THE ANALYSIS OF NATURAL AND SULFITED COMMERCIAL QUEBRACHO (*SCHINOPSIS LORENTZII*) AND ACACIA (*ACACIA MEARNSII*) PROATHOCYANIDIN EXTRACTS WITH ELECTROSPRAY IONISATION MASS SPECTROMETRY

A thesis submitted to meet the requirements for the degree

PHILOSOPHIAE DOCTOR

in the

Department of Chemistry
Faculty of Natural and Agricultural Sciences

at the

University of the Free State
Bloemfontein

by

MARYAM AMRA JORDAAN

Promoter

Prof. J.H. van der Westhuizen

January 2013

ACKNOWLEDGEMENTS

I would like to thank the almighty "ALLAH" (GOD) for the strength and perseverance that he has given me to complete this study.

Prof. J.H. van der Westhuizen as supervisor and mentor for the invaluable assistance and guidance that he has given me in achieving and overcoming all obstacles in the quest for education;

Dr. S.L. Bonnet for her professional research guidance;

I wish to express my sincere gratitude to the following people:

My husband Yasar and daughters Aminah and Aatikah and my son Umar for their support, patience and love during difficult circumstances;

Prof. N. Heideman for incomparable assistance, guidance, encouragement and financial support that he has given me;

Prof. A. Roodt for valuable assistance, guidance and financial support that he has given me;

The NRF, THRIP, UFS for financial support, especially "GOOT" who assist the previously disadvantaged people in achieving their goals;

To my mother and father as well as the rest of the Jordaan family for their encouragement;

To the Amra, Ibrahim, Dada and Docrat families for their support;

To my fellow colleagues at the Bloemfontein and Qwaqwa campuses and postgraduate students in the Chemistry department for their advice and assistance.

Maryam Amra Jordaan

Table of Contents

Abbreviations

AOAC: Association of Official Agricultural Chemists

aDP: Average Degree of Polymerisation

DP: Degree of Polymerisation

DP_n: Number average degree of polymerisation

CCC: Craig Counter current Chromatography

CP-MAS NMR: Cross Polarisation-Magic Angle Spinning Nuclear Magnetic Resonance

ESI: Electrospray Ionisation

EU REACH: European Union, Registration, Evaluation, Authorisation and Restriction of Chemical substances

HPLC: High Performance Liquid Chromatography

MALDI-TOF: Matrix-Assisted Laser Desorption Ionization-Time of Flight

MS: Mass Spectrometry

NMR: Nuclear Magnetic Resonance

PAC: Proanthocyanidins

Summary

Quebracho (*Schinopsis lorentzii* and *Schinopsis balansae*) heartwood and black wattle (*Acacia mearnsii*) bark extracts are important renewable industrial sources of proanthocyanidins (PACs). These extracts are used industrially in leather tanning and adhesive manufacturing. These applications are derived from their chemical properties. The poly hydroxy groups of PACs complex with proteins *via* hydrogen bonds and thus transforms raw skin into leather. The phloroglucinol or resorcinol type A-rings are nucleophilic and polymerise with aldehydes to form natural adhesives. The *ortho* hydroxy group on the B-ring form insoluble complexes with heavy metals and can be used in water purification applications. The extracts are often treated with sodium hydrogen sulphate (sulfitation) to enhance their industrial usefulness. From a literature search and discussions with role players in the black wattle and quebracho PAC extract manufacturing industry, it became evident that knowledge on the composition of commercial PACs extracts and chemical changes that takes place during sulfitation is unsatisfactory.

These PAC extracts are complex due to variable hydroxylation patterns of the constituent flavan-3-ol aromatic rings, different configurations of the C-2, C-3 and C-4 stereogenic centres, different degrees of polymerisation, and the existence of angular oligomers. Gel or paper chromatography fractionations of the complex extracts are hampered by poor resolution due to their hydrophilic polyphenolic nature and efforts to isolate pure compounds have been restricted to the isolation of mainly monomers and a few dimers and trimers.

PACs of the commercially important quebracho (*Schinopsis lorentzii* and *Schinopsis balansae*) and black wattle (*Acacia mearnsii*) extracts have a strong and stable interflavanyl bond. This stability is important from an industrial point of view as it leads to durable leather and adhesive products. It is attributed to the absence of 5-OH groups in the aromatic moieties of the extender fisetinidol and robinetinidol flavan-3-ols units. However, from an analytical point of view it is not advantageous. The high temperatures thus required to hydrolyse the interflavanyl bonds with weak acids; leads to decomposition of the intermediate monomers that renders conventional thiolysis and phloroglucinolysis based analytical methods unreliable.

In this thesis we used electrospray mass spectrometry (ESI-MS) to investigate the composition of PACs in black wattle extract and the changes that takes place in the chemical composition of quebracho PACs during sulfitation. We furthermore use all the information available from literature on the phytochemistry of flavan-3-ols and PACs and the syntheses of flavan-3-ol oligomers to guide us in our ESI-MS interpretations.

Previous research in our group established that quebracho PACs always consist of a catechin starter unit to which one, two or more fisetinidol extender units are attached. The first and second extender units are always attached to the relatively reactive phloroglucinol A-ring of the catechin starter unit to form predominantly dimers and angular trimers. Further extender units are attached to the relatively less reactive resorcinol A-rings of already incorporated fisetinidol extender units. This explains the relatively short degree of polymerisation of quebracho PAC extracts and their popularity as a tanning agent. Large PACs will not penetrate the spaces between skin proteins and cannot act as a tanning agent.

In this thesis we established that black wattle PACs have, in addition to catechin starter units, also gallocatechin starter units and, in addition to fisetinidol extender units, also robinetinidol extender units. Acacia PACs are thus more complex combinations of catechin, gallocatechin, fisetinidol and robinetinidol monomers. This contrasts with quebracho PACs that only contain catechin and fisetinidol monomers. The higher degree of hydroxylation of gallocatechin and robinetinidol explains the higher water solubility of black wattle PACs and the less frequent need for sulfitation.

We also established that during sulfitation of quebracho PACs, a sulfonic acid moiety is introduced in both the C-2 and C-4 position of the pyran heterocyclic C-ring. In the case of C-2 sulfitation, the heterocyclic ring is opened. This enhances the reactivity of the A-ring towards the reaction with formaldehyde (adhesive formation) and increases water solubility due to removal of rigidity and introduction of a polar sulfonic acid group. In the case of C-4 sulfitation, the interflavanyl bond is broken. Polarity and water solubility is thus not only

increased *via* an additional sulfonic acid moiety, but due to the presence of shorter oligomers and a smaller average chain length. We also developed a chromatographic method to estimate the degree of sulfitation of quebracho PAC extract.

We believe that we have made a valuable contribution towards a better understanding of the composition of black wattle and sulfited quebracho PAC extracts and have identified a number of misconceptions.

Keywords: quebracho, acacia, electrospray ionisation mass spectrometry (ESI-MS), proanthocyanidins, catechin, fisetinidol, gallocatechin, robinetinidol, adhesives, leather tanning.

Opsomming

Quebracho (*Schinopsis lorentzii* en *Schinopsis balansae*) kernhout en swartwattel (*Acacia mearnsii*) bas ekstrakte is baie belangrike industriële bronne van proantosianidiene (PACs). Hierdie ekstrakte word vir industriële leerlooiery en die vervaardiging van kleefmiddels aangewend. Hierdie toepassings is afgelei van hul chemiese eienskappe. Die hidroksie groepe van PACs komplekseer met proteïne *via* waterstofbindings om sodoende rou vel in leer te omskep. Die floroglusinol of resorsinol tipe A-ringe is nukleofilies van aard en polimeriseer met aldehiede om natuurlike kleefmiddels te vorm. Die *orto*-hidroksie groepe op die B-ring vorm onoplosbare komplekse met swaarmetale wat vir watersuiwering aangewend kan word. Die ekstrakte word dikwels met natriumwaterstofsulfiet behandel om hul industriële toepassings te verbeter. Dit was duidelik uit ondersoeke in die literatuur en gesprekke met invloedryke bronne uit die industrie van swartwattel en quebracho PACs, dat insig in die samestelling van industriële PACs en chemiese veranderinge tydens sulfitering onvoldoende is.

Hierdie PAC ekstrakte is kompleks as gevolg van wispelturige hidroksileringspatrone van die flavan-3-ol aromatise ringe, verskillende konfigurasies van C-2, C-3 en C-4 stereogeniese sentrums, die gemiddelde lengtes van die kettings en die voorkoms van die vertakte oligomere. Gel of papier chromatografie fraksies van die kompleks-ekstrakte word benadeel deur swak resolusie as gevolg van hul hidroksie fenoliese natuur en pogings om suiwer verbindings te isoleer was beperk tot die isolasie van hoofsaaklik monomere en slegs ‘n paar dimere en trimere.

PACs van die kommersieel belangrike quebracho (*Schinopsis lorentzii* en *Schinopsis balansae*) en swartwattel (*Acacia mearnsii*) ekstrakte het ‘n sterk en stabiele interflavaniel binding. Hierdie stabiliteit is veral belangrik uit ‘n industriële oogpunt aangesien dit tot duursame leer en kleefmiddels lei. Dit word toegeskryf aan die afwesigheid van 5-OH groepe in die aromatise eenhede van die verlengde fisetinidol en robinetinidol flavan-3-ols eenhede, maar van ‘n analitiese oogpunt is dit nie voordelig nie. Die hoë temperature wat nodig is om hidrolise van die interflavaniel binding met swak sure te veroorsaak, lei tot die

ontbinding van die intermediêre monomere en dus is metodes soos tiolise en floroglusinolise waarmee PACs konvensioneel geanaliseer word, onbetroubaar.

Ons het gebruik gemaak van elektrosproei-ionisasie massaspektrometrie (ESI-MS) in hierdie tesis om die molekulêre samestelling van die PACs in swartwattel ekstrakte te ondersoek, asook die veranderinge wat plaasvind in die chemiese samestelling van quebracho PACs gedurende sulfitering. Ons het reedsbestaande fito- en sintetiese chemiemetodes gekombineer met ESI-MS om lig op die chemiese samestelling van wattel PACs te werp.

Vorige navorsing in ons groep het vasgestel dat quebracho PACs altyd katesjien as begineenhede bevat waaraan een, twee of meer fisetinidol verlengingseenhede aan gebind is. Die eerste en tweede verlengingseenhede is altyd aan die relatief reaktiewe floroglusinol A-ring van die katesjien begineenheid gebind om hoofsaaklik dimere en trimere te vorm. Dit verduidelik die lae graad van polimerisasie van quebracho PAC ekstrakte en hul gewildheid as leerlooimiddel. Groot PACs kan nie die spasies tussen velproteïene binnedring nie en kan dus nie vir leerlooiery gebruik word nie.

In hierdie tesis het ons vasgestel dat swartwattel PACs nie net katesjien begineenhede bevat nie, maar ook gallokatesjien begineenhede. Dit bevat benewens fisetinidol, ook robinetinidol verlengingseenhede. Acacia PACs is dus ‘n baie meer komplekse kombinasie van katesjien, gallokatesjien, fisetinidol en robinetinidol monomere. Dit is in teenstelling met quebracho PACs wat slegs katesjien en fisetinidol monomere bevat. Die hoër graad van hidroksilering van gallokatesjien en robenitinidol verduidelik die verhoogde wateroplosbaarheid van swartwattel PACs en die minder gereelde behoefte aan sulfitering.

Ons het ook vasgestel dat met sulfitering van quebracho PACs, ‘n sulfoonsuurgroep aan beide die C-2 en C-4 posisie van die piraan heterosikliese C-ring gevoeg word. In die geval van C-2 sulfitering, word die heterosikliese ring geopen. Dit verhoog die reaktiwiteit van die A-ring teenoor formaldehied (kleefmiddelformasie) en verhoog ook wateroplosbaarheid as gevolg van ‘n minder rigiede struktuur en die toevoeging van ‘n addisionele

sulfoonsuurgroep. In die geval van C-4 sulfitering, word die interflavanielbinding gebreek. Polariteit en wateroplosbaarheid word dus nie slegs *via* ‘n addisionele sulfoonsuurgroep verhoog nie, maar ook as gevolg van die teenwoordigheid van korter oligomere en korter gemiddelde kettinglengte. Ons het ook ‘n chromatografiese metode ontwikkel om die graad van sulfitering van gesulfiteerde quebracho PAC ekstrakte mee te bepaal.

Ons glo dat ons ‘n waardevolle bydrae gelewer het ten opsigte van die samestelling van swartwattel en gesulfiteerde quebracho PAC ekstrakte en dat ons het ‘n aantal wanbegrippe uit die weg geruim het.

Chapter 1

Introduction

The complex nature of tannins, some of which are of economic importance, has stimulated research into their chemistry, composition and properties for almost a century. Tannins are classified into condensed and hydrolysable tannins. Hydrolysable tannins are esters of sugar, mostly glucose, and gallic acid or gallic acid derivatives. Condensed tannins are oligomers and polymers of flavan-3-ol flavonoid monomers. Polymers with a chain length of up to 223 have been described. The covalent interflavanyl bond between the flavan-3-ol monomers are uncharacteristically labile and can be hydrolysed with weak acid to give incipient benzylic carbocations that are further oxidised to coloured anthocyanidins. Hence the term proanthocyanidin (PAC) which is used synonymous with the term condensed tannin.

The word tannin comes from the Celtic word for oak tree. Skins and oak bark were left together in water for long periods. The water soluble PACs slowly migrated from the bark to the skin and reacted with the skin proteins to form leather. Leather production, probably the oldest human industry, played an essential role in human survival, particularly in cold and wet climates. Leather also resists water penetration and has a soft feel that makes it comfortable to wear as clothing. This reaction is believed to be hydrogen bonding between the polyphenols and amino acids. Leather is, in contrast with dried skin, resistant to bacterial and fungal degradation. The same interaction explains astringency in foods that contains tannins. Human taste buds are proteins. Mild astringency is important in the taste of beverages such as tea and red wine. The biological task of tannins in plants is probably protection against herbivores and other organisms *via* complexation with protein based digestives enzymes. Fungi, bacteria, and viruses also contain proteins that are destroyed.

The industrial use of hydrolysable tannins is currently limited by the small quantities that are commercially available. Oak and chestnut extracts are still used to produce high-value speciality leathers. In contrast, PACs are more readily available. The wattle extract is

obtained from the bark harvested from agricultural plantations of *Acacia mearnsii*. It thus represents a sustainable source of industrial raw material. These plantations are mostly in South Africa and create employment in poor rural areas. The heartwood of *Schinopsis balansae* and *Schinopsis lorentzii* from natural forests in South America are extracted to obtain quebracho extract, which represents an alternative commercial source of PACs. Mineral tanned leathers, that competes with wattle bark or quebracho heartwood extract tanned leathers, contains toxic metals, predominantly chromium, and may represent a serious environmental threat. This is particularly relevant when old leather products, such as car seats, are disposed. The reactive phloroglucinol and resorcinol A-rings present in PACs, react with formaldehyde to form CH_2 links between PAC molecules. This forms the basis of an adhesive manufacturing industry. Adhesives currently consume similar amounts of PACs than the leather industry.

PACs are extremely complex and variables include the chain length, stereochemistry of the heterocyclic pyran C-rings, and the degree of oxygenation (number of hydroxyl groups on the aromatic rings). PACs can be classified as 5-oxy and 5-deoxy PACs. The presence of a 5-hydroxy group on the A-ring (phloroglucinol type A-ring) of the constituent flavan-3-ol monomers imparts stability to the incipient benzylic carbocation on the heterocyclic C-4 carbon during acid catalysed hydrolysis. This renders the interflavanyl bond labile and facilitates analysis of these tannins *via* depolymerisation methods. This lability allows plants to transform astringent 5-oxy PAC polymers, important to protect green fruit with immature seed from herbivores, to non-astringent colored anthocyanidin monomers. These monomers are also colored and furthermore advertise that the fruit is ripe and ready to be eaten. In this way only seeds from ripe fruit are consumed and distributed.

The resorcinol type A-ring is much less reactive than phloroglucinol towards extender units during polymerisation. This probably explains the low aDP of 5-deoxy PACs (about 5) compared with 5-oxy-PACs, where aDP's of more than 200 has been described. The small polymer/oligomer size is however important for leather tanning as it allows the PACs to penetrate between skin protein fibres and cross link the fibres to transform the skin into leather. The 5-deoxy PACs have stable interflavanyl bonds because the resorcinol type A-ring is less able than phloroglucinol to stabilise the incipient carbocation during acid-

catalysed hydrolysis. The higher temperatures thus required to hydrolyse this bond makes depolymerisation methods unreliable and 5-deoxy extracts have not been analysed successfully with thiolysis, phloroglucinolysis, or other depolymerisation methods. The stability of the interflavanyl bond of 5-deoxy PACs is thus essential for the stability and the durability of the resulting leather. The complexity of PACs has so far prevented successful chromatographic analysis of PAC extract as far as total composition and nature of higher oligomers present are concerned.

A large portion of the extracts are sold as sulfited extracts. The natural extract is obtained *via* treating acacia bark or quebracho heartwood chips with boiling water. Further boiling of this extract with different levels of bisulfite yielded sulfited products with properties that are attractive to the industry. The chemical compositions of these sulfited extracts have been poorly investigated and little is known about the chemical changes that takes place during sulfitation. This is mostly due to the difficulty of purifying PACs with silica based chromatography, including reversed-phase materials, due to strong interactions of polyphenols with silica gel. Even the amount of sulfur incorporated during sulfitation has never been established satisfactorily. The water soluble bisulfite starting material cannot be separated from the water soluble unsulfited or sulfited PACs.

The need thus exists to develop improved methods to analyse complex PAC extracts, particularly the industrially important quebracho and acacia 5-deoxy extracts. A better understanding of the composition will have many benefits including:

1. Improved certificates of analyses that will satisfy regulatory authorities that commercial natural and sulfited acacia and quebracho extracts are safe. This is particularly relevant since EU REACH regulations are becoming more stringent and threatens access of commercial PAC extracts to Europe.
2. Manufacturing of standardised extracts where the composition of different batches manufactured at different times from trees from different regions and plantations have a constant composition.

3. A better understanding of the chemical changes that takes place during sulfitation will lead to more efficient manufacturing of sulfited products.
4. Identification of new plant sources of raw materials.
5. A better understanding of the chemistry of leather tanning and adhesive manufacturing and thus more efficient processes and better standardised products.
6. Possible new applications such as improved water purification resins that will be more acceptable to the market.
7. The ability to identify adulterated PACs extracts. This is becoming a problem as even small quantities of PACs in an extract react positively to the currently used tests.
8. Identification of natural products that are adulterated with PACs. For example PAC extracts are sometimes used to improve the taste of poor quality wines.

Mass spectrometry (MS) is a technique with a very high resolution that can easily distinguish between molecules that differs only one Dalton in mass. It can thus easily distinguish between oligomers that contain flavan-3-ol building blocks that differ in the number of hydroxyl groups present (e.g. catechin, gallocatechin, fisetinidol, and robinetinidol). Unfortunately MS does not differentiate between stereoisomers with the same *m/z* values. Daughter ion analysis (MS^2 etc.) may, however, differentiate between configurational isomers.

We thus analysed whole (unchromatographed) 5-deoxy PAC extracts (normal and sulfited acacia and quebracho) with MS to establish their composition and investigated the changes that take place during sulfitation. We used perspectives developed from existing phytochemical analysis (structures of monomers, dimers, and trimers isolated from acacia bark and quebracho heartwood) and synthetic chemistry (synthesis of dimers, trimers, and tetramers) to guide our MS interpretation. We thus postulated the following:

1. The PAC oligomers present in acacia and mimosa bark extract will be based on the monomers that are present in the bark, and the dimers and trimers detected by MS will mirror the dimers and trimers that have already been isolated *via* phytochemical investigations. We thus made a thorough analysis of all the monomers, dimers, and trimers that have so far been reported from the literature. No tetramer or higher oligomers have been reported as pure compounds

2. In contrast with commercial quebracho heartwood extract, which consists of catechin starter units and fisetinidol extender units, the starter unit in black wattle will be either catechin or gallocatechin angularly bonded to fisetinidol or predominantly robinetinidol extender units.

3. Sulfitation of quebracho will occur *via* opening of the heterocyclic pyran ring to give M+82 products (addition of a sulfonic acid moiety and two protons due to ring opening) or *via* fission of the interflavanyl bond to give an M-fisetinidol and fisetinidol + 82 product.

Investigating the literature revealed that the dimers and trimers isolated so far from phytochemical investigations closely resemble the dimers and trimer synthesized *via* biomimetic methods in terms of configurational- and stereochemistry. *In vitro* and *in vivo* synthesis thus follows the same rules.

Our MS results with dimers and trimers closely mirror what we would expect from phytochemical and synthetic considerations. We extrapolated these results to higher oligomers and believe we have made a valuable contribution to the knowledge of the chemistry and composition of acacia and quebracho PAC extracts. This work has resulted in two publications in *Phytochemistry*:

Chapter 3

Venter, P.B.; Senekal, N.D.; Amra-Jordaan, M.; Bonnet, S. L.; van der Westhuizen J. H. Analysis of Commercial Proanthocyanidins. Part 2: An Electrospray Mass Spectrometry

Investigation into the Chemical Composition of Sulfited Quebracho (*Schinopsis lorentzii and Schinopsis balansae*) Heartwood Extract, *Phytochemistry*, **2012**, *78*, 156-169.

Chapter 4

Venter, P.B.; Senekal, N.D.; Amra-Jordaan, M.; Khan, P.; Kemp, G..; Bonnet, S. L.; van der Westhuizen J. H. Analysis of Commercial Proanthocyanidins. Part 3: The Chemical Composition of Wattle (*Acacia mearnsii*) bark extract, *Phytochemistry*, **2012**, *83*, 153–167.

Chapter 2

Literature Review

2.1. Introduction to Vegetable Tannins

Vegetable tannins are astringent, water soluble, polyphenolic secondary metabolites with a relatively high molecular weight (500 to over 3000 Da). They occur ubiquitously in plants. They characteristically bind and precipitate proteins and carbohydrates (Eberhardt *et al*., 1994; Serrano *et al*., 2009; Haslam, 1998; Yanagida *et al*., 2003). The name "tannin" is derived from the ancient Celtic word "tan", for oak trees (Haslam, 1998). The bark of oak trees was used to convert animal hides to leather. This practice was employed by primitive tribes to increase the longevity of their hides and skin clothes, and improves the feel and renders them water repellent. The capability of tannins to complex with proteins *via* hydrogen bonds explains the use of tannins for leather tanning (Khanbabee and van Ree., 2001; Haslam, 1998). The term tannin refers to both hydrolysable tannins, polyesters of gallic acid or hexahydroxydiphenic acid and D-glucose (Figure 1), and condensed tannins (oligomers of flavan-3-ol monomers) (Figure 2) (Haslam, 1977; Khanbabee and van Ree. 2001; Pizzi, 2008). The term condensed tannin and proanthocyanidin (PACs) are synonymous. The term proanthocyanidin refers to the red color that develops upon treatment of condensed tannins with dilute acid. The interflavanyl bond is hydrolysed and colored anthocyanidins are formed (Scheme 1). Hydrolysable tannins do not form colored compounds under the same conditions (Serrano *et al*., 2009; Schofield *et al*., 2001; Santos-Buelga and Scalbert, 2000; Roux, 1992).

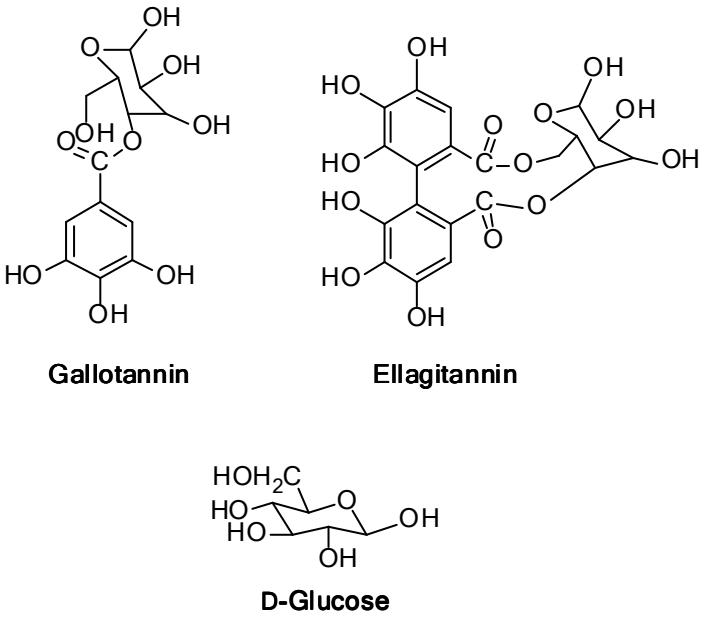


Figure 1: Examples of hydrolysable tannins

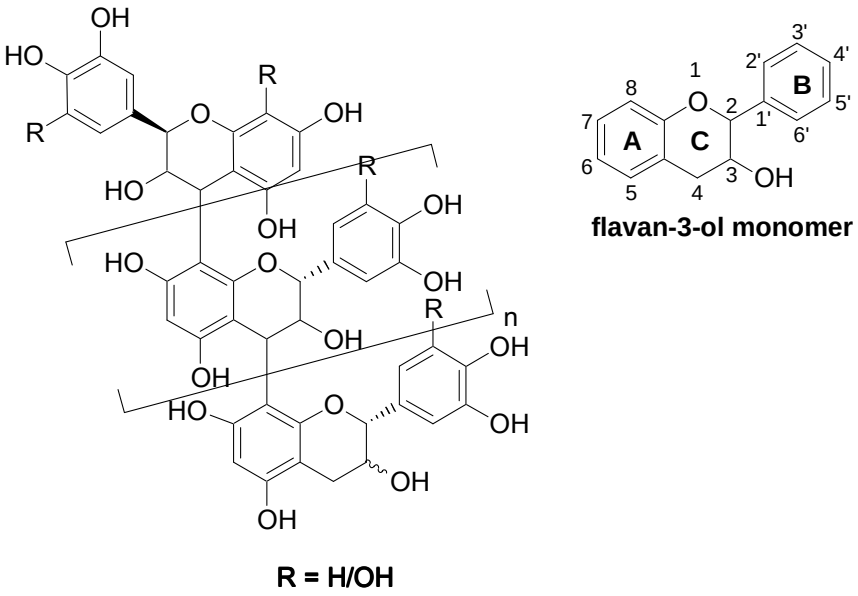
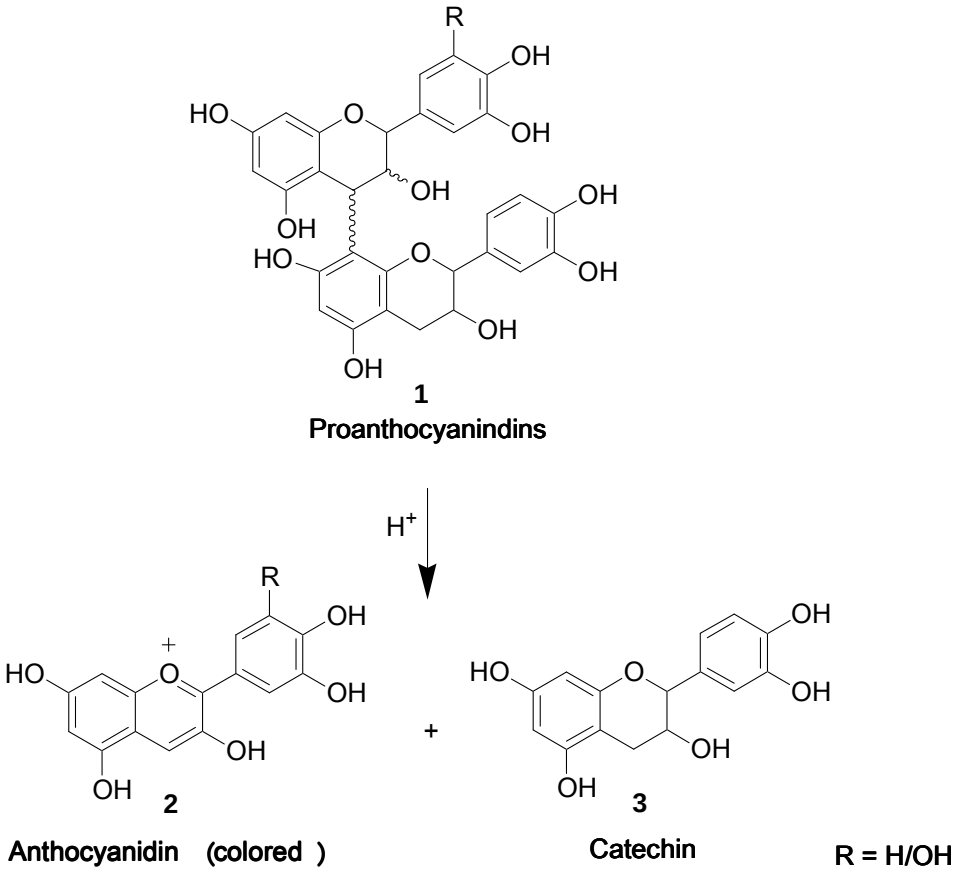


Figure 2: General structure of a proanthocyanidin

PACs are nowadays commercially more important than hydrolysable tannins due to the latter's inadequate worldwide production and elevated price (Pizzi, 2008).



Scheme 1: Hydrolysis of the interflavanyl bond to form anthocyanidins

Although PACs and hydrolysable tannins differ significantly in terms of the monomer constituents that comprise their oligomeric structures, both are polyphenols with large numbers of hydroxyl groups. These hydroxyl groups dominate their physical and chemical properties and explain the considerable overlap in biological functions and industrial applications between the two classes of tannins (Santos-Buelga and Scalbert, 2000).

Vegetable tannins have to a large extent been substituted by mineral tanning agents i.e. aluminium, chromium, zirconium salts in the commercial tanning of animal skins. Chromium salts, the more important mineral tanning agent (Sundar, 2001, 2002), is however toxic and its derisory disposal causes long term negative effects on human health and the environment stimulating renewed interest in vegetable tannins (Belay, 2010).

Other industrial uses of tannins include adhesive manufacture (Pizzi 2003, 2008), water purification resins (Beltran-Heredia *et al*., 2009; Beltran-Heredia and Sanchez-Martin, 2008, 2009), as mud additives for oil well drilling (Haslam, 1988; Herrick, 1980), and as iron anti-corrosion agents (Jaén *et al*., 1999; Matamala *et al*., 2000; Seavell, 1978). These applications are based on the *ortho* B-ring OH groups of the constituent flavan-3-ol monomers, which form insoluble complexes with heavy metals (Venter *et al*., 2012, Haslam, 1998).

Most trees contain tannins, mostly in the bark. Those of economic and industrial importance include:

1. PACs from black wattle bark (*Acacia mearnsii*), quebracho heartwood (*Schinopsis balansea* or *lorentzii*), *Tsuga* (hemlock bark extract), *Rhus* (sumach extract), and several species of pine and firs (*Pinus radiate* and *Pinus nigra*). PAC extracts from the bark of wattle trees (*Acacia mearnsii,* South Africa) and heartwood of quebracho (*Schinopsis lorentzii,* South America) are important industrial raw materials for leather tanning and adhesive manufacturing (Khanbabee and van Ree, 2001; Haslam, 1998; Pizzi, 2003, 2008).

2. Hydrolysable tannins include chestnut (*Castanea sativa*), myrabolans (*Terminalia* and *Phyllantus* tree species), divi-divi (*Caesalpina coraria*), tara, algarobilla, valonea, and oak (*Quercus spp*.) (Pizzi, 2003).

Tannins have traditionally also been used as medicines, especially in Asian constituencies. These tannin-containing plant extracts are used as astringents, against diarrhoea, diuretics, against stomach and duodenal tumours, and as anti-inflammatory, antibacterial, and haemostatic pharmaceuticals (Khanbabee and van Ree, 2001). They precipitate heavy metals and alkaloids (with the exception of morphine) and can thus be used as an antidote in poisoning with these substances (Khanbabee and van Ree, 2001; Pizzi, 2008).

Tannins are important in the food and beverage industry. Tannins are responsible for the astringent taste of Indian tea, a universal beverage prepared *via* fermentation of the leaves of

Camellia sinensis, a tropical ever-green plant. Chinese tea, also known as green tea, comprises unfermented *Camellia sinensis* leaves (Yang *et al*., 2007, 1993). They are added to poor quality red wine (Roux *et al*., 1962, 1975; Bate-Smith, 1954) to enhance mouth feel properties and even play a role in the taste of beer (Outtrup, 1992).

2.2. Classification of Tannins

As mentioned above, PACs (Figure 2) yield coloured anthocyanidins on heating with mineral acid *via* cleavage of a C-C interflavanyl bond (Scheme 1) (Serrano *et al*., 2009; Schofield *et al*., 2001; Santos-Buelga *et al*., 2000; Roux, 1992). The flavan-3-ol monomer units have the characteristic C_6-C_3-C_6 flavonoid skeleton and differ structurally according to the hydroxylation pattern in ring A and ring B and configuration at C-2, C-3, and C-4 (Serrano *et al*., 2009; Santos-Buelga and Scalbert, 2000).

2.2.1. A-Type Proanthocyanidins

A-Type PACs contain in addition to the C-C interflavanyl bond an ether interflavanyl bond between the aromatic D-ring of the lower monomer and C-2 of the heterocyclic C-ring of the top monomer. Figure 3 illustrates two examples of A-type PACs **4** and **5** (Serrano *et al*., 2009; Santos-Buelga and Scalbert, 2000; Achilonu, 2009).

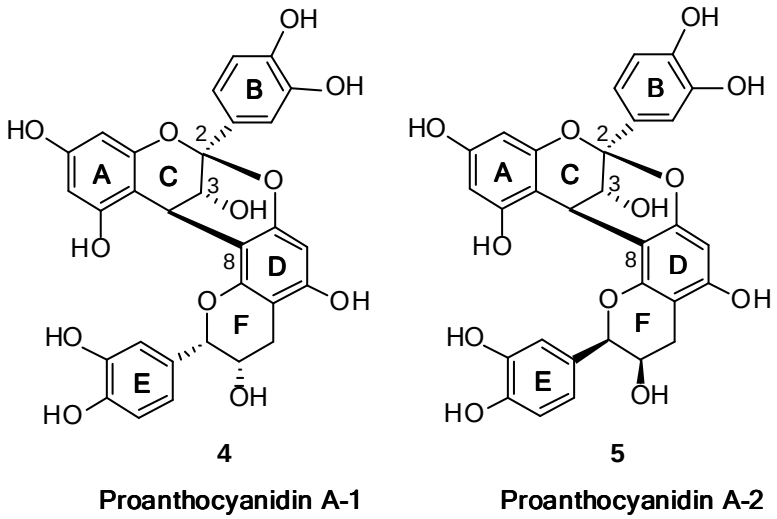


Figure 3: Examples of A-type proanthocyanidins

2.2.2. B-Type Proanthocyanidins

B-type PACs have a single C-C bond between the benzylic position on the flavan-3-ol monomer and the aromatic 6- or 8-position on the other constituent monomer as described above. They are classified according to the hydroxylation pattern of the aromatic rings and the stereochemistry of the heterocyclic C-ring. Procyanidins (R = H) and prodelphinidins (R = OH) are prevalent (Figure 4) (Haslam, 2007; Serrano *et al*., 2009). The most common dimers are the B1-B4 procyanidins, (Figure 4) (Serrano *et al*., 2009; Santos-Buelga and Scalbert, 2000).

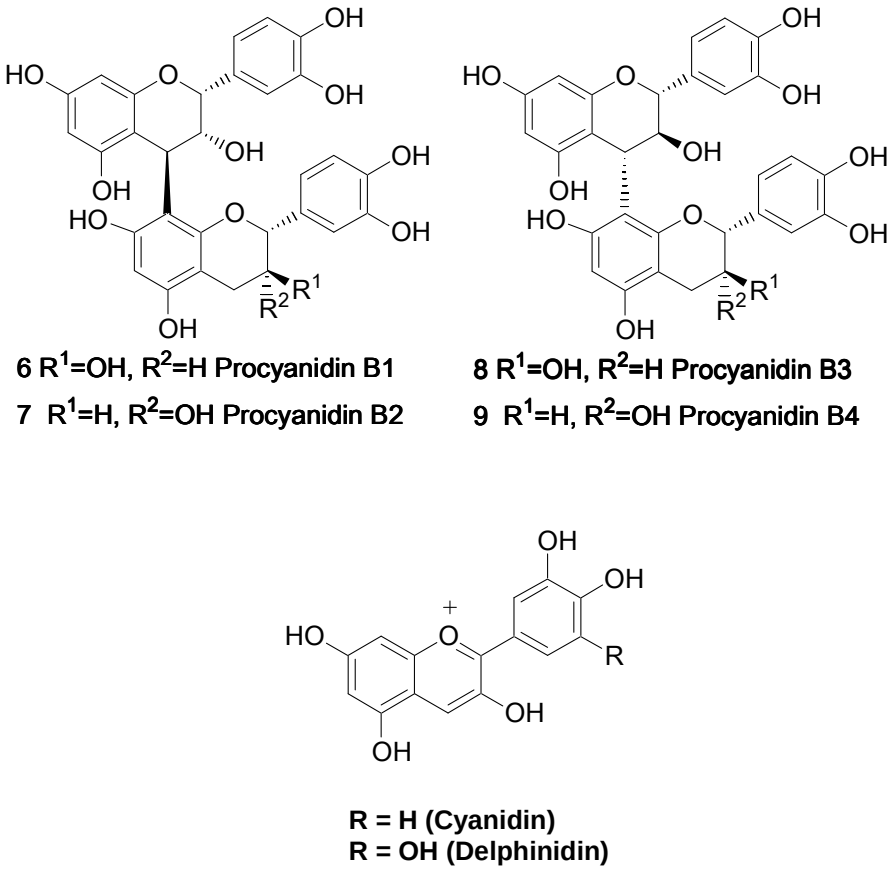


Figure 4: Examples of B-Type proanthocyanidin dimers

2.2.3. C-Type Proanthocyanidins

C-type proanthocyanidins are trimers (Figure 5). These trimers consist of C-C bonds between the C-4 of one flavan-3-ol monomer and the C-8 or C-6 of another flavan-3-ol monomer **(**Santos-Buelga and Scalbert, 2000**).**

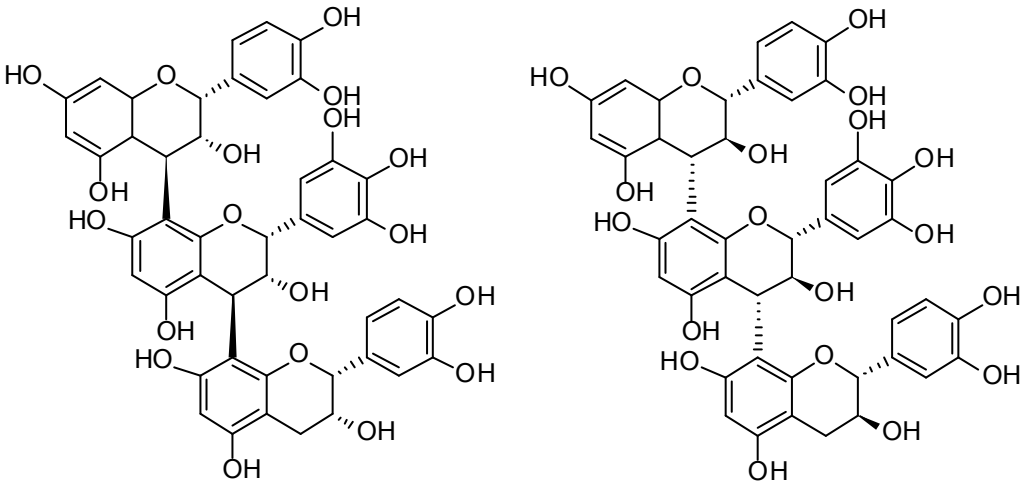
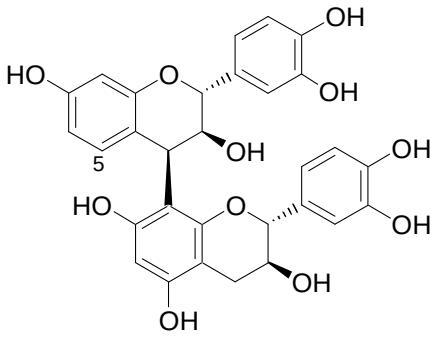


Figure 5: Examples of C-Type proanthocyanidins

2.2.4. 5-Deoxyproanthocyanidins

The commercially important PACs from wattle bark and quebracho heartwood do not have a hydroxyl group in the 5-position of the extender units (Figure 6). This renders the interflavanyl bond stable to acid-catalysed hydrolysis. These PACs still develop a red color upon heating with acid but higher temperatures are required. They can be profisetinidins with fisetinidol extender units, or prorobinetinidins with robinetinidol extender units. The absence of a 5-OH group and the higher temperatures required for hydrolysis have important consequences as far as the analysis of these PACs and their industrial applications are concerned (see below) (Roux, 1992; Venter *et al.*, 2012).



5-deoxy dimer

Figure 6: Example of a 5-deoxy PAC dimer.

2.2.5. Bi- and triflavonoids

The term bi- or triflavonoids are sometimes reserved for dimeric or trimeric flavonoids that are not attached *via* the C-4 position of the heterocyclic ring. The interflavanyl bond thus resists hydrolysis and does not form anthocyanidins upon heating with dilute acid. The C-4 position is often a carbonyl. The basic structure of a biflavonoid is illustrated in Figure 7 (Ferreira *et al*., 2006; Achilonu, 2009).

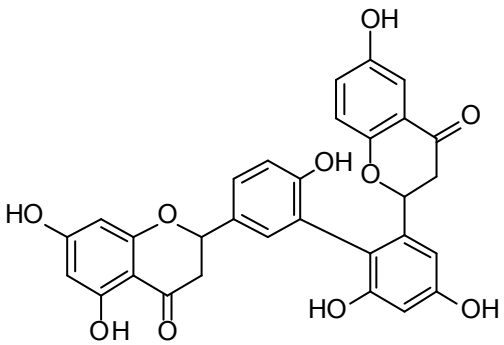


Figure 7: Example of a biflavonoid

2.3. Hydrolysable Tannins

Serrano and co-workers (2009) define hydrolysable tannins as polyesters of sugar moieties and organic acids. Figure 1 illustrates gallotannins (galloyl esters of glucose) and ellagitannins (hexahydroxydiphenic acid esters). Figure 8 shows an example of a complex hydrolysable tannin (gallotannin or ellagitannin linked *via* a C-C bond to a flavan-3-ol).

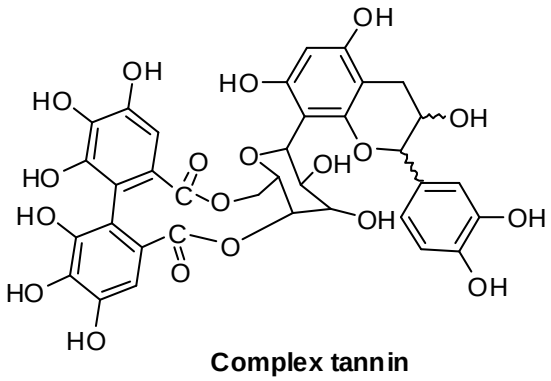


Figure 8: Example of a complex hydrolysable tannin

2.4. Analysis of Proanthocyanidins

The analysis of tannins has been challenging. This is due to the structural complexity of a heterogeneous mixture of hydrolysable or condensed tannins of different chain lengths, different substitution patterns, and different stereochemistry on the C-ring. The polyphenolic nature furthermore renders them difficult to purify with conventional silica gel based chromatography, including reversed-phase. Wide variations occur between tannins from different plant species. Other organic molecules such as gums and sugars may also interfere with analysis (Schofield *et al*., 2001; Venter *et al*., 2012). Furthermore, the extraction method and state of the sample may lead to wide variations in results (Scalbert, 1992).

The flavan-3-ol monomer building block base of proanthocyanidins was only generally accepted after 1951. Freudenberg (1934) was the first to suggest that PACs consisted of a complex mixture of flavan-3-ol monomers, condensed to form oligomers with variations in the average degree of polymerization (aDP). Paper chromatographic studies confirmed this hypothesis (Asquith, 1951; Roberts and Wood, 1951; White *et al*., 1951, 1952). This was followed by rapid progress in the isolation and characterisation of flavan-3-ol monomers and other flavonoid monomers that are not precursors of PACs. Paper chromatography, although tedious and time consuming, was suited to hydrophilic polyphenols and PACs up to tetramer level was obtained and characterised as pure compounds (Roux, 1958).

Wide variations in the average degree of polymerization (aDP) of PACs have been reported. According to Jones and co-workers (1976) who analysed the leaves of *Trifolium affine*, it may be 20-30. Souquet and co-workers (1996) analysed grape skins and it was determined at 83. Guyot and co-workers (2001) analysed cider apples and determined it at 190 units (Sun and Spanger, 2005).

At this stage we should distinguish between research aimed at the isolation and characterisation of pure compounds and synthesis based structure elucidation (phytochemistry), and the determination of aggregate polymer characteristics of whole unchromatographed PAC extracts (e.g. aDP). We will not give further attention to hydrolysable tannins, except where they interfere with the analysis of PACs. We assume that aggregate polymer characteristics are caused by the structure and number of the constituent monomers.

Quantitative PAC assays have traditionally been based on their ability to form complexes with alkaloids, proteins, or metals (gravimetric methods), the chemical reactivity and UV absorbance of their constituent phenolic rings (colorimetric methods), and depolymerisation (e.g. thiolysis) (Schofield, *et al*., 2001). Due to technological advancements in chromatographic methods, NMR and MS techniques have more recently been developed to analyse PACs.

2.4.1. Colorimetric Assays For Total Phenolics:

A variety of methods have been developed, based on the chemical transformation of the aromatic hydroxyl groups into coloured compounds and the measurement of the quantity of light absorbed by these compounds (Roux, 1957; Schofield *et al*., 2001). These quantitative methods and a good understanding of the underlying chemistry, have over the years, been a valuable source of information on the composition of PACs. For example, in the early days, when it was not evident that PACs contain flavan-3-ol subunits, some of these tests proved that PACs contain phenolic building blocks. The lead acetate method later proved that some of the aromatic rings have *ortho* hydoxy substitution patterns etc. It is furthermore important to know what percentage of the crude extract consists of PACs.

2.4.1.1. Measurement Of The Total Phenol Content

These methods are not specific for PACs and quantify the total concentration of phenolic hydroxyl groups in the plant extract (Schofield *et al*., 2001). Most of these methods do not distinguish between PACs and hydrolysable tannins. These methods rely on oxidation of the phenolate ion with $Fe(CN)_6^{3-}$ (The Prussian Blue Method) (Price and Buttler, 1977), or phosphotunstic-phosphomolybdic compounds (the Folin-Denis assay). Many improvements and modifications including the Folin-Ciocalteau method have been reported in an effort to enhance precision (Schofield *et al*., 2001).

2.4.1.1.1. Prussian Blue Assay

Polyphenols react with a mixture of $K_3Fe(CN)$ and $FeCl_3$ to give $Fe_4[FeCN_6]_3$ (Prussian Blue). The amount of Prussian blue is proportional to the amount of polyphenols present and forms the basis of an easy and cheap colorimetric method to quantify the total phenolics (Graham, 1992; Schofield *et al*., 2001; Santos-Buelga and Scalbert, 2000). Drawbacks include the formation of a precipitate and increase in color intensity with time.

$$\text{Polyphenol} + 2Fe(CN)_6^{3-}\ (\text{ferricyanide ion}) \longrightarrow Fe_4[Fe(CN)_6]_3\ (\text{Prussian Blue})$$

2.4.1.1.2. The Folin-Ciocalteau Assay

The Folin-Ciocalteau assay is an improved version of the Folin-Denis method. It was developed to measure tyrosine in proteins but all phenols will react. The chromophore is a phosphotunstic-phosphomolybdic complex of unknown structure and the chemistry of the reaction is not well understood (Tsao and Yang, 2003; Lapornik *et al*., 2005; Schofield *et al*., 2001; Ignat *et al*., 2011)**.**

2.4.2. Acid-Butanol Colorimetric Assay

This reaction involves the use of acid-catalysed oxidative depolymerisation of proanthocyanidins to yield red colored anthocyanidins. It relies on the labile nature of the interflavanyl bond that can be hydrolysed easily with weak acid (Scheme 1). This assay is often used qualitatively to confirm the presence of proanthocyanidins in plant tissues. Its quantitative use is however limited by many factors including the following (Gina-Chavez *et al.*, 1997; Schofield *et al*., 2001):

a) The amount of water present influences the yield of anthocyanidins.

b) The strength of the interflavanyl bond is determined by the nature of the A-ring and whether 4→6 or 4→8 bonds are involved. All PACs thus do not give the same cyanidin yields. Quebracho and wattle tannins for example, with no hydroxyl groups in the 5-position, are known to resist acid hydrolysis and cannot be quantified reliably with this method (Gina-Chavez *et al*., 1997).

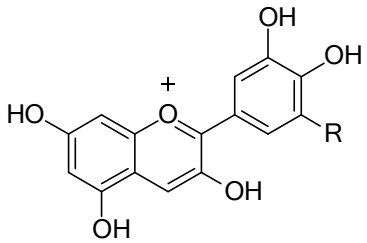
c) The acid-butanol ratio may influence the anthocyanidin yield.

d) The number of hydroxyl groups on the A- and B-rings may influence the wavelength of the absorbance maximum and extinction coefficient. For example, cyanidin and delphinidin (Figure 9) have λ_{max} at 545 and 557 respectively (Hemmingway, 1989).

e) Color yield is not always linear with the amounts of PACs present.

f) Trace amounts of metal ions may influence the color yield (Hagerman *et al*., 1997; Scalbert, 1992; Porter *et al*., 1986).

g) Anthocyanidins are known to be unstable and efforts to isolate them give poor yields.

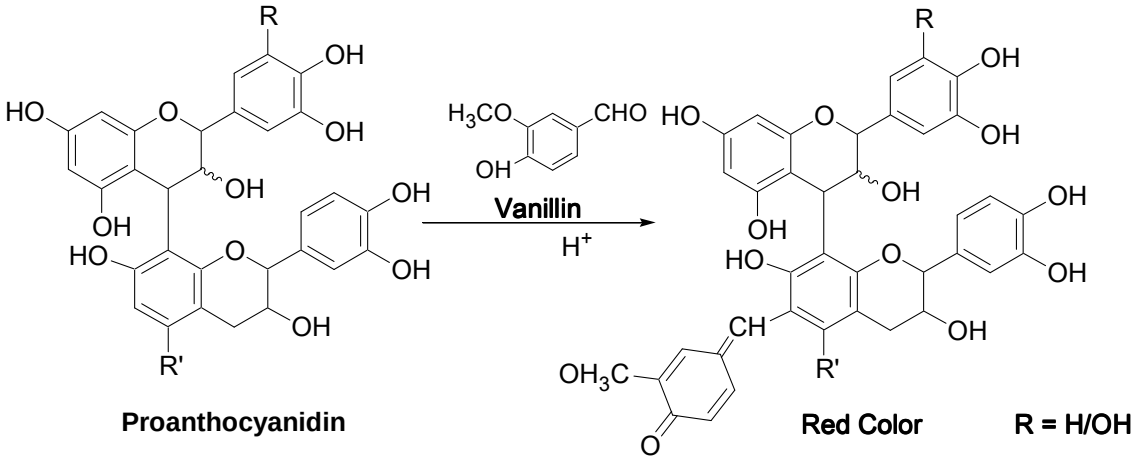


12 R = H (Cyanidin)
13 R = OH (Delphinidin)

Figure 9: Cyanidin and Delphinidin Structures

2.4.3. Vanillin Assay

PACs react with vanillin under acidic conditions to form colored complexes (Scheme 2).



Scheme 2: Vanillin Reaction

Factors such as the type of solvent used, concentration of the acid, temperature, vanillin concentration, etc. may influence the colour intensity. The vanillin assay is not specific for PACs as some monomeric flavanols also react with vanillin. The reactivity of monomers (catechin) towards vanillin is higher in an acidic environment than PACs. Catechin can thus be used as a reference standard (Sun and Spranger, 2005). Many of the problems associated with this method seem to parallel those associated with the butanol-HCl assay (Schofield *et al*., 2001; Scalbert *et al*., 1992; Hagerman, 1998; Sun *et al*., 1998; Naczk and Shahidi, 2006).

2.4.4.Precipitation Methods

Precipitation methods may be used to purify the PAC fraction in plant extracts and remove other molecules that may interfere with subsequent gravimetric or colorimetric assays. The well known interaction between PACs and proteins, which form the basis of their leather tanning ability and their anti-feedant, anti-bacterial and anti-fungal activity, has been used to precipitate and purify PACs. Kaolin, PEG, lead, and polyvinylpyrrolidone have also been used as precipitating agents (Makkar, 1989; Schofield *et al*., 2001; Venter *et al*., 2012).

2.4.4.1. Protein Precipitation Assays

Methods based on the precipitation of proteins have been reviewed by Makkar (1989). Leather chemists use a method based on percolating a tannin solution in a column filled with hide powder and measuring the increase in weight of the powder. The most obvious problem is that the composition of hide powder is difficult to standardise and it is time consuming. Improvements consist of replacing the hide powder with protein solutions. The accuracy of these methods can be questioned and it has been shown that the type of protein used and the nature of the extract may influence the results.

2.4.4.1.1. The Bate-Smith (1973) Method is based on the precipitation of the haemoglobin of haemolyzed blood and the colorimetric determination of the remaining unprecipitated haemoglobin at 578 nm. It requires fresh blood and does not discriminate between condensed and hydrolysable tannins.

2.4.4.1.2. The Hagerman and Butler (1978) Method is based on the precipitation of tannins with bovine serum albumin (BSA). The protein-tannin complex precipitate is subsequently dissolved in a detergent system consisting of 1% sodium dodecyl sulphate and 5% triethanolamine in water and the tannins are measured spectrophotometrically at 510 nm after oxidation to coloured compounds with ferric chloride.

2.4.4.1.3. The AOAC (1965) Method (Association of Official Agricultural Chemists) is based on the precipitation of tannins by gelatine, hide powder, or koaline and oxidation of the precipitated tannin with potassium permanganate. It has been standardized for leather tanning purposes as the official hide powder method.

The precipitation methods were popular due to the simple laboratory equipment required. It, however, requires tedious procedures and often gives unreliable results with low precision. It furthermore does not distinguish between PACs and hydrolysable tannins. It may be useful if tannins from the same source and with similar composition are quantified. It is of particular interest to the leather tanning industry as the extracts analysed do not contain hydrolysable tannins. Monomers and dimers are not precipitated as they cannot link two collagen strands in leather. Large oligomers are also not precipitated as they cannot penetrate between collagen strands. The components that are not precipitated by hide power are referred to as non-tans.

2.4.4.2. Polyvinylpyrrolidone Precipitation Method

Polyvinylpyrrolidone binds irreversibly with PACs and is often used to remove tannins from plant extracts before bioassays are performed. Since tannins precipitate and deactivate proteins, the presence of tannins in plant extracts denatures enzymes and results are false. Makkar and co-workers (1989) used the tannin binding property of polyvinylpyrrolidone to purify PACs (Schofield *et al*., 2001).

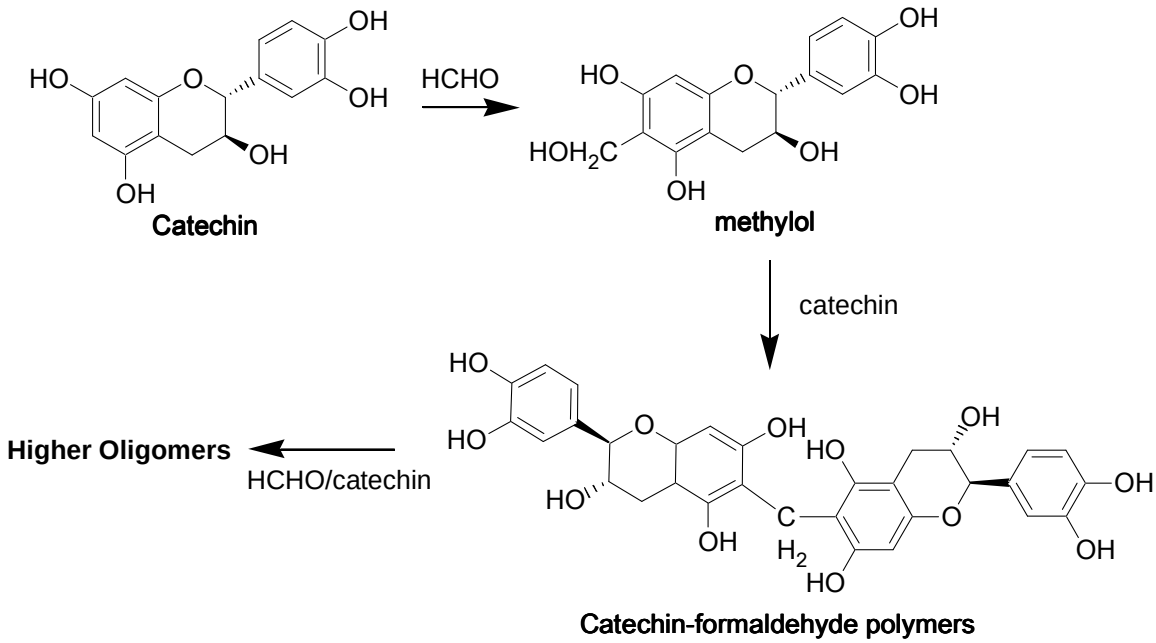
2.4.4.3. Lead Acetate Method

Lead complexes selectively with the catechol moiety present in all PAC B-rings. Roux developed a method to separate gums, PACs, and sugars. The gums are insoluble in absolute ethanol and are precipitated by addition of ethanol to an aqueous PAC solution. The PACs are subsequently precipitated with lead acetate. What

remains in solution represents the sugar fraction. This method gives an accurate value for the PAC content and was used to establish that *Acacia mearnsii* bark extract contains about 75% pure PACs, 13 % sugars, and 11 % ethanol insoluble gums and that quebracho heartwood extract contains about 95 % PACs (Roux, 1952, 1953). Alternate precipitation agents include polyethylene glycol (PEG) and trivalent ytterbium (Schofield *et al*., 2001).

2.4.4.4. Formaldehyde Method

The formaldehyde precipitation method selectively precipitates PACs *via* the 6- or 8-position on the A ring. Formaldehyde reacts with reactive hydroxyl substituted A-rings to form a benzylic methylol derivative that will attach to another 6- or 8-position on another reactive A-ring. The insoluble polymer that forms is removed *via* filtration. The same reaction forms the basis of adhesive manufacturing from PACs (Scheme 3) (Pizzi, 2008). The difference in total phenolic compounds before and after precipitation is determined with the Folin–Ciocalteau method and quantifies the PAC content. Non-PAC flavonoids may also precipitate. (Schofield *et al*., 2001; Kramling and Singleton, 1969; Katalinic *et al*., 2004; Pizzi, 2003)



Scheme 3: Formaldehyde reaction

2.4.5.Depolymerisation

Treatment of PACs with mineral acids leads *via* cleavage of the interflavanyl bond and autoxidation of the resulting carbocation flavan-3-ol monomers to colored anthocyanidins. These absorb at about 550 nm and the intensity of the color can be used to estimate the PAC content (Porter *et al*., 1986). Side reactions that lead to red-brown polymers, referred to as phlobatannins that absorb at about 450 nm, may interfere (Swain *et al*., 1959). The extent of these side reactions are, however, influenced by a variety of conditions that lead to unreliable results.

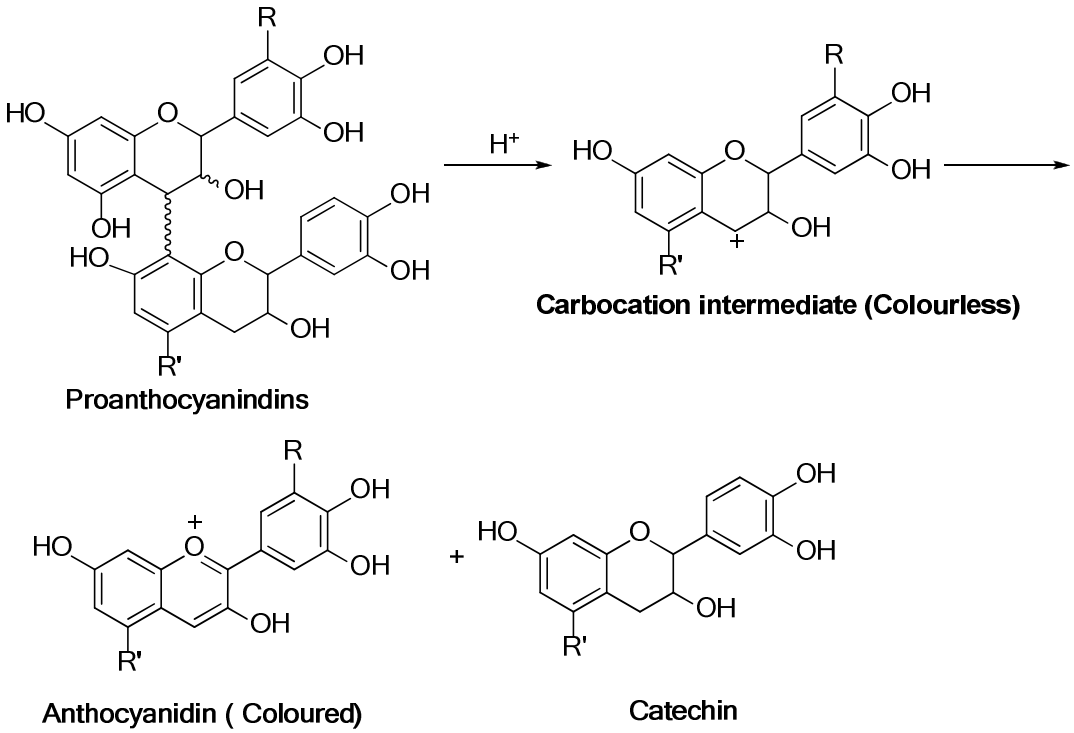
The proportion of water in the reaction mixture is important. Swain and co-workers (1986) reported that replacement of water with isopropyl alcohol or *n*-butanol increased the 550 nm absorption dramatically whilst reducing the 450 nm absorption. 6% water content gives the best results (Govindarajan and Matthew, 1965; Scalbert, 1992). It is assumed that these alcohols stabilise the 4-carbocation *via* ether formation.

The strength of the acid (maximum 20% HCl), temperature (95 °C), and reaction time (15 min) are considered critical (Govindarajan and Matthew, 1965; Scalbert *et al*., 1989; Scalbert, 1992; Jennings, 1981). As only the extender unit and not the starter unit forms anthocyanidins, PACs with shorter chain lengths gives lower absorbance. For example, in the case of dimers, the anthocyanidin formed will represent only 50% of the amount of dimer present. The nature of the extender unit is also important as prorobinetinidins, profisetinidins, and prodelphinidins does not give anthocyanidins, which is normally used as a standard (Scalbert, 1992; Govindarajan and Matthew, 1965).

Structural features are important. It has been reported that 4β→8 linkages are more labile that 4β→6 linkages, that extender units with 2,3-*cis* configuration are converted faster to anthocyanidins than extender units with 2,3-*trans* configuration, and that the hydroxylation pattern of the A-ring is important (Hemingway and Mcgraw, 1983; Govindarajan and Matthew, 1965; Scalbert, 1992).

2.3.5.1. Colorimetry

This method is based on the depolymerisation (cleavage of the interflavanyl bond) of PACs *via* hydrolysis with mineral acids. Autoxidation of the resulting carbocation flavan-3-ol monomers forms colored anthocyanidins. These absorb UV at about 550 nm and the intensity of the colour can be used to estimate the PAC content (Scheme 4) (Porter *et al*., 1986). The UV absorption curve data is obtained by plotting absorption density against concentration.



Scheme 4: Depolymerisation followed by oxidation

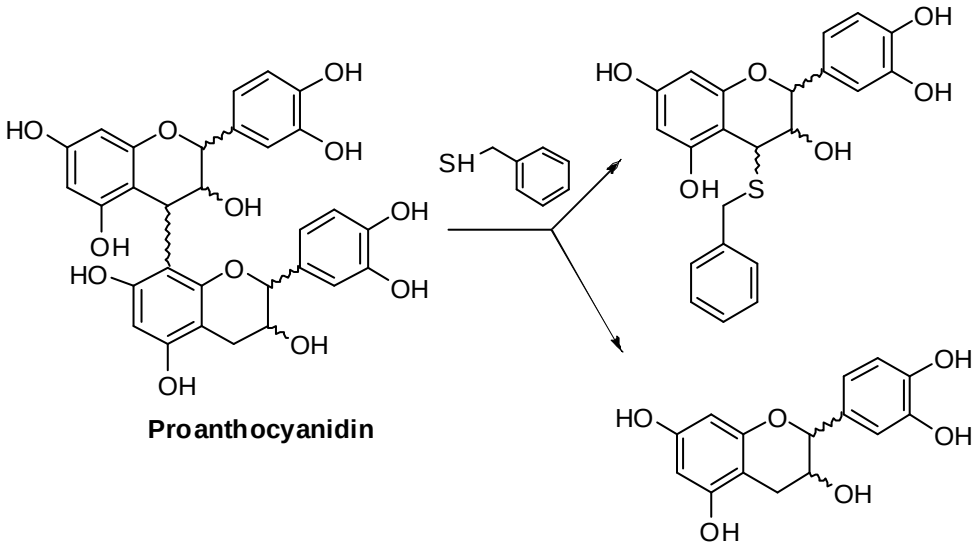
2.3.5.2. Thiolysis and Phloroglucinolysis

This has become a standard technique to analyse PACs. As discussed above, interflavanyl bonds can be hydrolysed with acid to form a benzylic carbocation that is oxidised to anthocyanidins. The incipient carbocations can be trapped if phloroglucinol or benzylmercaptan (toluene-α-thiol) are present in the reaction mixture, stable monomers are formed that can be analysed with HPLC. The terminal unit is released as an unsubstituted flavanol and the extender units as 4-phloroglucinol or 2-mercaptobenzyl substituted flavan-3-ols. The structure of these can be determined *via* comparison with standards or NMR to obtain an accurate picture of the PAC building blocks. The ratio between the unsubstituted flavan-3-ol and the 4-substituted flavanol give the average chain length (degree of polymerisation). Phloroglucinol may be preferred to toluene-α-thiol because it is odourless. Although

toluene-α-thiol is toxic and has an unpleasant odour, it gives higher yields (Scheme 5) (Matthews *et al*., 1997; Sun and Spranger, 2005).

Although this has become the method of choice to analyse PACs with a 5-OH hydroxy group, the reliability of this method is based on assumption. It requires qualitative fission of all interflavanyl bonds and no decomposition of anthocyanidins and the availability of internal standards.

The commercially important quebracho (*Schinopsis balansea* or *lorentzii*) and black wattle (*Acacia mearnsii*) PACs with 5-deoxy extender units, however, have acid resistant interflavanyl bonds that require higher temperatures for hydrolysis and has not been successfully analysed with this method. The method also does not give an indication of the amount of different oligomers present but only the aDP (Schofield *et al*., 2001; Venter *et al*., 2012; Santos-Buelga and Scalbert, 2000; Matthews *et al*., 1997; Guyot *et al*., 1998).



Scheme 5: Thiolysis degradation

2.4.6. Chromatographic Separation Techniques

The low molecular-mass polyphenols, including low molecular mass flavonoids and PACs, have been extensively investigated with a variety of chromatographic techniques, including paper, thin layer normal phase, reversed-phase HPLC, size exclusion, and countercurrent chromatography. The most commonly used columns in chromatography include: Sephadex LH-20, Toyopearl TSK HW-40 (F), Toyopearl TSK HW-40 (S), Toyopearl TSK HW-50 (S), Lichroprep RP-18, and solid phase extraction on C18 Sep-Pak cartridges (Lea and Timberlake, 1974; Boukharta *et al*., 1998; Fulcrand *et al*., 1999; Sun *et al*., 1999b; Ricardo-da-Silva, 1991; Saint-Cricq de Gaulejac *et al*., 1998; De Freitas *et al*., 1998; Meirelles *et al*., 1992; Vidal et al., 2002, Sun et al., 1994, 1998a, 1999b, Jarworski and Lee, 1987; Oszmianski *et al*., 1998; Revilla *et al*., 1991; Sun and Spranger, 2005). The polyphenolic nature, however, often interferes with the chromatographic separation and higher oligomers are not resolved. Due to poor resolution and irreversible binding to the chromatographic oligomer and polymer materials that are identical to the extender units, these methods generally give poor results and high levels of analytical skills are thus required (Schofield *et al*., 2001; Ignat *et al*., 2011; Yanagida *et al*., 2003, Flamini, 2003).

2.4.6.1. Paper Chromatography

This is the oldest chromatographic technique. Despite its time consuming nature it was responsible for the first isolation and characterisation of flavonoid monomers. It is well adapted to smaller PACs, particularly in qualitative 2D mode. Due to resolution problems larger oligomers and polymers cannot be purified. It is still used in industry as a crude analytical tool (Roux, 1952).

2.4.6.2. Conventional Phase TLC and HPLC

This method has been widely used to purify and isolate smaller flavonoids and smaller oligomers. The isolation and quantification of specific PACs are however challenging in comparison to other phenolic compounds due to the variety of isomers and oligomers present (Sun and Spranger, 2005; Yanagida *et al*, 2003). Derivitization (methylation and acetylation) is often required. Irreversible binding between polyphenols and silica gel gave poor recovery and poor resolution with higher oligomers (Rigard *et al*., 1993; Sun *et al*., 1999; Hammerstone *et al*., 1999; Guyot *et al*., 2001).

2.4.6.3. Size Exclusion Chromatography

Material such as sephadex and toyopearl has been extensively used to purify free underivitised polyphenols, and flavonoids and PACs. It generally gives better results than TLC and paper chromatography. The action in the case of PACs is often based on adsorption and not size exclusion. A recent report indicates that urea in the eluting solvent interferes with adsorption and allows size exclusion to become the prominent chromatographic action (Sun and Spranger, 2005).

2.4.6.4. Countercurrent Chromatography

This technique has recently received much attention. Its major advantage is that all the starting material is recovered as no solid stationary phase is involved that may irreversibly bind to the polyphenols. The major disadvantage is poor resolution. CCC thus mainly serves as a pre-chromatographic technique (Putman and Butler, 1985; Berthod *et al*., 1999; Cao *et al*., 2009; Yanagida *et al*., 2006).

2.4.6.5. Reversed-Phase Chromatography

This technique is well suited to smaller water soluble polyphenols and flavonoids as the hydroxy groups on silica gel that bind irreversibly with these compounds are capped with hydrophobic alkane groups, often C-18. It furthermore is compatible with water as eluting solvent. Amide and diol columns have recently become prominent for polyphenol chromatography. Unfortunately, resolution of higher oligomers and polymers remains a problem that limits the use of this technique. Isolation of PACs up to trimeric level has been accomplished with reversed-phase HPLC whilst the higher oligomers are co-eluted as a large unresolved peak. (Rigard *et al*., 1993; Sun and Spranger, 2005) Thiolysis and phloroglucinolysis discussed above are efforts to overcome these resolution challenges (Jaworski and Lee, 1987; Oszmianski *et al*., 1988).

2.4.7. NMR Methods

NMR has been extensively used to elucidate the structures of flavonoids and PACs (Kolodziej, 1992). The limitation has been the availability of pure oligomers due to the chromatographic limitations discussed above. The biggest known PAC that has been purified and characterised is a tetramer (Picinelli *et al*., 1997**;** Escribano-Bailón *et al*., 1992). This has prompted efforts to analyse PAC mixtures with NMR.

2.4.7.1. Liquid State NMR Analysis of PAC Mixtures

Thompson and Pizzi (1995) used ^{13}C NMR of concentrated aqueous solutions of commercial PAC extracts to determine the relative proportion of phloroglucinol *vs* resorcinol A-rings, pyrogallol *vs.* catechol B-rings, and the average degree of polymerisation. Unreacted free aromatic A-ring C-H carbons (C-6 or C-8) resonate

between 95 and 98 ppm. When these positions take part in an interflavanyl bond they resonate at 110 to 111 ppm. Integration of the two regions thus gives an indication of the proportion of C-6 or C-8 bonds involved in polymerisation and thus the degree of polymerisation. The quaternary (C-OH) carbons on the A-ring (C-5, C-7 or C-9) resonate at 156 to 158 ppm, whilst the quaternary equivalents (C-3′, C-4′, and C-5′) on the B-ring resonate at 146-148 ppm. The C-1′ resonates at 130-132 ppm for catechol and 132-135 ppm for pyrogallol, respectively.

Czochanska and co-workers (1980) used ^{I3}C NMR in $^{2}[H_6]$ acetone-water solvent to estimate the ratio of procyanidin to prodelphinidin and the average heterocyclic ring stereochemistry of the monomer substituents as 22 in isolated PACs. The ratio of monomers to chain-terminating units was also determined.

2.4.7.2. Solid-State NMR Analysis of PAC mixtures

Solid-state NMR has the same chemical shifts as observed in liquid state NMR, albeit with less resolution. It thus represents a useful way to analyse condensed tannin extracts with minimal sample preparation. Newman and Porter (1992) used it to determine the amount of PACs in plant fractions and to obtain an indication of the procyanidin: prodelphinidin ratio. Moubarik and co-workers (2009) investigated the cornstarch: quebracho ratio in phenol-formaldehyde plywood resins using ^{13}C solid state NMR.

Romer and co-workers (2011) and Senekal (2011) used solid-state ^{13}C-NMR to analyse PACs from four diverse extracts from mimosa, quebracho, chestnut and tara. These methods gave spectra that are readily differentiated from each other. They also used the technique to analyse leather directly and developed a method to distinguish mineral, PAC, and hydrolysable tannin tanned leather and distinguished quebracho and mimosa tanned leather. Solid-state CP-MAS ^{13}C NMR thus provided an easily

distinguishable and precise spectral fingerprint of the products of vegetable and alternate tanning procedures.

Hoong and co-workers (2010) published a CP-MAS ^{13}C NMR spectrum of *Acacia mangium* PACs. From a low relative intensity (30%) of the C-6/C-8 resonances (94-98 ppm) they deducted a high degree of polymerisation. From the C4-C8 (115-110 ppm): C4-C6 (105 ppm) ratio they deducted a predominantly C4-C6 profisetinidin and prorobinetinidin PAC content. From the significant 110-115 ppm resonance they concluded a high proportion of "catechin-like" building blocks. Fisetinidol or robinetinidol can, however, also give C6-C8 interflavanyl bonds and it is not clear to us how they came to this conclusion, or how the ratios were calculated as the 94-98, 105, and 110-115 ppm resonances appear together as a single broad resonance in the published spectra. The relatively low intensity of the carbohydrate resonances at 65-85 ppm suggested that *Acacia mangium* contains fewer carbohydrates than *Acacia mearnsii*. Their conclusions are however supported by their MALDI-TOF results.

2.4.8. Mass Spectrometry (MS)

Mass spectrometry techniques are extremely sensitive compared to NMR and minute quantities can be analysed. It is furthermore very selective and oligomers that differ by one Dalton (Da) or even less can be distinguished. MS can fractionate a mixture of oligomers such as a PAC extract into fractions of different degrees of polymerisation (monomers, dimers, trimers, tetramers etc). The major disadvantage is that standards are required for quantification. These are seldom available for PAC oligomers. However, PACs are oligomers based on an increment number of similar flavan-3-ol monomers and ionisation should be similar. MS and NMR thus potentially complement each other. Modern soft ionisation techniques such as electrospray ionisation (ESI), atmospheric pressure chemical ionisation (APCI), and matrix-assisted laser desorption ionisation (MALDI) combined with a quadrupole or time of

flight (TOF) analyser have been extensively used to analyse fruit and wine tannins (Flamini, 2003).

MALDI-TOF is a soft ionisation technique that is often used to analyse synthetic polymers. Due to this highly sensitive and selective technique, a complex extract containing impurities can be directly analysed (Yanagida *et al*., 2003). PAC polymers up to a DP of 20-30 have been successfully analysed with this technique (Jones *et al*., 1976; Takahata *et al*., 2001). PACs are detected as their sodium or potassium adducts, with a difference of 16 Da between them. The potassium adduct can thus be mistaken for a sodium adduct of an oligomer with an extra oxygen atom (eg. a robinetinidol instead of fisetinidol building block) (Hoong, 2010).

Electrospray ionization mass spectrometry (ESI-MS) is an alternative soft ionization method that has proved to be a formidable instrument for the characterisation of PACs. It can thus be used to identify individual PACs in heterogeneous mixtures (Sun and Spranger, 2005; Guyot *et al*., 1997; Le Roux *et al*., 1998; Hayasaka *et al*., 2003). It cannot reach the same single ion fragment masses that can be analysed with MALDI-TOF. However, Mouls (2011) demonstrated that PACs with a DP of up to 20 can be analysed and doubly charged ions may extend the reach further. ESI-MS with enhanced resolution has been used to detect single ($[M-H]^-$), double ($[M-H]^{2-}$), and triple ($[M-H]^{3-}$) charged ions of procyanidins in grape seed fractions with molecular sizes of up to 28 units (Sun and Spranger, 2005; Hayaska *et al*., 2003). A major advantage of ESI is that it can be used with a quadrupole analyser to do MS/MS. This allows monomers and oligomers with the same mass, but different chemical composition, to be distinguished (Flamini, 2003).

Mouls and co-workers (2011) compared direct ESI results with thiolysis derived results of cider apple HPLC fractions (Avrolles and Kermerrein cultivars) and concluded that mass spectrometry gave lower aDP results (Table 1). The difference increased with an increase in aDP. He concluded that larger oligomers have a lower intensity than smaller oligomers.

Table 1: Comparison between aDP of apple cider PAC fractions estimated by thiolysis and calculated from the mass spectrum

Tannin Fraction	aDP (Thiolysis)	aM_w	aDP (ESI-MS)
K1	6.7	1931.6	5.2
K2	15.5	4466.0	11.1
A1	20.9	6021.2	5.7
A2	49.5	14 258.0	11.8

Vivas and co-workers (2004) used the thioacidolysis/LC/ESI-MS method to analyse condensed tannins from seeds, skins, and stems of grapes (*Vitis vinifera*) and from the heartwood of quebracho and determined the following:

(i) the nature of flavan-3-ols

(ii) the degree of galloylation

(iii) average degree of polymerization (aDP)

The chromatographic profiles of different commercial enologic tannins were (*via* MALDI-TOF MS) compared to the standard PACs and thus origin could be determined.

Fulcrand and co-workers (2008) applied direct mass spectrometry approaches to characterize polyphenol composition of complex extracts. They concluded that it was a powerful tool for structure elucidation but that quantification and molecular weight distribution were compromised by mass discrimination. Monagas and co-workers (2007) reviewed the suitability of MALDI-TOF MS to analyse PACs and came to similar conclusions. They suggested that MALDI-TOF is better than ESI due to the absence of multiple charged ions.

Mane and co-workers (2007) proposed that the problem of mass discrimination can be overcome *via* complexation of PACs with serum albumin proteins and they used MALDI-TOF MS to analyse the molecular weight distribution of tannin fractions from protein-tannin complexes. Parameters determined were the number-average molecular weight (M_n), weight-average molecular weight (M_w), and the polydispersity index (PI) from the tannin bovine serum albumin (BSA) complex. Three PAC fractions with average degrees of polymerization of 3, 9, and 28, respectively, and one gallotannin fraction (tara tannin) were analysed.

Pasch and co-workers (2001) analysed commercially important quebracho (*Schinopsis lorentzii*) and mimosa (*Acacia mearnsii)* oligomers with MALDI-TOF MS. They concluded that both quebracho and mimosa extracts consist of flavan-3-ol monomer based PACs. They assumed that mimosa PACs are angular (branched) and quebracho PACs are linear.

Oligomers to a maximum of decamer level (2798 Da) were observed for the quebracho PACs. As the dominant repeating unit, with a mass of 272 Da, correlated with fisetinidol **(14)** (Figure 10), quebracho PACs were described as profisetinidins. The mimosa extract consists of a series of oligomeric flavan-3-ol units up to the octamer (n = 8) level (2333 Da). This correlates with the average degree of polymerisation determined by other methods (Thompson & Pizzi, 1995; Fechtal and Riedl, 1993). As the major repeat unit has a mass of 288 Da, mimosa PACs were described as a prorobinetinidin **(17)** (Figure 11) consisting of robinetinidol repeat units **(15)** (Figure 10)**.**

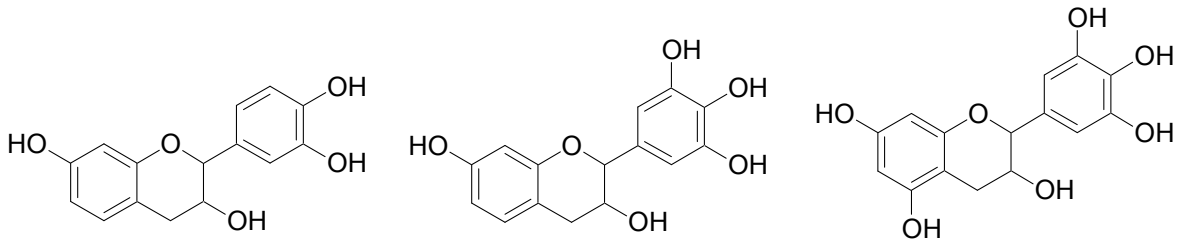


Figure 10: Examples of quebracho and mimosa PAC monomers

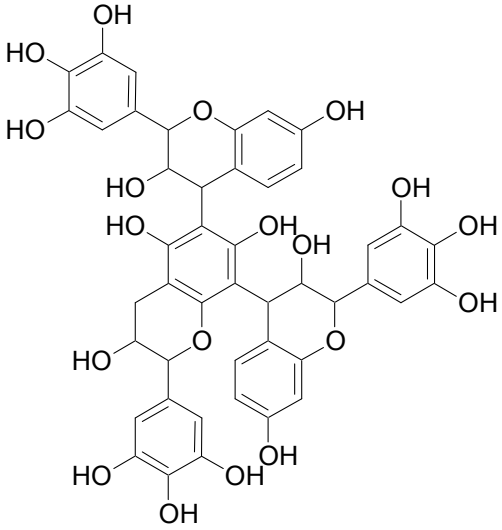


Figure 11: The structure of Acacia PACs (prorobinetinidins) proposed by Pasch

Hoong and co-workers (2010) used MALDI-TOF-MS to analyse *Acacia mangium* PACs. They observed oligomers that contain up to 11 flavan-3-ol repeat units (3200 Da). From the frequency distribution of the oligomers (based on the percentage intensity of the peaks) they calculated an aDP of between 7.0 and 7.4. This is higher than the aDP of between 4.9 and 5.4 reported for *Acacia mearnsi* PACs. The major peak-to-peak mass increments were 273, 289, and 304 Da, corresponding with fisetinidol (profisetinidin), robinetinidol (prorobinetinidin), and gallocatechin

(prodelphinidin) monomer building blocks. The prominent repeat unit was 288 which suggest that *Acacia mangium* is predominantly a prorobinetinidin. Since M+K peaks (which are visible in the MALDI-TOF MS spectra of PACs) may be misinterpreted as the Na adduct of an $[M+OH]^-$ ion, NaCl was added in an effort to reduce the M+K peaks. They identified angular (branched) PACs in their spectra but did not explain how they distinguished between these and linear PACs.

Venter and co-workers (2012) developed a methodology to analyse hot water-soluble quebracho PAC extract (unsulfited) with ESI-MS. They attributed intense *m/z* values at 561.1 and 833.1 Da, and less intense ions at *m/z* 1105 and 1377 (Figure 12) to fisetinidol-catechin dimers, angular fisetinidol-catechin-fisetinidol trimers, angular fisetinidol-fisetinidol-catechin-fisetinidol tetramers, and a pentamer with one catechin and four fisetinidol moieties (Table 2). These assignments are in accordance with the isolation of catechin **(3)** and *ent*-fisetinidol-4β-ol **(18)** (Figure 13) monomers, two diastereoisomers *ent*-fisetinidol-(4β→8)-catechin **(19)** and *ent*-fisetinidol-(4α→8)-catechin **(20)** (*m/z* 562) (Figure 14), and the angular trimer *ent*-fisetinidol-(4β→8)-catechin-(6→4β)-*ent*-fisetinidol **(23)** (*m/z* 834) (Figure 15) from quebracho heartwood (Viviers et al., 1983). These findings were supported by the fact that no robinetinidol **(15)** was ever isolated from quebracho (Roux and Evelyn, 1960).

Table 2: SI (negative mode) ions for hot water-soluble quebracho extract.

Oligomer	***m/z* value**	**Catechin**	**Fisetinidol**
Dimer	561	1	1
Trimer	833	1	2
Tetramer	1105	1	3
Pentamer	1377	1	4

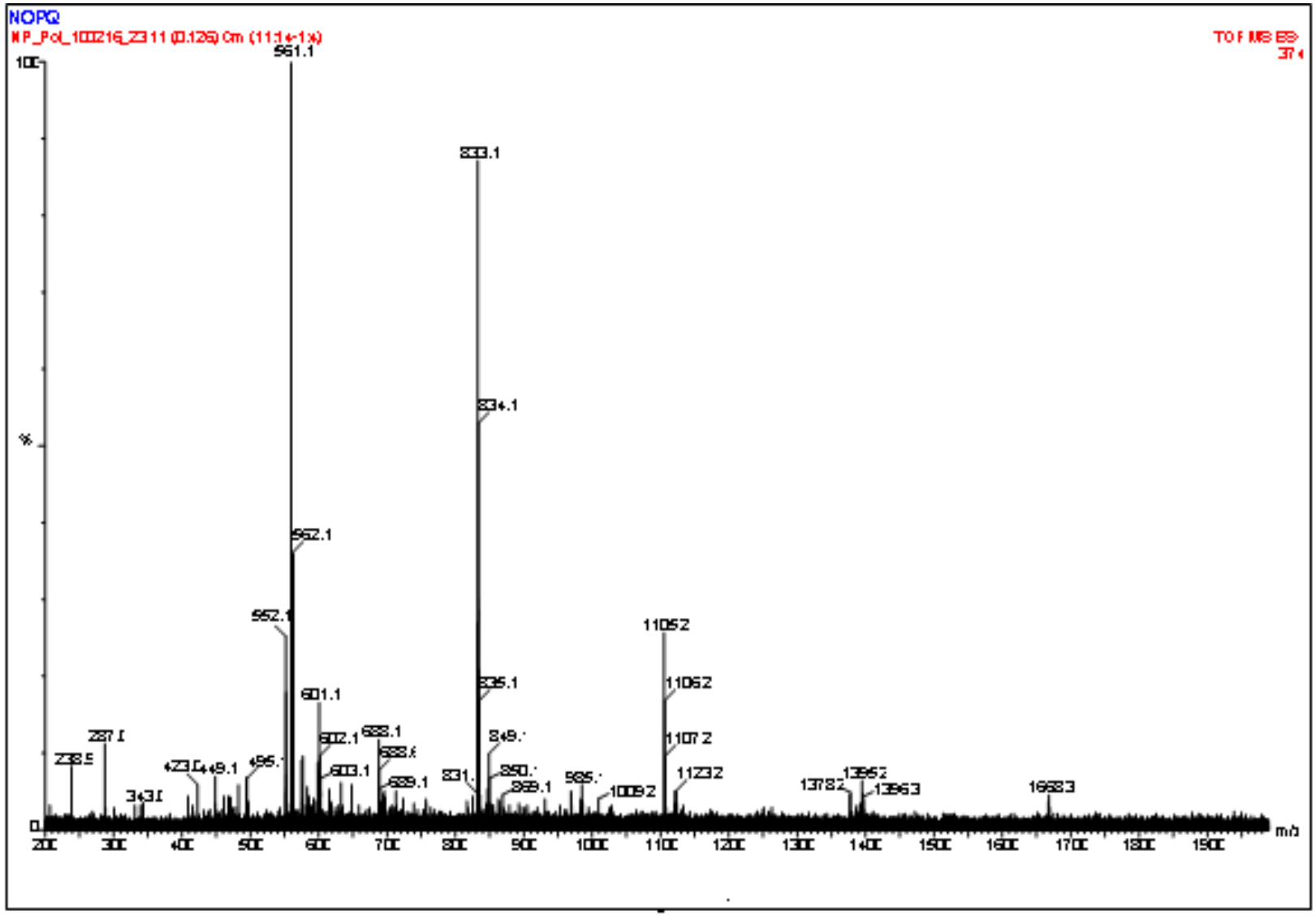


Figure 12: ESI-MS spectrum of quebracho heartwood

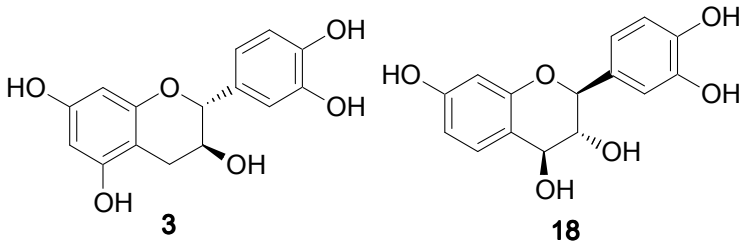


Figure 13: Flavan-3-ol and flavan-3, 4-diol monomers from quebracho heartwood (putative building blocks of quebracho PACs).

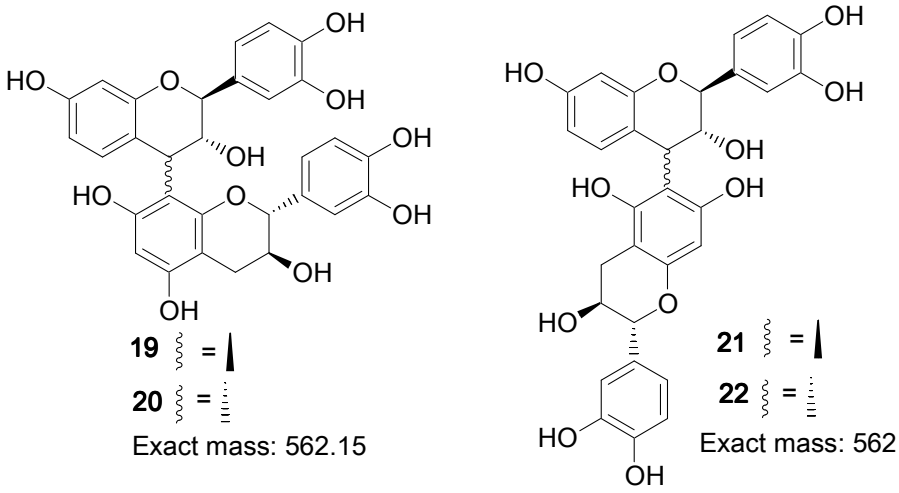


Figure 14: Quebracho heartwood dimers

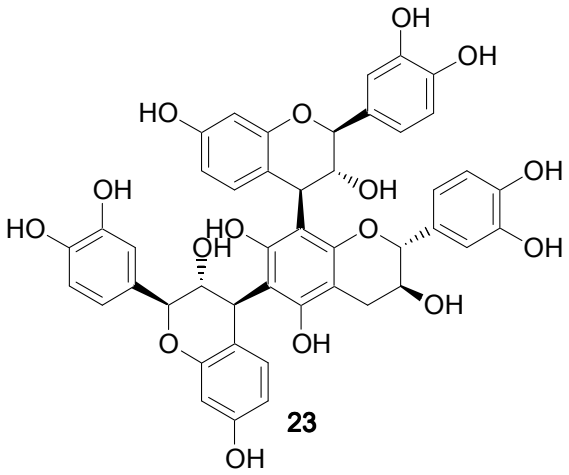


Figure 15: Quebracho heartwood trimer

Product ion scans (APCI in the negative mode) of the *m/z* 561.2 (dimer) (Figure 16a) and *m/z* 833.3 (trimer) (Figure 16b) peaks both yield the *m/z* 289.4 product ion as base peak as would be expected from a catechin containing dimer or trimer. The complementary *m/z* 273 ion, associated with fisetinidol, was not observed. The proposed fragmentation pattern for the dimer is given in Scheme 6.

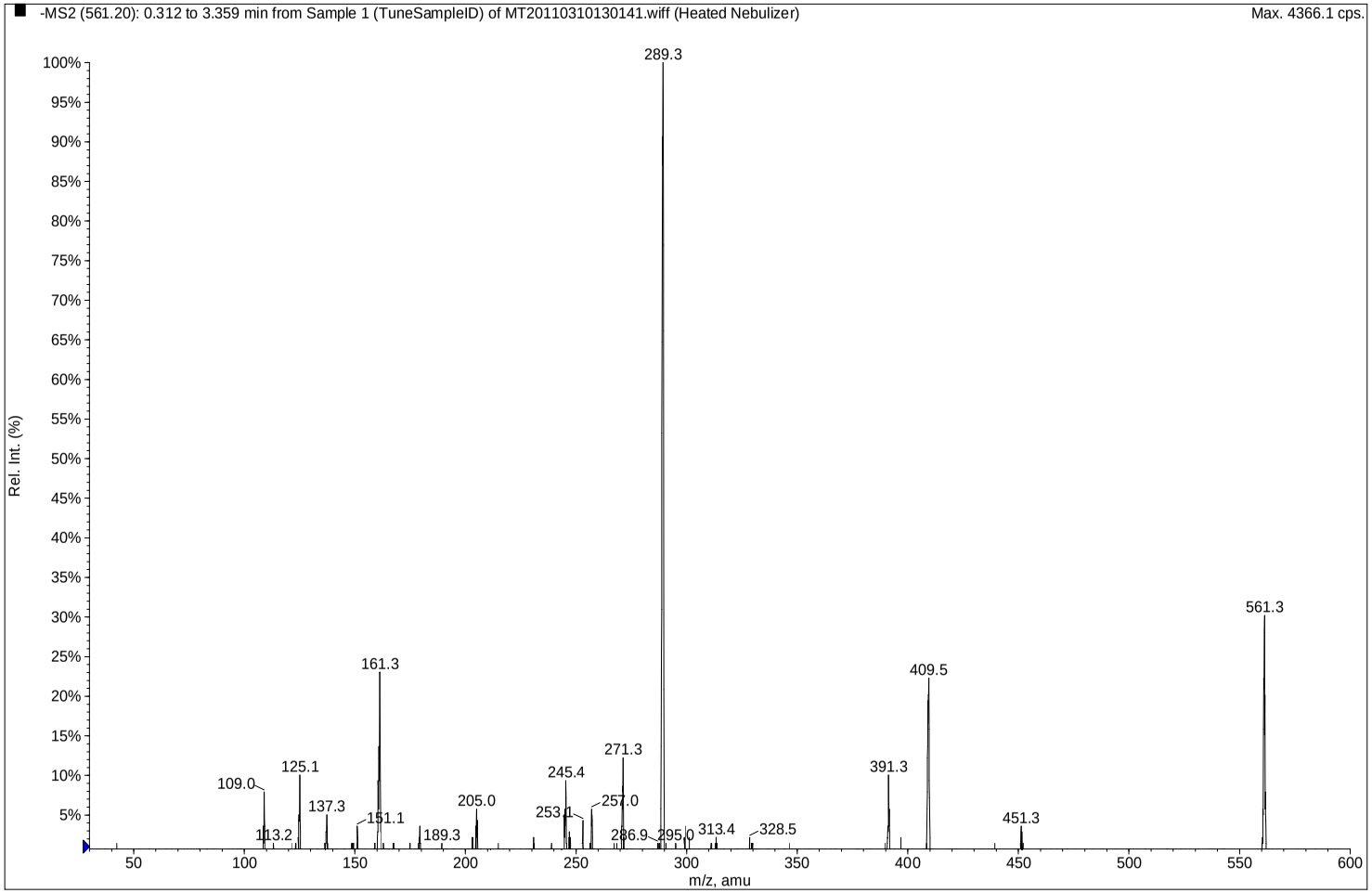


Figure 16a. MS^2 of *m/z* 561 quebracho dimer ion

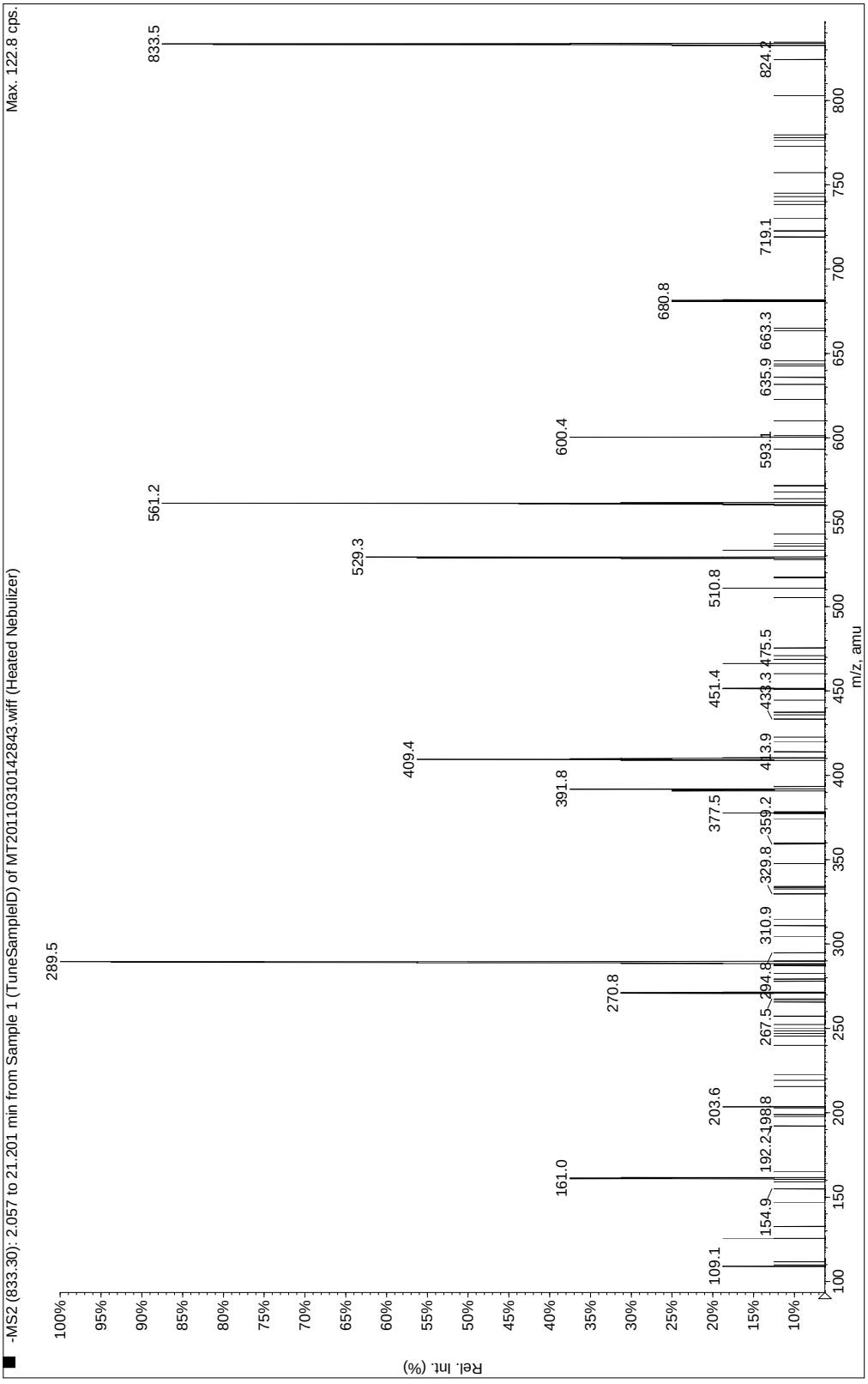
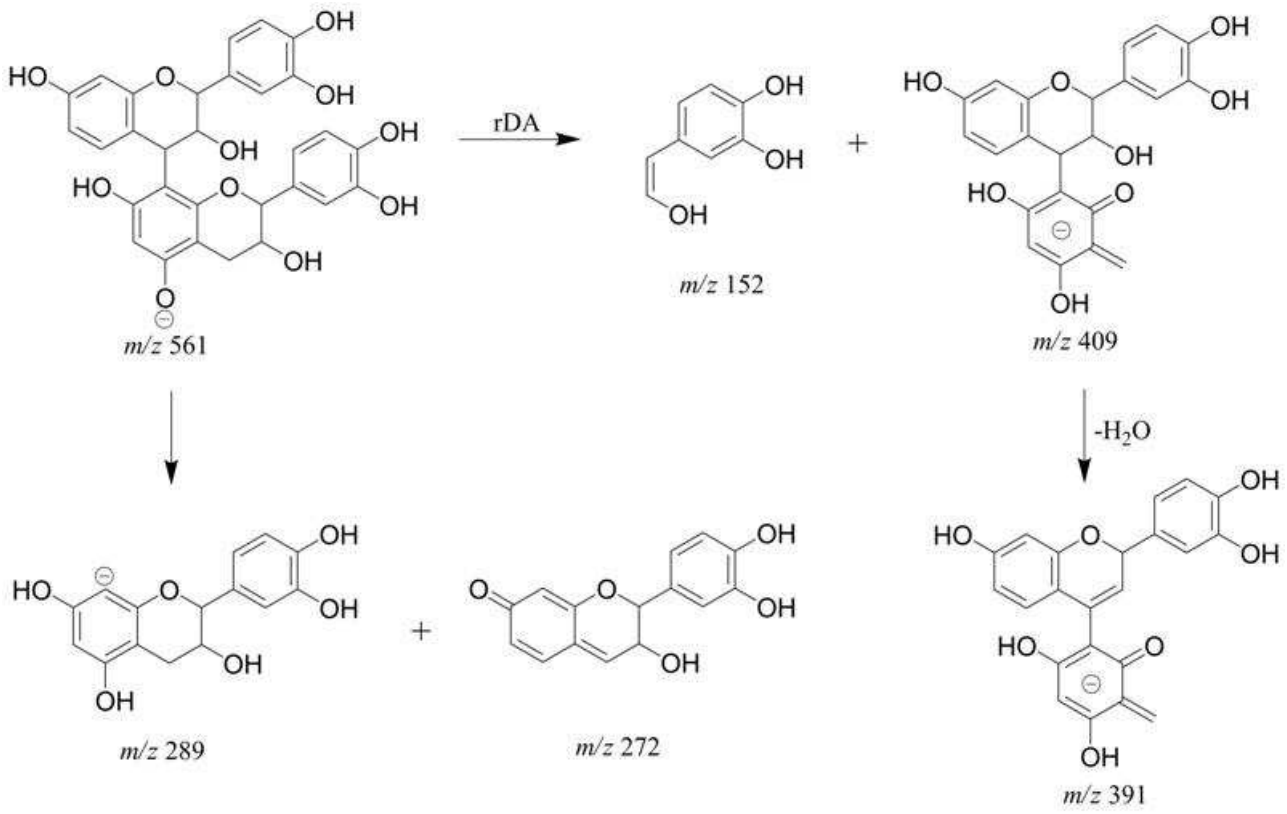


Figure 16b: MS^2 of *m/z* 833 quebracho trimer



Scheme 6: Fragmentation of *m/z* 561 quebracho dimer $(M-H)^-$

They compared the product ion scan (MS^2) of the *m/z* 289 fragment (Figure 17a) with the MS of pure catechin **3** (Figure 17b) and robinetinidol **15** (Figure 17c) and concluded that the *m/z* 289 fragment is catechin and not robinetinidol as was previously reported by Pasch (2001) and Vivas (2004). This conclusion is supported by the absence of robinetinidol monomers in the extracts studied by Roux and Evelyn (1960) and Viviers and co-workers (1983). They estimated the composition of quebracho extract as consisting of about 33% dimers, 37% trimers, 21% tetramers, 8% pentamers, and 1% heptamers.

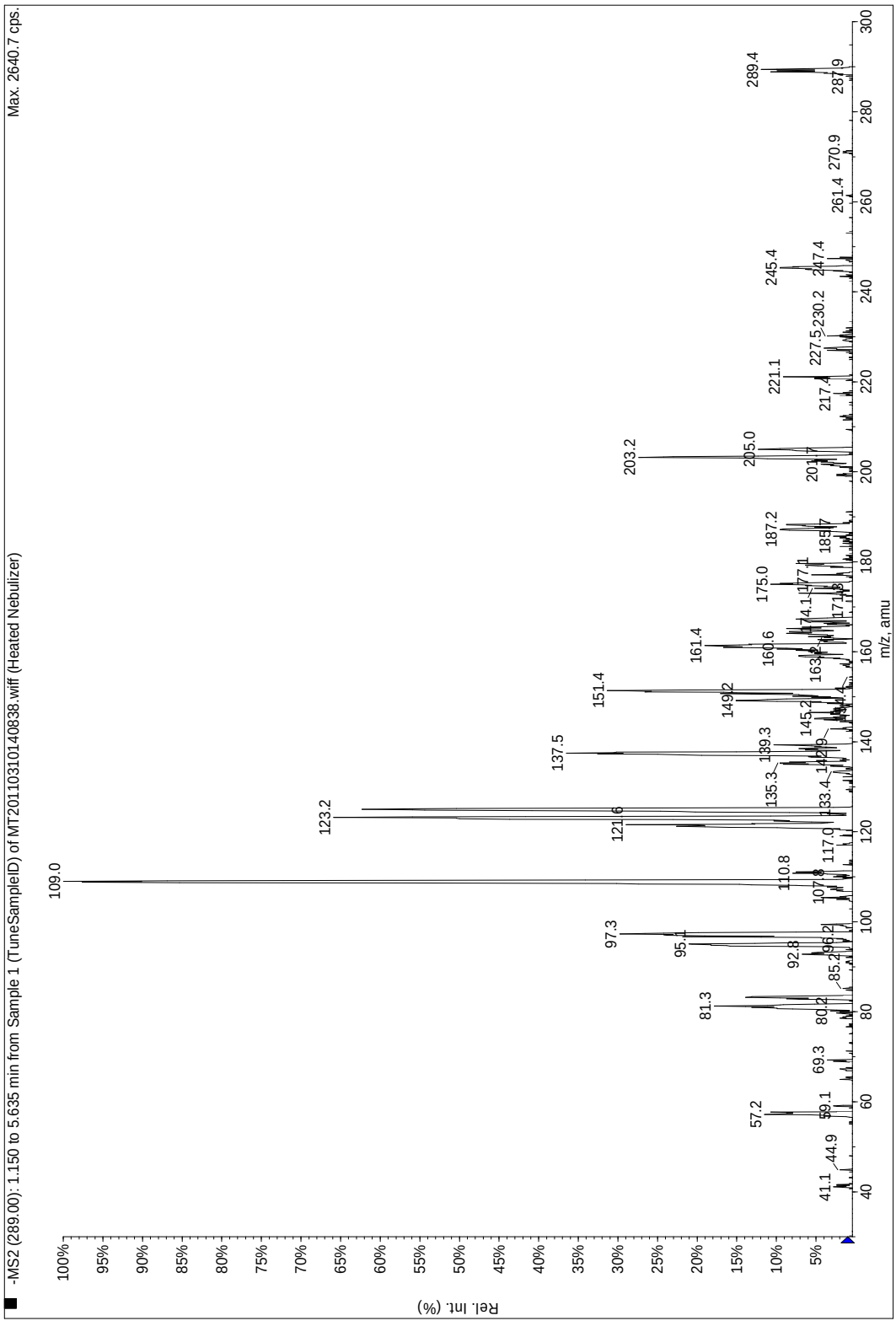


Figure 17a. Product ion scan of the *m/z* 289 fragment in Figure 12

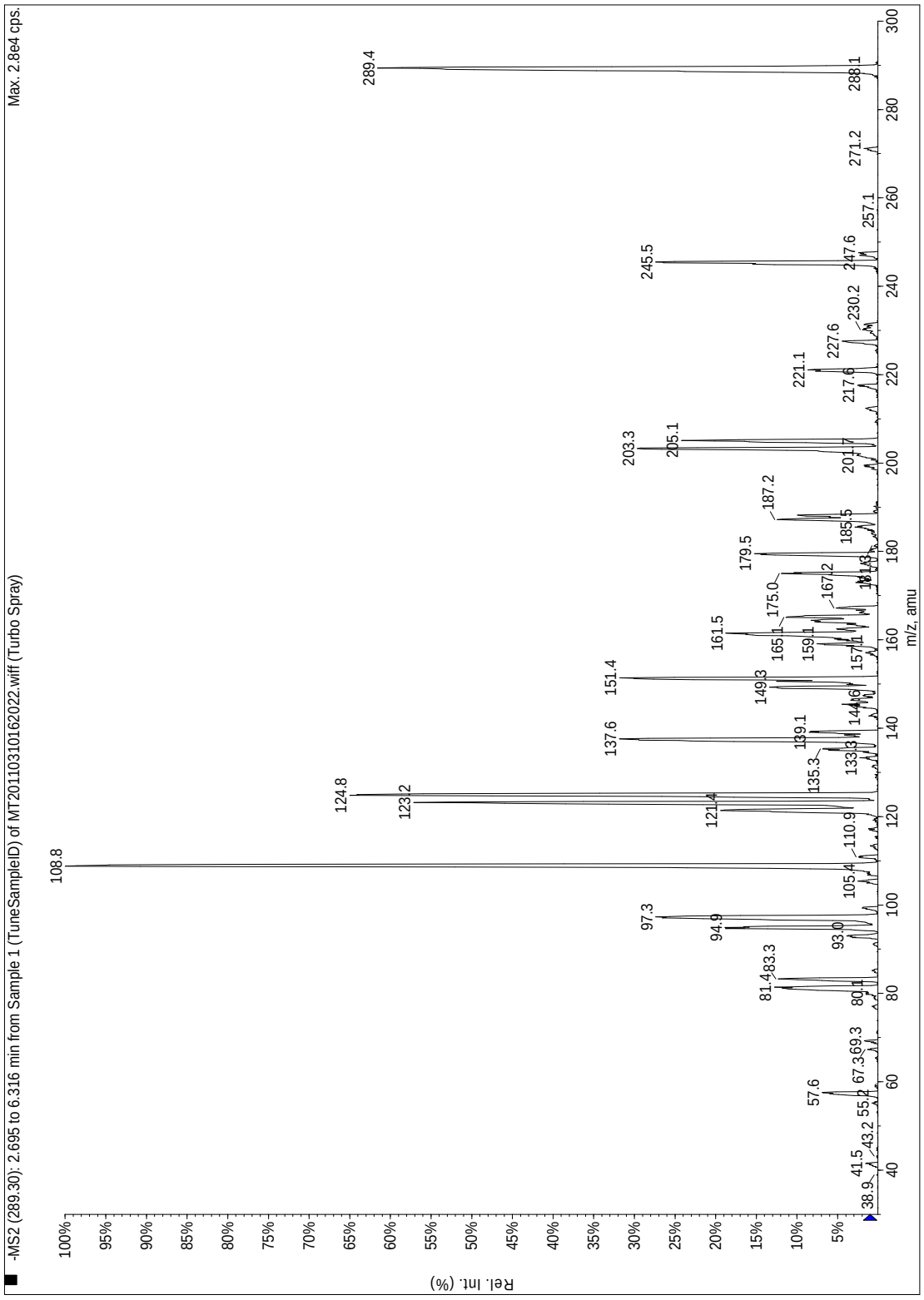


Figure 17b. Product ion scan of an authentic catechin sample

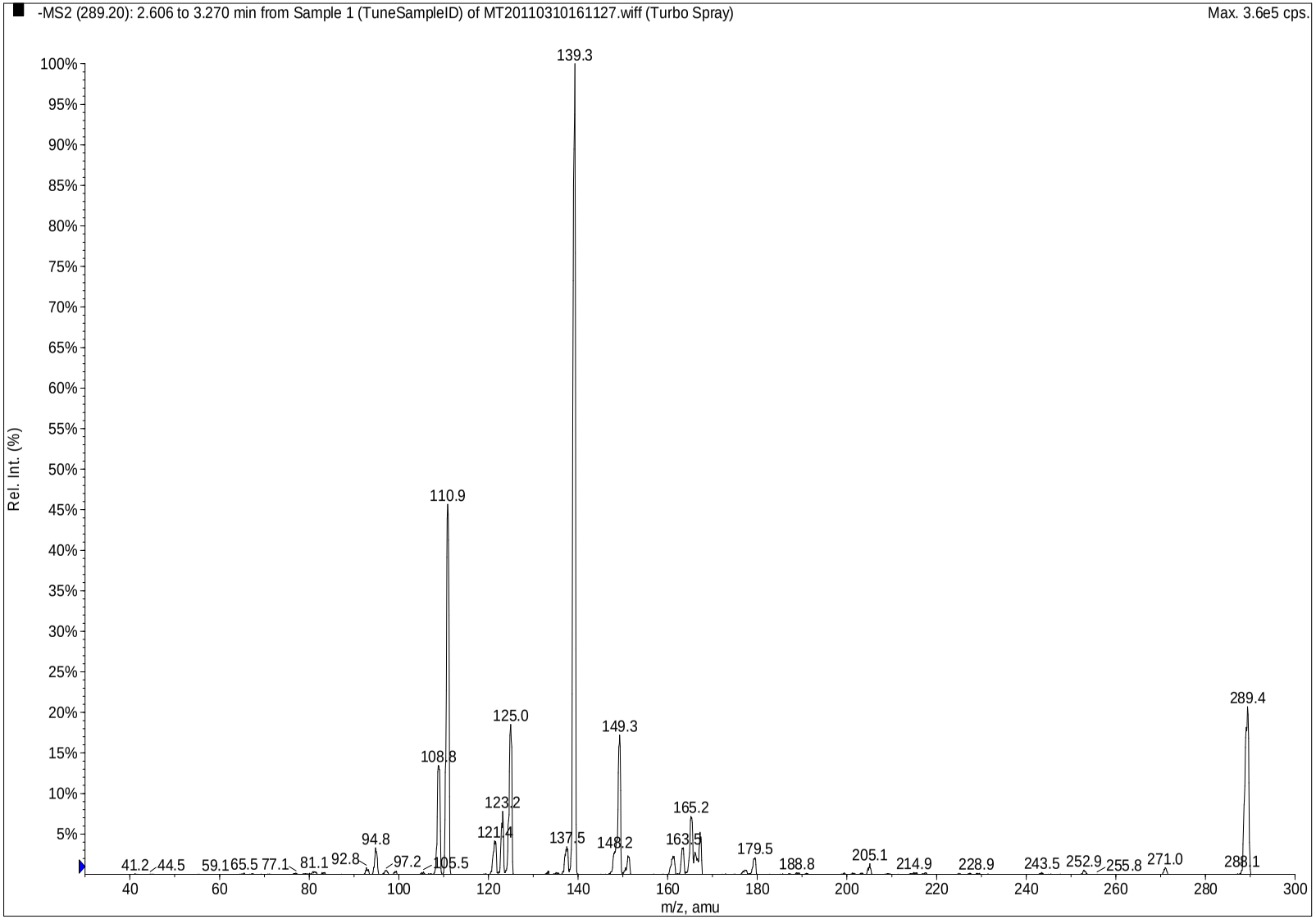
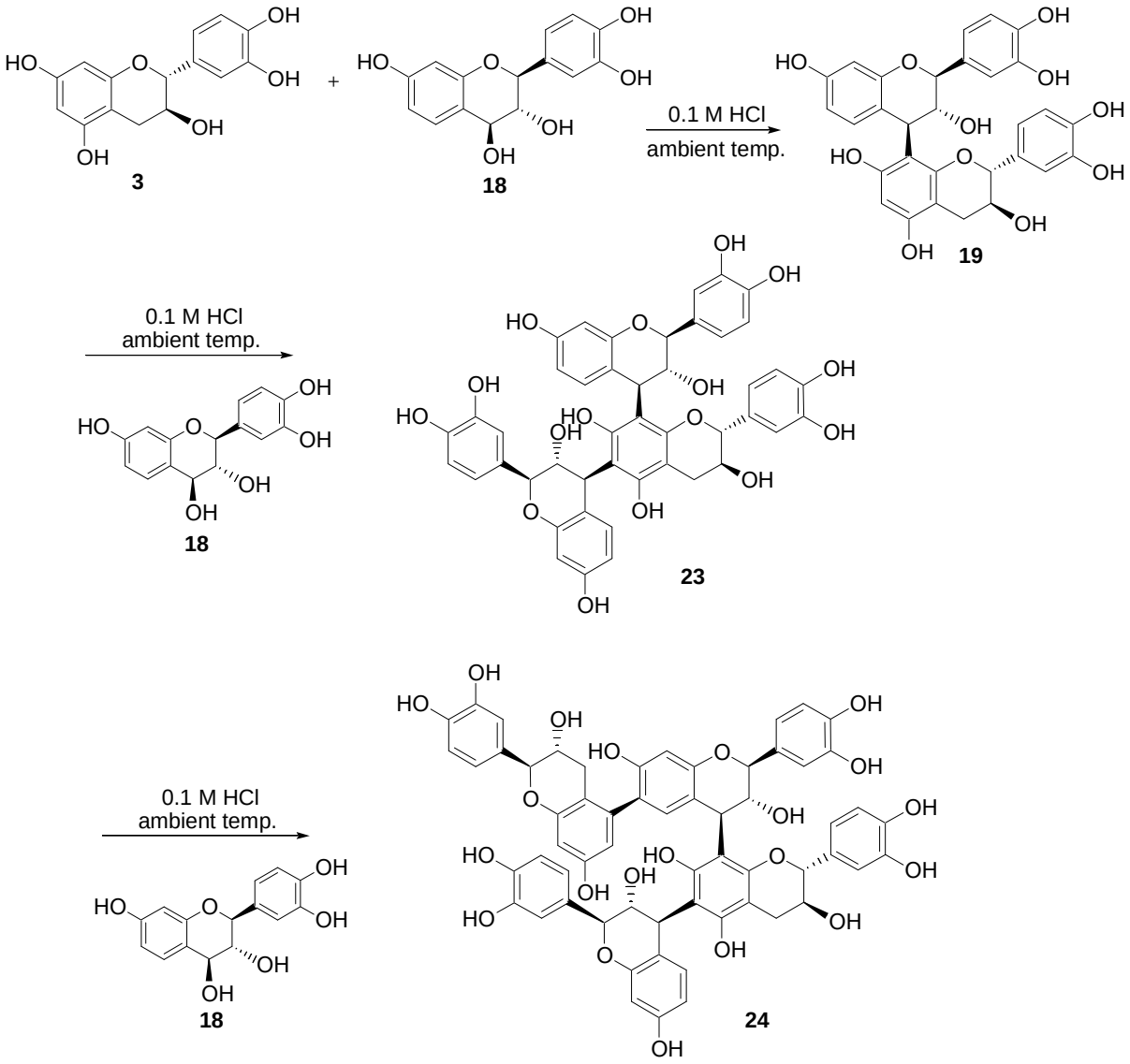


Figure 17c. Product ion scan of an authentic robinetinidol sample

The postulated biosynthesis of quebracho heartwood PACs *via* condensation of a catechin starter unit and one or more fisetinidol-4-ol extender unit precursors was replicated in the laboratory with mild acid catalysis at room temperature. As the C-8 position of the phloroglucinol type A-ring of catechin is more reactive than the alternative C-6 position, synthetic dimers are predominantly C-8 coupled fisetinidol-catechin. The C-6 position of catechin is more reactive than the nucleophilic positions on the resorcinol type A-ring of fisetinidol. The second fisetinidol-4-ol is thus crafted onto this position and trimers are exclusively fisetinidol-catechin-fisetinidol. Further extension takes place *via* the less reactive fisetinidol extender units, probably explaining the relative low occurrence of tetramer and higher oligomers. The C-6-position of the fisetinidol unit is sterically less hindered than the C-8 position and the third extender unit on a tetramer is coupled at this position. The synthesis of tetramers and higher fisetinidol-catechin PACs is summarised in Scheme 7. The close correlation between synthetic PACs and those isolated from quebracho heartwood suggest that their biosynthesis parallels their laboratory synthesis and support structural MS assignments of the PACs in natural extracts.



Scheme 7: Synthesis of quebracho heartwood PACs

2.5. Industrial Applications of PACs

Plant derived PACs have been used since antiquity to tan leather. It represents a renewable source of polymers, and is non-toxic and biodegradable, and thus has received renewed industrial interest to supplement or replace oil derived chemical products and toxic chrome and other mineral based tanning agents (Pizzi, 2008). The industrially useful PACs are 5-deoxy PACs and these are available in commercially

viable quantities. The absence of a 5-OH groups imparts stability to the interflavanyl bonds and hence durability to the products manufactured from it.

Their industrial application is derived from their chemical properties which can be classified as follows:

1. The ability to form complexes with proteins *via* hydrogen bonds. This forms the basis for their leather tanning properties and imparts resistance to microbial and bacterial attack as it complexes with their proteins (Haslam, 1974, 1988, 1997; Venter *et al*., 2012).
2. The electron donating OH groups impart high electron density to the aromatic rings, particularly the phloroglucinol or resorcinol-type A-rings. These rings are thus nucleophilic and polymerise with aldehydes to form natural adhesives (Pizzi, 1978; Venter *et al*., 2012).
3. The two or three *ortho* hydroxy groups on the B-rings of constituent flavan-3-ol monomers form insoluble complexes with heavy metals and thus explains their water purification and other colloidal applications (Beltran-Heredia and Sanchez-Martin, 2008; Venter *et al*., 2012).

2.5.1. Leather Tanning

Both hydrolysable tannins and PACs can be used to produce vegetable tanned leathers. Quebracho and wattle PACs are commercially produced in large quantities and have largely replaced the older hydrolysable tannins in leather tanning. Small quantities of hydrolysable tannins from chestnut bark are still used for speciality leathers such as sole leather tannage (Covington, 1997; Shuttleworth, 1955). Nowadays mineral agents such chromium has largely replaced vegetable tanned leather. The toxicity of chrome and the environmental issues associated with the disposal of chromium tanned leather has however stimulated renewed interest in

vegetable tannins (Belay, 2010). About 50% of commercial PAC production is used for leather tanning.

The skin of any animal is made up of three layers: the epidermis, the corium and the flesh layer. The corium, which is used to produce leather, consists of collagen fibres, which are comprised of helically-twisted protein biopolymers, chemically linked to permit strength and elasticity (Dirksen, 1997; Haslam, 1998). The tanning process is comprised of cross linking adjacent protein fibres with PACs *via* hydrogen bonds between protein amide (NHCO) groups and PAC hydroxy groups. The result is leather, which in contrast with skin, is durable and resists bacterial and fungal degradation. It is also water repellent since the hydrophylic amine groups have been saturated with PAC OH groups. The rigidity and hardness of dried skin is replaced by a supple product with a soft touch. The product assumes the reddish-brown colour of the PAC extract (Cassano *et al*., 2003, Haslam 1998). Large amounts of PACs are involved and vegetable tanned leather may contain about 50% PACs by weight.

The degree of polymerisation of the PAC oligomers is important. Monomers and dimers cannot link two collagen strands in leather. Longer oligomers are too big to penetrate the intermolecular spaces within the collagen causing poor penetration and thus surface tanning only. These surface tanned leathers are normally hard and used for belts and shoe soles (Shuttleworth, 1955; Roux, 1992; Slabbert, 1992; Haslam, 1998).

Vegetable extracts (e.g. quebracho and wattle) used to tan leather, contains in addition to PACs, also so called non-tans. These are mainly sugars and gums, but also minor amounts of acids and their salts, hemicelluloses, pectin, and unknown compounds containing nitrogen and phosphorus. The larger non-tans may block intermolecular spaces and interfere with PAC penetration, whilst some of the smaller ones may be beneficial in controlling astringency. It has been found that removal of non-tans from vegetable tannins used for book cover leathers, reduce the acid resistance (from sulfur pollutants in the air) and longevity of these leathers (Covington, 1997; Haslam, 1998,

Roux, 1992; Dirksen, 1997). Quebracho consists of about 95% PACs and 5% non-tans and mimosa about 65% PACs and 35% non-tans (Venter *et al*., 2012).

2.5.2. Adhesive Manufacturing

The development and industrial use of PAC based adhesives began in the late 1940s (Yazaki and Collins, 1997). PAC-based adhesives are considered as renewable resource adhesives and are aimed at replacing more expensive synthetic phenolic resins (Pizzi, 1998 and 2003; Grisby *et al*., 2004). The polyphenolic A-ring reacts with formaldehyde to produce polymers (Refer to Scheme 5) linked *via* methylene bridges. *Acacia mearnsii* PAC adhesives have been commercially produced in South Africa for many years (Yazaki and Collins, 1997) and consume about 50% of the total extract production.

The limited number of reactive sites and rapid polymerisation (fast gelling and curing times and shorter pot lives compared to synthetic phenolic resins) causes weak and brittle adhesives and considerable effort has been made to overcome this. Sulfitation improves the situation. Applied research involves efforts to generate additional sites for polymerisation for cold-setting adhesive applications *via* cleavage of the interflavanyl bond and the introduction of more flexibility into the rigid PAC ring *via* opening of the heterocyclic C-ring (Pizzi, 1998, 2003; Grisby *et al*., 2004).

PAC extracts with resorcinol A-rings (quebracho and wattle) require hot-pressing to manufacture adhesives. This contrasts with the more reactive phloroglucinol A-ring type PAC extracts (e.g. pine species) that has an additional OH group on the A-ring and polymerises at ambient temperature. Resorcinol-type tannin extract adhesives thus have a slower reactivity than phloroglucinol tannins (Figure 18).
An alternative approach involves the addition of resorcinol and phenol to the extract. These commercial resorcinol based adhesives include tannin-resorcinol-formaldehyde

(TRF) and synthetic phenol-resorcinol-formaldehyde (PRF) which are cold-setting adhesives used for curing, cold-set finger jointing, or laminating applications. These are, however, expensive products compared to PAC extracts (Pizzi, 2003, 2008).

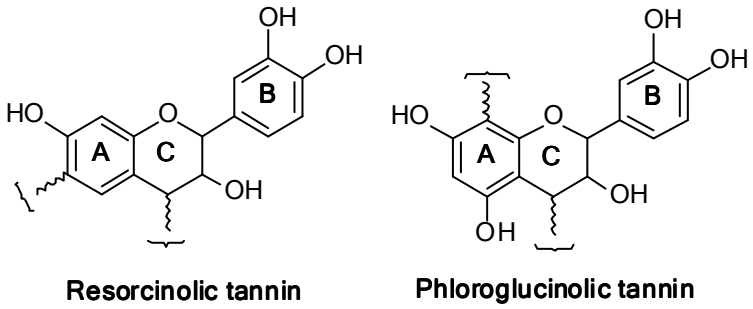


Figure 18: Tannin extracts adhesive composition

2.5.3. Dyes

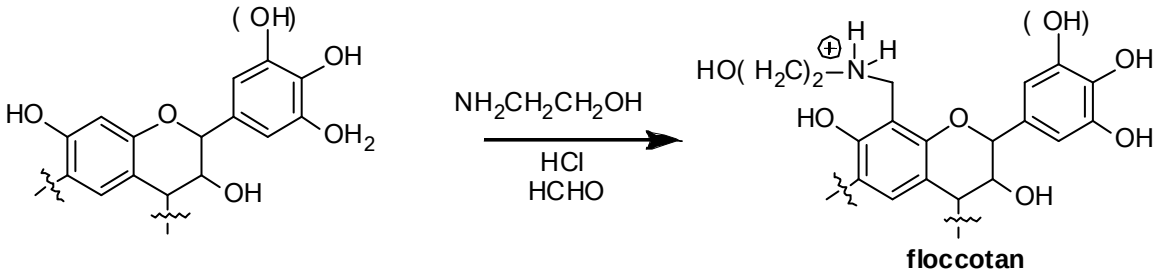
PACs have been used in the manufacture of ink (iron gallate ink) and as caustics for cationic dyes (tannin dyes) (Khanbabee and van Ree, 2001). PACs are used as mordants to increase the uptake of cationic dyes onto cotton. The cotton is initially treated with PAC extract and then with a metal salt preceding dyeing. This technique imparts wash and light fastness to the dyed fabrics (Vankar *et al*., 2007).

2.5.4. Mineral Flotation Agents (Coagulants and Flocculants)

Suspended positively charged colloidal particles such as clay and organic matter in water that may be responsible for unpleasant appearance, taste, and smell clarify muddy water (Roux *et al*., 1975). PACs are negatively charged and coagulate and precipitate these particles to obtain pure water on an industrial scale.

PACs are anionic polymers used in mineral flotation agents (Roux *et al*., 1975). Quebracho tannic acid derivatives are the most common polymers used in flotation. Although normal and sulfited PACs can be used, the most common is aminated quebracho, a commercial product named Floccotan. The PAC is converted into an amphoteric (iso-electric point at pH 7) positively charged polymer containing a methyl amino group.

Scheme 8 illustrates the Mannich-type reaction between acacia PACs, ethanolamine, and formaldehyde in acidic aqueous medium to form "floccotan" (Roux *et al*., 1975).



Scheme 8: Formation of "floccotan"

Roussy and co-workers (2005) have used a combination of chitosan (i.e. polymer composed of glucosamine and acetylglucosamine units) and tannins for the coagulation and flocculation of industrial solutions containing dark violet ink.

Beltran-Heredia and co-workers (2009) described the use of a tannin-based coagulant and flocculant agent manufactured from quebracho (*Schinopsis balansae*) for the removal of hazardous sodium dodecylbenzenesulfonate (SDBS) from wastewater. SilvaFLOC is an anionic surfactant-removal agent that efficiently removed 80% SDBS.

Beltran-Heredia and Sanchez-Martin (2009) have also tested another tannin-based coagulant and flocculant agent, TANFLOC, obtained from *Acacia mearnsii* bark. Chemical modifications of the extract include a quaternary nitrogen that imparts a cationic character. TANFLOC was effective in treating urban wastewater and the removal of anionic surfactants and organic matter.

2.5.5. Corrosion Inhibitors

PAC extracts react with iron salts to form a blue-black iron tannate complex. This reaction has been used for centuries to prevent the corrosion of steel and copper surfaces of steam boilers (Brannt and Wahl, 1886; Moresby *et al*., 1978). PACs inhibit corrosion by reacting with Fe^{3+} forming an insoluble, reticulate shielding layer on the rusted steel surface (Jaén *et al*., 1999; Moresby *et al*., 1978; Seavell *et al*., 1978).

Jaén and co-workers (1999) studied the use of PACs to hinder corrosion *via* the formation of amorphous iron-tannin complexes with X-ray. Matamala and co-workers (2000) compared the effect on steel when treated with tannin primers formulated with natural tannins extracted from Chilean radiata pine species and Brazilian black acacia. The reactivity of the pine tannins exceeded that of the acacia tannins thus giving better corrosion inhibition.

2.5.6. Fluidifying and Superplasticizing Additives

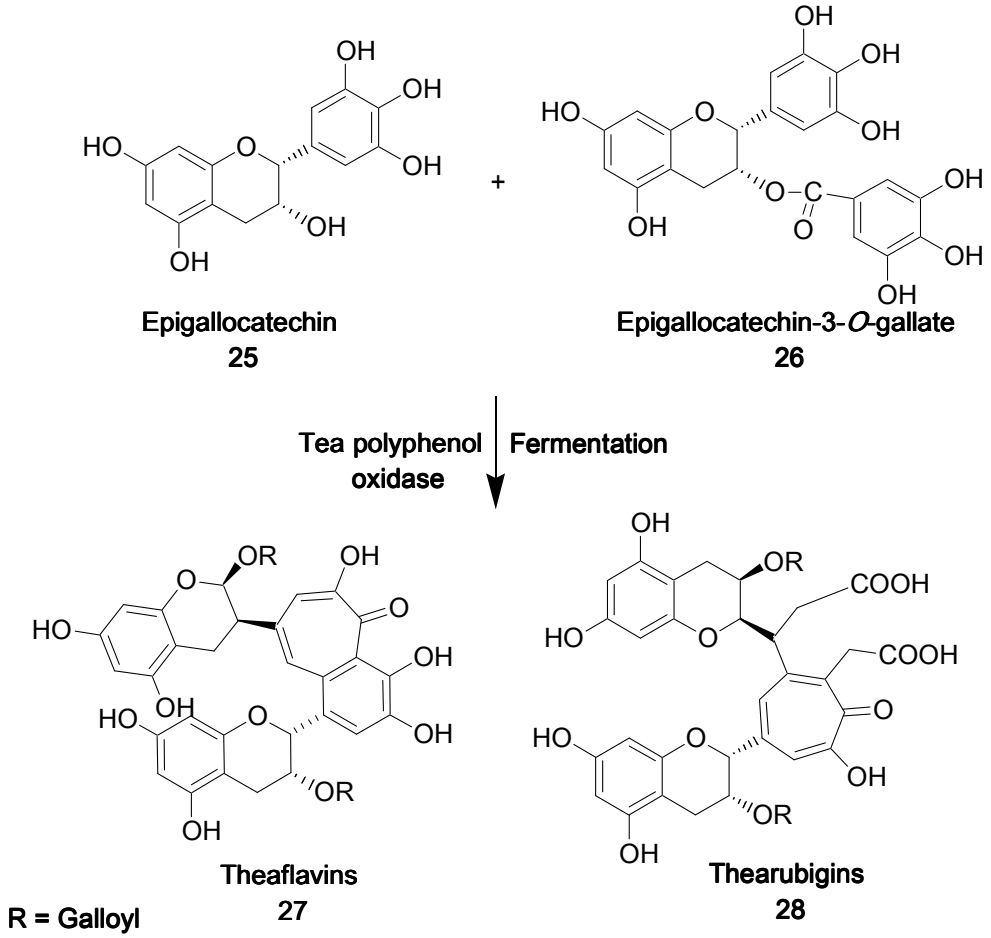
The addition of PACs increases the fluidity and decreases the viscosity of mud that forms during the oil drilling process (Haslam, 1998; Herrick, 1980). The process probably involves the hydration of the mud hydrophilic groups thus weakening the clay network and liberating free water. The addition of small quantities of PACs to cement improves its workability and improves strength development. Large amounts, however, impede the setting and strength development of concrete (Pizzi, 2008; Zaisong, 1997).

2.5.7. Food and Beverage Industry

PACs are important taste components in food and beverages. The protein-polyflavonol hydrogen bond interaction with protein based taste receptors in the mouth imparts astringency. This complements the volatile essential oil based flavour component that acts *via* receptors in the nose (Joslyn and Goldstein, 1964). PACs may also act as anti-feedants due to high astringency (see discussion below) (Rhodes and Cates, 1976).

PACs are of particular importance in tea, a PAC containing hot water-soluble extract of the leaves of *Camellia sinensis*, a tropical ever-green plant. During the production of green tea, the fresh leaves are heated immediately after picking followed by drying to deactivate enzymes and thus conserving the monomeric constituents. Chinese tea consists mainly of an abundance of epigallocatechin monomers. Manufacturing of black tea involves grinding the tea leaves after picking to endorse enzymatic oxidation and subsequent condensation of the tea polyphenols (theaflavins and thearubigins) through a fermentation process (Scheme 9). Addition of milk to the tea reduces astringency by precipitating the tannins with milk proteins. The flavonoid monomers in tea are considered to be anti-oxidants and particularly Chinese tea is considered a

health beverage with important anti-aging, anti-cancer, and anti-inflammatory properties (Jankun *et al*., 1997; Garbisa *et al*., 1999; Yang and Ruan, 2009; Wang *et al*., 2007, 1993, Haslam, 1998).



Scheme 9: Major components of green and black tea

PACs are important taste components in red wine. In white wine the juice is separated from the PAC-containing grape skins before fermentation. White wine thus contains little or no PACs. In contrast, red wine is fermented in the presence of the skins (Ignat *et al*., 2011, Haslam, 1998). The alcohol that forms during fermentation assists in the extraction of PACs from the skins. Red wine thus contains high levels of PACs. Young red wines may be undrinkable and ageing may be required to reduce astringency. Wine ageing is still a poorly understood process (Bate-Smith, 1954, Haslam 1998; Hofmann *et al*., 2006) that not only reduces astringency but also introduces taste and flavour components that greatly enhance wine quality.

Oak barrels are used during the ageing process of red, and some white wines, and brandy. Hydrolysable tannins are extracted from the wood during the process and impart important taste and associated quality components. These are enhanced with further ageing. Hydrolysable tannins thus also undergo poorly understood chemical changes during ageing (Sanz *et al*., 2012).

Wood aged red wine thus contains both PACs and hydrolysable tannins whilst brandy and wood aged white wine contains only hydrolysable tannins. Whisky and many other alcoholic drinks are also aged in oak wood. In cheaper versions, wood chips serves as the tannin source (Glabasnia and Hofmann, 2006).

Commercial tannin containing powders are often used to improve taste qualities of poor red wines. Quebracho heartwood extract is particularly popular since it is difficult to detect analytically.

Regular moderate drinking of PAC containing red wine is considered important for health and longeativity. Other components in red wine (eg. stibenes, phenolic alcohols etc.) (Makris *et al*., 2007; Ignat *et al*., 2011) are also involved.

Much of the beneficial effects like anti-ageing, anti-cancer, anti-inflammatory properties associated with fresh fruit and vegetables, including cereals and nuts have been attributed to the anti-oxidant and free radical scavenging properties of PACs (Santos-Buelga and Scalbert, 2000).

2.5.8. Medicinal and Pharmaceutical applications

A plethora of biological activities has been reported for pure flavonoids, flavan-3-ols, and PACs. These include antitumor and anticancer activity (Chung *et al*., 2010; Serrano *et al*., 2009; Santos-Buelga and Scalbert, 2000; Pizzi, 2008). We, however, must point out that many enzymes are proteins and *in vitro* inhibition by tannin extracts may be due to irreversible denaturation of proteins, similar to leather tanning (Pizzi, 2008; Ignat *et al*., 2011).

2.5.9. Biological Functions of Tannins

The purpose of tannins in plants is controversial and actively debated. It is unlikely that plants would spend a considerable amount of effort to synthesise the large quantities of tannins that are normally present if there was no evolutionary benefit. It is generally assumed that tannins protect plants against herbivores, micro-organism and viruses. This is presumably *via* their ability to complex and denature proteins. Proteins in the digestive system of herbivores are thus rendered indigestible either *via* destroying digestive enzymes or *via* forming indigestible food-tannin complexes. Hydrolysable tannins and procyanidins occur complementary. In plants were PACs are absent, hydrolysable tannins dominate and *vice versa*. This supports their protective role *via* complexation of proteins with the hydroxyl groups on either hydrolysable tannins or PACs (Clausen *et al*., 1992).

The 5-oxy PACs with labile interflavanyl bonds impart astringency to immature fruit and protect them and the seeds they contain against herbivores. As soon as the seeds are mature, however, these 5-oxy PACs are hydrolysed and the fruit becomes edible and the seeds are distributed by herbivores. At the same time the monomers that are the products of hydrolysis of the interflavanyl bonds, are further oxidised to anthocyanidins that actually advertises to herbivores that the fruit are ready to be

eaten. The 5-deoxy PACs in bark and wood that are of a more permanent nature have stable interflavanyl bonds (Lea, 1992; Haslam, 1998).

It has also been postulated that flavonoids and PACs protect plants against ultraviolet rays in sunlight *via* quinone methide and anthocyanidin formation. This is important in young plants that do not contain sufficient quantities of chlorophyll, presumed to be a major protecting agent against UV damage. The autumn colors associated with aged leaves may well be part of the process to transfer valuable leave components back to the tree without UV damage in the absence of chlorophyll (Solovchenko and Schmitz-Eiberger, 2003; Flint *et al*., 1985).

2.6. References:

Achilonu, M. C. Novel synthetic approaches toward biflavanoids and procyanidins. PhD Thesis, University of the Free State, **March 2009**, pp.15-16.

AOAC. Official Methods of Analysis, 10th ed.; Association of Official Agricultural Chemists. Washington DC, **1965**.

Asquith, R. A. Paper chromatography of the pyrogallol tannins. *Nature*, **1951**, *168*, 738.

Bate-Smith, E. Astringency in foods. *Food Proc. Packag*. **1954**, *23*, 124-127.

Bate-Smith, E. Haemanalysis of tannins: The concept of relative astringency. *Phytochemistry*, **1973**, *12*, 907-912.

Belay, A.A. Impacts of chromium from tannery effluent and evaluation of alternative treatment options. *J. Environ. Prot.*, **2010**, *1*, 53-58.

Beltran-Heredia, J.; Sanchez-Martin, J. Municipal wastewater treatment by modified tannin flocculent agent. *Desalination*, **2009**, *249*, 353-358.

Beltran-Heredia, J.; Sanchez-Martin, J. Removing heavy metals from polluted surface water with tannin-based flocculant agent. *J. Hazardous Mat.* **2008**, *165*, 1215-1218.

Beltran-Heredia, J.; Sanchez-Martin, J.; Frutos-Blanco, G. *Schinopsis balansae* tannin-based flocculant in removing dodecyl benzene sulfonate. *Sep. Purif. Technol.*, **2009**, *67*, 295-303.

Berthod, A.; Billardello, B.; Geoffroy, S. Polyphenols in countercurrent chromatography. An example of large scale separation. *Analusis*, **1999**, *27*, 750-757.

Boukharta, M.; Girardin, M.; Metche, M. Procyanidines galloylées du sarment de vigne (*Vitis vinifera*). Séparation et identification par chromatographie liquide haute performance and chromatographie en phase gazeuse. *J. Chromatogr*., **1988**, *455*, 406-409.

Brannt, W. T.; Wohl, W. H. (Eds), The Techno-Chemical Receip(e)t Book, U.S.A.: Henry, Carey, Baird and Co., Philadelphia, **1886**, pp.39-40.

Cao, X.; Wang, C.; Pei, H.; Sun, B. Separation and identification of polyphenols in apple pomace by high-speed counter-current chromatography and high-performance liquid chromatography coupled with mass spectrometry. *J. of Chromatogr. A*, **2009**, *1216*, 4268–4274.

Cassano, A.; Adzet, J.; Molinari, R.; Buonomenna, M. G.; Roig, J.; Drioli, E. Membrane treatment by nanofiltration of exhausted vegetable tannin liquors from the leather industry. *Water Res.*, **2003**, *37*, 2426.

Chung, K. T.; Wong, T. Y.; Wei, C-I.; Huang, Y. W.; Lin, Y. Tannins and human health: A review. *Crit. Rev. Food Sci. Nutr.*, **1998**, *38(6)*:421–464.

Clausen, T. P.; Reichardt, P. B.; Bryant, J. P.; Provenza, F. Introduction in: plant polyphenols. Hemingway, R.W., Laks, P.E. (Eds), Plenum Press, New York, **1992,** pp. 639-647.

Cooper, K.G.; Hanlon, L.G.; Smart, G.M.; Talbot, R.E. 25 years experience in the development and application of scale inhibitors. *Desalination*, **1979**, *31*, 243.

Covington, A. D. Modern tanning chemistry. *Chemical Society Reviews*, **1997**, 111-126.

Covington, A. D. Tanning Chemistry: The Science of Leather. The Royal Society of Chemistry, Cambridge, **2009,** pp.304-309.

Covington, A. D.; Lilley, T. H.; Song, L.; Evans, C. S. Collagen and polyphenols: new relationships and new outcomes. Part 1. Flavonoid reactions for new tanning processes. *J. Am. Leather Chem. Ass.*, **2005,** *100*, 325.

Czochanska, Z.; Foo, L. Y.; Newman, R. H.; Porter, L.J. Polymeric proanthocyanidins. Stereochemistry, structural units, and molecular weight. *J.C.S. Perkin I*, **1980**, 2278-2286.

De Freitas, V.; Glories Y.; Bourgeois, G.; Vitry, C. Characterization of oligomeric and polymeric procyanidins from grape seeds by liquid secondary ion mass spectrometry. *Phytochemistry*, **1998,** *49*, 1435-1441.

Dirksen, V. The degradation and conservation of leather. *JCMS*, **1997**, *3*.

Eberhardt, T. L.; Young, R. A. Conifer seed cone proanthocyanidin polymers: characterization by ^{13}C NMR spectroscopy and determination of antifungal activities. *J. Agric Food Chem.*, **1994**, *42*, 1704-1708.

Escribano-Bailón, T.; Gutiérrez-Fernández, Y.; Rivas-Gonzalo, J. C.; Santos-Buelga, C. Characterization of procyanidins of *Vitis vinifera* variety tinta del país grape seeds. *J. Agric. Food Chem.*, **1992**, *40*, 1794−1799.

Fechtal, M., Riedl, B. Use of eucalyptus and *Acacia mollissima* bark extract-formaldehyde adhesives in particleboard manufacture. *Holzforschung*, **1993,** *47*, 349.

Ferreira, D.; Slade, D.; Marais, J. P. J. Flavanoids chemistry, biochemistry and applications, CRC press, **2006**, pp. 553-615; 1101-1128.

Flamini, R. Mass spectrometry in grape and wine chemistry, Part 1: polyphenols. *Mass Spectrom. Rev.*, **2003**, *22*, 218-250.

Flint, S. D.; Jordan, P. W.; Caldwell, M. M. Plant protective response to enhanced UV-B radiation under field conditions: Leaf optical properties and photosynthesis. *Photochem. and Photobio.*, **1985**, *41*, 95-99.

Freudenberg, K.; Maitland, P. Tannins and similar substances. XXVII. Quebracho tannin. *Liebig's Ann.*, **1934**, *510*, 193-205.

Fulcrand, H.; Remy, S.; Souquet, J.M.; Cheynier, V.; Moutounet, M. Study of wine tannin oligomers by on-line liquid chromatography electrospray ionization mass spectrometry. *J. Agric. Food Chem.*, **1999**, *47*, 1023-1028.

Fulcrand, H; Mané, C; Preys, S.; Mazerolles, G.; Bouchut, C.; Mazauric, JP.; Souquet, J. M.; Meudec, E.; Li, Y.; Cole, R. B.; Cheynier, V. Direct mass spectrometry approaches to characterize polyphenol composition of complex samples. *Phytochemistry*, **2008**, *69* , 3131–3138.

Garbisa, S; Biggin, S.; Cavallarin, N.; Sartor, L.; Benelli, R.; Albini, A. Tumor invasion: molecular shears blunted by green tea. *Nat. Med.*, **1999**, *5*, 1216.

Glabasnia, A; Hofmann, T. Sensory-Directed Identification of taste-active Ellagitannins in American (*Quercus alba* L.) and european oak wood (*Quercus robur* L.) and quantitative

analysis in bourbon whiskey and oak-matured red wines. *J. Agric. Food Chem.*, **2006**, *54* (9), 3380–3390.

Govindarajan, V. S.; Matthew, A. G. Anthocyanidins from leucoanthocyanidins. *Phytochemistry*, **1965**, *4*, 985.

Grigsby, W.; Warnes, J. Potential of tannin extracts as resorcinol replacements in cold cure thermoset adhesives. *Holz als Roh- und Werkstoff*, **2004**, *62*, 433-438.

Guyot, S.; Marnet, N.; Drilleu, J. F. Thiolysis-HPLC characterization of apple procyanidins covering a large range of polymerization states. *J. Agric. Food Chem.*, **2001**, *49*, 14-20.

Hagerman, A. E.; Robbins, C. T.; Weerasuriya, Y.; Wilson, T. E.; Mcarthur, C. J. Tannin chemistry in relation to digestion. *Range Manage.*, **1992,** *45*, 57-62.

Hammerstone, J. F.; Lazarus, S. A.; Mitchell, A. E.; Rucker, R.; Schmitz, H. H. Identification of procyanidins in cocoa (*Theobroma cacao*) and chocolate using high-performance liquid chromatography/mass spectrometry. *J. Agric. Food Chem.*, **1999**, *47* (2), 490–496.

Haslam, E. Polyphenol-protein interactions. *Biochem. J.*, **1974,** *139*, 285-288.

Haslam, E. Practical polyphenols. From structure to molecular recognition and physiological action, Cambridge University Press, Cambridge, **1998**, pp. 10-407.

Haslam, E. Twenty-second Procter memorial lecture: Vegetable tannins - renaissance and reappraisal. *J. Soc. Leather Technol. Chem.*, **1988,** *72*, 45.

Haslam, E. Vegetable tannage: where do the tannins go? *J. Soc. Leather Technol. Chem.*, **1997,** *82*, 45.

Haslam, E. Vegetable tannins-Lessons of a phytochemical lifetime. *Phytochemistry*, **2007**, *68*, 2713-2721.

Hayasaka, Y.; Kennedy, J. A. Mass spectrometric evidence for the formation of pigmented polymers in red wine. *Aust. J. Grape Wine Res.*, **2003**, *9 (3)*, 210-220.

Hemingway, R. W.; Mcgraw, G. W. Kinetics of acid-catalyzed cleavage of procyanidins. *J. Wood Chem. Technol.*, **1983**, *3*, 421.

Herrick, F. W. Chemistry and utilization of western hemlock bark extractives. *J. Agric. Food Chem.*, **1980**, *28*, 228–237.

Hofmann, T.; Glabasnia, A.; Schwarz, B.; Wisman, K. N.; Gangwer, K. A.; Hagerman, A. E. Protein binding and astringent taste of a polymeric procyanidin, 1, 2, 3, 4, 6-penta-O-galloyl-b-D-glucopyranose, castalagin, and grandinin. *J. Agric. Food Chem.*, **2006** *54*, 9503–9509.

Hoong, Y. B.; Pizzi, A.; Tahir, P. M.; Pasch, H. Characterization of *Acacia mangium* polyflavanoid tannins by MALDI-TOF mass spectrometry and CP–MAS ^{13}C NMR. *Eur. Polym. J.*, **2010**, *46*, 1268-1277.

Ignat, I.; Volf, I.; Popa, V. I. A critical review of methods for characterization of polyphenolic compounds in fruit and vegetables. *Food Chem.*, **2011**, *126*, 1821-1835.

Jaén, J. A.; García de Saldaña, E.; Hernández, C. Characterization of reaction products of iron and iron salts and aqueous plant extracts. *Hyperfine Interact.*, **1999**, *122*, 139.

Jankun, J.; Selman, S. H.; Swiercz, R.; Skrypczak-Jankun, E. Why drinking green tea could prevent cancer. *Nature*, **1997**, *387*, 561.

Jaworski, A. W.; Lee, C. Y. Fractionation and HPLC determination of grape phenolics. *J. Agric. Food Chem.*, **1987**, *35*, 257−259.

Jennings, A. C. The determination of dihydroxyphenolic compounds in extracts of plant tissues. *Anal. Biochem.*, **1981**, *118*, 396.

Jones, W. T.; Broadhurst, R. B.; Lyttleton, J. W. The condensed tannins of Pasture legume species. *Phytochemistry*, **1976**, *15*, 1407-1409.

Joslyn, M. A., Goldstein, L. L. Astringency of fruits and fruit products in relation to phenolic content. *Adv. Food Res.*, **1964**, *13*, 179–317.

Katalinić, V.; Miloš, M.; Modun, D.; Musić, I.; Boban, M. Antioxidant effectiveness of selected wines in comparison with (+)-catechin. *Food Chem.*, **2004**, *86*, 593–600.

Kennedy, J. A.; Taylor, A. W. Analysis of proanthocyanidins by high performance gel permeation chromatograph**y**. *J. Chromatogr. A*, **2003,** *995*, 99-107.

Khanbabee, K; van Ree, T. Tannins: Classification and definition. *Nat. Prod. Rep*., **2001,** *18*, 641-649.

Kolodziej, H. Isolation, structural identification and NMR spectroscopy of proanthocyanidins. In: Polyphenols 2000. Marten S., Treutter D. and Forkmann G. (Eds); freisingweihenstephan, **2002**; pp. 98-119.

Kramling, T. E.; Singleton, V. L. An estimate of nonflavonoid phenols in wines. *Am. J. Enol. Vitic*., **1969**, *20*, 86-92.

Lapornik, B.; Prosek, M.; Wondra, A. G. Comparison of extracts prepared from plant by-products using different solvents and extraction time. *J. Food Eng.*, **2005**, *71*, 214–222.

Le Roux, E.; Doco, T.; Sarni-Manchado, P.; Lozano, Y.; Cheynier, V. A-type proanthocyanidins form pericarp of *Litchi chinensis*. *Phytochemistry*, **1998,** *48*, 1251-1258.

Lea A. G. H., Timberlake C. F. The phenolics of cider. I. procyanidins. *J. Sci. Food Agric.*, **1974**, *25*, 1537-1545.

Lea, A. G. H. Introduction in: Plant polyphenols. Hemingway, R.W., Laks, P.E. (Eds), Plenum Press, New York, **1992,** pp. 827-847.

Makkar, H. P. S. Protein Precipitation methods for quantification of tannins: A Review. *J. Agric. Food Chem*. **1989**, *37*, 1197-1202.

Mané, C.; Sommerer, N.; Yalcin, T.; Cheynier, V.; Cole, R.B.; Fulcrand, H. Assessment of the molecular weight distribution of tannin fractions through MALDI-TOF MS analysis of protein-tannin complexes. *Anal. Chem.*, **2007**, *79*(6), 2239-48.

Matamala, G.; Smeltzer, W.; Droguett, G. Comparison of steel anticorrosive protection formulated with natural tannins extracted from acacia and from pine bark. *Corros. Sci.,* **2000**, *42*, 1351.

Matthews, S.; Mila, I.; Scalbert, A.; Brigitte Pollet, B.; Lapierre, C.; Herve´ du Penhoat, C. L. M.; Rolando, C.; Donnelly, D. M. X. Method for estimation of proanthocyanidins based on their acid depolymerization in the presence of nucleophiles. *J. Agric. Food Chem*., **1997**, *45*, 1195-1201.

Meirelles, C.; Sarni, F.; Ricardo-da-Silva, J. M.; Moutounet, M. Evaluation des procyanidines galloylées dans les vins rouges issue de différents modes de vinification. In*:* Proceedings of International Polyphenolic Group Convention. Lisbon, **1992**, *16*, Tome II, pp 175-178.

Monagas, M.; Quintanilla-López, J. E. ; Gómez-Cordovésa, C.; Bartoloméa, B.; Lebrón-Aguilar, R. MALDI-TOF MS analysis of plant proanthocyanidins. *J. of Pharm. and Biomedical Anal.*, **2010**, *51*, 358–372.

Morera, J. M.; Barcardit, A.; Olle, L.; Bartoli, E.; Borras, M. D. Minimization of the environmental impact of chrome tanning: A new process with high chrome exhaustion. *Chemosphere*, **2007**, *69*, 1728-1733.

Moresby, J. F. Black Wattle and its Utilisation Abridged English Edition, Brown, A.G., Ko, H.C. (Eds), Rural Industries Research & Development Corporation (RIRDC), **1997,** pp. 145-148.

Moubarik, A.; Pizzi, A.; Allal, A.; Charriera, F.; Charriera, B. Cornstarch and tannin in phenol–formaldehyde resins for plywood production. *Ind. Crop. Prod.*, **2009**, *30*,188–193.

Mouls, L.; Mazauric, J. P.; Sommerer, N.; Fulcrand, H.; Mazerolles, G. Comprehensive study of condensed tannins by ESI mass spectrometry: average degree of polymerisation and polymer distribution determination from mass spectra. *Anal. Bioanal. Chem*., **2011,** *400*, 613-623.

Naczk, M.; Shahidi, F. Phenolics in cereals, fruits and vegetables: Occurrence, extraction and analysis. *J. Pharm. Biomed. Anal.*, **2006**, *41*, 1523–1542.

Newman, R. H.; Porter, L. J. Introduction in: Plant polyphenols. Hemingway, R.W., Laks, P.E. (Eds), Plenum Press, New York, **1992**, pp. 339-347.

Oszmianski, J.; Ramos, T.; Bourzeix, M. Fractionation of phenolic compounds in red wine. *Am. J. Enol. Vitic*., **1988**, *39*, 259−262.

Outtrup, H. Introduction in: Plant polyphenols. Hemingway, R.W., Laks, P.E. (Eds), Plenum Press, New York, **1992,** pp. 849-858.

Pasch, H.; Pizzi, A.; Rode, K. MALDI-TOF mass spectrometry of polyflavonoid tannins. *Polymer*, **2001**, *42*, 7531-7539.

Picinelli, A.; Suárez, B.; Mangas, J. J. Analysis of polyphenols in apple products. *Z. Lebensm. Unters. Forsch. A*, **1997**, *204*, 48−51.

Pizzi, A.**,** Handbook of Adhesive Technology, 2nd Edition Revised and Expanded, Marcel Dekker Inc., New York, **2003**, pp. 1-15.

Pizzi, A. Monomers, Polymers and Composites from Renewable Resources, Belgacem, M. N.; Gandini, A. (Eds), Elsevier, **2008**, pp. 179-199.

Pizzi, A. The chemistry and development of tannin-based adhesives for exterior plywood. *J. Polymer Sci*., **1978,** *22*, 2397-2399.

Porter, L. J.; Hrstich, L. N.; Chan, B. G. The conversion of procyanidins and prodelphinidins to cyanidin and delphinidin. *Phytochemistry*, **1969**, *8*, 677.

Putnam, L. J.; Butler, L. G. Fractionation of Condensed Tannins by Counter-Current Chromatography. *J. Chromatogr.*, **1985,** *318*, 85-93.

Reed, J. D.; Krueger, C.G.; Vestling, M.M. MALDI-TOF mass spectrometry of oligomeric food polyphenols. *Phytochemistry*, **2005,** *66*, 2248–2263.

Revilla, E.; Alonso, E.; Bourzeix, M.; Heredia, N. Dosage des catéchines et des proanthocyanidols dans les vins. Feuillet Vert O. I. V., **1991**, 829.

Rhodes, D.F., Cates, R.G. Toward a general theory of plant antiherbivores chemistry. Recent Adv. *Phytochem*., **1976**, *10*, 168–213.

Ricardo-da-Silva, J. M.; Rigaud, J.; Cheynier, V.; Cheminat, A.; Moutounet, M. Procyanidin dimers and trimers from grape seeds. *Phytochemistry*, **1991**, *4*, 1259-1264.

Rigaud, J.; Escribano-Bailon, M. T.; Prieur, C.; Souquet, J. M.; Cheynier, V. Normal-phase high-performance liquid chromatographic separation of procyanidins from cacao beans and grape seeds. *J. Chromatogr*., **1993**, *654*, 255-260.

Rigaud, J.; Perez-Ilzarbe, J.; Ricardo da Silva, J. M.; Cheynier, V. Micro method for the identification of proanthocyanidin using thiolysis monitored by high-performance liquid chromatography. *J. Chromatogr. A*, **1991,** *540*, 401-405.

Roberts, E. A. H.; Wood, J. D. A study of the polyphenols in tea leaf by paper chromatography. *Biochem. J.* **1951**, *49*, 414-422.

Roussy, J.; Chastellan, P.; van Vooren, M.; Guibal, E. Treatment of ink-containing wastewater by coagulation/flocculation using biopolymers, *Water SA*. **2005**, *31*, No. 3, 369-376.

Roux, D. G. Methods of fractionation and identification of constituents of condensed tannins, *JALCA*, **1958**, *(7)*, 384-395.

Roux, D. G. Photometric methods of tannin analysis for black wattle tannin, *J. Soc. Leath. Trade's Chem.*, **1951**, *35*, 322.

Roux, D. G. Ultraviolet photometric methods of tannin estimation in relation to wattle extract utilization. *J. Am. Leather Chemists' Assoc*., **1957**, *52***,** 319-329.

Roux, D. G.; Ferreira, D.; Botha, J. J. Structural considerations in predicting the utilization of tannins. *J. Agric. Food Chem*., **1980**, *28*, 216-222.

Roux, D. G. The fractionation and paper chromatography of black wattle tannins or polyphenols. *J. Soc. Leather Trades' Chem*., **1953**, *37*, 229.

Roux, D. G.; Ferreira, D.; Hundt, H.K.L.; Malan. E. Structure, stereochemistry, and reactivity of natural condensed tannins as basis for their extended industrial application. *App. Polym. Simp*. **1975,** *28*, 335-353.

Roux, D. G.; Paulus, E. Polymeric leuco-fisetinidin tannins from the heartwood of *Acacia mearnsii*. *Biochem. J.*, **1962,** *82*, 320-324.

Roux, D. G. Introduction in: Plant polyphenols. Hemingway, R.W., Laks, P.E. (Eds), Plenum Press, New York, **1992,** pp. 7-39.

Saint-Cricq De Gaulejac, N.; Freitas, V.; Glories, Y.; Bourgeois, G.; Vivas, N. Fractionement et dosage des procyandines oligomère des raisins et des vins: relation avec la qualité des vins. *Sciences des Aliments*, **1998**, *18*, 59-76.

Santos-Buelga, C.; Schalbert, A. Review, Proanthocyanidins and tannin-like compounds-nature, occurrence, dietary intake and effects on nutrition and health. *J. Sci. Food Agric*., **2000**, *80*, 1094-1117.

Sanz, M.; Fernández de Simón, B.; Esteruelas, E.; Mu˜noz, A. M.; Cadahía, E.; Hernández, M. T.; Estrella, I.; Martinez, J. Polyphenols in red wine aged in acacia (*Robinia pseudoacacia*) and oak (*Quercus petraea*) wood barrels. *Analytica Chimica Acta* , **2012**, *732*, 83–90

Scalbert, A. Introduction in: Plant polyphenols. Hemingway, R.W., Laks, P.E. (Eds), Plenum Press, New York, **1992,** pp. 259-280.

Scalbert, A.; Monties, B.; Janin, G. Tannins in wood: comparison of different estimation methods. *J. Agr. Food Chem*., **1989**, *37*, 1324.

Schofield, P.; Mbugua, D. M.; Pell, A. N. Analysis of condensed tannins: A review, *Anim. Feed Sci. Technol*., **2001**, 91, 21-40.

Seavell, A. J. Anti corrosive properties of mimosa (wattle) tannin. *Journal of the Oil and Colour Chemists' Association*, **1978**, *61*, 439.

Senekal, N. D. A Solid State NMR and MS Characterisation of the Chemical Composition of Mimosa Bark Extract, MSc Thesis**,** University of the Free State, **January 2011**, pp. 45-122.

Serrano, J.; Puupponen-Pimia, R; Dauer, A.; Aura, A-M. ; Saura-Calixto, F. Tannins: Current knowledge of food sources, intake, bioavailability and biological effects. *Mol. Nutr. Food Res*., **2009**, *53*, S310-S329.

Shuttleworth, S. G. Wattle Tannin and Mimosa Extract, Leather Industries Research Institute, Scott & Sherry Printers, Grahamstown, South Africa, **1955**, pp. 33-47.

Slabbert, N. Introduction in: Plant polyphenols. Hemingway, R.W., Laks, P.E. (Eds), Plenum Press, New York, **1992,** pp. 7-39.

Solovchenko, A.; Schmitz-Eiberger, M. Significance of skin flavonoids for UV-B-protection in apple fruits. *J. Exp. Bot.*, **2003**, *54 (389)*, 1977-1984.

Souquet, J.; Cheynier, V.; Brossaud, F.; Moutounet, M. Polymeric proanthocyanidins from grape skins. *Phytochemistry*, **1996**, *43*, 509 -512.

Sun, B.S.; Belchior, G. P.; Ricardo-da-Silva, J. M.; Spranger, M. I. Isolation and purification of dimeric and trimeric procyanidins from grape seeds. *J. Chromatogr. A*, **1999b**, *841*, 115-121.

Sun, B. S.; Leandro, M. C.; Ricardo-da-Silva, J. M.; Spranger, M. I. Separation of grape and wine proanthocyanidins according to their degree of polymerization. *J. Agric. Food Chem.*, **1998a**, *46*, 1390-1396.

Sun, B. S.; Spranger, M. I.; Roque-do-Vale, F.; Ricardo-da-Silva, J. M. Thioacidolysis-HPLC characterization of oligomeric and polymeric procyanidin fractions from grape seeds. In: *Actas do 1 Encontro Nacional de Cromatografia*, Lisboa, **1999c**, 34-35.

Sun, B.; Spranger, I. Review: Quantitative extraction and analysis of grape and wine proanthocyanidins and stilbenes. *Ciência Téc. Vitiv.* , **2005**, *20* (2), 59-89.

Sundar, V. J.; Rao J. R.; Muralidharan, C. Cleaner chrome tanning-emerging options. *J. Cleaner Prod.*, **2002**, *10*, 69-74.

Swain, T.; Hillis, W. E. The phenolic constituents of *Prunus domestica. I.* The quantitative analysis of phenolic constituents. *J. Sci. Food Agric.*, **1959**, *91*, 92.

Takahata, Y.; Ohnishi-Kameyama, M.; Furuta, S.; Takahashi, M.; Suda, I. Highly polymerized procyanidins in brown soybean seed coat with a high radical-scavenging activity. *J. Agric. Food Chem.*, **2001,** *49*, 5843-5847.

Taylor, A. W.; Barofsky, E.; Kennedy, J. A.; and Deinzer, M. L. Hop *(Humulus lupulus* L.) proanthocyanidins characterized by mass spectrometry, acid catalysis, and gel permeation chromatography. *J. Agric. Food Chem.*, **2003,** *51*, 4101-4110.

Thompson, D., Pizzi, A. Simple ^{13}C-NMR methods for quantitative determinations of polyflavonoid tannin characteristics. *J. Appl. Polym. Sci.*, **1995,** *55*, 107.

Thompson, R. S.; Jacques, D.; Haslam, E.; Tanner, R. N. J. Plant proanthocyanidins. Part I. Introduction; the isolation, structure, and distribution in nature of plant procyanidins. *J. Chem. Soc., Perkin Trans. 1*, **1972,** 1387-1399.

Tsao, R.; Yang, R.; Young, J. C.; Zhu, H. Polyphenolic Profiles in Eight Apple cultivars using high-performance liquid chromatography (HPLC). *J. Agric. Food Chem.,* **2003**, *51,* 6347-6353.

Vankar, P. S.; Shanker, R.; Verma, A. Enzymatic natural dyeing of cotton and silk fabrics without metal mordants. *J. Cleaner Prod.*, **2007**, *15*, 1441-1450.

Venter, P. B.; Sisa, M.; van der Merwe, M. J.; Bonnet, S. L.; van der Westhuizen, J. H. Analysis of commercial proanthocyanidins. Part 1: The chemical composition of quebracho (*Schinopsis lorentzii* and *Schinopsis balansae*) heartwood extract. *Phytochemistry*, **2012**, 73, 95-106.

Vidal, S.; Cartalade, D.; Souquet, J. M.; Fulcrand, H.; Cheynier, V. Changes in proanthocyanidin chain length in winelike model solutions. *J. Agric. Food Chem.*, **2002**, *50,* 2261-2266.

Vivas, N.; Nonier, M. F.; Vivas de Gaulejac, N.; Absalon, C.; Bertrand, A.; Mirabel, M. Differentiation of proanthocyanidin tannins from seeds, skins and stems of grapes (*Vitis vinifera*) and heartwood of Quebracho (*Schinopsis balansae*) by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry and thioacidolysis/liquid chromatography/electrospray ionization mass spectrometry. *Analytica Chimica Acta* **2004,** *513*, 274-256.

Viviers, P. M.; Kolodziej, H.; Young, D. A.; Ferreira, D.; Roux, D. G. Synthesis of condensed tannins. Part 11. Intramolecular enantiomerism of the constituent units of tannins from the Anacardiaceae: Stoicheometric control in direct synthesis: Derivation of ^{1}H nuclear magnetic resonance parameters applicable to higher oligomers. *J. Chem. Soc., Perkin Trans 1*, **1983,** 2555-2562.

Wang, K.; Ruan, J. Analysis of chemical components in green tea in relation with perceived quality, a case study with Longjing teas. *Int. J. of Food Sci. Technol.*, **2009**, *44*, 2476–2484.

Yanagida, A.; Shoji, T.; Shibusawa, Y. Separation of proanthocyanindins by degree of polymerisation by means of size-exclusion chromatography and related techniques. *J. Biochem. Biophys. Methods*, **2003**, 56, 311-322.

Yang, C. S.; Lambert, J. D.; Ju, J.; Lu, G.; Sang, S. Tea and cancer prevention: Molecular mechanisms and human relevance. *Toxicol. Appl. Pharmacol*. **2007**, *224*, 265-273.

Yang, C. S.; Wang, Z. Y. A Review- Tea and Cancer. *J. Natl Cancer Inst*. **1993**, *85*, 1038-1049.

Yazaki, Y.; Collins, P. J. Black Wattle and its Utilisation Abridged English Edition, Brown , A. G., Ko, H. C. (Eds), Rural Industries Research & Development Corporation (RIRDC) , **1997,** pp. 127-129.

Young, D. A., Ferreira, D., Roux, D. G. Synthesis of condensed tannins. Part 15. Structure of natural 'angular' profisetinidin tetraflavanoids: asymmetric induction during oligomeric synthesis. *J. Chem. Soc*., *Perkin Trans 1*, **1985,** 2529-2535.

Zaisong, W., Black Wattle and its Utilisation Abridged English Edition, Brown , A. G., Ko, H. C. (Eds), Rural Industries Research & Development Corporation (RIRDC), **1997,** pp. 150-151.

Chapter 3

3. Analysis of Commercial Proanthocyanidins. Part 2: An Electrospray Mass Spectrometry Investigation into the Chemical Composition of Sulfited Quebracho

3.1. Introduction

Vegetable tannins are plant extracts that have traditionally been used to tan leather. The term tannin refers to both hydrolysable tannins i.e. polyesters of gallic or hexahydroxydiphenic acid and D-glucose and proanthocyanidins (PACs), i.e. flavan-3-ol oligo-/ polymers. PACs are also referred to as condensed tannins. Owing to greater availability from natural and planted forests, PACs have virtually replaced hydrolysable tannins in the industrial applications of vegetable tannins (Pizzi, 1982).

Important industrial sources of PACs are mimosa bark extract (*Acacia mearnsii*) and quebracho heartwood extracts (*Schinopsis lorentzii* and *Schinopsis balansae*). Apart from the tanning of leather (Roux *et al*., 1975), PACs are also used to manufacture adhesives (Pizzi, 1977), as water purification resins (Beltran-Heredia *et al*., 2009; Beltran-Heredia and Sanchez-Martin, 2008, 2009), and as mud additives for oil well drilling (Haslam, 1988; Herrick, 1980). Their potential uses as iron anti-corrosion agents (Jaén *et al*., 1999; Matamala *et al*., 2000; Seavell, 1978), scale inhibitors in water pipes (Cooper *et al*., 1979), and beneficiation of mineral ores (Moscovici *et al*., 1961; Roux *et al*., 1980) have been reported.

PACs are often sulfited to reduce viscosity and increase solubility in water. Many of the properties and industrial applications of vegetable tannins are attributed to their ability to form complexes with proteins *via* hydrogen bonds (Covington *et al*., 2005; Haslam, 1974). These include astringency in tea and red wine *via* interactions between tannins and protein-based taste receptors in the mouth (Joslyn and Goldstein, 1964), anti-feeding properties due

to the indigestibility of tannin-protein complexes (Rhodes and Cates, 1976), and growth inhibition of many micro-organisms *via* irreversible deactivation of enzymes (Akin, 1982). The complexation of vegetable tannins with hide proteins *via* hydrogen bonds transforms biodegradable raw hide into leather which resists bacterial degradation, has a pleasant feel to it, and is abrasion-, heat-, and water-resistant (Haslam, 2005). PACs are used to manufacture adhesives and resins *via* cross linking of the nucleophilic aromatic A-rings of the constituent flavan-3-ol monomers with formaldehyde (Pizzi, 1980). PACs with *ortho*-dihydroxy substitution on the B-ring can complex with heavy metals and this explains some of their water purification applications (Beltran-Heredia and Sanchez-Martin, 2008).

Slow progress in defining the composition of the PAC extract has been attributed to the complexity of the extracts and poor resolution and recovery with silica gel based chromatography. The interflavanyl carbon–carbon bonds in proanthocyanidins can be hydrolysed with dilute acid to give unstable intermediates that form colored anthocyanidins, hence the term proanthocyanidin (Roux, 1992). This process gives rise to autumn colors in deciduous trees and the ripening of fruit. The astringency that prevents green fruit from being consumed disappears as interflavanyl bonds are hydrolysed and the resulting monomers are oxidized to colored anthocyanidins that signal that the fruit is ripe and ready for eating (Jacques *et al*., 1977). The classical method to analyse PACs consists of trapping hydrolysis intermediates with toluene-a-thiol or phloroglucinol (thiolysis and phloroglucinolysis) before anthocyanidins are formed (Kennedy and Taylor, 2003; Rigaud *et al*., 1991) and analysis of the trapped intermediates with HPLC. This has shed light on uncertainties such as the different hydroxylation patterns of the constituent flavan-3-ol aromatic rings, the different configurations at the C-2, C-3 and C-4 stereogenic centres, the possibility of a second ether-type interflavanyl bond (A-type PACs), the average chain length (degree of polymerisation), and the presence of angular oligomers.

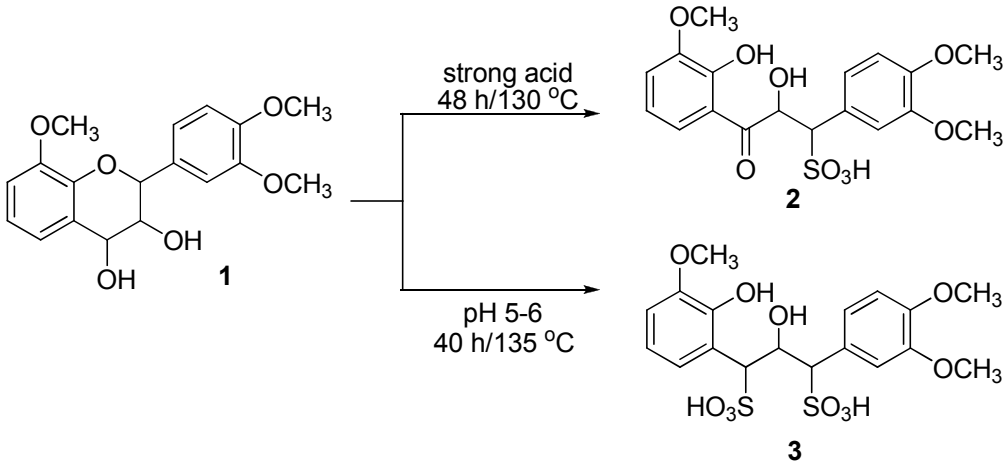
The absence of 5-OH groups in the constituent monomers of commercially important PAC extracts such as mimosa and quebracho, however, imparts stability to the interflavanyl bond against acid hydrolysis (Roux *et al*., 1980, 1975). The consequent higher temperatures required to hydrolyse the interflavanyl bond decompose the intermediates and trapped products and render thiolysis and phloroglucinolysis unreliable. For example, Vivas *et al*. (2004) did not isolate any known flavan-3-ol/toluene-α-thiol adducts upon thiolysis of

quebracho tannins. The absence of 5-OH groups in commercially important PACs is probably not a coincidence, as the resulting stable interflavanyl bonds impart longevity to leather, adhesives, and other products derived from these PACs.

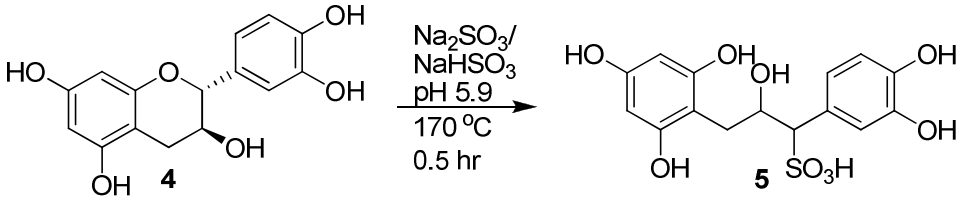
The two main sources of quebracho extract are forests of *S. balansae* (red ‘‘chaqueno’’ quebracho) from the eastern Chaco region and *S. lorentzii* (red ‘‘santiagueno’’ quebracho) from the western Chaco region of Argentina, Bolivia, and Paraguay. The extract consists of about 95% PACs and 5% water-soluble sugars on a dry basis. A warm water-soluble (unsulfited) quebracho vegetable tannin extract is obtained from chipped quebracho heartwood (after removal of the bark and outer white wood) by boiling with water in pressurized vessels. A cold water-soluble extract (sulfited extract) is obtained upon sulfitation of the warm water-soluble extract (treating the warm water soluble extract with $NaHSO_3$ or direct extraction of wood chips with a boiling aqueous bisulfite solution). Higher extraction rates are obtained with boiling aqueous bisulfite solution than with boiling water alone.

The relative affinity of PACs for collagen, the rate of penetration into hides and skins during commercial tannage, mobility within leather, and desorption from finished leather under moist conditions are determined by oligomer composition (Covington, 2009). The relative insoluble nature of hot water-soluble quebracho extract limits its tanning applications in sole-leather pit processes. Cold water-soluble (sulfited) quebracho extract has a much enhanced pelt penetration. High viscosity is a problem in adhesive manufacturing from condensed tannin extracts. Early formation of methylene bridges (derived from formaldehyde) immobilizes the tannin–formaldehyde network. The rigid network, associated with long distances between constituent PACs and high viscosity, prevents further methylene bridge formation and cross linking. This leads to adhesives with high viscosity, poor water resistance, and low strength (Pizzi, 1978). Sulfitation changes both the physical and chemical properties of PACs. The reaction solubilises relatively water-insoluble PACs and reduces the viscosity of PAC extracts (Pizzi, 1982). Sulfitation also increases the moisture retention of tannin adhesives and allows the adhesive film to dry more slowly (Pizzi, 1979) although the presence of hydrophilic sulfonic acid moieties reduces the adhesives’ water resistance. A better understanding of the molecular composition of PACs in general and sulfited PACs in particular will enhance their industrial applications. The commonly held view is that the

heterocyclic pyran ring is opened upon sulfitation leading to sulfonic acid derivatives (Pizzi, 1972). This is supported by the work of Richtzenhain and Alfredsson (1955), who obtained **2** upon sulfitation of **1** in strong sulfuric acid, and **3** at pH 5–6 after prolonged heating (Scheme 1). Similar results were obtained by Sears (1972), who isolated **5** which contains a sulfonic acid group at C-2 *via* opening of the pyran ring upon sulfitation of catechin **(4)** at high temperatures (Scheme 2).



Scheme 1: Sulfitation of a flavan-3, 4-diol



Scheme 2: Sulfitation of catechin

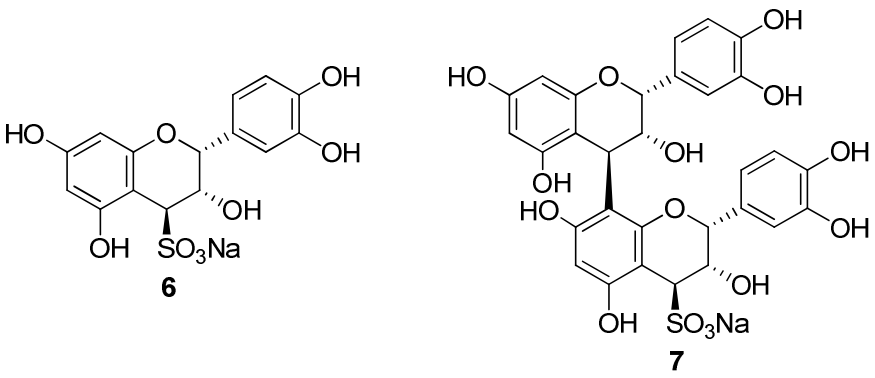
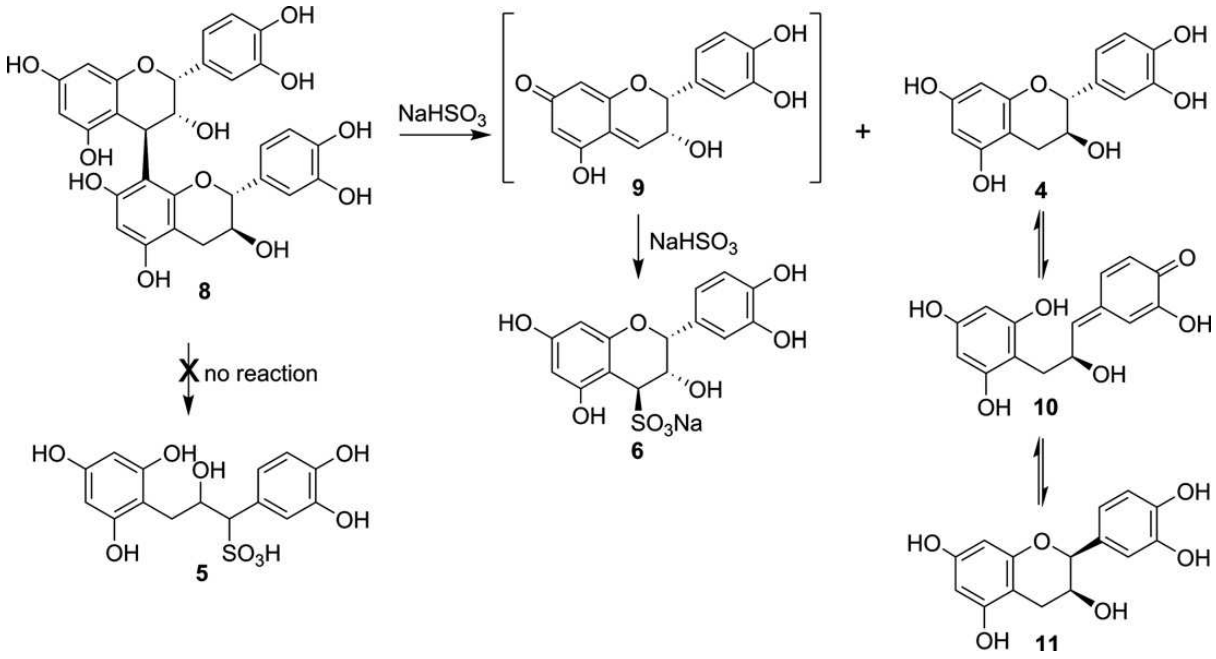
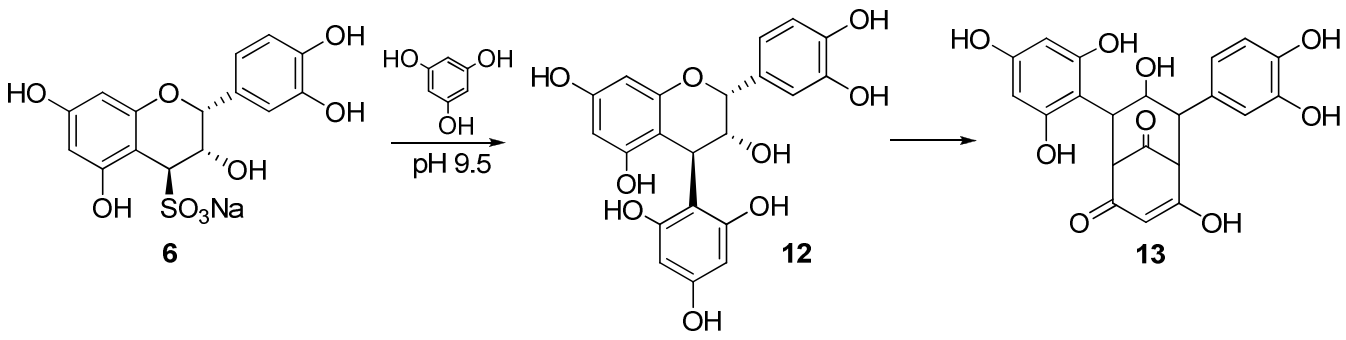


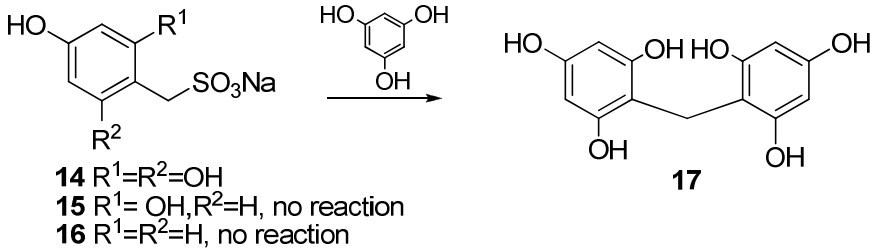
Figure 1: Products from the sulfitation of loblolly pine bark extract



Scheme 3: Sulfitation of epicatechin-(4β→8)-catechin 8



Scheme 4: Reaction of epicatechin-4β-sulfonate with phloroglucinol



Scheme 5: Reaction of hydroxylated benzylsulfonic acids with phloroglucinol

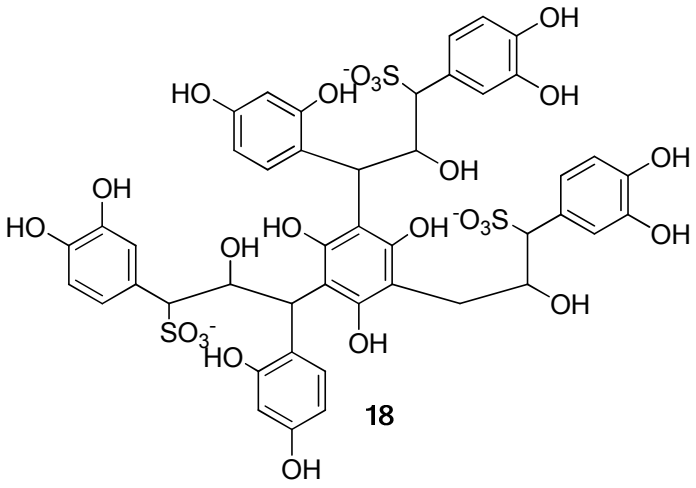


Figure 2: Hemingway's proposed structure of a sulfited quebracho PAC

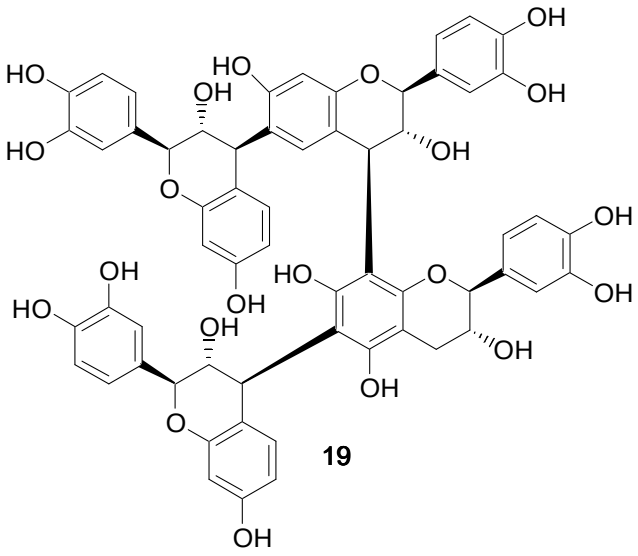


Figure 3: Proposed structure of an angular quebracho tetramer

Ring-opening will be beneficial to adhesive manufacturing as it changes the A-ring of the constituent PAC monomers from a less nucleophilic alkoxybenzene into a more nucleophilic hydroxybenzene. The additional nucleophilicity promotes cross linking with formaldehyde on the periphery of oligomers, resulting in lower curing time and pot life of thermosetting PAC-based adhesives. The ring-opening theory was, however, refuted by Hemingway's group (Foo *et al*., 1983; McGraw and Hemingway, 1988) who isolated predominantly epicatechin-4β-sulfonate **6** (20%) and epicatechin-(4β→8)-epicatechin-4β-sulfonate **7** (6%) (Figure 1), and only small quantities (1%) of the pyran ring-opened **5** upon sulfitation of loblolly pine bark extract.

Table 1: Oligomers observed in cold water-soluble (sulfited) and hot water-soluble (unsulfited) quebracho extract (Figures 4a and 4b, negative mode m/z values)

Oligomer	Monosulfited oligomer molecule (m/z+82)	Unsulfited oligomer molecule (m/z)
Dimer	643	561
Trimer	915	833
Tetramer	1187	1105

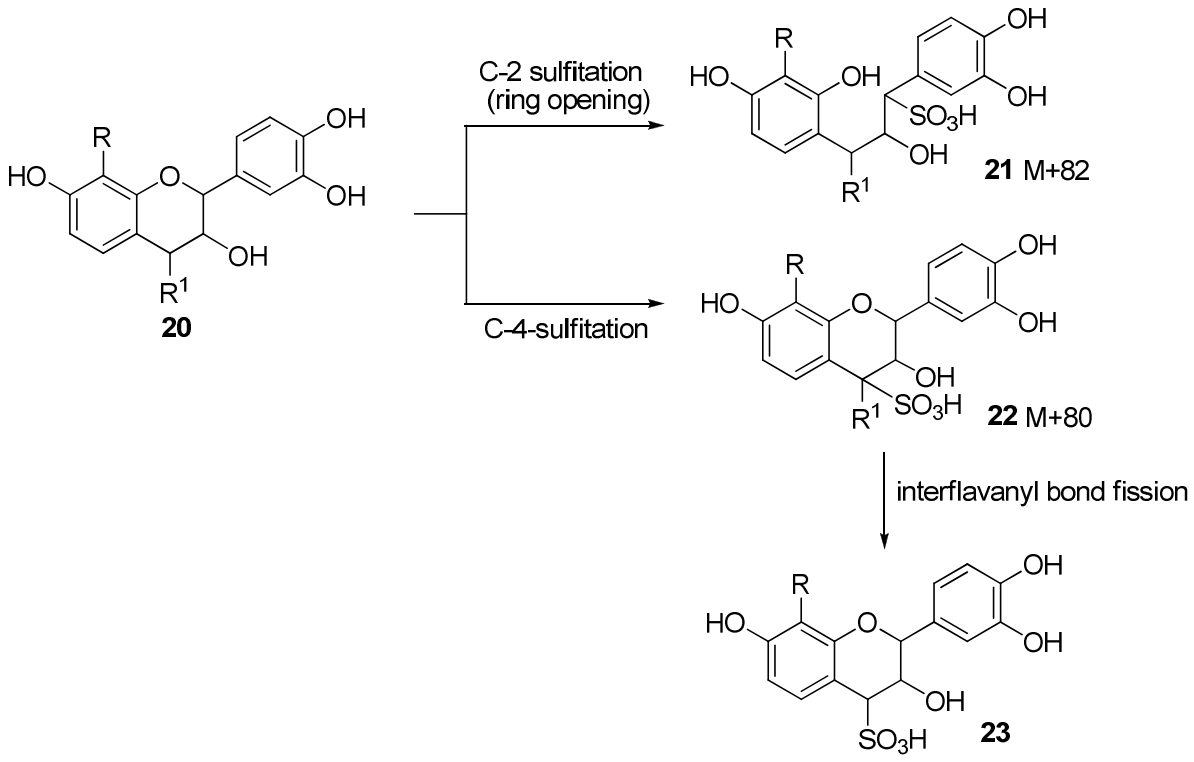
Hemingway's conclusion is further supported by the isolation of **6, 4,** and **11** upon sulfitation of procyanidin B1 **8** (McGraw and Hemingway, 1988). Isolation of **11** and not **5**, presumably *via* the B-ring quinone methide **10**, indicates that C-2 epimerization takes place in preference to C-2 sulfitation (Scheme 3). In contrast with catechin- and epicatechin-derived PACs, where the interflavanyl bond is weakened by the presence of a 5-OH group (phloroglucinol A-ring), quebracho and mimosa PACs are based on flavan-3-ols with a resorcinol A-ring and have interflavanyl bonds that resist fission. The work described above is thus not directly applicable to sulfitation of quebracho extracts. We thus assume that sulfitation at C-4 and the accompanying ring fission will take place less readily with quebracho and mimosa PACs. This assumption is, however, not supported by the reduction of the number average molecular weight of mimosa bark extract from 1355 to 1016 Da after sulfitation (determined with size exclusion chromatography), which was attributed to cleavage of interflavanyl bonds (Fechtal and Riedl, 1993). Hemingway demonstrated that the 4-sulfonic acid group in **6** is readily replaced by phloroglucinol to yield a 4β-phloroglucinol–epicatechin adduct **12** (McGraw and Hemingway, 1988). This product rearranges to the catechinic acid derivative **13** (major product) (Scheme 4). The transformation of **6** into **12** demonstrates the good nucleofugic attributes of a 4-sulfonic acid substituent and the reactivity of C-2 towards sterically accessible nucleophiles. To investigate the influence of the ring hydroxylation pattern on the stability of the C-4 sulfonic acid group, Hemingway treated model compounds **14, 15,** and **16** with phloroglucinol (Scheme 5) (McGraw and Hemingway, 1988). Phloroglucinol reacted at room temperature with 14 at pH 9.5 and above to form **17** in high yields. In contrast, **15** and **16** were unreactive under all pH conditions. These results suggest

that the C-4 sulfonic acid group of sulfited PACs with phloroglucinol A-rings, e.g. loblolly pine bark extract, will be nucleofugic whilst sulfonic acid groups at C-4 of sulfited PACs with resorcinol A-rings, e.g. mimosa and quebracho, will be non-nucleofugic. Hemingway's results correspond well with the observation above, i.e. the presence of a 5-OH substituent weakens the interflavanyl bond towards acid-catalysed hydrolysis. This suggests that C-4 sulfited quebracho and mimosa PACs will form less readily than C-4 sulfited phloroglucinol PACs such as loblolly pine bark extract and wine tannins, but once formed will be more stable. Hemingway suggests compound **18** as a possible structure for sulfited quebracho PAC trimers (Figure 2) (McGraw and Hemingway, 1988). Electrospray ionisation (ESI) and matrix-assisted laser desorption ionisation (MALDI) are soft ionisation techniques that can be used in mass spectrometry (MS) to fractionate a mixture of oligomers, such as quebracho PAC extract, and determine the molecular mass of each fraction. MALDI allows the investigation of larger oligomers, i.e. the detection of m/z values with a higher degree of polymerisation than ESI. Both techniques suffer from mass discrimination. Bigger oligomers have progressively smaller MS response factors and are thus underestimated (Mané *et al*., 2007; Mouls *et al*., 2011; Taylor *et al*., 2003). Modern ES-MS equipment allows charged MS fragments to be isolated and further fragmented, allowing structural isomers with the same m/z values to be distinguishable. Mimosa and quebracho extracts consist of relatively small oligomers (aDP of about 5), well within the range of ESI.

3.2. Results and Discussion

We recently reported (Venter *et al*., 2012a) that quebracho PAC oligomers consist of a homologous series of flavan-3-ol based oligomers. The starter unit is always catechin and the extender units are always ent-fisetinidol. The first two extender units are angularly bonded *via* C-4 to C-6 and C-8 of the catechin starter unit. Further oligomerisation takes place *via* C-6 of the ent-fisetinidol extender units. Figure 3 gives the proposed structure of a tetrameric ent-fisetinidol-(4β→6)-ent-fisetinidol-(4β→8)-catechin-(6→4β)-ent-fisetinidol **(19)**. We based the stereochemical assignments of **19** on the stereochemistry of the catechin and ent-fisetinidol-4β-ol monomers isolated from quebracho heartwood during phytochemical

investigations (Roux and Evelyn, 1960; Viviers *et al*., 1983) and phytochemical and synthetic organic perspectives (Venter *et al*., 2012a). For simplicity and because MS data do not give stereochemical information, we refer to the tetramer **19** as fisetinidol–fisetinidol–catechin–fisetinidol. There is little empirical information on the chemical changes that take place during sulfitation of quebracho extract. The presumed sulfonic acid moiety-containing products have not been isolated and their structures have not been determined. MALDI–TOF investigations of commercial sulfited quebracho extract have not even detected sulfonic acid-containing monomers or oligomers (Pasch *et al*., 2001; Vivas *et al*., 2004). Karchesy *et al*. (1989) detected sulfonic acid moieties with fast atom bombardment mass spectrometry in sulfited conifer bark extract, a 5-OH PAC extract. Vidal *et al*. (2002) detected flavanol units substituted with sulfonic acid at C-4 (M+80) with ESI-MS in sulfited grape seed extract, also a 5-OH PAC extract. Herein we expand our published ESI-MS methodology on the composition of unsulfited quebracho extract to sulfited quebracho extract. When comparing our ESI-MS with published MALDI data, it should be taken into account that MALDI-ionisation is observed *via* sodium $[M+23]^+$ or potassium $[M+39]^+$ adducts. The corresponding m/z values are 16 Da apart and can be misinterpreted in PAC mass spectra as evidence of the presence of oligomers with additional OH-groups (Reed *et al*., 2005).



Scheme 6: Influence of the position of sulfitation (C-2 or C-4) on the *m/z* values observed in the MS spectrum of sulfited quebracho extract. R and R^1 indicate H, or the remaining flavan-3-ol building blocks

3.2.1. ESI-MS Investigation of Sulfited Quebracho Extract

Inspection of the negative mode ESI mass spectrum of cold water-soluble sulfited quebracho PAC extract (Figure 4a) reveals not only the dimers, trimers, and tetramers (m/z 561, 833, and 1105) observed in the ESI mass spectrum of unsulfited quebracho PAC extract (Figure 4b) (Venter *et al.*, 2012a), but also their m/z +82 sulfited analogues (m/z 643, 915, and 1187) (Table 1). Sodium adducts of the sulfited dimers and trimers are detected at m/z 665 and 937. The absence of similar adducts for unsulfited dimers and trimers anticipated at m/z 583 and 855, respectively, implies that sulfonic acid moieties are essential in the formation of these adducts. The presence of [M+82] molecules (e.g. 21) and the absence of [M+80] molecules (e.g. 22) are significant and indicate that sulfited dimers, trimers, and tetramers are formed

via cleavage of the pyran ring and the introduction of a C-2 sulfonic acid moiety [M+82] (Scheme 6). C-4 sulfitation would lead to [M+80] molecules. Of particular interest in the negative mode ESI spectrum of cold water-soluble (sulfited) quebracho PAC extract (Figure 4a) are the m/z 353 (273 + 80) and 435 (273 + 80 + 82) ions. The m/z 353 (273 + 80) ion indicates that the monosulfited species is C-4 sulfited, i.e. derived *via* interflavanyl bond fission. The m/z 435 (353 + 82) ion, however, corresponds with the addition of a second sulfite ion which contributes 82 Da *via* heterocyclic ring opening, i.e. C-2 sulfitation (Scheme 7). The fisetinidol (m/z = 273) ion is absent in the MS of unsulfited quebracho extract (Figure 4b), suggesting that mono- and di-sulfited fisetinidol (m/z 353 and 435, respectively) observed in the sulfited extract (Figure 4a) are not formed from fisetinidol monomers in the unsulfited extract and are the result of interflavanyl bond fission during sulfitation. A mechanism explaining the formation of the m/z 353 and 435 monomers during sulfitation of a PAC tetramer, e.g. m/z 1105, trimer, e.g. m/z 833, and dimer, e.g. m/z 1106, is proposed in Scheme 7. The m/z 353 ion can be directly formed via C-4 sulfitation of an unsulfited terminal extender unit or indirectly *via* a C-2 sulfited terminal extender unit.

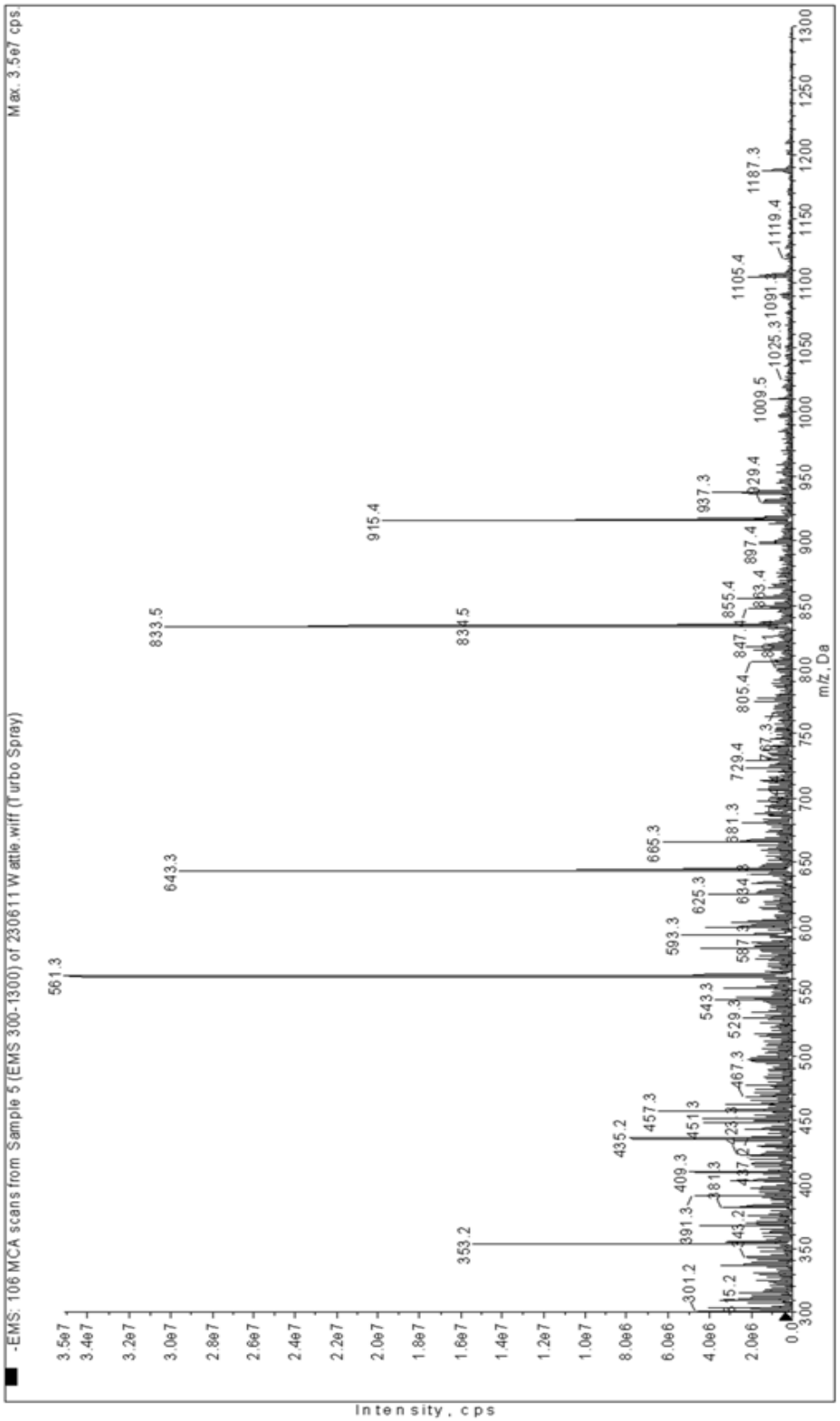


Figure 4a: Negative mode ESI spectrum of cold water-soluble (sulfited) quebracho PAC extracts (m/z 300–1300 range)

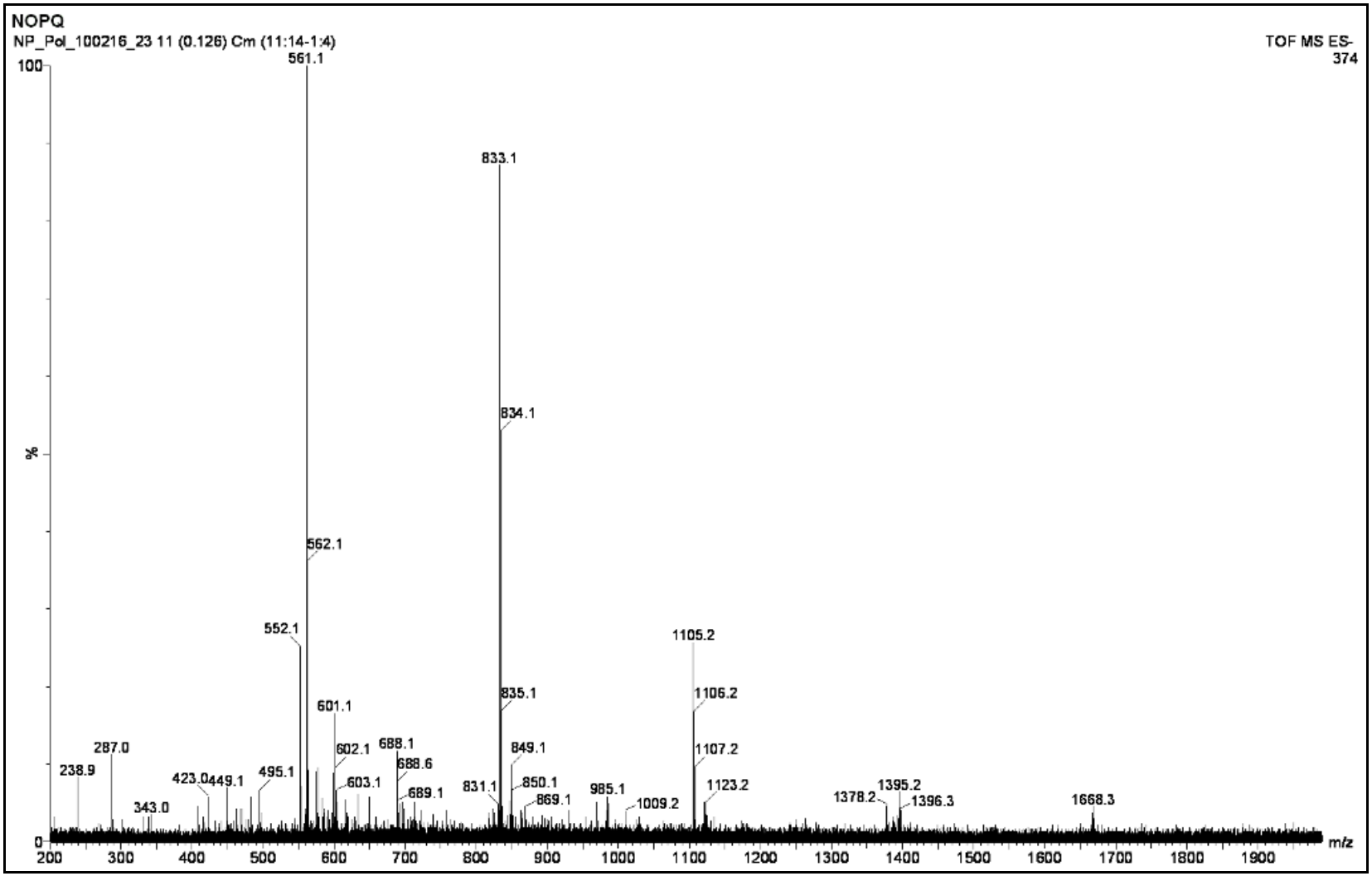
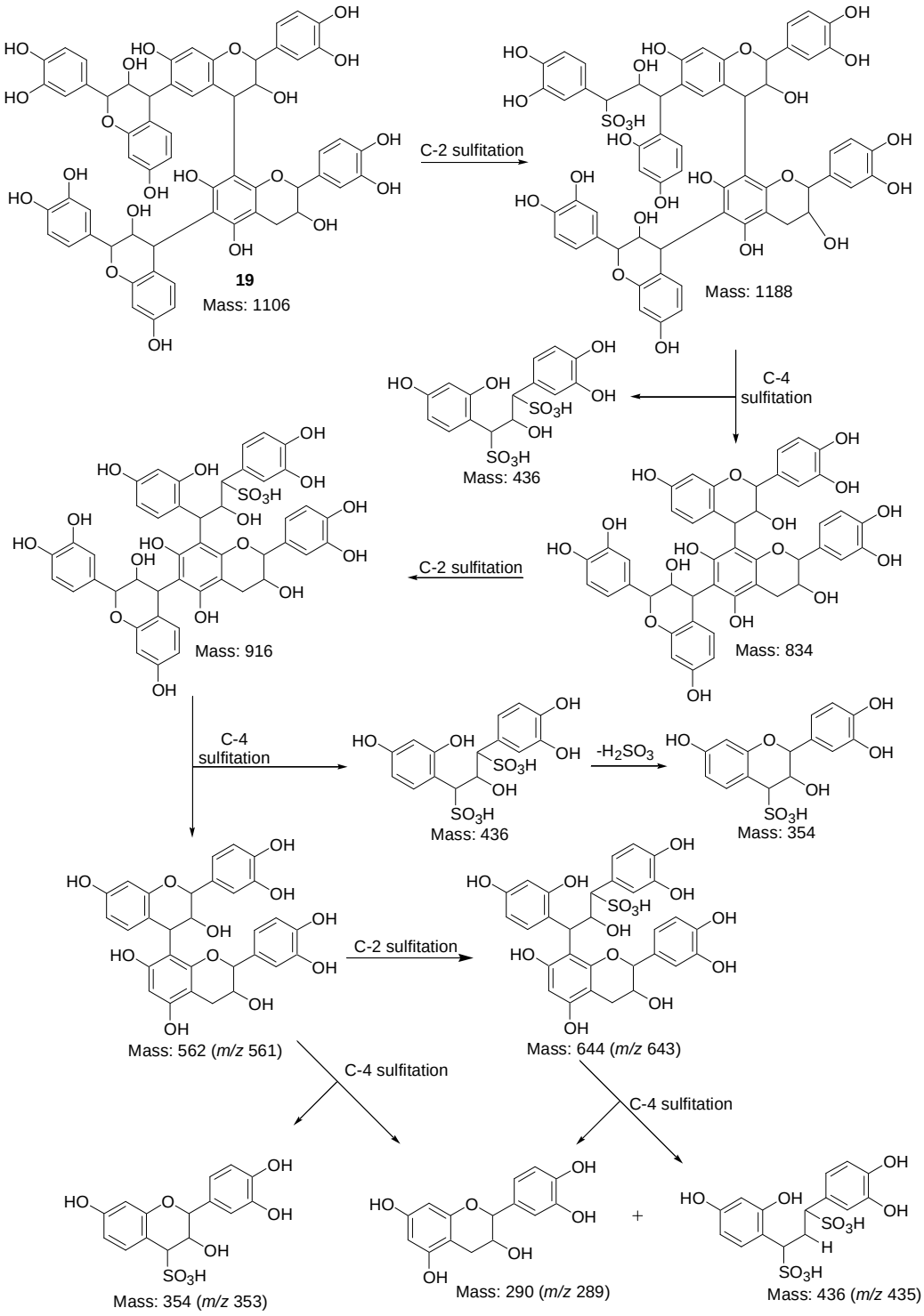


Figure 4b: Negative mode ESI spectrum of hot water-soluble (unsulfited) quebracho PAC extracts (m/z 200–2000 range) (Venter et al., 2012a)



Scheme 7: Products of C-4 sulfitation and interflavanyl bond fission of a sulfited dimer

3.2.2. MS^2 Investigation of the m/z 353, 435, 643, and 915 ions

MS^2 experiments were performed on the monosulfited fisetinidol ion (m/z 353), the disulfited fisetinidol ion (m/z 435), the sulfited dimer ion (m/z 643), and the sulfited trimer ion (m/z 915). The MS^2 results are given in figures 5–8, respectively, and the associated fragmentation patterns in schemes 8–11, respectively. These confirm our structure assignments in scheme 7. In oligomers with more than 90 carbons (tetramers), the ^{13}C isotope peak is bigger than the ^{12}C peak. Owing to the poor resolution of the equipment used for MS^2 experiments, these two peaks are not always well resolved and the ^{13}C isotope is sometimes automatically annotated - giving a fragment that is 1 Da heavier than expected. In the Q1 experiments (e.g. figures 4a and 4b), performed with a higher resolution mass spectrometer, both ions are present. No evidence of C-2 sulfited catechin 5 (m/z 371 ion, scheme 2) is found in any of the ESI-MS spectra studied. Neither was any evidence of robinetinidol (m/z 305), sulfited robinetinidol (m/z 369), disulfited robinetinidol (m/z 451), or any robinetinidol-containing oligomers (m/z = M+16) observed in any of our spectra. This confirms our previously published results (Venter *et al*., 2012a) that quebracho extract contains no robinetinidol extender units and that robinetinidol moieties identified with MALDI–TOF represent potassium adducts (16 Da heavier than the corresponding sodium adduct) of fisetinidol.

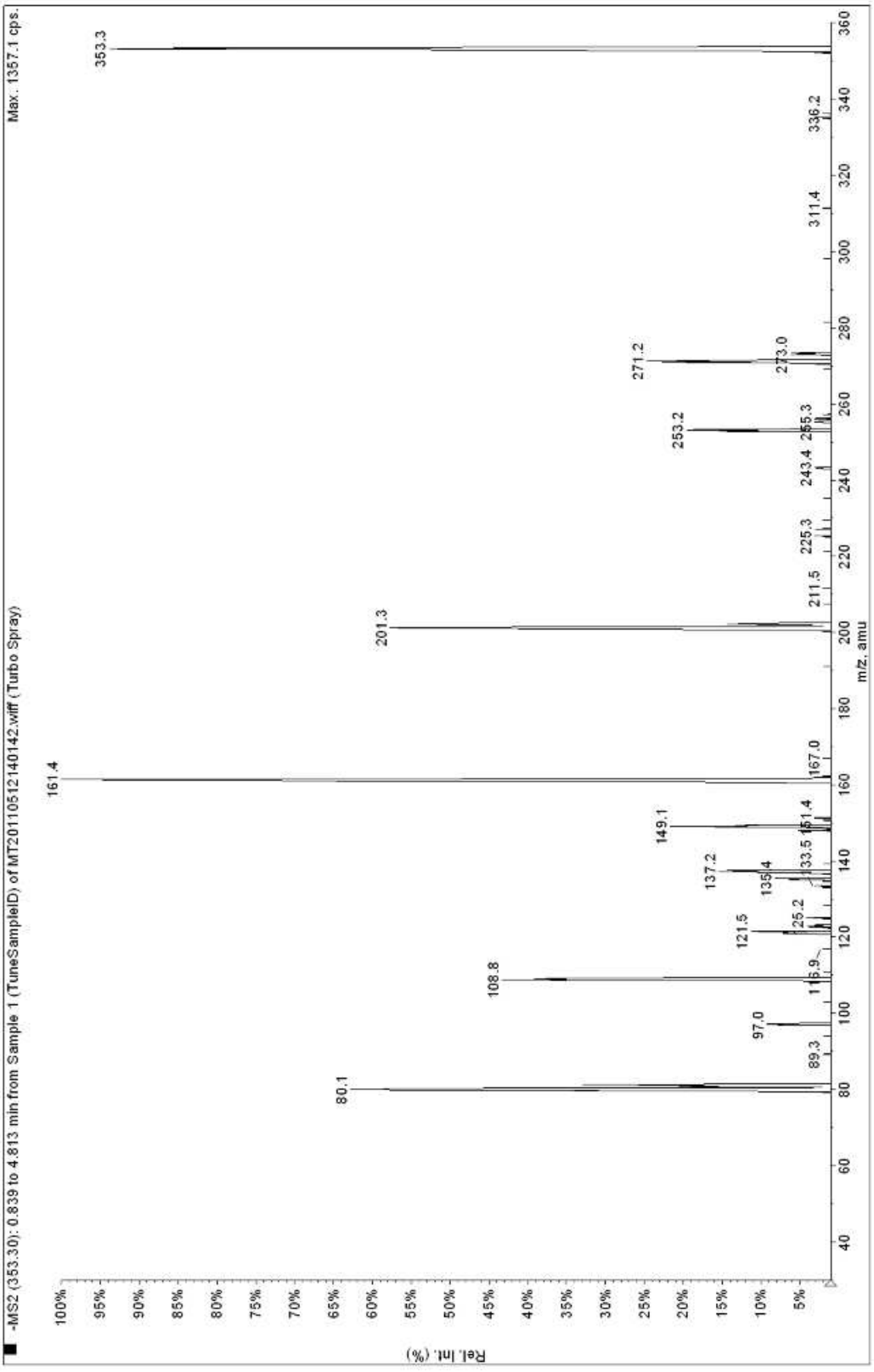


Figure 5: Negative mode MS^2 of m/z 353 fragment (monosulfited fisetinidol)

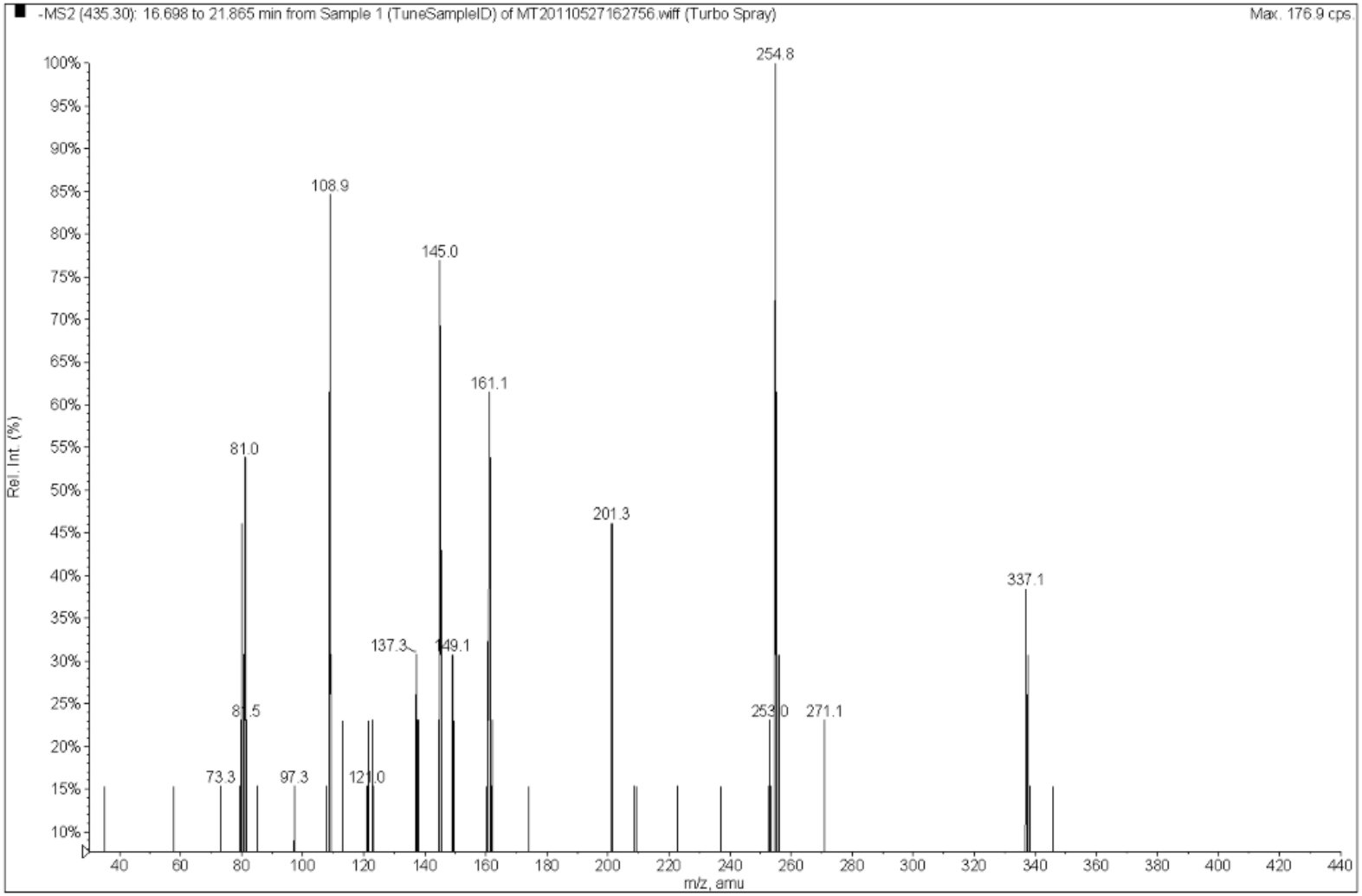


Figure 6: MS^2 fragmentation of the m/z 435 ion (disulfited fisetinidol)

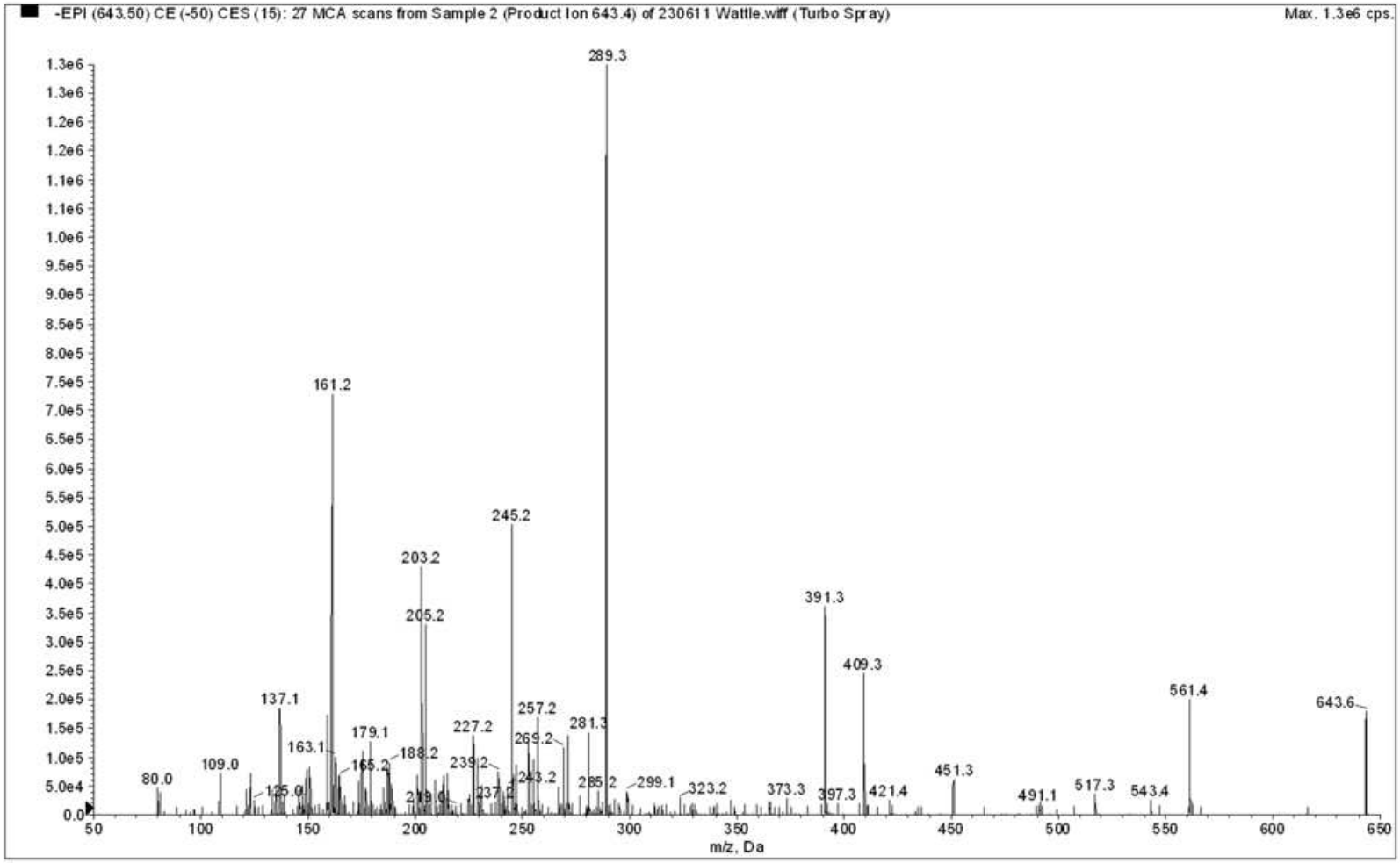


Figure 7: MS^2 fragmentation of the m/z 643 ion (sulfited dimer)

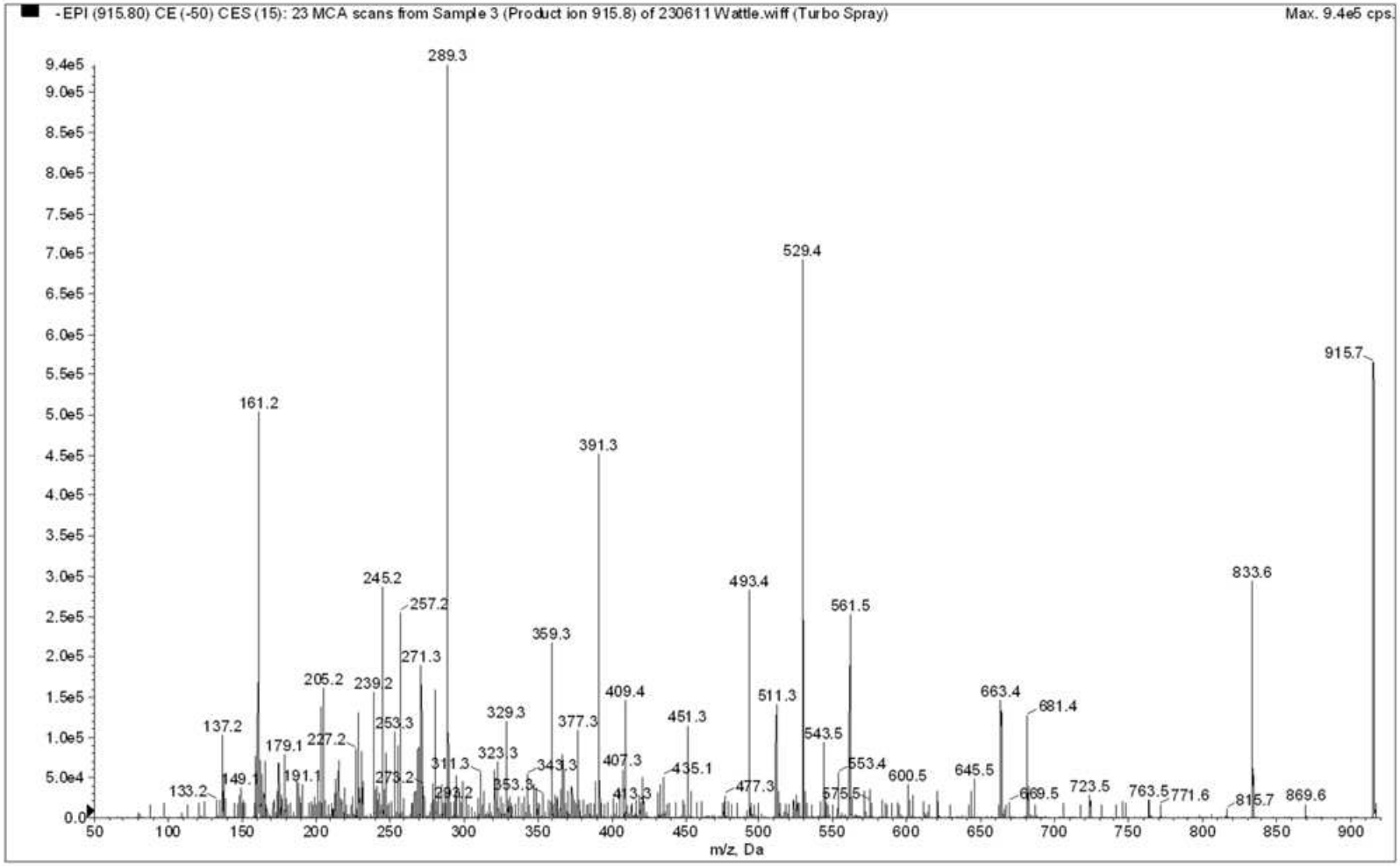
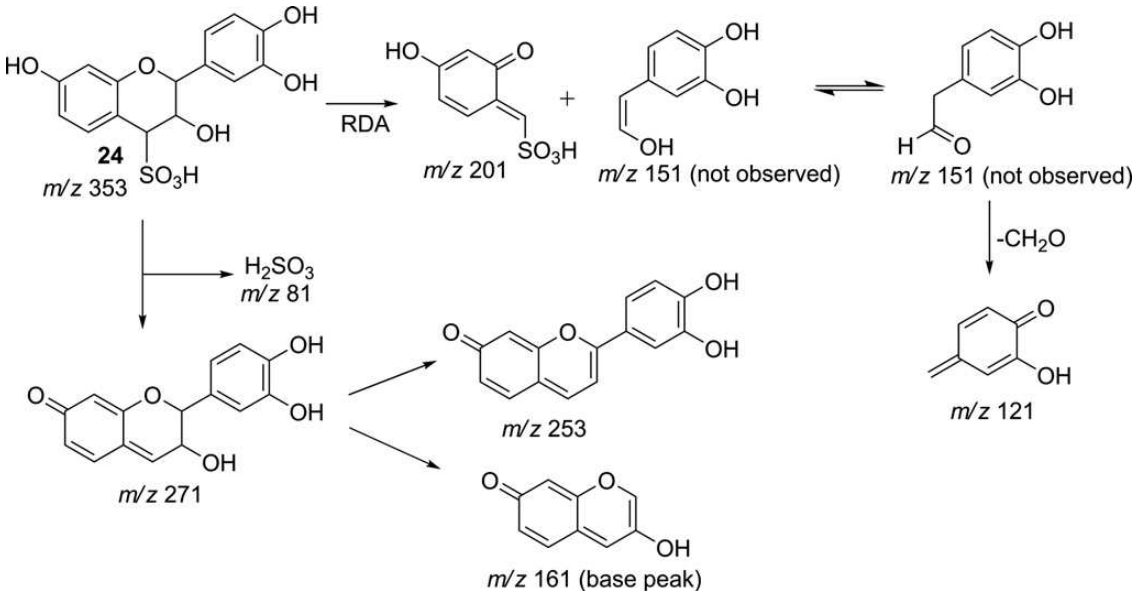
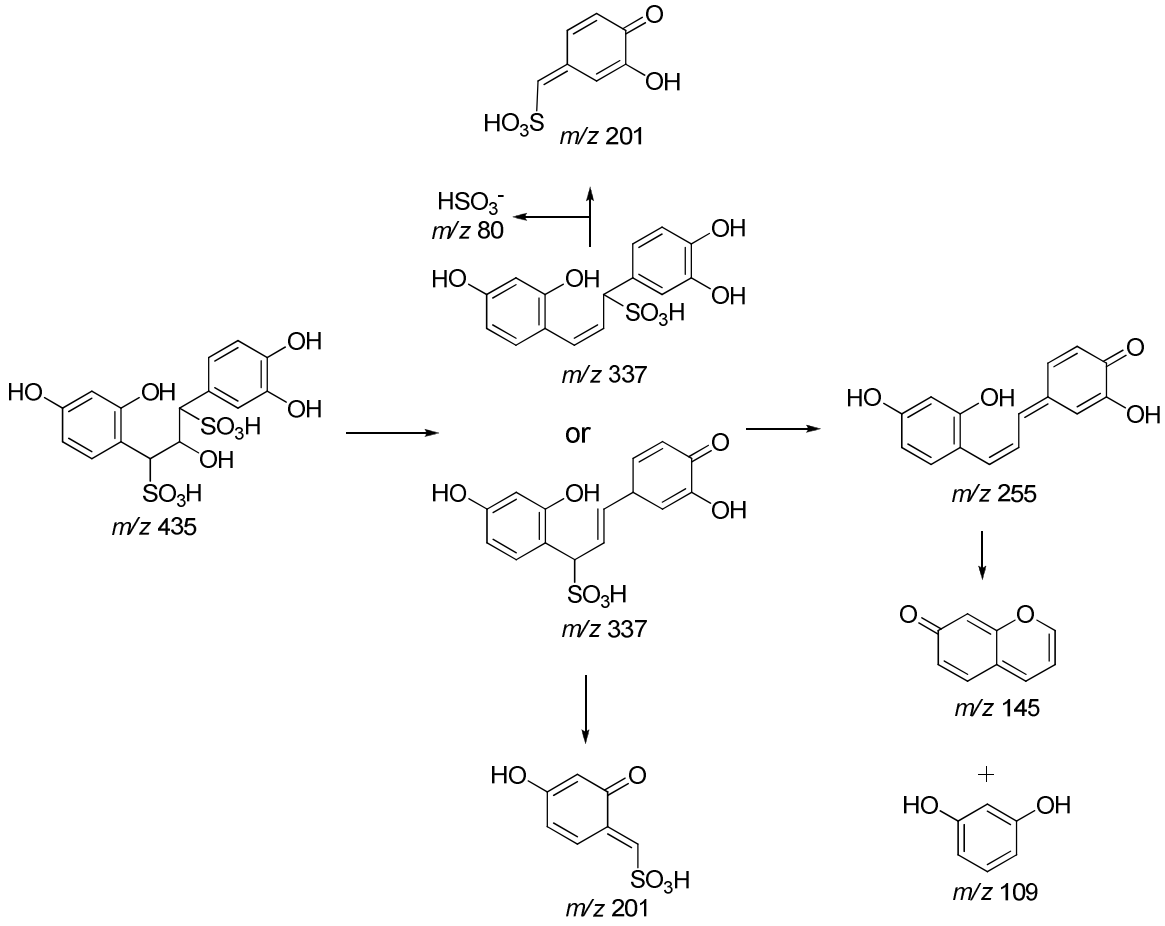


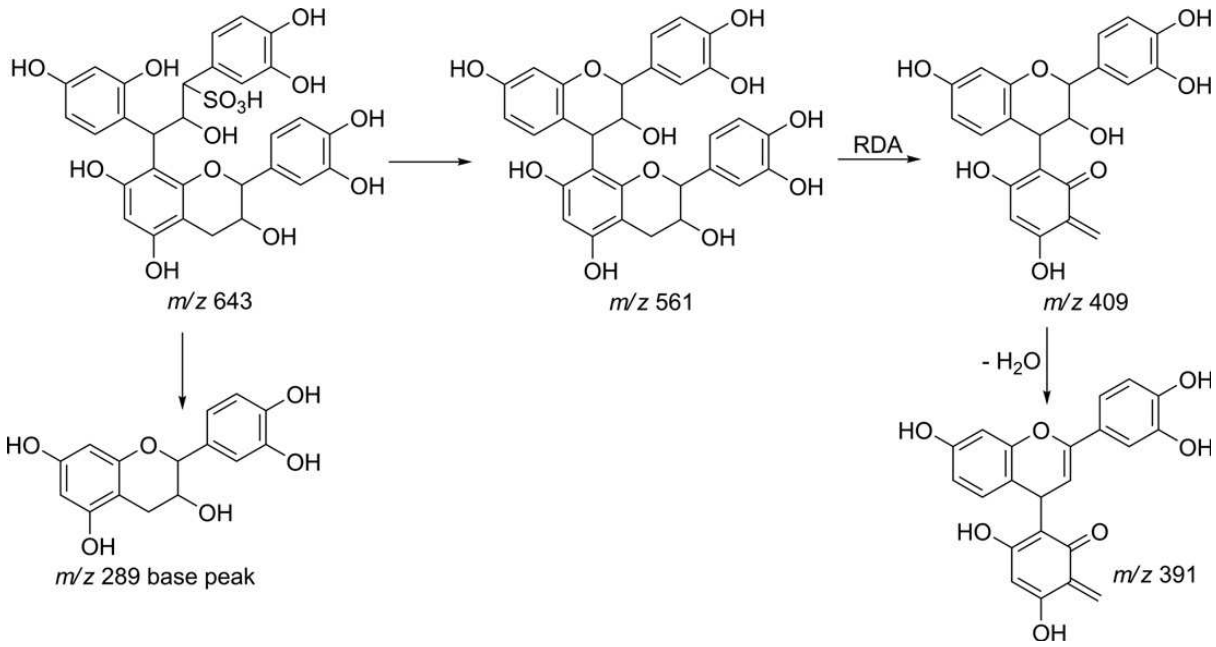
Figure 8: MS^2 fragmentation of the m/z 915 ion (sulfited trimer)



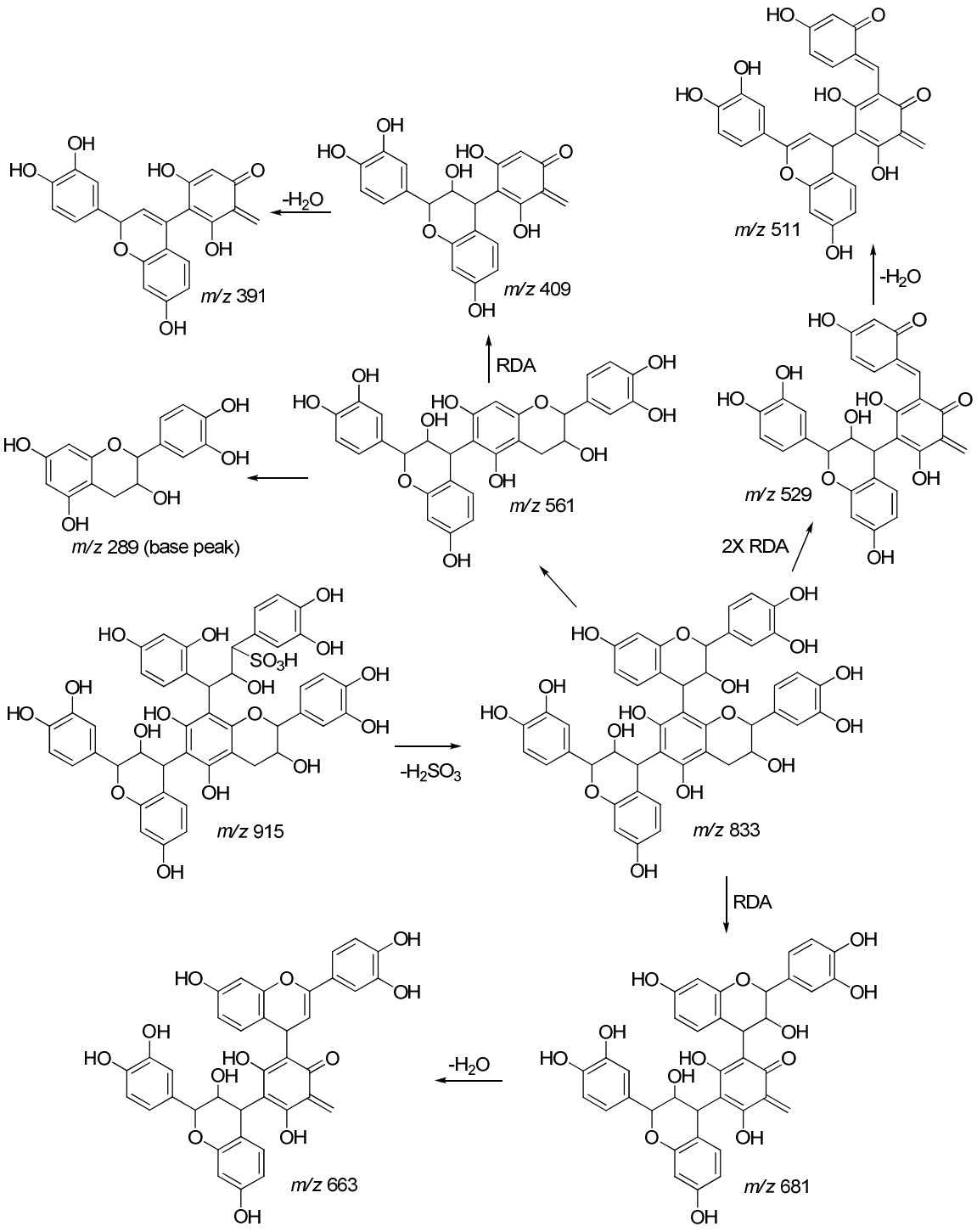
Scheme 8: MS^2 fragments of the *m/z* 353 ion (monosulfited fisetinidol)



Scheme 9: MS^2 fragments of the *m/z* 435 ion (disulfited fisetinidol)



Scheme 10: MS^2 fragmentation of the *m/z* 915 ion (sulfited trimers)



Scheme 11: MS^2 fragments of the *m/z* 915 ion (monosulfited trimer)

3.2.3. Chromatography of Sulfited Quebracho Extract

Reversed-phase chromatography of sulfited quebracho extract with an amide column (Figure 9) gave two well separated peaks (total ion current detection). The second eluting peak in the chromatogram is absent in the chromatogram of unsulfited quebracho extract (Figure 10). Diode array (UV) detection gives similar results (Figures S1 and S2). Integration of the UV peak areas suggests that the sulfited extract consist of a 1:1 ratio of sulfited and unsulfited PACs. A combined mass spectrum of the components in the second eluting sulfited peak in Figure 9 (Figure 11) clearly shows the presence of sulfited species [M+82] only, whilst the combined mass spectrum of the first eluting unsulfited peak in Figure 9 (Figure 12) contains only unsulfited PACs (c.f. Table 1). These contrast with Figure 4a (unchromatographed sulfited quebracho extract) where both sulfited and unsulfited species are present. Salient in the MS of the sulfited fraction are sodium adducts [M+22].

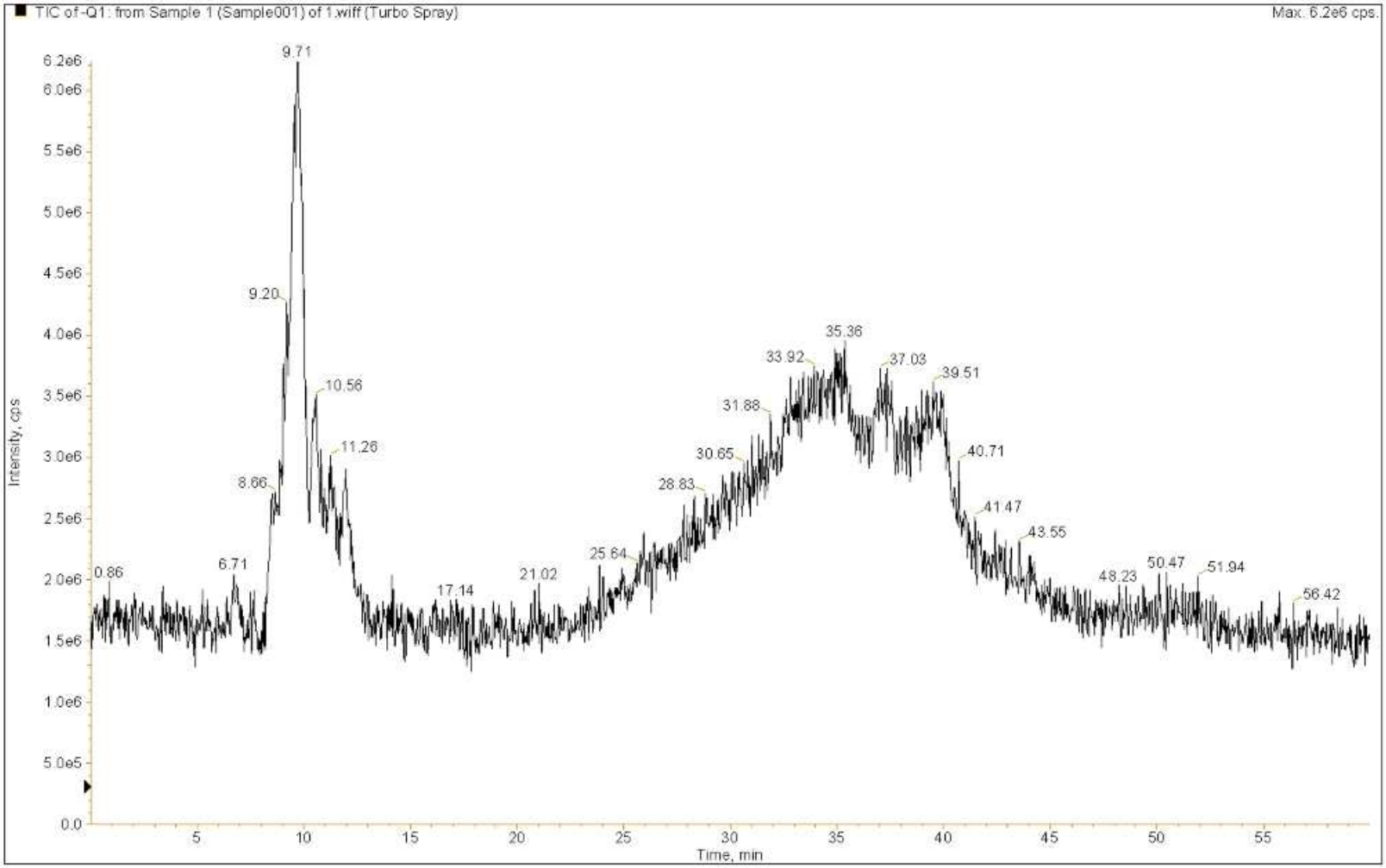


Figure 9: Total ion current amide column chromatogram of sulfited cold water-soluble quebracho extract

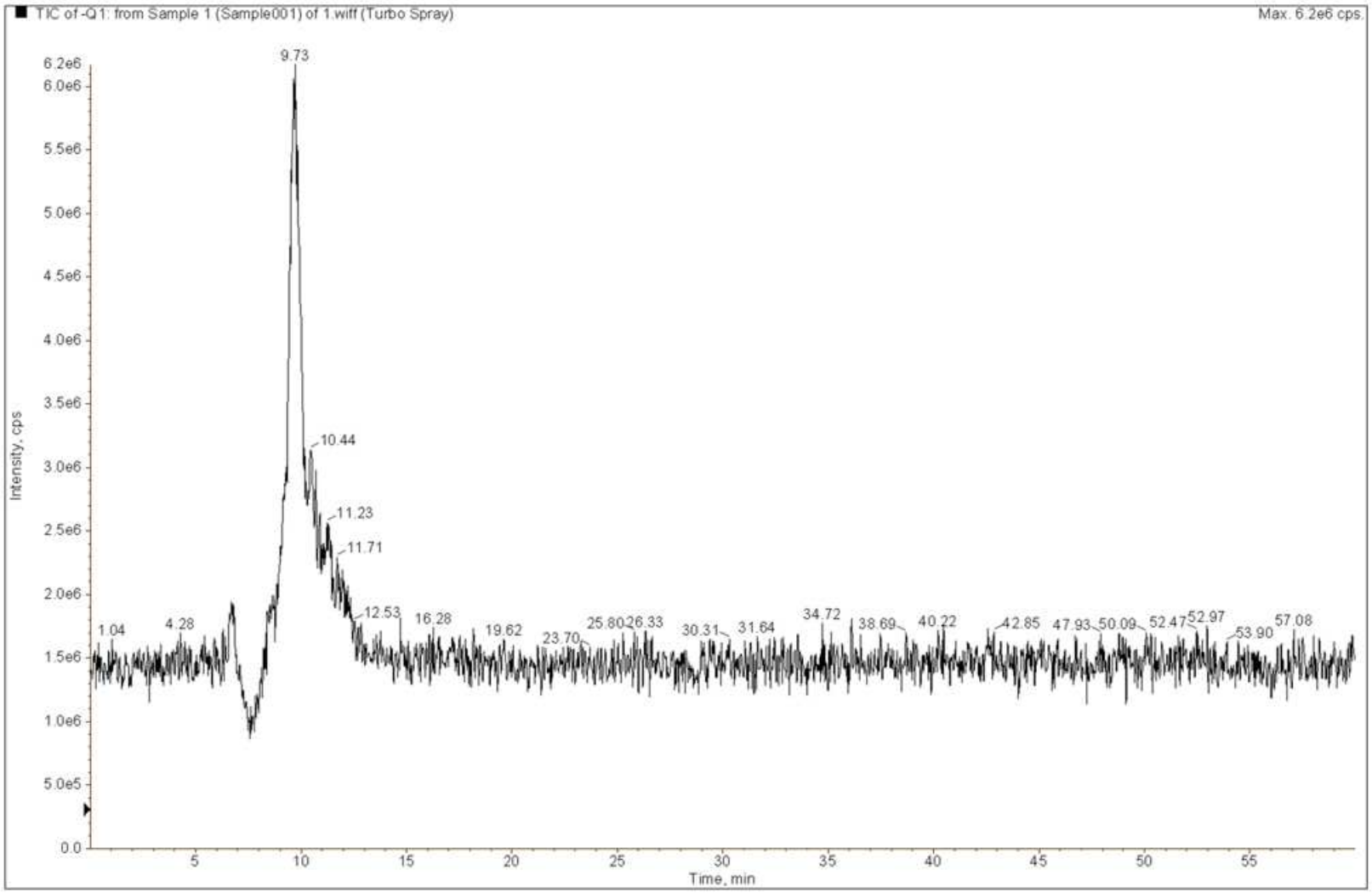


Figure 10: Total ion current amide column chromatogram of unsulfited hot water-soluble quebracho extract

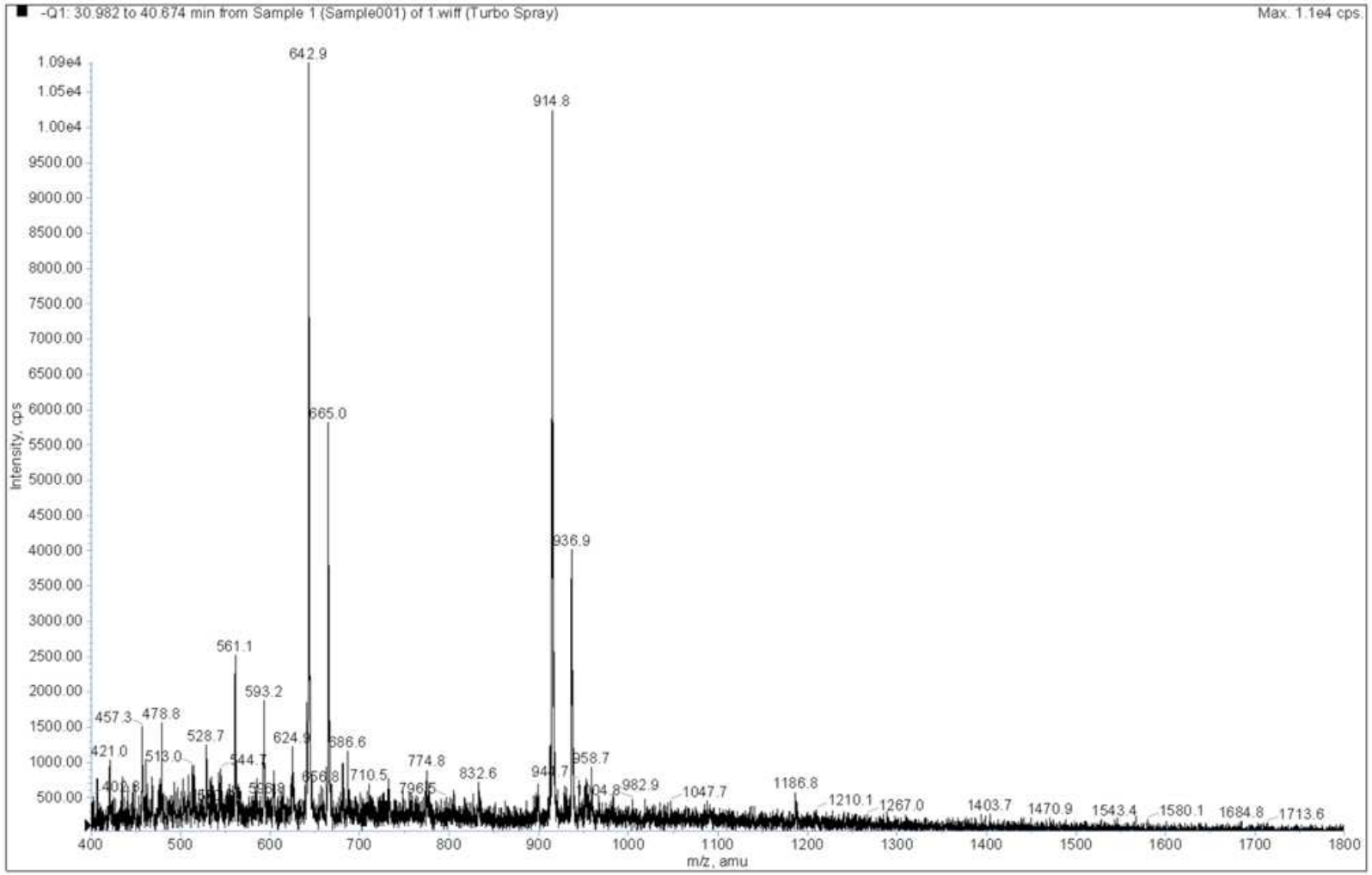


Figure 11: Combined mass spectrum of the sulfited peak

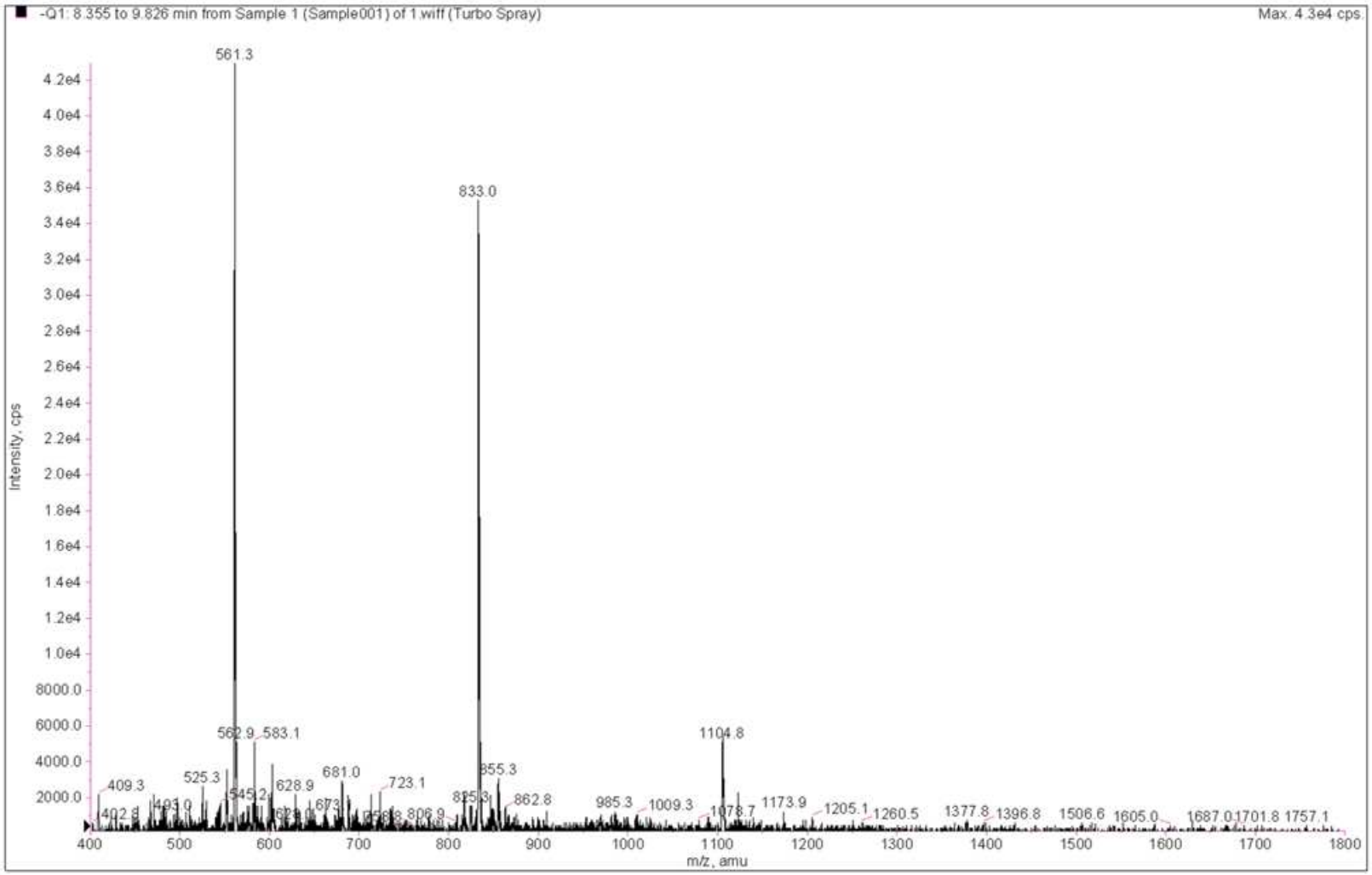
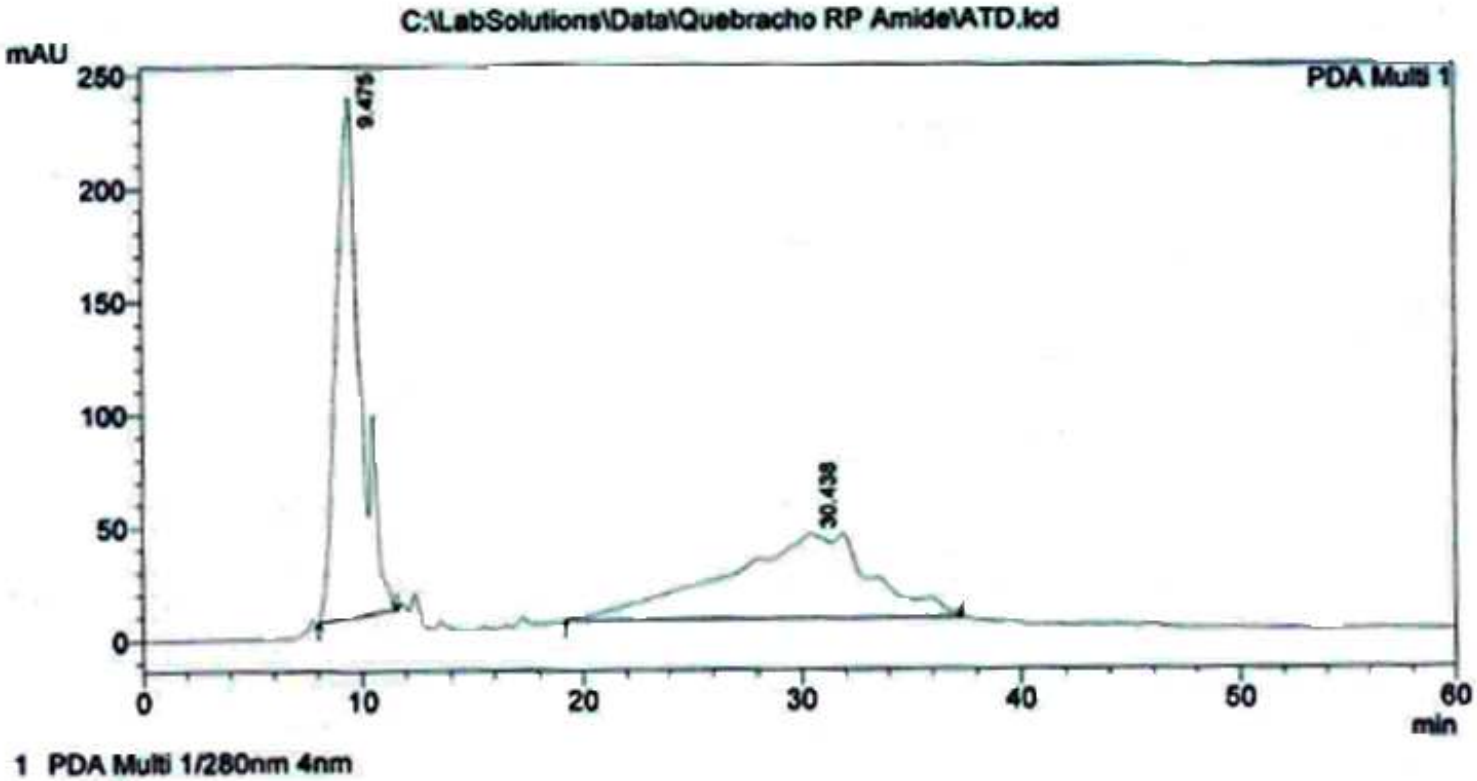


Figure 12: Combined mass spectrum of the unsulfited peak

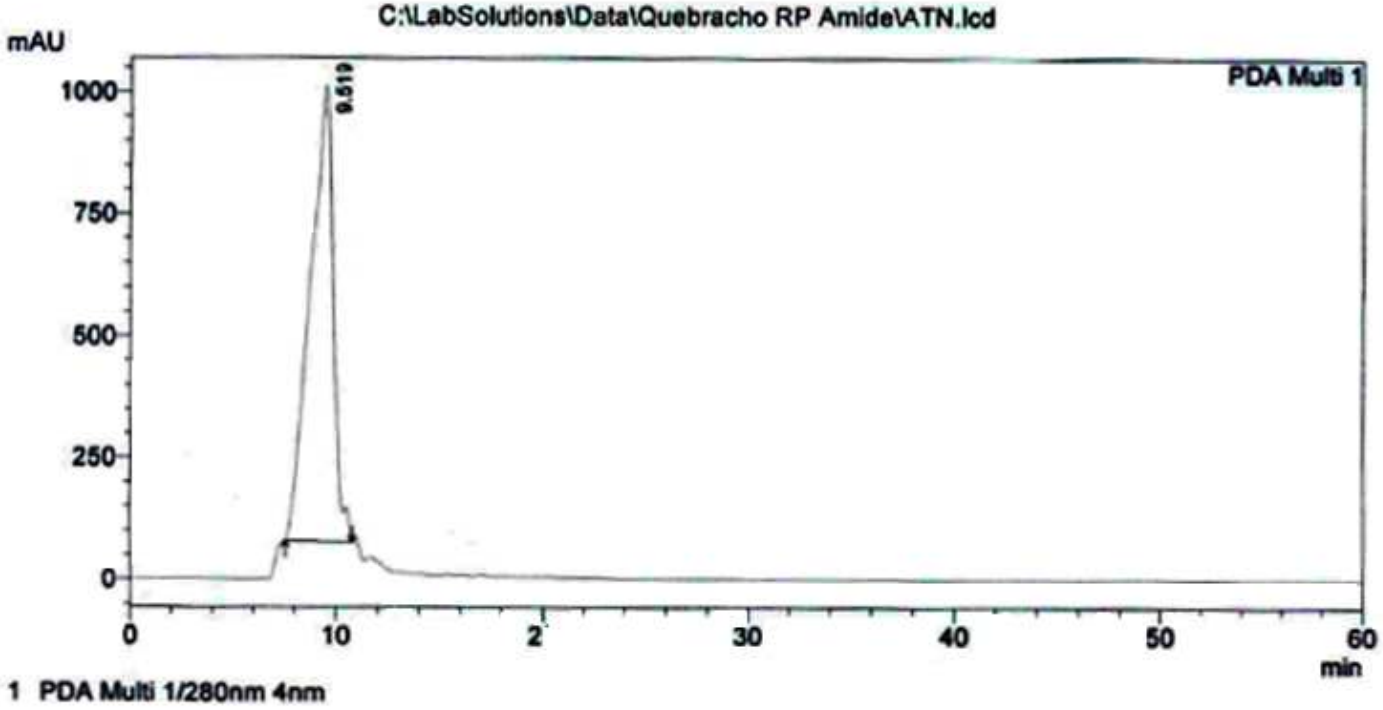


PeakTable

PDA Ch1 280nm 4nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	9.475	17519391	229841	50.102	86.136
2	30.438	17448367	36994	49.898	13.864
Total		34967758	266835	100.000	100.000

Figure S1. Diode array (UV) detection chromatogram of cold water-soluble (sulfited) quebracho extract



PeakTable

PDA Ch1 280nm 4nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	9.519	73006603	936970	100.000	100.000
Total		73006603	936970	100.000	100.000

Figure S2. Diode array (UV) detection chromatogram of hot water-soluble (unsulfited) quebracho extract

3.3. Conclusion

An ESI-MS investigation of sulfited (cold water-soluble) quebracho extract PACs indicates that during sulfitation, sulfite ions (SO_3^{2-}) are introduced at both C-2 and C-4 of the constituent fisetinidol monomeric moieties. In the case of C-2 sulfitation, the pyran ring is opened and M+82 ions associated with C-2 sulfited dimers, trimers, and tetramers are observed at m/z 643, 915, and 1187, respectively. The acyclic sulfonate-containing molecules react further with an additional bisulfite ion at C-4. This is associated with fission of the interflavanyl bond, explaining the presence of m/z 353 and 435 ions and represents exhaustive sulfitation, i.e. fission of all the interflavanyl bonds. No sulfited catechin (starter unit) was observed. In the commercial sample that we investigated (Venter *et al*., 2012a), the sulfitation process was incomplete and the unsulfited oligomers that we identified in the unsulfited (hot water-soluble) extract are still present in reduced intensity at m/z 561, 833, and 1105. Analytical liquid chromatography using an amide column separates the fraction that contains sulfonic acid moieties from the unsulfited fraction. A combined mass spectrum of the components in the sulfited fraction clearly shows the presence of sulfited species [M+82] only whilst the combined mass spectrum of the unsulfited fraction contains only unsulfited PACs (c.f. Table 1). The integration of the two peaks suggests that sulfited quebracho consists of about 50% sulfited and 50% unsulfited PACs. The coveted industrial properties associated with sulfited quebracho heartwood extract, i.e. solubility in cold water and better penetration of leather, are thus attributed to the introduction of hydrophilic sulfonic acid moieties, the cleavage of the pyran rings, and the reduction of the average chain length *via* interflavanyl bond fission.

3.4. Experimental

Sulfited cold water-soluble quebracho extract from *S. lorentzii* was supplied by Mimosa Extract Company (Pty.) Ltd., 24 van Eck Place, Pietermaritzburg 3201, South Africa. HPLC grade (≥99.9% purity) methanol and water were purchased from Merck. An API 2000 triple-quadrupole mass spectrometer (Applied Biosystems/MDS Sciex), coupled with an

electrospray ionisation (ESI) source, was used. Biosystems/MDS SCIEX Analyst software version 1.4.2 was used for data acquisition. The ESI interface was operated in the negative ionisation mode. The ionspray voltage (IS) was set at –4500 V, the declustering potential was set to –30.0 V, and the curtain gas (N_2) was set to 30 (arbitrary units). The nebulizer gas (N_2) and auxiliary gas (N_2) were set to 30 and 75 psi, respectively, with the heater temperature at 400 °C. HPLC analysis was performed using an Agilent 1200 system interfaced to an API 2000 with an Ascentis–RP–Amide column (250 mm, 4.6 mm i.d., 5.0 µm). An isocratic mobile phase was used, consisting of 80% solvent A (0.1% formic acid in water) and 20% solvent B (0.1% formic acid in methanol), with a flow rate of 250 µL/ min. The extract was dissolved in 1.0 mL of mobile phase. A QTRAP 3200 triple-quadrupole mass spectrometer (Applied Biosystems/MDS Sciex), coupled with an electrospray ionisation (ESI) source, was used for the acquisition of product ions. The ionspray voltage (IS) was set at –4500 V, the curtain gas was set to 15 (arbitrary units), and the declustering potential was set to –126.0 V. The nebulizer gas (GAS 1) and auxiliary gas (GAS 2) were set to 20 and 10 psi, respectively with the heater temperature at 300 °C. The collision energy was set to –10 eV.

3.5. References

Akin, D. Forage cell wall degradation and p-coumaric, ferulic, and sinapic acids. *Agron. J.*, **1982**, *74*, 424–428.

Beltran-Heredia, J.; Sanchez-Martin, J. Municipal wastewater treatment by modified tannin flocculent agent. *Desalination*, **2009**, *249*, 353–358.

Beltran-Heredia, J.; Sanchez-Martin, J. Removing heavy metals from polluted surface water with tannin-based flocculant agent. *J. Hazardous Mat*., **2008**, *165*, 1215–1218.

Beltran-Heredia, J.; Sanchez-Martin, J.; Frutos-Blanco, G. *Schinopsis balansae* tannin-based flocculant in removing dodecyl benzene sulfonate. *Separ. Purif. Technol*., **2009**, *67*, 295–303.

Cooper, K. G.; Hanlon, L. G.; Smart, G. M.; Talbot, R. E. 25 years experience in the development and application of scale inhibitors. *Desalination*, **1979**, *31*, 243.

Covington, A.D. Vegetable Tanning. In: Tanning Chemistry: The Science of Leather, The Royal Society of Chemistry, Cambridge, UK, **2009,** pp. 288–309.

Covington, A. D.; Lilley, T. H.; Song, L.; Evans, C. S. Collagen and polyphenols: new relationships and new outcomes. Part 1. Flavonoid reactions for new tanning processes. *J. Am. Leather Chem. Ass*., **2005**, *100*, 325.

Fechtal, M.; Riedl, B. Use of eucalyptus and *Acacia mollissima* bark extract formaldehyde adhesives in particleboard manufacture. *Holzforschung*, **1993**, *47*, 349.

Foo, L. Y.; McGraw, G. W.; Hemingway, R. W. Condensed Tannins: Preferential substitution at the interflavanoid bond by sulfite ion. *J. Chem. Soc. Chem. Commun*., **1983**, 672–673.

Haslam, E. Polyphenol–protein interaction. *Biochem. J*., **1974**, *139*, 285–288.

Haslam, E. Polyphenols, Collagen and Leather. In: Practical Polyphenolics. From Structure to Molecular Recognition and Physiological Action. Cambridge University Press, Cambridge, England, **2005,** pp. 378.

Haslam, E. Twenty-second Procter memorial lecture: Vegetable tannins - renaissance and reappraisal. *J. Soc. Leather Technol. Chem*. **1988**, *72*, 45.

Herrick, F. W. Chemistry and utilization of western hemlock bark extractives. *J. Agric. Food Chem*., **1980**, *28*, 228–237.

Jacques, D.; Opie, C. T.; Porter, L .J; Haslam, E. Plant proanthocyanidins. Part 4. Biosynthesis of procyanidins and observations on the metabolism of cyanidin in plants. *J.C.S. Perkin I*, **1977**, 1637–1643.

Jaén, J. A.; García de Saldaña, E.; Hernández, C. Characterization of reaction products of iron and iron salts and aqueous plant extracts. *Hyperfine Interac*., **1999**, *122*, 139.

Joslyn, M. A.; Goldstein, L. L. Astringency of fruits and fruit products in relation to phenolic content. *Adv. Food Res*., **1964**, *13*, 179–317.

Karchesy, J. J.; Foo, L. Y.; Hemingway, R. W.; Barofsky, E.; Barofsky, D. F. Fast atom bombardment mass spectrometry of condensed tannin sulfonate derivatives. *Wood Fiber Sci*., **1989**, *21 (2)*, 155–162.

Kennedy, J. A.; Taylor, A. W. Analysis of proanthocyanidins by high performance gel permeation chromatography. *J. Chromatogr. A*, **2003**, *995*, 99–107.

Mané, C.; Sommerer, N.; Yalcin, T.; Cheynier, V.; Cole, R. B.; Fulcrand, H. Assessment of the molecular weight distribution of tannin fractions through MALDI-TOF MS analysis of protein-tannin complexes. *Anal. Chem*., **2007**, *79 (6)*, 2239–2248.

Matamala, G.; Smeltzer, W.; Droguett, G. Comparison of steel anticorrosive protection formulated with natural tannins extracted from acacia and from pine bark. *Corrosion Sci*., **2000**, *42*, 1351.

McGraw, G. W.; Hemingway, R. W. Condensed tannins: desulfonation of hydroxybenzylsulfonic acids related to proanthocyanidin derivatives. *J. Wood Chem. Technol.*, **1988**, *8*, 91–109.

Moscovici, A.; Balaes, E.; Fruchter, M. Germanium tannin precipitation. *Rev. Chim.,* **1961**, *12*, 508.

Mouls, L.; Mazauric, J. P.; Sommerer, N.; Fulcrand, H.; Mazerolles, G. Comprehensive study of condensed tannins by ESI mass spectrometry: average degree of polymerisation and polymer distribution determination from mass spectra. *Anal. Bioanal. Chem*., **2011**, *400 (2*), 613–623.

Pasch, H.; Pizzi, A.; Rode, K. MALDI-TOF mass spectrometry of polyflavonoid tannins. *Polymer*, **2001**, *42*, 7531–7539.

Pizzi, A. Condensed tannins for adhesives. *Ind. Eng. Chem. Prod. Res. Dev.*, **1982**, *21*, 359–369.

Pizzi, A. Hot-setting tannin-urea-formaldehyde exterior wood adhesives. *Adhes. Age* **1977**, *20*, 27–29.

Pizzi, A. Phenol and tannin-based adhesive resins by reaction of coordinated metal ligands. Part 1. Phenolic chelates. *J. Appl. Polym. Sci*., **1979**, *24*, 1247–1256.

Pizzi, A. Sulphited tannins for exterior wood adhesives. *J. Org. Chem*., **1972**, *37*, 3546–3547.

Pizzi, A. Tannin-based adhesives: new theoretical aspects. *Int. J. Adhes. Adhes*., **1980**, *1*, 13–16.

Pizzi, A. Wattle based adhesives for exterior grade particleboards. *Forest Prod. J.*, **1978**, *30 (4)*, 38–42.

Reed, J. D.; Krueger, C. G.; Vestling, M. M. MALDI-TOF mass spectrometry of oligomeric food polyphenols. *Phytochemistry*, **2005**, *66*, 2248–2263.

Rhodes, D. F.; Cates, R. G. Toward a general theory of plant antiherbivores chemistry. Recent Adv. *Phytochemistry*, **1976**, *10*, 168–213.

Richtzenhain, H.; Alfredsson, B. Chem. Uber Ligninmodellsubstanzen Chem. Ber. **1955**, *89*, 378–385.

Rigaud, J.; Perez-Ilzarbe, J.; Ricardo da Silva, J. M.; Cheynier, V. Micro method for the identification of proanthocyanidin using thiolysis monitored by high performance liquid chromatography. *J. Chromatogr. A*, **1991**, *540*, 401–405.

Roux, D. G**.** Introduction. In: Plant polyphenols Hemingway, R.W., Laks, P.E. (Eds.), Plenum Press, New York, **1992,** pp. 7–39.

Roux, D. G.; Evelyn, S. R. The distribution and deposition of tannins in the heartwoods of *Acacia mollissima* and *Schinopsis spp*. *Biochem. J*., **1960**, *76*, 17–23.

Roux, D. G.; Ferreira, D.; Botha, J. J. Structural considerations in predicting the utilization of tannins. *J. Agric. Food Chem*., **1980**, *28*, 216.

Roux, D. G.; Ferreira, D.; Hundt, H. K. L.; Malan, E. Structure, stereochemistry and reactivity of natural condensed tannins as basis for their extended industrial exploitation. *J. Appl. Polym. Sci*., Polymer Symposium, **1975**, *28*, 335–353.

Sears, K. D. Sulfonation of catechin. *J. Org. Chem*. **1972**, *37*, 3546.

Seavell, A. J. Anti corrosive properties of mimosa (wattle) tannin. *Journal of the Oil and Colour Chemists' Association*, **1978**, *61*, 439.

Taylor, A. W.; Barofsky, E.; Kennedy, J. A.; Deinzer, M. L. Hop (*Humulus lupulus L*.) proanthocyanidins characterized by mass spectrometry, acid catalysis, and gel permeation chromatography. *J. Agric. Food Chem*., **2003**, *51*, 4101–4110.

Venter, P. B.; Sisa, M.; Van der Merwe, M. J.; Bonnet, S. L.; Van der Westhuizen, J. H. Analysis of commercial proanthocyanidins. Part 1: The chemical composition of Quebracho (*Schinopsis lorentzii* and *Schinopsis balansae*) heartwood extract. *Phytochemistry*, **2012a**, *73*, 95–105.

Venter, P. B.; Senekal, N.D.; Amra-Jordaan, M.; Bonnet, S. L; van der Westhuizen, J. H. Analysis of commercial proanthocyanidins. Part 2: An electrospray mass spectrometry investigation into the chemical composition of sulfited quebracho (*Schinopsis lorentzii* and *Schinopsis balansae*) heartwood extract, *Phytochemistry*, **2012b**, *78*, 156-169.

Vidal, S.; Cartalade, D.; Souquet, J.-M.; Fulcrand, H.; Cheynier, V. Changes in proanthocyanidin chain length in winelike model solutions. *J. Agric. Food Chem*., **2002**, *50*, 2261–2266.

Vivas, N.; Nonier, M. F.; Vivas de Gaulejac, N.; Absalon, C.; Bertrand, A.; Mirabel, M. Differentiation of proanthocyanidin tannins from seeds, skins and stems of grapes (*Vitis vinifera*) and heartwood of Quebracho (*Schinopsis balansae*) by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry and thioacidolysis/liquid

chromatography/electrospray ionization mass spectrometry. *Anal. Chim. Acta*, **2004**, *513*, 256–274.

Viviers, P. M.; Kolodziej, H.; Young, D. A., Ferreira, D.; Roux, D. G. Synthesis of condensed tannins. Part 11. Intramolecular enantiomerism of the constituent units of tannins from the Anacardiaceae: Stoichiometric control in direct synthesis: Derivation of ^{1}H nuclear magnetic resonance parameters applicable to higher oligomers. *J. Chem. Soc. Perkin Trans. 1*, **1983**, 2555–2562.

Chapter 4

4. Analysis of Commercial Proanthocyanidins. Part 3: The Chemical Composition of Wattle (*Acacia mearnsii*) bark extract

4.1. Introduction

Proanthocyanidins (PACs) are ubiquitous in plants and their structure comprise of oligomerized/polymerized flavan-3-ol monomers. The term PAC refers to the characteristic red color that develops upon heating with dilute acid (Roux, 1992). PACs are also referred to as condensed tannins to distinguish them from hydrolysable tannins (oligomeric gallic acid esters of glucose and other sugars), which do not produce a red color upon heating with aqueous acid.

Many of the properties of PACs are based on their ability to form hydrogen bonds with proteins. Astringency in tea and red wine is based on interactions between PACs and the protein-based taste receptors in the mouth (Joslyn and Goldstein, 1964). The same PAC-protein interaction transforms biodegradable raw hide into leather which resists bacterial degradation, has a nice pleasant feel, and is abrasion, heat, and water resistant (Haslam, 2005). The anti-feeding properties that protect plants against herbivores are based on the indigestibility of PAC-protein complexes and the growth of micro-organisms is inhibited by the irreversible deactivation of enzymes (Akin, 1982; Rhodes and Cates, 1976).

PAC extracts, including wattle bark extract, are used industrially to produce vegetable tanned leather (Roux *et al*., 1975), adhesives *via* cross linking of the aromatic A-rings of the constituent flavan-3-ol monomers with formaldehyde (Pizzi, 1977), water purification resins through complexation of the B-ring catechol moiety with heavy metals (Beltran-Heredia *et*

al., 2009a, 2009b, 2008), and for additives in drilling (Haslam, 1988; Herrick, 1980). They are also used to supplement tannins in red wine (oenological tannins) (Parker *et al*., 2007). Important industrial sources of PACs are wattle bark and quebracho heartwood extract. Sources of hydrolysable tannins are tara pods, chestnut bark and oak gall extracts.

The bark from commercially grown black wattle trees (*Acacia mearnsii*; previously *A. mollissima*) is harvested, extracted with hot water, and then either spray-dried to give powdered wattle extract, or concentrated further to yield a solid/block product on cooling. These chemically unmodified wattle extracts are known commercially as wattle extract, and as *Acacia mearnsii*, ext. by ECHA (EC No. 272-777-6; CAS No. 68911-60-4). The spray-dried product contains typically 5% moisture and the solid/block product, 15% moisture (personal communication, Dr. N. P. Slabbert).

Wattle bark extract consists of about 75% PACs and 25% sugars and insoluble gums on a dry basis (Roux, 1953). Determination of the wattle PAC composition has been slow, mainly due to the complexity of the extracts and the difficulty of purifying polyphenols with silica-gel-based chromatography materials and the poor resolution of the alternative gel or paper chromatography. Uncertainties include the hydroxylation patterns of the constituent flavan-3-ol aromatic rings, different configurations at the C-2, C-3, and C-4 stereogenic centres, the possibility of a second ether interflavanyl bond (A-type PACs), the average chain length (degree of polymerisation), and the possibility of branching (formation of angular oligomers). The characteristic absence of 5–hydroxy groups in the chain extender units of wattle PACs imparts stability to the interflavanyl bond against acid hydrolysis (Roux and Paulus, 1962; Roux *et al*., 1975). The high temperatures thus required to hydrolyse this bond render the classical method to analyse PACs *via* acid hydrolysis of the interflavanyl bond and subsequent trapping of intermediates with toluene-α-thiol or phloroglucinol (Thompson *et al*., 1972; Foo *et al*., 1978; Kennedy *et al*., 2003; Rigaud *et al*., 1991) and analysis of such trapped intermediates with HPLC (Shen *et al*., 1986; Koupai-Abyazani *et al*., 1993; Rigaud *et al*., 1991; Kennedy *et al*., 2003), unreliable.

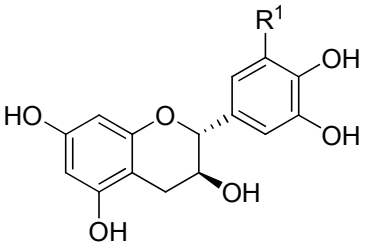
Roux (1953) established the complexity of the PAC fraction of wattle extract with 2D paper chromatography. At least seven distinct heterogeneous PAC groups of high molecular weight were observed. The sugar fraction consists of sucrose, glucose and fructose. The gum fraction consists of carbohydrate polymers with an average molecular weight of up to 92 000 Da and hydrolysed predominantly to galactose and arabinose.

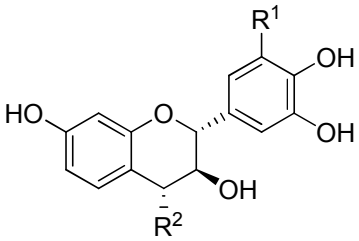
Evelyn (1954) obtained 68.75 % pure PAC polymers from the wattle extract *via* lead salt precipitation. This correlates with a PAC content value of 62.5% obtained with the Roux colorimetric method (Roux, 1957). Roux and Evelyn (1960) determined the average molecular weight of wattle PACs with ray ebulliometry to be about 1250 Da.

Thompson and Pizzi (1995) used ^{13}C NMR methods to determine the relative proportion of C4-C8 and C4-C6 interflavanyl bonds to the proportion of free C6 and C8 sites. These values gave a number average degree of polymerization (aDP) of 4.9 for the PACs in wattle extract. It corresponds with a molecular weight of 1343 - 1406 Da.

Pizzi (1982) reported that the PAC fraction of wattle tannins consisted of 5% prodelphinidin (polymers with gallocatechin 1 extender units), 25% profisetinidin (fisetinidol 3 extender units), and 70% prorobinetinidin (robinetinidol 5 extender units) analogues (Figure 1).

Roux and Mais (1960) isolated gallocatechin **1**, catechin **2**, and robinetinidol **3** from mature wattle bark (Figure 1). Drewes and Roux (1963) additionally isolated fisetinidol **5**, and observed traces of robinetinidol-4α-ol **4** and fisetinidol-4α-ol **6** (Figure 1). Saayman and Roux (1965) demonstrated that immature *Acacia mearnsii* bark contains gallocatechin **1**, catechin **2**, robinetinidol-4α-ol **4**, and fisetinidol-4α-ol **6** accompanied by a low concentration of robinetinidol **3** (Figure 1). They also determined that catechin and gallocatechin occur in a 3:1 ratio in mature bark. The concomitance of **1, 2, 4**, and **6** with PACs was regarded as evidence of the biogenesis of PACs from flavan-3,4-diols.





1 R^1 = OH: gallocatechin (Mass 306 Da)
2 R^1 = H: catechin (Mass 290 Da)

3 R^1 = OH, R^2 = H: robinetinidol (Mass 290 Da)
4 R^1 = OH, R^2 = OH: robinetinidol-4α-ol (Mass 306 Da)
5 R^1 = R^2 = H: fisetinidol (Mass 274 Da)
6 R^1 = H, R^2 = OH: fisetinidol-4α-ol (Mass 290 Da)

Figure 1: Flavan-3-ol and flavan-3, 4-diol monomers isolated from *Acacia mearnsii* bark

Drewes and co-workers (1967) reported three dimers **7**, **8**, and **9** (Figure 2) from freshly stripped wattle bark extracted with ethyl acetate at ambient temperature. These dimers had a low affinity for hide powder and low tanning properties and were classified as "phenolic half tannins". The detection of the same three dimers with analytical 2D paper chromatography upon adding dilute HCl to a mixture of either **4** or **6** with **1** or **2** (same R_f and colour development with spray reagents) suggests that the dimers were the product of the reaction of a C-4 carbocation of a flavan 3, 4-diol **(4** or **6)** with the nucleophilic A-ring of gallocatechin **(2)** or catechin **(1)**. Later work indicated that **7, 8**, and **9** were not the C-6 but the C-8 coupled dimers **10, 11**, and **12** (Figure 2) (Hundt and Roux, 1981, 1978; Botha *et al*., 1981, 1978).

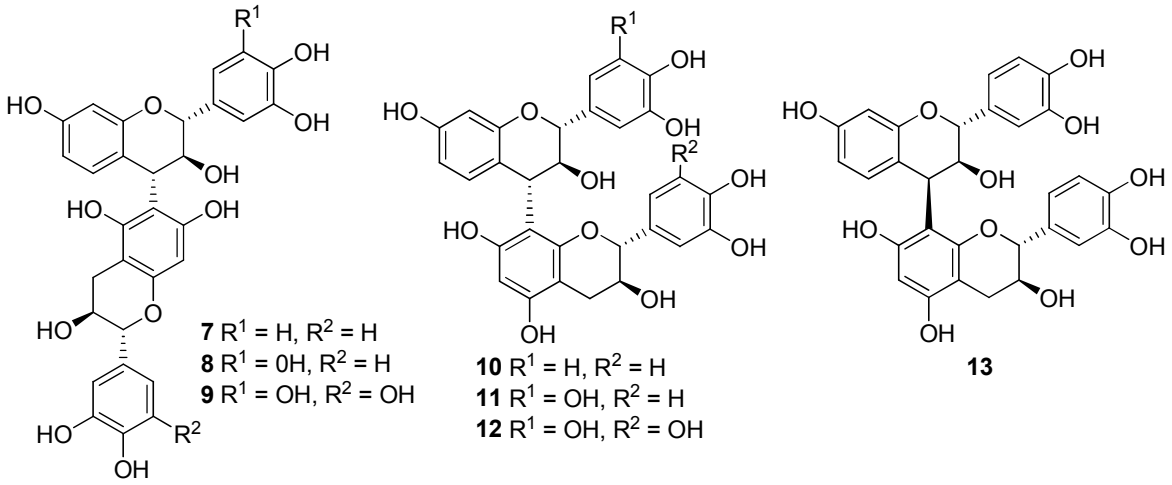
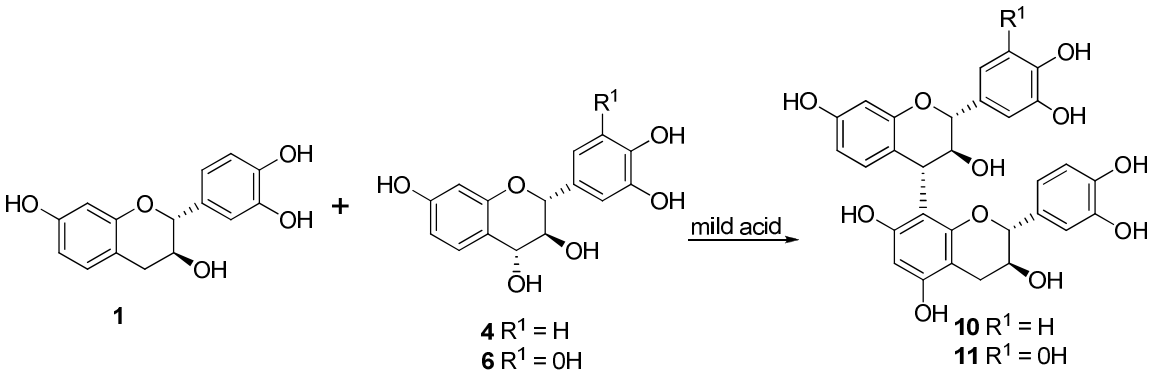


Figure 2: Dimers isolated from *Acacia mearnsii* bark

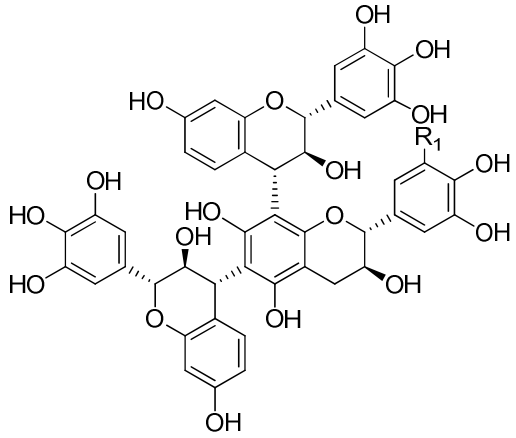
Botha *et al*. (1981) isolated four 4,8-linked dimers **10, 11, 12,** and the previously undetected **13** (Figure 2) and confirmed these structures synthetically *via* a mild acid-catalysed coupling of gallocatechin **1** or catechin **2** to robinetinidol-4α-ol **4** or fisetinidol-4α-ol **(6)** (Scheme 1). Interflavanyl bond formation was found to occur preferentially *via* the sterically less hindered and more potent C-8 position of the catechin or gallocatechin starter units. Botha *et al*. (1981) found that **13**, with 3,4-*cis* configuration, was the major component in the natural bark extract whilst **10, 11**, and **12** with 3,4-*trans* configuration, were the major synthetic components.

Whereas Hundt and Roux (1981) used chemical shifts to distinguish between C4-C8 and C4-C6 interflavanyl bonds, Balas and co-workers (1994) developed a method based on 2D HMBC NMR experiments for this purpose. Recently, Esatbeyoglu *et al*. (2011) developed a low temperature ^{1}H NMR procedure for the characterisation of underivatised procyanidin dimers and trimers and to determine the position of the interflavonoid bond.

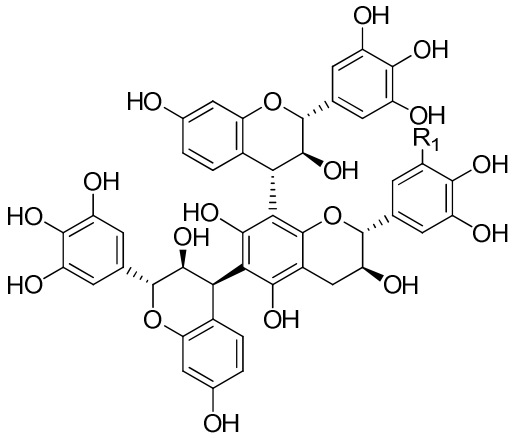


Scheme 1: Examples of the syntheses of PAC dimers from catechin and fisetinidol-4α-ol or robinetinidol-4α-ol

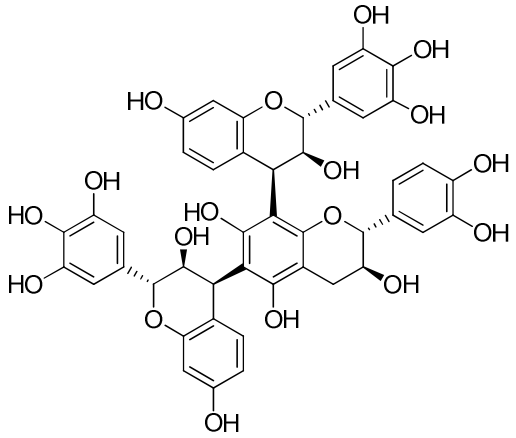
Viviers and co-workers (1983) investigated the trimers in the ethyl acetate extract of freshly stripped bark extracted at ambient temperature. They isolated five angular prorobinetinidin trimers **14, 15, 16, 17,** and **18** (Figure 3) with both the C-6 and C-8 position of the catechin or gallocatechin starter units attached to extender units using Craig counter current and preparative paper chromatography. Synthetic proof was provided for three of the structures **14, 16**, and **18**. Whilst isolated dimers were a mixture of prorobinetinidins with robinetinidol extender units and profisetinidins with fisetinidol extender units, the trimers were exclusively prorobinetinidins. This change in selectivity at the sterically more hindered and less potent nucleophilic C-6 position, agrees with the finding by Viviers and co-workers (1983) that robinetinidol-4α-ol **(6)** is more reactive at C-4 than fisetinidol-4α-ol **(4)**. This was attributed to additional stabilisation of the incipient C-4 carbocation *via* the more electron-rich pyrogallol B-ring in the A-conformation of the carbocation. It may, however, also indicate that the presumably less readily available fisetinidol-4α-ol **(6)** has been depleted at the dimer stage.



14 R^1 = H; Exact Mass: 866
15 R^1 = OH; Exact Mass: 882



16 R^1 = H; Exact Mass: 866
17 R^1 = OH; Exact Mass: 882

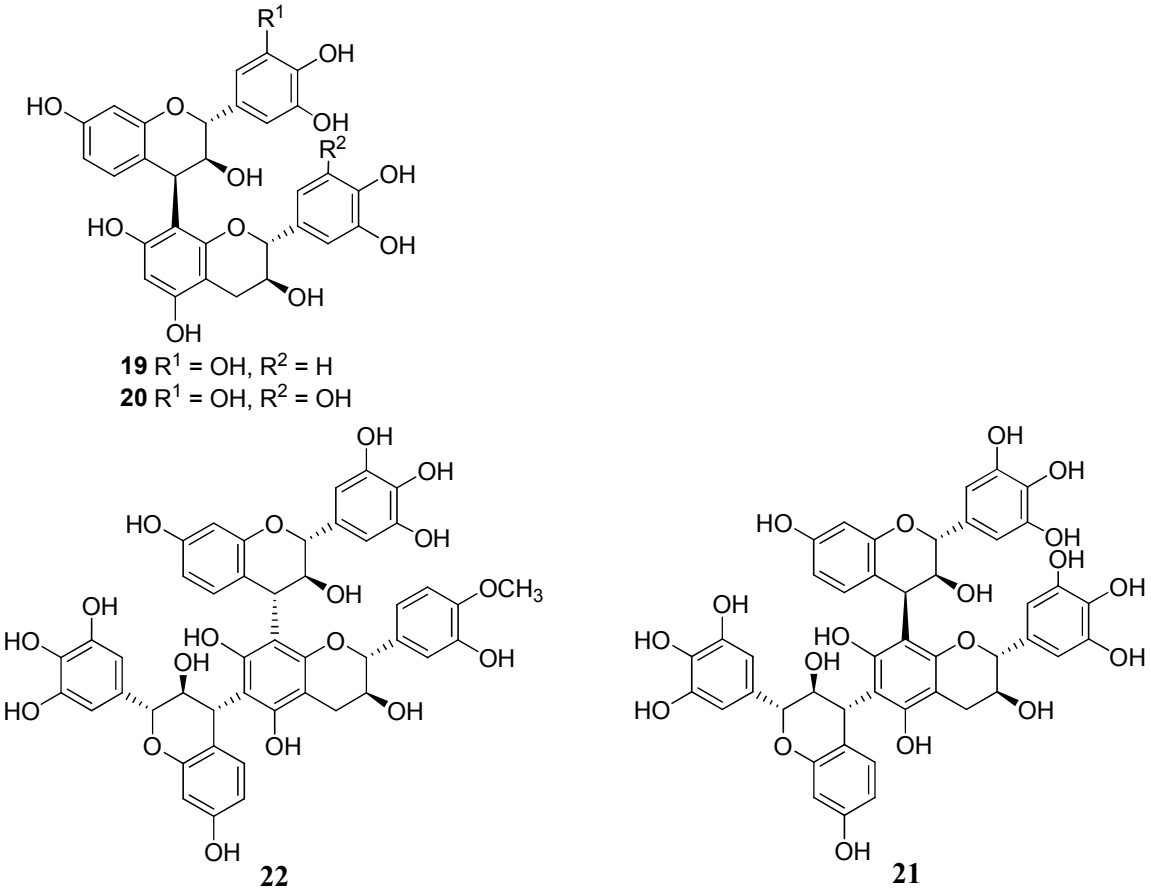


18 Exact Mass: 866

Figure 3: Trimers isolated by Viviers and co-workers

Cronje and co-workers (1993) investigated spray-dried aqueous wattle bark extract and isolated, in addition to the dimers **10, 11 , 12**, and **13** first isolated by Drewes and Roux (1967), and Botha et al. (1981), the 3,4-*cis* prorobinetinidin dimers **19** and **20** (Figure 4). They concluded that prorobinetinidin dimers with their robinetinidol extender units predominates profisetinidin dimers with fisetinidol extender units in a 3:1 ratio. Cronje also isolated the two prorobinetinidin trimers **21** and **22** and four dimeric (**23, 24, 25, 26**) and a single trimeric phlobatannin **(27)** (Figure 5). These are rearrangement products of the prorobinetinidins described above, where the more reactive phloroglucinol A-ring of the starter unit replaces the less reactive resorcinol A-ring of the prorobinetinidin extender unit

(Steynberg *et al*., 1986). The absence of these phlobatannins in the ethyl acetate extract indicates formation during the high temperature aqueous extraction process. The absence of profisetinidin phlobatannin analogues correlates with the lower reactivity of a C-2 position next to a catechol B-ring than the C-2 position adjacent to a pyrogallol B-ring.



19 R^1 = OH, R^2 = H
20 R^1 = OH, R^2 = OH

22 **21**

Figure 4: Additional dimers and two trimers isolated by Cronje

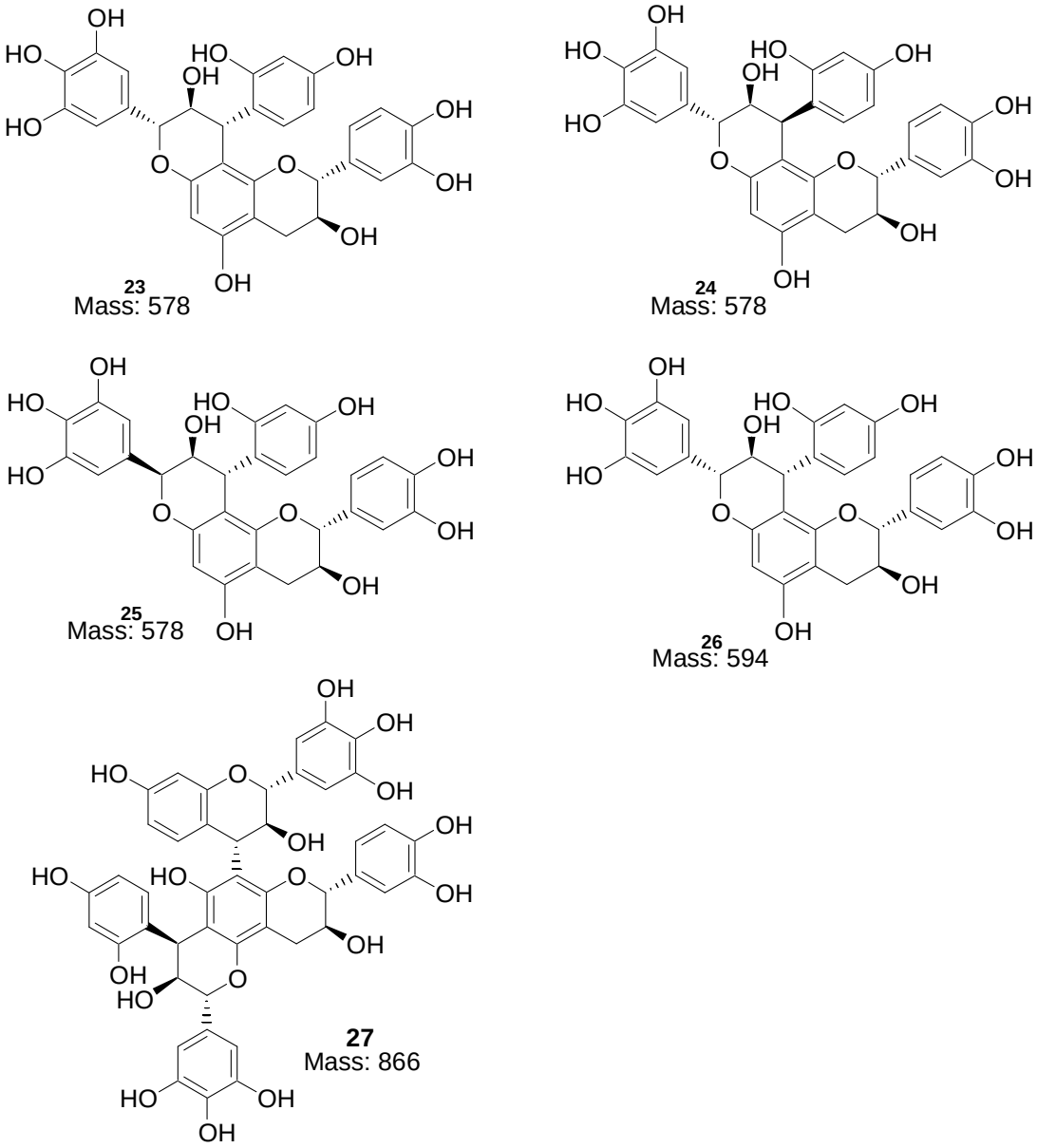


Figure 5: Phlobatannins isolated by Cronje

They also isolated a 6-methyl-containing dimer [fisetinidol-(4α,8)-6-methyl-catechin] **(28)** and the first 5-deoxy A-type dimer and the first A-type dimer with a 3,4-*cis* C-ring configuration [robinetinidol-(2α→7;4β→ 8)-catechin] **(29)** (Figure 6)**.**

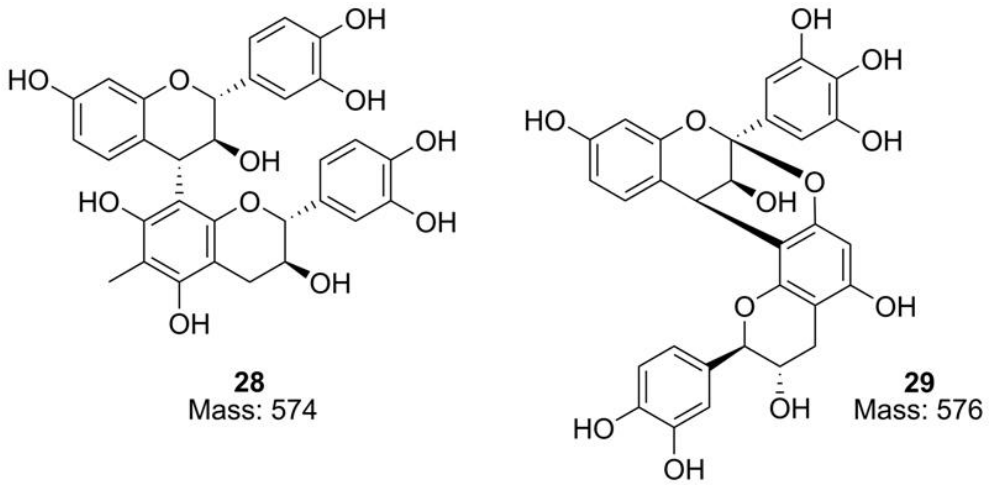


Figure 6: 6-methyl substituted and A-type dimers

In summary, six of the eight theoretically possible dimer diastereoisomers have been isolated. They all contain 3*R* absolute configuration. The two gallocatechin containing profisetinidin dimers were not detected. The spray dried water extract also contains phlobatannin dimers that are probably formed during high temperature aqueous extraction. The predominant 3,4-*cis* stereochemistry of the extender unit of the isolated dimers (Botha *et al*., 1981), in contrast with the predominant *trans* stereochemistry of the synthetic analogues, indicates enzymatic preferences. The five trimers isolated so far are exclusively catechin- or gallocatechin-based prorobinetinidins. The isolated trimers contain predominantly 3,4-*trans* extender units. The interflavanyl bonds *via* the C-8 position of the catechin or gallocatechin extender units predominate, but C4-C6 dimers have also been isolated, albeit in low concentrations (Botha *et al*., 1981). No evidence of prodelphinidin-type dimers or trimers, or their gallocatechin-4-ol extender unit precursor could be found.

We thus conclude that wattle proanthocyanidins are biosynthesized according to a protocol that limits the number of PAC structures:

1. The more reactive nucleophilic phloroglucinol A-ring of either catechin or gallocatechin acts as starter unit and initiates the polymerisation process by reacting at C-4 of either fisetinidol- or robinetinidol-4-ol extender units (Scheme 1). Each PAC molecule will thus contain only one catechin or gallocatechin moiety.

2. The first interflavanyl bond is formed *via* C-8 of the reactive phloroglucinol A- ring resulting in the preferential formation of C-8 coupled dimers (Figures 1, 2 and 4).

3. The second flavanyl moiety will be added at the vacant C-6 position of the phloroglucinol A-ring leading to angular structures (Figures 3 and 4).

4. The third and subsequent extender units will be added at the less reactive resorcinol A-rings of the fisetinidol or robinetinidol moieties. The C-6 position is more reactive and sterically less hindered than the alternative C-8 position (Young *et al*., 1985).

Mass spectrometry (MS) fractionates a complex mixture, such as wattle extract, into fractions of different *m/z* values. The intensity of each peak is related to the amount of the corresponding oligomer present. Exact quantification requires internal standards, which are not available in the case of complex PAC mixtures. Wattle PACs, however, represent a homologous series of closely related compounds and mass spectrometry is often used to estimate the percentage composition of a complex mixture. Mouls and co-workers (2011) compared aDP values of 5-oxy-PACs derived from ESI-MS peak intensities with values obtained from thiolysis and concluded that MS derived values underestimate the aDP. Mass spectrometry thus underestimates the concentration of higher molecular weight oligomers.

Pasch and co-workers (2001) investigated commercial wattle tannin extract with MALDI-TOF MS and observed oligomers to a maximum of octamers (2333 Da). They observed major peaks at 906 and 1195 Da. This is in line with the aDP of 4.9 found by Thompson and co-workers (1995) and Fechtal and Riedl (1993). The oligomers were observed as clusters of peaks 16 Da apart. For example, the tetramers consisted of peaks at 1147, 1163, 1179, 1195, and 1211. They concluded that wattle PACs are combinations and permutations of fisetinidol (*m/z* 274) and robinetinidol (*m/z* 290) and a smaller fraction of gallocatechin (*m/z* 306) flavan-3-ol monomers. Catechin was not detected.

A better understanding of the constituents of vegetable tannins will not only lead to a better understanding of their biosynthesis and biological function in plants, but will also enhance industrial applications. The relative affinity for collagen, rate of penetration into hides and skins during commercial tannage, and mobility within leather and desorption from finished leather under moist conditions are important leather tanning parameters that are determined by the molecular composition of the PACs involved. The availability of nucleophilic centres for cross linking with formaldehyde on the periphery of oligomers/polymers determines the curing time of thermosetting PAC-based adhesives.

4.2. Results and Discussion

Herein we report our results of the investigation of the composition of wattle PAC extract with ESI mass spectrometry. We used the aforementioned biosynthetic assumptions as guideline to interpret our results. Precursor and product ion scans and a comparison of MS^2 spectra with the MS of authentic monomer samples permitted differentiation of isomers with the same *m/z* values in many cases.

It is often assumed that matrix-assisted laser desorption ionisation (MALDI) is superior to ESI for investigating polymers. Electrospray ionisation (ESI), however, does not require a matrix for ionisation and provides more reliable information on smaller molecules. ESI also permits product and precursor ion investigations. A potential problem with MALDI-TOF is that ions are not detected directly but as sodium or potassium adducts. The mass difference between a sodium and potassium adduct of 16 Da may be misinterpreted as indicating the presence of an additional oxygen atom in a MS fragment. ESI does not detect these M+16 artefacts (Reed *et al.*, 2011).

4.2.1. Q1 Scan of Wattle Bark Extract

The negative mode ESI scans of wattle bark extract are given in Figures 7a and 7b and the *m/z* values and their proposed structures are summarised in Table 1. We assume, from the phytochemical results discussed above, that interflavanyl bonds consist of both 4α- and 4β-3,4-*cis* and 3,4-*trans* stereochemistry in MS-derived structures.

Whereas similarly investigated quebracho heartwood extract (Venter *et al*., 2012a) has only one possible starter unit (catechin) and one possible extender unit (fisetindol), the starter unit in wattle can be either catechin (*m/z* 289) or gallocatechin (*m/z* 305) and the extender units either fisetinidol (*m/z* 273) or robinetinidol (*m/z* 289) with masses 16 Da apart. Wattle PAC oligomers, thus, do not appear as single peaks at *m/z* values of 561 (dimer), 833 (trimer), 1105 (tetramer), and 1377 (pentamer) etc., as was observed with quebracho extract, but as clusters 16 Da apart. Two monomer fragments are detected at *m/z* 289 and 305.

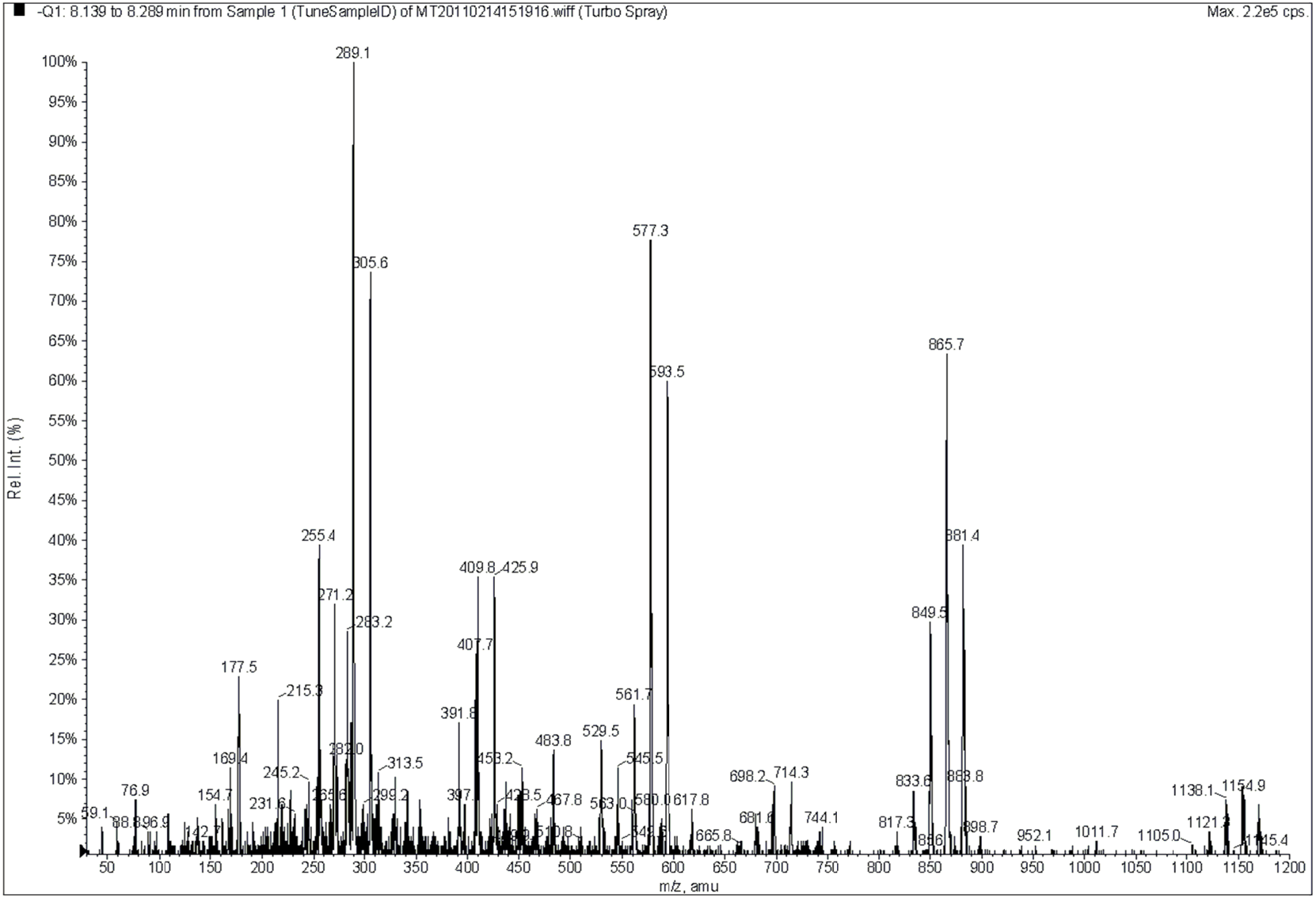


Figure 7a: Negative mode ESI spectrum (Q1) of wattle bark extracts (*m/z* 30 - 1200 *m/z*)

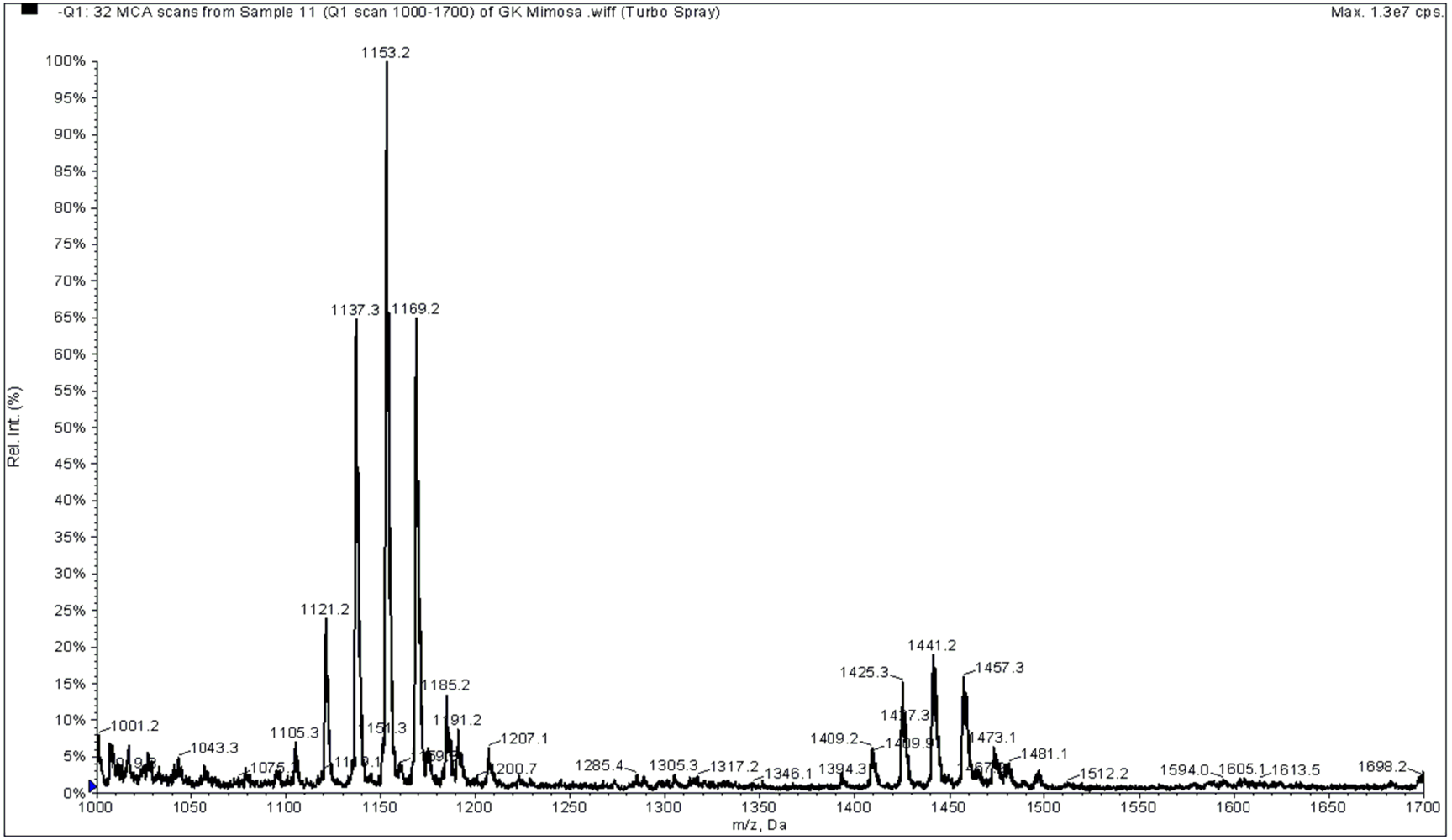


Figure 7b: Negative mode ESI spectrum (Q1) of wattle bark extract (*m/z* 1000 - 1700 *m/z*)

Table 1: Proposed structures and prevalence of PAC monomers, dimers, trimers, and tetramers that occur in wattle bark extract

Oligomer	**Starter unit**	**Starter unit**	**Extender unit**	**Extender unit**	***m/z* value**	**Composition* (% by weight)**
	Catechin	Gallocatechin	Fisetinidol	Robinetinidol		
Monomers	1				289	1
		1			305	4
			1		273	0
				1	289	4
Dimers			2		545	1
	1		1		561	5
	1			1	577	18
		1	1		577	3
		1		1	593	16
Trimers	1		2		833	2
	1		1	1	849	8
		1	2		849	1
	1			2	865	13
		1	1	1	865	5
		1		2	881	11
Tetramers	1		3		1105	0.6
	1		2	1	1121	0.5
	1		1	2	1137	1.5
	1		0	3	1153	1.5
		1	3		1121	0.5
		1	2	1	1137	0.5
		1	1	2	1153	1.5
		1		3	1169	2

* Estimate based on multiplying peak intensity with MW.

Since PACs are a homologous series comprising mainly catechin, gallocatechin, fisetinidol and robinetinidol constituent units, we assumed similar ionisation potentials and used peak intensities to estimate the composition and aDP. This indicates that wattle PAC extract consists of about 9% monomers, 42% dimers, 40% trimers, 9% tetramers and 1% pentamers and higher oligomers by mass. More than 50% of the PACs in the extract thus consist of trimeric and higher oligomers, and the ESI-MS-derived aDP approximates three.

The aDP results obtained by Pizzi (1982), Pasch (2001) and Roux and Evelyn (1960) measure about five. These higher values are supported by Mouls's (2011) conclusion that MS underestimates the aDP of 5-oxy PACs by less than two in the heptamer range and more with higher oligomers, compared to thiolysis-derived values.

4.2.2. Identification of PAC Monomers with Product Ion Scans

The fragmentation spectrum (MS^2) (Figure 8a) of the *m/z* 305 ion in Figure 7a matches the MS of an authentic gallocatechin sample (Figure 8b), indicating that the *m/z* 305 ion consists exclusively of gallocatechin.

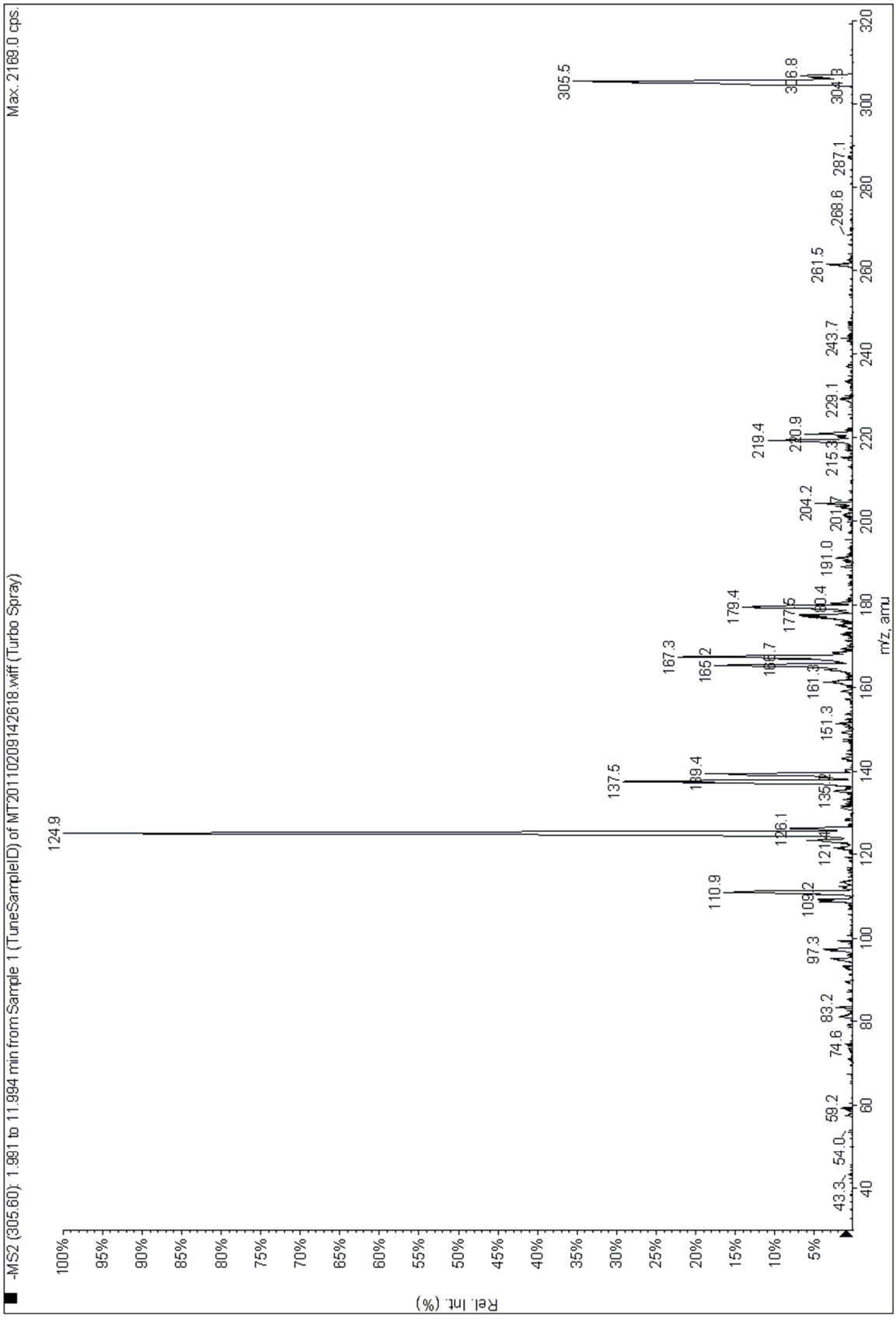


Figure 8a: Product ion scan (MS^2) of the *m/z* 305 fragment in the MS of wattle bark extract

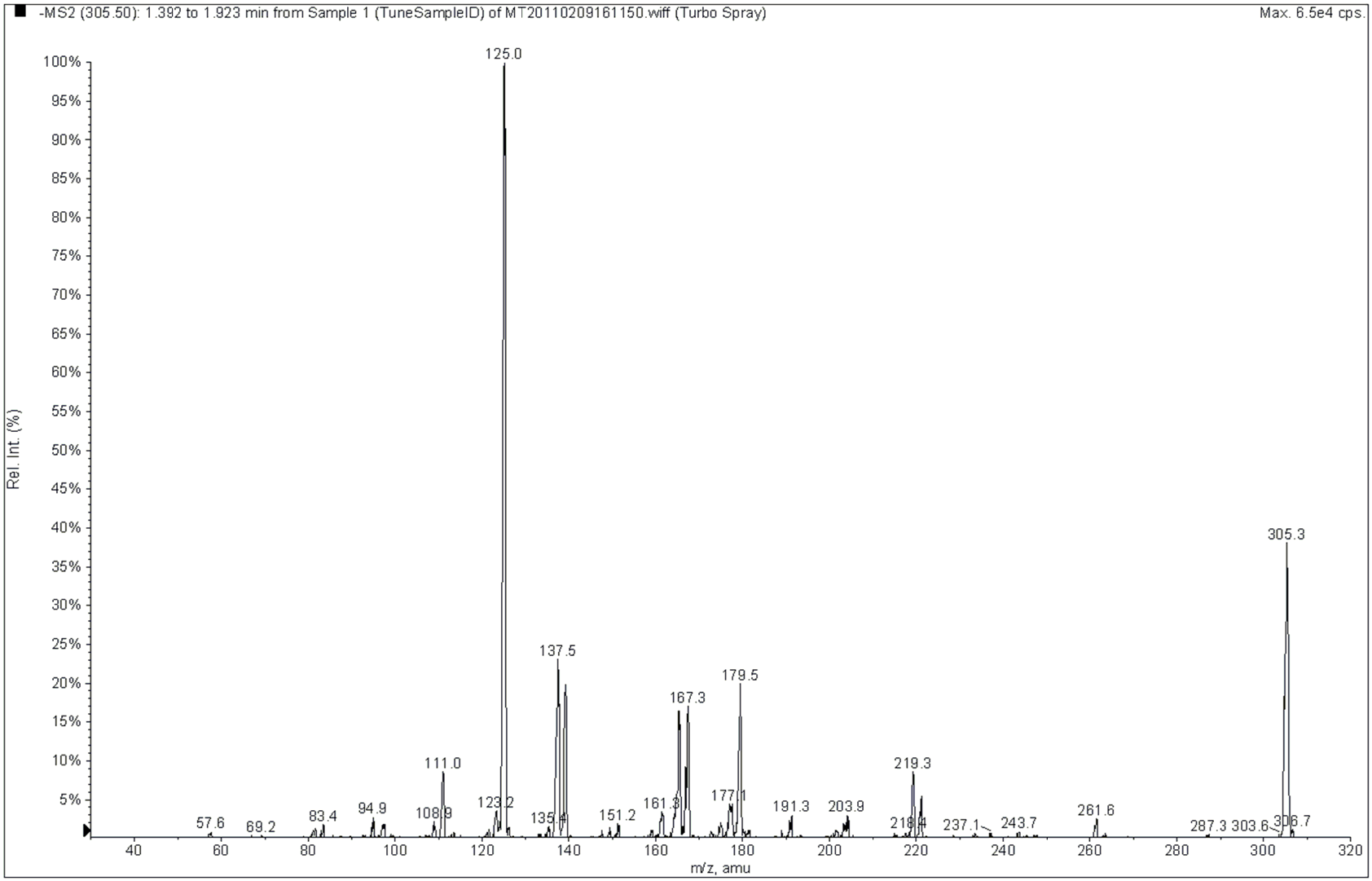


Figure 8b: Product ion scan (MS) of an authentic gallocatechin

Comparison of the MS^2 spectra of authentic catechin (Figure 8d) and authentic robinetinidol (Figure 8e) suggests that the *m/z* 245 peak is diagnostic for catechin and the *m/z* 139 peak is diagnostic for robinetinidol. Comparing the intensities of these two peaks in the MS^2 (Figure 8c) of the *m/z* 289 fragment in Figure 7a and taking the 245/289 and 139/289 ratios in figures 8d and 8e into account, we conclude that the *m/z* 289 fragment in the MS of wattle bark extract consists of *ca*. 20% catechin and 80% robinetinidol.

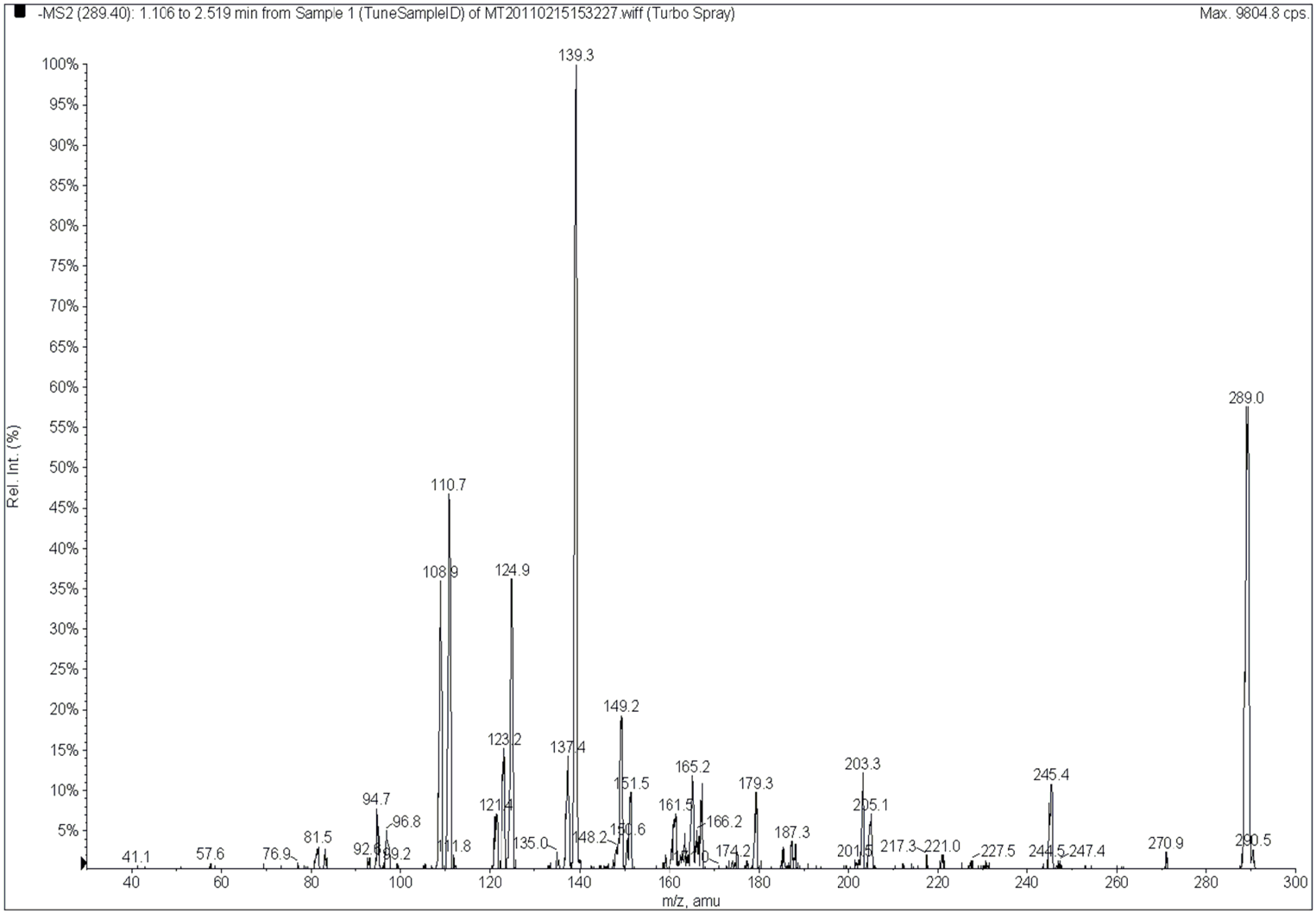


Figure 8c: Product ion scan (MS^2) of the *m/z* 289 fragment in the MS of wattle bark extract

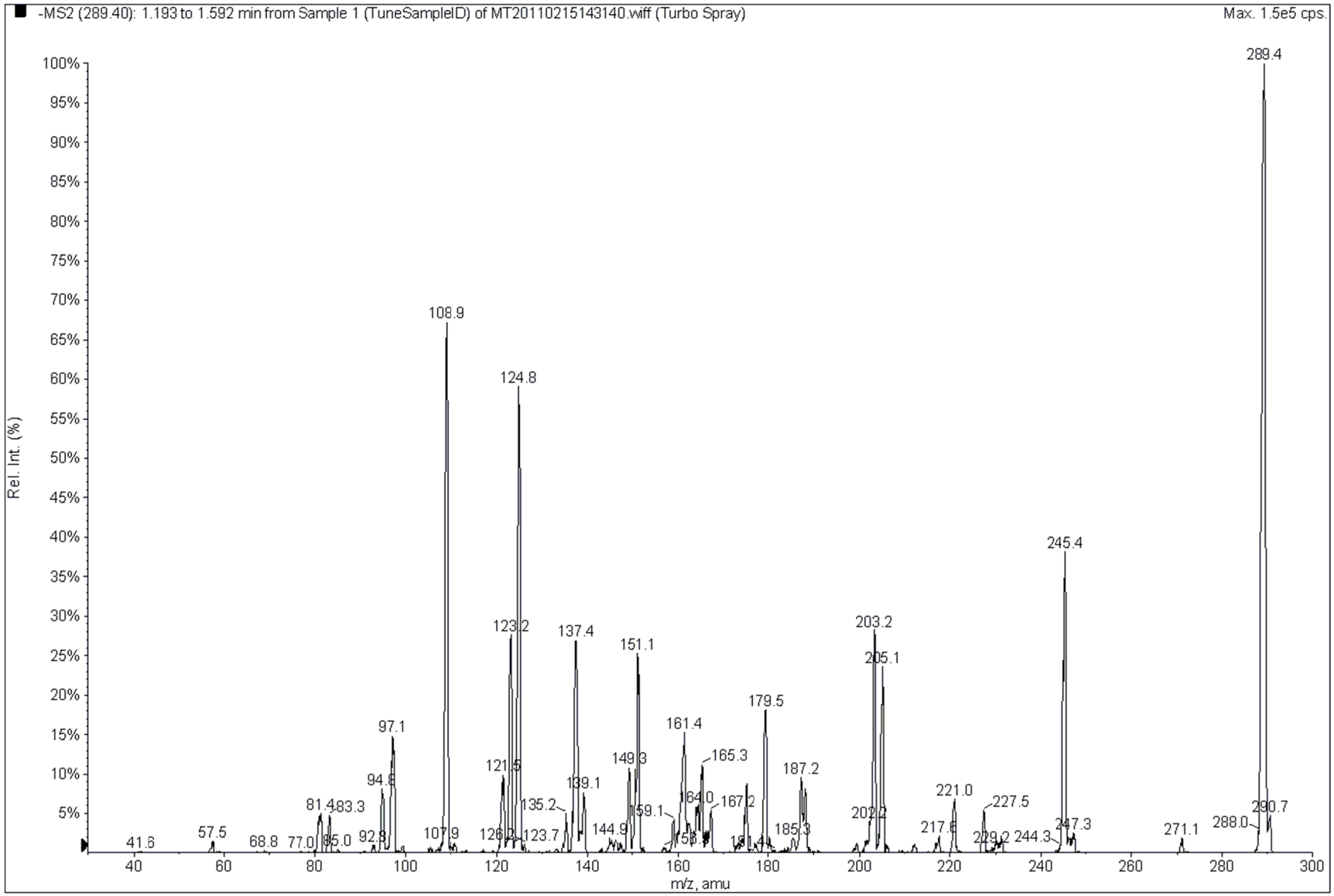


Figure 8d: Product ion scan (MS) of an authentic catechin sample

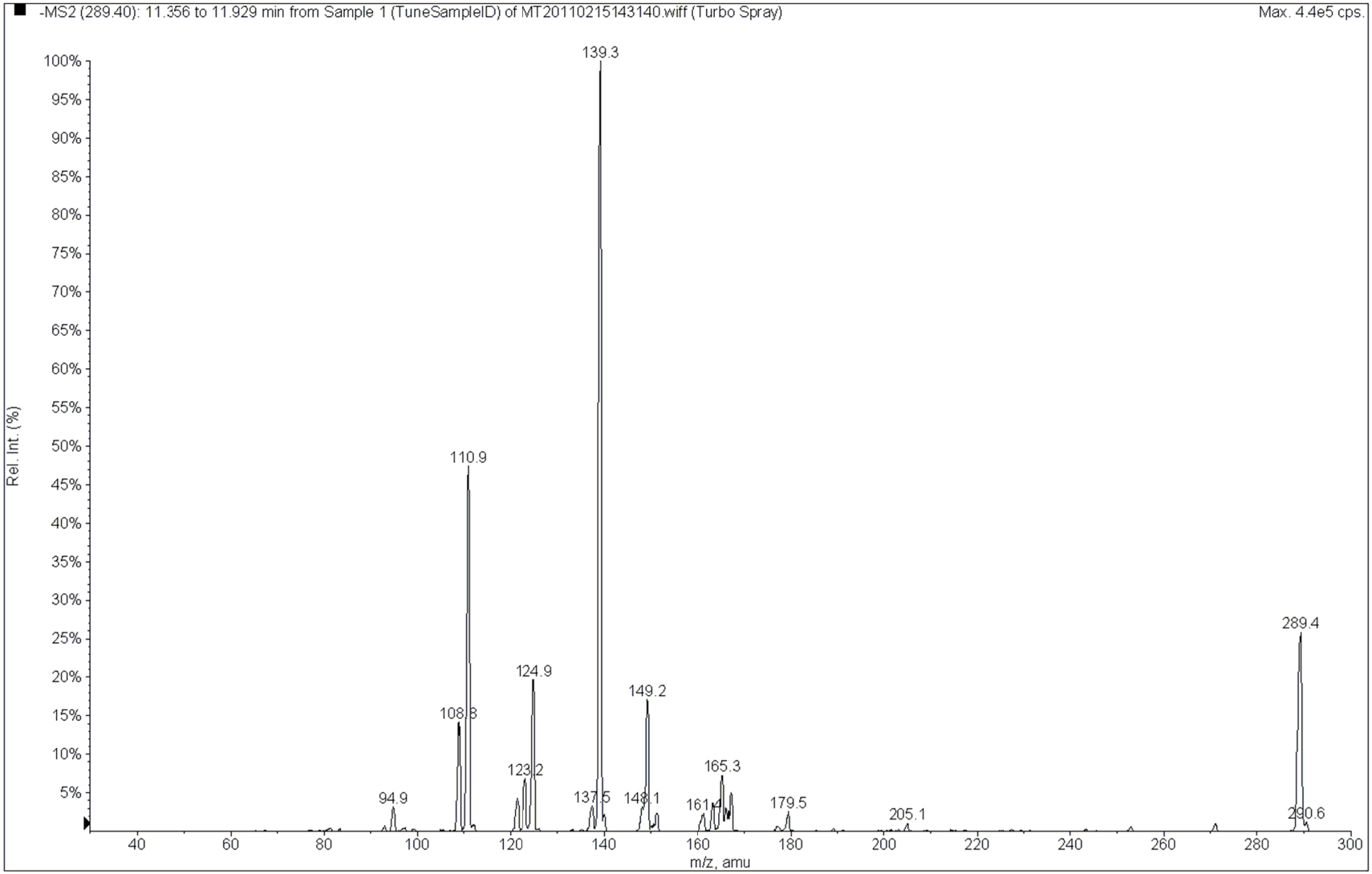


Figure 8e: Product ion scan of an authentic robinetinidol

In summary, product ion scan mass spectrometry (MS^2) indicates that the *m/z* 305 ion in Figure 7a is exclusively a gallocatechin fragment and *m/z* 289 consists of about 80% robinetinidol and 20% catechin. It is uncertain whether these are monomer fragments or whether they are derived *via* fragmentation from higher oligomers. Thus, in contrast with MALDI-TOF MS, ESI-MS supports the presence of catechin monomers, as would be expected from its isolation from wattle bark (Saayman and Roux, 1965). The absence of an *m/z* 273 (fisetinidol) fragment correlates with the low occurrence of profisetinidins, both in the isolated dimers and trimers and our MS results.

4.2.3. Precursor Ion Scans of Monomers and Monomer Fragments

A precursor ion scan of the *m/z* 305 fragment (gallocatechin) (Figure 9a) indicates unambiguously that the *m/z* 578 and 594 dimers and *m/z* 850, 866, and 881 trimers contain gallocatechin moieties. This is in agreement with the conclusions that are based on product ion scans of these dimers and trimers (below) and phytochemical investigations.

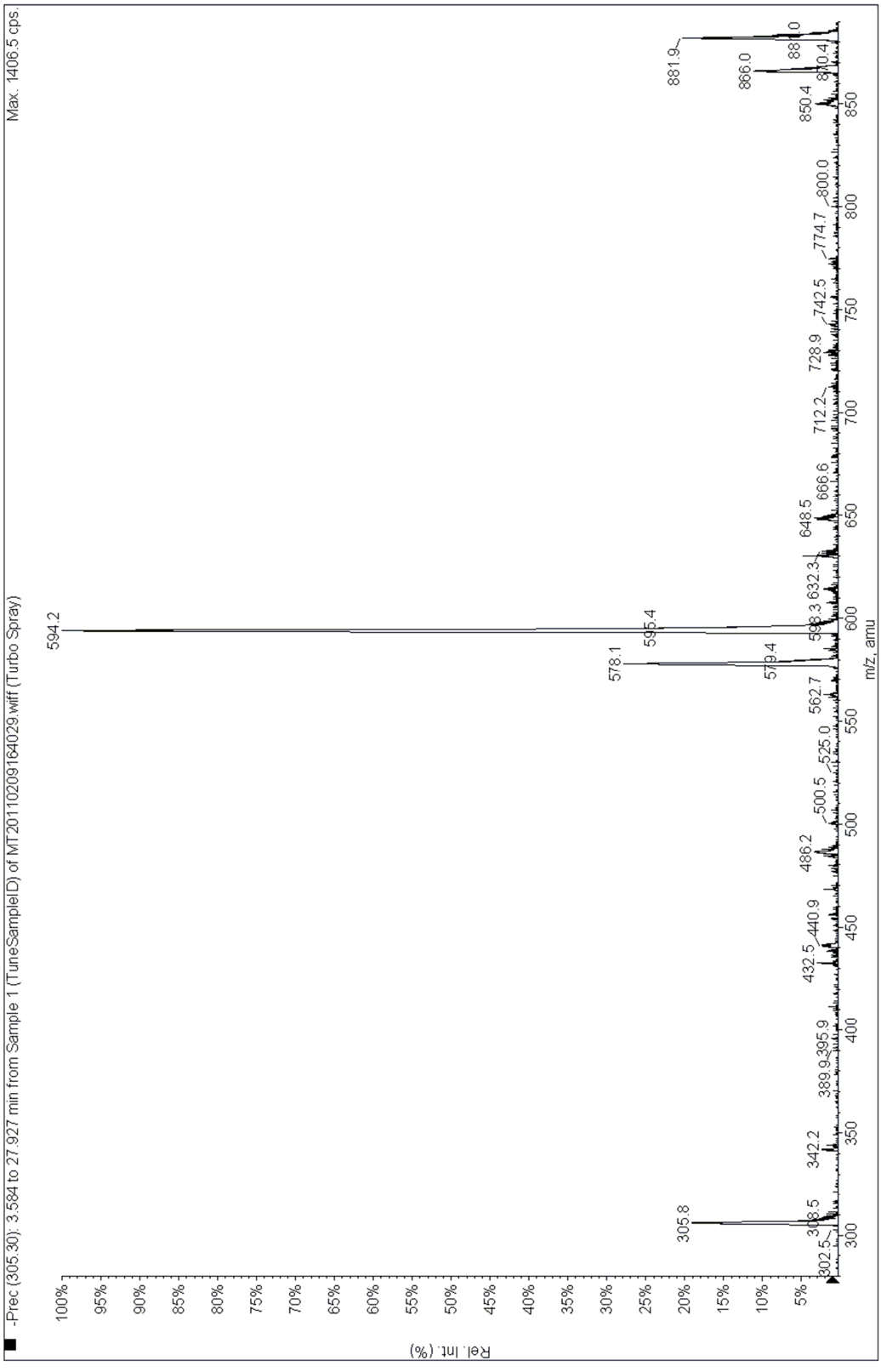


Figure 9a: Precursor ion scan of the *m/z* 305 fragment

A precursor ion scan of the *m/z* 289 fragment (Figure 9b) has a strong correlation with the *m/z* 577 and less with the *m/z* 561 and 593 dimers, and 833, 849, and 865 trimers. Since *m/z* 289 is a mixture of robinetinidol and catechin (Figure 8c), these correlations, however, do not allow unambiguous assignment of the structures of the corresponding dimers and trimers.

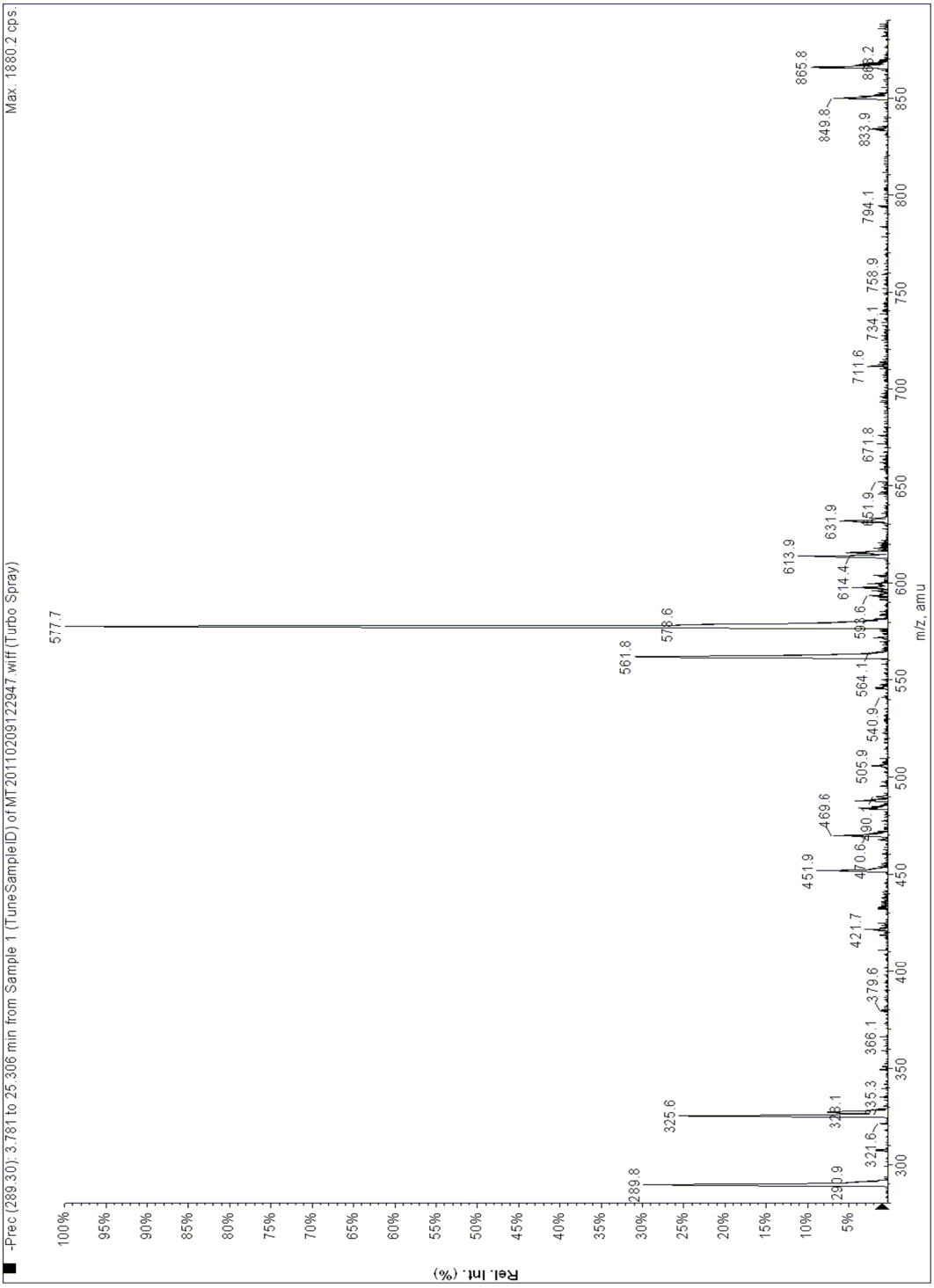


Figure 9b: Precursor ion scan of the *m/z* 289 fragment

The precursor ion scan correlation of the *m/z* 245 fragment with the exclusively catechin-fisetinidol *m/z* 561 and exclusively gallocatechin-robinetinidol 593 dimers invalidates the diagnostic use of the *m/z* 245 fragment for catechin in a PAC sample that also contains gallocatechin moieties (Figure 9c).

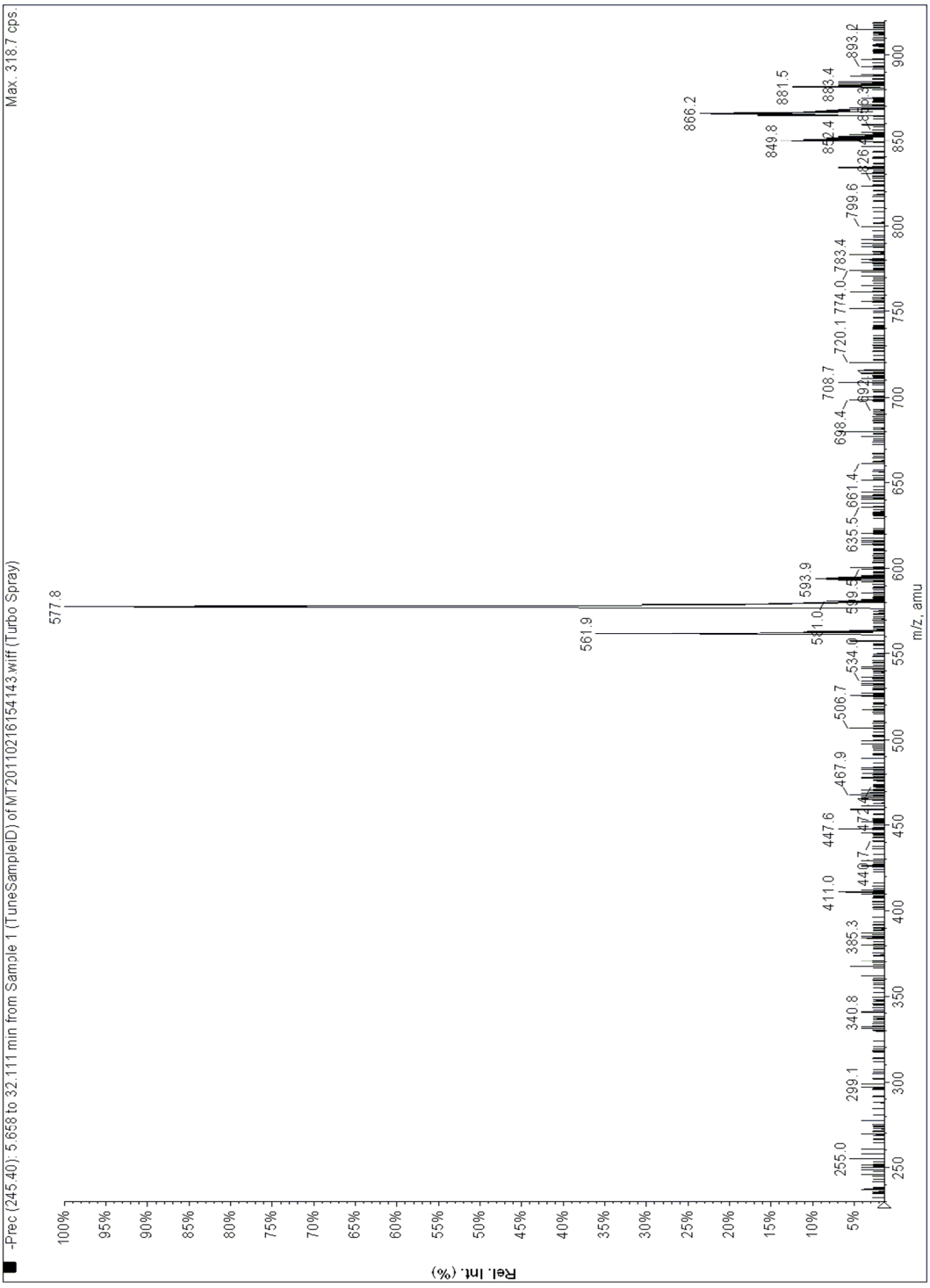


Figure 9c: Precursor ion scan of the *m/z* 245

4.2.4. Fragmentation of Dimers and Higher Oligomers

Dimers and higher oligomers demonstrate characteristic fragmentation patterns:

1. Retro-Diels-Alder (RDA) fragmentation of the heterocyclic C-ring of the starter-unit-derived moieties, catechin or gallocatechin with phloroglucinol type A-rings, is common. The fisetinidol and robinetinidol extender units do not seem to undergo RDA fragmentation, probably due to their resorcinol-type A-rings. Because catechin loses 152 Da and gallocatechin 168 Da during RDA fragmentation, dimers and trimers with the same mass but different starter units, e.g., fisetinidol-gallocatechin and robinetinidol-catechin, are distinguishable. The complementary *m/z* 152 and 168 fragments are not observed. We assume that the ploroglucinol-containing A-ring captures the charge. The intensities of the RDA fragments in MS^2 increase as the PAC becomes larger, probably due to intramolecular thermal stabilisation of fragmentation energy. The monomers show no RDA fragmentation and the trimers have an almost 1:1 ratio between the molecular ion and the RDA fragment. The RDA fragment loses water (18 Da). A gallocatechin starter unit shows more intense RDA fragmentation than catechin. This leads to an ambiguous result when *m/z* 152/168 ratios are compared to other ratios. The RDA fragmentation of trimers is summarised in Scheme 3.
2. Interflavanyl-bond fission is common. The moiety with the starter unit carries the charge and the lost extender unit is not detected. Trimers fragment to dimers and dimers to catechin (*m/z* 289) or gallocatechin (*m/z* 305) monomers. As robinetinidol is not detected, the *m/z* 289/305 ratio is very useful and is used, e.g., to estimate the ratio of gallocatechin-fisetinidol to catechin-robinetinidol in an *m/z* 577 mixture. Salient in the fragmentation spectra of tetramers is the absence (Figures 12d and 12e) or low occurrence (Figure 12c) of trimer fragments. This suggests that tetramers fragment directly to dimers *via* the loss of a linear robinetinidol-robinetinidol, robinetinidol-fisetinidol or fisetinidol-fisetinidol moiety.
3. Comparison of robinetinidol, catechin, gallocatechin, and fisetinidol fragmentation patterns indicates that the *m/z* 177 ion is diagnostic for a robinetinidol extender unit

and *m/z* 161 for a fisetinidol extender unit. The structures of these fragments are not clear and probably involves rearrangement of the B-ring.

4. Since no regiochemical or configurational information can be gleaned from MS, we cannot unequivocally distinguish between C-6 and C-8 coupled dimers and between 4α- and 4β-interflavanyl bonds with MS, and no stereochemistry is indicated in our MS-bases structural assignments (e.g. Scheme 3). However, based on the structure of isolated dimers, we assume that C-8 coupled PACs predominate.

4.2.5. Wattle PAC Dimers

We observed three dimer peaks (*m/z* 561, 577, and 593) in the MS of wattle bark extract (Figure 7a) in a ratio of 1:4:3.5. The product ion scan (ESI in the negative mode) of the *m/z* 593 peak (Figure 10a) that corresponds unambiguously with gallocatechin-robinetinidol gave gallocatechin (*m/z* 305) as base peak. The complementary robinetinidol fragment expected at *m/z* 289 was not observed. We also observed a RDA fragmentation of the gallocatechin starter unit at *m/z* 425 with the robinetinidol extender unit intact and a RDA-H_2O fragment at *m/z* 407. The fragment at *m/z* 177 (absent from Figure 10b) is considered diagnostic of the robinetinidol extender unit. The fragmentation pattern is summarised in Scheme 2. Since MS gives no regiochemical or configurational information, we assume C-8 coupling based on the structure of isolated products and do not give C-4 configurational assignments.

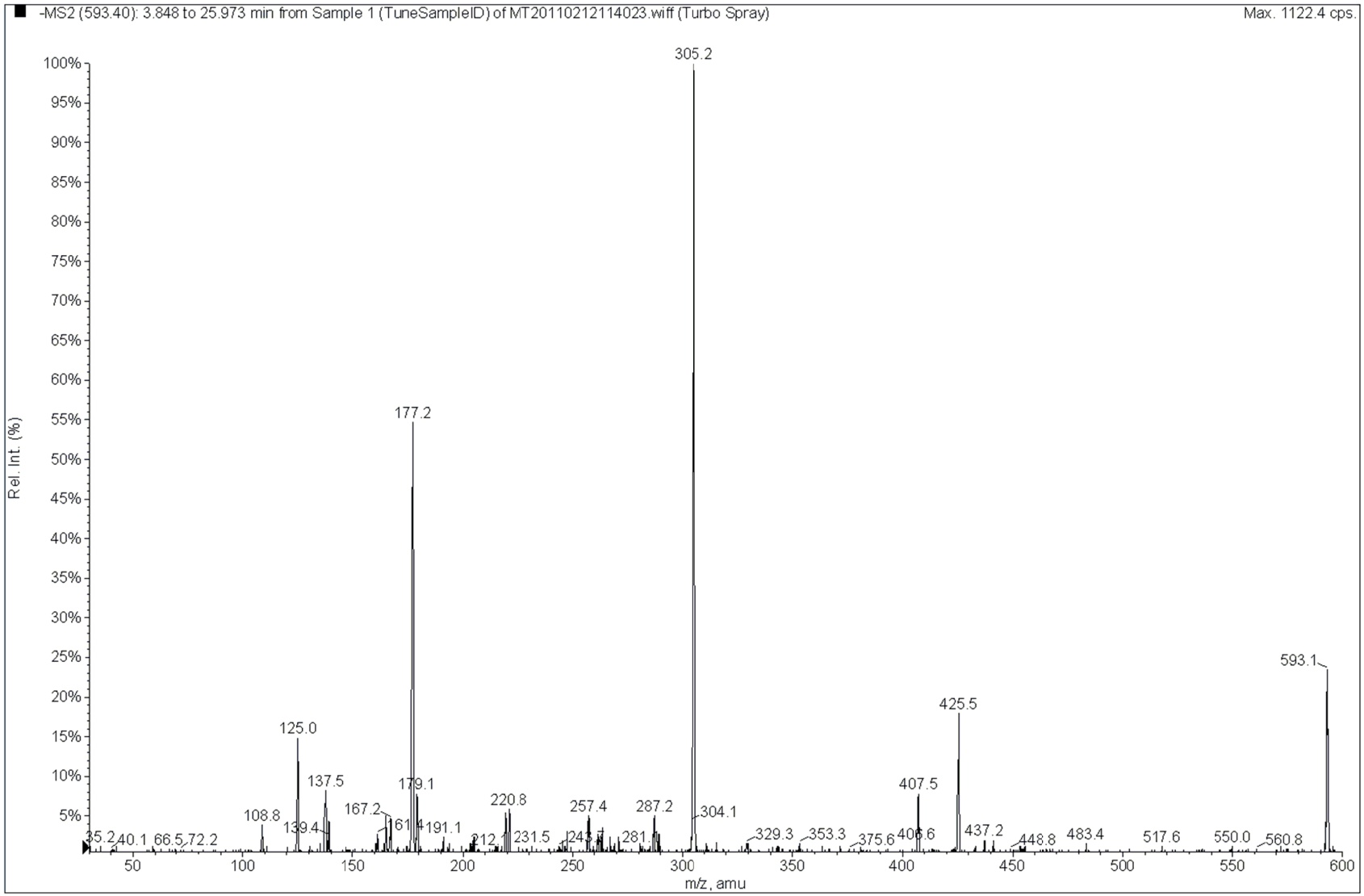
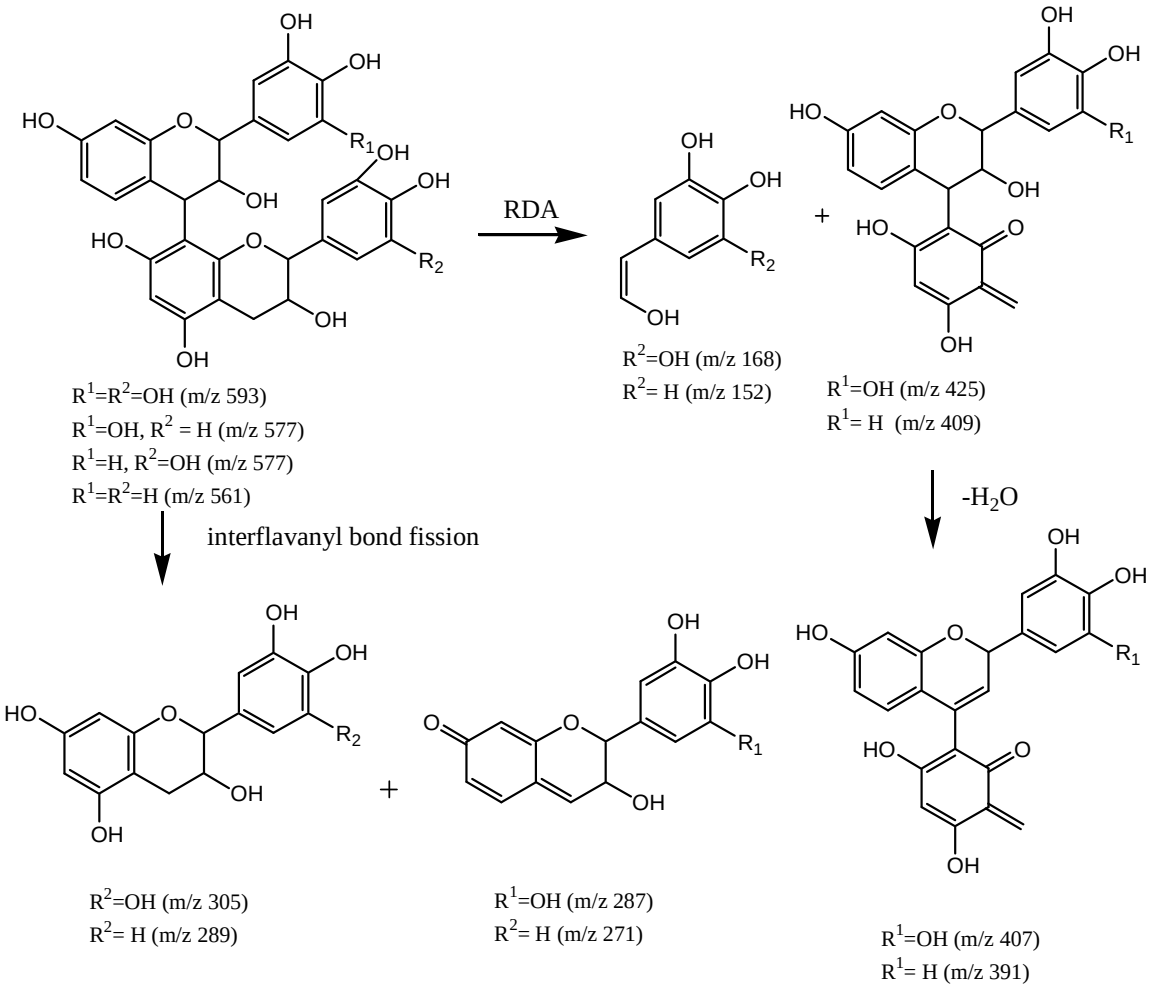


Figure 10a: Product ion scan of the *m/z* 593 dimer (robinetinidol-gallocatechin) (ESI in the negative mode)



Scheme 2: Fragmentation of wattle dimers [M-H]$^+$

Similarly, the product ion scan of the *m/z* 561 ion (Figure 10b) that corresponds unambiguously with catechin-fisetinidol gave the catechin *m/z* 289 fragment as the base peak, as would be expected from fission of a catechin-fisetinidol interflavanyl bond, and no complementary fisetinidol (*m/z* = 273) fragment. We also observe the RDA fragment of the catechin starter unit with the fisetinidol extender unit intact at *m/z* 409 and an RDA-H_2O fragment at *m/z* 407. The fragment at *m/z* 161 (absent in Figure 10a) is considered diagnostic for the fisetinidol extender unit. The fragmentation pattern is summarised in Scheme 2.

The *m/z* 577 fragment can be either gallocatechin-fisetinidol or catechin-robinetinidol. As extender units are not observed in MS^2 (Figure 10c), we assume that *m/z* 305 and 289 (1:6 ratio) originate from the respective gallocatechin and catechin starter units. The diagnostic fisetinidol and robinetinidol extender unit fragments at *m/z* 161 and 177 (1:6.5 ratio) confirm the mixture of gallocatechin-fisetinidol and catechin-robinetinidol dimers. From the fragment intensity ratios, we conclude that the *m/z* 577 wattle PAC dimers consist of about 15% gallocatechin-fisetinidol and 85 % catechin-robinetinidol. The low occurence of gallocatechin-fisetinidol is in agreement with its non-detection by phytochemical methods (see introduction). The ratio between the RDA fragments at 425 (M-152) and 409 (M-168), assigned to catechin-robinetinidol and gallocatechin-fisetinidol, respectively, is not used as the gallocatechin moiety is susceptible to a more intense RDA fragmentation than the catechin moiety (see above).

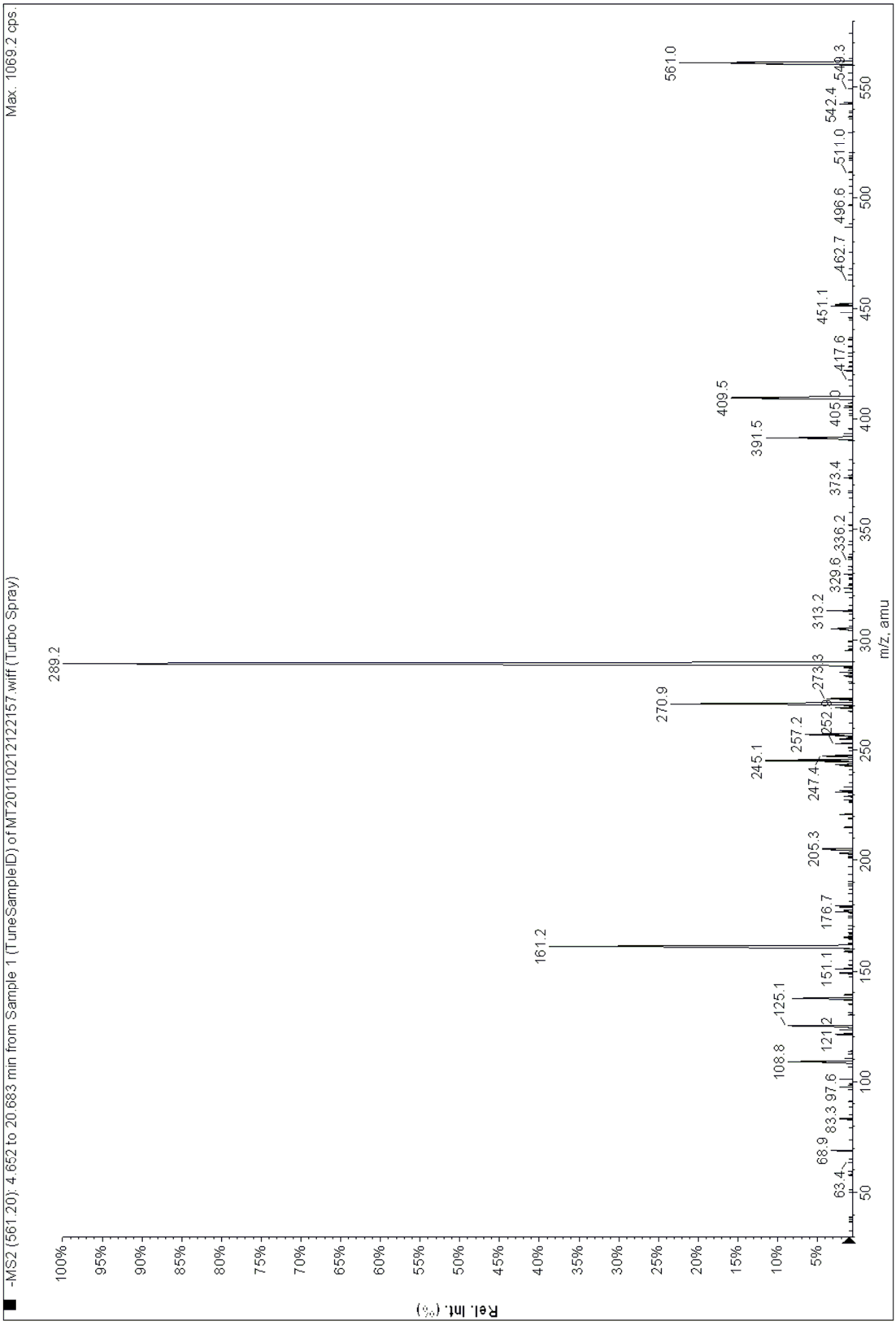


Figure 10b. Product ion scan of the *m/z* 561 dimer (catechin-fisetinidol)

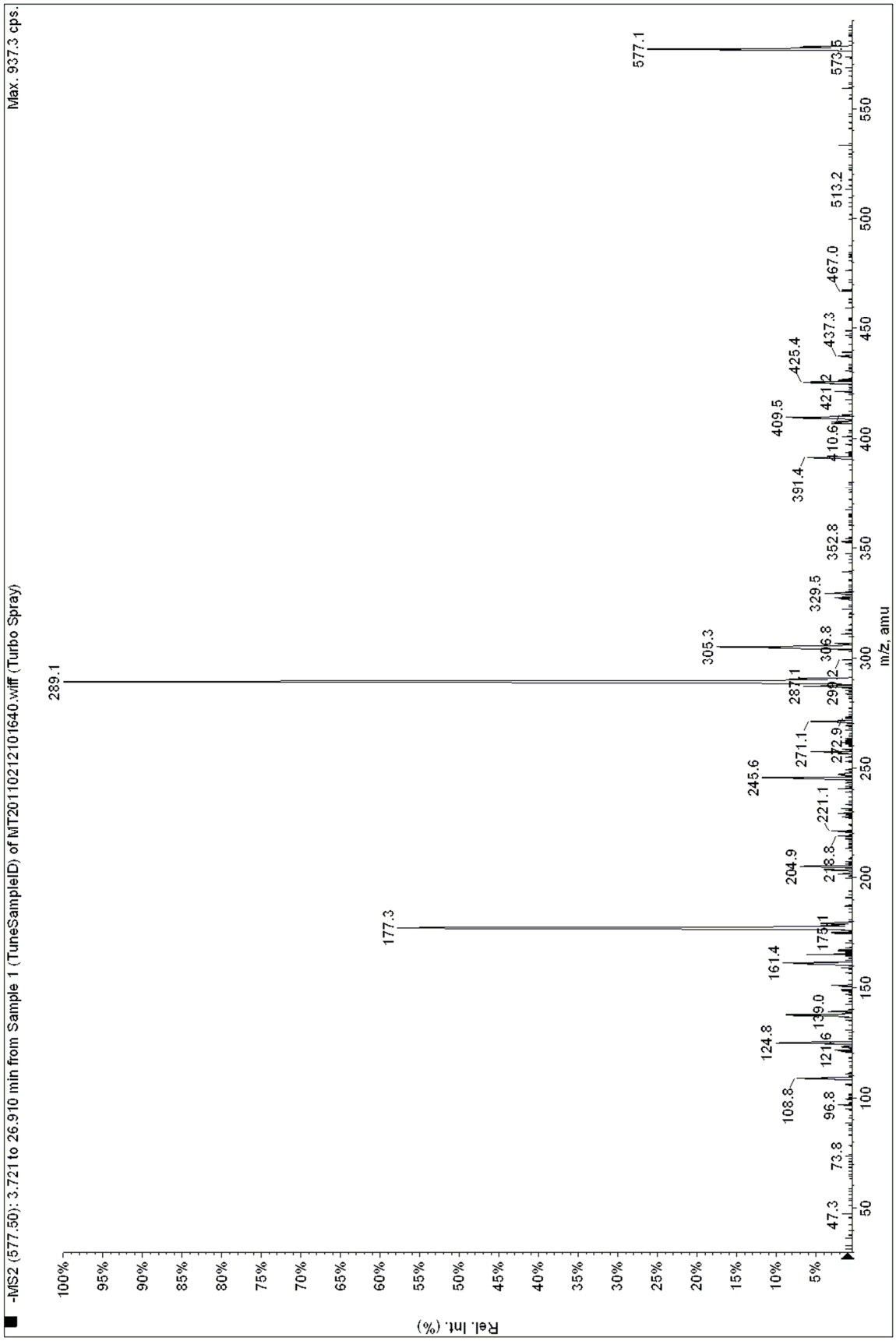


Figure 10c: Product ion scan of the *m/z* 577 dimer (gallocatechin-fisetinidol and catechin-robinetinidol)

In summary, wattle extract PAC dimers consist of gallocatechin-robinetinidol (39%), catechin-robinetinidol (42%), gallocatechin-fisetinidol (7%), and catechin-fisetinidol (12%). The dimers are thus predominantly prorobinetinidins, consisting of a catechin or gallocatechin starter unit and robinetinidol extender units. We did not detect MS fragments that we could unequivocally attribute to phlobatannin dimers or A-type prorobinetinidins.

4.2.6. Wattle PAC Trimers

Four trimer peaks at *m/z* 833, 849, 865, and 881 are observed in the MS of wattle bark extract (Figure 7a) in a ratio of 1:4:8:5. The major *m/z* 881 peak corresponds unambiguously with a robinetinidol-gallocatechin-robinetinidol trimer. The MS^2 of this fragment (Figure 11a) gave the expected diagnostic gallocatechin (*m/z* 305) and robinetinidol (*m/z* 177) and [M-168] RDA (*m/z* 713) fragments (Scheme 3). Since MS gives no regiochemical or configurational information, we assume C-8 coupling based on the structure of isolated products and do not give C-4 configurational assignments. Salient is the *m/z* 593 fragment (gallocatechin-robinetinidol) that corresponds with the loss of one robinetinidol moiety.

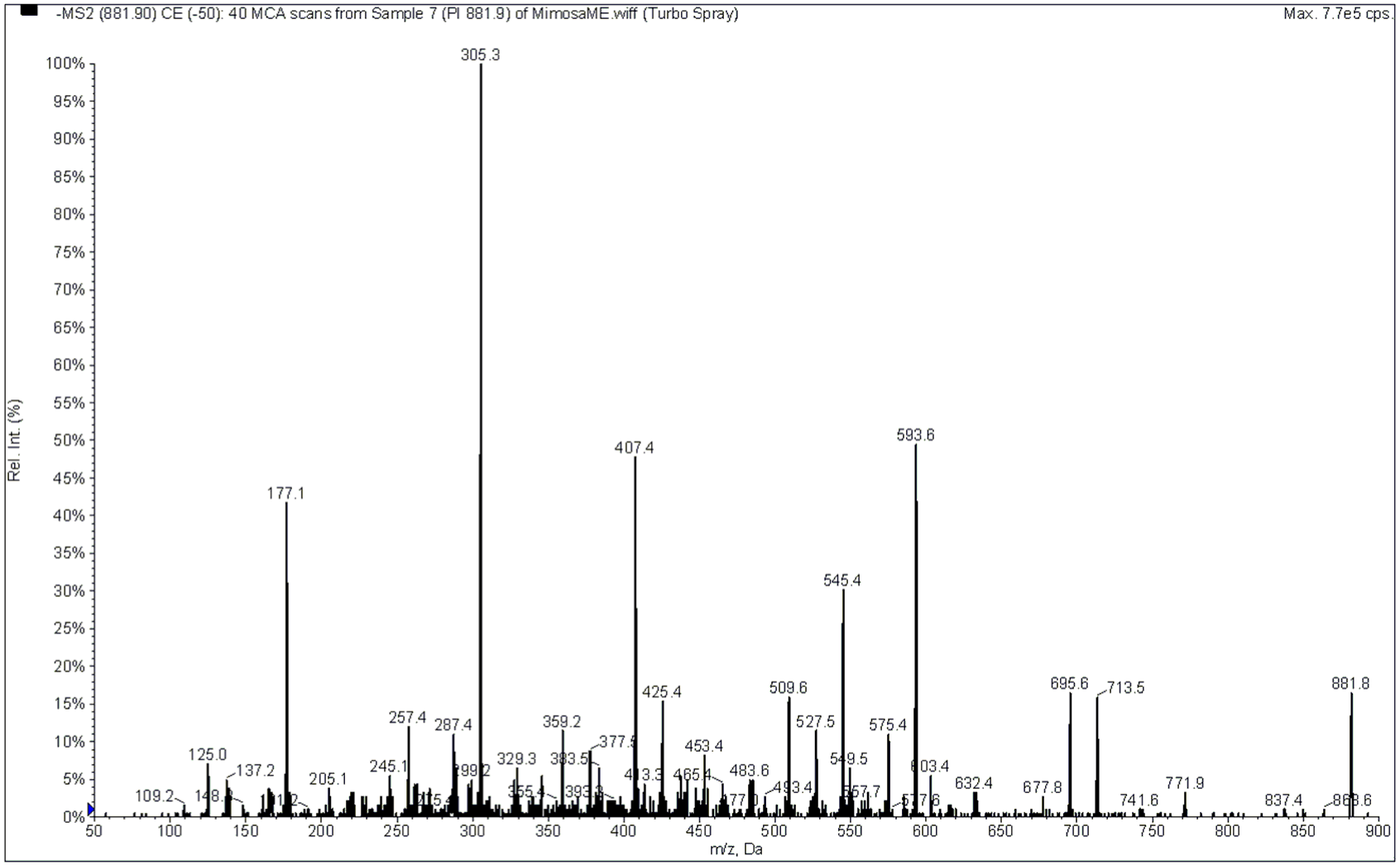


Figure 11a: Product ion scan of the *m/z* 881 trimer (robinetinidol-gallocatechin-robinetinidol)

The minor *m/z* 833 peak in the ESI-MS (Figure 7a) corresponds unambiguously with the fisetinidol-catechin-fisetinidol trimer. An MS^2 of this fragment (Figure 11b and Scheme 3) gave the expected diagnostic catechin (*m/z* 289), fisetinidol (*m/z* 161), and [M-152] RDA (*m/z* 681) fragments. Salient is the *m/z* 561 fragment (catechin-fisetinidol) that corresponds with the loss of a fisetinidol moiety.

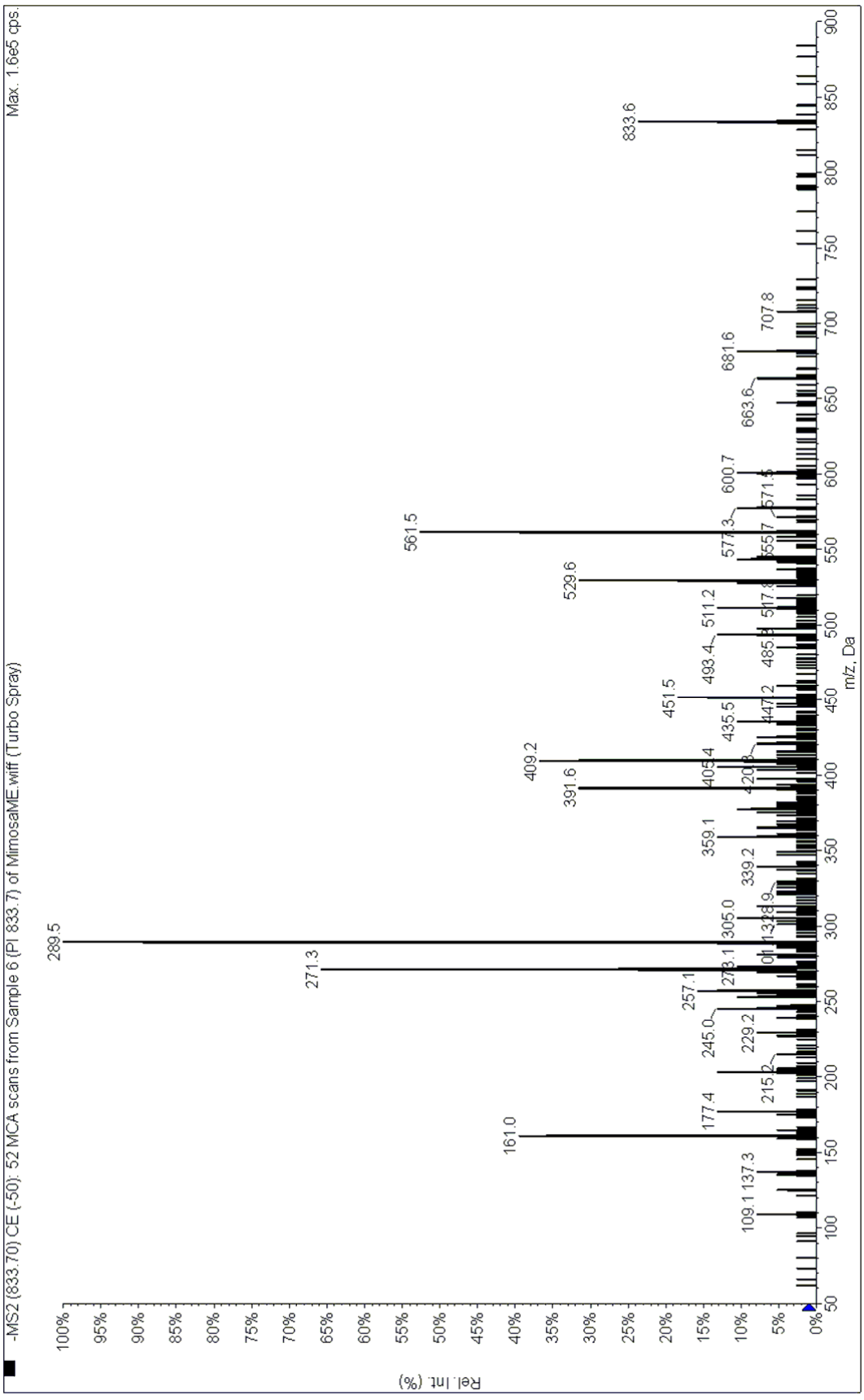


Figure 11b: Product ion scan of the *m/z* 833 trimer (fisetinidol-catechin-fisetinidol)

The predominant *m/z* 865 trimer peak corresponds with fisetinidol-gallocatechin-robinetinidol or with robinetinidol-catechin-robinetinidol. The RDA fragments at *m/z* 713 and 697 corresponding with the loss of 152 and 168 Da, respectively, in the MS^2 of the *m/z* 865 trimer (Figure 11c), indicate the presence of both gallocatechin and catechin starter units. The 2.2:1 ratio between catechin (*m/z* 289) and gallocatechin (*m/z* 305) and the 2.6:1 ratio between the robinetinidol-catechin *m/z* 577 and robinetinidol-gallocatechin *m/z* 593 MS^2 fragments indicate that the *m/z* 865 trimer consists of about 70% robinetinidol-catechin-robinetinidol and 30% fisetinidol-gallocatechin- robinetinidol.

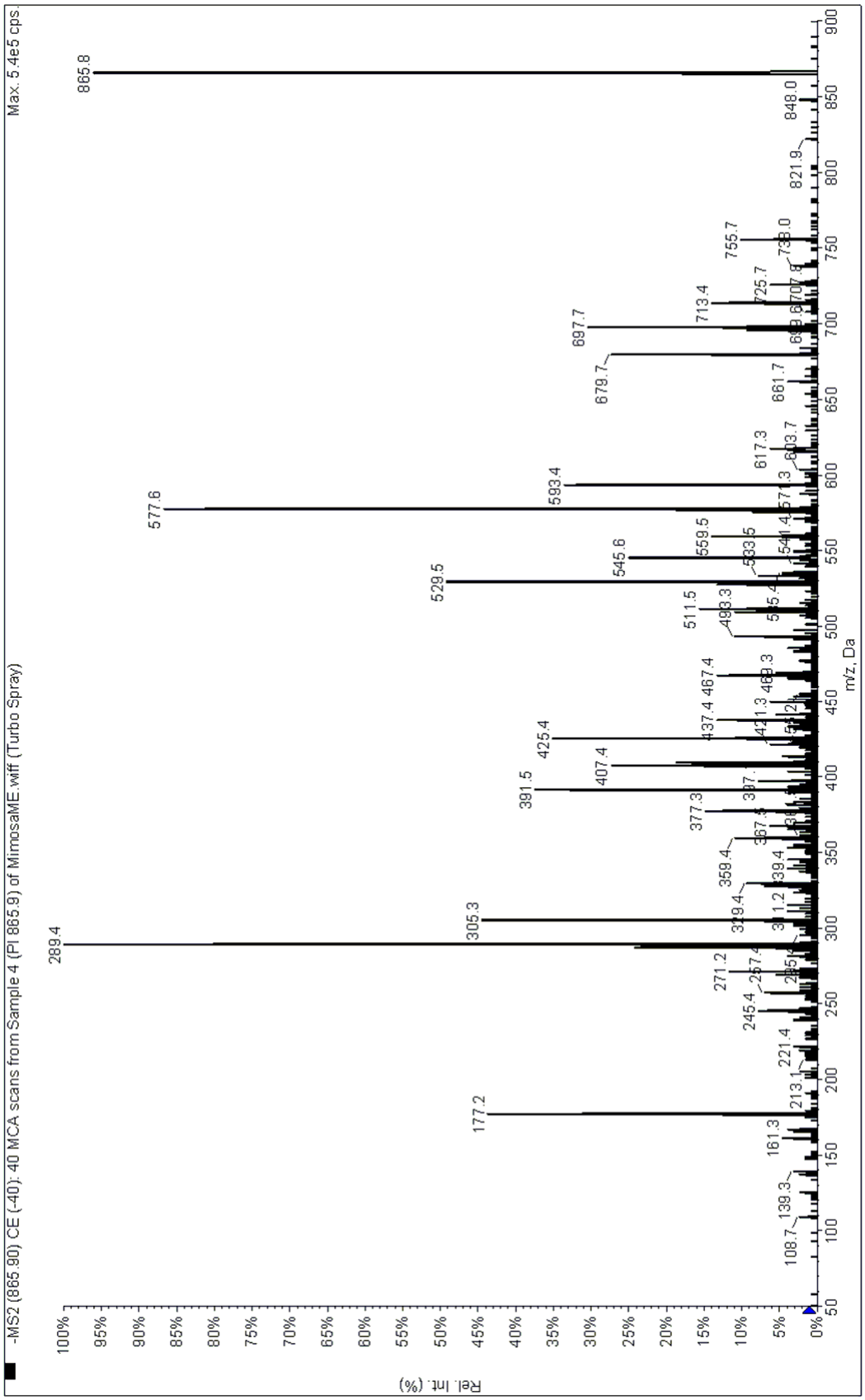


Figure 11c: Product ion scan of the *m/z* 865 trimer (robinetinidol-catechin-robinetinidol and robinetinidol-gallocatechin-fisetinidol)

The prominent *m/z* 849 trimer peak corresponds with fisetinidol-catechin-robinetinidol or fisetinidol-gallocatechin-fisetinidol. RDA fragments at *m/z* 697 and 681 in the MS^2 of the *m/z* 849 trimer (Figure 11d) corresponding with loss of 152 and 168 Da, respectively, indicate the presence of both catechin and gallocatechin starter units. The 1:7 ratio between catechin (*m/z* 289) and gallocatechin (*m/z* 305) suggests that the *m/z* 849 trimers consist of *ca*. 88% fisetinidol-catechin-robinetinidol and 12% fisetinidol-gallocatechin-fisetinidol.

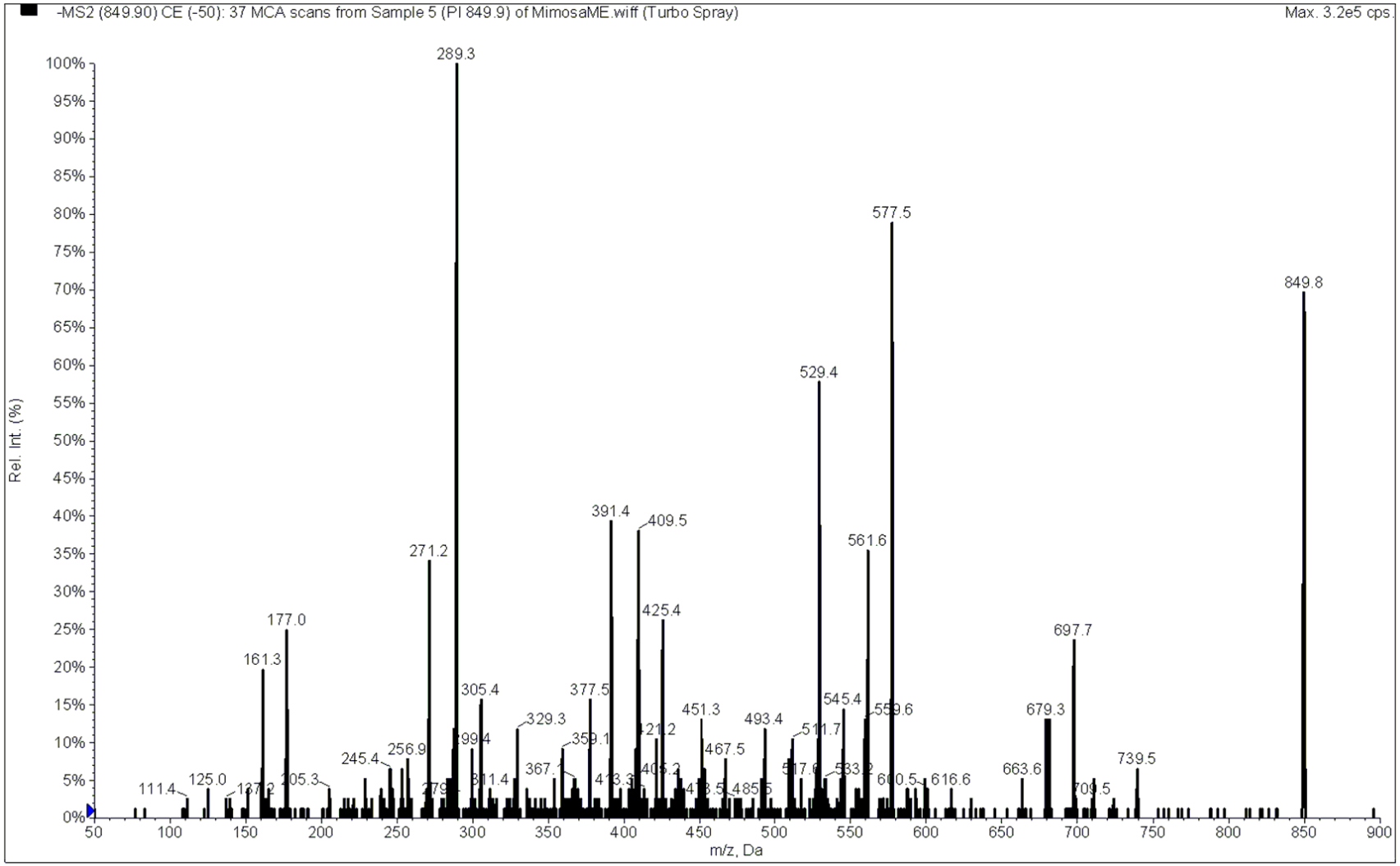
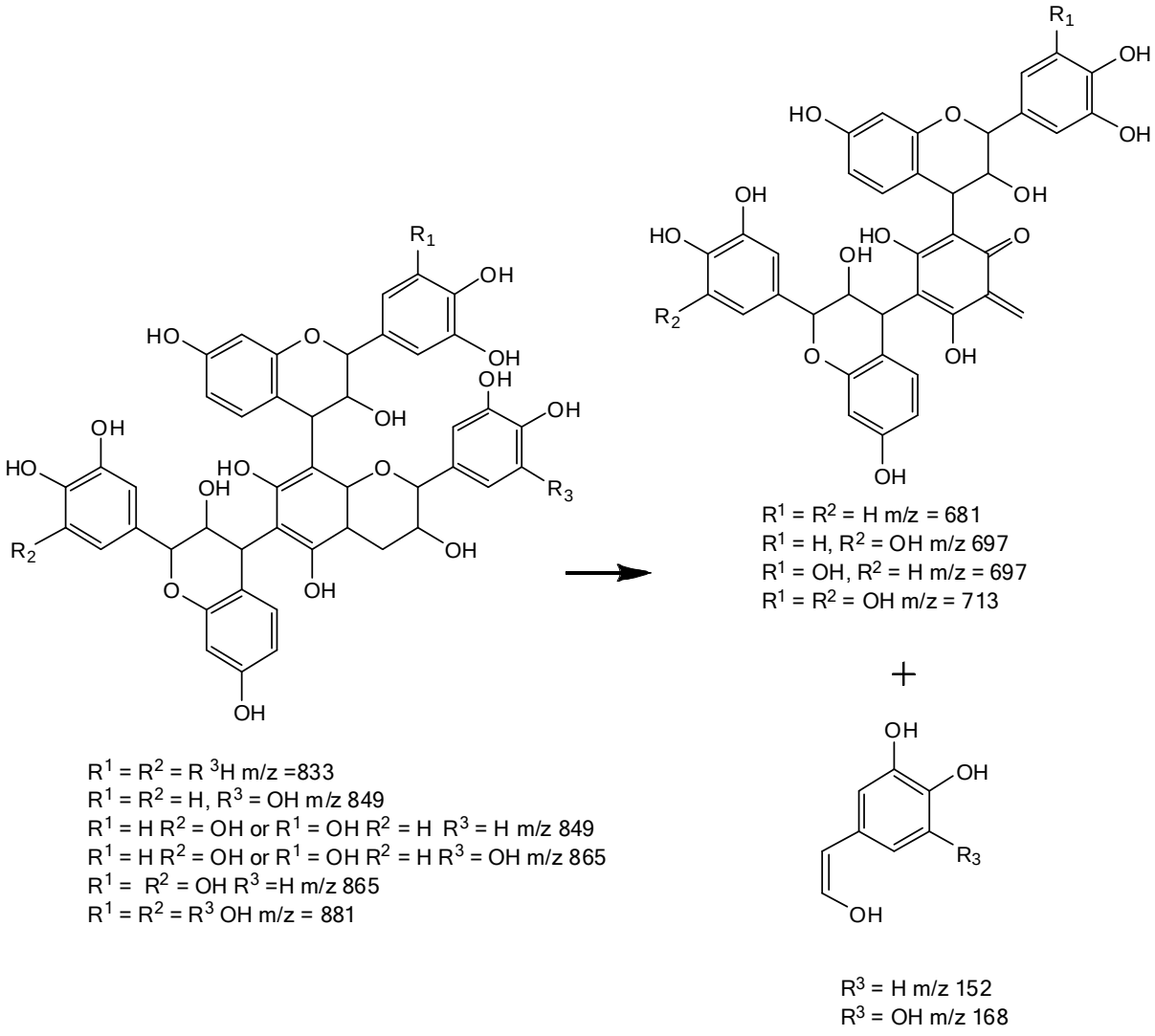


Figure 11d: Product ion scan of the *m/z* 849 trimer (fisetinidol-catechin-robinetinidol or fisetinidol-gallocatechin-fisetinidol)



Scheme 3: Structures and *m/z* values of trimer-derived RDA fragments

In summary, wattle extract PAC trimers consist of robinetinidol-catechin-robinetinidol (32%), robinetinidol-gallocatechin-robinetinidol (27%), robinetinidol-catechin-fisetinidol (20%), robinetinidol-gallocatechin-fisetinidol (13%), fisetinidol-catechin-fisetinidol (5%), and fisetinidol-gallocatechin-fisetinidol (3%). The trimers are, thus, predominantly prorobinetinidins, consisting of a catechin or gallocatechin starter unit and robinetinidol extender units. We did not detect MS fragments that we could unequivocally attribute to phlobatannin or A-type trimers.

4.2.7. Tetramer and Pentamer Oligomers

Mimosa bark PAC tetramers and pentamers, similar to dimers and trimers, occur as clusters, 16 Da apart (Figures 12a and 12b), corresponding to all possible combinations and permutations of catechin and gallocatechin starter units and robinetinidol and fisetinidol extender units (Table 1). As ^{13}C has a natural abundance of 1.1%, the M+1 ion has a relative intensity of 60% in tetramers and 82% in pentamers. M+2 and M+3 ions are also observed.

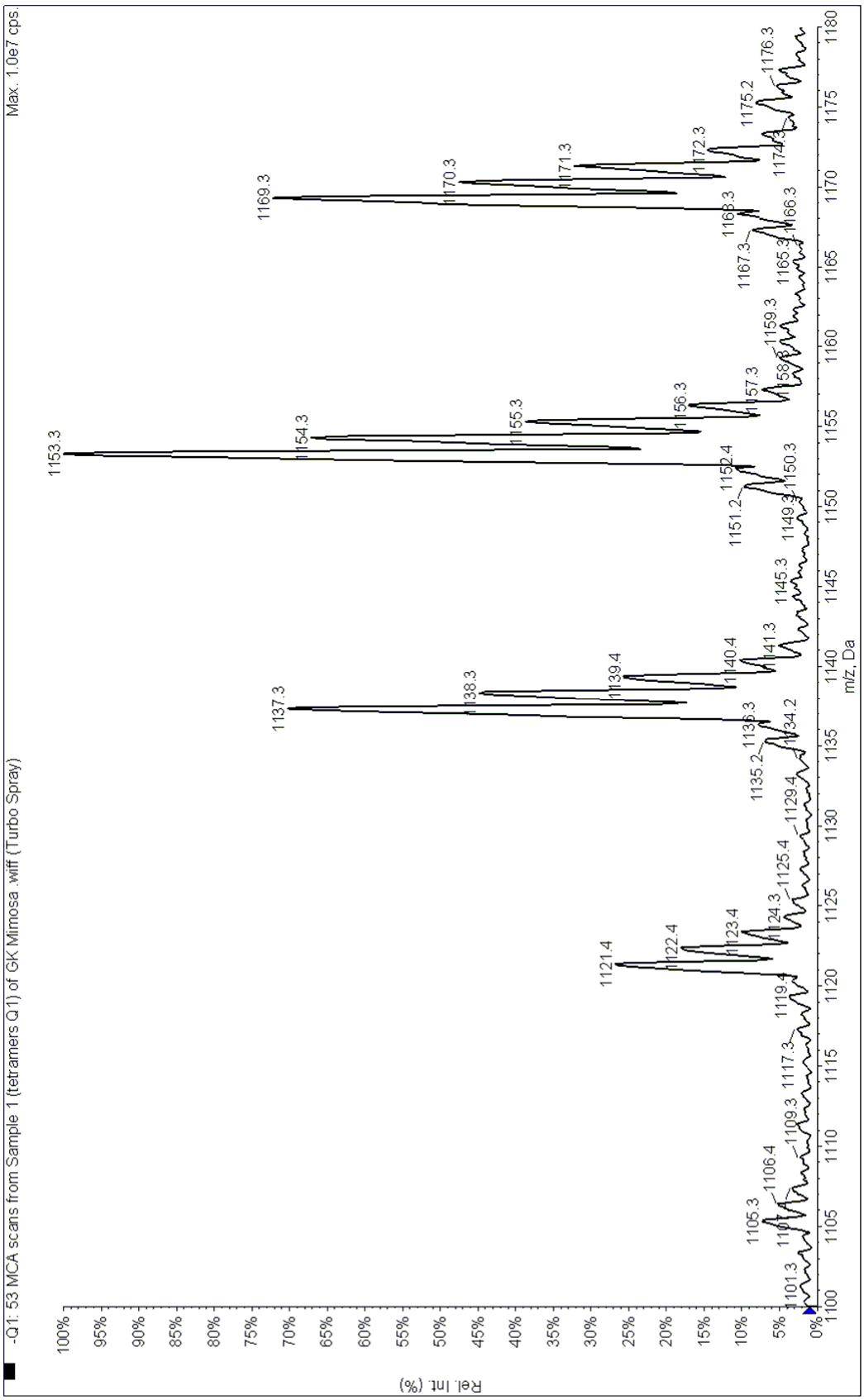


Figure 12a: Cluster of wattle bark PAC tetramers and ^{13}C isotopomers

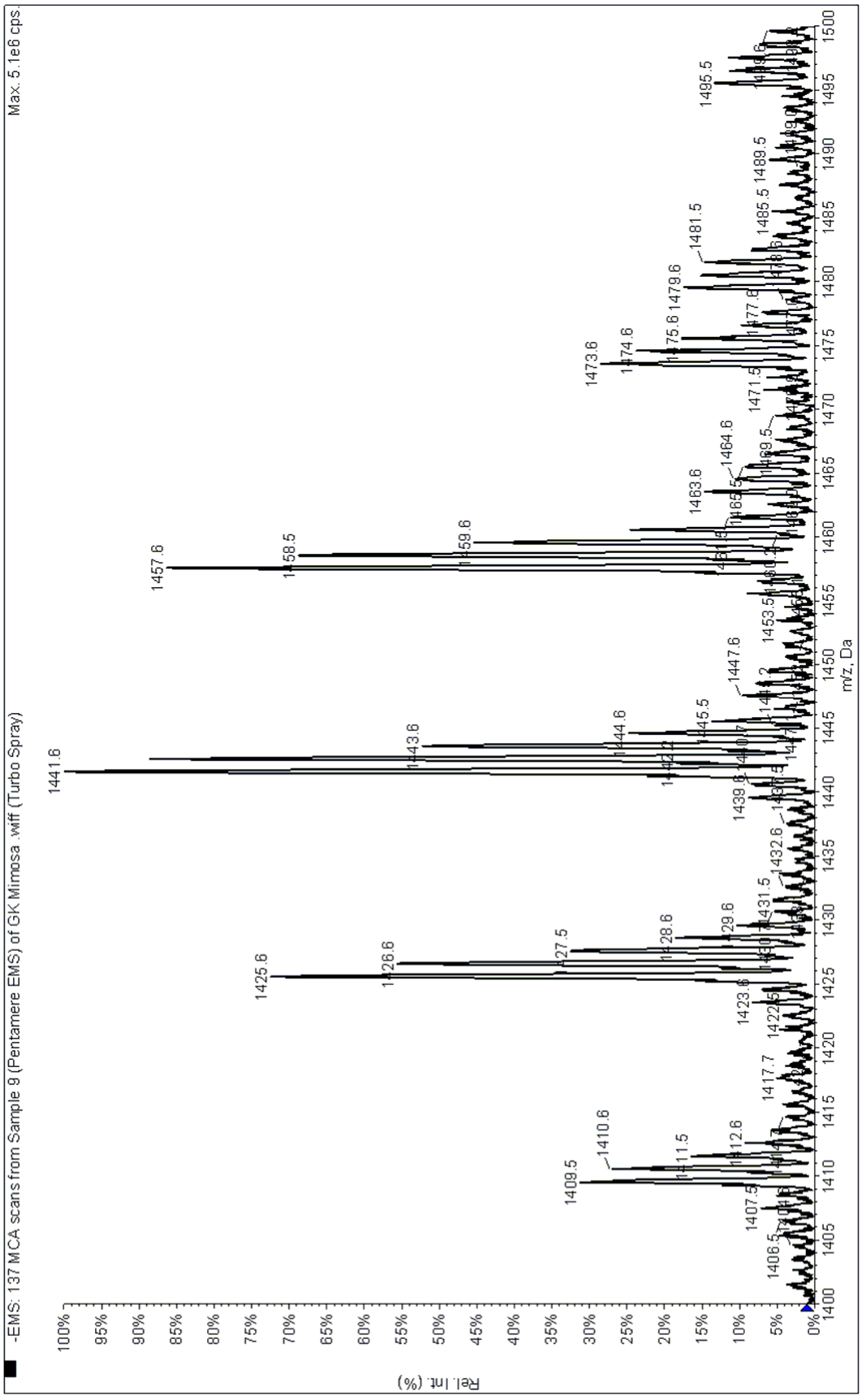


Figure 12b: Cluster of wattle bark PAC pentamers and ^{13}C isotopomers

In contrast with 5-oxy containing PACs (Pezet *et al*., 2001), based on catechin-4-ol and gallocatechin-4-ol extender units, where polymers with more than 20 monomeric units have been reported, and where a gradual drop in intensity is observed as one proceeds along the homologous series, a pronounced drop in intensity from trimers to tetramers is evident; and polymers larger than the hexamer level are barely observable.

The reactive positions on the phloroglucinol A-ring of the catechin and gallocatechin starter units are occupied at the trimeric level. Additional interflavanyl bond formation to form tetramers and higher oligomers is only possible *via* the relatively unreactive C-6 of the resorcinol A-ring of a fisetinidol or robinetinidol extender unit of the trimers. This, and not mass discrimination, probably explains the low occurrence of tetramers and higher oligomers. We would have expected the acid-hydrolysis-resistant interflavanyl bond to resist MS fragmentation.

The *m/z* 1105 PAC representing the tetramer with the lowest MW corresponds uniquely with a catechin starter unit and three fisetinidol extender units. Owing to the low intensity, MS^2 of this fragment is not possible with our equipment. It constitutes about 5% of the total tetramers.

The tetramer with the highest MW at *m/z* 1169 corresponds uniquely with a gallocatechin starter unit and three robinetinidol extender units. It constitutes about 10% of the total tetramer composition. The MS^2 (Figure 12c, Scheme 4) shows the expected loss of one robinetinidol moiety at *m/z* 881, two robinetinidol moieties at 593 and three robinetinidol moieties at 305 (gallocatechin). Since MS gives no regiochemical or configurational information, we assume C-8 coupling based on the structure of isolated products and do not give C-4 configurational assignments in scheme 4. Salient is the low intensity of the *m/z* 881 trimer fragment and the absence of its associated RDA (*m/z* 881-168) ion. The dimer fragment associated RDA is prominent at 407 (*m/z* 593-168-18).

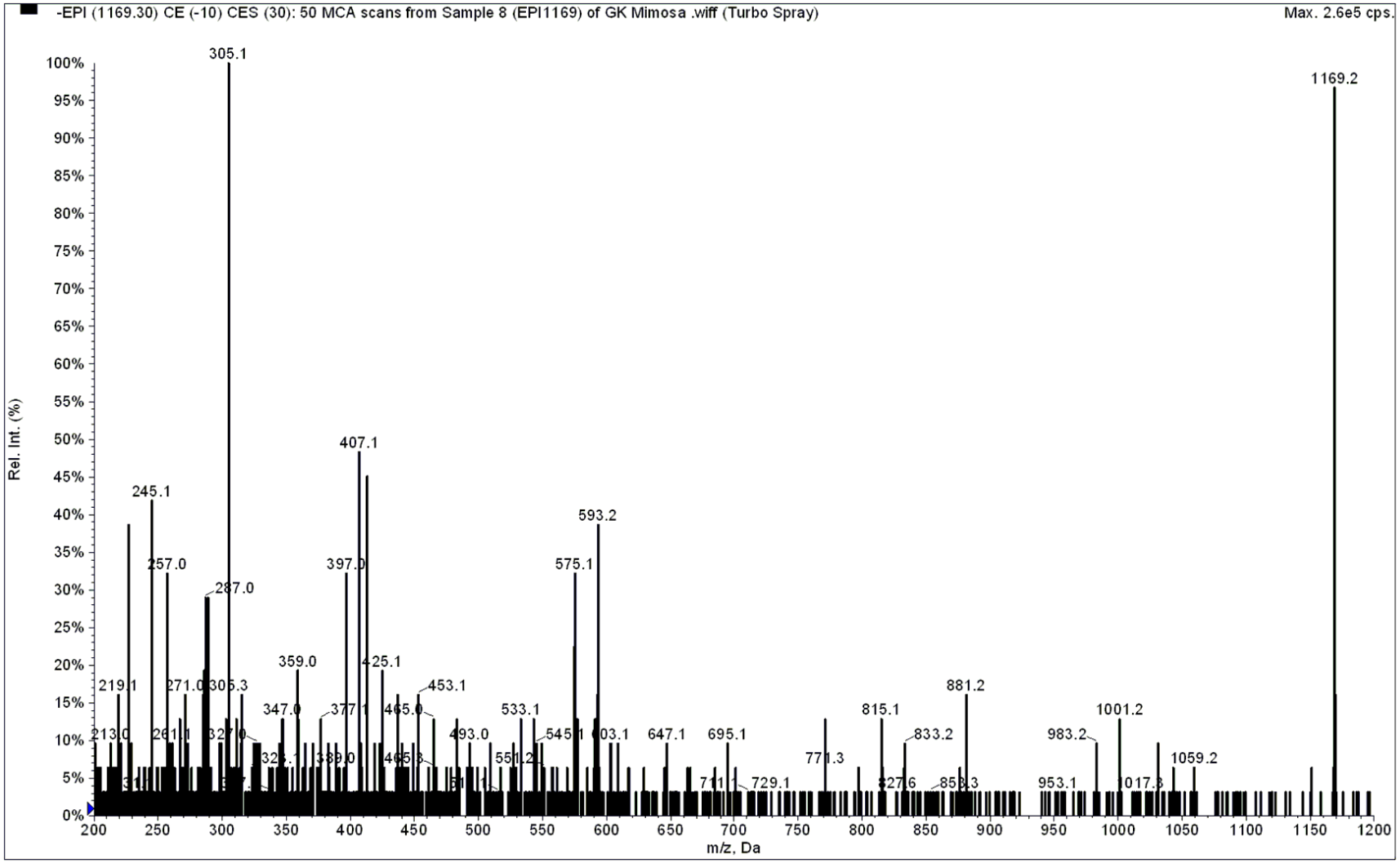


Figure 12c: MS^2 of the *m/z* 1169 tetramer comprising a gallocatechin starter unit and three robinetinidol extender units

Since catechin-robinetinidol and gallocatechin-fisetinidol moieties have the same *m/z* value, the composition of the three remaining tetramers, *i.e. m/z* 1121 (10%), 1137 (40%), and 1155 (35%) cannot be unambiguously assigned without MS^2. The *ca.* 1:1 ratio of the *m/z* 305 (gallocatechin) and 289 (catechin) ions in the *m/z* 1153 fragmentation spectrum (Figure 12d) indicates an almost equal proportion of robinetinidol-catechin-robinetinidol-robinetinidol and robinetinidol-gallocatechin-robinetinidol-fisetinidol in this tetramer. This ratio is about 1:2 in the *m/z* 1137 tetramer (Figure 12e) indicating that fisetinidol-catechin-robinetinidol-fisetinidol is more common than fisetinidol-gallocatechin-fisetinidol-fisetinidol. Our equipment was not sensitive enough to analyse the small *m/z* 1121 fragment with MS^2.

Salient in the MS^2 of tetramers is the absence or low intensity of trimeric ions originating *via* loss of a single monomer fragment. This suggests that the loss of two extender units is a favoured fragmentation pattern, i.e., loss of a fisetinidol-fisetinidol, fisetinidol-robinetinidol, or robinetinidol-robinetinidol moiety and that an interflavanyl bond to a starter unit is weaker than an interflavanyl bond between two extender units. This corroborates the observation that a 5-oxy group, present in the starter units and absent in the extender units, weakens interflavanyl bonds. The expected *m/z* 545, 561, and 577 fragments are, however, not observed in our MS^2 spectra. An *m/z* 545 ion corresponding with a fisetinidol-fisetinidol moiety is visible in the MS of the extract (Figure 7a, Table 1).

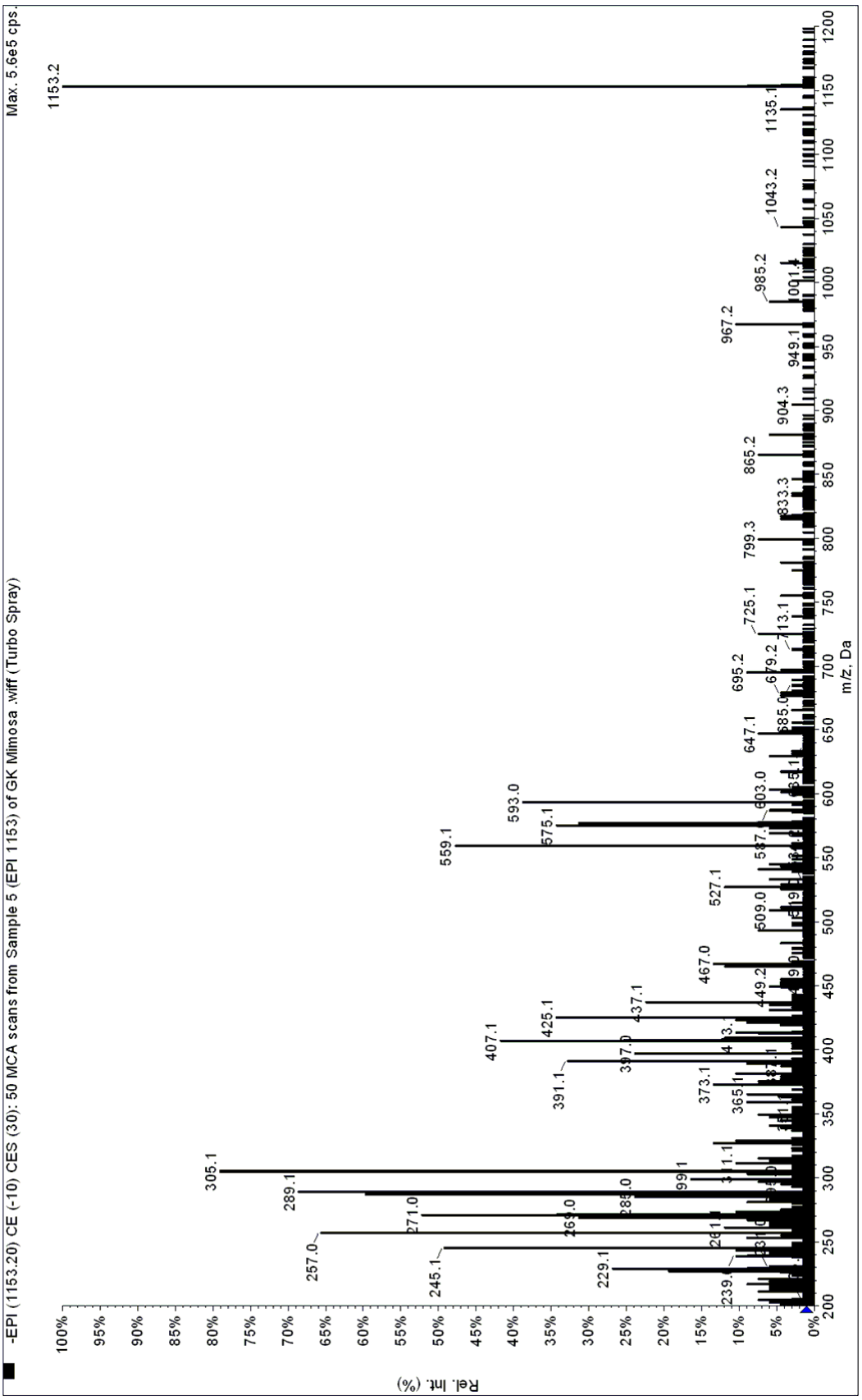


Figure 12d: MS^2 of the *m/z* 1153 tetramer

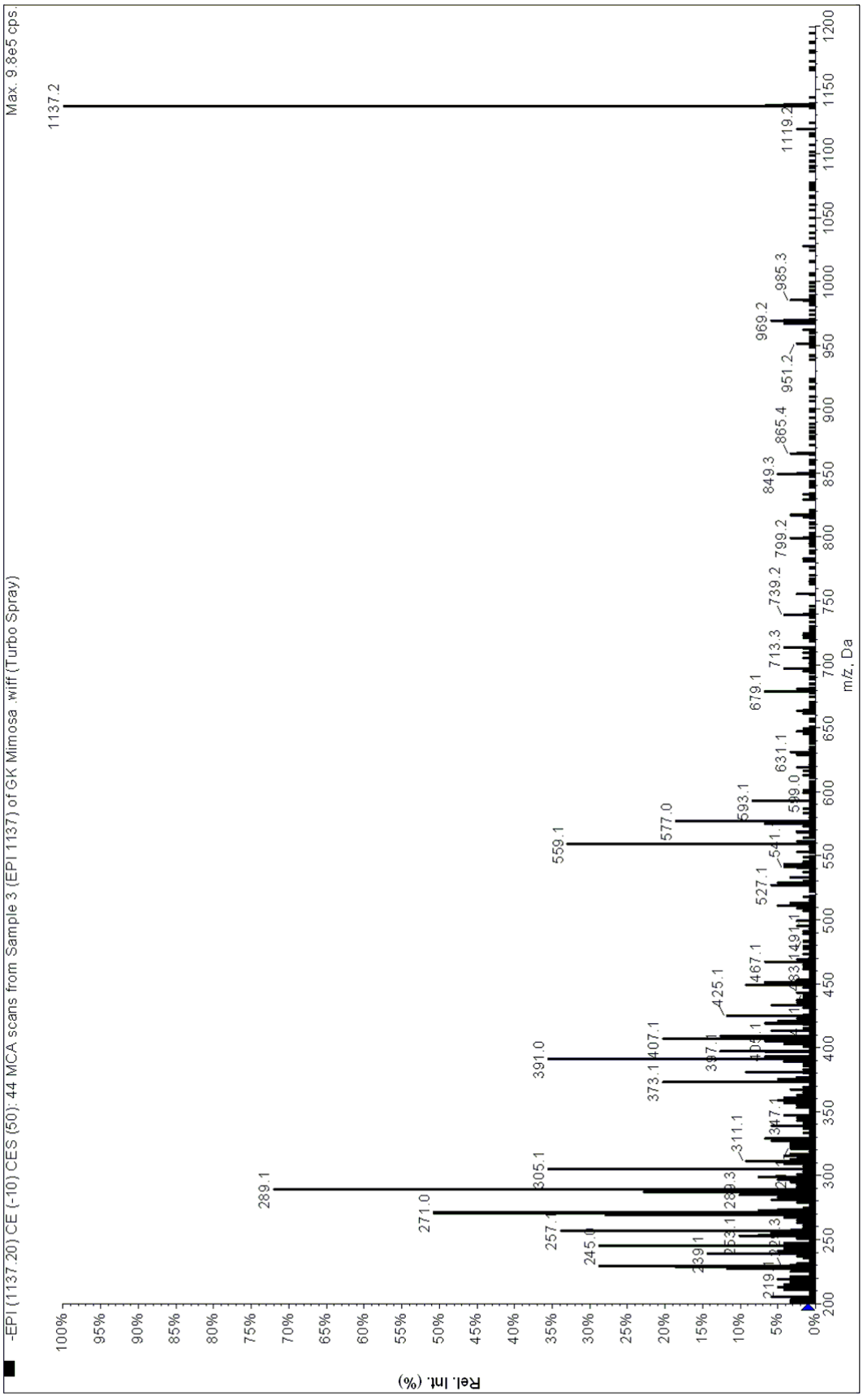
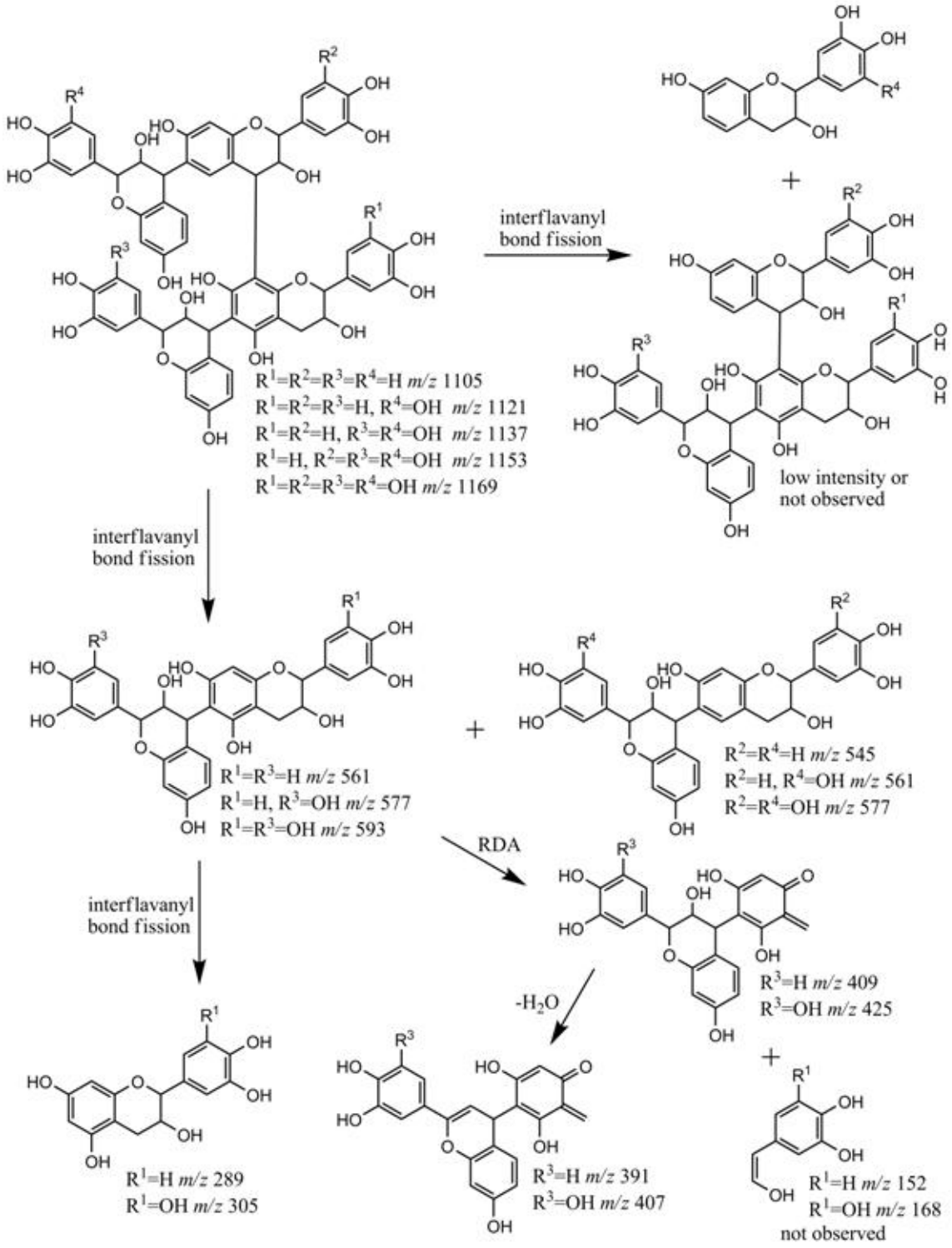


Figure 12e. MS^2 of the *m/z* 1137 tetramer



Scheme 4: Structures and *m/z* values of tetramer fragment ions

The highest MW singly charged oligomers observed in our ESI spectra are a faint cluster around *m/z* 1441, representing pentamers. This contrasts with a faint cluster of octamers

identified by Pasch with MALDI-TOF. However, we observe a cluster of doubly charged PACs (8 Da apart, z = 2) at *m/z* 1554 to 1612 (Figure 13) that corresponds to undecamers. Wattle PACs thus undoubtedly contain polymers that are not observed in MS or phytochemical observations due to low concentrations. These probably have a negligible effect on the industrial properties of wattle extract.

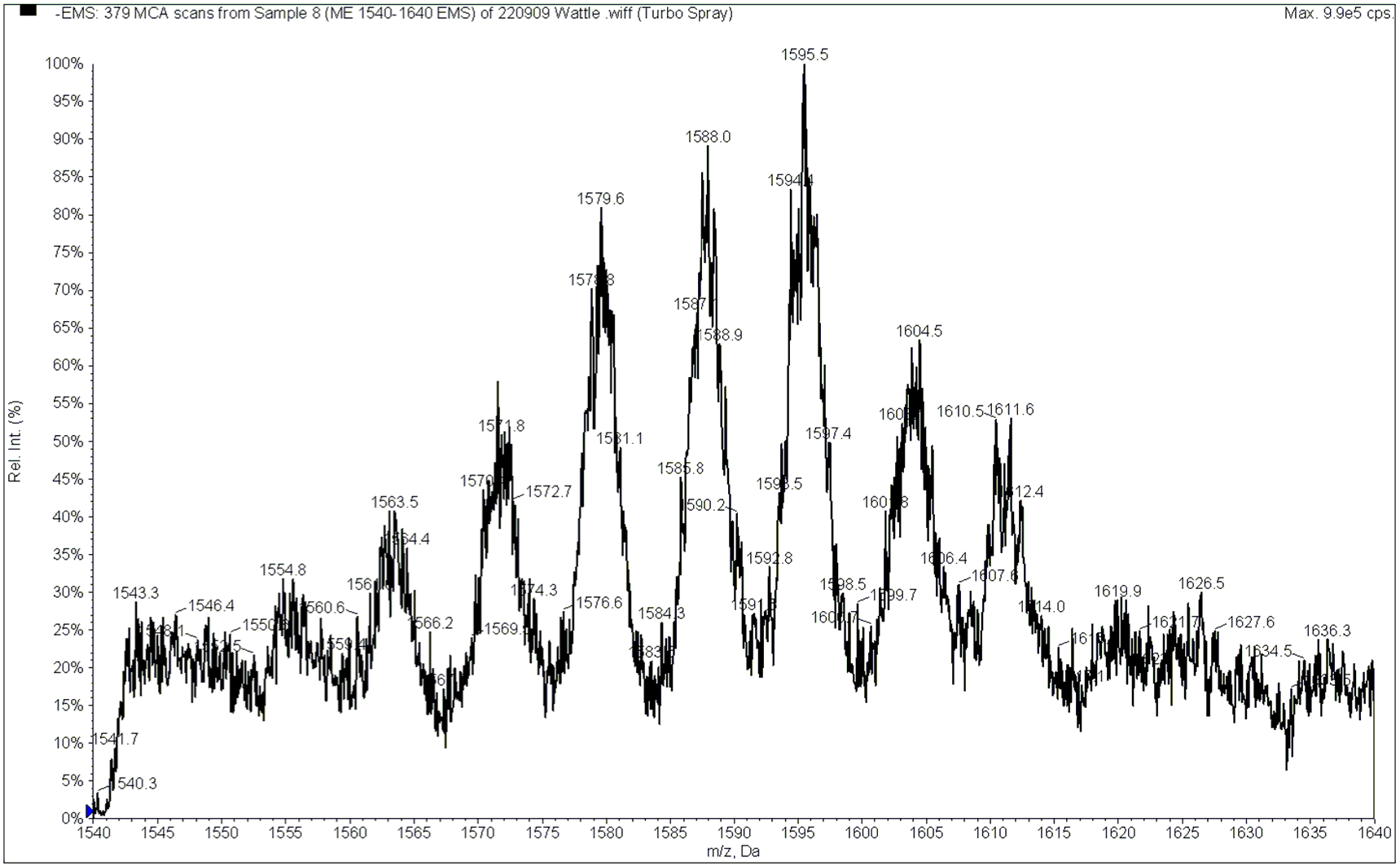


Figure 13: Doubly charged ions indicating the presence of undecamer (eleven) flavan-3-ol units

A calculation of the number average degree of polymerisation (aDP) of wattle extract PACs based on the prevalence of monomers, dimers, trimers, and tetramers (Table 1) gave a value of 2.5. This is lower than the values of 4.3, 4.9, 5.4, and 4.7 determined with Ray ebulliometry (Roux and Evelyn, 1960), ^{13}C NMR (Thompson and Pizzi, 1995), MALDI-TOF (Pasch *et al*., 2001), and size exclusion chromatography (Fechtal and Riedl,1993), respectively, and suggests that the relative prevalence of higher oligomers is underestimated by ESI, due to mass discrimination. Mouls and co-workers (2011) reported that a PAC extract with an aDP of 6.7 determined by thiolysis gave an aDP value of 4.9 with ESI. Adjusting our ESI value by this difference of 1.8 gave an aDP of 4.3 for wattle extract PACs.

4.3. Conclusion

Phytochemistry and synthetic organic chemistry perspectives were combined with an ESI-MS and precursor ion and fragmentation analytical investigation to probe the chemical composition of the PACs in commercial wattle extract. The starter unit is either catechin or gallocatechin and the extender units, fisetinidol or robinetinidol. The second extender unit is always linked to the starter unit to give angular trimers. At the tetrameric level, the reactive positions on the phloroglucinol A-ring of the catechin or gallocatechin starter units are occupied. Tetramer formation is thus only possible *via* reaction of a relatively unreactive resorcinol A-ring of one of the grafted extender units at C-4 of a fourth extender unit. This probably explains the predominance of dimers and trimers and the virtual absence of higher oligomers. This predominance of trimers is essential for leather tanning properties as high MW polymers would not be able to penetrate leather. The absence of 5-oxy catechin or gallocatechin extender units that would introduce weak links in the oligomer chain explains the relative stability of wattle extract during acid hydrolysis, the durability of products like leather and adhesives derived from wattle PACs, and the much simpler fragmentation spectrum below 289, compared to other PACs. In contrast with commercial quebracho heartwood extract, where the *m/z* 289 fragment is always catechin (Venter *et al*., 2012a), this fragment represents a mixture of catechin and robinetinidol in wattle bark extract.

The aDP of 2.5, calculated from the intensity of MS ions, suggests that the prevalence of higher oligomers is underestimated by ESI. Adjusting this value by 1.8, in accordance with Mouls's *et al*. (2011) conclusions on ESI and thiolysis derived values, gave an aDP of 4.3. The adjusted value agrees well with published aDPs of 4.3, 4.9, 5.4, and 4.7 determined by Ray ebulliometry (Roux and Evelyn, 1960), ^{13}C NMR (Thompson and Pizzi, 1995), MALDI-TOF (Pasch *et al*., 2001), and size exclusion chromatography (Fechtal and Riedl, 1993).

The higher oxygenated nature of bark PACs like commercial wattle extract compared to heartwood PACs like commercial quebracho extract presumably reflects the higher protective (enhanced complexation with digestive and other enzymes associated with herbivores, insects and microbial attackers) first line of defense properties required by bark, compared to heartwood.

4.4. Experimental

Wattle bark extract from *A.mearnsii* was supplied by Mimosa Extract Company (Pty) Ltd, 24 van Eck Place, Pietermaritzburg, 3201, South Africa.

HPLC grade (≥99.9 % purity) methanol and water were purchased from *Merck*. An API 2000 triple-quadrupole mass spectrometer (Applied Biosystems/MDS Sciex), coupled with an electrospray ionisation (ESI) source, was used. Biosystems/MDS SCIEX Analyst software version 1.4.2 was used for data acquisition.

The ESI interface was operated in the negative ionisation mode. The ionspray voltage (IS) was set at –4500 V, the declustering potential was set to –30.0 V, and the curtain gas (N_2) was set to 30 (arbitrary units). The nebulizer gas (N_2) and auxiliary gas (N_2) were set to 30 and 75 psi, respectively, with the heater temperature at 400 ^0C.

A QTRAP 3200 triple-quadrupole mass spectrometer (Applied Biosystems/MDS Sciex), coupled with an electrospray ionisation (ESI) source, was used for the acquisition of product ions. The ionspray voltage (IS) was set at –4500 V, the curtain gas was set to 15 (arbitrary units), and the declustering potential was set to –126.0 V. The nebulizer gas (GAS 1) and auxiliary gas (GAS 2) were set to 20 and 10 psi respectively with the heater temperature at 300 ^{0}C. The collision energy was set to –10 eV.

4.5. References

Akin, D. Forage cell wall degradation and ρ-coumaric, ferulic, and sinapic acids. *Agron. J.*, **1982**, *74*, 424-428.

Balas, L.; Vercauteren, J. Extensive high-resolution reverse 2D NMR analysis for the structural elucidation of procyanidin oligomers. *Magn. Reson. Chem.*, **1994**, *32*, 386-393.

Beltran-Heredia, J.; Sanchez-Martin, J. Removing heavy metals from polluted surface water with tannin-based flocculant agent. *J. Hazardous Mat.*, **2008**, *165*, 1215-1218.

Beltran-Heredia, J.; Sanchez-Martin, J. Municipal wastewater treatment by modified tannin flocculent agent, *Desalination*, **2009,** *249*, 353-358.

Beltran-Heredia, J.; Sanchez-Martin, J.; Frutos-Blanco, G. *Schinopsis balansae* tannin-based flocculant in removing dodecyl benzene sulfonate. *Sep. Purif. Technol.*, **2009**, *67*, 295-303.

Botha, J. J.; Ferreira, D.; Roux, D. G. Condensed tannins: Direct synthesis, structure, and absolute configuration of four biflavonoids from black wattle bark ('Mimosa') extract. *J. Chem. Soc. Chem. Comm.*, **1978**, 700-702.

Botha, J. J.; Ferreira, D.; Roux, D. G. Synthesis of condensed tannins. Part 4. A direct biomimetic approach to [4,6]- and [4,8]-biflavonoids. *J. Chem. Soc., Perkin I,* **1981**, 1235-1245.

Cronjé, A.; Steynberg, J. P.; Brandt, E. V.; Young, D. A.; Ferreira, D. Oligomeric flavanoids. Part 16. Novel prorobinetinidins and the first A-type proanthocyanidin with a 5-deoxy A and a 3, 4-*cis* C-ring from the maiden investigation of commercial wattle bark extract. *J. Chem. Soc., Perkin I,* **1993**, 2467-2477.

Drewes, S. E.; Roux, D. G. Condensed tannins. 15. Interrelationships of flavonoid components in wattle-bark extract. *Biochem. J.*, **1963**, *87*, 167-172.

Drewes, S. E.; Roux, D. G.; Saayman, H. M. Some stereochemically identical biflavanols from the bark tannins of *Acacia mearnsii*. *J. Chem. Soc. (C),* **1967**, 1302-1308.

Esatbeyoglu, T.; Jaschok-Kentner, B.; Wray, V.; Winterhalter, P. Structure elucidation of procyanidin oligomers by low-temperature ^{1}H NMR spectroscopy. *J. Agric. Food Chem.*, **2011**, *59*, 62-69.

Evelyn, S. R. The molecular weight of black wattle tannin. Part 1. The application and standardization of apparatus for accurate measurement. *J. Soc. Leather Trades' Chem.*, **1954**, *38*, 142.

Fechtal, M.; Riedl, B. Use of eucalyptus and *Acacia mollissima* bark extract-formaldehyde adhesives in particleboard manufacture. *Holzforschung*, **1993**, *47*, 349.

Foo, L. Y.; Porter, L. J. Prodelphinidin polymers: definition of structural units. *J. Chem. Soc. Perkin Trans. I*, **1978**, 1186–1190.

Haslam, E. Polyphenol–protein interactions. *Biochem. J.*, **1974**, *139*, 285–288.

Haslam, E. Twenty-second Procter memorial lecture: Vegetable tannins - renaissance and reappraisal. *J. Soc. Leather Technol. Chem.*, **1988**, *72*, 45.

Haslam, E. Vegetable tannage: where do the tannins go? *J. Soc. Leather Technol. Chem.* **1997**, *82*, 45.

Haslam, E. Polyphenols, Collagen and Leather. In *Practical Polyphenolics. From Structure to Molecular Recognition and Physiological Action.* Cambridge University Press: Cambridge, England, **2005**, pp. 378.

Herrick, F. W. Chemistry and utilization of western hemlock bark extractives. *J. Agric. Food. Chem.*, **1980**, *28*, 228-237.

Hundt, H. K. L.; Roux, D. G. Condensed tannins: determination of the point of linkage in 'terminal' (+)-catechin units and degradative bromination of 4-flavanylflavan-3, 4-diols. *J. Chem. Soc. Chem. Comm.*, **1978**, 696-698.

Hundt, H. K. L.; Roux, D. G. Synthesis of condensed tannins. Part 3. Chemical shifts for determining the 6- and 8-bonding positions of 'terminal ' (+)-catechin units, *J. Chem. Soc., Perkin I*, **1981**, 1227-1234.

Joslyn, M. A.; Goldstein, L. L. Astringency of fruits and fruit products in relation to phenolic content. *Adv. Food Res***. 1964**, *13*, 179-317.

Kennedy, J. A.; Taylor, A. W. Analysis of proanthocyanidins by high performance gel permeation chromatography. *J. Chromatogr. A*, **2003**, *995*, 99-107.

Koupai-Abyazani, M. R.; Muir, A. D.; Bohm, B. A.; Towers, G. H. N.; Gruber, M. Y. The proanthocyanidin polymers in some species of *Onobrychis*. *Phytochemistry*, **1993**. *34*, 113-117.

Mouls, L.; Mazauric, J. P.; Sommerer, N.; Fulcrand, H.; Mazerolles, G. Comprehensive study of condensed tannins by ESI mass spectrometry: average degree of polymerisation and polymer distribution determination from mass spectra. *Anal. Bioanal. Chem.*, **2011**, *400(2)*, 613-623.

Parker, M.; Smith, P. A.; Birse, M., Francis**,** I. L.; Kwiatkowski, M. J.; Lattey, K. A.; Liebich, B.; Herderich, M. J. The effect of pre- and post-ferment additions of grape derived tannin on Shiraz wine sensory properties and phenolic composition. *Aust. J. Grape Wine Res*., **2007**, *13*, 30–37.

Pasch, H.; Pizzi, A.; Rode, K. MALDI-TOF mass spectrometry of polyflavonoid tannins. *Polymer*, **2001**, *42*, 7531-7539.

Pezet, R.; Peret, C.; Tabbachi, R. Analysis of oligomeric and polymeric tannins of grape berries by liquid chromatography/electrospray ionization multiple-stage tandem mass spectrometry. *Eur. J. Mass Spectrom*., **2011**, *7*, 419-426.

Pizzi, A. Hot-setting tannin-urea-formaldehyde exterior wood adhesives. *Adhes. Age*, **1977**, *20*, 27–29.

Pizzi, A. Condensed tannins for adhesives. *Ind. Eng. Chem. Res. Dev*. **1982**, *21*, 359 – 369.

Reed, J. D.; Krueger, C. G.; Vestling, M. M. MALDI-TOF mass spectrometry of oligomeric food polyphenols. *Phytochemistry*, **2005**, *66*, 2248-2263.

Rhodes, D. F.; Cates, R. G. Toward a general theory of plant antiherbivores chemistry. *Recent Adv. Phytochem*., **1976**, *10*, 168-213.

Rigaud, J.; Perez-Ilzarbe, J.; Ricardo da Silva, J. M.; Cheynier, V. Micro method for the identification of proanthocyanidins using thiolysis monitored by high-performance liquid chromatography. *J. Chromatogr. A*, **1991**, *540*, 401-405.

Roux, D. G. Determination of gums in black wattle extract. *J. Soc. Leather Trades' Chem.*, **1953**, *37*, 374.

Roux, D. G. The fractionation and paper chromatography of black wattle tannins or polyphenols. *J. Soc. Leather Trades' Chem.*, **1953**, *37*, 229.

Roux, D. G. Ultraviolet photometric methods of tannin estimation in relation to wattle extract utilization. *J. Am. Leather Chemists' Assoc.*, **1957**, *52***,** 319-329.

Roux, D. G., Evelyn, S.R. The distribution and deposition of tannins in the heartwoods of *Acacia mollissima* and *Schinopsis spp. Biochem. J.*, **1960**, *76*, 17-23.

Roux, D. G.; Maihs, E. A. Isolation and estimation of (-)-7:3′:4′:5′-tetrahydroxyflavan-3-ol, (+)-catechin and (+)-gallocatechin from black-wattle-bark extract. *Biochem. J.* **1960**, *74*, 44 – 48.

Roux, D. G.; Paulus, E. Polymeric leuco-fisetinidin tannins from the heartwood of *Acacia mearnsii*. *Biochem. J.* **1962**, *82*, 320-324.

Roux, D. G.; Ferreira, D.; Hundt, H. K. L.; Malan, E. Structure, stereochemistry and reactivity of natural condensed tannins as basis for their extended industrial exploitation. *J. Appl. Polym. Sci.*, Polymer Symposium, **1975**, *28*, 335-353.

Roux, D. G. Introduction. In Plant polyphenols, Hemingway, R. W., Laks, P.E. (Eds), Plenum Press, New York, **1992**, pp. 7-39.

Saayman, H. M.; Roux, D. G. The origin of tannins and flavonoids in black-wattle barks and heartwoods, and their associated "non-tannin" components. *Biochem. J.* **1965**, *97*, 794 – 801.

Shen, Z.; Haslam, E.; Falshaw, C. P.; Begley, M. J. Procyanidins and polyphenols of *Larix gmelini* bark. *Phytochemistry*, **1986**, *25*, 2629-2635.

Steynberg, J. P.; Young, D. A.; Burger, J. F. W.; Ferreira, D.; Roux, D. G. Phlobatannins via facile ring-isomerizations of profisetinidin and prorobinetinidin condensed tannin units. *J. Chem. Soc., Chem. Commun*., **1986**, 1013.

Thompson, D.; Pizzi, A. Simple ^{13}C-NMR methods for quantitative determinations of polyflavonoid tannin characteristics. *J. Appl. Polym. Sci*., **1995**, *55*, 107.

Thompson, R. S.; Jacques, D.; Haslam, E.; Tanner, R. N. J. Plant proanthocyanidins. Part I. Introduction; the isolation, structure, and distribution in nature of plant procyanidins. *J. Chem. Soc., Perkin Trans. 1,* **1972**, 1387-1399.

Venter, P. B.; Sisa, M.; Van der Merwe, M. J.; Bonnet, S. L.; Van der Westhuizen, J. H. Analysis of commercial proanthocyanidins. Part 1: The chemical composition of quebracho (*Schinopsis lorentzii* and *Schinopsis balansae*) heartwood extract. *Phytochemistry*, **2012a**, *73*, 95–105.

Viviers, P. M.; Botha, J. J.; Ferreira, D.; Roux, D. G. Synthesis of condensed tannins. Part 7. Angular [4,6:4,8]-prorobinetinidin triflavanoids from black wattle ("Mimosa') bark extract. *J. Chem. Soc., Perkin I*, **1983**, 17 – 22.

Young, D. A.; Ferreira, D.; Roux, D. G. Synthesis of condensed tannins. Part 15. Structure of natural 'angular' profisetinidin tetraflavanoids: asymmetric induction during oligomeric synthesis. *J. Chem. Soc., Perkin Trans 1*, **1985**, 2529-25