublet	3.70
4-$H_{[C]}$	2.85	doub,doub	$\Sigma J = 18.78$
4-$H_{[B]}$	2.65	doub,doub	$\Sigma J = 17.45$
OAc	2.25 - 2.00	----	----

The ^{13}C NMR spectrum was allocated as follows: (App. B NMR#7)

Table #18:

CHEM SHIFT	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12	C13
Catn	20.61	20.64	20.77	20.95	21.08	23.9	68.27	77.64	107.68	108.78	110.2	121.76	123.7
"	C14	C15	C16	C17	C18	C19	C20	C21	C22	C23	C24	---	---
"	124.4	136.13	142.07	142.1	149.41	149.85	154.36	168.08	168.39	168.98	170.15	---	---

From this spectrum it was observed that there were 5 carbons bound to oxygens, there were 4 aromatic carbons bound to oxygens, 4 aromatic carbons, 1 aliphatic carbon bound to oxygen and 6 carbons assigned for the CH_3 groups of the acetylated compound. To augment these findings a DEPT 45 projection for the carbons was obtained. As suspected from the ^{13}C trace there were 5 peaks between 21.0-20.5 ppms that corresponded to the CH_3 groups. There was a CH group at 25.0 ppms and further CH, CH_2 groups at 124.35, 123.70, 121.70, 108.60, 107.60, 77.60 and 68.20ppms. These peaks were the carbons bound to oxygens and aromatic carbons bound to hydrogen that were expected. In all 13 out of the 25 carbons were bound to hydrogens with 5 bound to oxygens leaving a total of 7 bound to other carbons.

Finally, a C-H correlation was used to further determine the positions at which carbon was bound to hydrogen. The following table details those couplings: (App. B NMR#8).

Table #19: Summary of C-H Correlation Data

$^{13}C/^{1}H$	H1(2.15)	H2(2.9-2.55)	H3(5.05)	H4(5.15)	H5(6.40)	H6(6.55)	H7(7.10)
C1(20.6-21.0)	X						
C2 (23.9)		X					
C3 (68.3)				X			
C4 (77.6)			X				
C5(107.6)						X	
C6(108.7)					X		
C7(121.7)							X
C8(123.7)							X
C9(124.3)							X

From the findings observed from the acetylation of (+)-catechin it appeared that this method was both effective and successful and that the assay developed did in fact work in the separation of the acetylated compounds. An experiment using 400 mgs input (+)-taxifolin was undertaken to provide acetylated material for purification. Once the material was isolated post acetylation it was examined by HPLC and pyridine was observed to be still present. The material was further washed and dried under vacuum but the interferrence on HPLC was seen to remain causing doubt as to whether this was in fact pyridine. The material was subjected to a double purification by semi-preparative HPLC. However, following the two separations insufficient quantities of isolated material were recovered. As a result of this it was decided to repeat the acetylation using the method of Fonknechten *et al* to yield the crude products ingreater yield.

The crude product post acetylation had a broad peak at a retention time of 8.4 mins at 36.13%II and a peak at 12.96 mins at 56.35%II. Further analysis of this compound showed a late eluting peak at 25 mins and this was assumed to be procyanidin B3. It was therefore assumed that the first broad peak was most likely residual pyridine. Once again the product was dried using vacuum to try and remove this peak.

The re-dried material was analysed by HPLC which resulted in the observation of 3 main peaks with retention times at 12.80, 30.50, and 59.90 mins respectively (App.A Chrom#6). It was decided to collect the later two peaks since the first peak was identified as acetylated catechin by spiking with this standard. A summary of the reaction profile was as follows:

Table #20: Reaction Profile for Isolated Acetylated Compound

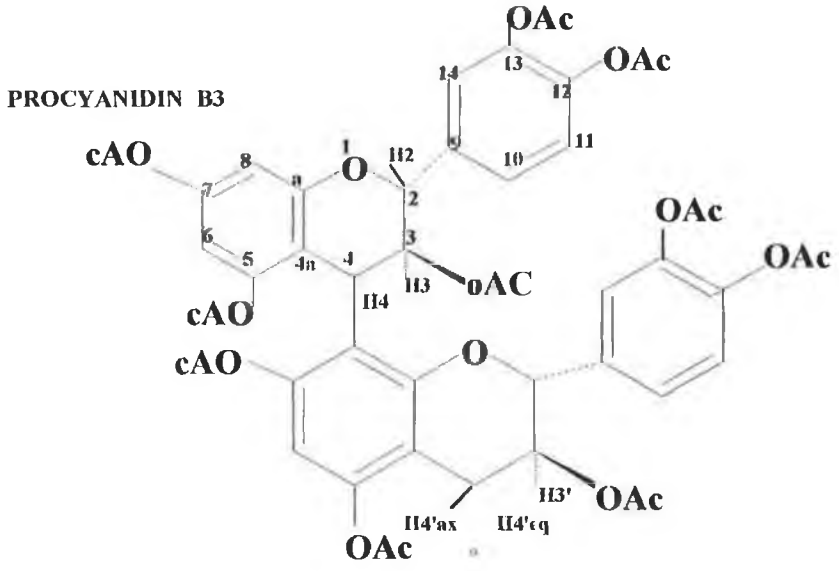
Sample/Ret Time	uk1 7.65	uk2 10.5	uk3 11.8	uk4 12.4	Cat'n 12.8	uk5 17.8	uk6 28.1	B3? 30.5	uk7 33.4	uk8 36.9	uk9 54.0	uk10 59.9
Acet'd Pdt	0.87	0.08	1.92	0.69	24.7	0.85	0.4	24.3	2.56	3.18	3.3	23.9

This material was purified using an initial concentration of 65.60 mgs/ml. The peak at 30.50 mins was fractionated into 40 tubes in order to improve its purification. Analysis of the tubes 20 through 40 showed a good quality for the desired peak and these fractions were combined giving a compound with 83.50%HI purity prior to extraction. The late eluting peak was collected as one fraction since no other peaks were observed to interfere with it. This had a % HI purity greater than 90%. Both of these compounds were isolated in the usual manner and both were subjected to analysis by 400mHz NMR.

NMR Analysis of 30.54min. peak:

(i) ^{1}H NMR Spectra Data.

The following structure was used to detail the proton positions of acetylated B3.



From the values reported by Fonknechten *et al*(table 21) for acetylated B3 a table for the recovered material was compiled with all key proton resonances listed:

Table #21: Fonknechten *et al* Acetylated B3.

Proton Type	Chemical Shift (ppms)	Signal Type	Splitting (Hz)
H-Ar	7.40 - 6.45	multiplet	----
3-$H_{[C]}$	5.06	triplet	----
3'-$H_{[F]}$; 2'-$H_{[F]}$	4.95	multiplet	----
2-$H_{[C]}$	4.78	doublet	9.80
4-$H_{[C]}$	4.50	doublet	9.80
4'-Heq	2.96	doub,doub	$\Sigma J = 16.5$
4'-Hax	2.66	doub,doub	$\Sigma J = 16.5$

Note 1: All other aliphatic protons were attributed to acetyl groups.

Note 2: Resolution of spectrum suggested 80mHz NMR was used although this was not stated.

Table #22: Recovered peak ^1H NMR data (App B NMR#9).

Proton Type	Chemical Shift (ppms)	Signal Type	Splitting (Hz)
H- Ar	7.20 - 6.45	multiplet	----
3-$H_{[C]}$	5.55	triplet	----
3'-$H_{[F]}$; 2'-$H_{[F]}$	4.90	multiplet	----
2-$H_{[C]}$	4.65	doublet	11.40
4-$H_{[C]}$	4.40	doublet	10.00
4'-Heq	2.85	doub,doub	$\Sigma J = 17.28$
4'-Hax	2.60	doub,doub	$\Sigma J = 21.88$

Note 3: Again all alaphatic protons were attributed to acetyl groups.

Analysis and confirmation of the isolated structure was further supported with the aid of a COSY spectrum. Here all the J coupled protons were identified giving an indication as to which protons were coupled to each other. (App. B NMR#10). Here some of the bonding patterns of the aromatic region were clearly defined. The doublet at 4.40ppms was coupled to the triplet at 5.55ppms and also the doublet at 4.65ppms. This tended to confirm the positions for protons as proposed by Fonknechten *et al* were the 3-$H_{[c]}$ was splitting the 4-$H_{[c]}$ into a doublet and the 2-$H_{[c]}$ into a doublet with each of these splitting the other into a doublet. Likewise both of these protons split the 3-$H_{[c]}$ into a triplet.

From comparison of the isolated product to the product presented by Fonknechten *et al* it was apparent that their method did, in fact, produce procyanidin B3. The peak at 30.54 mins was confirmed as acetylated B3 thereby confirming the synthesis worked. However, two of the claims made by this group may not be correct. Firstly, it would appear that the recovery of procyanidin B3 in a 50% yield was not possible since a third late eluting peak with equal % area by HPLC (assuming similar response factor) was observed. Secondly, it was stated that this method gave no evidence for the production of the trimer procyanidin C2. However, the following NMR data detailing the spectral findings for the compound isolated at a retention time of 60 mins would appear to confirm the production of this trimer. The first table summarised the ^{1}H NMR data for procyanidin C2 as proposed by Delcour *et al*, while the second presented the spectral data of the isolated material.

Table #24: ^{1}H NMR data for procyanidin C2.

Proton Type	Chemical Shift (ppms)	Signal Type	Splitting (Hz)
9 X H-Ar$_{[B/E/H]}$	7.33 - 6.44	multiplet	----
6-H$_{[D]}$	6.65	singlet	----
6-H$_{[G]}$	6.61	singlet	----
8-H$_{[A]}$	6.53	doublet	2.50
6-H$_{[A]}$	6.20	doublet	2.30
3-H$_{[CorF]}$	5.57	doub,doub	ΣJ = 19.0
3-H$_{[ForC]}$	5.45	doub,doub	ΣJ = 18.5
2-H$_{[I]}$; 3-H$_{[I]}$	5.23	singlet(Broad)	----
2-H$_{[CorF]}$	4.73	doublet	10.25
2-H$_{[ForC]}$	4.61	doublet	10.00
4-H$_{[CorF]}$	4.56	doublet	8.25
4-H$_{[ForC]}$	4.13	doublet	9.00

Note 1: All other protons were aliphatic and acetyl related.

Table#25: ^{1}H NMR data for 60.00min isolated product (App. B NMR#11).

Proton Type	Chemical Shift (ppms)	Signal Type	Splitting (Hz)
9 X H-$Ar_{[B/E/H]}$	7.25 - 6.45	multiplet	----
6-$H_{[D]}$	6.65	singlet	----
6-$H_{[G]}$	6.61	singlet	----
8-$H_{[A]}$	6.53	doublet	----
6-$H_{[A]}$	6.20	doublet	----
3-$H_{[CorF]}$	5.55	doub,doub	$\Sigma J = 19.50$
3-$H_{[ForC]}$	5.45	doub,doub	$\Sigma J = 19.00$
2-$H_{[I]}$; 3-$H_{[I]}$	5.23	singlet(Broad)	----
2-$H_{[CorF]}$	4.73	doublet	----
2-$H_{[ForC]}$	4.61	doublet	----
4-$H_{[CorF]}$	4.56	doublet	----
4-$H_{[ForC]}$	4.13	doub,doub	----

As can be seen both the tables are closely identical which suggests that the late eluting peak is procyanidin C2. Again a COSY spectrum was run and the J coupled protons showed which protons were inter- related. Here the 3 doublets between 4.73-4.56 ppms were linked to the 2 doublet of doublets between 5.55-5.45 ppms (App. B NMR#12). Again 1 proton split the other into a doublet while the doublets themselves were observed to split 3-$H_{[C\ or\ F]}$ into a doublet of doublets while 3-$H_{[C\ or\ F]}$ split 2-$H_{[I]?[C]}$ or $_{[F]}$ into a doublet.

Finally, what this work showed was firstly the synthesis of acetylated procyanidin B3 and procyanidinC2 did occur but, secondly, that these were present in approximately 30% yields and this did not represent a major improvement on the proposed synthesis of Delcour *et al* which was far more rigorous. The acetylation of these products proved useful for their identification but was of no use in the long term as the aim of this work was to isolate procyanidin B3 in its free form. Since the gain in % terms of the method proposed by Fonknechten *et al* was little better than the original method of Delcour *et al* it was decided that attempts at isolating procyanidin B3 in its free form would be better suited to the more rigorous reaction of Delcour *et al*. Also it was felt that with this better control of the synthesis, procyanidin C2 production was less likely thereby making the separation of procyanidin B3 by semi-preparative HPLC an easier prospect.

Attempted isolation of procyanidin B3 in its free form:

In order to aid the identification of procyanidin B3 in its free form an authentic sample of procyanidin B3 was supplied by Guinness Group Research as a methanol solution stored at -20°C. This standard was extracted from barley and was analysed by both HPLC (App. A Chrom#7) and 400mHz NMR. The standard was evaporated from the methanol and reconstituted in deuterated acetone to obtain a ^{1}H NMR (App.2 NMR#13). Some of the solution was also diluted with mobile phase and was used as a HPLC peak marker.

Table #26: ^{1}H NMR characteristics of authentic B3.

Proton Type	Chemical Shift (ppms)	Splitting (Hz)
H- Ar	7.38 - 6.65	multiplet
8-$H_{[A]}$	6.50	doublet
6-$H_{[A]}$	6.48	doublet
3-$H_{[C]}$	6.28	doublet
3-$H_{[F]}$	6.26	doublet
2-$H_{[F]}$	6.20	singlet
2-$H_{[C]}$	6.09	singlet
4-$H_{[C]}$	5.94	doublet
4-$H_{[F]}$	5.85	multiplet
	4.72	doublet
	4.62 - 4.35	multiplet
	4.92	doublet

The HPLC profile was summarised as follows

Table #27: In process HPLC Profile.

Sample/Ret Time	uk1 0.04	uk2 2.40	uk3 2.83	uk4 3.76	uk5 4.14	uk6 4.84	uk7 5.72	uk8 7.68	B3? 9.10	uk9 10.86
B3 Std	0.02	0.87	1.2	0.07	0.42	0.24	1.76	0.33	94.5	0.54

This represented the first unambigous identification for the retention time of free procyanidin B3. Having established this retention time a large scale (500 mg input (+)-taxifolin) reaction was undertaken, initially following the procedure of Fonknechten *et al.* The synthesis appeared to go to completion in the usual manner and the crude material was isolated as all previous reactions had been. This product was subjected to purification by semi-preparative HPLC and analysis of the collected fraction indicated the presence of a peak that shared the same retention time as procyanidin B3 with a purity greater than 91.00%HI (App. A Chrom #8). The profile for the combined fractions was summarised as follows:

Table #27: Combined Fraction Reaction Profile

Sample/Ret Time	uk1 2.41	uk2 5.60	uk3 8.05	B3? 9.10	uk4 10.46	Catechin 14.83	uk5 17.04
Frac'ns	1.68	0.4	0.53	91.01	5.93	0.23	n/d

This material was extracted into ethyl acetate and evaporated to dryness. It was immediately reconstituted in $CDCl_3$ and analysed by 400mHz NMR. The collected spectrum showed that this material was not of sufficient purity to yield definite proof as to the presence of procyanidin B3. Since the purity appeared to be good by HPLC two questions again arose. The first concern was that an impurity may have been present that was not apparent on HPLC. The second concern was one already expressed, which

was the potential lability of the compound. It was likely that the isolated compound had degraded during its extraction. Some small evidence that procyanidin B3 was present was presented by some key chemical shift values as follows:

Table #28: Key Procyanidin B3 Chemical Shifts.

Proton No:	Chemical Shift (ppms)	Signal Type (Isolated Product)	Signal Type (Procyanidin B3)
1	6.40	doublet	doublet
2	6.29	doublet	doublet
3	6.20	singlet	singlet
4	6.05	singlet	singlet
5	5.95	doublet	doublet
6	5.85	multiplet	multiplet
7	5.67	singlet	singlet

A second attempt at isolating B3 in its free form was undertaken this time on a 200 mg input scale. Once again no pure sample was obtained although again there was evidence of the presence of procyanidin B3 when analysed by 400mHz NMR. Another attempt on a even smaller scale (100 mgs) was undertaken. In order to ensure that the retention times for the compounds on both the analytical and semi-preparative columns were identical a new 30cm x 3.9 mm id μBondapak C18 analytical column was used and the following assay was employed for both analytical and semi-preparative work:

Column: μBondapak C18 30cm x 3.9mmid; 10μm.

Mobile Phase: 90:10 H2O: CH3CN (ApH = 3.0)

Flow Rate: 1.0 ml/min.

Detection: λ = 220nm.

Retention Time:	Time (Mins.)
Unknown#1	12.48
Unknown#2	15.92
Unknown#3	18.11
B3	20.27
Unknown#4	23.28
(+)-Catechin	25.15
Unknown#5	34.62
Unknown#6	38.27
Unknown#7	44.19
Unknown#8	48.30

From analysis of the freeze-dried product it was evident that this reaction produced many products and that as for the acetylated reaction it was apparent that the yield was much less than the stated 50%. The reaction profile for this product was summarised as follows:

Table #29: Reaction Profile for Free Hydroxy (Fonknechten Reaction).

Sample/Ret Time	uk1 12.48	uk2 15.92	uk3 18.11	B3 20.27	uk4 23.28	Cat'n 25.15	uk5 34.62	uk6 38.27	uk7 44.19	uk8 48.30
Product	0.14	0.41	0.19	21.99	20.2	32.3	11.03	2.45	3.26	7.03
Standard	0.19	n/d	n/d	96.15	n/d	0.21	n/d	n/d	n/d	n/d

The scale up to semi-preparative HPLC gave the same retention times for the peaks of interest, thus a direct comparison was possible. The collected fractions were analysed and those collected over the first 25 fractions gave the best results with the first 15 being combined to give a peak with a % HI purity of 97.80%. Analysis of the waste aqueous fraction, after ethyl

acetate extraction showed that a significant quantity of procyanidin B3 was not extracted.

The isolated product was analysed by 400mHz NMR and again an impure spectrum was obtained. Analysis of the crude product which had beenretained in solution at ambient for several days showed the profile to be unchanged therefore suggesting that the compound was not degraded when in contact with mobile phase. This further reinforced the suggestion that the compound may have degraded during the ethyl acetate extraction procedure.A comparison of the ^{1}H NMR for the authentic B3 and the collected fraction is summarised in the following table:

Table #30: Comparison of Isolated Fraction and procyanidin B3 Standard.

Proton No:	Chemical Shift (ppms) B3Std : Suspect	Peak Type B3Std : Suspect
1	7.35 : 7.35	singlet : singlet
2	7.20-7.10 : 7.20-7.10	doub,doub:doub,doub
3	6.90 : 6.90	doublet : doublet
4	6.87 : 6.87	doublet : doublet
5	6.85-6.70 : 6.85-6.70	multiplet : multiplet
6	6.50 : 6.50	doublet : multiplet
7	6.46 : 6.46	doublet : doublet
8	6.29 : 6.29	doublet : doublet
9	6.27 : 6.27	doublet : doublet
10	6.20 : 6.20	doublet : doublet
11	6.05 : 6.05	singlet : singlet
12	6.00 : 6.00	singlet : singlet*
13	5.91 : 5.91	---- : doublet
14	5.89-5.80: 5.85-5.80	multiplet : multiplet

* Impurity at 6.00ppms due to ethyl acetate interference

The more rigorous method proposed by Delcour *et al* was then investigated as it was felt that this synthesis should lead to fewer impurities, making isolation of pure product easier. This reaction was carried out on a small scale (100mgs) and from in process HPLC data only 2 peaks were observed. (App.1 Chrom#9), one for procyanidin B3 and the other for catechin. The procyanidin B3 was present at 16.40%II and this was very close to the expected 20.00% yield. The isolated product had the following profile:

Table # 31: Isolated Crude Product (Delcour *et al).*

Sample/Ret Time	uk1 8.46	uk2 10.05	EtoAc 11.82	uk3 13.28	uk4 17.23	B3 19.90	uk5 22.0	Cat'n 24.03
Crude Product	0.02	0.06	2.48	0.40	0.10	16.47	1.03	79.37

None of the later eluting peaks observed in the Fonknechten *et al* method were present in this profile and this served to confirm that the more controlled reaction would give a cleaner profile (App. A Chrom#10). The product was passed down the semi-preparative column but analysis of the collected fractions showed that none were of sufficient purity to warrant further isolation. The remaining portion of this crude was purified using a two step isolation, the first simply to remove the excessive (+)-catechin, the second to isolate the procyanidin B3. The collected material had a purity of 85.00%HI. This was compared to the authentic procyanidin B3 as follows (App.B NMR#14):

Table # 32: Comparison of procyanidin B3 Std and Product.

Proton No:	Chemical Shift (ppms) B3Std : Product	Peak Type B3Std : Product
1	7.35 : ----	singlet : ----
2	7.20-7.10 : 7.05-6.95	doub,doub:doub,doub
3	6.90 : 6.90	doublet : doublet
4	6.87 : 6.87	doublet : doublet
5	6.85-6.70 : 6.85-6.70	multiplet : multiplet
6	6.50 : 6.50	doublet : multiplet
7	6.46 : 6.46	doublet : doublet
8	6.29 : 6.29	doublet : doublet
9	6.27 : 6.27	doublet : doublet
10	6.20 : 6.20	doublet : doiblet
11	6.05 : 6.05	singlet : singlet
12	---- : 6.00	---- : multiplet*
13	5.91 : 5.91	---- doublet
14	5.89-5.80: 5.85-5.80	multiplet . multiplet
15	5.65 : 5.65	singlet : singlet

* Peak due to Ethyl Acetate Interference

This was the last of the attempted syntheses of procyanidin B3 in its free form by these synthetic methods. In all cases these reactions probably suffered from small scale input of (+)-taxifolin due to the expense of this material. The poor return for procyanidin B3 makes either of these procedures examined impractical. However, it should be noted that scale up of both procedures may have an advantageous effect on the synthesis.

As a result of this work it became clear that a more suitable form of synthesis would be one that employed an enzyme to effect dimerisation of catechin. This is a common reaction in nature where catechin is oxidised to its higher molecular weight oligomers by the action of several oxidase enzymes. Based upon literature it was decided to attempt a bio-mimetic reaction to form procyanidin B3. Much work has been done into the identification of the mode of action of these enzymes and it has been suggested that one of the peaks present in these reactions was procyanidin B3 [4].

Procyanidin B3 by enzymatic oxidation:

The initial procedure that was examined for the enzymatic oxidation of (+)-catechin was that described by Oszmianski *et al*. This group reacted chlorogenic acid, catechin and a mixture of both with tyrosinase, a polyphenol oxidase enzyme extracted from mushrooms. The reaction was allowed to proceed for a 12 hour period and was sampled at T = 0, T= 1hr., T = 2hrs. and T = 12 hrs. The reaction was monitored by HPLC at both 220 and 280nm and a comparison between these wavelengths was made. The pH of the acetic acid solution in which this reaction was carried out was adjusted to 3.50 as it was felt a more controlled reaction would take place at lower pH's. The reaction profile for this experiment was summarised as follows:

Table #33: Enzymatic Oxidation Reaction Profile λ = 220nm.

Sample/Ret Time	uk1 6.24	uk2 10.17	uk3 11.40	uk4 12.85	uk5 14.70	Cat'n 20.32	uk6 28.48	uk7 29.28
T0 post Add'n	0.22	n/d	0.04	n/d	n/d	98.60	0.96	0.15
T = 1Hr hold	n/d	1.60	n/d	n/d	n/d	98.40	n/d	n/d
T = 2Hr hold	n/d	n/d	1.86	0.81	n/d	95.92	n/d	n/d
T = 12Hr hold	n/d	n/d	2.65	1.30	0.51	95.54	n/d	n/d

Table #34: Enzymatic Oxidation Reaction Profile λ = 280nm.

Sample/ Ret Time (mins).	uk1 5.54	uk2 10.62	uk3 18.11	Cat'n 18.72	uk4 20.70
T0 post Add'n	n/d	n/d	n/d	100.0	n/d
T = 1Hr hold	3.73	n/d	n/d	96.27	n/d
T = 2Hr hold	12.32	n/d	n/d	85.47	n/d
T = 3Hr hold	6.95	n/d	n/d	93.05	n/d
T = 12Hr hold	n/d	n/d	n/d	100.0	n/d
B3/Cat'n Spike	1.41	15.07	1.90	81.11	0.50

From this initial experiment it was clear that very little reaction had taken place and that procyanidin B3 might not have been present. Since the experiment produced very little reaction products at pH 3.5 it was decided to repeat the procedure at the higher pH of 6.5. It was also observed that the wavelength of 220nm gave a better response for all products monitored and this was now the wavelength of choice.

Table #35: Reaction Profile for Oxidation @ pH 6.5.

Sample/Ret Time(mins).	uk1 5.10	uk2 5.80	uk3 7.50	uk4 8.20	uk5 9.50	uk6 10.28	uk7 11.20	uk8 12.70	uk9 13.80	Cat'n 16.30
T0 post Addn	n/d	n/d	n/d	n/d	0.04	0.12	n/d	0.1	0.02	97.32
T = 5mins	n/d	n/d	n/d	n/d	6.41	1.94	n/d	0.3	0.55	90.2
T= 1Hr hold	n/d	n/d	n/d	n/d	7.26	1.66	0.29	0.6	1.35	86.9
B3/Cat spike	0.06	0.93	0.22	0.38	22.2	1.12	6.4	1.3	0.8	66.6
T = 2Hr hold	n/d	n/d	n/d	n/d	7.2	1.8	0.25	n/d	0.75	89.6
T =O/N hold	n/d	n/d	n/d	0.4	6.7	2	n/d	n/d	0.4	88.9

The pH adjustment to a value close to neutral did appear to have a positive effect on the reaction. A peak with the same retention time as procyanidin B3 was observed after just 5mins. contact with the enzyme. However, it was also observed that this peak did not appear to grow significantly with time. Yet another experiment used the same parameters with the exception of the enzyme concentration which was doubled. The reaction was observed to form a dark yellow colour almost immediately which turned to brown over time. The reaction profile for this experiment was summarised as follows:

Table #36: Reaction Profile for Increased Enzyme Concentration.

Sample/ Ret Time(mins).	uk1 5.27	uk2 9.57	uk3 12.54	uk4 13.26	uk5 14.56	Cat'n 17.14
T0 post Add'n	0.32	0.34	0.14	n/d	n/d	98.40
T = 5mins hold	0.37	8.76	2.46	n/d	1.47	86.93
T= 2Hr hold	1.56	6.56	0.95	0.90	167	88.35

The results obtained suggested that although colouration did appear to be more rapid for higher concentrations of enzymes this did not mean a corresponding increase in the desired product peak was obtained. A comparison for the two 5min. traces showed a similar reaction profile in both cases. (App.A Chrom 11).

Another feature of these experiments was the fact that a slight decrease in the % area for the unknown product was observed over time. This may have been due to the fact that the enzyme was further reacting with this formed product to form higher oligomers. Following on from these experiments it was decided to scale up the reaction and to attempt a separation of the unknown peak by semi-preparative HPLC. For the reaction 600 mgs. of (+)-catechin was used. The peak of interest was observed to have a %II of 12% in the isolated crude material following its extraction from ethyl acetate. Following the first purification attempt it was observed that a large concentration of catechin still remained and that this material warranted a further purification. The reaction profile after the first purification was summarised as follows:

Table #37: Reaction Profile of Purified Enzymatic Oxid'n Product.

Sample/ RetTime	uk1 5.36	uk2 5.62	uk3 6.33	uk4 7.18	uk5 8.48	uk6 10.2	uk7 11.3	uk8 13.1	uk9 14.0	uk10 14.8	Cat'n 17.9	uk11 18.7
pdt	1.17	4.81	1.73	0.2	1.05	28.8	2.49	0.44	0.49	7.46	44.7	6.31

Even though the reaction appeared to proceed to yield a product which corresponded to procyanidin B3 retention time, there was insufficient concentrations of it to allow proper isolation by HPLC. This reaction was repeated with the same results, except that the isolated product following the two extractions had a % HI figure of 94% by HPLC. The material was immediately analysed by 400mHz NMR and the ^{1}H spectrum showed that the product was not as pure as first thought. However, from this spectrum it was not possible to identify any of the key chemical shift resonances

attributable to procyanidin B3. This sample was evaporated to dryness and reconstituted with mobile phase and analysed by HPLC in order to try and identify some of these impurities. It was clear that about 5%II of these impurities was due to the presence of catechin and since this was not apparent in the fractionated samples it was most probably due to degradation. (App.A Chrom #12).

Since this reaction appeared only to produce the peak of interest in 8 to 10% yield another approach was needed in order to identify this peak. It was decided to allow the reaction to proceed in an NMR tube thereby enabling constant monitoring. On this occasion the reaction was also monitored by HPLC by removing small aliquots from the NMR tube. Running the reaction on this scale highlighted one of the problems that had already been observed in some of the experiments. Under these conditions catechin did not fully go into solution prior to the addition of the enzyme thereby somewhat reducing the quantity of starting material available to be acted upon by the enzyme. To allievate this problem approximately 10%v/v acetone was used to fully dissolve the catechin before the deuterated water was added. The in process HPLC analysis for this reaction was as follows:

Table #38: Reaction Profile of Acetone Soluabilised Compounds.

Sample/Ret Time(mins).	Act'n 5.15	uk1 6.06	uk2 6.64	uk3 7.58	uk4 7.98	uk5 8.80	uk6 11.15	uk7 12.14	uk8 12.88	Cat'n 13.63
T = 20mins	0.7	0.06	0.05	0.16	0.19	26.24	2.42	0.48	1.36	67.9
T = 40mins	0.64	0.21	0.24	0.1	0.05	37.6	2.34	0.36	1.5	55.26
T = 60mins	0.61	0.19	0.32	0.13	0.1	29.3	4.5	1.3	2.79	58.45

It would appear that a product had formed with a retention time of 8.8mins. but it was not certain whether this peak was solvent related or a legimate product. The NMR spectra that accompanied these traces did not show any sign of resonances associated with procyanidin B3. (App.B NMR#15). The sample was subsequently held at ambient for a considerable period of time and was analysed after 20 hrs. and 6 days respectively but failed to show any further reaction. The reaction was repeated without using acetone to dissolve the catechin in order to verify that the use of acetone was not a problem. The same reaction profiles and NMR spectra were generated for this experiment. (App.B NMR#16). Finally, even though there was no evidence for the production of procyanidin B3 the fact still remained that a product was being produced and an effort was made to try and identify what composition this unknown possessed.

Following on from the experience gained in the isolation of procyanidin B3 as its acetylated derivative it was decided to utilise the same methodology. The reaction profile for the isolated compounds following acetylation was as follows:(App.A Chrom #13)

Table #39: Reaction Profile for Acetylated Enzyme Products.

Sample/Ret Time(mins).	Cat'n 12.99	uk1 17.66	uk2 18.08	uk3 18.67	uk4 21.04	uk5 21.92	uk6 25.52	uk7 29.57	uk8 30.74	uk9 33.10
Acety'd Pdt	91.66	0.07	0.15	0.49	0.26	0.06	0.94	1.45	4.7	0.22

The retention times observed for the peaks of the enzyme reaction agreed closely to those observed following the acetylation of the condensation products formed from the method of Fonknechten *et al.* The product was subjected to purification by semi-preparative HPLC. The collected fractions were analysed but no evidence as to the purity of the collected peak was obtained due to the low dilution at which the compound was collected. Analysis by 400mHz NMR showed firstly a lack of product and,

secondly, what product was present appeared impure. However, some of the resonances may have been attributed to acetylated B3. (App.2 NMR#17).

It was felt that if any product was to be isolated from the enzymatic reaction then a higher proportion of the compound of interest would have to be formed. Further experiments were undertaken with the aim of increasing the % of the product formed. Three experiments that varied the enzyme concentration were attempted. The first was a control using the concentration as described, the second used four times the concentration while the third used ten times the concentration. All three were started at the same time and allowed to stand at ambient for 2 hours with constant agitation. They were all quenched in the usual manner and acetylated. HPLC analyses for the three isolated materials was summarised as follows:

Table #40: Reaction Profiles of Alternative Enzyme Conc. Products.

Sample/ Ret Time(mins).	syst'm 1.82	Cat'n 11.20	uk1 16.48	uk2 22.68	uk3 25.68	uk4 26.60	uk5 28.76	uk6 37.76	uk7 47.82	uk8 60.03
Control	n/a	65.9	0.8	10.89	1.09	7.89	1.44	0.6	1.67	4.11
4 X enzyme	n/a	28.55	1.16	17.34	n/d	6.24	3.15	6.3	23.63	9.49
10X enzyme	n/a	34.85	3.01	9.59	n/d	8.53	5.27	7.82	17.57	n/d

It was apparent from these results that the addition of extra enzyme did not lead to an increase in the peak of interest but rather an increase in later eluting peaks assumed to be higher oligomers. In all cases the %II area of the peak was similar and suggested that a finite level for this peak had been reached. It was also noted that a higher concentrations of the enzyme

caused a rapid precipitation of the products upon quenching thus limiting the quantity of material recovered. The material from the control experiment was analysed by 400mHz NMR and yet again no evidence of the appearance of procyanidin B3 was found.

Two more attempts at isolating this unknown product were undertaken. The first of these was a repeat of the previous reactions in every detail with the exception of the extraction of the crude product into ethyl acetate. In this case the aqueous mother liquor was removed by rotary evaporation directly in an effort to limit product degradation. Analyses of this mother liquor with 50% of its volume removed showed that the product was present at 61%HI, with catechin at 10%II and several other peaks making up the remainder. Instead of subjecting this recovered material to a second purification it was analysed directly by 400mHz NMR. There was no evidence to suggest that the peak of interest was procyanidin B3 although some of the resonances common to catechin were found as would be expected. (App.B NMR#18). The second experiment did employ a second purification, however, as had already been seen the concentration of the collected fractions was too dilute to allow analyses. The collected product was of a good purity and the ^{1}H NMR failed to show any of the resonances associated with B3. The profile of this products was as follows:

Table #41: ^{1}H NMR Profile for Isolated Enzyme Product.

Peak Type	Chemical Shift (ppms)	Signal Type
1 X H-Ar	8.60	doublet
H-Ar	7.80-6.65	multiplet
----	6.60	singlet
----	6.45	singlet
----	5.90	doublet
----	5.50	doub,doub
----	5.20-5.05	multiplet
----	4.20-4.00	multiplet

Some of the shifts observed were also seen in traces for procyanidin B3 but the characteristic doublets at 4.78 and 4.30ppm were not present further reinforcing the thought that procyanidin B3 was not present. Finally, two alternative experiments were carried out whereby the starting material was dissolved in 10%v/v CH_3CN prior to enzyme addition. The reason for using this solvent was to maintain all the compounds of interest in solution. The first of these experiments used a mild acid wash to remove pyridine post actylation while the second was a repeat but omitted this wash just to confirm that the acid had not caused degradation. No evidence for the presence of procyanidin B3 was obtained in either of these experiments. Ancillary to these experiments reaction blanks which omitted (+)-catechin and the enzyme respectively were undertaken.

These two experiments served to prove that the mere presence of either catechin or enzyme was insufficient to produce the observed peak. All of the experiments completed, seemed to produce a reaction product with a retention time similar to procyanidin B3 in 8 to 10% yield. Isolation of this material proved difficult but NMR evidence served to discount the suggestion that this peak was in fact procyanidin B3. Following a review of the literature a reference which suggested to the identity of this compound was obtained. In the 1960's Weignes *et al* exposed (+)-catechin to peroxidase enzymes and proposed that the resulting product was in fact dehydrodicatechin A, a (2β-O-7) linked dimer of (+)-catechin. The supposition that this was the unknown product made some sense as this dimer would probably have similiar retention time on HPLC as procyanidin B3 but would certainly have a different NMR profile.

The first attempt at this reaction was undertaken in duplicate and was done so to compare the enzymes tyrosinase and horseradish peroxidase. Both reactions failed to produce the yellow precipitate as described after standing for 14 days. Re-examination of theliterature indicated that during the 14 day hold it was necessary to add fresh enzyme and hydrogen peroxide on a daily basis. The following experiment recreated the method as laid down and after 14 days a yellow precipitate did form. HPLC analysis of the reaction after 7 days showed primarily unreacted catechin but also a peak with a similar retention time to that of the unknown product and procyanidin B3. (App.A Chrom#14). Having followed the extraction procedure the isolated product was analysed by HPLC and was found to mainly contain (+)-catechin. (App.A Chrom#15). Analysis of the waste mother liquor showed that what product had formed had failed to extract into the organic layer thereby explaining why so little of this material was isolated (App. A Chrom#16).

Some question as to the activity of the enzyme was raised as this material had been stored at -20°C for a considerable period of time. The in process HPLC details for this reaction were summarised as follows:

Table #42: Inprocess Reaction Profile for Enzyme Mediated Products.

Sample/ Ret Time(mins).	uk1 2.59	uk2 3.08	uk3 6.40	uk4 10.78	uk5 12.91	uk6 17.15	uk7 18.86	uk8 20.59	Cat'n 23.18	uk9 28.08
T = 7 days	n/d	n/d	n/d	n/d	0.17	0.18	6.89	1.72	89.61	1.41
Isolated Pdt	0.12	1.77	0.4	0.28	n/d	n/d	1.54	0.43	95.17	n/d
Waste M/L	0.16	n/d	n/d	0.21	n/d	n/d	10.77	2.18	82.74	2.52

The enzymatic formation of dehydrodicatechin A did appear to produce a peak with the same retention time as procyanidin B3. Although this material was not isolated and identified it is possible that this was the product produced by the other enzyme reactions.

CONCLUSION:

From all of the experiments carried out in the examination of the synthesis of procyanidin B3,a few interesting observations were made regarding its isolation. Firstly as had been stated in the literature the synthesis of the condensed tannin dimers and trimers was not a trivial procedure, in fact very few groups had ventured into this area of chemistry, as it was frought with difficulties. In its free form the isolation of procyanidin B3 was not achieved using the appartus and conditions investigated. This does not mean that it was not possible to isolate it but rather that it was a labile material that required special treatment during extraction. Secondly the analytical conditions that were developed worked well for separating the compounds and even aided isolation of the acetylated derivatives, but they may be further enhanced with the use of gradient elution facilities. Also it was observed that the percentage yield obtained in these syntheses did not justify the type of cost in equipment and solvent that would be involved in the recovery of the dimer. The use of 400mHz NMR was of great assistance for the identification of the compounds made and also gave a good indication of the purity of any isolated compounds. Finally we were unable to confirm literature reports of the presence of procyanidin B3 in the enzymatic oxidation of catechin.

If any further synthetic work was to be carried out on these compounds a ready supply of the starting compound (+)-taxifolin would be necessary. To this end it may be benificial to try and synthesise this compound initially, with the possible aim of telescoping the procedure and purifying the resultant products by commercial scale preparative chromatography. If the procyanidin B3 could be improved it would be of interest to try and identify the decomposition products of the dimer in alcoholic solutions possibly by LC-MS, as this would go a long way towards understanding the modes of action involved in chill haze formation in beers and the ageing of wine.

REFERENCES:

[1] Delcour J.A., Ferreira D., Roux D.G., *J. Chem. Soc. Perkin Trans. 1*, (1983), 1711.

[2] Fonknechten G., Moll M., Cagniant D., Kirsch G., Muller J.F., *J. Inst. Brew.*, 89,(1983), 424.

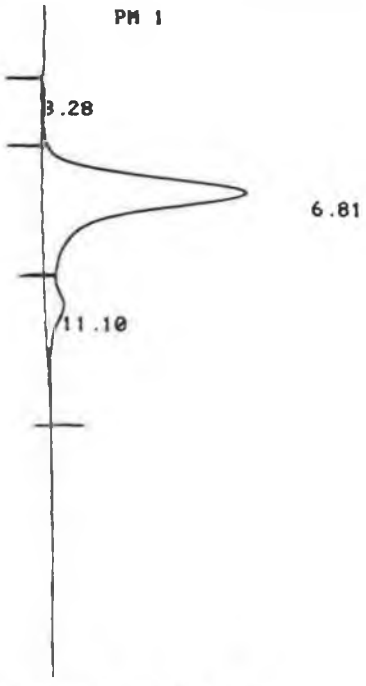
[3] Delcour J.A., Vercruysse S.A.R., *J.Inst. Brew.*, 92, (1986), 244.

[4] Oszmianski J., Lee C.Y., *J. Agric. Food. Chem.*, 38, (1990), 1202.

[5] Weignes K., Ebert W., Huthwelker D., Mattauch H., Perner J., *Liebigs Ann. Chem.*, 726, (1969), 114.

[6] Outtrup H., Schaumburg K., *Carlsberg Res. Commun.*, 46,(1981), 43.

APPENDIX #A: HPLC CHROMATOGRAMS.



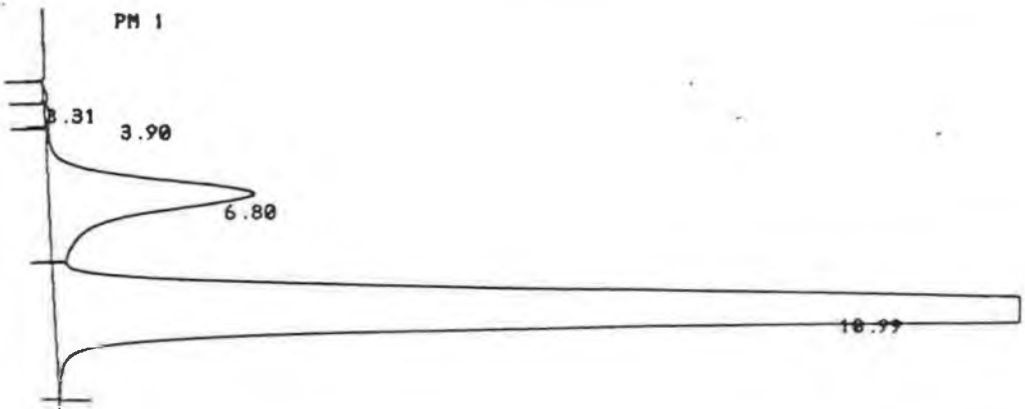
DATA SAVED TO BIN # 21

CATHECIN 1,4,1 12/01/91 11:54:03 CH= "A" PS= 1.

FILE 1. METHOD 0. RUN 24 INDEX 24 BIN 21

PEAK#	AREA%	RT	AREA BC
1	2.229	3.28	119477 02
2	87.02	6.81	4664062 02
3	10.751	11.1	576241 03
TOTAL	100.		5359780

App. A, Chrom #1: Isolated unknown post semi-preparative purification.



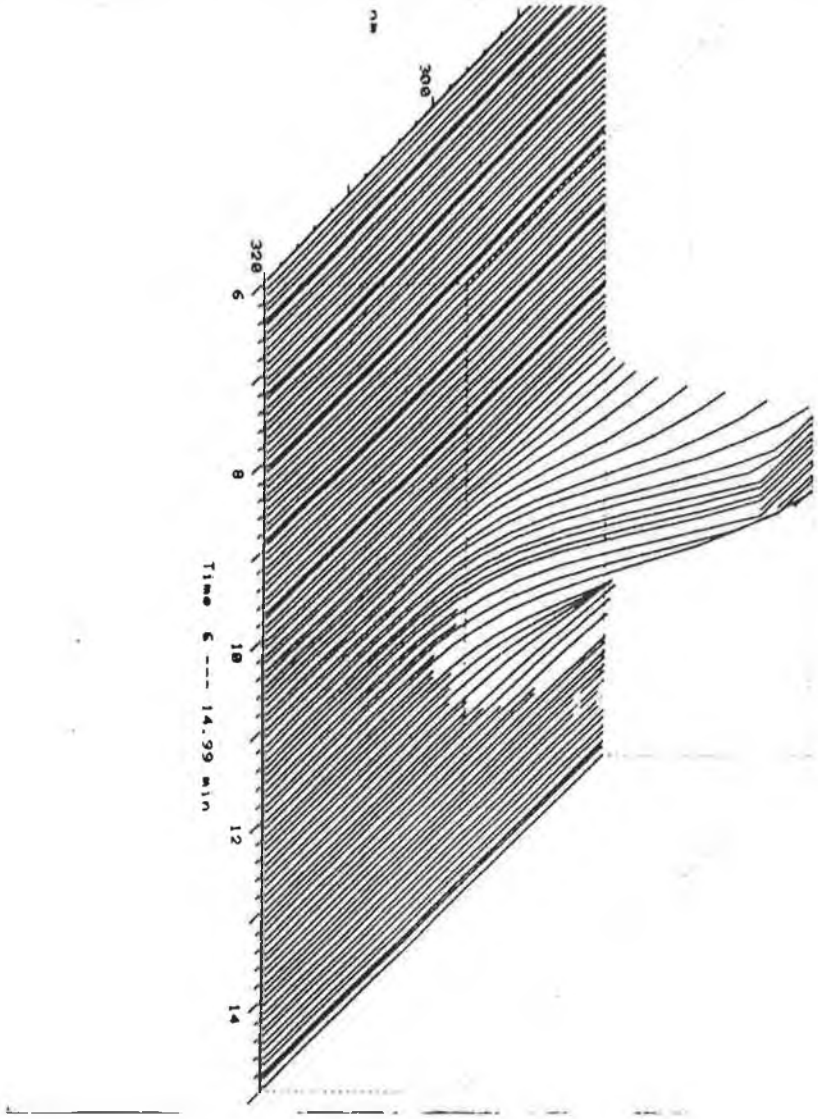
App. A, Chrom #1: Unknown spiked with catechin.

ER 0
DATA SAVED TO BIN # 22

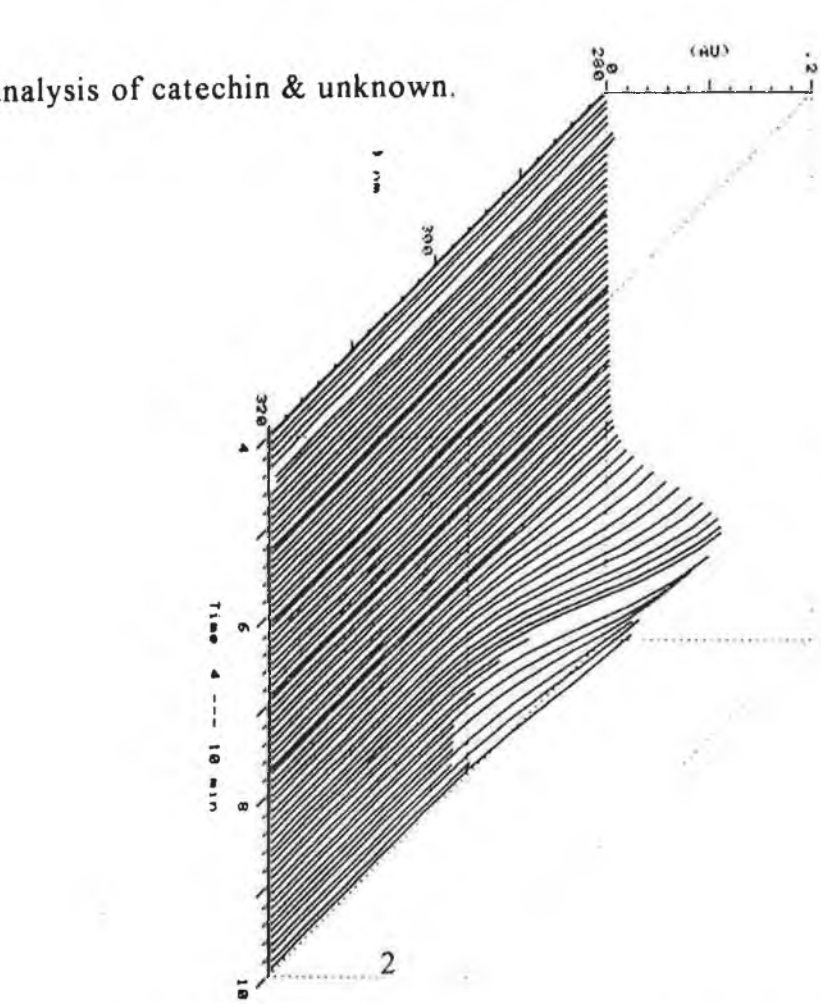
CATHECIN 1,4,1 12/01/91 12:26:59 CH= "A" PS= 1.

FILE 1. METHOD 0. RUN 25 INDEX 25 BIN 22

PEAK#	AREA%	RT	AREA	BC
1	0.091	3.31	30154	02
2	0.179	3.9	59685	02
3	13.871	6.8	4613772	02
4	85.859	10.99	28558585	03
TOTAL	100.		33262196	

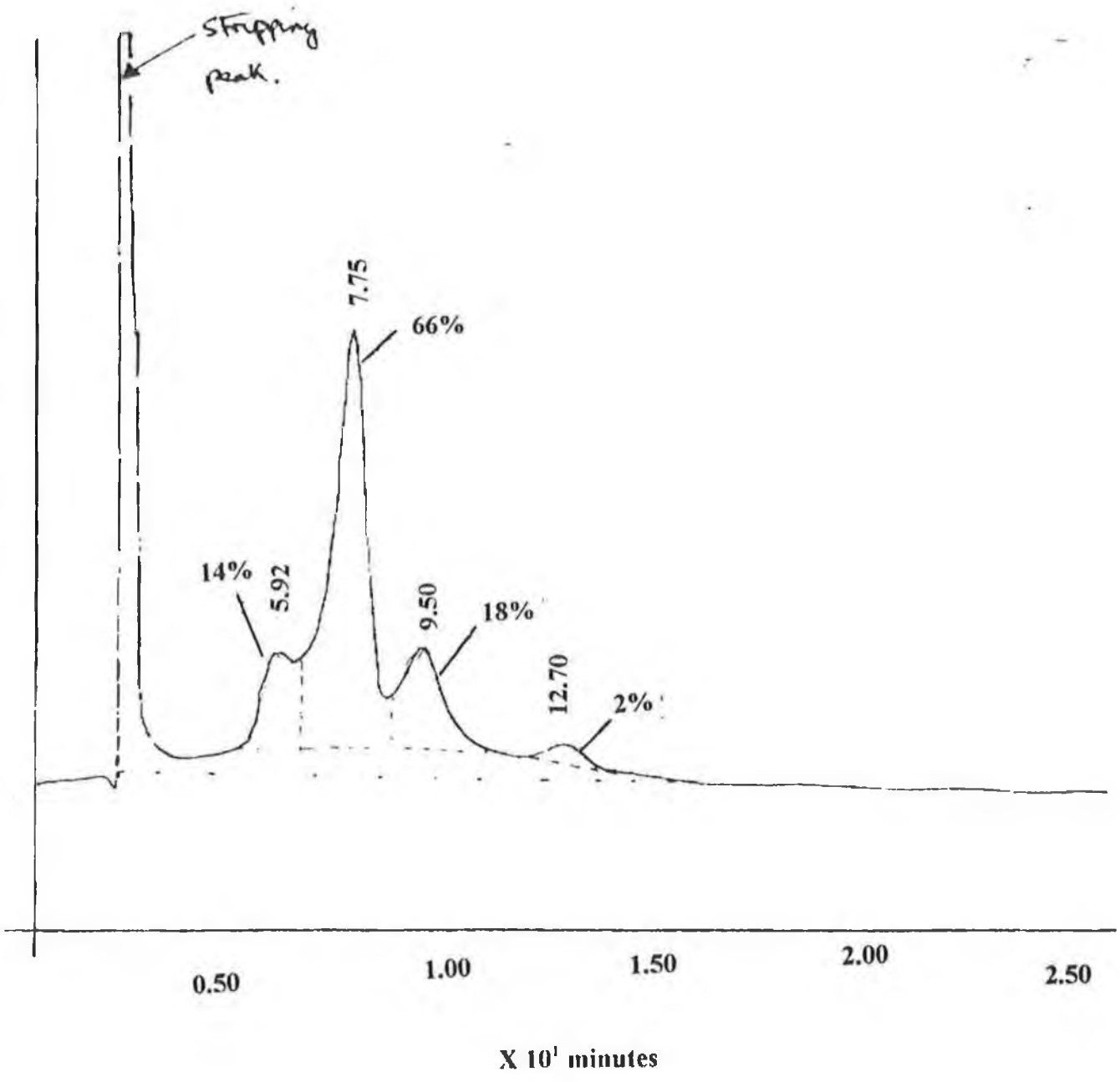


p. A, Chrom #2: PDA analysis of catechin & unknown.

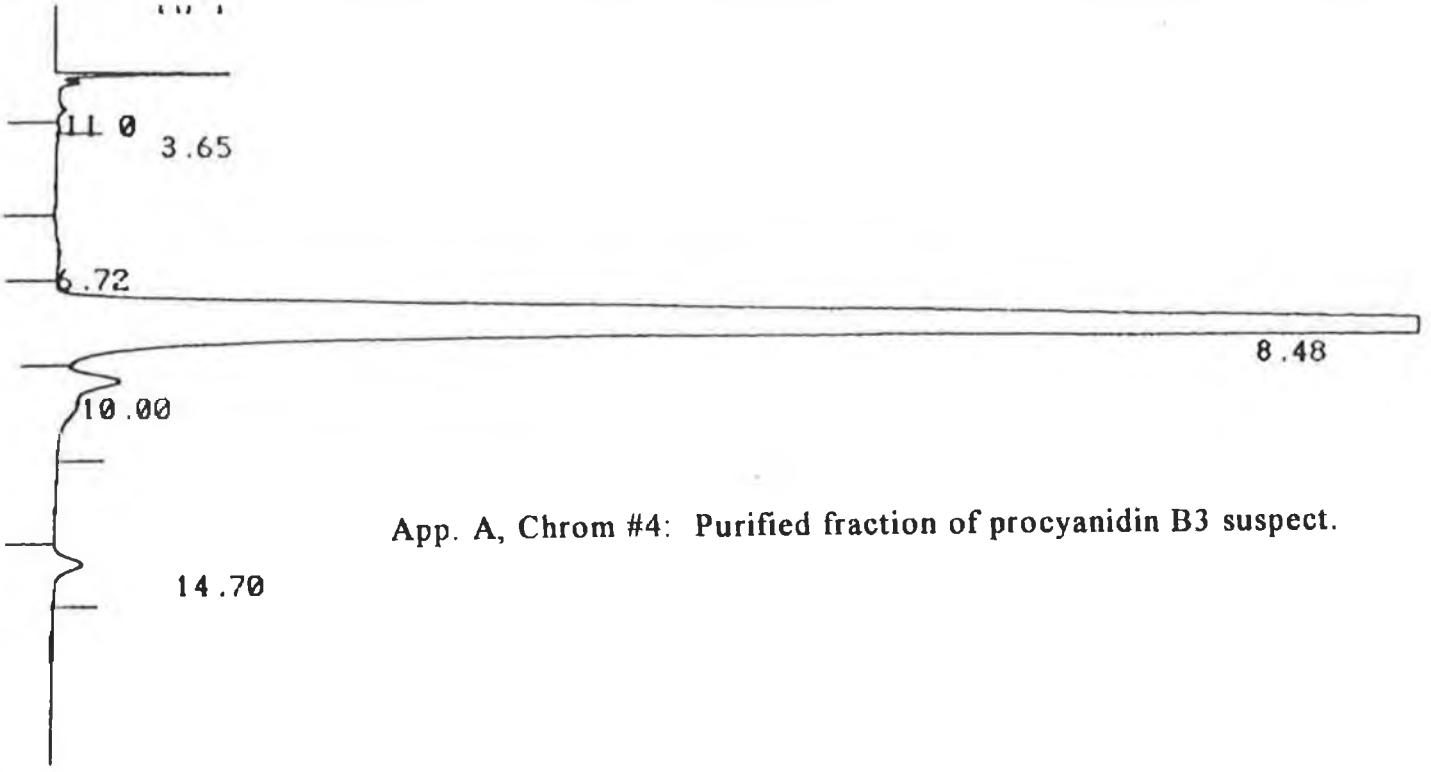


Waters 996

*** Three dimensional plot ***



App. A, Chrom #3: Guinness Group Research analysis of unknown.



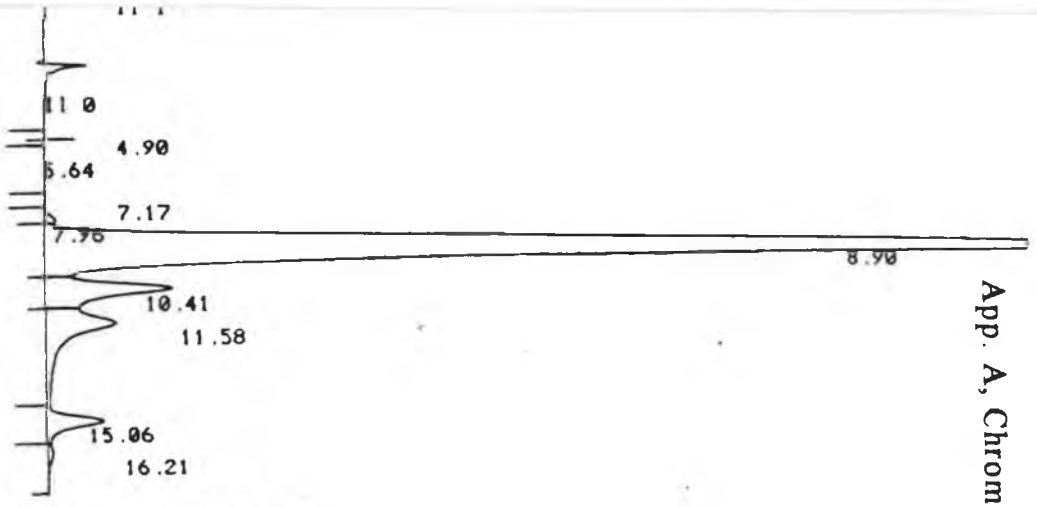
App. A, Chrom #4: Purified fraction of procyanidin B3 suspect.

DATA SAVED TO BIN # 23

CATHECIN 1,7 02/25/92 17:30:35 CH= "A" PS= 1.

FILE 1. METHOD 0. RUN 26 INDEX 26 BIN 23

PEAK#	AREA%	RT	AREA	BC
1	0.019	3.65	2690	01
2	0.244	6.72	33700	02
3	95.648	8.48	13203595	02
4	3.179	10.	438813	03
5	0.909	14.7	125513	01
TOTAL	100.		13804311	



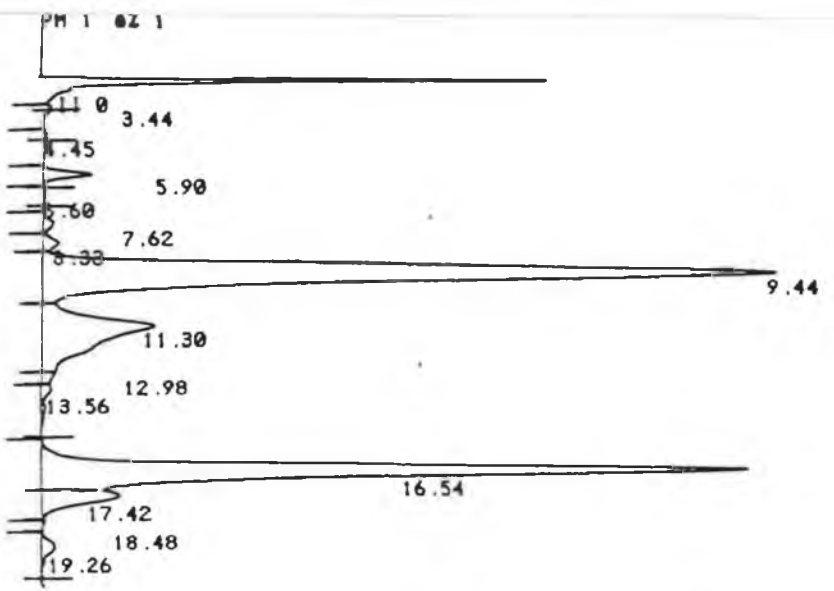
DATA SAVED TO BIN # 24

CATHECIN 1.7 03/20/92 11:04:52 CH= "A" PS= 1.

FILE 1. METHOD 0. RUN 25 INDEX 25 BIN 24

PEAK#	AREA%	RT	AREA	BC
1	0.008	4.9	1234	01
2	0.114	5.64	16960	02
3	0.059	7.17	8797	02
4	0.332	7.96	49613	02
5	82.706	8.9	12349935	02
6	7.153	10.41	1068153	02
7	6.484	11.58	968181	02
8	2.735	15.06	408365	02
9	0.409	16.21	61074	03
TOTAL	100.		14932312	

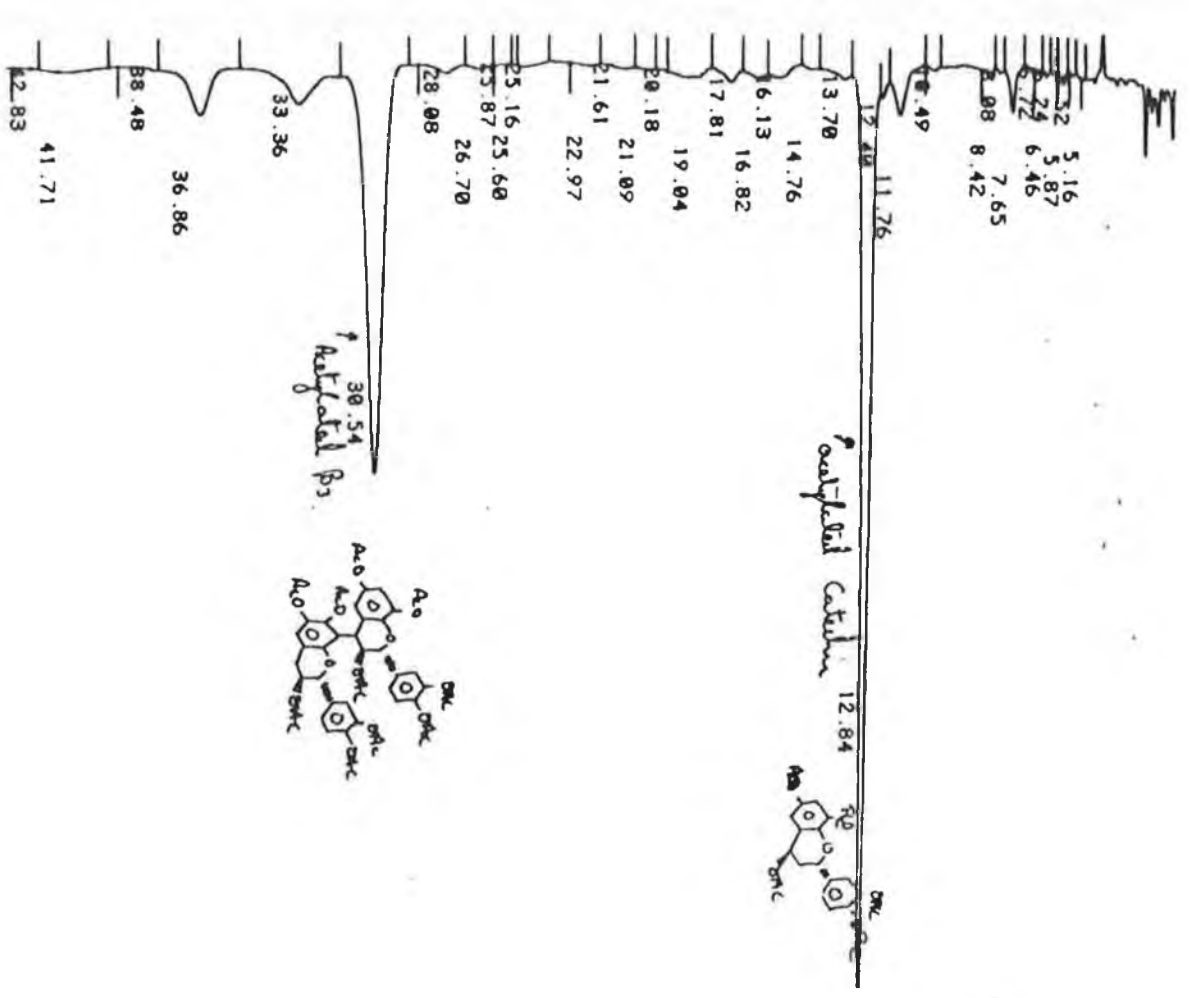
App. A, Chrom #5: Isolated product post 1st analysis & after ethyl acetate extraction.



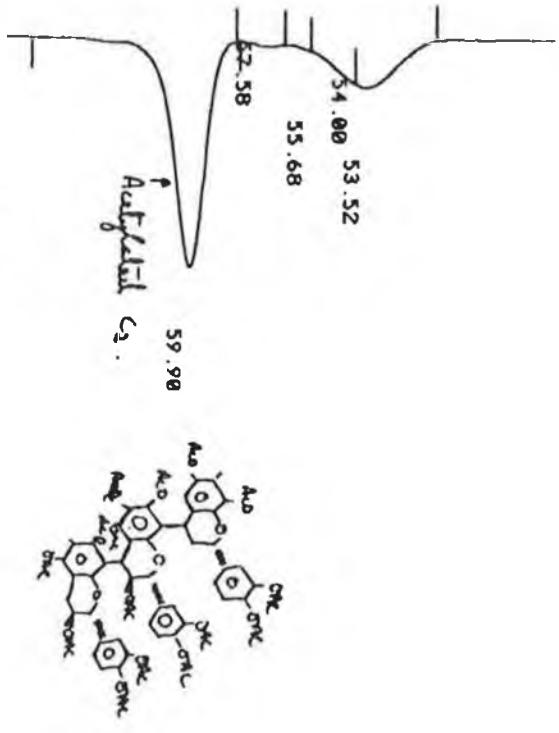
CATHECIN 1.7 03/27/92 18:37:11 CH= "A" PS= 1.

FILE 1. METHOD 0. RUN 2 INDEX 2 BIN 1

PEAK#	AREA%	RT	AREA	BC
1	0.032	3.44	1252	01
2	0.033	4.45	1318	01
3	1.162	5.9	46081	01
4	0.079	6.6	3134	01
5	0.346	7.62	13720	02
6	0.546	8.33	21652	02
7	46.634	9.44	1849957	02
8	11.33	11.3	449461	02
9	0.392	12.98	15539	02
10	0.571	13.56	22634	03
11	34.194	16.54	1356477	02
12	3.854	17.42	152894	02
13	0.084	18.48	3351	02
14	0.744	19.26	29519	03
TOTAL	100.		3966989	



App. A, Chrom #6: Acetylated products ex Fonknechten reaction.



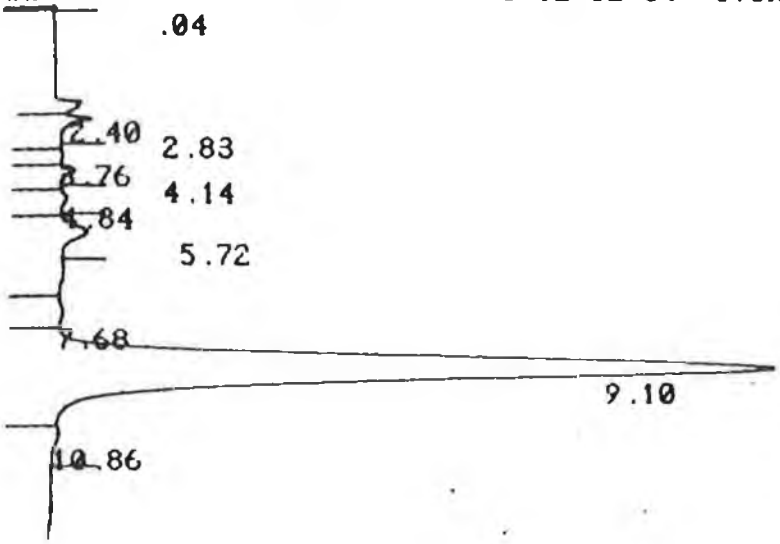
DATA SAVED TO BIN # 6

CAT 1.13 ACETYLATION 10/17/92 14:25:17 CH= "A" PS= 1.

FILE 1. METHOD 0. RUN 6 INDEX 6 BIN 6

PEAK#	AREAX	RT	AREA BC
1	0.008	5.16	1457 01
2	0.024	5.52	4389 01
3	0.139	5.87	25807 01
4	0.039	6.24	7300 02
5	0.058	6.46	10777 02
6	0.019	6.72	3563 03
7	0.873	7.65	162333 02
8	0.088	8.08	16330 02
9	0.1	8.42	18596 03
10	0.082	10.49	15238 01
11	1.926	11.76	358312 02
12	0.689	12.4	128090 02
13	24.67	12.84	4589165 02
14	0.763	13.7	142024 02
15	0.19	14.76	35434 02
16	0.806	16.13	149854 02
17	0.728	16.82	135438 02
18	0.85	17.81	158049 02
19	1.309	19.04	243473 02
20	0.168	20.18	31275 02
21	0.296	21.09	54970 02
22	0.352	21.61	65516 02
23	0.111	22.97	20731 03
24	0.214	25.16	39781 02

CHANNEL A INJECT 03/08/93 12:02:54 STORED TO BIN # 3

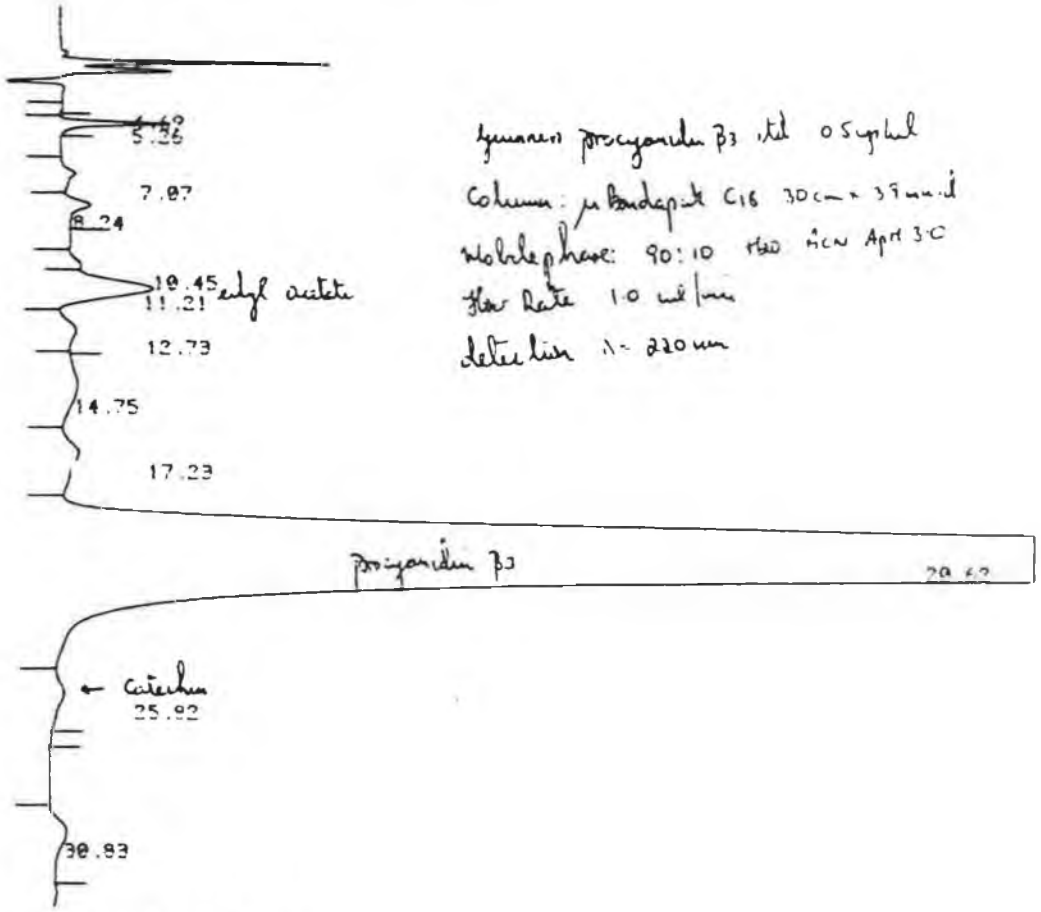


DATA SAVED TO BIN # 3 App. A, Chrom #7: Authentic procyanidin B3 peak marker.
(Guinness Group Research)

FREE PROCYANIDIN B3 03/08/93 12:02:54 CH= "A" PS= 1.

FILE 1. METHOD 0. RUN 3 INDEX 3 BIN 3

PEAK#	AREA%	RT	AREA	BC
1	0.023	0.04	1334	01
2	0.873	2.4	51517	02
3	1.199	2.83	70702	03
4	0.071	3.76	4183	02
5	0.421	4.14	24861	03
6	0.236	4.84	13946	01
7	1.764	5.72	104048	01
8	0.33	7.68	19462	02
9	94.541	9.1	5576494	02
10	0.542	10.86	31961	03
TOTAL	100.		5898508	



Synthesis procyanidin B3 std 0.5 g/ml
Column: µ Bondapak C18 30 cm × 3.9 mm id
Mobile phase: 90:10 H2O MeCN Apr 3 C
Flow Rate 1.0 ml/min
Detection λ = 220 nm

DATA SAVED TO BIN # 6

FREE B3 ASSAY 11/20/93 14:15:07 CH= "A" PS= 1.

FILE 1. METHOD 0. RUN 0 INDEX 0 BIN 6

PEAK#	AREA%	RT	AREA BC
1	0.006	4.69	4219 01
2	0.434	5.26	292180 01
3	0.111	7.07	74711 02
4	0.216	8.24	145501 03
5	0.131	10.45	88461 02
6	1.394	11.21	939651 02
7	0.193	12.73	129804 03
8	0.346	14.75	232915 02
9	0.472	17.23	317907 02
10	96.153	20.62	64794045 08
11	0.209	25.82	140942 05
12	0.335	30.83	225892 01

DATA SAVED TO BIN # 11

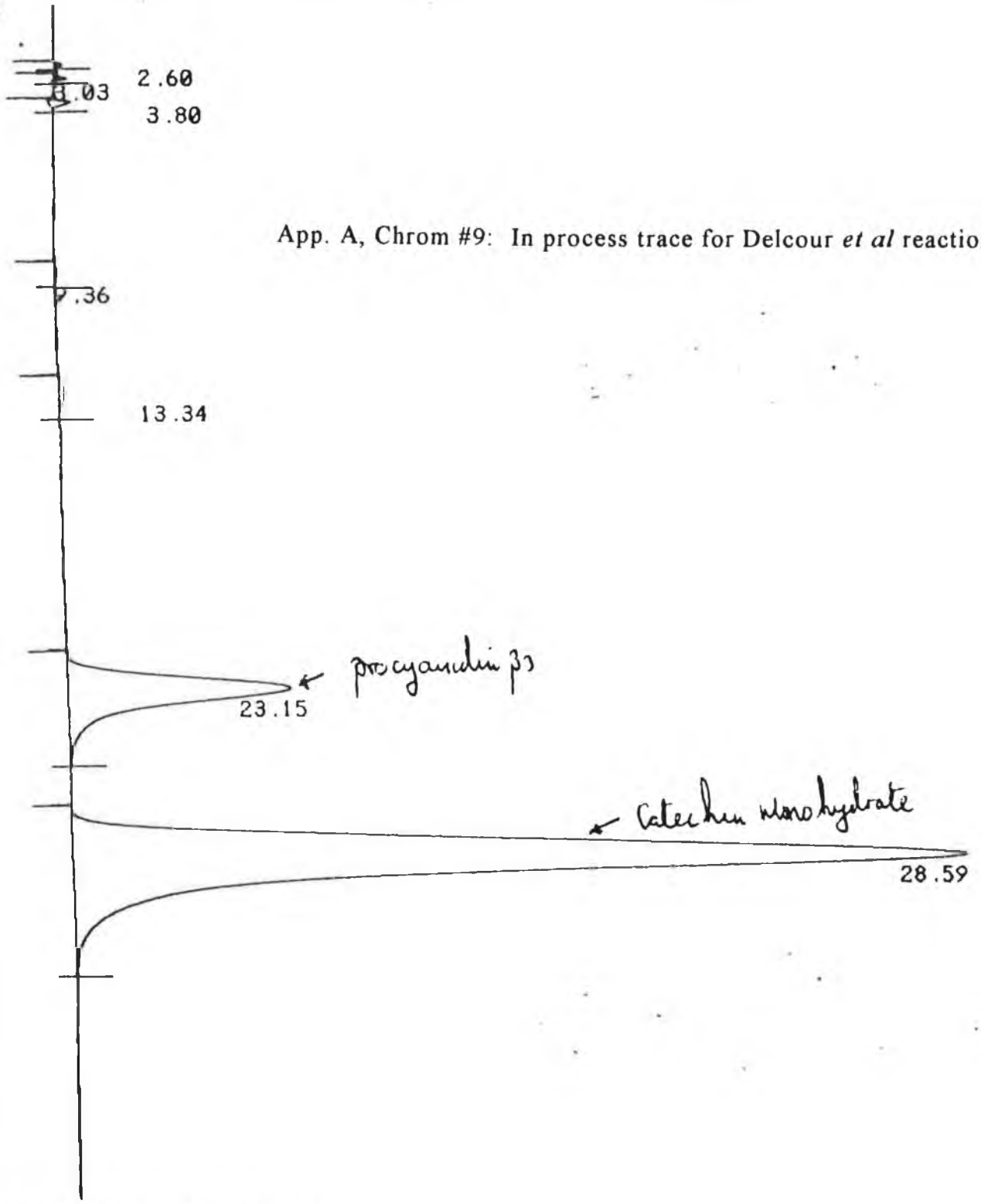
FREE B3 ASSAY CH= "A" PS= 1.

FILE 1. METHOD 0. RUN 11 BIN 11

PEAK#	AREA%	RT	AREA BC
1	1.392	2.66	23085 02
2	1.051	2.78	17432 02
3	1.073	2.96	17786 02
4	3.206	3.05	53167 02
5	0.309	3.28	5121 03
6	2.596	3.5	43046 03
7	1.963	6.82	32559 01
8	88.41	19.18	1466060 01
TOTAL	100.		1658256

System peaks subtracted

App. A, Chrom #8: Purified fraction of procyanidin B3 in free form, compared to authentic peak marker.



App. A, Chrom #9: In process trace for Delcour *et al* reaction.

DATA SAVED TO BIN # 1

DELCOUR FREE B3 01/08/94 12:50:58 CH= "A" PS= 1.

FILE 1. METHOD 0. RUN 1 INDEX 1 BIN 1

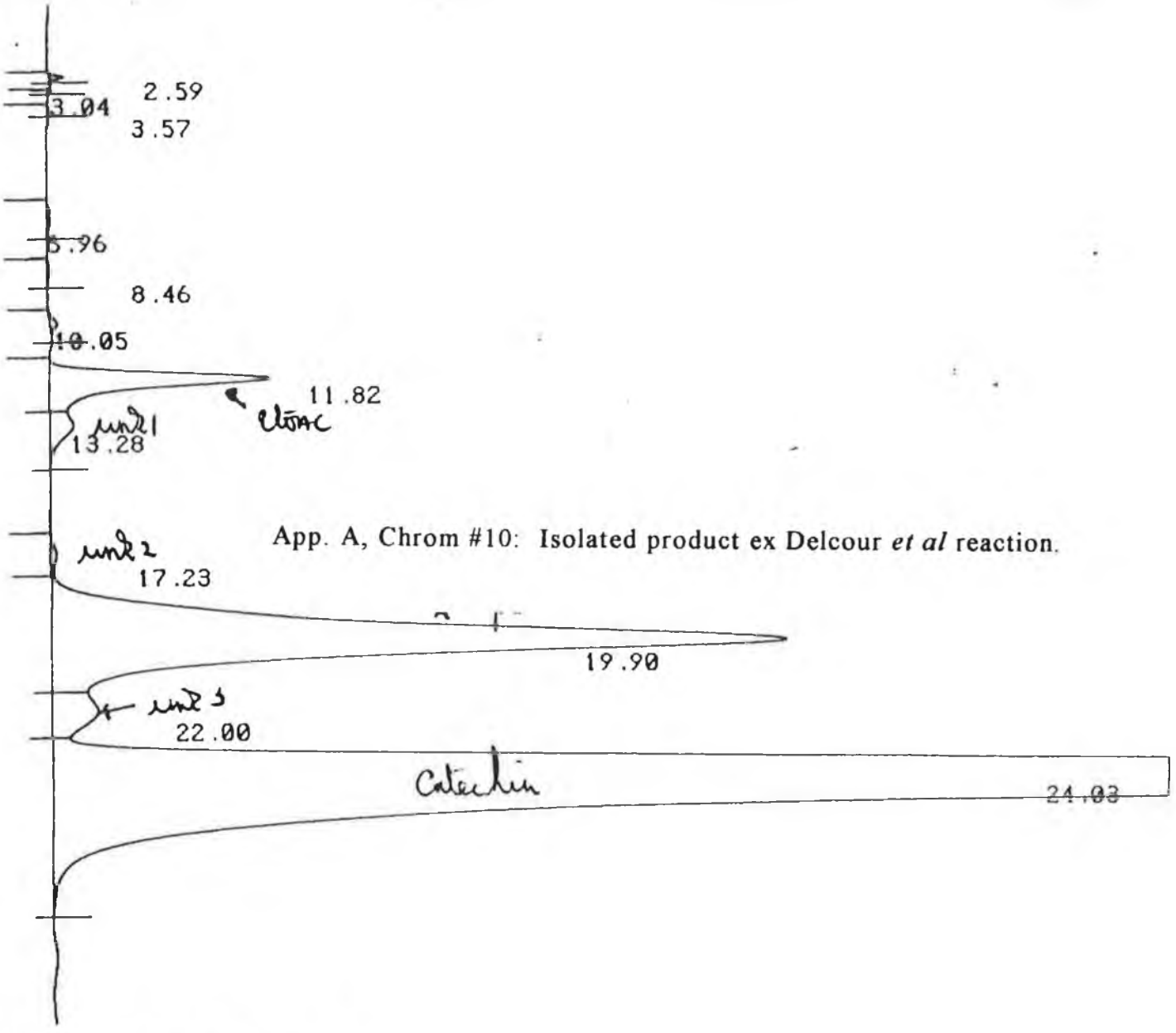
PEAK#	AREA%	RT	AREA	BC
1	0.055	2.6	10428	01
2	0.059	3.03	11070	01
3	0.289	3.8	54406	01
4	0.06	9.36	11393	01
5	0.255	13.34	48096	01
6	16.36	23.15	3080911	01
7	82.921	28.59	15615708	01
TOTAL	100.		18832012	

11

← B3 : 16.36%

catechin = $\frac{1505706}{5419672} \times 100 \times 0.35 = 10.1$ mg/ml

243 → 434.3 ml



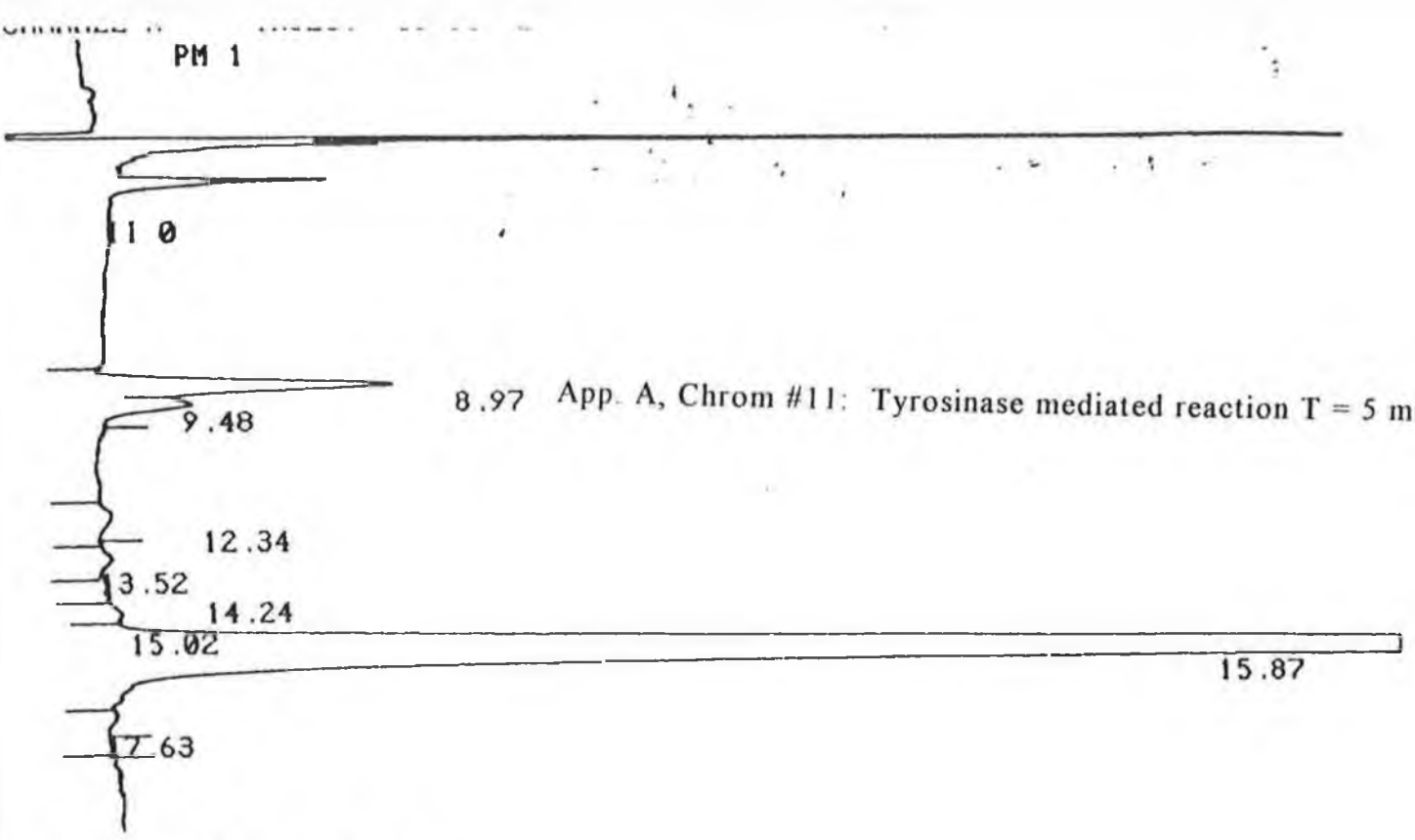
App. A, Chrom #10: Isolated product ex Delcour *et al* reaction.

DATA SAVED TO BIN # 4

DELCOUR FREE B3 01/10/94 17:20:58 CH= "A" PS= 1.

FILE 1. METHOD 0. RUN 4 INDEX 4 BIN 4

PEAK#	AREA%	RT	AREA	BC
1	0.033	2.59	21042	01
2	0.005	3.04	3145	01
3	0.005	3.57	3147	01
4	0.034	6.96	21721	01
5	0.021	8.46	13643	01
6	0.058	10.05	36776	01
7	2.48	11.82	1583865	02
8	0.404	13.28	258199	03
9	0.097	17.23	61897	02
10	16.464	19.9	10516001	02
11	1.034	22.	660443	02
12	79.366	24.03	50694678	03
TOTAL	100.		63874557	



App. A, Chrom #11: Tyrosinase mediated reaction T = 5 min

DATA SAVED TO BIN # 3

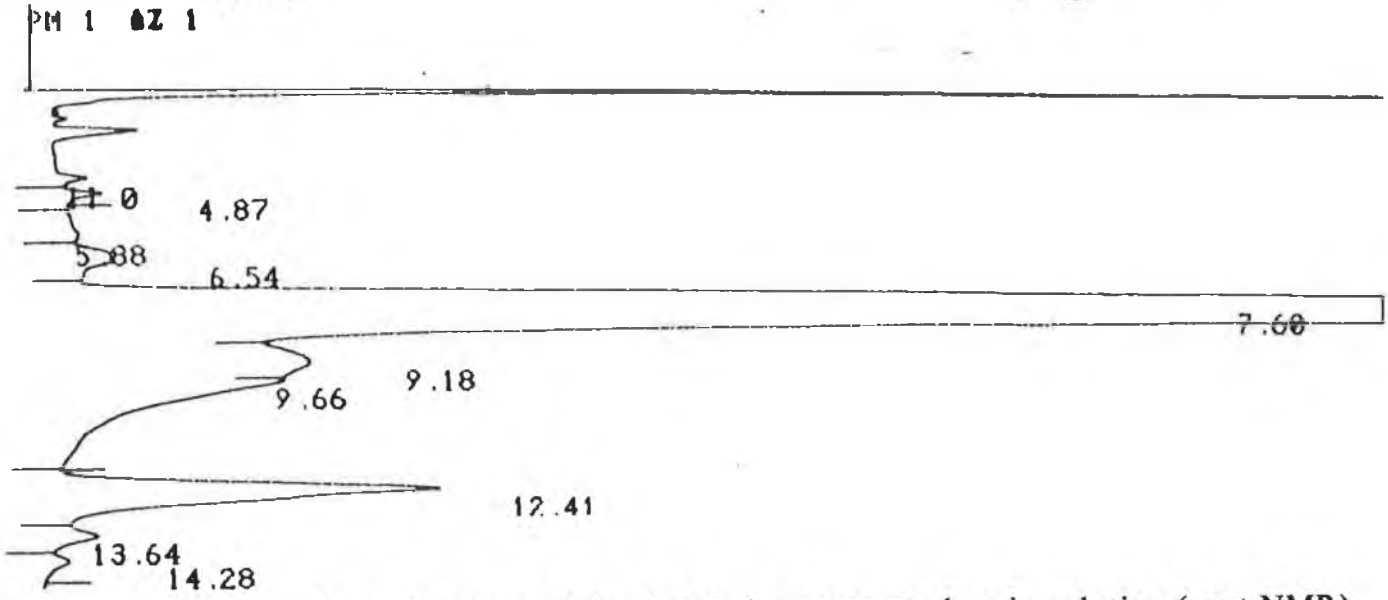
INPUT OVERRANGE AT RT= 2.58

ENZYME CAT 1,3 05/15/92 16:29:06 CH= "A" PS= 1.

FILE 1. METHOD 0. RUN 3 INDEX 3 BIN 3

PEAK#	AREA%	RT	AREA BC
1	6.414	8.97	275885 02
2	1.943	9.48	83580 03
3	0.299	12.34	12870 01
4	0.311	13.52	13362 02
5	0.241	14.24	10384 02
6	0.536	15.02	23034 02
7	90.181	15.87	3878704 08
8	0.075	17.63	3207 05

TOTAL 100. 4301026

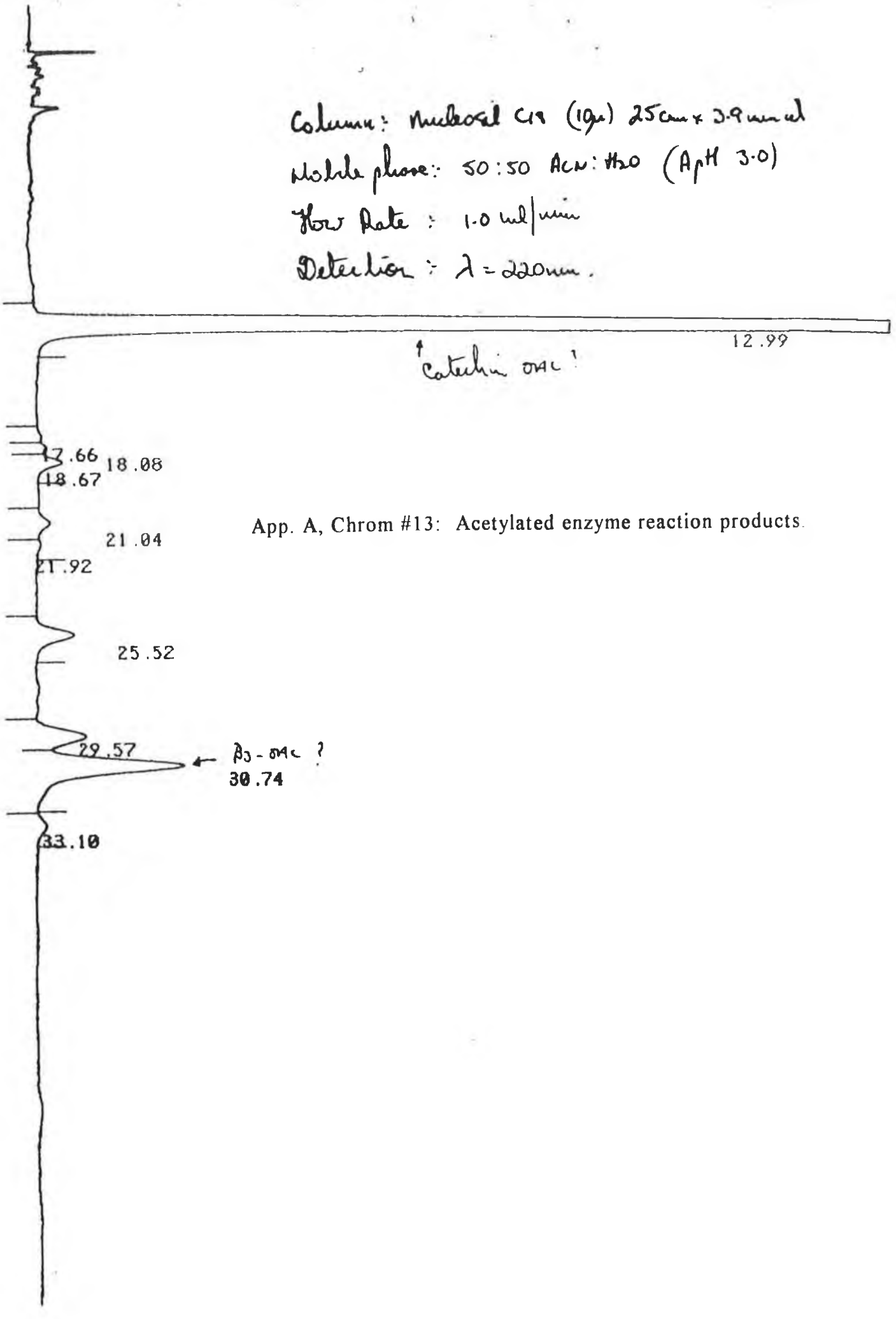


App. A, Chrom #12: Isolated enzyme product in solution (post NMR).

ECAT 1,6 PREP2 06/13/92 16:20:27 CH= "A" PS= 1.

FILE 1. METHOD 0. RUN 30 INDEX 30 BIN 27

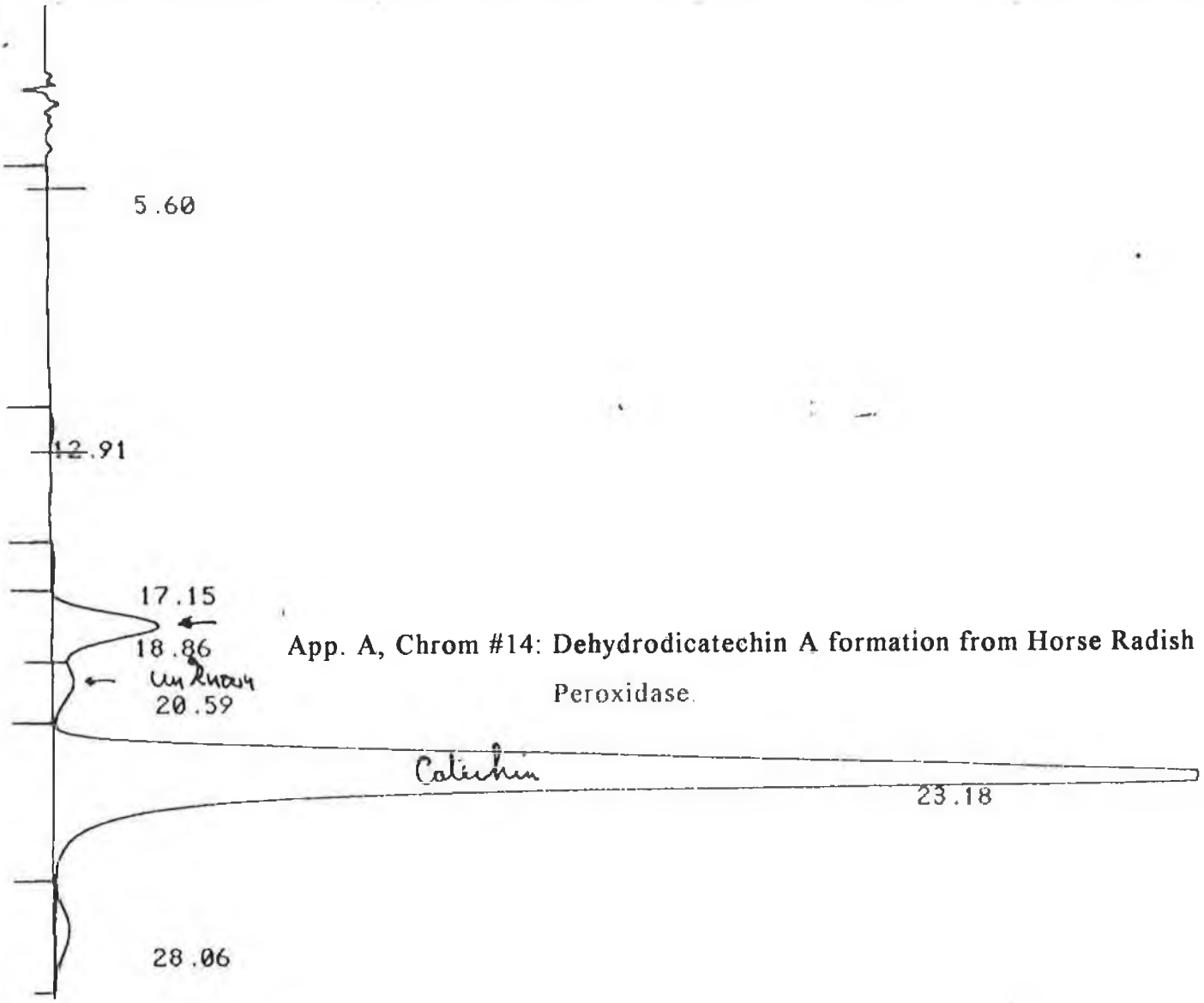
PEAK#	AREA%	RT	AREA	BC
1	0.142	4.87	58165	01
2	0.147	5.88	60378	02
3	0.723	6.54	297077	02
4	84.	7.6	34497279	02
5	5.345	9.18	2194915	02
6	3.879	9.66	1593225	03
7	5.133	12.41	2107912	02
8	0.447	13.64	183462	02
9	0.184	14.28	75582	03
TOTAL	100.		41067995	



App. A, Chrom #13: Acetylated enzyme reaction products.

ECAT ACETYLATION 10/21/92 16:43:46 CH= "A" PS= 1.

RUN 2 INDEX 2 BIN 1

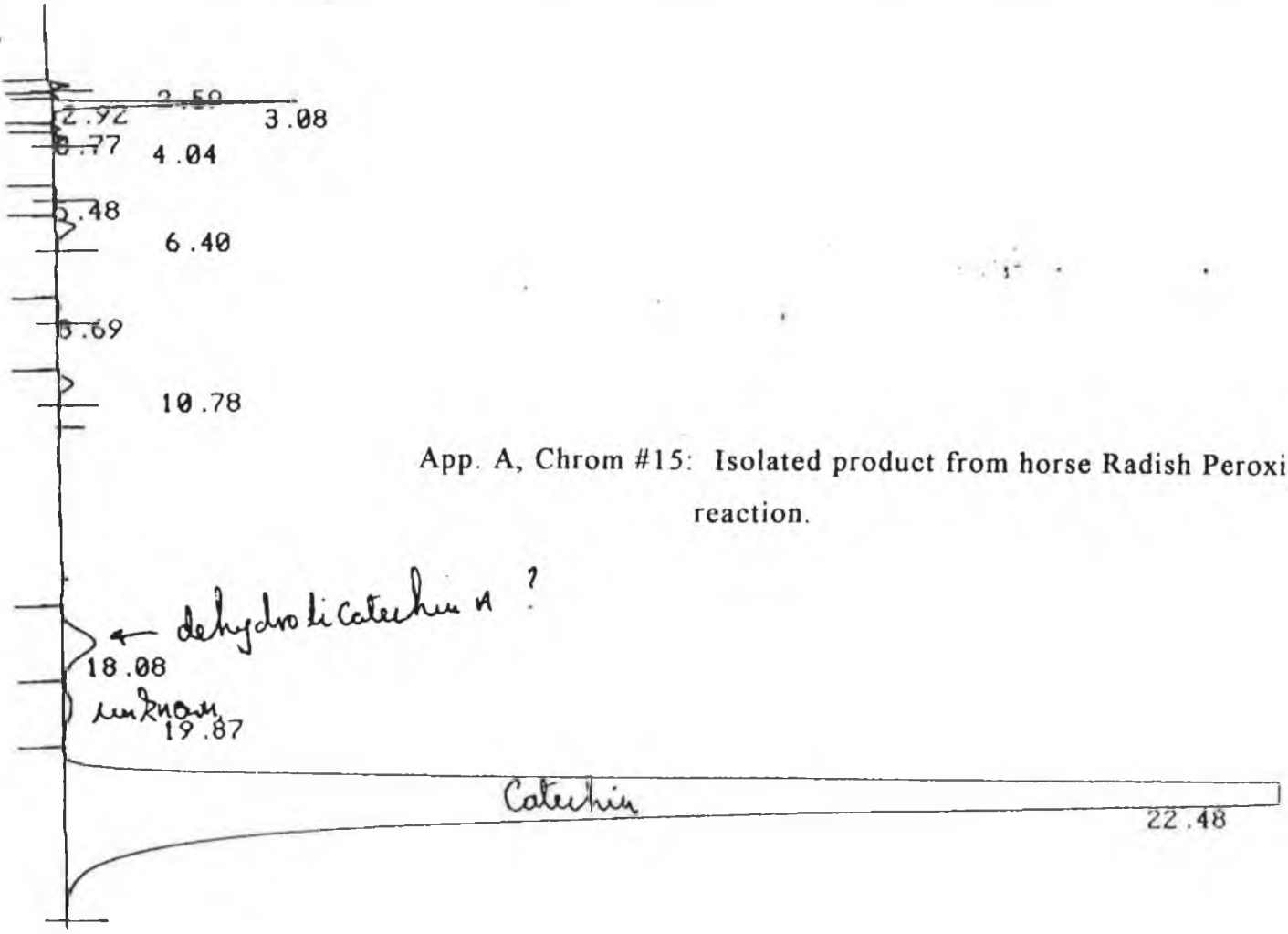


App. A, Chrom #14: Dehydrodicatechin A formation from Horse Radish Peroxidase.

DEHYDRODICATECHIN A ASSAY 02/04/94 16:59:27 CH= "A" PS= 1.

FILE 1. METHOD 0. RUN 2 INDEX 2 BIN 1

PEAK#	AREA%	RT	AREA	BC
1	0.018	5.6	3377	01
2	0.166	12.91	31148	01
3	0.182	17.15	34143	02
4	6.892	18.86	1294396	02
5	1.722	20.59	323503	02
6	89.613	23.18	16831447	08
7	1.408	28.06	264382	05
TOTAL	100.		18782396	



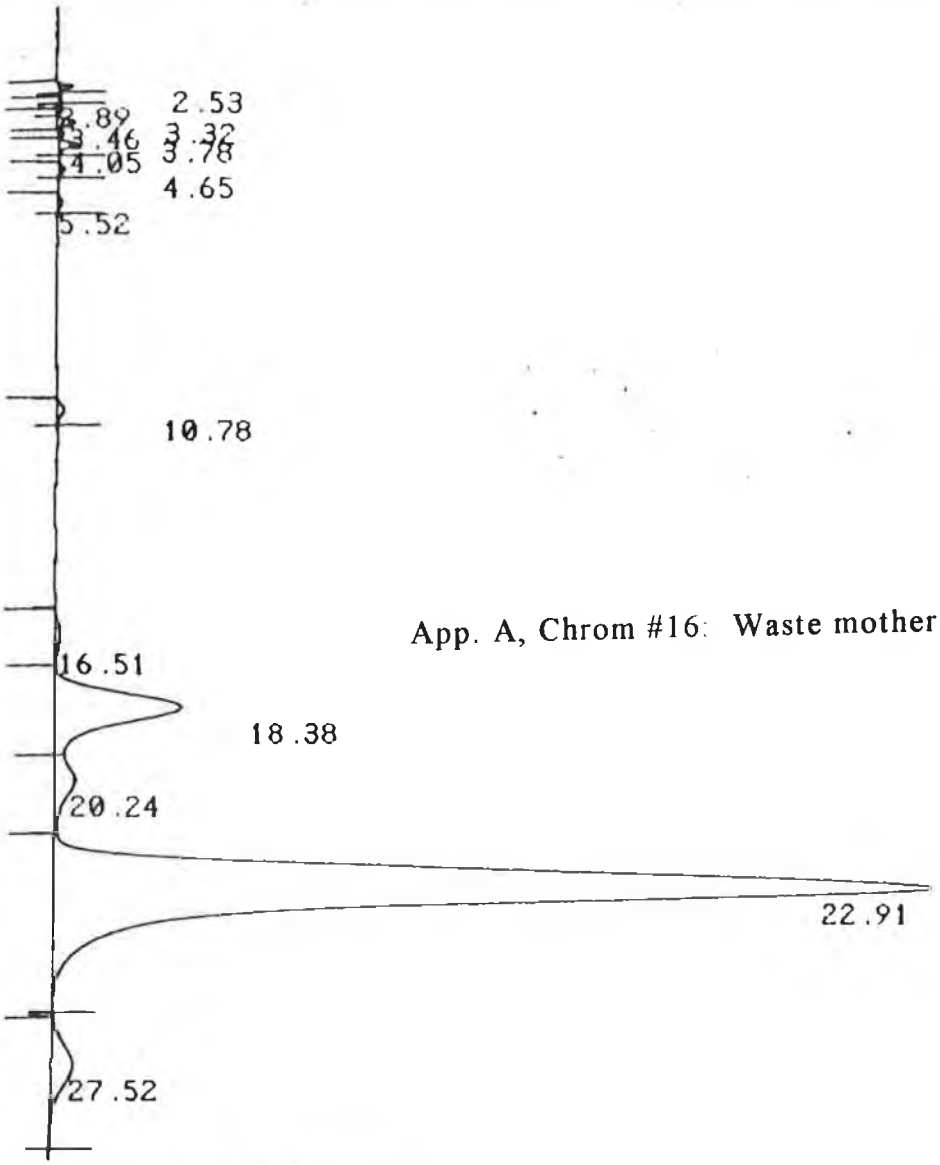
App. A, Chrom #15: Isolated product from horse Radish Peroxida reaction.

DATA SAVED TO BIN # 1

DEHYDRODICATECHIN A ASSAY 02/18/94 16:15:58 CH= "A" PS= 1.

FILE 1. METHOD 0. RUN 1 INDEX 1 BIN 1

PEAK#	AREA%	RT	AREA	BC
1	0.124	2.59	25799	01
2	0.022	2.92	4530	02
3	1.775	3.08	369090	08
4	0.054	3.77	11229	06
5	0.101	4.04	21081	07
6	0.029	5.48	6004	01
7	0.398	6.4	82827	01
8	0.069	8.69	14408	01
9	0.285	10.78	59238	01
10	1.546	18.08	321357	02
11	0.427	19.87	88709	02
12	95.169	22.48	19784892	03
TOTAL	100.		20789164	



App. A, Chrom #16: Waste mother liquor post ethyl acetate extractio

DATA SAVED TO BIN # 2

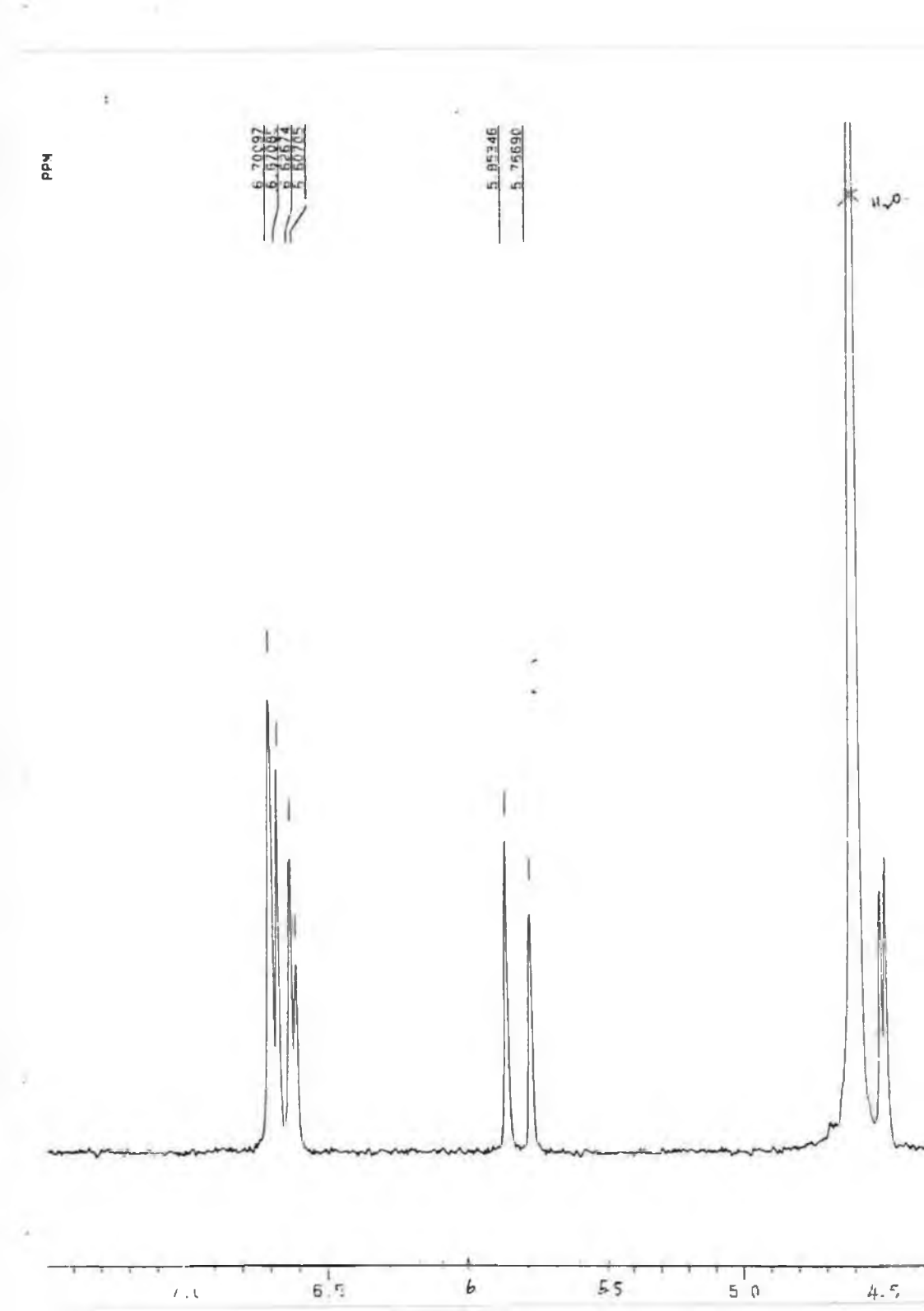
DEHYDRODICATECHIN A ASSAY 02/18/94 16:47:24 CH= "A" PS= 1.

FILE 1. METHOD 0. RUN 2 INDEX 2 BIN 2

PEAK#	AREA%	RT	AREA	BC
1	0.156	2.53	17522	01
2	0.008	2.89	861	01
3	0.028	3.32	3166	02
4	0.326	3.46	36680	02
5	0.085	3.78	9521	02
6	0.312	4.05	35190	03
7	0.095	4.65	10702	01
8	0.092	5.52	10386	01
9	0.212	10.78	23839	01
10	0.466	16.51	52454	02
11	10.774	18.38	1213678	02
12	2.185	20.24	246118	02
13	82.741	22.91	9321097	03
14	2.522	27.52	284125	01

TOTAL 100. 11265339

APPENDIX #B: 400 mHz NMR SPECTRA.


PPM
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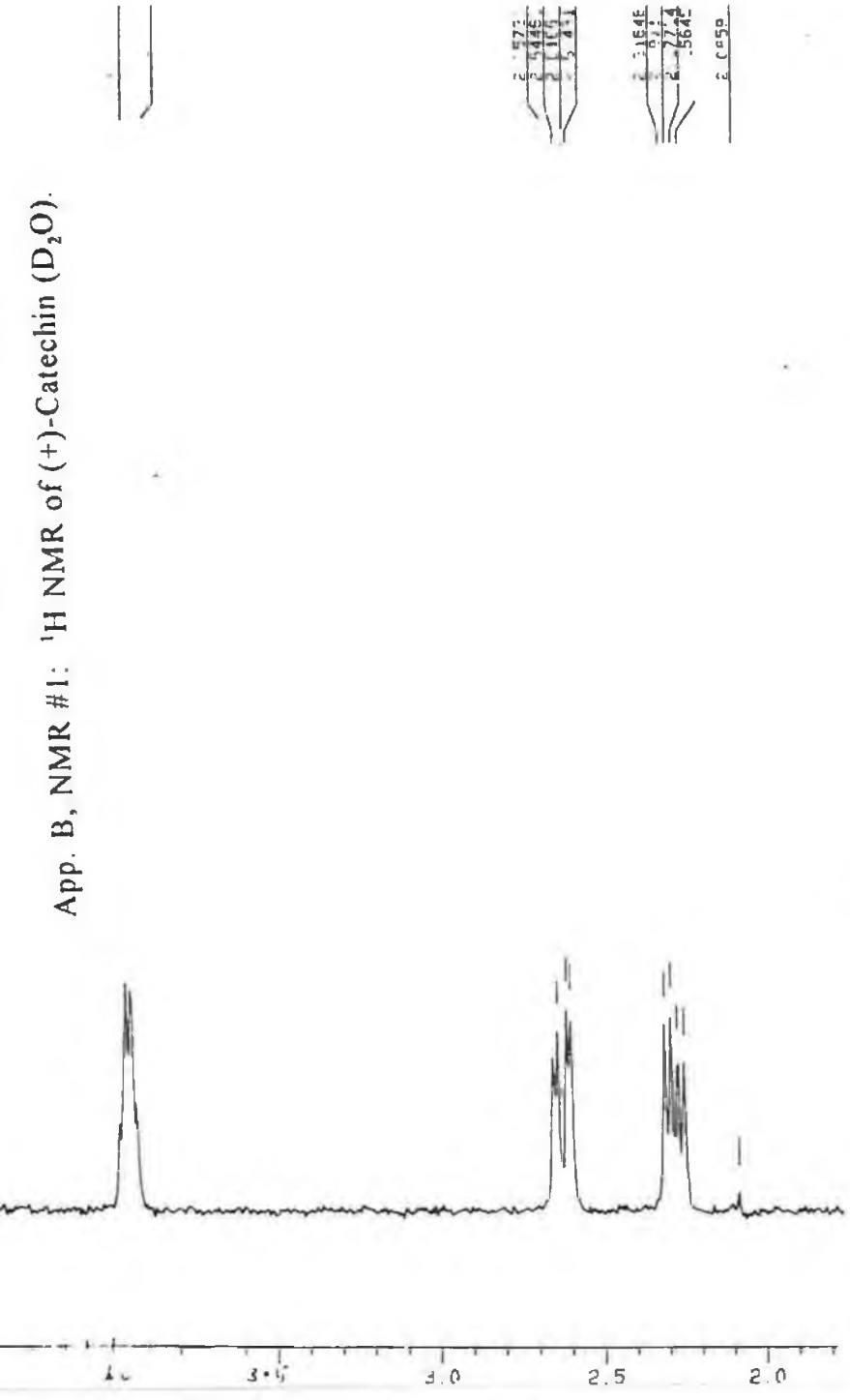
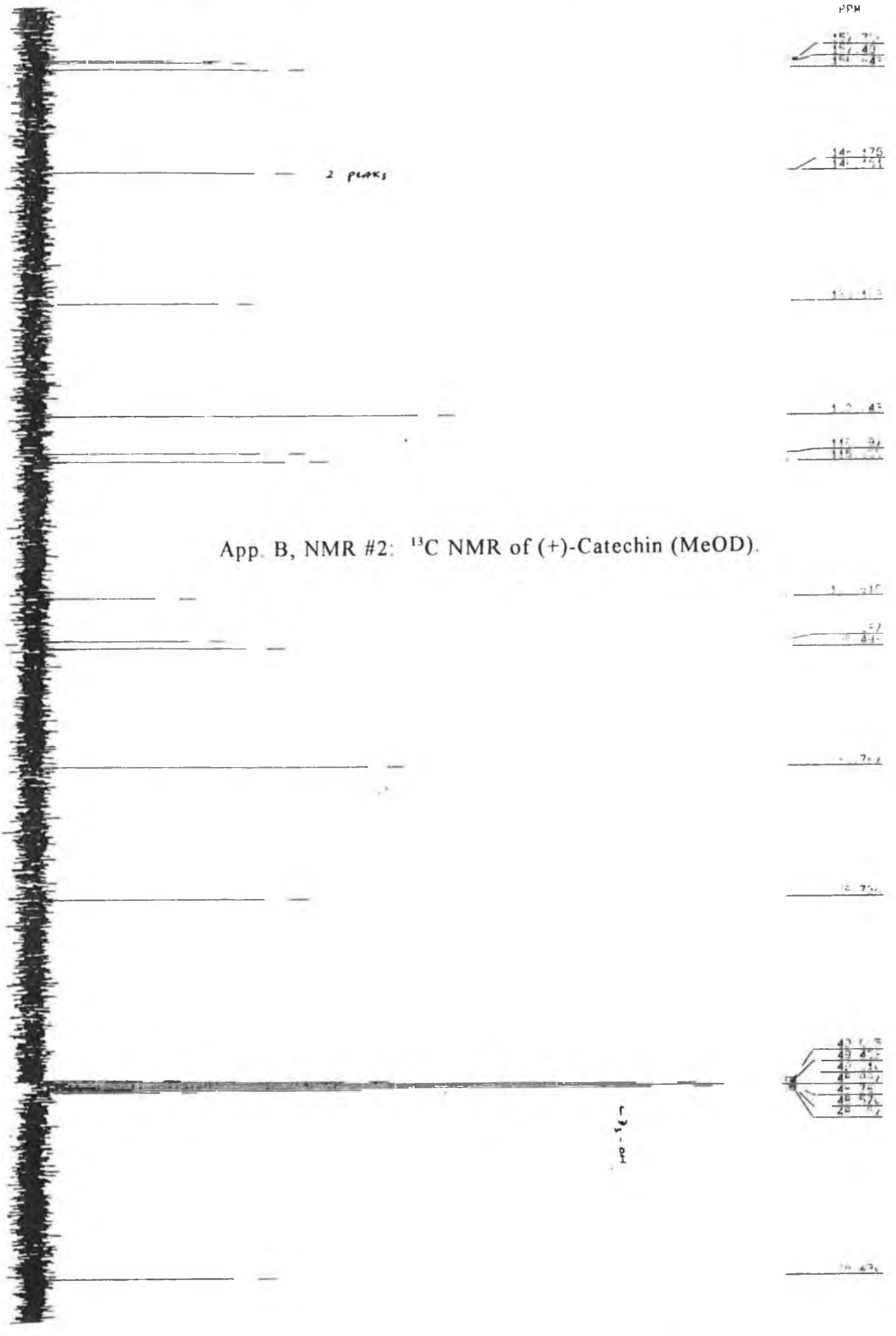
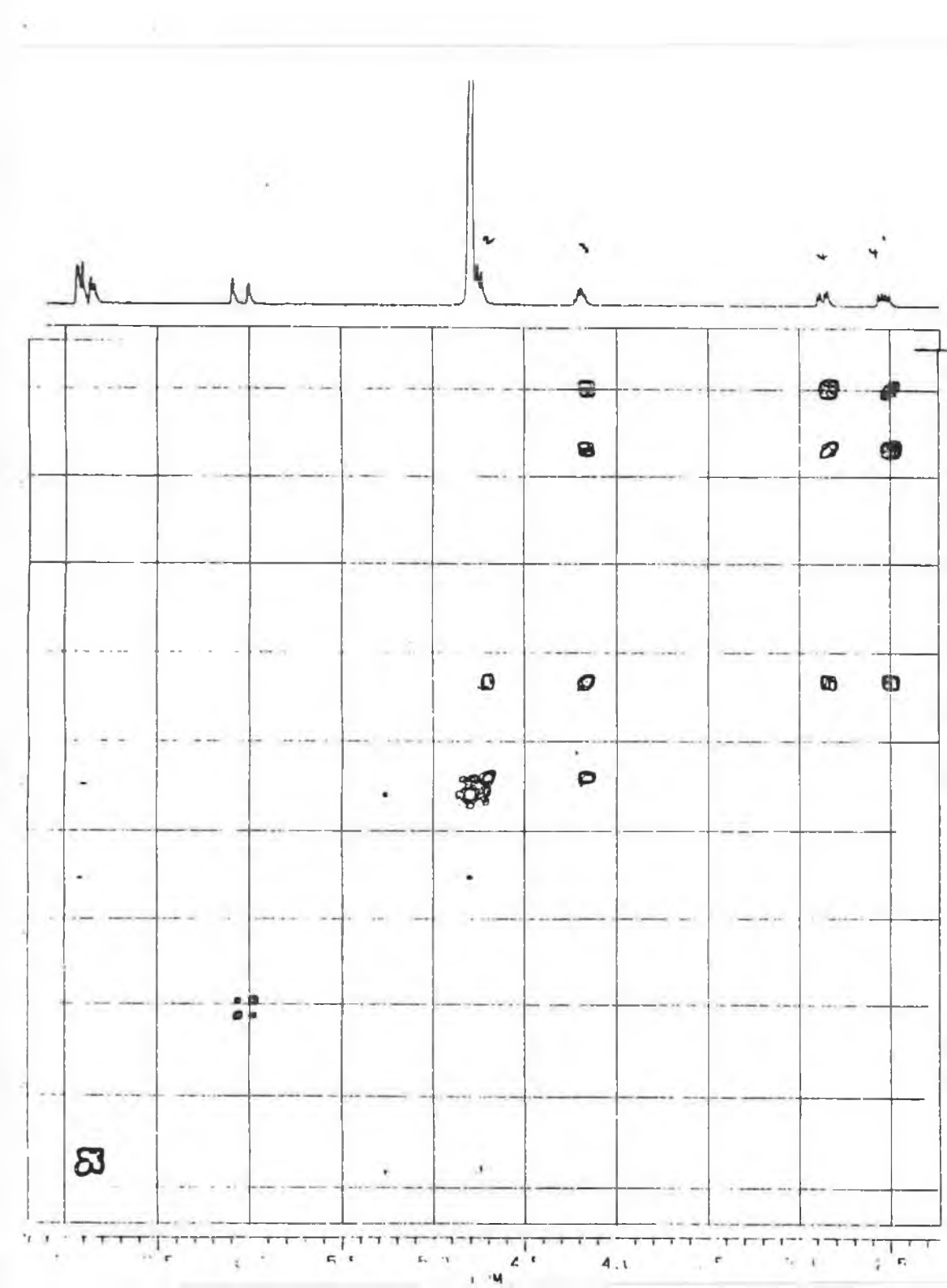
6.67085
6.62574
6.59765
5.85346
5.75690
1.00
6.5
6
5.5
5.0
4.5

App. B, NMR #1: ^{1}H NMR of (+)-Catechin (D_2O).





App. B, NMR #2: ^{13}C NMR of (+)-Catechin (MeOD).



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AU PROG.
  ZG7.AU
DATE 27-11-91

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SI1    512
SW2  1992.032
SW1   996.016
ND0      1


WDW2     S
WDW1     S
SSB2     0
SSB1     0
MC2      M
PLIM ROW.
F1       7.198P
F2       2.229P
AND COLUMN.
F1       7.198P
F2       2.229P

D1      .8700000
P1        9.00
RGA
RD    0.0
PW          0.0
DE        316.30
NS             4
DS             1
D0      0.000030
P3         4.50
NE          128
.N      .0005000
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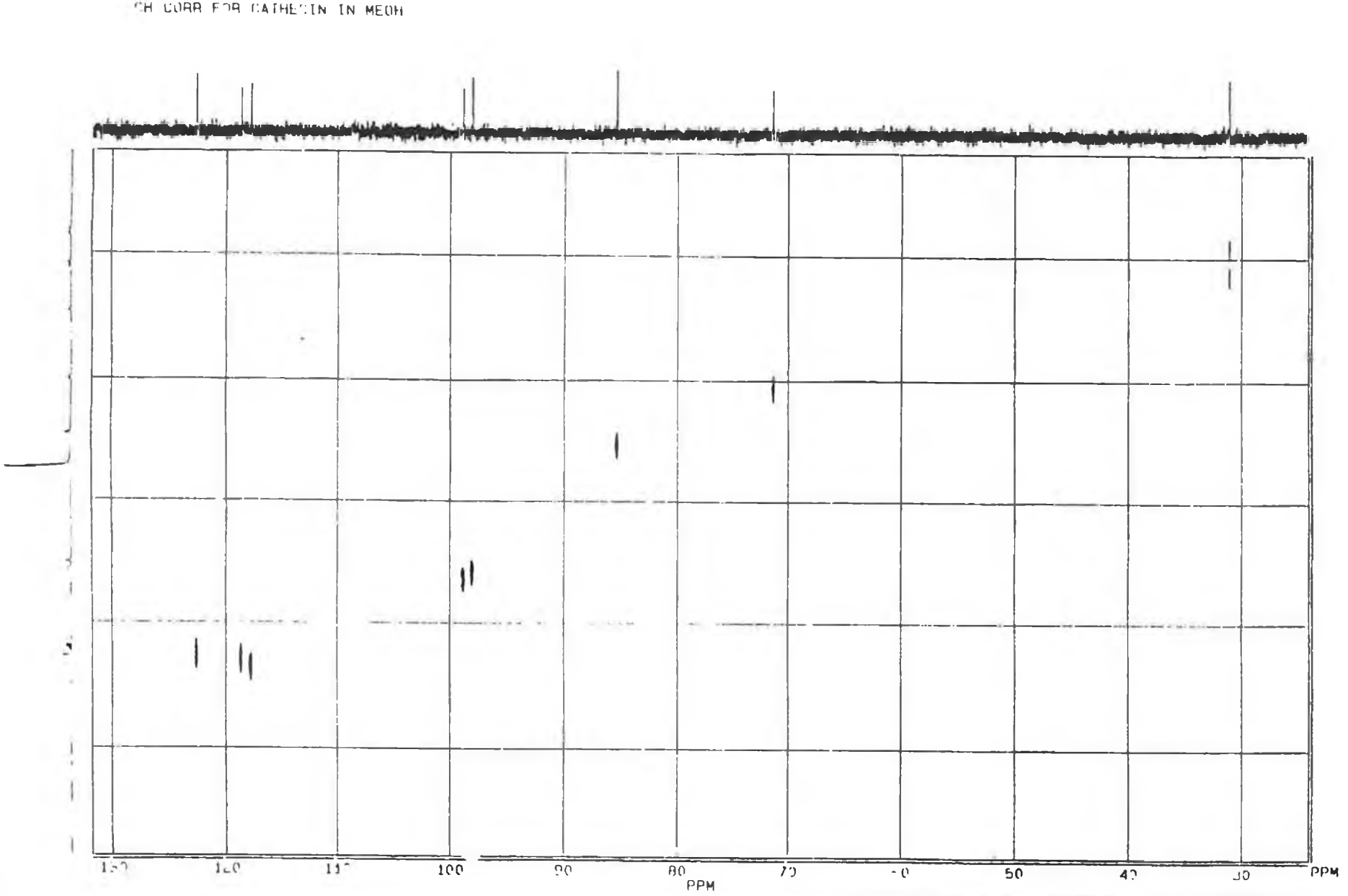
App. B, NMR #3: 2D-COSY of (+)-Catechin.



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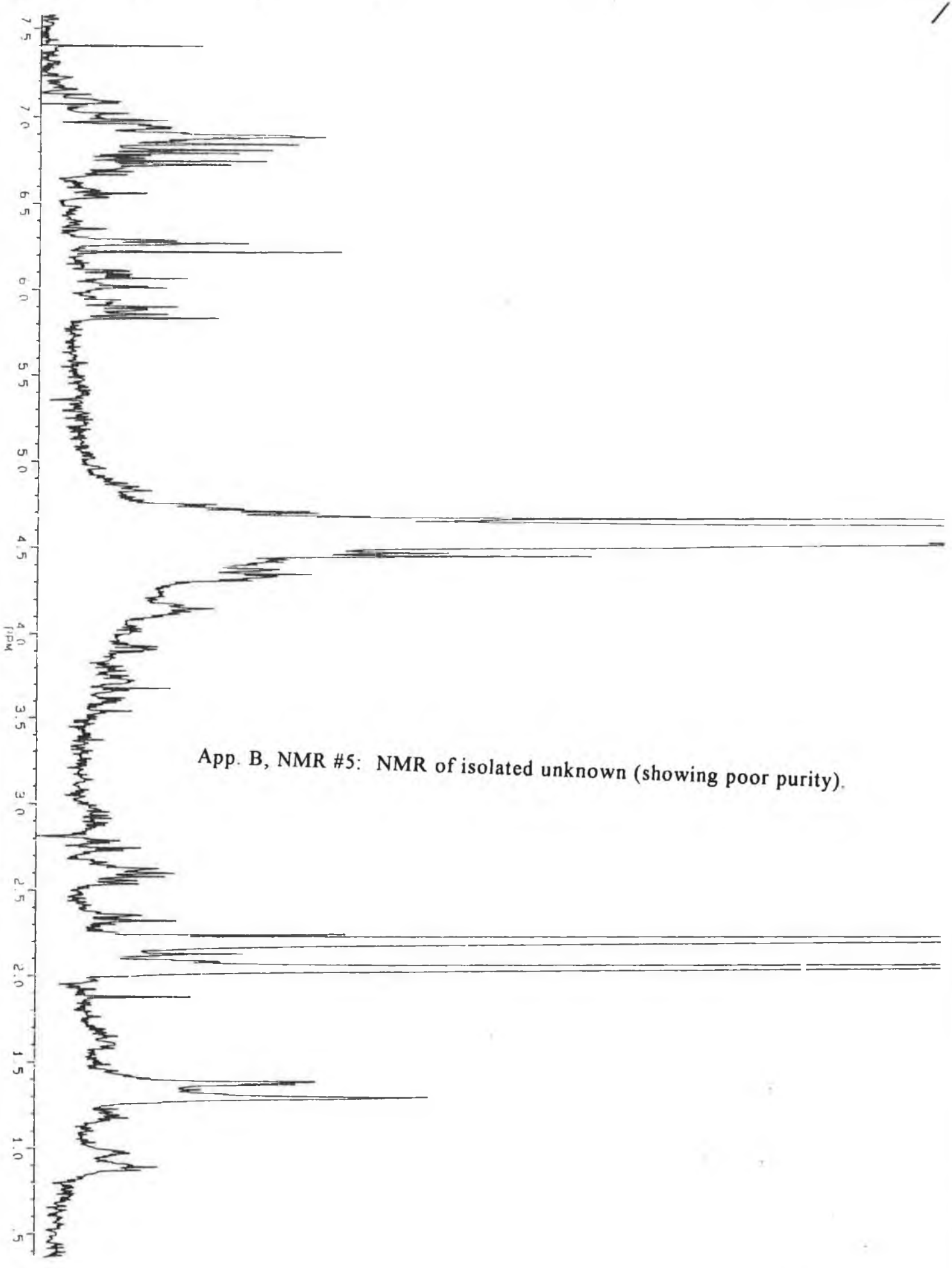
PROJH1.001
F2 PROJ.
PROJX.001
AU PROG
XHCORR AU
DATE 30-11

SI2 4096
SI1 256
SW2 18518.5
SW1 3787.8
ND0 2

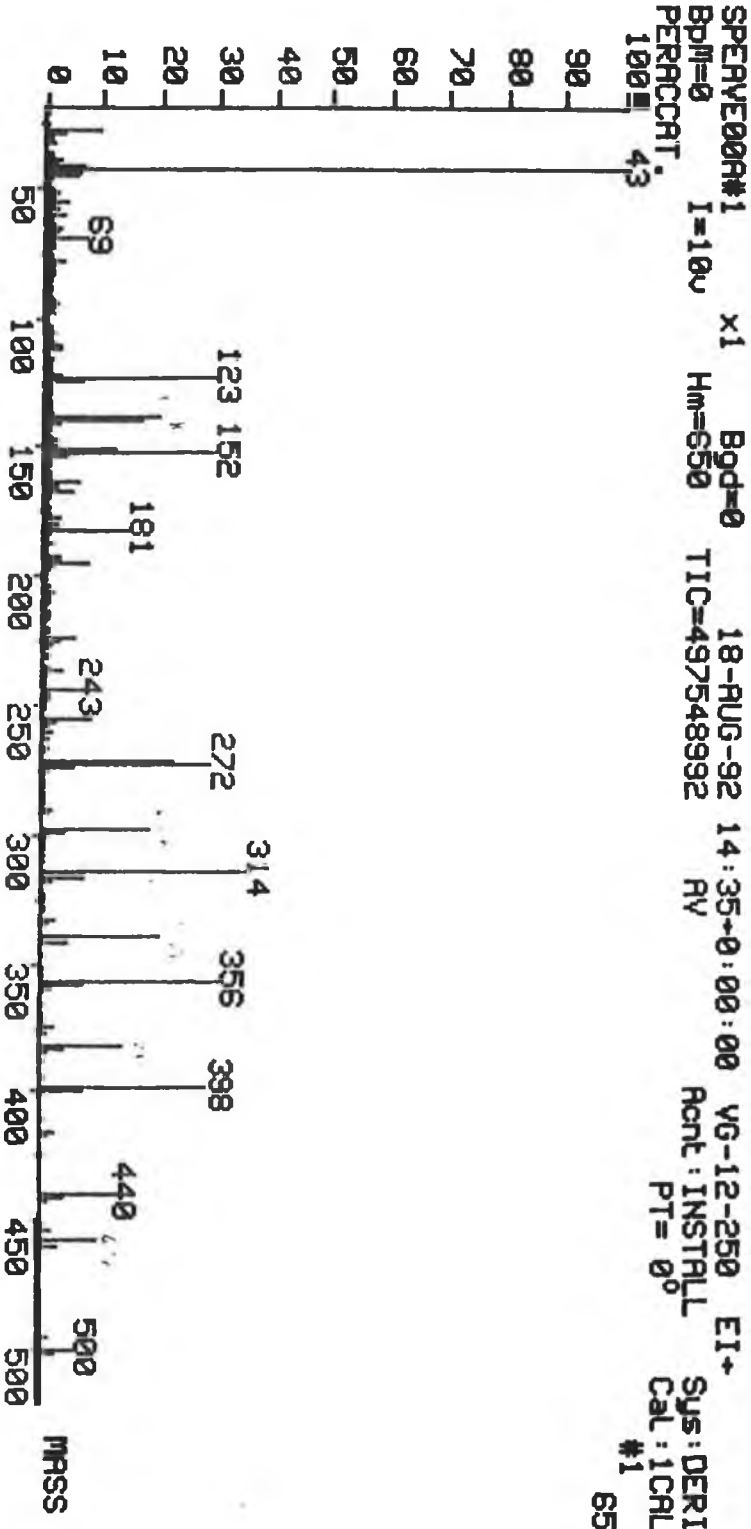
WDW2 G
WDW1 S
LB2 2.0
GB2 0.0
GOB1 10
MC2 M
PLIM ROW.
F1 131.6
F2 24.0
AND COLUMN.
F1 64.3
F2 5.77

D1 2.00000
S1 DH
P1 9
D0 0000
P4 11
D6 00345
P2 5.5
D4 .00172
S2 3DH
BD 0.0
PW 9
DE 30.
NS 8
DS 1
P9 95.
NE 128
IN .00006

App. B, NMR #4: C-H Correlation of (+)-Catechin.

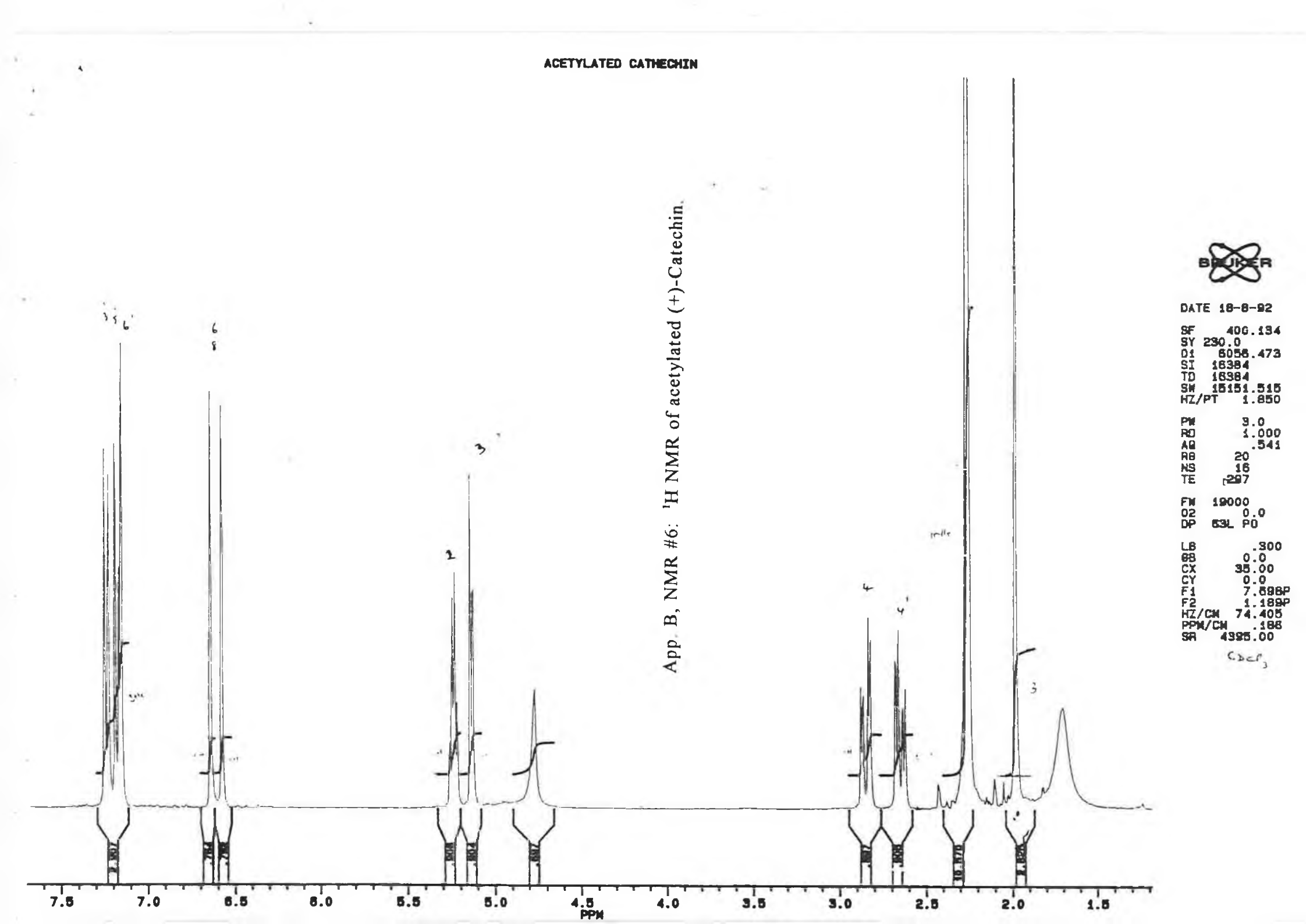


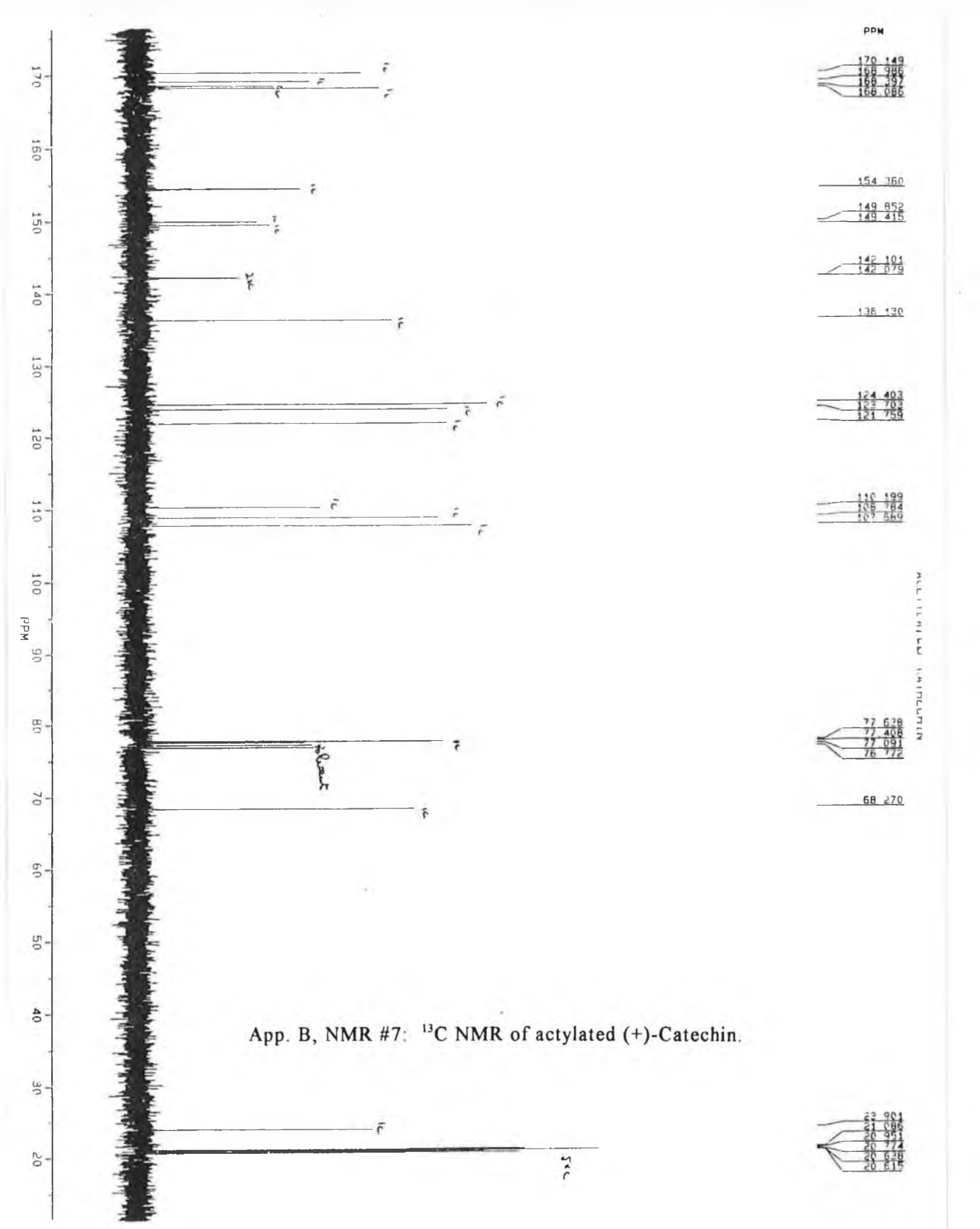
App. B, NMR #5: NMR of isolated unknown (showing poor purity).



App. B, Spec #1: Mass spectrum of acetylated (+)-Catechin.

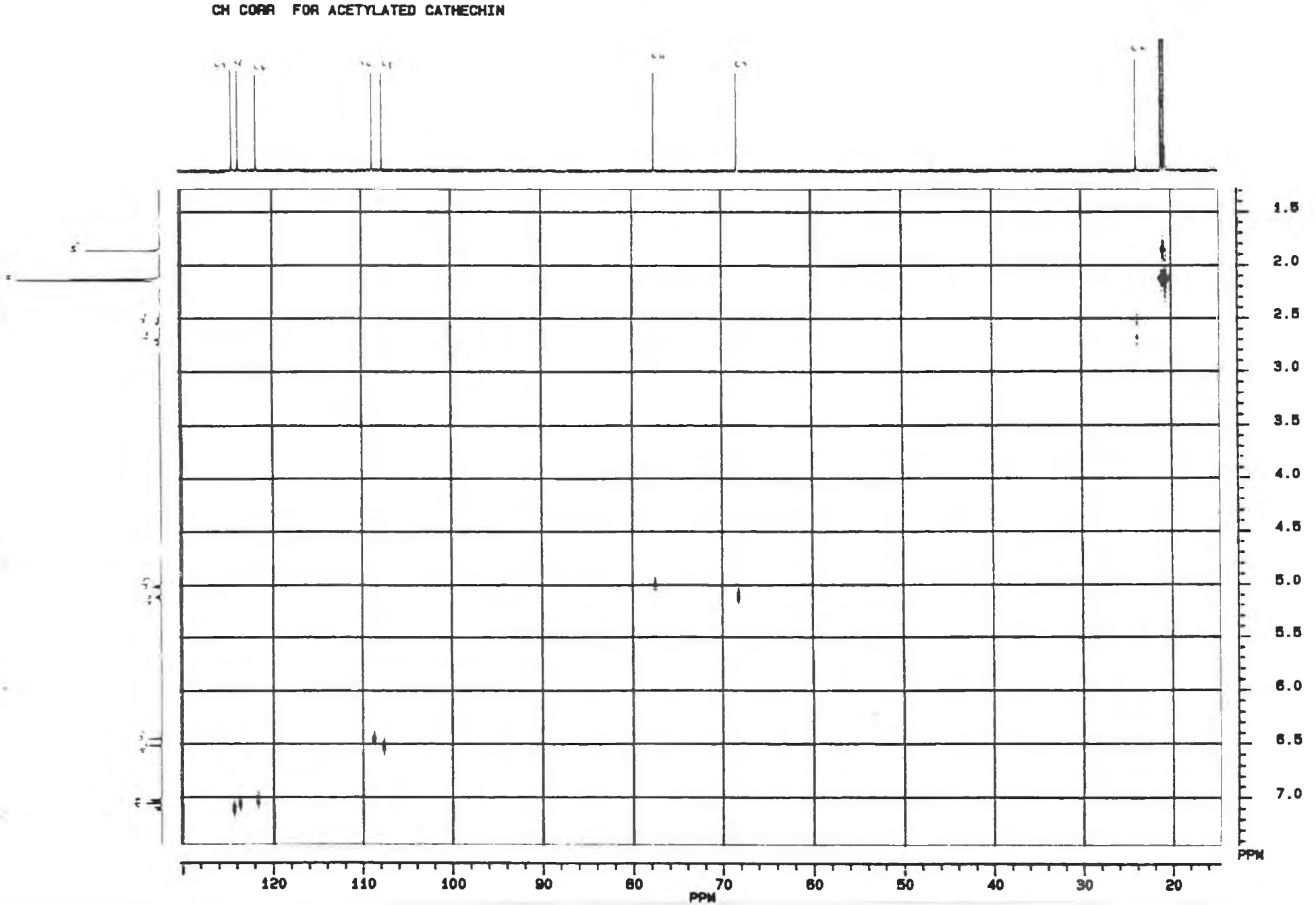
App. B, NMR #6: ^{1}H NMR of acetylated (+)-Catechin.





App. B, NMR #7: ^{13}C NMR of actylated (+)-Catechin.

App. B, NMR #8: C-H Correlation of acetylated (+)-Catechin.



AU182101.SMX
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PROJH1.001
F2 PROJ:
PROJX.001
AU PROG:
Z28.AU
DATE 18-8-92

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SW2	11627.907
SW1	1292.742
NDO	2
WDW2	E
WDW1	E
LB2	2.000
LB1	2.000
GB2	0.0
GB1	0.0
MC2	M
PLIN ROW:	
F1	130.212F
F2	14.642F
AND COLUMN:	
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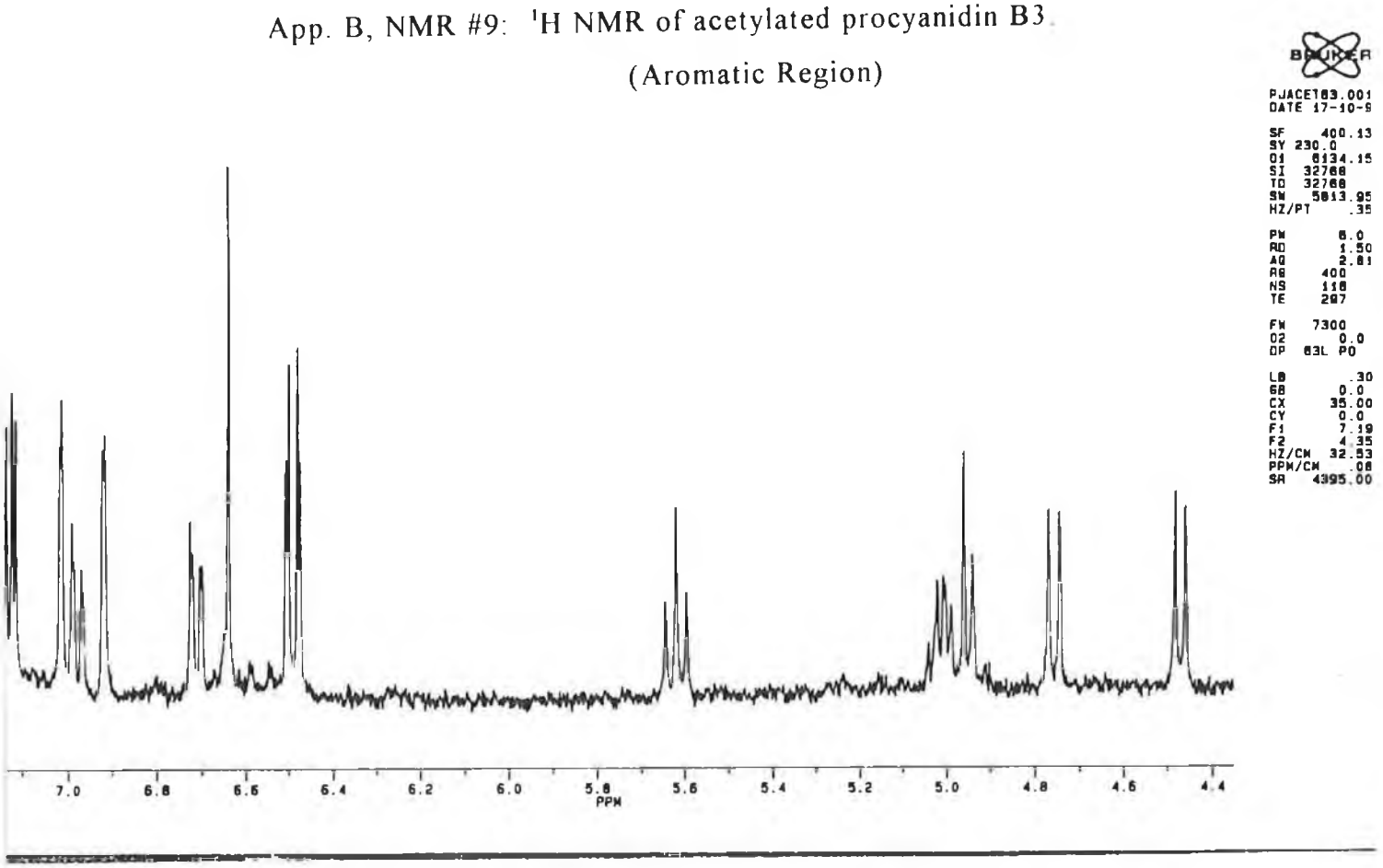
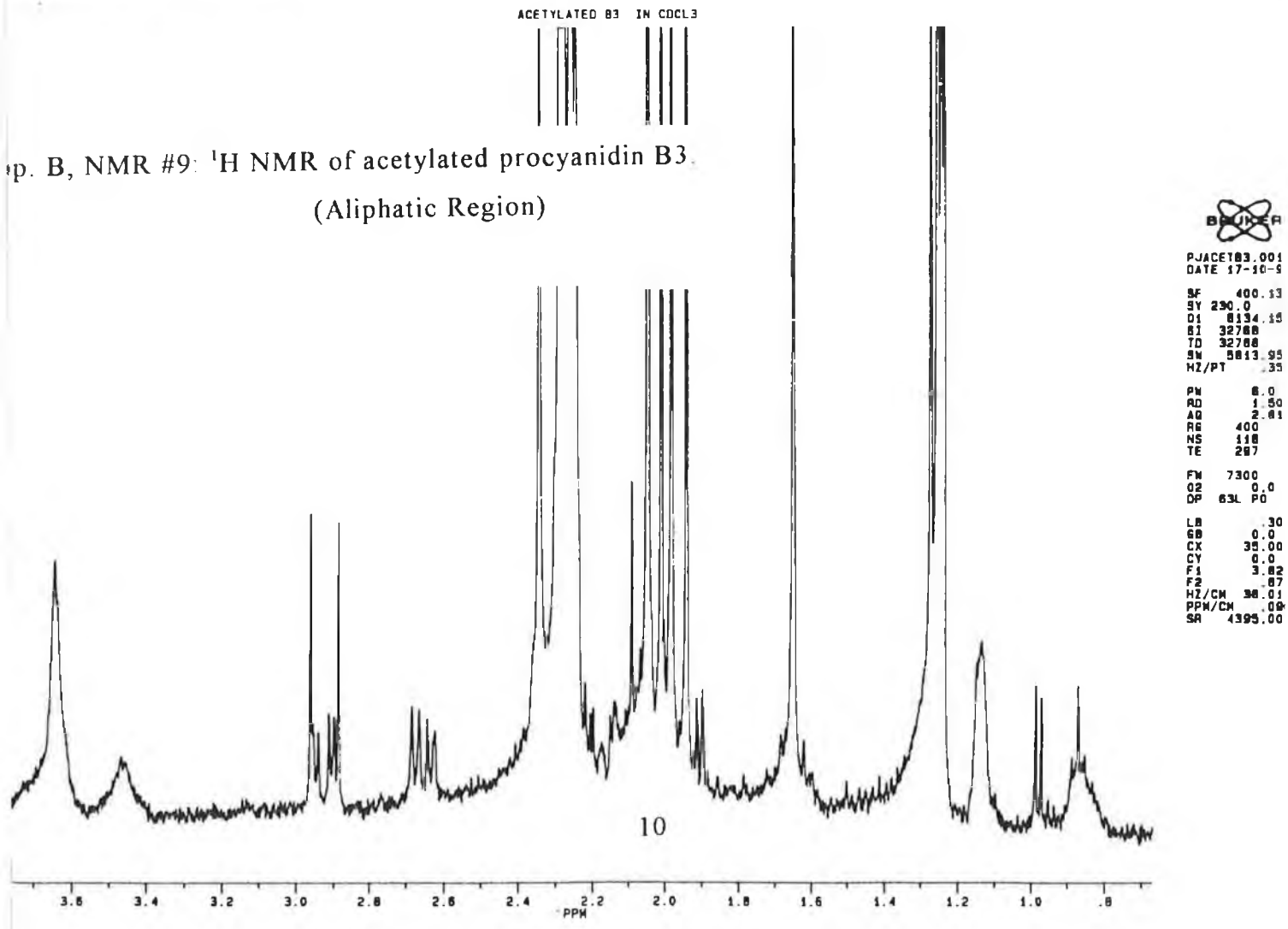
F2	1.277F
D1	1.9180000
S3	0H
P1	11.80
D0	.0000030
P6	11.00
D2	.0034500
P5	5.50
D4	.0017200
S2	17H
RGA	
RD	0.0
PW	0.0
DE	56.30
NS	64
DS	2
P9	95.00
NE	128
IN	.0002026

App. B, NMR #9: ^{1}H NMR of acetylated procyanidin B3.

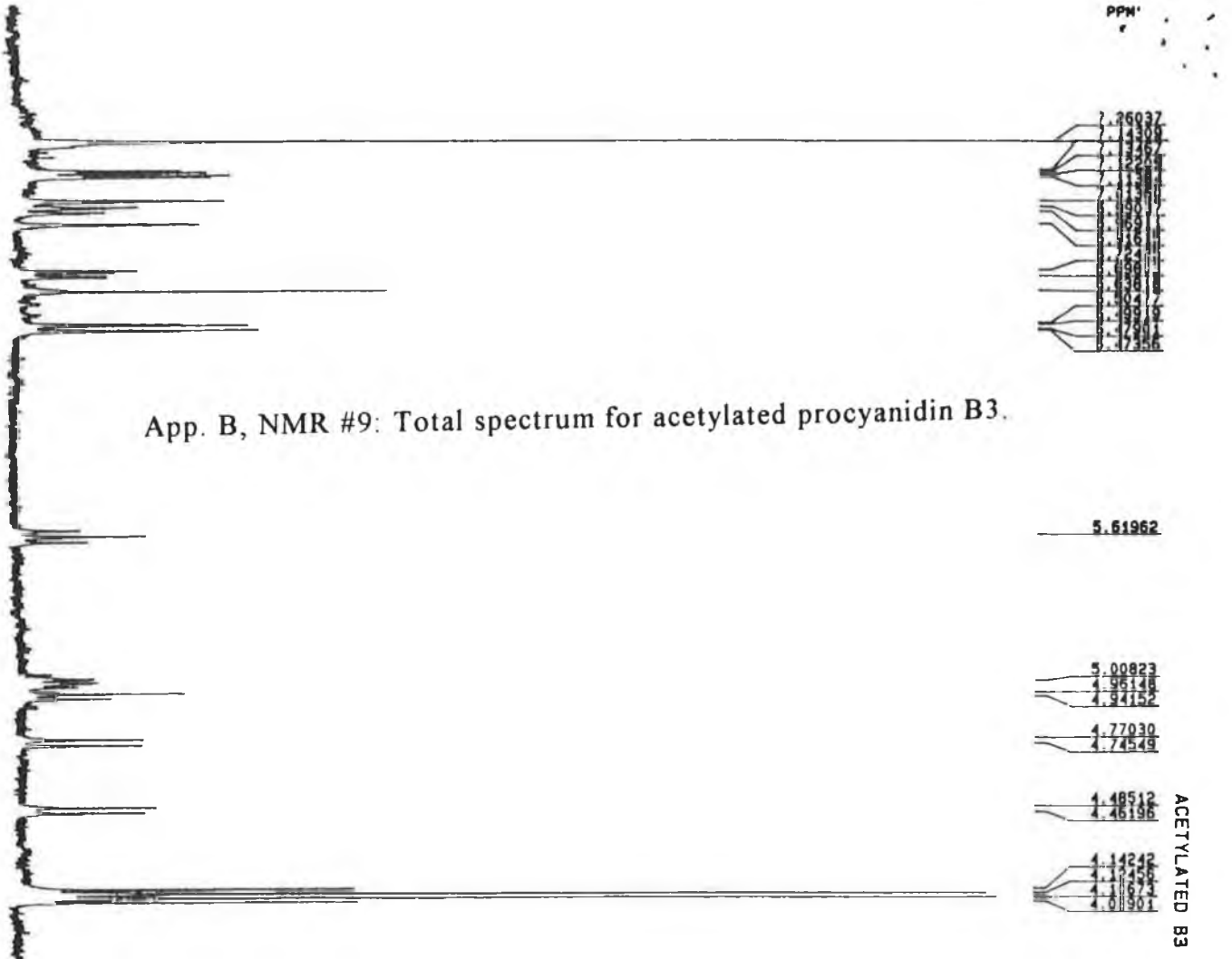
(Aromatic Region)





App. B, NMR #9: ^{1}H NMR of acetylated procyanidin B3.

(Aliphatic Region)



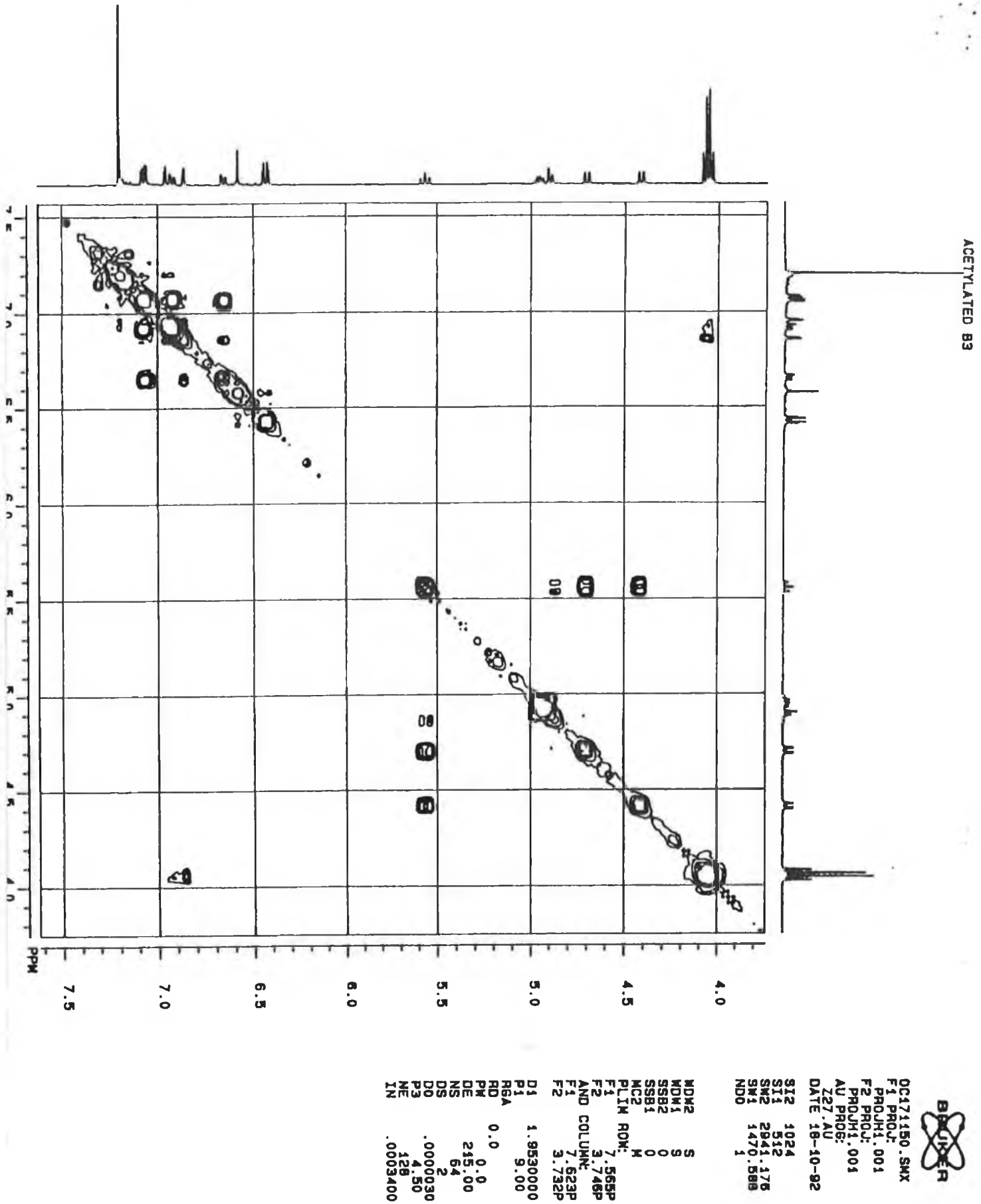
App. B, NMR #9: Total spectrum for acetylated procyanidin B3.

IN CDCL3

BRUKER

DC150S.150
DATE 17-10-92

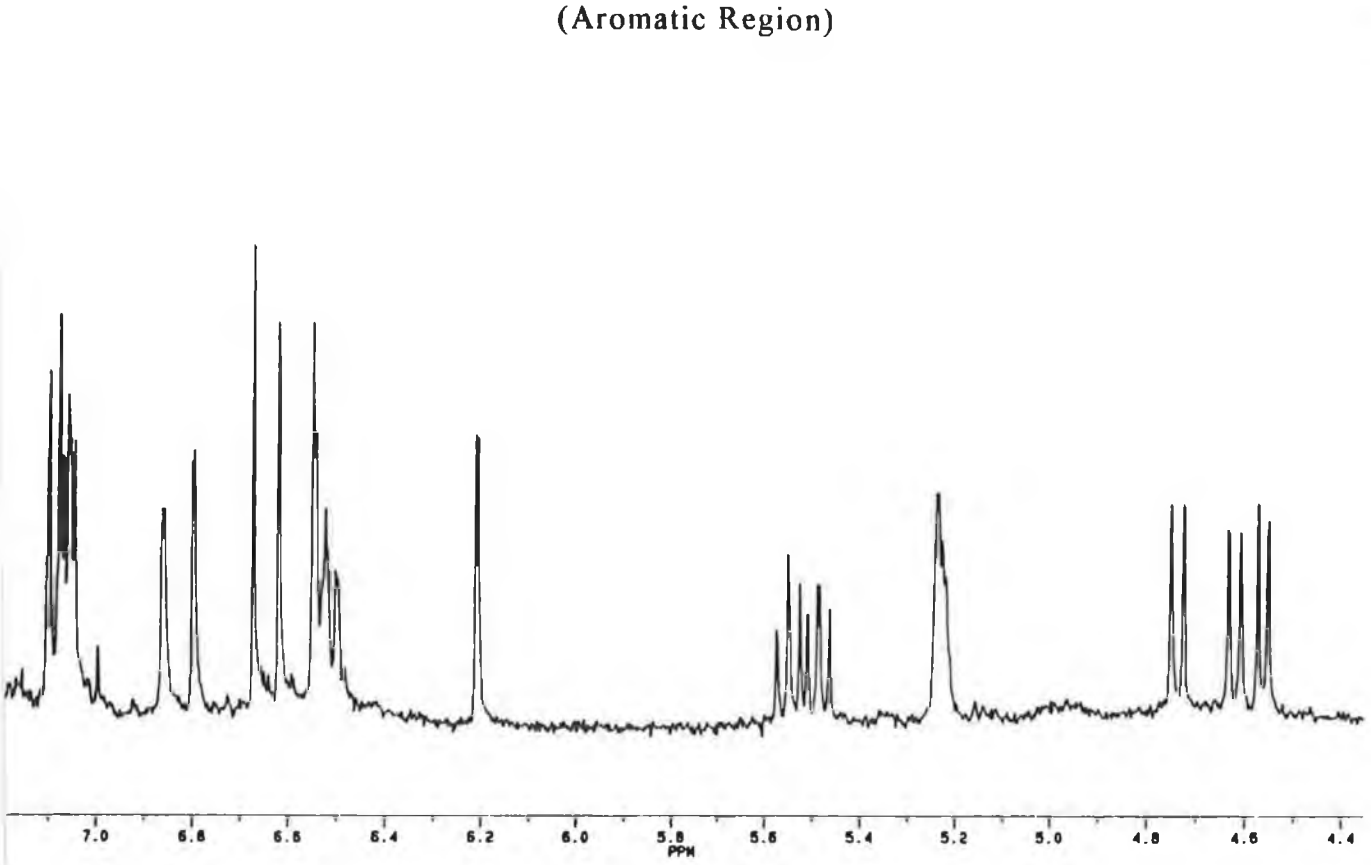
SF	400.134
SY	230.0
O1	6134.154
SI	32768
TD	32768
SW	5813.953
HZ/PT	.355
PW	6.0
RD	1.500
AQ	2.818
RG	400
NS	116
TE	297
FW	7300
O2	0.0
DP	63L PO
LB	.300
GB	0.0
CX	35.00
CY	0.0
F1	7.832P
F2	.511P
HZ/CM	83.695
PPM/CM	.209
SR	4395.00

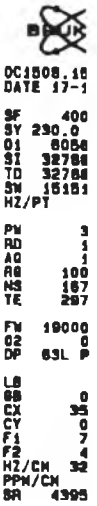


App. B, NMR #10: 2D COSY of acetylated procyanidin B3.

App. B, NMR #11: ^{1}H NMR of acetylated procyanidin C2.

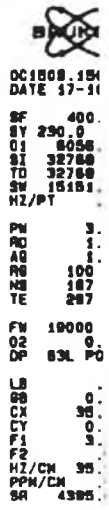
(Aromatic Region)

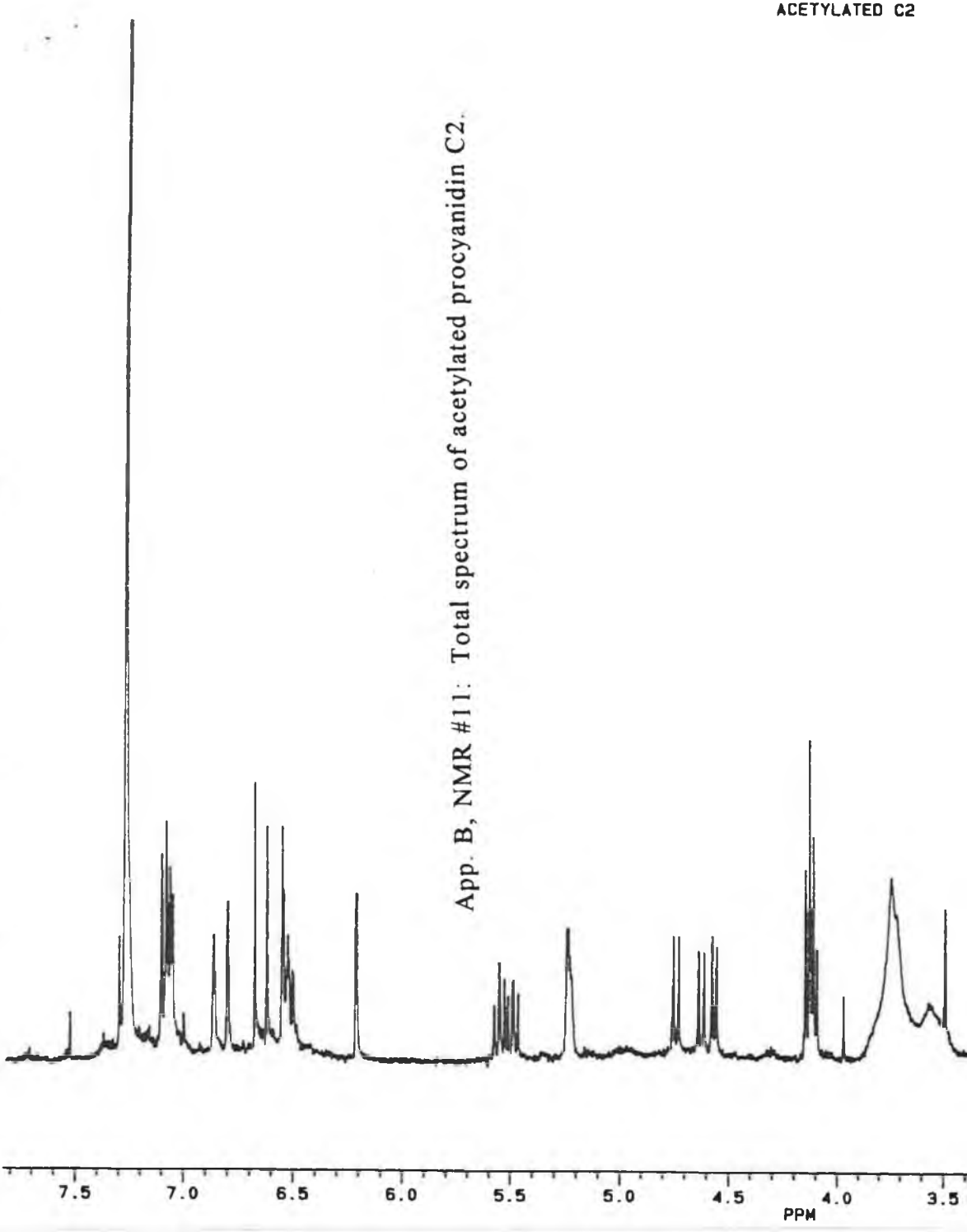




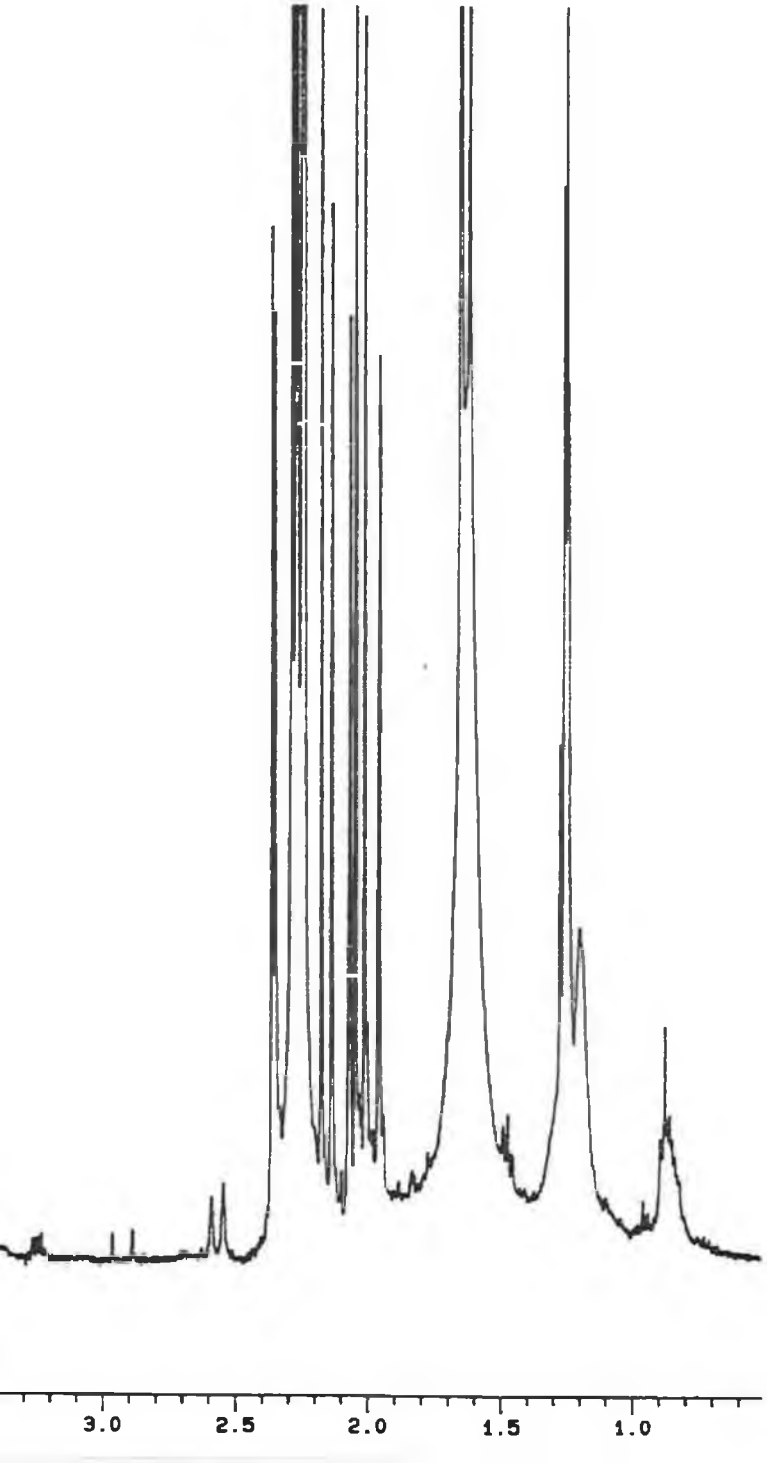
App. B, NMR #11: ^{1}H NMR of acetylated procyanidin C2.

(Aliphatic Region)





App. B, NMR #11: Total spectrum of acetylated procyanidin C2.



BRUKER

OC150S.150
DATE 17-10-92

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DP	63L PO
LB	.300
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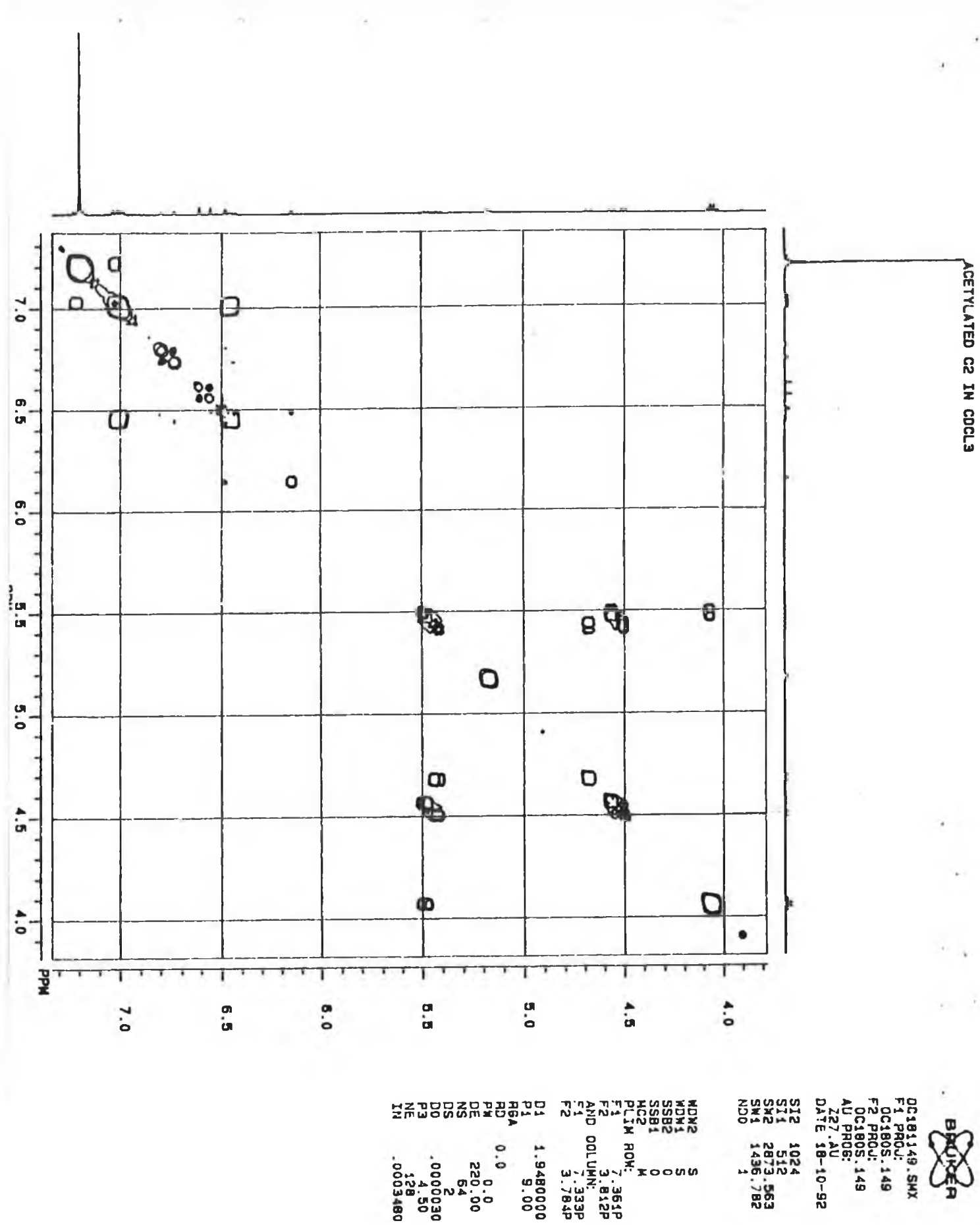
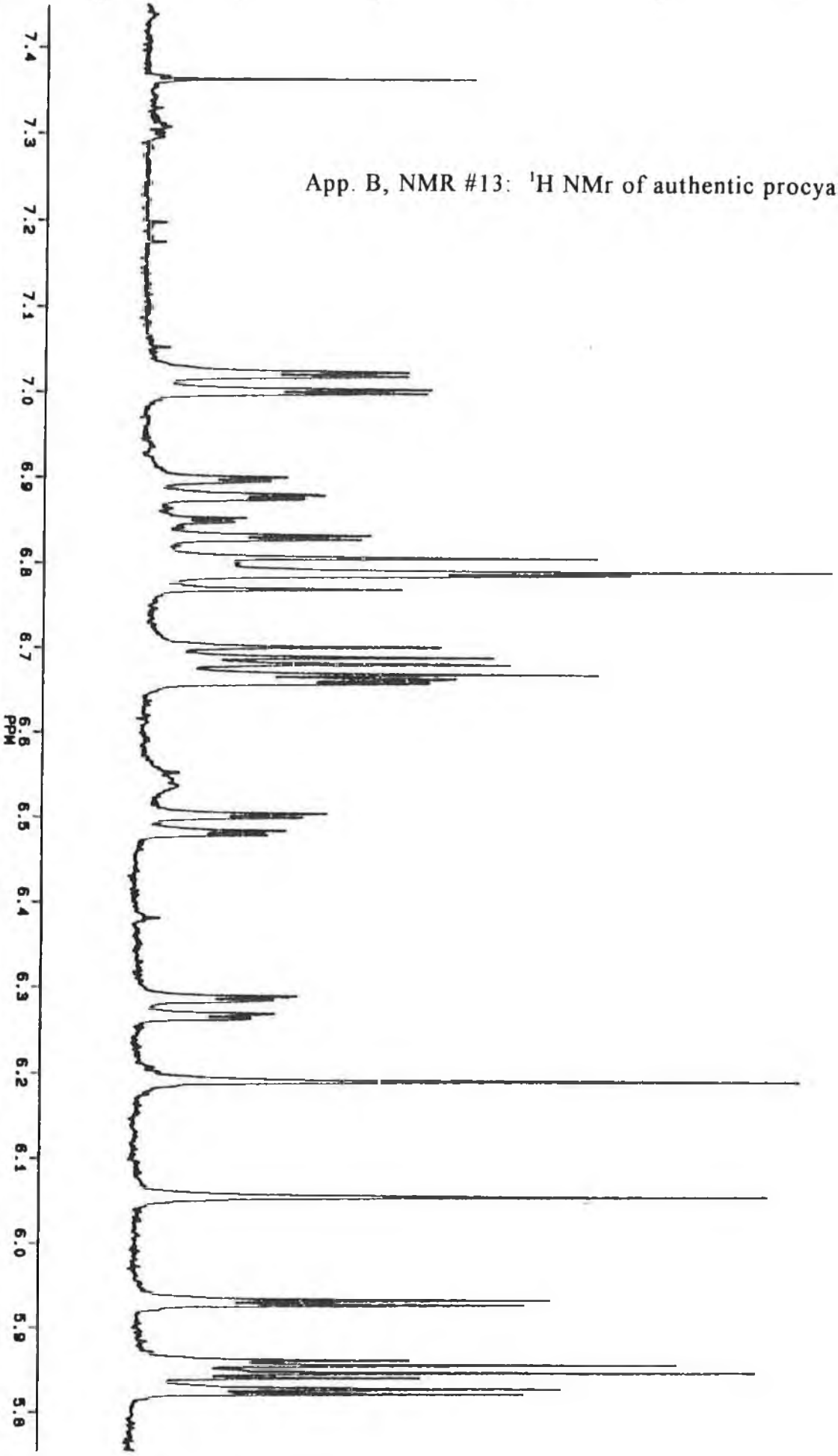
F1	7.832P
F2	.512P
HZ/CM	83.679
PPM/CM	.209
SR	4395.00

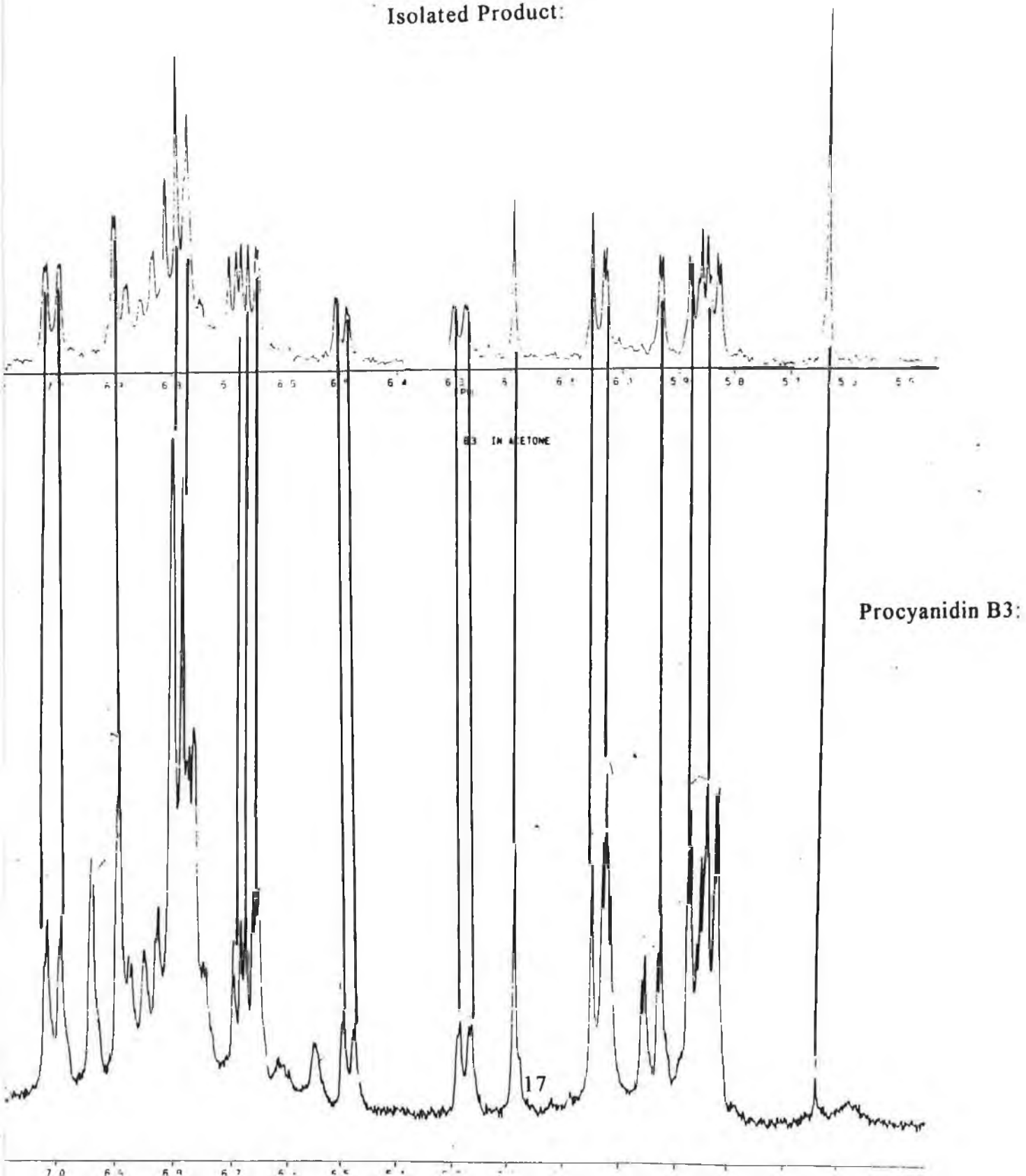


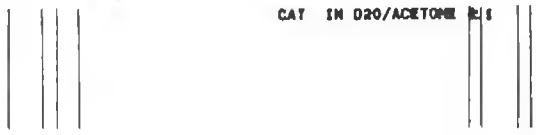
App. B, NMR #12: 2D COSY of acetylated procyanidin C2.

App. B, NMR #13: ^{1}H NMr of authentic procyanidin B3 (CD_2O).



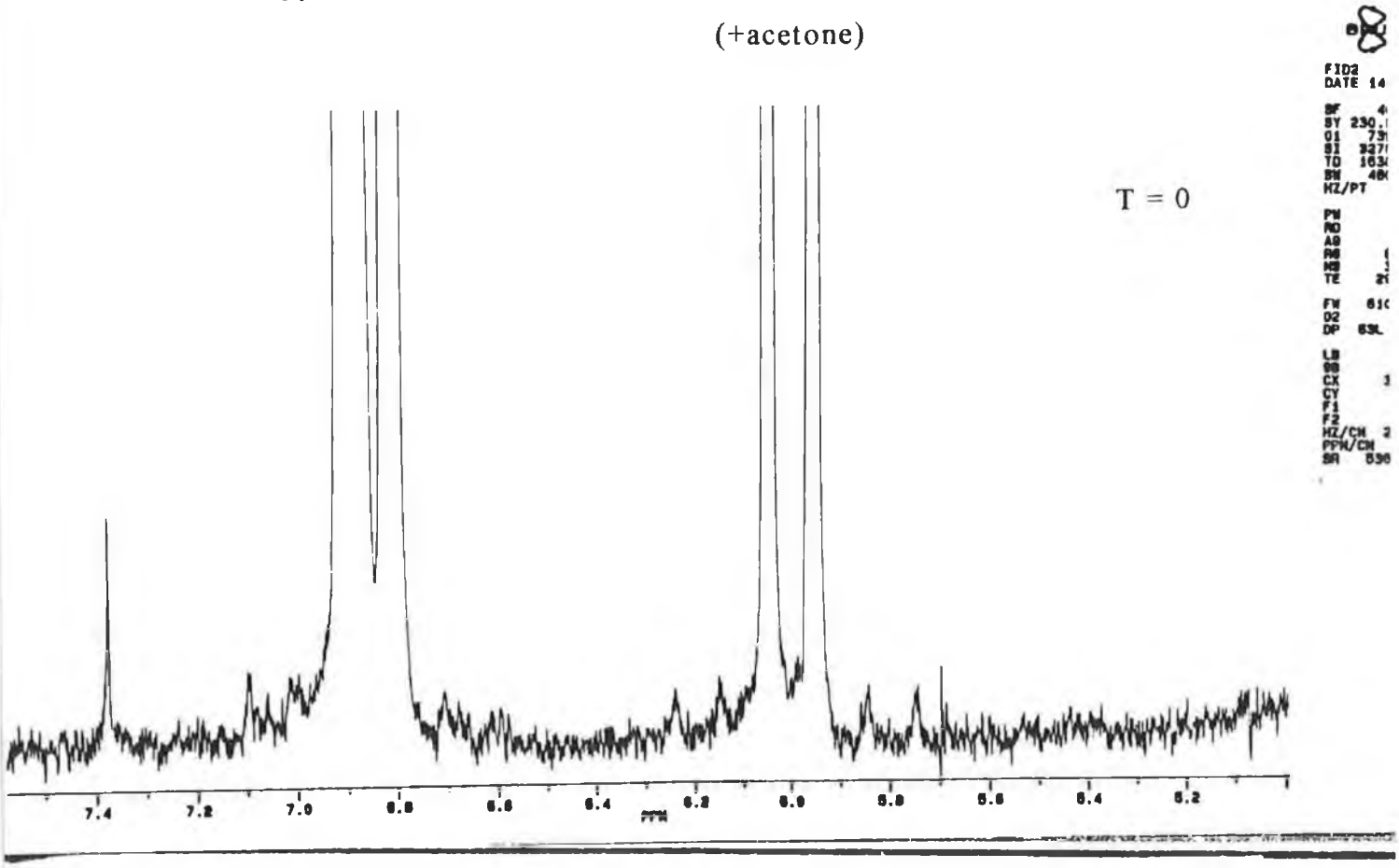
App. B, NMR #14: Comparison of procyanidin B3 & isolated product.

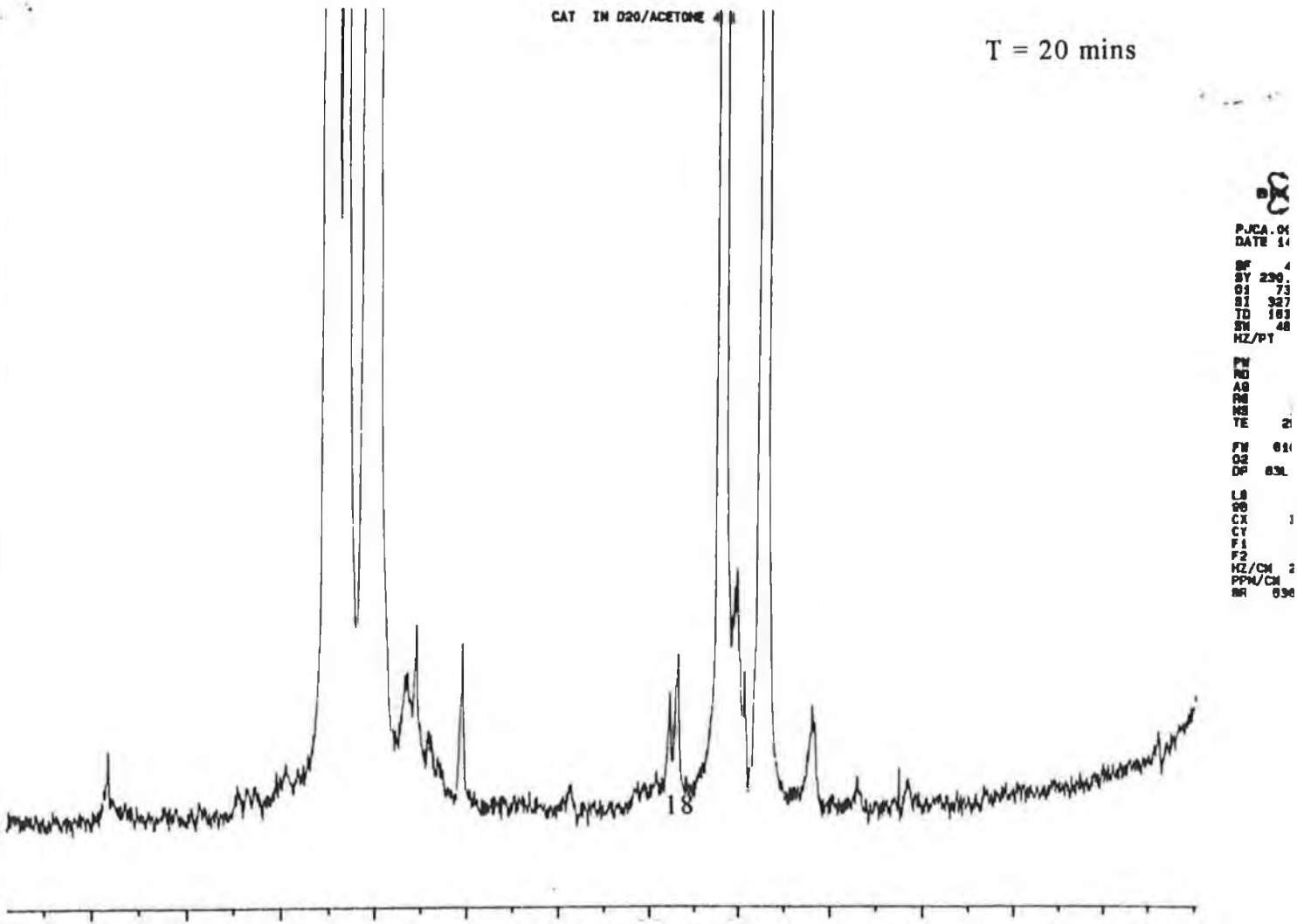




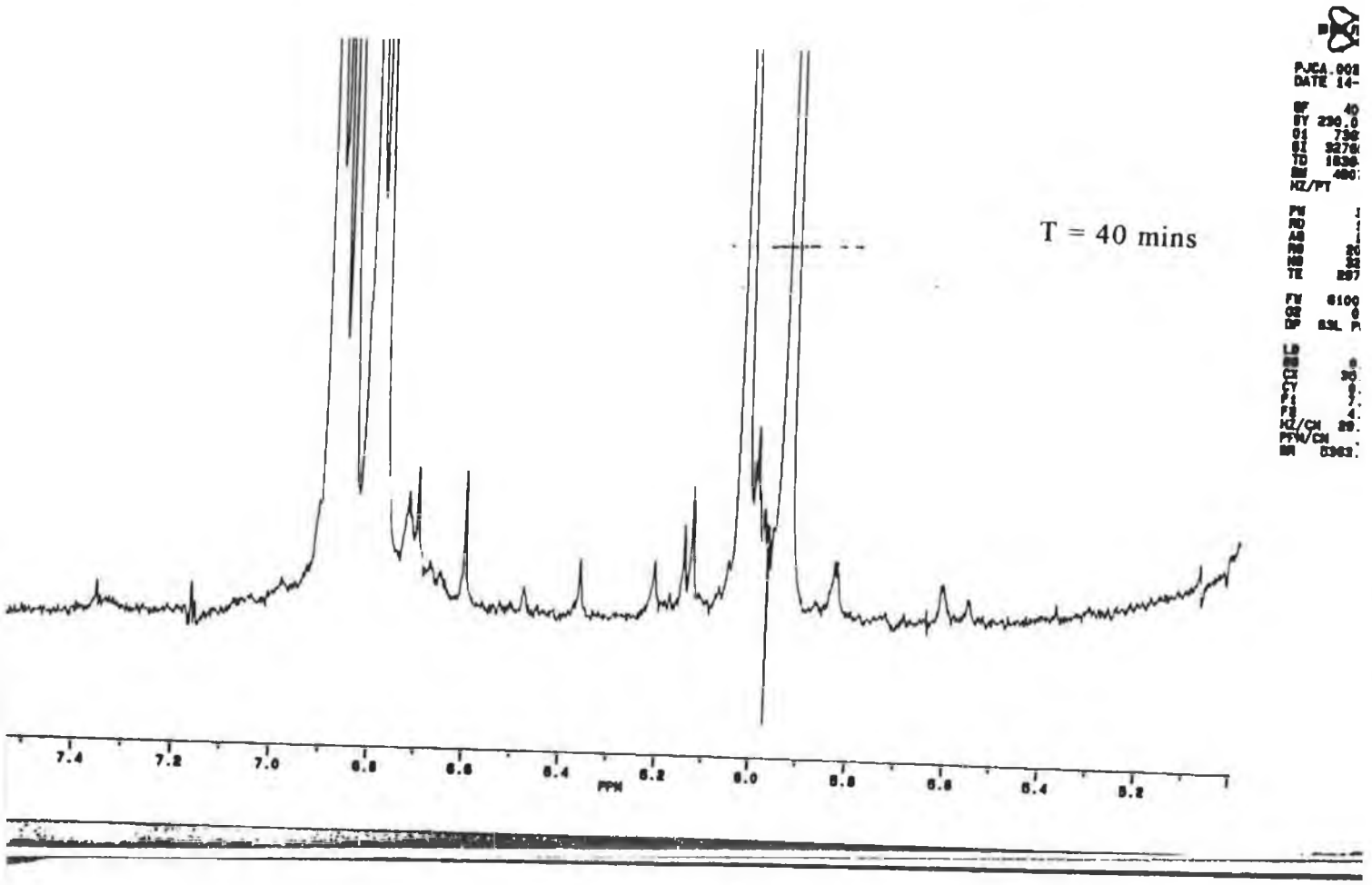
App. B, NMR #15(A): In process reaction profile for enzyme reaction.

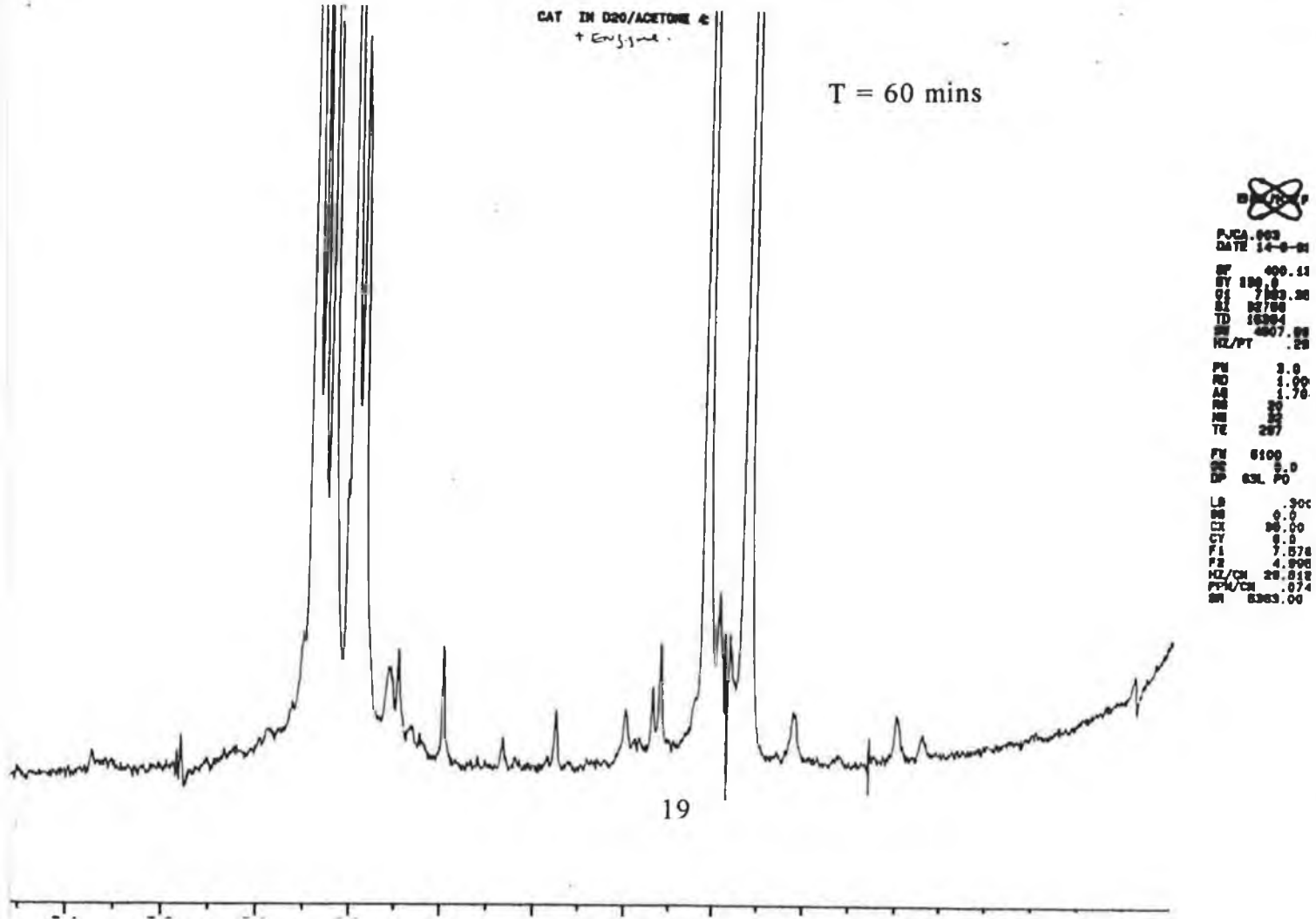
(+acetone)

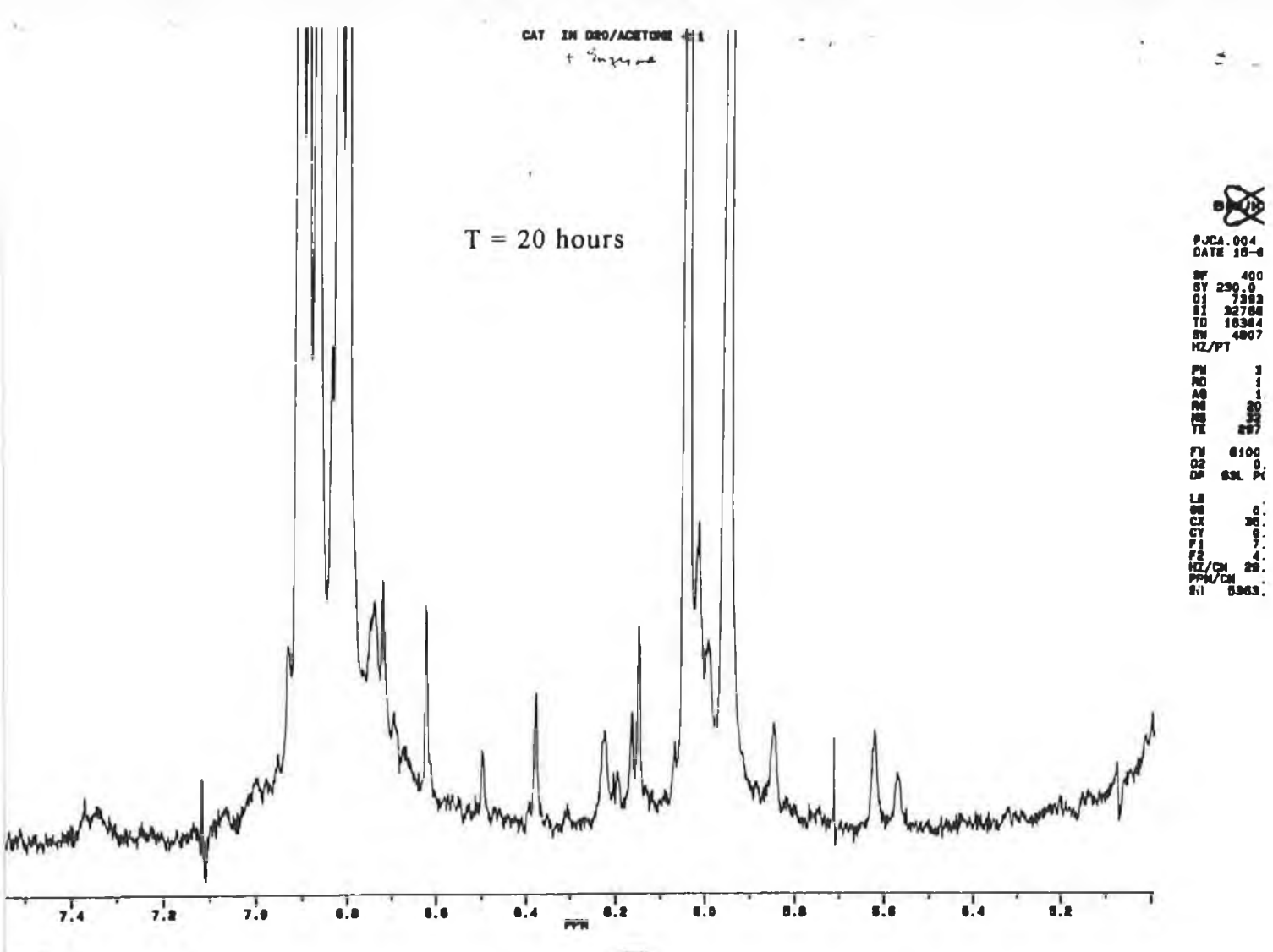




App. B, NMR #15(B): Enzyme reaction (+acetone).



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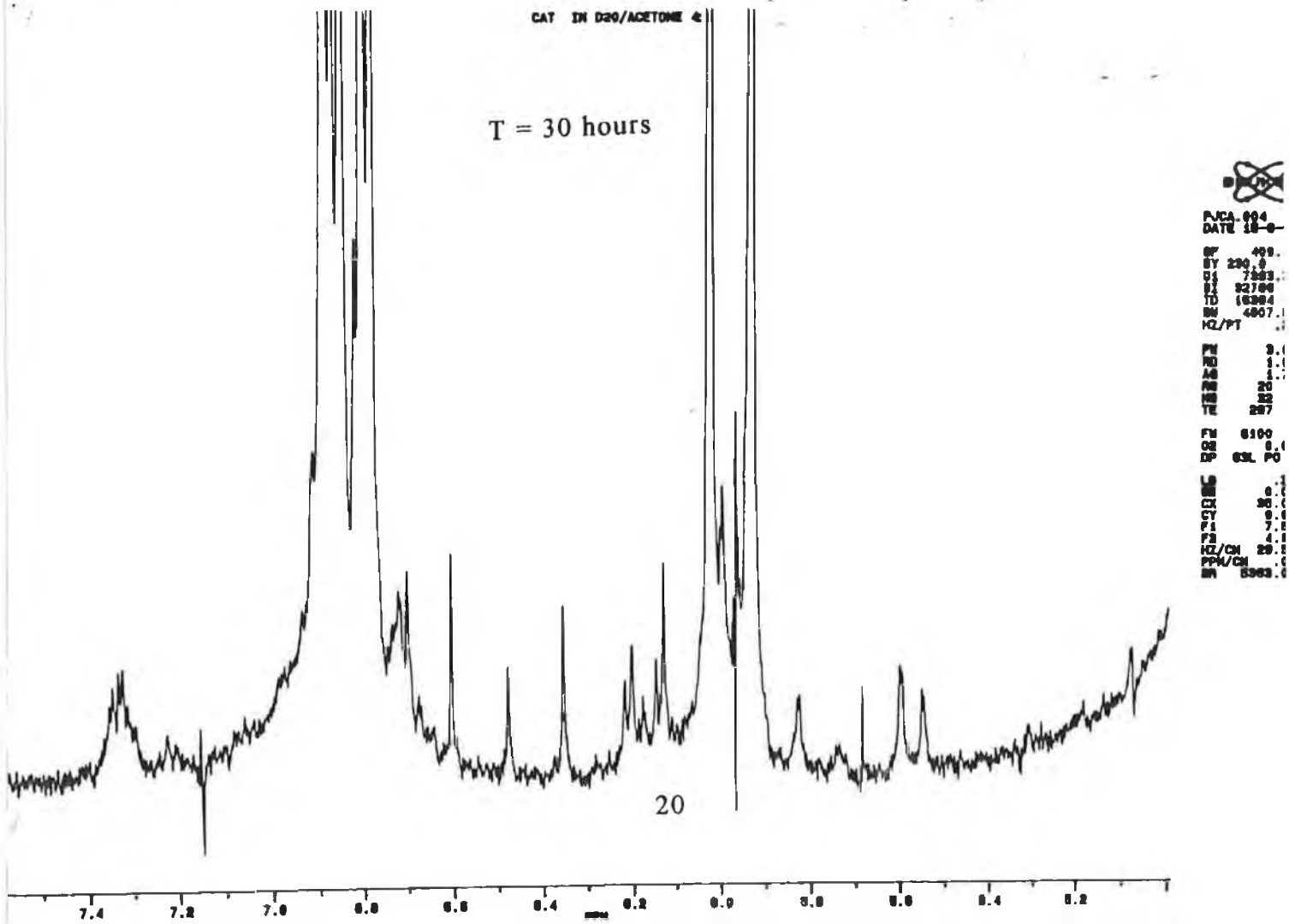
6.8
6.6
6.4
6.2
6.0
5.8
5.6
5.4
5.2
PPM


CAT IN D20/ACETONE
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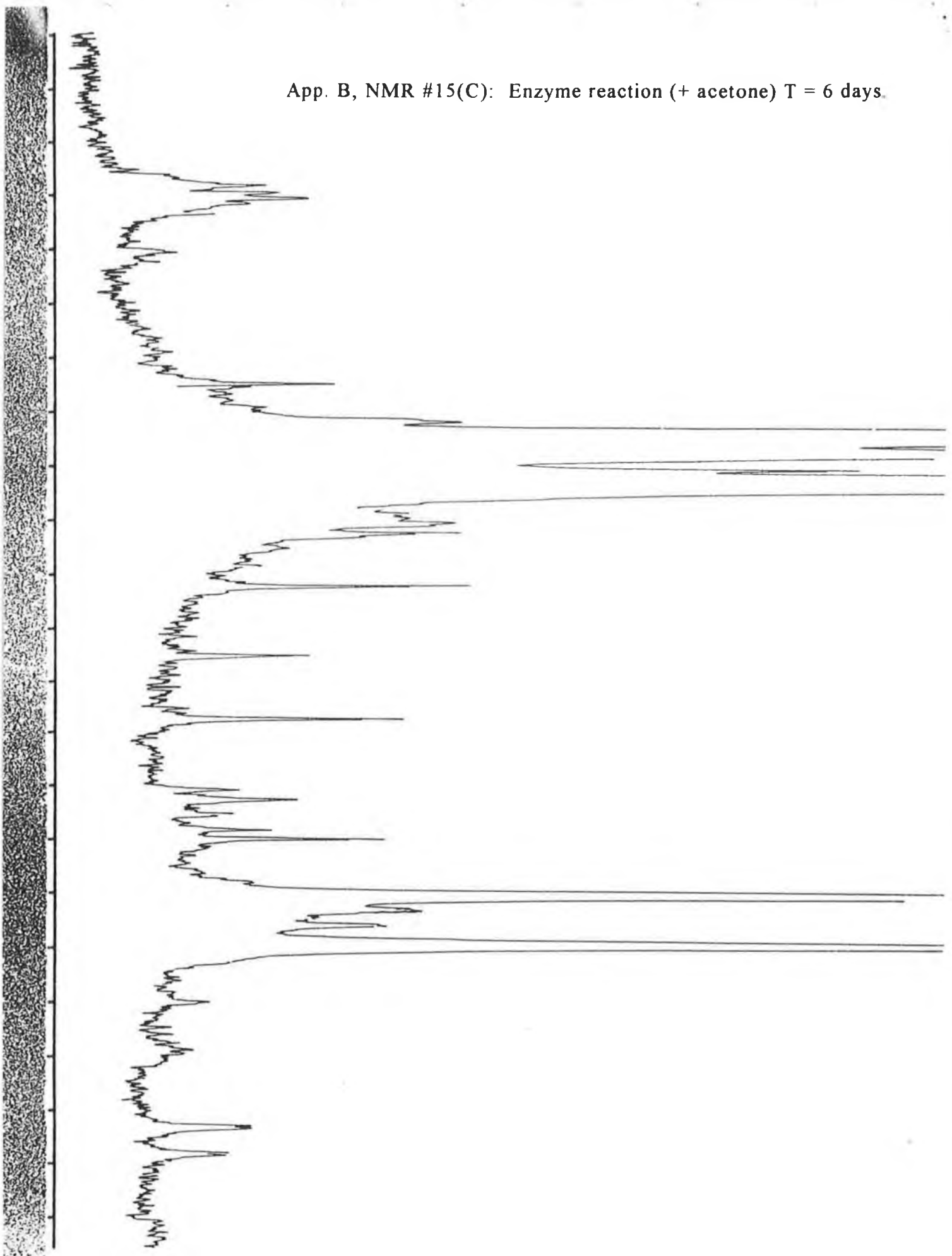
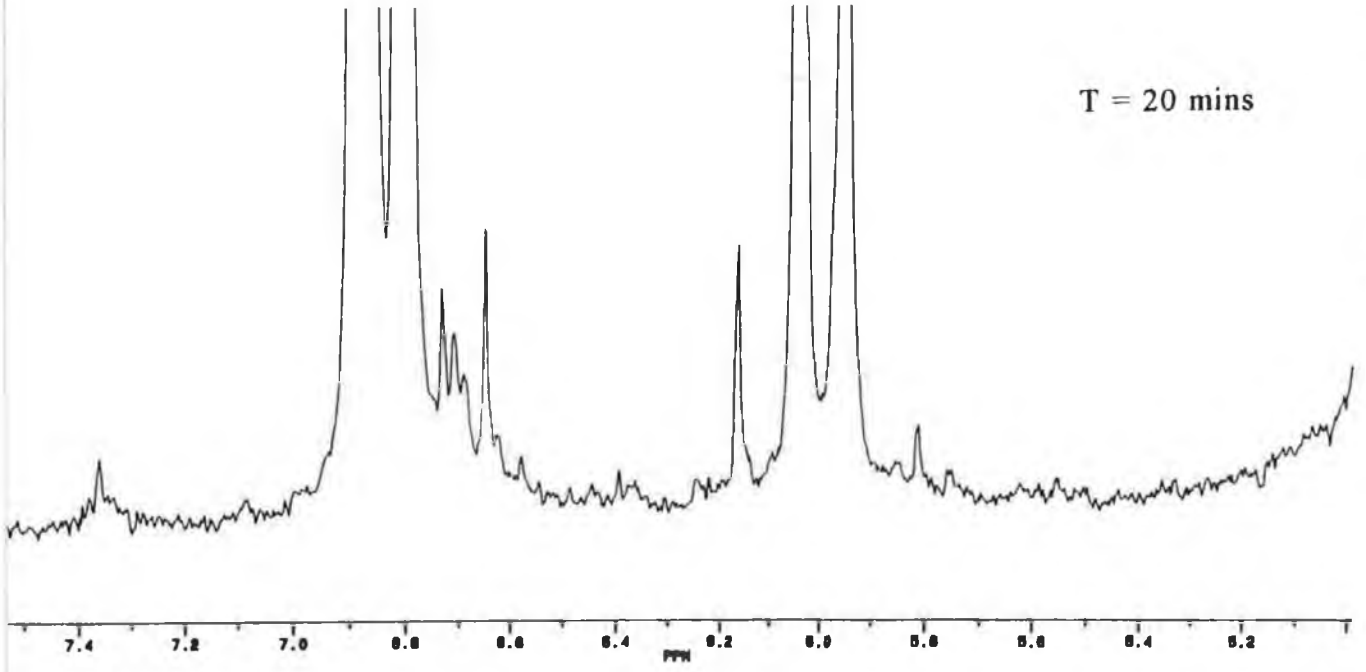
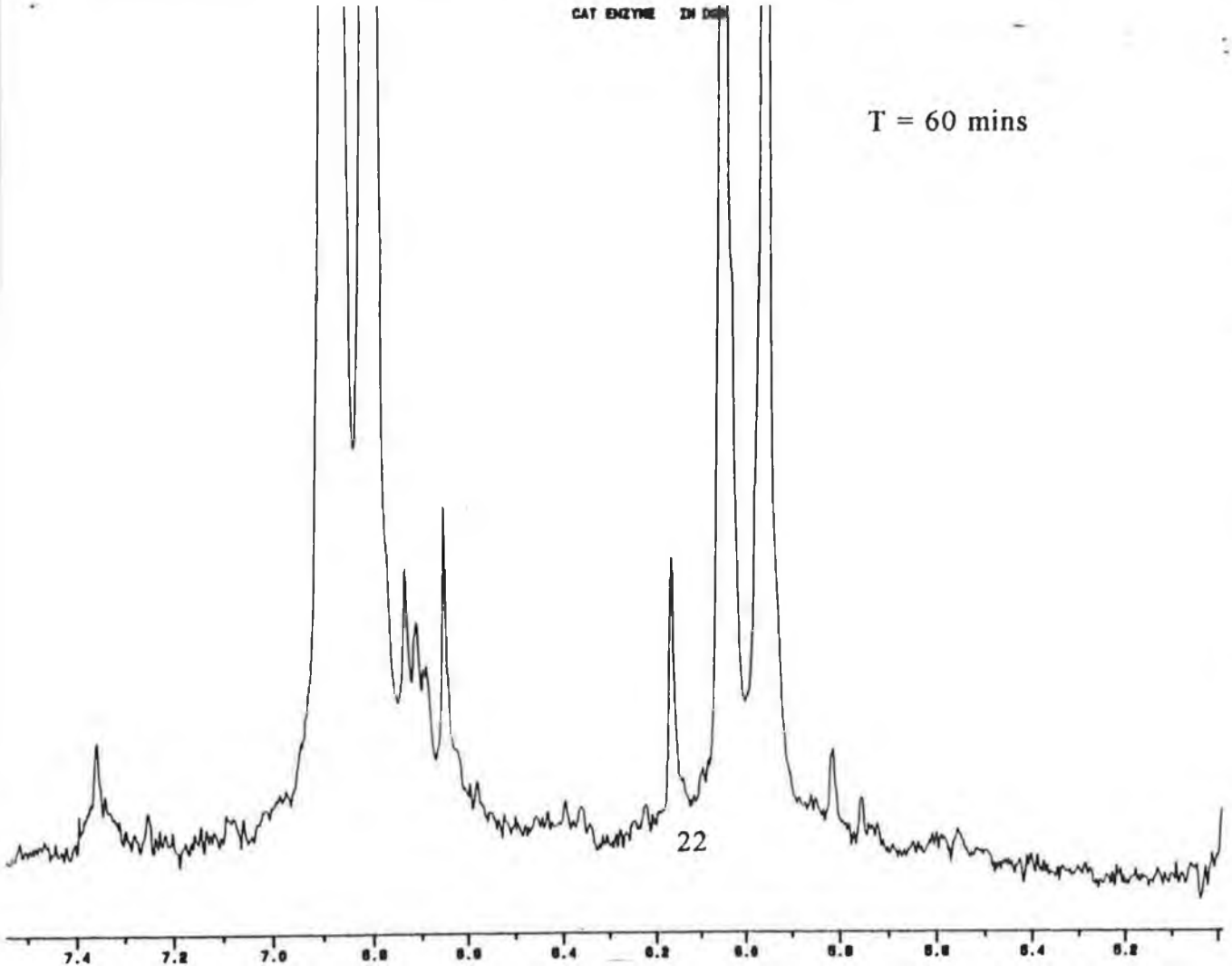
7.2
7.0
6.8
6.6
6.4
6.2
6.0
5.8
5.6
5.4
5.2

App. B, NMR #15(C): Enzyme reaction (+ acetone) T = 6 days.

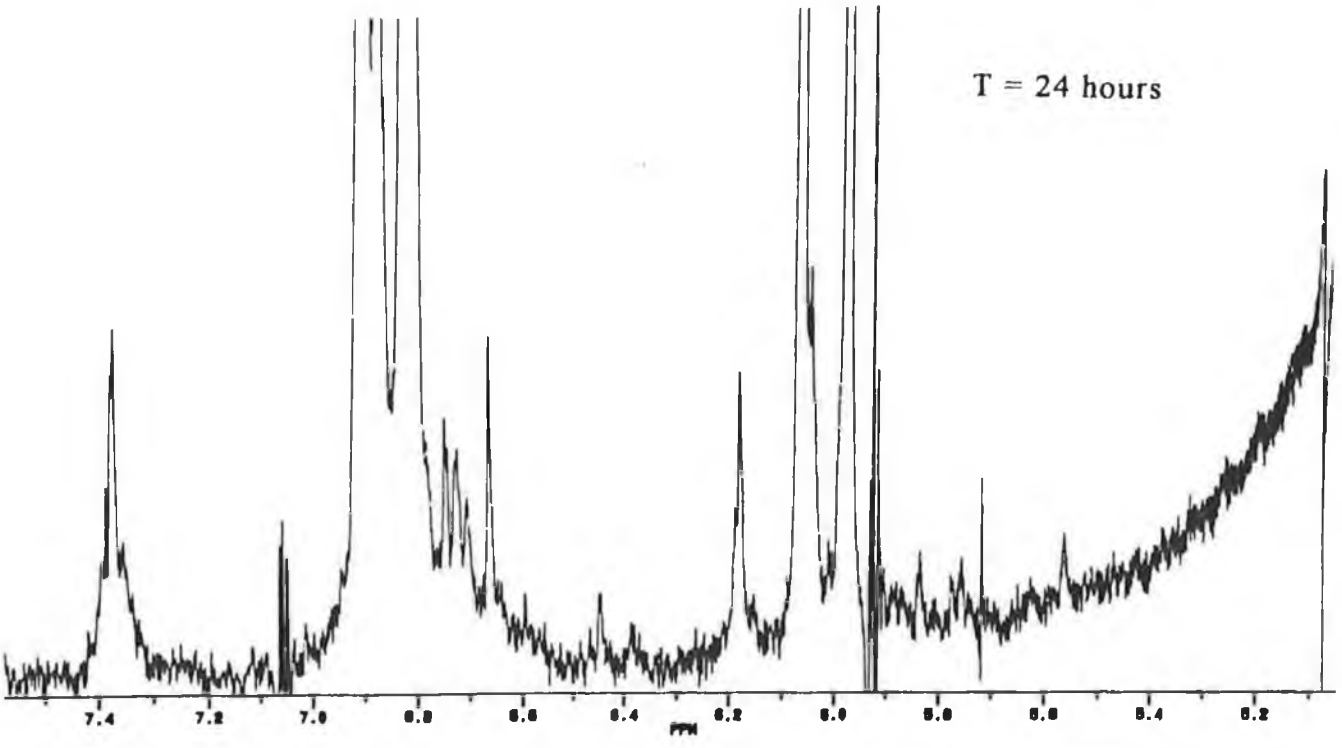


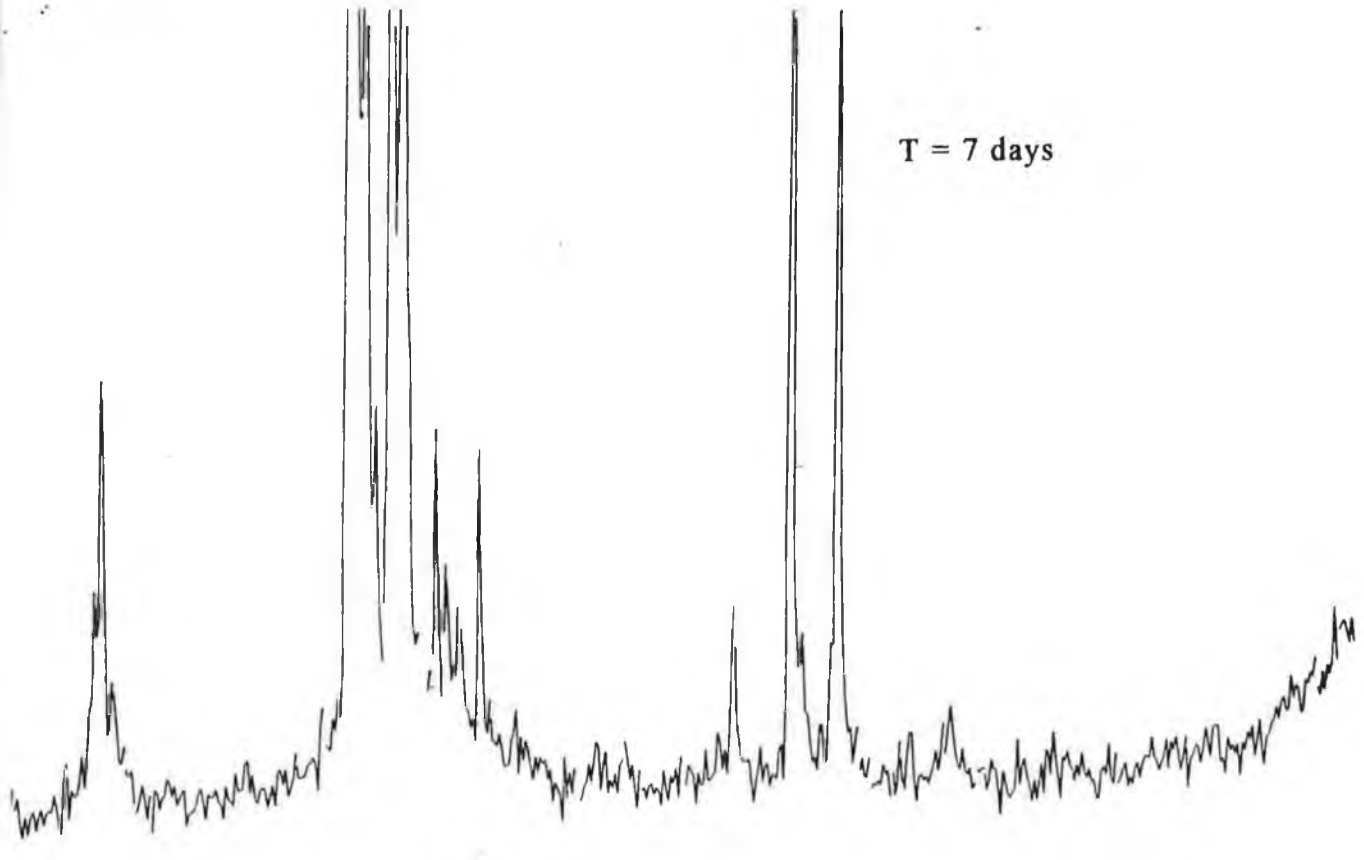
App. B, NMR #16(A): Enzyme reaction (No acetone).

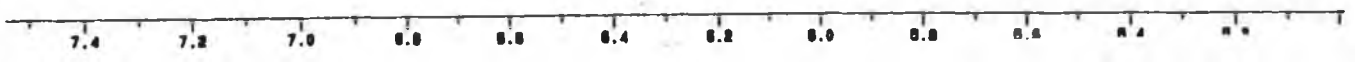


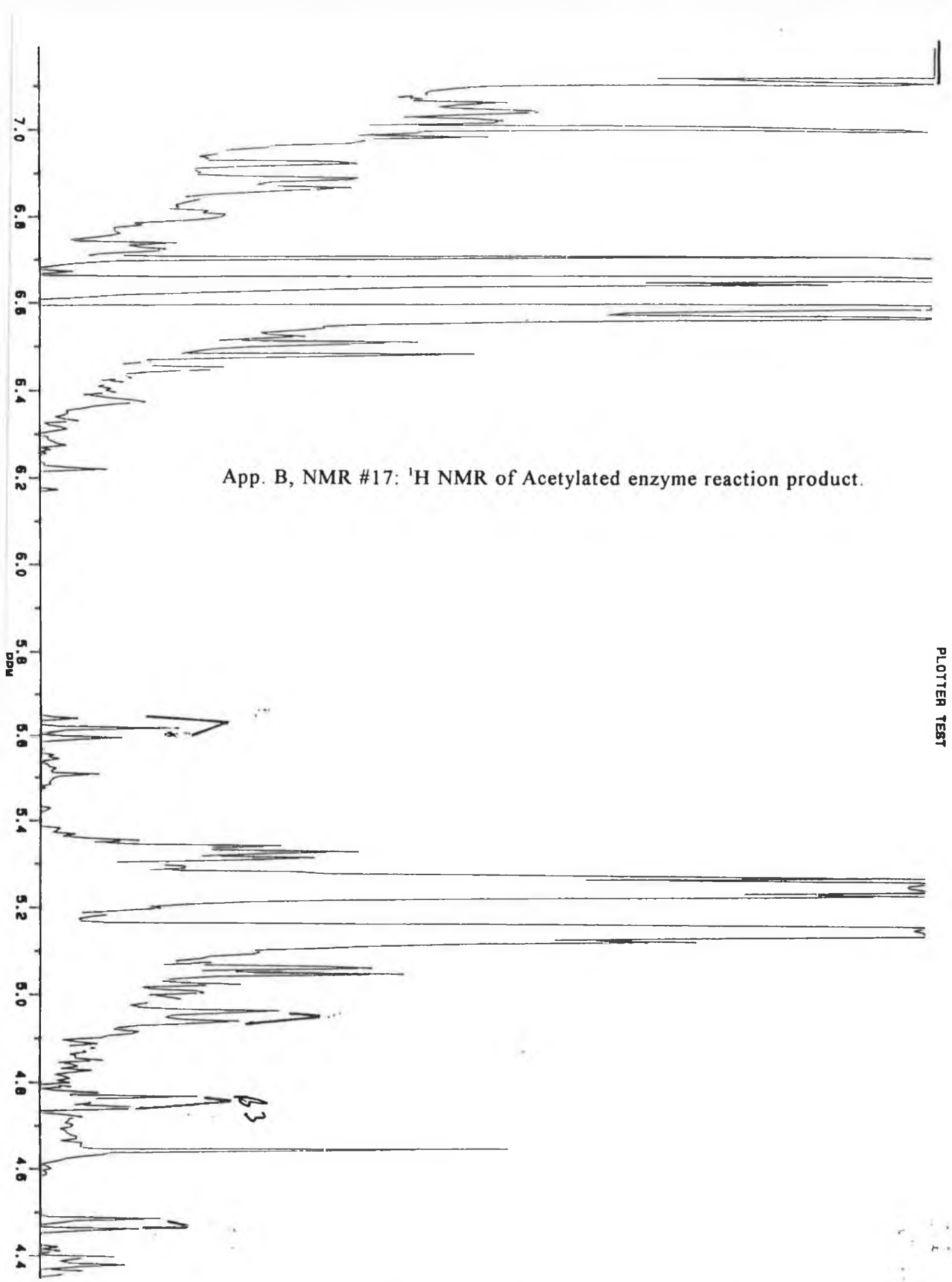


App. B, NMR #16(B): Enzyme reaction (No acetone).

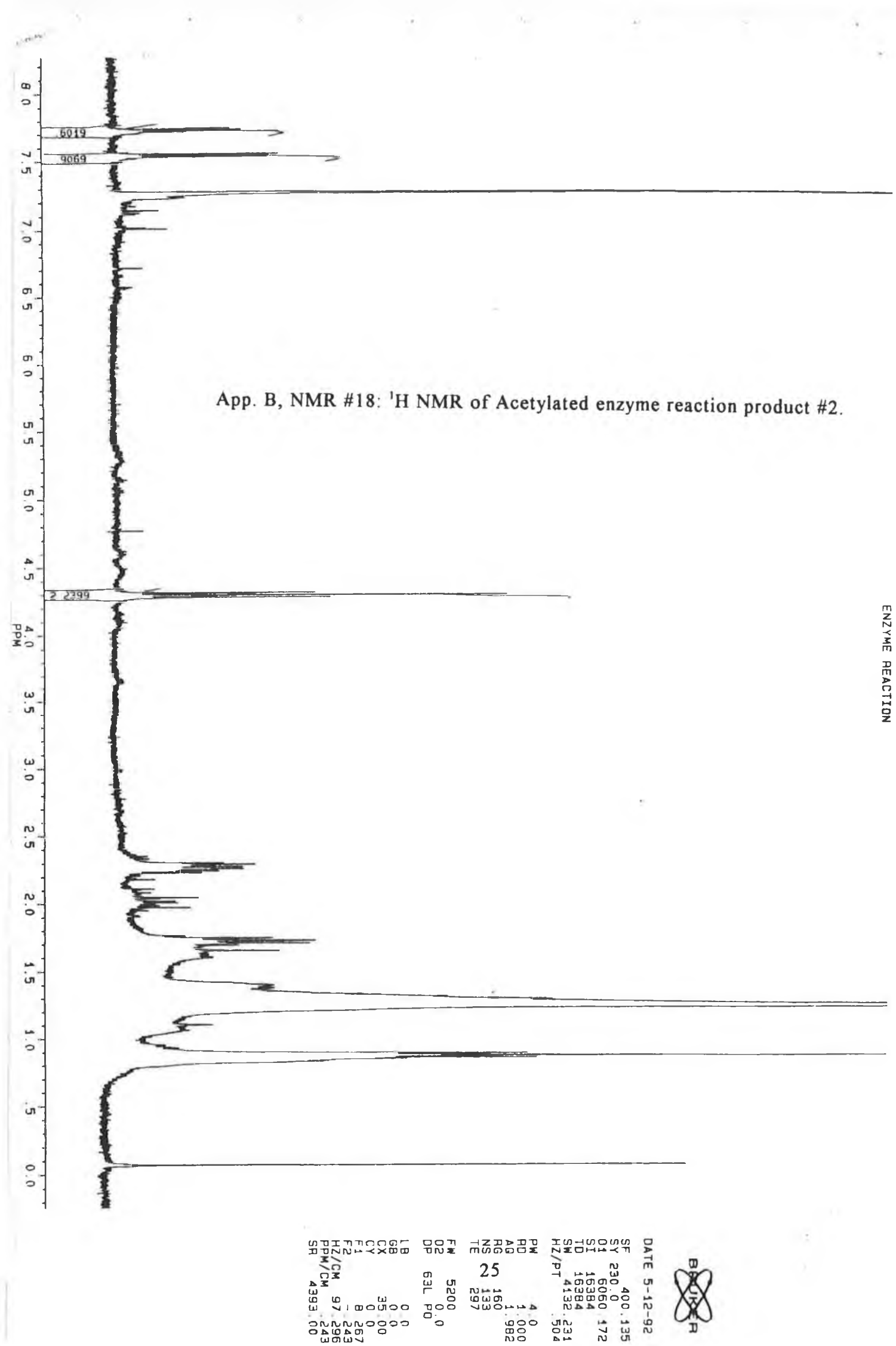








App. B, NMR #17: ^{1}H NMR of Acetylated enzyme reaction product.



App. B, NMR #18: ^{1}H NMR of Acetylated enzyme reaction product #2.