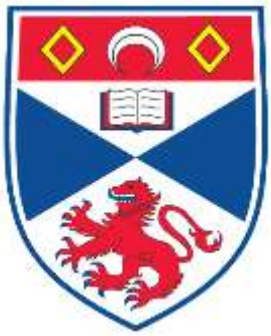
STUDIES ON THE PRESERVATION OF FLOWERS

Jennifer Elliott

A Thesis Submitted for the Degree of PhD
at the
University of St. Andrews



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Studies on the Preservation of Flowers

By Jennifer Elliott

A thesis submitted for the degree of

Doctor of Philosophy

in the Faculty of Science at

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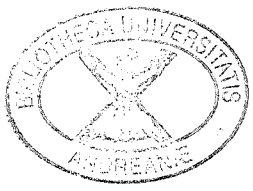

October 2001

Abstract

A known method for the preservation of green foliage was adapted in order to preserve floral tissues, retaining the colour and texture, thereby providing a method suitable for the preservation of whole flowers.

Initially, the effects of the existing foliage preservation process on floral tissues were studied and the resulting problems of limp sticky petals and colour loss were identified. Subsequently, with a knowledge of basic plant anatomy and of the properties of the main floral pigments, the anthocyanins, a series of experiments on petals and whole flowers were carried out in an attempt to rectify these problems and to incorporate the remedies into a method for preserving whole flowers.

The problem of improving the texture and firmness of flower heads was tackled by investigating the effects of adding bulking or setting ingredients to the process fluid and establishing their optimum concentrations. In the case of flower colour, the addition of acid was required in order to maintain the bright anthocyanin colours and a range of acids was investigated. Furthermore, since it is known that in nature the anthocyanin pigments are stabilised by metal ions and copigments, the use of these agents in the preservation process was also considered.

This empirical work was then validated by confirming the identity of the main pigments involved and by studying various aspects of the new

preservation process. Factors examined included acid concentration, temperature, solvent composition and the addition of metal ions and copigments to solutions of petal extracts containing anthocyanin pigments. Physical changes resulting from processing, including process fluid content and the moisture absorption properties of processed petals were also measured.

Finally, the application of a selection of coating materials was assessed in an attempt to increase the life span of the processed flowers by providing extra protection against environmental stresses.

Declaration

I, Jennifer Elliott, hereby certify that this thesis, which is approximately 40,000 words in length, has been written by me, that is the record of work carried out by me and that it has not been submitted in any previous application for a higher degree.

date 29.10.01.. signature of candidate [redacted]

Declaration

I was admitted as a research student in March 1998 and as a candidate for the degree of the degree of Doctor of Philosophy in June 1999; the higher study for which this is a record was carried out in the University of St.Andrews between 1998 and 2001.

date 29:10:01 signature of candidate [redacted]

Declaration

I hereby certify that the candidate has fulfilled the conditions of the Resolution and Regulations appropriate for the degree of Doctor of Philosophy in the University of St.Andrews and that the candidate is qualified to submit this thesis in application for that degree.

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To my family

(including Lizzie)

and friends.

Contents

Abbreviations

General

LC = liquid chromatography

GLC = gas liquid chromatography

HPLC = high performance liquid chromatography

PC = paper chromatography

TLC = thin layer chromatography

HPTLC = high performance thin layer chromatography

UV = ultraviolet

VIS = visible

NMR = nuclear magnetic resonance

MS = mass spectrometry

MALDI = matrix-assisted laser desorption/ionization

TOF = time-of-flight

CD = circular dichroism

RR = resonance Raman

PDA = photodiode array

SEM = scanning electron microscopy

R_f = mobility relative to front

m/z = mass to charge ratio

ODS = octadecylsilane

SEPAL = Scottish Everlastings Patenting and Licensing

Polidene = Polidene 33-004 (vinylidene chloride/acrylate copolymer emulsion)

Abbreviations (continued)

Chemicals

MeOH = methanol

EtOH = ethanol

n-BuOH = *n*-butanol

HCO_2H = formic acid

HOAc = glacial acetic acid

EtOAc = ethyl acetate

PTSA = *p*-toluenesulphonic acid

DHAP = 2,6-dihydroxyacetophenone

3AQ = 3-aminoquinoline

PEG = polyethylene glycol

Solvent systems

Forestal = conc.HCl-HOAc-H_2O (3:30:10)

Formic = conc.HCl-HCO_2H-H_2O (2:5:3)

EAW = EtOH-HOAc-H_2O (10:1:9)

MAW = MeOH-HOAc-H_2O (10:1:9)

BAW = n-BuOH-HOAc-H_2O (4:1:5)

BuHCl = n-BuOH-2M HCl (1:1, top layer)

CHAPTER 1

Chapter 1

Introduction

1.1 Background

This research project arose as a result of a Teaching Company Scheme between the University of St. Andrews and the Fife based company Scottish Everlastings Patenting and Licensing Ltd, otherwise known as SEPAL.

SEPAL was founded in 1997, having emerged from its partner company Scottish Everlastings, which had been in existence for just over 10 years and had been involved in the preservation of plant material by traditional drying techniques. Originally the scope of the operation had ranged from growing, through to packaging and wholesale, but latterly, as the demand for dried floral arrangements grew and new suppliers entered the market, it became more economical to import the dried plant material from other countries where labour costs were low and the growing conditions favourable. Having reduced operations considerably, whilst still maintaining profits, the company then moved on, to keep one step ahead of the market, into the area of innovation by attempting to develop new techniques for the preservation of plant material. Existing techniques had been based largely on either drying (e.g. by hanging, pressing or bedding in silica gel) or by treating the plant material chemically, either systemically (by capillary action), or by coating. Systemic methods involved either adding nutrients via the stem in an attempt to extend the life of the cut plants, or they involved replacing the plant fluids with substances less likely to evaporate than water (e.g. glycerol, diols or polyols) thereby

maintaining the plant body. Coating with materials such as lacquers and resins was also an attempt to maintain body by preventing water loss.

All of these existing methods, however, had the disadvantage of being lengthy and often prone to producing undesirable colour and textural changes. In the case of dried material mould growth was also a problem and methods to extend the life of fresh plants (i.e. by either adding nutrients or coating to prevent water loss) were only capable of prolonging life for up to about a week. Furthermore, the systemic methods were very much dependent on the condition of the plant and the time lapse since cutting, since it appeared that only freshly cut, healthy plants were capable of successfully drawing viscous fluids up through the stem. Consequently, this time restraint led to practical difficulties, particularly if fresh plant material had to be imported and it also meant that post-harvesting conditions had to be tightly controlled. [1]

SEPAL, bearing these difficulties in mind, developed a new method which took advantage of the positive attributes of replacing the plant fluids with polyhydric alcohols (i.e long lasting body and more realistic appearance than dried plants) but overcame the problems associated with the systemic method of introducing these preservatives. This new method (which will be discussed further in Chapter 4) involved submerging the entire plant in the preservation fluid at elevated temperatures and allowing the fluid to enter the plant via the epidermal tissues. The advantages of this method over the existing methods was that it was quick, easy to perform and could tolerate considerable delays from harvesting to processing (e.g. in the case of the Noble Fir up to eight weeks).

Having developed this method for the preservation of a wide range of green foliage, Scottish Everlastings then patented the technology [2] and sold licenses to leading

companies in the preserved flower industry in America and Holland, hence the birth of SEPAL.

SEPAL then intended to expand the range of patents on offer in order to meet market demands, which were very much in favour of preserved flowers, with a potential retail market size in Europe alone in excess of £25 million per year. In particular, SEPAL hoped to serve the potentially lucrative red rose market.

1.2 Aim

Unfortunately, attempts to utilise the 'foliage technology' in order to preserve whole flowers were unsuccessful in that the flower heads lost all colour and became limp and sticky. It was these problems that this research project aimed to address, with the ultimate aim of developing a theoretical, full-scale, commercially viable process for the preservation of whole flowers.

Given the commercial nature of the project several factors had to have a considerable bearing on the direction of the research. In the first instance, time was limited since the market demands were not static and competition was always a threat. Secondly cost was of vital importance in almost every aspect of the work, from the more obvious chemical costs to the investment in equipment and the price of labour. This meant that any process developed was required to be as simple as possible in order to avoid lengthy processing times and the need to employ a large, highly skilled, expensive workforce. In addition, the issue of safety had to be considered. Not only safety in the workplace, which in most developed countries is a legal requirement (e.g controlled in the U.K. by The Health and Safety at Work Act and by The Occupational Safety and Health Administration in the U.S.A) but also the safety of

the ultimate consumer, which meant that the end product had to comply with the relevant consumer safety legislation. With increasing concerns world wide over environmental issues factors such as waste disposal and chemical emissions also had to be borne in mind when planning the operation.

1.3 Thesis

This thesis commences with a brief description of the anatomical structure of flowering plants, concentrating on the histology of leaves and petals, before moving on to discuss the structures and properties of floral pigments and their contribution to the wide range of observed flower colours. With this insight into the possible factors affecting flower structure and pigment stability a description is then given of the application of this information to solving the problems in hand i.e. preventing colour loss in petals and improving overall firmness of flower heads. Initially the investigations concerned the effects of various speculative treatments aimed at improving the appearance of whole petals and flowers. This approach was deemed necessary since the company, due to time constraints, favoured an all-encompassing approach to solving the problems. Once the foundations for the basic process had been laid down, however, more quantitative work concerning the behaviour of the pigments *in vitro* not only confirmed the previous qualitative work but also allowed refinements to the process to be made. One other area, which had not originally been considered necessary, was the search for a suitable coating material as it transpired from reports from the company pioneering the foliage process in America, that the coating material recommended was not performing satisfactorily.

Finally, in order to assess the durability of the preserved flowers in a typical consumer environment, a trial run in a local restaurant was set up and constructive criticism and comments reported.

1.4 References

1. L. Carstairs and I. Carstairs (Scottish Everlastings Ltd), personal communication.
2. British Patent, *Methods of Preserving Plant Material* (GB228 7637A).

CHAPTER 2

Chapter 2

The anatomy of flower bearing plants

2.1 Introduction

Plant anatomy deals with the form, variability and structure of the tissues comprising the plant body. It includes the study of minute structures and thus includes histology. This chapter briefly presents the general structure of the higher plants before moving on to describe the cell and the cell components of relevance to the preservation process. The remaining sections deal with structure of the leaf and the flower.

2.2 General structure of the plant

The plant, in its botanical sense, includes every form of vegetable life from pond-weed to trees. The angiosperms, or the flower-bearing seed plants, represent the most recently evolved group and form the main part of present day cultivated vegetation. In the more perfect angiosperms the main organs (portions of the plant which have a distinct part or function) are the root, the stem, the leaves, the flower and the fruit.[1] The first three of these organs, whose functions are to assist in the growth of the plant, are the organs of vegetation. The flower and the fruit, whose functions are the formation of the seed, are the organs of reproduction. The organs which will be dealt with in more detail are the leaf and the flower but firstly a mention will be given to the basic units from which all organisms are constructed; the cell.

2.3 The cell

2.3.1 General structure

The main components of the plant cell are the cell wall, cytoplasm and nucleus. The cytoplasm includes the endoplasmic reticulum, Gogli apparatus, mitochondria, plastids, microbodies, ribosomes, spherosomes, microtubules, vacuoles and ergastic substances (reserve and waste materials produced by the cell which include tannins and pigments) (Fig. 2.1).

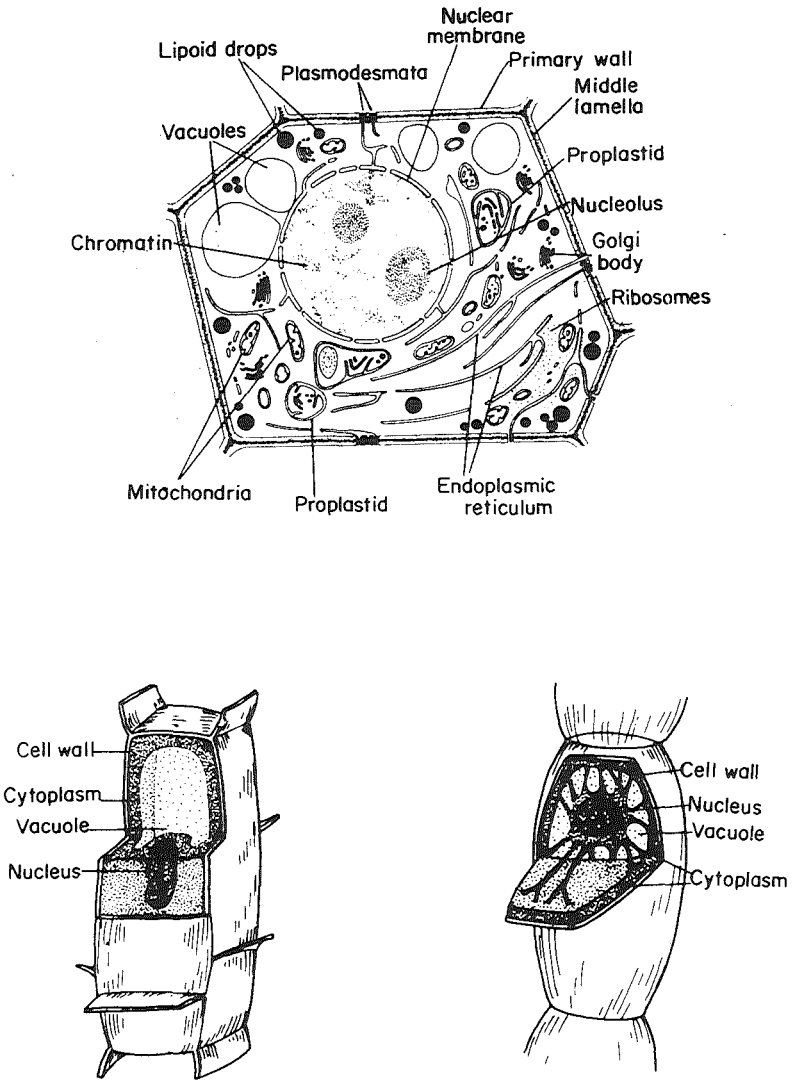


Fig. 2.1 The plant cell.[1]

The cytoplasm is encapsulated by the plasmalemma, which is the boundary membrane adjacent to the cell wall (Fig. 2.2). As plant pigments are normally located in the plastids and in the vacuoles these are the cell components of interest in the study of floral pigments.[1, 2, 3]

The structure and properties of the plasmalemma and the cell wall also have some relevance to the investigation into the effects of the preservation process on the plant material since the process involves the penetration of this barrier by the preservation medium.

2.3.2 Plastids

Plastids are organelles (groups of structures occurring within the cell) unique to plants. They are viscous membrane bound bodies varying in form, size and pigmentation. The principal types of plastids are chloroplasts (plastids in which photosynthesis takes place), chromoplasts (plastids containing pigments other than chlorophyll) and leucoplasts (plastid which store plant products e.g. starch). Chloroplasts are green as a result of the pigment chlorophyll which predominates in them. Chromoplasts are usually yellow, orange or red due to carotenoid pigments. Leucoplasts are non-pigmented plastids usually located in tissues not exposed to light. Leucoplasts of tissues which become exposed to light may develop into chloroplasts eg. in the potato tuber.[1]

2.3.3 Vacuoles

Vacuoles occupy more than 90% of the volume of most mature plant cells. A vacuole is a watery cell compartment surrounded by a membrane and containing a

variety of organic and inorganic substances such as sugars, proteins, organic acids, phosphatides, tannins, flavonoid pigments and calcium oxalate. There may be many minute vacuoles which grow with the cell and eventually fuse to form a large central vacuole. The vacuoles function in regulation of the water and solute content of the cell ie. in osmo-regulation, in storage and in digestion. There is evidence that vacuoles contain digestive enzymes capable of breaking down cytoplasmic components. The amount of enzymes can change during the life of the cell and they may be produced to different extents in different cells. Some vacuoles may, however, completely lack digestive enzymes. [1,3]

2.3.4 Plasmalemma

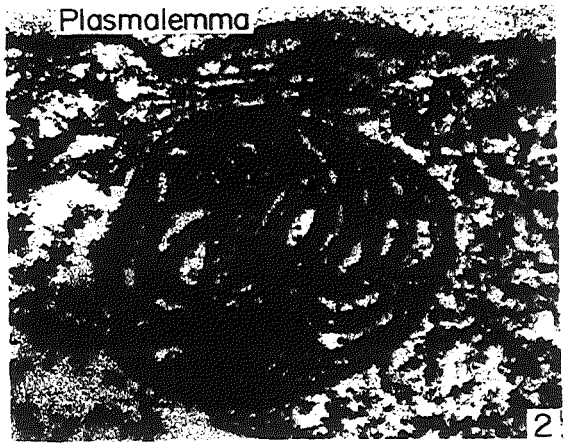


Fig. 2.2 Electron micrograph x 77,000 showing the plasmalemma.[1]

The plasmalemma is the boundary membrane of the cell, which regulates the passage of molecules between the cell and its surroundings. Like most cell membranes it consists of a lipid bilayer traversed by proteins as depicted in the basic fluid mosaic model (Fig. 2.3), with different intracellular membranes having slightly different

compositions. Some of the membrane proteins are involved in the transport of metabolites while others may be enzymes and display catalytic activity.[1, 3]

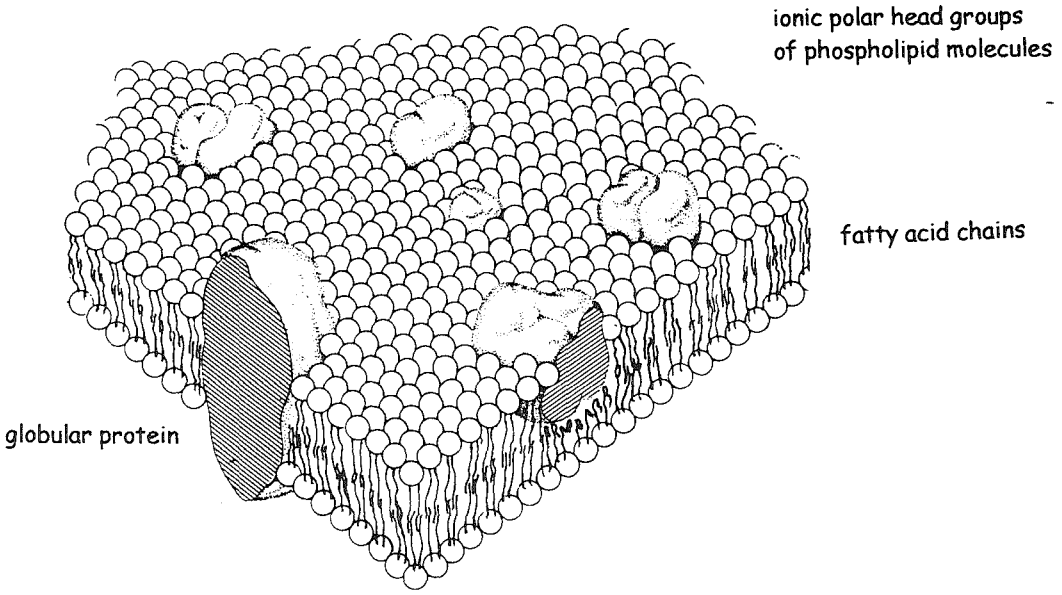


Fig. 2.3 A schematic 3-D drawing of the fluid mosaic model of a cell membrane.[4]

2.3.5 The cell wall

The cell wall is the structure external to the plasmalemma, secreted by the cell and enclosing it. It is typically tough, sometimes rigid, and protective or skeletal in function but with relatively little effect on solute influx or efflux, which are mainly controlled by the plasmalemma. The properties of the cell walls largely determine the shape of the cells and hence the morphology of plant and can depend on the age of the tissue. The cell wall typically consists of microfibrils of cellulose (greater than 40% of the dry mass) embedded in a matrix of other substances, rather like glass-fibre- reinforced plastic (Fig. 2.4).[1, 3, 5]

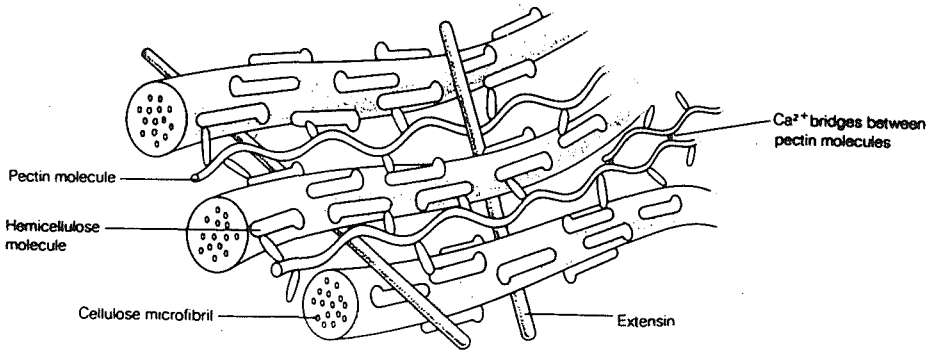


Fig. 2.4 Diagrammatic representation of a cell wall.[3]

The microfibrils are flattened threads, 5-8 nm wide and can be 1-7 micrometres long. Being made of cellulose they are insoluble in water and the ordinary organic solvents and are stable at low pH.[5,6]

The matrix includes polysaccharides such as hemicelluloses and pectins together with proteins and there is some evidence of chemical cross links between calcium ions and the carboxyl groups of adjacent pectin chains and between phenolic acids (ferulic and coumaric acid) and cell wall polysaccharides (Fig. 2.5), all of which serve to strengthen the cell wall.[3,7]

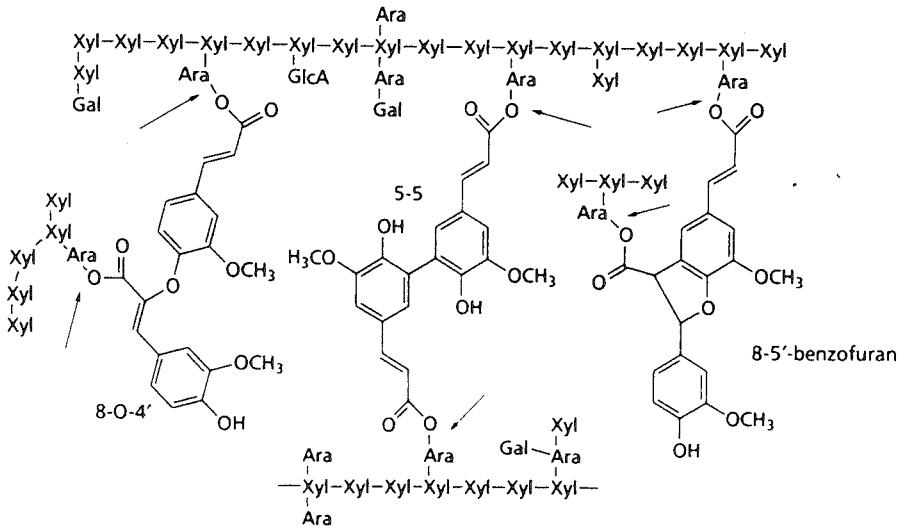


Fig. 2.5 Ferulate dimers linking polysaccharide chains in the plant cell wall.[7]

The wall contains much water and is porous, thus allowing free passage to water and to solutes less than about 4nm in diameter.[5] If, as is nearly always the case, the concentration of solutes in the cell is greater than in the medium, then the wall restrains the tendency of the cell to expand by osmotic uptake of water. Equilibrium occurs when the excess hydrostatic pressure, the turgor pressure, generated by the stretching of the wall, is equal to the difference of osmotic pressure inside and outside of the cell. Cell walls are typically flexible but turgor gives them rigidity and enables non-woody plants to stand erect.

2.4 The leaf

2.4.1 General Structure

The main function of the leaf is the synthesis of organic compounds using light as a source of energy. Histologically the leaf is composed of three types of tissue (groups of cells which carry on specific activities) namely the epidermis, the mesophyll and the vascular tissues (Fig. 2.6).

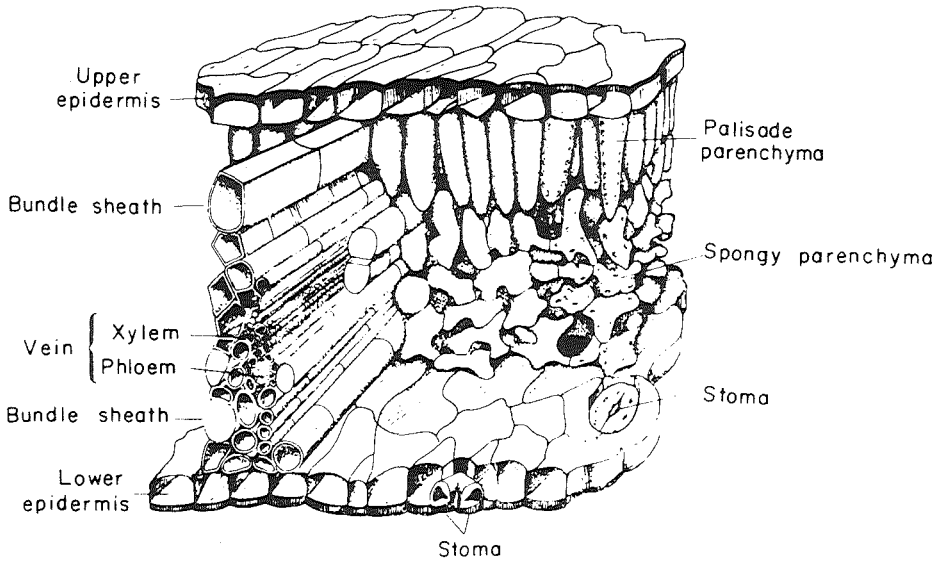


Fig. 2.6 A three-dimensional drawing of a leaf portion.[1]

2.4.2 The epidermis

The epidermis constitutes the outer most layer of cells and in different plants varies in the number of layers, shape, structure, arrangement of stomata, appearance and arrangement of trichomes and occurrence of specialised cells.

A distinction is made between the epidermal tissues of the two surfaces of the leaf; that surface which usually faces upwards is referred to as the adaxial surface and the other surface as the abaxial surface.[1]

The epidermal cells are always closely attached to form a compact layer normally devoid of intercellular spaces and are almost always held together by a layer of pectin which in turn is covered by the cuticle (a layer of fatty substances) and usually a layer of wax (Fig. 2.7). [2, 8]

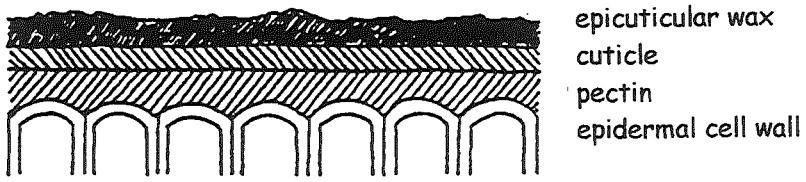


Fig. 2.7 Schematic representation of a transverse section of a plant cuticular membrane.[8]

This water-impermeable barrier is broken by stomata, which allow for gas exchange between the leaf tissues and the atmosphere and are also the main point of water loss through transpiration. Stomata are found in greatest concentration on the abaxial surface in order to reduce this water loss.[9]

2.4.3 The mesophyll

The mesophyll comprises the parenchymatous tissue internal to the epidermis. Two types of parenchyma can be distinguished in the mesophyll: the upper palisade parenchyma consisting of elongated cells and the lower spongy parenchyma consisting of irregularly shaped cells with large intercellular spaces. Of the two, the palisade parenchyma is more densely packed with chloroplasts.[1]

2.4.4 The vascular tissues

The veins seen in the leaf blade contain the vascular tissues, the xylem (with the prime function of water transport) and the phloem (with the main function of transporting metabolites such as sugars). The veins are normally surrounded by parenchyma. The larger veins are surrounded by parenchyma which is poor in chloroplasts and the smaller veins by a layer of tightly packed parenchyma cells (the bundle sheath) which are thin walled and may contain as many chloroplasts as the mesophyll cells.[1, 2, 9]

2.5 The flower

2.5.1 General Structure

The flowers are the reproductive organs of the angiosperms. The flower consists of a group of specialized leaves (the floral organs) namely the sepals, petals, stamens and carpels (Fig. 2.8). The sepals, which are the outermost organs of the flower and are usually green, constitute the calyx. The petals, which are usually coloured, constitute the corolla. These two organs together constitute the perianth. The perianth, or floral

envelope, encloses the stamens and the carpels. Each stamen consists of a stalk, the filament, bearing an anther in which the pollen grains develop. The carpels occur singly or in groups and they comprise the pistil. Basically the carpels consist of the ovary, the style and the stigma.[2,9]

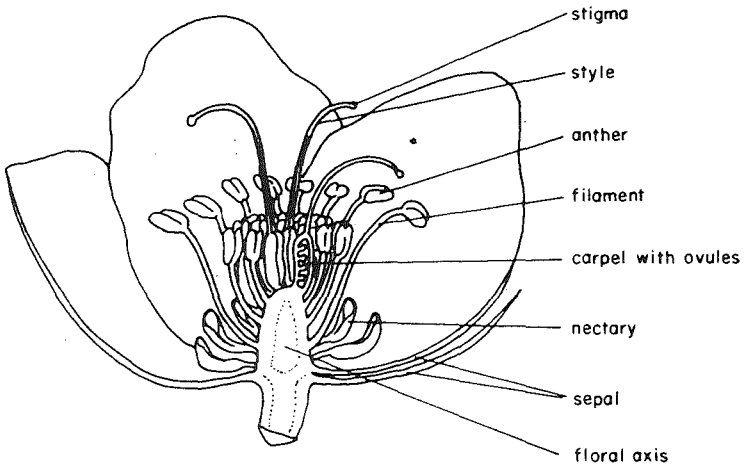
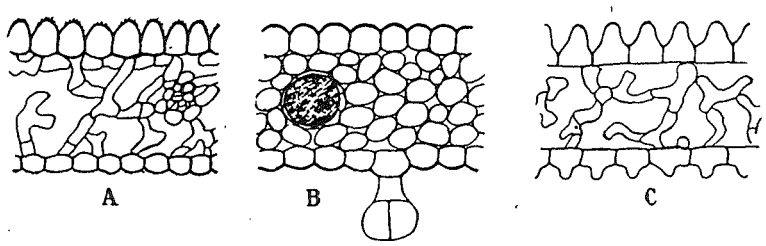


Fig. 2.8 The flower.[9]

2.5.2 Histology of sepals and petals

Although the external structure of sepals and petals may be similar to that of leaves, the internal structure of only green sepals resembles that of foliage leaves and the internal structure of coloured sepals and petals is often quite different.

In the perianth the vascular system is usually greatly reduced in amount and in supporting tissues. The mesophyll is simple in structure, commonly consisting of a few cell layers of spongy parenchyma only (no palisade layer) and containing chromoplasts or pigmented cell sap or both (Fig. 2.9).

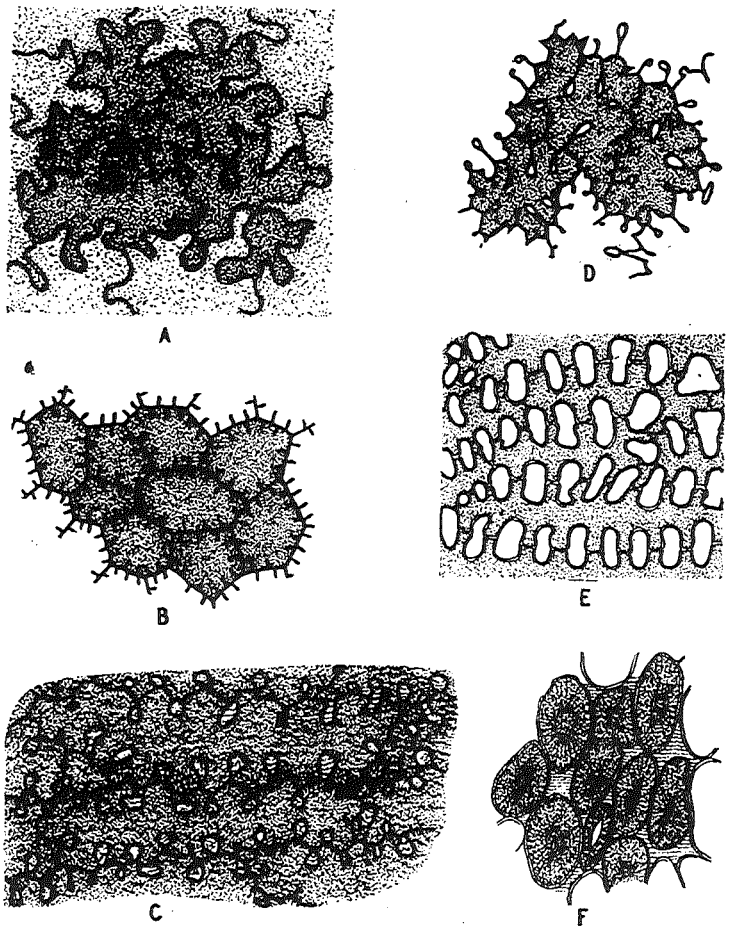


Cross sections of petals. *A*, *Amelanchier laevis*; *B*, *Lysimachia Nummularia*, showing glandular hair and secretory chamber; *C*, *Pinguicula vulgaris*.

Fig. 2.9 Cross sections of petals.[2]

The epidermis is more complicated than that of leaves. Usually the epidermal cell walls are thin and the anticlinal walls of these cells are often undulate or irregularly lobed in outline and are arranged so that the projections are dovetailed with one another, a feature that strengthens the epidermis. The outer walls of the epidermal cells commonly have papillae, which make the petals glisten. Stomata, if present, are non-functioning and trichomes may also be present. Intercellular spaces, usually lacking in the epidermis of leaves, are frequent. These intercellular spaces when filled with air give an opaque quality to petals, even those which are devoid of pigments. They lie in loops or lobes of the cell wall but are always covered by cuticle (Fig. 2.10). The cuticle varies in thickness, being very thin on delicate, ephemeral petals. Various brightly coloured water-soluble pigments such as the anthocyanins are found in the cell sap of the epidermal cells.[2,9]

The extreme types of petals show many variations from and exceptions to the typical structure described above. The less specialized the petals are, the more their structure resembles that of foliage leaves. The more highly specialized type having a weak vascular system, with veinlets and most of the main bundles lost and a mesophyll consisting of one to three poorly defined layers of widely spaced cells which are lacking along the margins so that part of the petal consists of epidermal layers only.[9]



Epidermis of corolla. *A, Calceolaria; B, Pelargonium; C, Clarkia; D, Anchusa; E, Linum; F, Erythrina.*

Fig. 2.10 Epidermis of corolla.[2]

2.6 Implications

Although the function of many of the structures discussed here will be redundant in the cut plant, knowledge of their location, physical properties and previous roles may go some way to explaining the successes and failures of the preservation process thereby making progress possible.

2.7 References

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CHAPTER 3

Chapter 3

Plant Pigments

3.1 Introduction

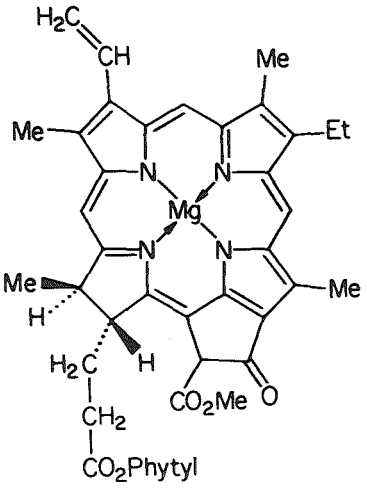
There are three structurally distinct groups of pigments responsible for observed colour in flowering plants: chlorophylls, carotenoids and flavonoids.[1,2,3]

It is the object of this chapter to briefly outline the structures of these three groups and then to discuss in more detail the properties of one particular sub-group of flavonoid pigments, the anthocyanins, since these were the particular floral pigments identified as requiring stabilisation during the preservation process. Bearing in mind that the ultimate aim was to stabilise these pigments as they exist *in vivo*, particular attention was paid to other cellular constituents with which they could be interacting.

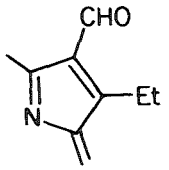
3.2 Chlorophylls

The lipid soluble chlorophylls are responsible for the green colouration in leaves and are found in the chloroplasts of all photosynthetic tissue where they act as the essential catalysts of photosynthesis. Chemically they each contain a porphyrin (tetrapyrrole) nucleus with a chelated magnesium atom in the centre and a long chain hydrocarbon (phytyl) side chain attached through a carboxylic acid group. There are at least five chlorophylls in plants; all with the same basic structure but which show variations in the nature of the aliphatic side chains attached to the porphyrin nucleus. For example, chlorophylls *a* and *b* are the chlorophylls occurring in higher plants,

ferns and mosses and differ only in that chlorophyll *b* has an aldehyde group instead of a methyl substituent attached to the top right hand pyrrole ring (Fig. 3.1).



Chlorophyll *a*



Chlorophyll *b*

Fig. 3.1 The structures of chlorophylls *a* and *b*.[1]

The other chlorophylls *c-e* are only found in algae with yet more chlorophylls confined to certain bacteria.[1, 2, 3]

Chlorophylls are relatively labile; for example the phytol side chain can be removed enzymatically with chlorophyllase or hydrolytically with acid or alkali to give chlorophyllides, or the central magnesium ion can be easily lost with the formation of protochlorophylls (in the case of chlorophyll *a*, an olive brown solid, phaeophytin). Alternative metal derivatives (e.g. copper, zinc, iron, silver) can then be prepared in acidic solutions, which in the case of copper gives a stable blue/green complex.[2]

3.3 Carotenoids

The carotenoids; the yellow, orange and sometimes red pigments, are a widely distributed group of pigments occurring in both plants and animals. In animals they are derived from the plant carotenoids by way of dietary intake and provide many brilliant animal colours, as seen in the flamingo, starfish and lobster. In plants the carotenoids are believed to have two functions, one being to act as accessory pigments in photosynthesis and the other being to provide colour to flowers and fruit.[1, 2] In green leaves the ratio of chlorophylls to carotenoids has been found to be approximately three to one (although in shade leaves this ratio is sometimes increased).[2] In flowers they appear mostly as deep yellows (as in daffodils, buttercups and marigolds) while in fruits they also provide oranges and reds (as in rosehips, tomatoes and paprika).[1]

As with the chlorophylls these lipid soluble pigments are also located in the plastids of plant cells (i.e. the chloroplasts and the chromoplasts) but the colours are often masked by chlorophyll and only become noticeable when there is little or no chlorophyll as is the case in chromoplasts.[4]

Of the several hundred known carotenoids, only a few are common in higher plants. The majority of these carotenoids have a C40 carbon skeleton and most possess a C18 central unit consisting of seven conjugated double bonds and four side chain methyl groups. The well-known carotenoids are either simple unsaturated hydrocarbons based on the chemical structure of lycopene, or they are based on their oxygenated derivatives known as xanthophylls (Fig. 3.2).[1, 2, 3] Almost all deep yellow flowers contain large amounts of xanthophylls whilst deep orange flowers are often characterised by the presence of large amounts of β-carotene.

Carotenoids are unstable pigments being easily oxidised on exposure to air, however, those containing a carboxylic acid group (e.g. Crocin, the pigment of saffron) are relatively stable.[1,2]

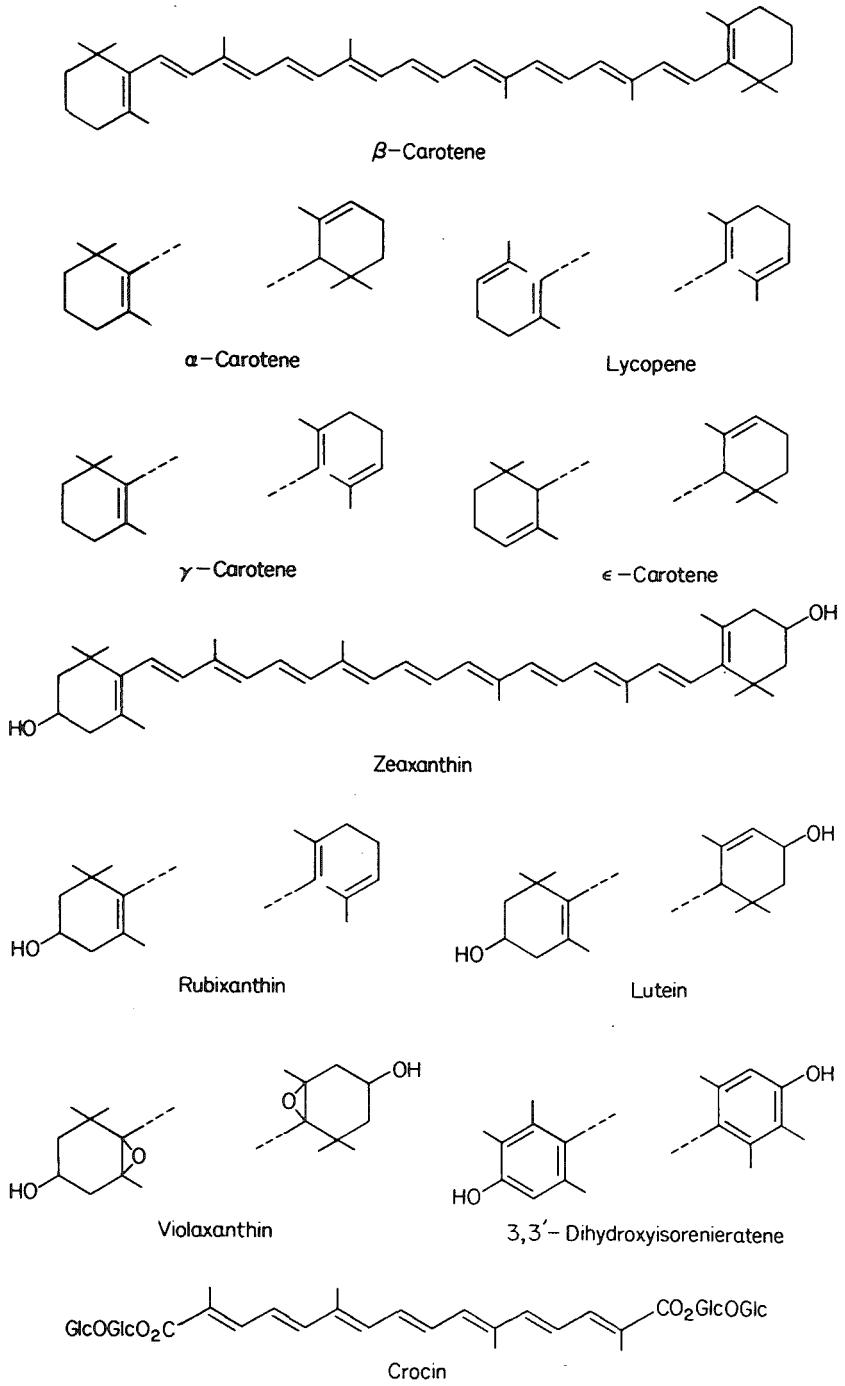


Fig. 3.2 Structures of some common carotenoids.[1]

3.4 Flavonoids

The Flavonoid pigments, which give rise to a whole range of colours from ivory to deep red, purple and blue, are water soluble and are generally present in the cell sap which occupies the vacuoles of the epidermal cells of petals.[4] The main groups of flavonoid pigments, which are involved directly in flower colouration, are the anthocyanins, the flavones, the flavonols, the chalcones and the aurones (Fig. 3.3).[2, 5]

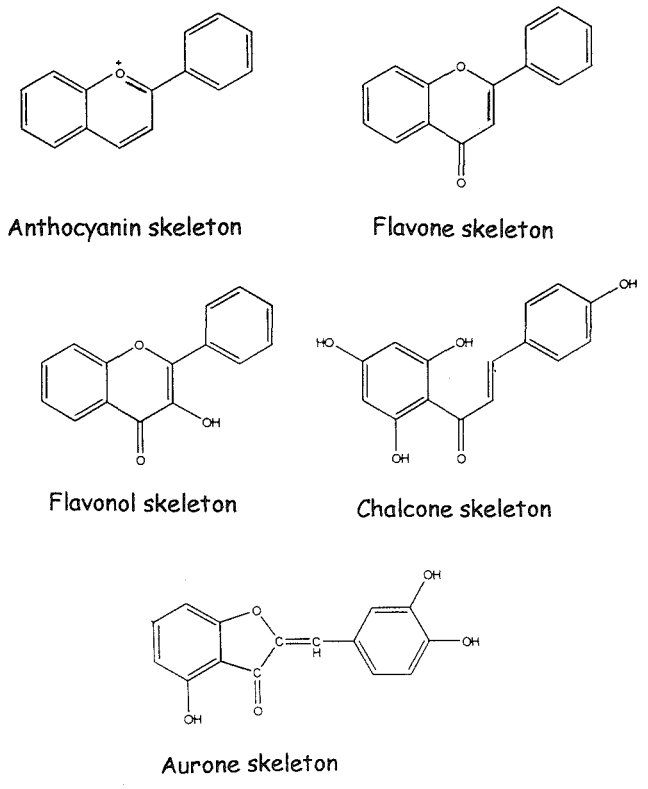


Fig. 3.3 Structures of the main groups of flavonoid pigments.[2, 3, 5]

In most cases the flavonoid compounds exist in the plant as glycosides, that is, one or more of their hydroxyl groups is joined by an acetal link to a sugar. The presence of sugars in the molecule infers sa*p*-solubility to the otherwise generally insoluble flavonoid compounds. The sugar free compounds are referred to as the aglycones

and although they have been reported as being present in plants it is most likely that they are produced during the course of extraction.[1]

The flavones, favonols, chalcones and aurones (formerly the anthoxanthins) are deep yellow to pale yellow or white in colour, the latter often making white petals look solid since without them they would appear translucent. Many of these pigments also absorb strongly in the ultraviolet and can be seen by insects.[2,4]

The most widely distributed of the anthoxanthins are the flavonols and flavones; quercetin, kaempferol and myricetin being the most common of the flavonol aglycones and apigenin and luteolin (corresponding in hydroxylation pattern to kaempferol and quercetin) being the most widespread aglycones of the the flavones. Other methylated derivatives are known e.g. isorhamnetin, azaleatin and chrysoeriol, but these are of more limited natural occurrence.

Over a hundred different glycosides of quercetin, the principal flavonol in plants, have been discovered and by far the commonest is quercetin 3-rutinoside (rutin). The range of different glycosides of the flavones is less than that of the flavonols but a common type is the 7-glucoside, exemplified by luteolin 7-glucoside. A series of glycosylflavones in which sugar is bound by a carbon-carbon bond also exists, one example being orientin (Fig. 3.4).[1,2]

Amongst the reactions of these phenolic compounds, one of those relevant to the flower preservation process is their tendency to form chelates with metal ions. For example, Cu (II) ions complex with quercetin in aqueous solution giving rise to shifts in the maximum absorbance of up to +60nm (depending on the relative concentrations of flavonol and metal ion). Their ability to form loose molecular complexes both with themselves (self-association) and with the anthocyanins

(copigmentation) is another relevant property.[6] Both of these phenomena will be discussed in section (3.5), in relation to the anthocyanins.

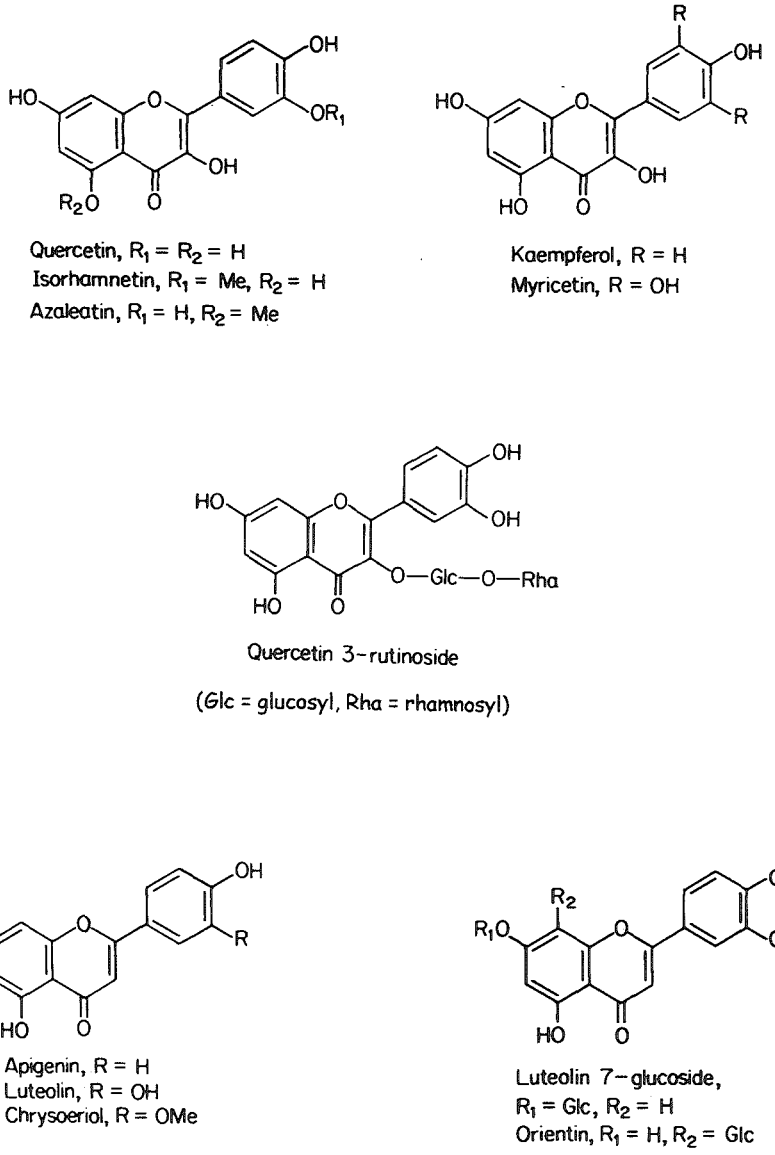


Fig. 3.4 Structures of some common flavonols and flavones.[1]

Finally, the anthocyanins; these are a particularly important group of the flavonoid pigments since they are responsible for most of the red, blue, mauve and purple colours found in flowers. The structure and properties of these compounds will now be discussed in more detail.[1,2,3,6,7]

3.5 The Anthocyanins

3.5.1 Structure

The anthocyanins are flavylium salts derived chiefly from six aglycones (anthocyanidins) namely, pelargonidin, cyanidin, delphinidin, peonidin, petunidin and malvidin (Fig. 3.5), by glycosidation of the hydroxyl group at position 3 and in many cases by a second sugar at position 5.

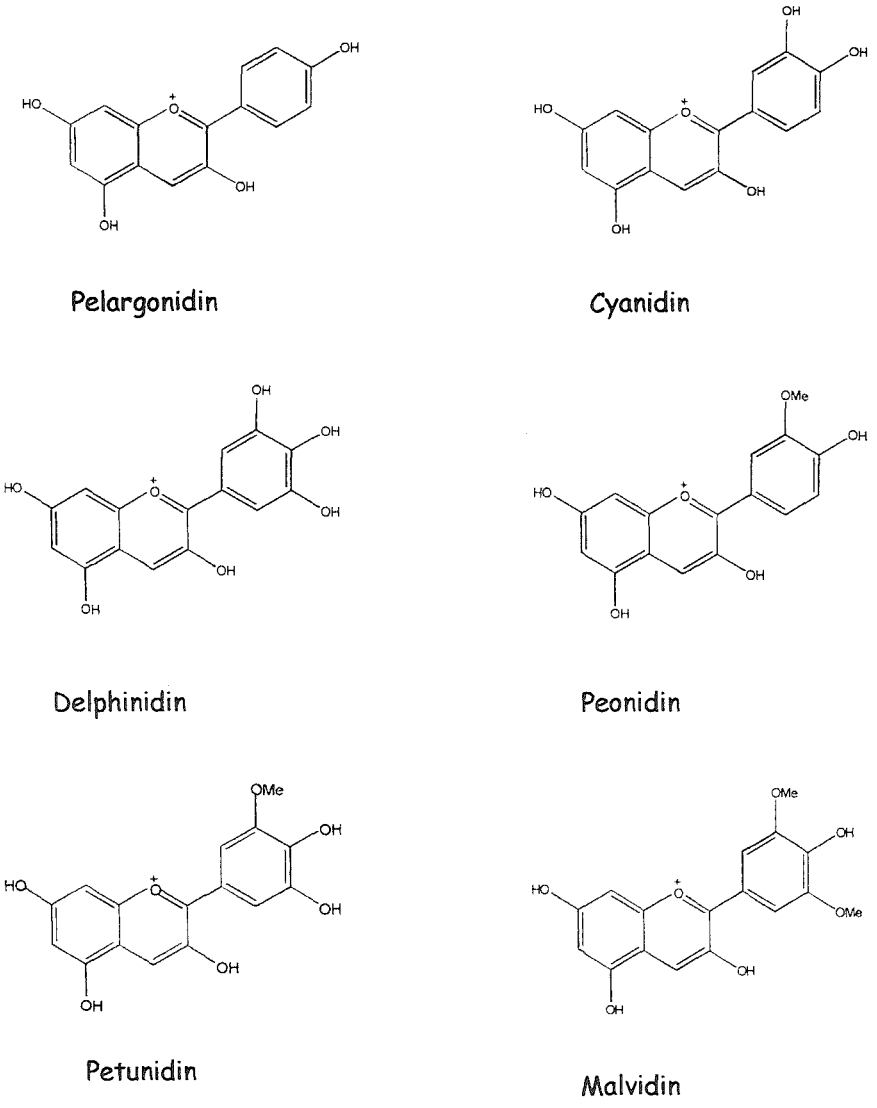


Fig. 3.5 Structures of anthocyanidins.[6]

The sugars present may be monosaccharides, disaccharides or trisaccharides. Furthermore, sugar hydroxyl groups in some anthocyanins are acylated by a variety of aliphatic acids, phenolic benzoic acids, or phenolic cinnamic acids (Fig. 3.6), giving rise to a considerable number of anthocyanins (Appendix 1).

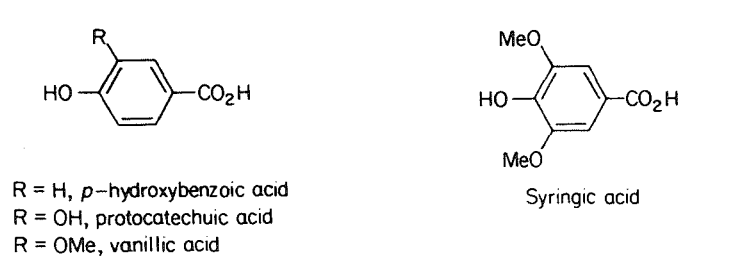


Fig. 3.6 Some acylating phenolic acids.[8]

With regards to roses, it should be noted that only glucose has been identified as being used for glycosidation and that the 3,5-diglucosides have been found to be the dominant pigments. Furthermore, to date, no acylated anthocyanins have been detected.[9]

3.5.2 Synthesis

The first synthesis of an anthocyanin was carried out by Willstätter and Mallison in 1914[10] who reduced quercetin in poor yield to cyanidin. In 1934 Robinson and his

workers devised more convenient methods for the synthesis of anthocyanins and anthocyanidins by condensing a series of 2-*o*-benzoyl- (or *o*-tetraacetylglucosyl)-phloroglucinaldehydes with a variety of ω-hydroxy (or *o*-tetraacetylglucosyl)-acetophenones to produce all the common anthocyanidins and their glycosides,for example, the synthesis of hirsutin (Fig. 3.7).[11, 2]

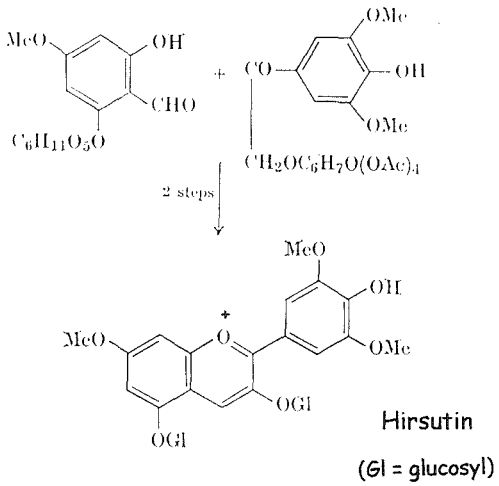


Fig. 3.7 Synthesis of Hirsutin.[3, 11]

3.5.3 Reactions of flavylium salts

3.5.3.1 General

In more recent years chemical studies using model flavylium salts have been undertaken in order to provide structural information on the types of reactions that anthocyanins undergo under various conditions. The reactivity, hence the instability of these compounds stems from the fact that they are electron deficient and therefore highly susceptible to attack by almost any nucleophilic reactant including water, peroxides and sulphur dioxide. One of the most significant properties, however, is that their colour is markedly affected by pH.

3.5.3.2 Effect of changes in pH

Studies into the effects of pH changes on flavylium salts when position 3 of the flavylium nucleus is (1) unsubstituted, as is rare in nature, (2) glycosylated, as in anthocyanins, or (3) hydroxylated, as in anthocyanidins, have been carried out. [12, 13, 14, 15, 16] These studies showed that on raising the pH from 1-4, flavylium compounds of type (1) form an equilibrium mixture with the corresponding trans-2-hydroxychalcones (Fig. 3.8). These chalcones are formed from intermediate anhydro bases *via* the highly unstable carbinol (pseudobase) and presumably the cis-chalcone. It was also observed that the flavylium-chalcone equilibrium is affected by light, in that the light shifts the equilibrium towards the flavylium cation.[17, 18, 19]

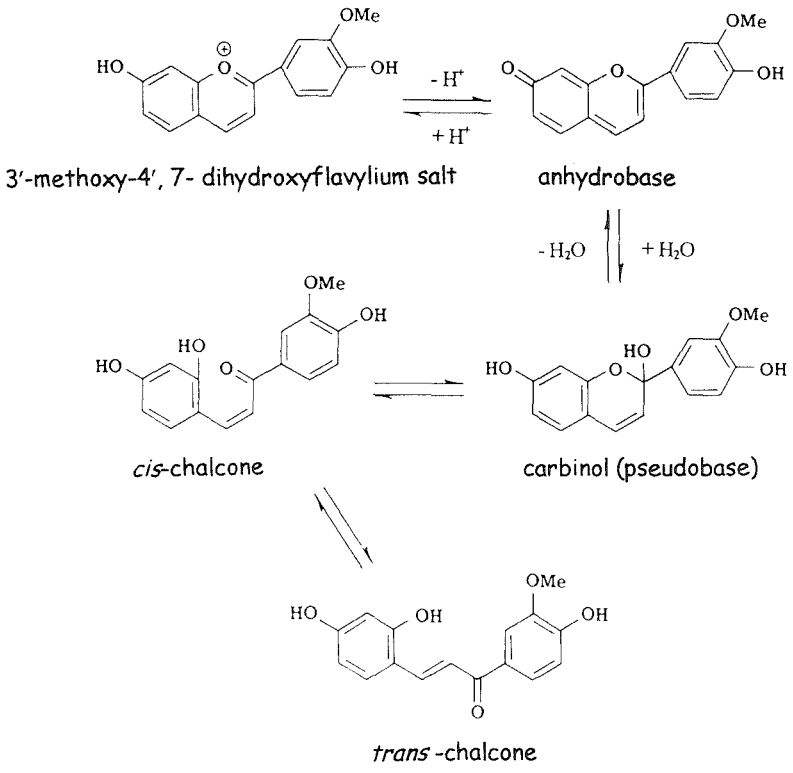


Fig. 3.8 The effects of pH on flavylium salts of type (1).[6]

Flavylium salts of type (2), the anthocyanins, which are stable in strongly acidic media (pH below 2.5) and are normally coloured red or orange, form very unstable coloured anhydro bases (normally purple) in the pH range 2-5, which rapidly hydrate (through the flavylium cation) with the formation of colourless carbinols (pseudobases).[20, 21, 22] These carbinols are fairly stable at room temperature (although irreversible decomposition does occur slowly on long standing) and they instantly regenerate the coloured anthocyanin cation on acidification (Fig. 3.9).

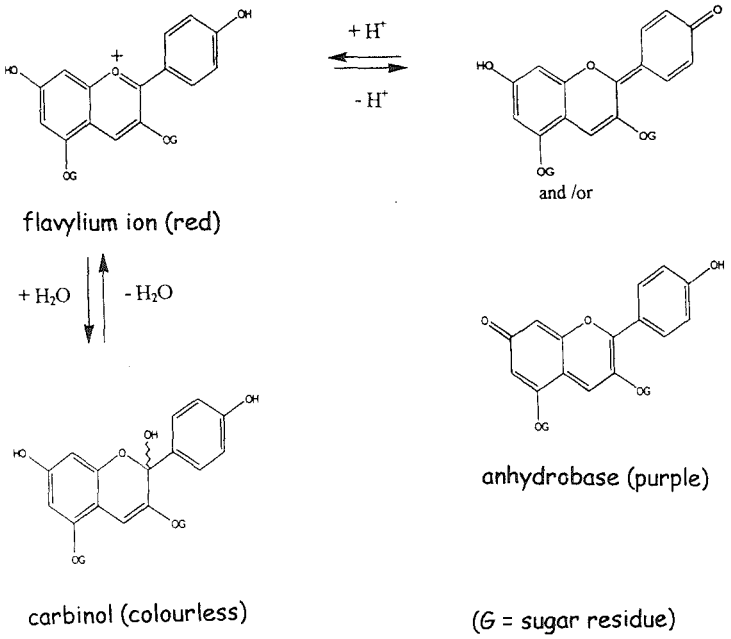


Fig. 3.9 The effects of pH on flavylium salts of type (2).[20]

In the case of acylated anthocyanins, it has recently been found that aqueous solutions of these pigments can retain their colour in slightly acidic or even neutral solutions.[23] It is also of interest that towards pH 7 there exists a mixture of the anyhdrobase and its blue ionic form.[24]

Type (3) compounds, the anthocyanidins, are very unstable and readily decompose irreversibly. In contrast to the stability of the carbinols derived from anthocyanins, carbinols derived from anthocyanidins are unstable on standing and are believed to undergo ring fission (possibly *via* the chalcone [25, 26]) to yield α-diketones in the pH range 2-5, [25] which are then hydrolyzed to yield phenolic benzoic acids (Fig. 3.10).

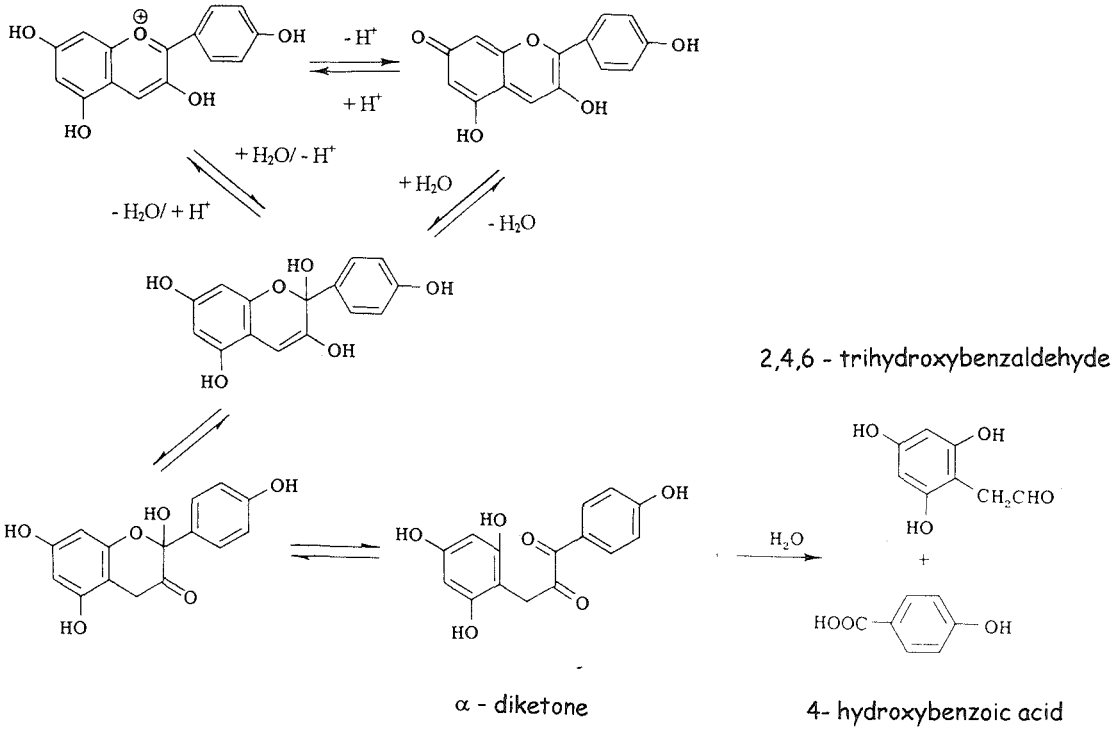


Fig. 3.10 The effects of pH on flavylium salts of type (3).[6]

3.5.3.3 Sulphite bleaching

The decolorisation of anthocyanins by sulphites has been attributed to nucleophilic attack on the flavylium cation by the negative ions of sulphurous acid. The product formed is probably chromen-4 (or 2-) sulphonic acid (analogous to the product formed by the nucleophilic attack of water) (Fig. 3.11).[6, 26, 27]

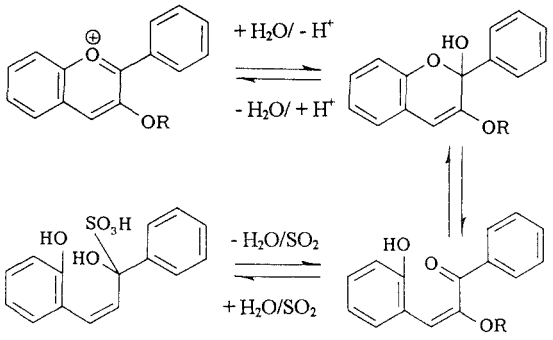
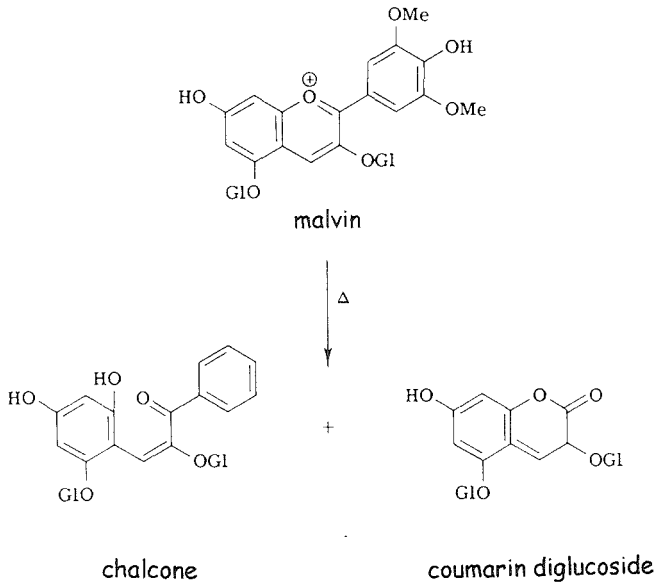


Fig. 3.11 The sulphite bleaching of flavylium salts.[6, 26]

3.5.3.4 Thermal decomposition

The reactions involved and the products formed in the thermal decomposition of anthocyanins are not clear. Progress in this area indicates that thermal decomposition of anthocyanins involves conversion of anhydrobases to the corresponding chalcones and coumarin glucoside (Fig. 3.12).[6, 23]



3.12 The thermal decomposition of anthocyanins.[6]

The formation of coumarin glucoside on heating not only occurs in model systems but has also been observed in grape juice and in wines containing these anthocyanin glucosides.[6]

In the case of the anthocyanidins, investigations into the effects of heat on the pigments cyanidin, pelargonidin, and delphinidin[28] have indicated that thermal degradation occurs *via* the corresponding chalcone which subsequently breaks down to yield 2,4,6-trihydroxybenzaldehyde from ring A (for all the anthocyanidins) and 3,4-dihydroxybenzoic acid, 4-hydroxybenzoic acid and 3,4,5-trihydroxybenzoic acid for cyanidin, pelargonidin and delphinidin respectively. The rate of the flavylium cation disappearance was found to be very pH dependent, being slower in more acidic media, largely as a result of the increased rate of formation of the chalcone at higher pH.

3.5.3.5 Photochemical degradation

Anthocyanins are known to exhibit very poor stability to light and have been found to breakdown to form the same products as those obtained by thermal degradation.[28] The reaction mechanism, however, is believed in part to involve the excitation of the flavylium cation and the direct conversion to products. Again glycosidation and acylation have been found to markedly increase the stability of aqueous solutions of these pigments on exposure to light.[23, 26, 28]

3.5.3.6 Enzymatic degradation

Enzyme systems capable of decolourising anthocyanins have been discovered in moulds, fruits and flowers. This enzymatic decolourisation may be caused by

glycosidases which hydrolyse the protective glycosidic groupings to yield the unstable aglycones, or by phenolases which require a catechol or other *o*-dihydroxyphenols for activation. These anthocyanin destroying enzymes may be inactivated by heat.[6, 26]

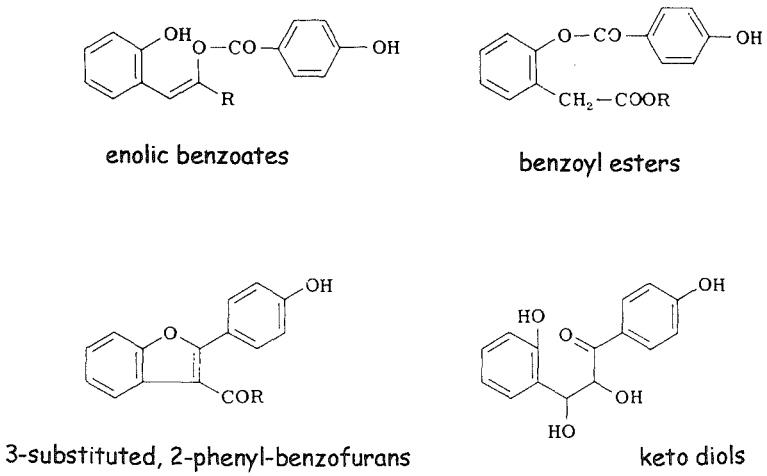


Fig. 3.13 The products of peroxide oxidation of flavylium salts under various conditions.[6, 26]

3.5.3.7 Oxidative deterioration

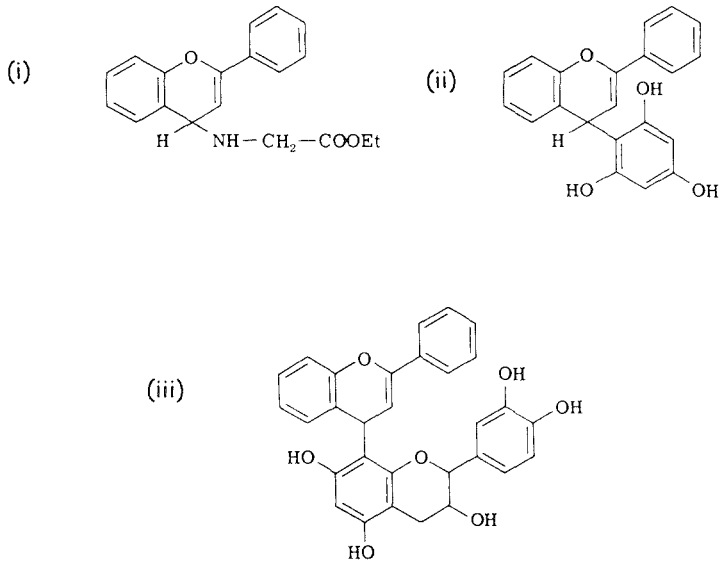
As already indicated, pH not only exerts a profound influence on the colour of anthocyanins, but it also affects their stability. It appears, however, that within certain pH ranges oxygen markedly weakens the stability of anthocyanins. For example, in the case of cyanidin 3-glucoside, pH in the range 2 to 4 has little effect on the breakdown of the pigment on heating under anoxic conditions, however, oxygen greatly accelerated the degradation in the same pH range.[26, 29]

The accelerated decomposition of anthocyanins in the presence of ascorbic acid has also been attributed to the presence of oxygen. It is thought that this ascorbic acid induced degradation is mediated by hydrogen peroxide, which is produced as a result

of the autoxidation of ascorbic acid. Flavylium salts have been shown to be highly suseptible to nucleophilic attack by hydrogen peroxide producing colourless enolic benzoates, benzoyl esters, 3-substituted 2-phenylbenzofurans or keto diols under various conditions (Fig. 3.13).[6, 26]

3.5.3.8 Condensation reactions

Anthocyanin breakdown on contact with air is known to be accelerated in the presence of normally co-occurring cellular constituents such as ascorbic acid, sugars and sugar derivatives and amino acids. One suggestion is that the accelerated decolourisation of anthocyanins in the presence of these constituents can be attributed to the condensation of the pigments with these constituents. It has been demonstated that flavylium salts condense easily with amino acids, phloroglucinol, catechin and other nucleophiles to yield colourless 4-substituted flav-2-enes (Fig. 3.14), which usually then undergo further changes, the net result being the loss of the flavylium pigment. [26, 30]



3.14 The condensation products of flavylium salts with ethylglycine (i), phloroglucinol (ii) and catechin (iii).[26]

3.6 Stabilzation of anthocyanins

3.6.1 General

As previously stated, anthocyanins form stable coloured flavylium ions in strongly acidic media and form colourless pseudo-bases (or carbinols) when neutralized to pH 4-6. Since the pH of flower cell sap is only weakly acidic (pH 3.8-5.8) the observed colour of anthocyanin pigmented flowers must be attributed to some form of stabilization of the anthocyanin molecule. Recent studies[20, 31] have indicated that anthocyanins may be stabilized by such means as self-association (vertical stacking of aromatic rings between anthocyanins), copigmentation (between anthocyanins and phenols such as flavonols, flavones and tannins), intra-molecular sandwich type stacking (intra-molecularly between anthocyanins and their aromatic acid residues) and by complex formation between anthocyanins, flavonoids and metal ions e.g. metalloanthocyanins commelinin (from *commelina communis*) and protocyanin (from *centaurea cyanus*) the blue cornflower pigment, each of which consist of six anthocyanin and six flavone molecules and two metal ions.[20] An outline of the theory behind these effects will now be given.

3.6.2 Molecular association complexes

In order to prevent the nucleophilic attack of the coloured flavylium ion by water, the presence in the medium of a species capable of forming strong interactions with the flavylium cation, or with its anhydro bases appears to be required. In this respect, structural and electronic features of the anthocyanin coloured forms (the orange or red flavylium ion, the purple anhydro base and the blue anionic species) make them

particularly suitable to form molecular (non covalent) complexes with other species, since they are all planar chromophores with extended electronic delocalisation.[20, 21, 23] Species that are able to associate with these forms can be the anthocyanin pyrylium ring itself (self-association) or in the special case of acylated anthocyanins, the aromatic acid residues of the anthocyanin (intramolecular copigmentation), or another structurally unrelated planar molecule (intermolecular copigmentation). In any case, the theory behind the phenomena is similar in that it involves providing efficient protection against loss of colour on hydration (by shifting the hydration equilibrium towards the highly coloured cation).[24, 31]

In order for a stable molecular complex to be formed, the interacting species must develop strong attractive forces between each other. These are electrostatic and dispersion forces including π-π overlap between aromatic residues (vertical stacking), dipole-dipole interactions and hydrogen bonding. Molecular association, whatever its nature, is accompanied by a relative desolvation of both interacting species, since their surfaces in contact with the solvent molecules are reduced upon complexation. In water, the consequent release of solvent molecules into the hydrogen bonded network of liquid water (bulk solvent), where strong cohesive forces are at work, provides a large positive contribution to the driving force behind complexation (the hydrophobic effect). Water, therefore, plays a major role in this type of molecular association and the addition of any other solvent disrupts the hydrogen bonded network of water molecules necessary for the hydrophobic effect to occur.[24, 31, 32] It appears that this type of molecular association is essentially an enthalpy driven phenomenon, since the entropy changes (i.e. the reduction in the degrees of freedom of both the interacting species when they associate and also the

more ordered state reached by the water molecules as they are released into the tetrahedral network of bulk water) are unfavorable.

To return to the case of the flavylium ion; in slightly acidic aqueous solution, the free flavylium ion is strongly hydrated, a situation that even leads to a true chemical reaction, the formation of the carbinol. However, when the flavylium ion is engaged in a stable complex, its resulting desolvation makes it less vulnerable to nucleophilic attack by water. It appears, therefore, that complexation efficiently competes with hydration. The resulting shift in the hydration equilibrium towards the highly coloured flavylium ion produces an increase in the visible absorption of the flavylium chromophore (hyperchromism) and a simultaneous shift to a longer wavelength (bathochroism) is also frequently observed (hydrophobic effect).[31, 32]

This stabilisation by stacking is mainly brought about by intermolecular or intramolecular hydrophobic interaction between aromatic nucleii, however, it is believed that such stackings are further stabilised by a superimposed layer of hydrophilic sugar moieties (hydrogen bonding) and possibly charge transfer interactions (Fig. 3.15). The presence of such stackings has been detected *in vivo* by visible light absorbance and light scattering techniques, namely circular dichroism (CD) and resonance Raman (RR) spectroscopy. [20, 32, 33, 34]

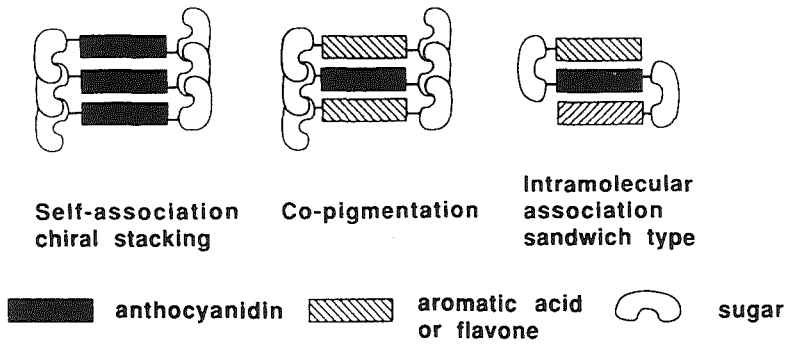


Fig. 3.15 Schematic representation of three stacking types.[20]

3.6.3 *Self-association*

In the case of self-association, this type of molecular association only occurs at high anthocyanin concentrations, typically 10^{-2} M or higher [20, 32] and does not usually occur at concentrations[20] below 10^{-4} M. Depending to some extent on the pH, self-association can be homo-self-association, where the species are similarly charged (i.e. flavylium ion with flavylium ion, anhydro base with anhydro base or anion with anion), or it can be hetero-self-association, where the species are of differing charge (e.g the anhydrobase and its anionic form with which it coexists at higher pH). The self-association of the neutral anhydrobases appears to be much stronger than that of the flavylium cations or the ionized anhydrobases, possibly because in both of the latter, electrostatic repulsion probably occurs.[32] This is significant in the case of flowers since the neutral anhydrobases are probably the anthocyanin forms that prevail in most of the cell vacuoles. At about pH 7 the anhydrobase and its anion are in equilibrium and on increasing the concentration, self-association is believed to suppress ionization of the anhydrobase, thus giving rise to a shift to shorter wavelength (hypochromic effect).[20, 32]

3.6.4 *Intramolecular copigmentation*

In the case of anthocyanins bearing at least two aromatic acyl groups linked to their glycosyl residues, the stabilisation phenomenon is known as intramolecular copigmentation. As mentioned previously (3.5.1), anthocyanins can be acylated by a variety of aliphatic and aromatic acids and many of the polyacylated anthocyanins, have been found to be exceptionally stable to colour loss on hydration, both in slightly acidic media and in neutral solutions.[35, 36] For example, in the case of

platyconin (Fig. 3.16), extracted from the petals of Chinese bell-flowers (*platycodon grandiflorum),* hydration and decolourisation only takes place after the aromatic acyl groups have been removed by hydrolysis suggesting that, in this case, pigment stability is not due to self-association, but to the presence of acyl groups.[23]

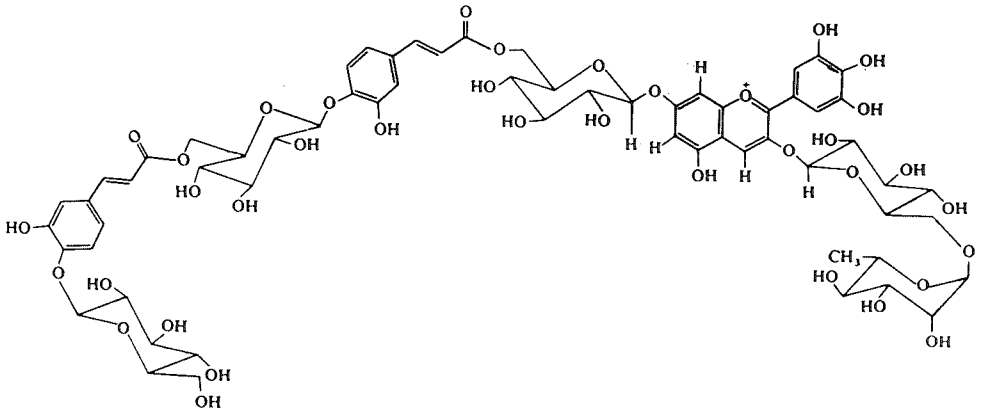


Fig. 3.16 Stereostructure of platoconin.[23] (3-*o* -(6-*o*- (α-L-rhamnopyranosyl)-*β* -D-glucopyranosyl)-7-*o* (6-*o*- (*trans*-4-*o*- (6-*o*- (*trans*-4-*o*- (*β*-D-glucopyranosyl)-caffeoyl-*β* -D-glucopyranosyl)- caffeoyl)-*β* -D-glucopyranosyl) delphinidin.)

The stability of such anthocyanins is currently being attributed to the formation of a sandwich type stacking in which the two aromatic acyl moieties stack above and below the pyrilium ring, thus providing efficient protection against nucleophilic attack by water.[36, 37] The presence of two aromatic acyl groups does not, however, imply protection against hydration, since other steric factors may determine whether such an intramolecular association can occur e.g. the structures of the aromatic acyl residues, their position of linkage to the sugar, their length and flexibility. It is also to be noted that stability to light has also been shown to be a property of these acylated anthoycanins.[23, 26]

3.6.5 Intermolecular copigmentation

Intermolecular copigments coexist alongside anthocyanins in the cell vacuole. They have little or no colour by themselves but acting as copigments to the anthocyanins they are able to stabilise and enhance the anthocyanin colours by intermolecular hydrophobically reinforced π-π stacking of their aromatic nucleii with those of the pigment. These copigments may include other flavonoids, polyphenols, alkaloids, amino acids and nucleotides, all of which can interact under suitable conditions with the anthocyanins giving rise to an increase in the absorbance in the visible (hyperchromism) and frequently a bathochromic shift (Appendix 2).[23, 26, 32]

Copigmentation is affected by several factors, among which pH is an important one. For example, at pH 1 the anthocyanin malvin exists in aqueous solution as the flavylium cation. The addition of the quercetin glycoside, spiraeoside results in a 15nm shift in spectral maximum due to the interaction of the flavylium ion with the copigment. There is, however, no increase in absorbance at the visible maximum on adding the copigment. At pH 2-3, there is a considerable loss of colour for the anthocyanin solution without copigment and a significant colour retention for the solutions containing the anthocyanin and the copigment. Thus the copigment effect is to reduce the production of the colourless carbinol. At pH 4-6, the solutions containing malvin alone are colourless, whereas the solutions with malvin and the copigment are still coloured. In this pH the range the anhydrobases are formed and again colour retention in the solution containing copigment is due to a decrease in the amount of the carbinol pseudobase in these solutions.[24, 32, 38]

The other main factors which affect copigmentation are: the chemical nature and concentration of both pigment and copigment, temperature and the role played by the solvent. [23, 24, 26]

To date, the effect of concentration of anthocyanin and copigment has been investigated for only a few compounds.[35] Nonetheless, the results so far reported suggest that, for most anthocyanins there is an optimum molar ratio of pigment to copigment at which intensity and stability are maximised. As with self-association the copigmentation effect is at its best at relatively high anthocyanin concentrations (i.e of the order 10^{-2} M).[20, 23, 39]

In recent investigations, copigmentation has been demonstrated to be extremely sensitive to temperature. For instance, weakly acidic solutions of anthocyanins containing copigments are observed to give a considerable decrease in absorbance in the visible range on heating, whereas the corresponding non-copigmented solutions remain almost unchanged over the temperature range 10-60 °C. On cooling the opposite effect occurs in that copigmented solutions show an increase in colour. This is a property that, *in vivo,* can be used to determine whether a flower contains copigmented pigments. For instance, by comparing the colour of a petal that has been refrigerated for a few minutes with that which has been kept at room temperature, the evidence for copigmentation being the darker colour of the copigmented petal.[32]

On the effect of the solvent on the copigmentation phenomenon very little work has been carried out, but the present indication is that no solvent is better than water (indicting that the strength of the copigment effect parallels the cohesion of the hydrogen bonded tetrahedral network of water molecules). [24]

The most efficient copigments discovered so far are the flavonols quercetin and its glycoside rutin, the aurone aureusidin and the C-glycosyl flavones such as swertisin (Appendix 2).[23, 31, 37, 40] To date, copigmentation is the most important means of stabilisation of rose anthocyanin chromophores and a series of quercetin and kaempferol glycosides have been identified as copigments.[9, 41, 42]

3.6.6 Interaction with Metals

Anthocyanins possessing a catechol group in their B ring (e.g. the glycosides of cyanidin, delphinidin and petunidin) are known to form blue coloured metal complexes (chelates) in aqueous solution with metal ions such as Al (III), Fe (III), Cr (III), Ti (III) Sn (IV) and also purple complexes with Sn (II). [32, 43] The presence of a chelating metal ion and a catechol group, however, does not always guarantee complexation and the presence of a suitable copigment is often required. For example, in model experiments, aluminium ions alone did not produce a stable blue colour with delphinidin 3-glucoside, but required the presence of the copigments 3-caffeoyl- and 3-*p*-coumaroyl-quinic acids.[32] Furthermore, the structure of the copigment was found to be important since on adding 3- or 5-caffoeylquinic acid to the above anthocyanin and aluminium mixture only gave rise to a blue colour for the 5-caffoeylquinic acid. In general, however, it was observed that adding chelating metal ions to those solutions containing pigments and copigments possessing the required structures for copigmentation not only provided a large bathochromic shift but also a large increase in visible absorbance. This would indicate a strengthening of the pigment-copigment interaction and thereby more efficient protection of the pyrilium ring from nucleophilic attack by water.

There is, however, substantial evidence to suggest that this is not the only possible type of metal-pigment interaction occurring. For instance, common plant metal ions such as Ca (II), Mg (II) and K (I), which have been implicated in the formation of anthocyanin complexes, do not form stable chelates with the anthocyanin catechol group.[43] Further evidence that metal ions are not necessarily forming chelates with the catechol group of the anthocyanins comes from studies on commelinin, which is known to contain magnesium but which also retains the cytochrome-*c*-reducing capacity of other anthocyanins possessing a free catechol group in the B-ring, intimating that other attachment mechanisms must be operating.[26] Indeed, the colour of commelinin has only been reproduced when Mg (II) ions have been added to solutions of the anthocyanin and flavonol components of the pigment complex.[26, 44]

3.7 Contribution of anthocyanins to flower colour

3.7.1 General

The enormous contribution of just six basic anthocyanidin structures to the vast array of flower colours observed in nature, is not only due to the various means of stabilisation and protection of the anthocyanin coloured forms, but also to the structure of the anthocyanins themselves. In this section the various aspects of anthocyanin structure and their relation to flower colour will be considered and some of the anthocyanin stabilising effects will be revisited in the context of flower colour.

3.7.2 Hydroxylation

Neglecting complications such as methylation, glycosylation and co-pigmentation, the pigments pelargonidin, cyanidin and dephinidin, which differ in structure only in

the number of their hydroxyl groups, provide a whole range of flower colours. In general, all pink, scarlet and orange-red flowers have pelargonindin as the predominating pigment, all crimson and magenta flowers have cyanidin and mauve and blue flowers have delphinidin. Varieties of garden plants containing mixtures of these anthocyanidin types are as common as those having single pigments (Appendix 3).[2] There are also plants such as the garden rose, which are not able to synthesise delphinidin and in which colour variation is more restricted.[9]

3.7.3 Methylation

	$\lambda max^{MeOH-HCl}$(nm)	$\Delta\lambda$
Delphinidin derivatives:		
Parent compound (delphinidin)	546	--
3'-*O*-methyl ether (petunidin)	546	0
3',5'-di-*O*-methyl ether (malvidin)	542	4
7,3',5'-tri-*O*-methyl ether (hirsutidin)	536	10
5,3',5'-tri-*O*-methyl ether (capensinidin)	538	8
Cyanidin derivatives:		
Parent compound (cyanidin)	535	--
3'-*O*-methyl ether (peonidin)	532	3
7,3'-di-*O*-methly ether (rosinidin)	524	11

Table 3.1 Visible absorption maxima of methylated anthocyanidins.[2]

Methylation of some of the hydroxyl groups of the anthocyanins has a slight reddening effect on colour.[45] This is apparent from a consideration of the absorption spectra of the known methylated pigments (Table 3.1).

This effect is illustrated in roses where cyanidin-peonidin mixtures are found almost exclusively in pinker varieties, whereas crimson and deeper red varieties have only cyanidin.[46]

3.7.4 Glycosylation

Although there is a hypsochromic effect (-15 nm) in the visible region when anthocyanidins are glycosylated in the 3-position, this is not an important contributor to flower colour because anthocyanidins always occur in flowers with at least one sugar attached to the 3-hydroxyl group. Furthermore the nature of the 3 substituted sugar is immaterial as all 3-glycosides of a particular anthocyanidin have the same visible spectra. 3-Glycosides and 3,5-glycosides have almost identical visible maxima, however, the substitution of a sugar in the 5-position does give rise to a slight fluorescence. Acylation of the sugar residues with *p*-coumaric or caffeic acid partly quenches this fluorescence giving rise to duller blooms.[2]

3.7.5 Concentration

The anthocyanin concentration in flower petals varies within the range 0.01-15% dry-weight. Such variations in concentration can have profound effects on colour giving at one end of the scale flowers with a faint pinkish blush of colour e.g. the rose "Madame Butterfly" and at the other end a deep purple/black colouration e.g. the tulip "Queen of the Night". [2]

3.7.6 Copigmentation

As stated previously (section 3.6), copigmentation, normally with a flavone glycoside or a hydrolyzable tannin is a phenomenon believed to be responsible for the blueing of flowers. It has been demonstrated *in vitro* that at room temperature, aqueous acid extracts of co-pigmented flowers are bluer in tone than those of non co-pigmented petals. Furthermore, on heating, the loose co-pigment complex is dissociated giving rise to a reddening in colour, which on cooling reverts back to the original blue shade. Some of these copigmented complexes may also be dissociated by addition of alcohol (or other non-aqueous solvents), although some stronger complexes, which are stable to alcohol, have been isolated.[2]

3.7.7 Metal complexing

Another factor responsible for the blueing of flowers is the chelation of anthocyanins with metal ions. For example, the same pigment cyanin, which is found in the red rose and in the blue cornflower, has been found to owe its observed blue colour in the latter case to the formation of the metal complex with Fe (III) and Mg (II) ions.[20] Studies have shown that the requirements for blueing by metal chelation are: a) the presence in the anthocyanin of a free *ortho*-dihydroxy grouping, (b) an internal mineral balance which promotes availability of the chelating metals and (c) in many cases the presence of suitable copigments.[43]

3.7.8 Effect of pH

In vitro the effect of pH on anthocyanin colour in aqueous solution is considerable. They appear red or orange in acid solution, violet changing to colourless in neutral solution and blue in alkali. It is also true that in the flower there is a rough correlation between the colour and the pH of the cell sap, red flowers often being a little more acidic than blue. However, the idea of a simple correlation between pH and flower colour must be discounted as the pH of flowers does not vary much and in some cases the order may be reversed e.g. the case of the red rose and the blue cornflower where the sap of the latter is the more acidic.[20]

3.7.9 Histological and structural effects

Observed flower colour is not only a result of chemical interactions involving the water soluble sap pigments occurring within individual cells. The vivid colours of some flowers result from the arrangement of the cells containing the different water soluble pigments e.g. the lurid colour of some delphiniums has been attributed to the presence of cells containing red anthocyanin side by side by cells containing blue pigment. The stripes seen in Crocus aureus are due to a combination of blue sap colour on the outer side of the perianth leaves and yellow soluble pigment on the inner side. The dark purple spot in some poppy flowers is due to cyanin superimposed on a pelargonin background.[47] Water soluble sap pigments such as the anthocyanins may also co-occur with the lipid soluble plastid pigments i.e. carotenoids and chlorophylls. There are many different ways in which these pigments can be arranged in the tissues and the great variety of such combinations accounts to a large extent for the numerous shades and tints.

Generally speaking the anthocyanins occur in the epidermis of the corolla and the plastids pigments in the inner tissues or the epidermis or both. When both the plastid and the sap pigments occur in the same cell, especially in the papillae of the epidermis, the plastids tend to occupy the base of the cell while the sap soluble pigment is uniformly diffused. The effect of an anthocyanin and a lipid soluble yellow pigment occurring in the same cell can be seen in the crimson flowers of Mirabilis Jalapa.[48] Brown colours formed by magenta cyanin on a yellow carotenoid background can be seen in the coffee-coloured rose "Café".[2] There are of course a great many other combinations of these pigments and a correspondingly large number of shades of brown, crimson or scarlet.

Recent work has also indicated that the shape of the epidermal cells influences the optical properties of the anthocyanins and thereby the perceived colour.[31, 49] One other factor, which may also play a role in determining the colour of many flowers, is the actual physical structure of the petal surface, but this has yet to be demonstrated.

3.8 Implications

In the angiosperms an infinite number of different flower colours exist and it is rare to find two plant species, even in the same genus, with exactly the same flower tone. As we have seen, this vast range of observed flower colour is a result of many modifying factors, several of which may operate at the same time. Any attempt to control the colouration of preserved flowers would therefore not only have to consider the chemistry of the individual pigments but also the interactions between these pigments and the other cell constituents.

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CHAPTER 4

Chapter 4

Process development
(a qualitative approach)

4.1 Introduction

This chapter will outline the various stages of development for the process of whole flower preservation based on the information obtained from the literature on plant anatomy and floral pigments. The chapter commences with a brief description of the existing foliage process and then goes on to describe the means by which this process has been adapted in order to render it suitable for the preservation of whole flowers, and in particular red roses.

The work described is essentially of a qualitative nature since it deals, for the most part, with the aesthetic qualities of the preserved plant material, which from a marketing and retailing perspective are of utmost importance.

Moreover, as mentioned previously (section 1.2) factors such as "cost" and "safety" have had a considerable bearing on any chemical developments made and subsequently on the overall direction of the project.

4.2 Equipment and materials

4.2.1 Heating

Petals were processed in solutions heated by a temperature controlled water bath; Grant instruments (Cambridge).

4.2.2 pH

pH measurements were recorded on a PHM182 standard pH meter from Radiometer A/S (Copenhagen), fitted with a combined glass electrode from Russell pH Ltd. (Auchtermuchty). Calibration was carried out using pH 4 and pH 7 buffer solutions (Russell pH Ltd.) and measurements made at R.T.

In the case of solutions containing diols, no correction of the pH values read on the meter were made.

4.2.3 Light exposure

Light exposure tests were performed using a 300-watt halogen lamp from Status International (UK) Ltd., and samples were positioned, using a light meter (model U-01588-24, from Cole-Parmer Instrument Company Ltd.) so as to receive a light intensity of 20,000 lux. Continuous exposure for 7 days at this level, without fading, was considered to guarantee colour stability in the preserved foliage for at least six months. A shorter test was also used as a quality control procedure for the foliage process, a failed batch being indicated by fading after 24 hours of light exposure.[1,2]

4.2.4 Chemicals

General chemicals were of laboratory grade and were supplied by Sigma-Aldrich Company Ltd, Lancaster Synthesis Ltd, Fluka Chemicals or Avocado Research Chemicals Ltd (Appendix 4).

Rutin, polygalacturonic carrageenan and ferulic acid were of analytical grade and were supplied by Sigma.

Polidene 33-004 (a vinylidene chloride copolymer emulsion) was supplied by Scott Bader Company Ltd.

4.3 The effects of the foliage preservation process on whole flowers

4.3.1 General

The process for the preservation of green foliage, prior to this work, involved immersing plant material at temperatures of between 60 °C and 95 °C, in an aqueous solution containing various proportions of monohydric, dihydric and polyhydric alcohols, chlorophyll and copper salt. The heating period ranged from 20 minutes up to 3 hours depending on the nature of the foliage.[1] The preserved plant material was then rinsed with water, dried and finally dipped in an aqueous polymer solution (Polidene 33-004) in order to provide a polymer coating (section 4.12).

It was believed that during processing an exchange took place between the cell sap and the process fluid resulting in preserved plant material having a colour and texture very close to that of the original plant. The dihydric and polyhydric alcohols were believed to provide lasting "body" to the plant material since they were less likely to evaporate than water. The chlorophyll was believed to boost the depleted pigment levels (due to a mass action effect on exchange) and the copper ions were thought to replace the magnesium ions in chlorophyll (possibly by complexing with phaeophytin-section 3.2) thereby preventing loss of the green colouration.[1,2]

As yet however, there has been no in-depth investigative work concerning the nature of the chemical and physical changes taking place either during or after treatment, therefore, the development work related to flowering plants has had to evolve based on this simple theory of fluid exchange.

The first experimental step, *en route* to developing a process capable of preserving flowers, was to assess the effect of the existing foliage process on whole flowers.

4.3.2 Experimental

Several different varieties of red rose were processed in the current commercial green foliage solution (consisting of 60% v/v propan-1,2-diol, 25% v/v water, 10% v/v butan-1,4-diol, 5% v/v propan-2-ol, 10 g/L copper sulphate pentahydrate and a non quantified chlorophyll boost) at a temperature of 80 °C for periods of between 30 and 60 minutes.

4.3.3 Results and discussion

The leaves were found to be generally of good appearance (with the exception of a slight brown deposit), however the rose heads were observed to turn almost colourless (although visually there did not appear to be any bleeding into solution) and become limp and sticky. This was confirmation that the existing foliage process was not entirely suitable for whole flower preservation and therefore required modification.

4.4 The effects of temperature, process time and fluid composition on the processing of red petals

4.4.1 General

It was decided to study the effects of the various physical aspects of the foliage process on petals and flower heads. In particular process temperature, process time

and the basic process fluid composition were considered since these were the most obvious problem areas. Initially the addition of copper sulphate and /or chlorophyll was not required since these additives were originally intended to retain the green colouration in leaves.

4.4.2 Experimental

4.4.2.1 Temperature

A solution containing 60% v/v propan-1,2-diol, 25% v/v water, 10% v/v butan-1,4-diol, 5% v/v propan-2-ol (pH 4) was prepared. Red rose petals (unknown variety) were processed in this solution for 5 minutes at temperatures of 20 °C, 40 °C, 50° C, 60° C, 70 °C and 80 °C.

4.4.2.2 Time

The above experimental work (4.4.2.1) was repeated but the process times were 1 minute, 3 minutes, 5 minutes, 10 minutes, 20 minutes and 30 minutes and the petals in the 20 °C solution were also allowed to soak overnight.

4.4.2.3 Composition

The following solutions, in which the proportions of the main ingredients were varied, were prepared:

i. 40% v/v butan-1,4-diol, 60% v/v propan-1,2-diol

ii. 50% v/v butan-1,4-diol, 50% v/v propan-1,2-diol

iii. 75% v/v butan-1,4-diol, 25% v/v propan-1,2-diol

iv. 100% water

v. 60% v/v propan-1,2-diol, 25% v/v water, 10% v/v butan-1,4-diol, 5% v/v propan-2-ol

Petals from red roses (unknown variety) were processed for 5 minutes (in the first instance) at 80 °C (the temperature generally used to process foliage) in each of these solutions.

4.4.3 Results and discussion

4.4.3.1 Temperature

At temperatures of 60 °C and above the colour of the petals initially changed from red to pale crimson before finally turning almost colourless. Some colour bleeding was observed but not to the extent that it could account for the significant colour loss in the petals. It was also noted that during processing the petals became translucent, with the translucency starting at the periphery and moving inwards (on processing leaves translucency also occurred, but it did so in a random fashion). Furthermore, it was observed that the higher the process temperature, the faster the sequence of colour changes.

When dried, the petals appeared to have retained their body and regained their opaqueness but had lost most of their colour.

At temperatures of less than 60 °C no colour changes were observed and on drying the petals became shrivelled. The indication here was that at temperatures of 60 °C and above an exchange between the cell sap and the process fluid had taken place (i.e. processing had taken place), whereas below this temperature the exchange was prevented. The immediate change in colour from, red to pale crimson on immersing

the petals into the hot process fluid could have been due to the effect of temperature on copigmented or self-associated pigments (section 3.6).

The fact that processing only appeared to take place at temperatures above 60 °C may have been because at this temperature the cuticular barrier (Fig. 2.7) was removed, thereby allowing the process fluid to penetrate the cell walls.

The translucency of the petals on ingress of the process fluid could have been due to the expulsion of air from the intercellular spaces. This effect was then reversed on drying, giving a return to opaque petals (section 2.5).

The observation that the process fluid entered from the periphery of the petals and moved inwards, as opposed to the apparently random fashion in which it entered the leaves, could have been due to the process fluid entering by the veinlets in petals in the absence of stomata (section 2.5).

4.4.3.2 Time

Processing times less than 5 minutes generally resulted in only partly processed petals (although this was to some extent related to the size of the petals in that smaller petals appeared to process after shorter times). Processing for periods of 5 minutes or longer did not appear to affect the end results. Soaking the petals overnight at 20 °C did not result in processed petals.

It appeared therefore that after processing times of 5 minutes, temperature not time was the main factor determining whether processing took place. In other words, at temperatures below 60 °C processing did not take place, whatever the processing time, possibly since the cuticle was still intact. Furthermore, the processing time for petals was much more dependent on size than process time for leaves, since in the

former the fluid was required to move from the periphery inwards, whereas for the leaves ingress was continuously occurring over the entire area at any given time (possibly entering via the stomata, which are generally absent in petals).

The effects of temperature and processing time on pigment concentration and stability are investigated more quantitatively in chapter 5.

4.4.3.3 Composition

As was observed previously, the colour of the petals changed from red to pale crimson on submersion in the hot process fluids. In the case of the water however, the colour did not fade to colourless and become translucent (after turning pale crimson) as was the case for the diol solutions. It was also observed that on drying, the petals processed in water regained their colour but became withered, whereas the petals processed in the remaining solutions were supple and of full body. The petals processed in the non-aqueous solutions were significantly firmer than those processed in the aqueous solution and there was little difference in the overall appearance of the petals processed in the different diol only solutions.

(The above work was repeated using a process time of 20 minutes and the results were identical. Processing in these solutions for periods of 5 and 20 minutes at a temperature of 60 °C indicated process times longer than 5 minutes were required at this temperature).

It appeared, therefore, that at high temperatures the arrangement of the pigment molecules in the petals was disrupted giving rise to an observed colour change (possibly a copigmentation or self-association effect). When water alone was used for processing, the original colour returned on cooling indicating that the pre-

processing arrangement of the pigments had also resumed. However, for the solutions containing diols the original colour was lost completely, indicating that the diol solution had largely replaced the aqueous cell sap and prevented the pigment molecules from reforming their pre-treatment arrangement. Again, this could be explained by the molecular association effects (e.g. copigmentation). In the case of water, the pigments would still be in a purely aqueous solution after processing in which event stabilisation by copigmentation would be at its best (section 3.6), giving rise to coloured petals even at higher pH (i.e. pH 3-5, the pH of cell sap). Whereas, in the case of the diol solutions, the pigments would be in a diol or diol/water solution after processing and stabilisation by copigmentation would be much less effective, if occurring at all. Since the pH of the diol solutions was approximately pH 4 and no stabilisation mechanism was in place, then the anthocyanins would be in their colourless form and the resulting processed petals would also be colourless.

4.5 The effects of acidification

4.5.1 General

Given that at pH<2 the anthocyanins exist in their stable, highly coloured form and that anthocyanins in their colourless carbinol form can regenerate the coloured flavylium ion on acidification (Fig. 3.9), it was decided to investigate the effects of acidification.

4.5.2 Experimental

4.5.2.1 Addition of acid to a processed petal

A petal which had been processed in the 60% v/v propan-1,2-diol, 25% water, 10% v/v butan-1,4-diol, 5%v/v propan-2-ol solution for 5 minutes at 80 °C was dipped in a 50% v/v HCl solution.

4.5.2.2 Acidification of the process solution with HCl

With the exception of the 100% water process medium, solutions as described in section (4.4.2.3) were prepared. To each process mix in turn was added concentrated HCl at levels of 125 g/L, 50 g/L, 25 g/L and 5 g/L (pH <2). Non-acidified solutions were run as controls. Red petals (unknown variety) were processed in these solutions for periods of between 1 and 30 minutes at a temperature of 80 °C.

4.5.2.3 Assessment of alternative acids

Various diol solutions were prepared and to each was added the following acids:

i. trifluoroacetic acid (10 g/L, 20 g/L)

ii. acetic acid (20 g/L, 50 g/L, 100 g/L)

iii. formic acid (10 g/L, 50 g/L, 125 g/L)

iv. *p*-toluenesulphonic acid (10 g/L, 20 g/L, 25 g/L, 50 g/L)

v. citric acid (20 g/L, 50 g/L, 100 g/L, 200 g/L)

vi. phosphoric acid (20 ml/L)

vii. sulphuric acid (20 ml/L)

viii. nitric acid (20 ml/L)

ix. 1-naphthalene sulphonic acid (20 g/L, 40 g/L)

x. 2-naphthalene sulphonic acid (20 g/L, 40 g/L)

xi. 1,8-naphthalene dicarboxylic acid (20 g/L, 50 g/L)

xii. 4-aminonaphthalene-1-sulphonic acid (20 g/L, 50 g/L)

xiii. 5-aminonaphthalene-1-sulphonic acid (20 g/L, 50 g/L)

Red rose petals (unknown variety) were processed in solutions containing the above acids at a temperature of 80 °C for periods of between 5 and 30 minutes.

4.5.3 Results and discussion

4.5.3.1 Addition of acid to a processed petal

The petal, which had been almost colourless, was observed to turn red after several seconds on immersion in the 50% v/v HCl solution.

The indication here was that the colour had not been lost as a result of bleeding but that the pigments had largely remained in the petal in a colourless form i.e. as the carbinol (Fig. 3.9). It appeared that the addition of acid regenerated the coloured flavylium cation (as predicted) and that although the colour of the processed petals was not identical to the original (possibly due to the disruption of the pre-treatment arrangement of the pigments) the colour was still strong and visually attractive.

The next logical step was to study the effects of acidifying the process mix and to try to determine suitable acids and optimum acid strengths.

4.5.3.2 Acidification of the process solution with HCl

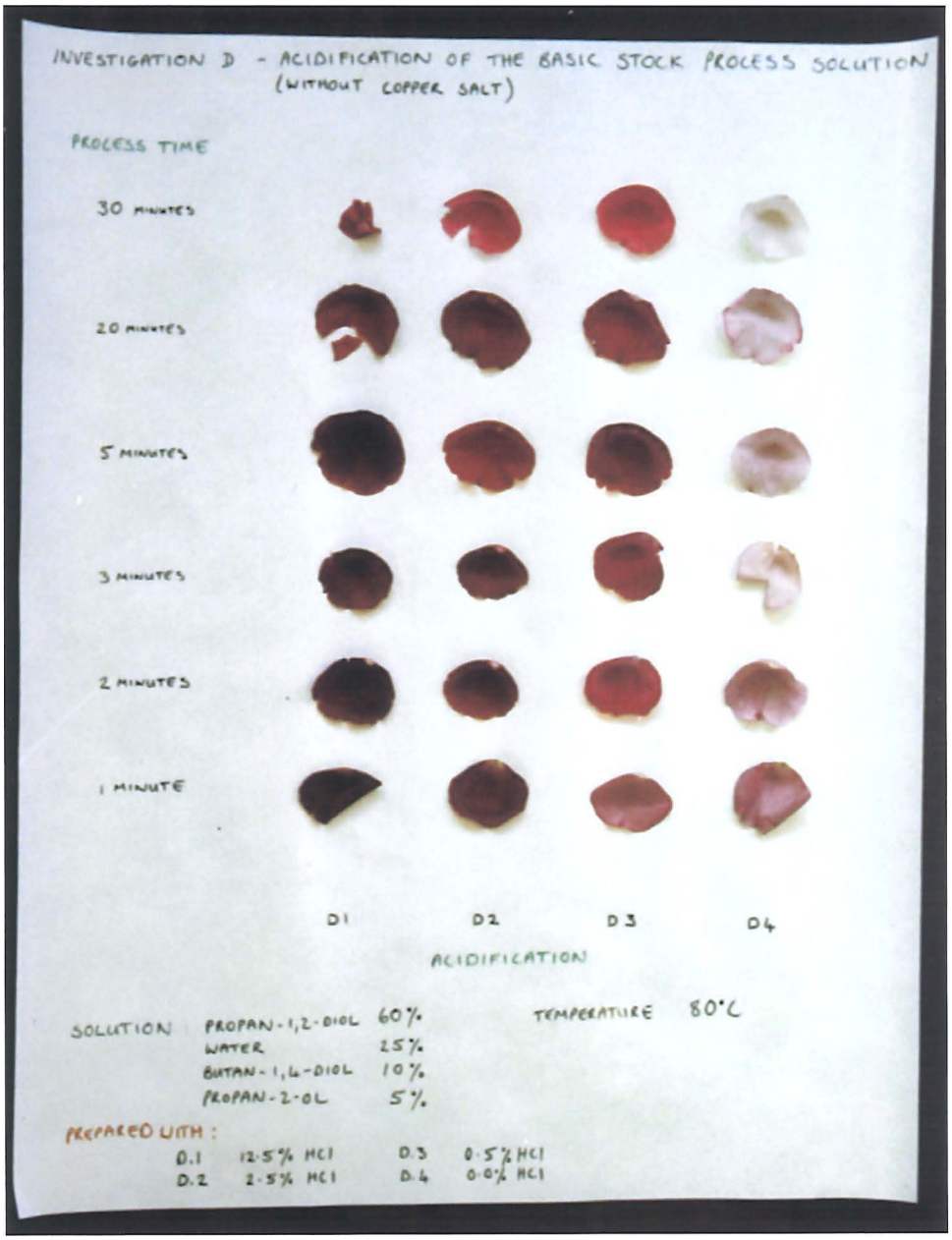
On submerging petals in the above solutions the petals were observed to initially turn pale in colour and then, in the case of the acidified solutions, the red colouration was seen to return as the process fluid entered the petals.

In general the best results with regards final colour were for acid strengths greater than 5 g/L. With regards body, suppleness and longevity 125 g/L HCl was found to destroy the tissue (particularly for process times greater than 20 minutes) and too short a process time (i.e.<5 minutes) gave rise to petals that dried out quickly (photograph 4.1). The best overall results were for the 75% v/v butan-1,4-diol, 25% v/v propan-1,2-diol mix to which had been added between 25 g/L and 50 g/L HCl at process times of 5-20 minutes.

Acidification of the process fluid, therefore, appeared to be a successful means of retaining colour in the processed petals (note: in general the colour of the petals was still reasonably strong 4 months after processing although the petals began to shrivel around the periphery). It remained, therefore, to assess other acids for commercial suitability.

4.5.3.3 Assessment of alternative acids

With the exception of the last 3 listed acids and nitric acid all the above mentioned acids successfully retained a red colouration in the petals. The naphthalene-sulphonic acids and the *p*-toluenesulphonic acids appeared to be particularly effective, possibly as a result of the acids contributing to the copigmentation effect (i.e. stabilisation by the hydrophobically reinforced π-π stacking of the aromatic nucleii of the acids with



Photograph 4.1 Acidification of the process solution with HCl.

D1. 12.5% v/v HCl D2. 2.5% v/v HCl D3. 0.5% v/v HCl D4. 0.0 % HCl

those of the pigment). Weaker acids such as citric acid, acetic acid and formic acid were required to be present in concentrations of greater than 100 g/L.

On considering cost, safety (Appendix 4) and overall effectiveness, *p*-toluenesulphonic acid, at concentrations of 20 g/L and above (pH <2), appeared to be comparable to hydrochloric acid as a suitable commercial ingredient.

In chapter 5 a more quantitative approach to colour stabilisation by the addition of *p*-toluenesulphonic acid is considered.

4.6 Processing of flower heads

4.6.1 General

Having successfully retained the red colouration of petals by acidification the next stage of the project was to produce whole flower heads which were not only of acceptable colour but also of good body, firmness and general appearance. It was to be noted that previous attempts to process whole flowers in the aqueous commercial solution (section 4.3) had not only resulted in a loss of colour but had also produced limp sticky heads. It was therefore decided to investigate the use of the acidified non-aqueous diol soutions (which had produced superior results for petals) for the processing of whole heads and to compare the results with those for the acidified commercial solution.

4.6.2 Experimental

Red rose heads (unknown variety) were processed for 25 minutes at 80 °C in the following solutions:

i. 60% v/v propan-1,2-diol, 25% v/v water, 10% v/v butan-1,4-diol with 25 ml/L HCl added

ii. 75% v/v butan-1,4-diol, 25% v/v propan-1,2-diol with 25 ml/L HCl added.

The heads were rinsed with water and then allowed to dry.

4.6.3 Results and discussion

The processed flower heads were all of a similar strong red colouration (although a slightly different shade than the unprocessed heads) for roses processed in both solutions. The rose heads processed in the aqueous solution (i) had become limp with the petals having become sticky and collapsed. The rose heads processed in the diol solution (ii) were, on the other hand, of good body and overall appearance although slightly oily.

4.7 Two-stage processing of whole flowers

4.7.1. General

It appeared that the most successful results for the processing of rose heads were for non-aqueous solutions. Attempts at processing other flowers such as carnations and irises also showed this to be the case. Therefore, a two-stage process in which the heads were processed in the acidified non-aqueous solution and the stems were processed in the commercial solution with copper salt and chlorophyll (section 4.3) appeared to be the way forward.

4.7.2 Experimental

A selection of red roses (variety: Sacha), carnations and tulips were processed in two stages. The heads were processed at 80 °C for periods of 20-45 minutes in the 75% v/v butan-1,4-diol, 25% v/v propan-1,2-diol, 25 g/L HCl solution and the stems and leaves were processed at 80 °C for 20-45 minutes in the aqueous commercial solution (section 4.3).

4.7.3 Results and discussion

The appearances of the individually processed parts of each flower were reasonably good (although the rose leaves dried out quickly and a slight brown deposit was observed on the underside of the leaves). In practice however, it proved difficult to process the green sepals of the roses and carnations in a two-stage process. Processing in the commercial foliage solution to below the sepals (i.e. to the top of the stem) resulted in the loss of their green colouration and made the slight staining from the petal pigments more obvious. A weakness in the "neck" of the stems, possibly due to double processing, or to the condition of the plant pre-processing, was also observed. Processing in the commercial solution to just above the sepals gave rise to over processed or non- processed areas of the petals.

4.8 One-stage processing of whole flowers

4.8.1 General

From a practical standpoint a two-stage process was going to prove challenging, therefore it was decided to investigate the possibility of using a one-stage process.

Initially this was to involve preparing a composite petal/foliage solution that was non-aqueous and contained hydrochloric acid, copper salt and chlorophyll and then studying the effects of processing leaves and petals in this solution. This was followed by the processing of whole flowers by total submersion and processing for 30 minutes.

This entire process was then repeated using slightly less butan-1,4-diol in an attempt to reduce oiliness and using *p*-toluenesulphonic acid for acidification since this had been the preferred acid in previous tests (section 4.5).

4.8.2 Experimental

4.8.2.1 Acidification using HCl

A solution containing 75% v/v butan-1,4-diol, 25% v/v propan-1,2-diol, 10 g/L copper sulphate, 25 ml/L hydrochloric acid and a chlorophyll boost (grass, 100 g/L) was prepared. Petals and leaves from red roses (variety: Sacha) were processed in this solution at 80 °C for periods of between 5 and 45 minutes.

Whole roses and carnations (unknown varieties) were then processed in this solution for 30 minutes.

4.8.2.2 Acidification with *p*-toluenesulphonic acid and reduction in butan-1,4-diol content.

A solution containing 50% v/v butan-1,4-diol, 50% v/v propan-1,2-diol, 10 g/L copper sulphate, 25 g/L *p*-toluenesulphonic acid and a chlorophyll boost (100 g/L

grass) was prepared. Petals and leaves from red roses (variety: Sacha) were processed in this solution for between 5-45 minutes at a temperature of 80 °C.

Whole roses and carnations (unknown varieties) were then processed in this solution for 30 minutes.

4.8.3 Results and discussion

4.8.3.1 Acidification with HCl

The colour of the rose petals was found to be a slightly darker red than for the petals processed without copper salt and chlorophyll (section 4.5) and the overall appearance of the petals was good.

A processing time of at least 30 minutes was required for the rose leaves giving rise to leaves of good green colouration and supple feel (more supple than for leaves processed in the aqueous commercial solution (section 4.5), either with or without added acid). It was also noted that the brown deposit (possibly a metal-tannin complex[3]) observed on the underside of leaves processed in the non-acidified commercial solution had not appeared. Subsequent processing of whole red roses and carnations under these conditions produced flowers of good colour and body, with no apparent weakness in the necks and with green coloured sepals. There was, however, oiliness to the petals, which was particularly noticeable in the larger petals of the roses.

4.8.3.2 Acidification with *p*-toluenesulphonic acid and reduction of butan-1,4-diol content.

Initially the colours of the processed petals and leaves appeared to be similar to those processed in HCl solutions. After a period of 4 months, however, the colour of the petals processed in the solution containing HCl had faded slightly and developed an orange tinge. This was possibly due to evaporation of the hydrochloric acid with time, but the superior results for the aromatic acid could also have been due increased stabilisation of the anthocyanin pigments as a result of the *p*-toluenesulphonic acid taking part in the copigmentation effect.

Long term, therefore, the results for acidification by *p*-toluenesulphonic acid were superior to those for HCl, but there still appeared to be a slight oiliness to the petals even although the butan-1,4-diol content had been reduced.

4.9 The use of propan-2-ol in the final rinse stage

4.9.1 General

Throughout the work carried out, a slight bleeding of the anthocyanin pigments into solution was observed during processing. This did not appear to detract from resulting visual colour of the petals, however, occasionally the sepals of roses became stained with red pigment. This staining appeared to take place during drying, before the rinse water had dried off. In an attempt to prevent this happening it was decided to dry the roses as quickly as possible and to facilitate this by using a volatile solvent in the final rinse stage and then oven drying. Propan-2-ol was chosen

as the solvent, mainly because it was already an ingredient in the foliage solution and the drying was conducted in an oven set at an arbitrary temperature of 50 °C.

4.9.2 Experimental

Red roses and carnations processed in a range of solvent mixtures were dipped in:

i. water at room temperature

ii. propan-2-ol at room temperature

iii. water followed by propan-2-ol at room temperature

All roses were dried in the oven for 48 hours at 50 °C (after which they appeared dry and opaque).

4.9.3 Results and discussion

Oven drying after rinsing with propan-2-ol (ii), or water followed by propan-2-ol (iii), reduced staining of the sepals. Furthermore, these two treatments had the effect of separating the petals and causing them to stiffen slightly. The slight oiliness of petals containing a high proportion of butan-1,4-diol was seen to persist.

4.10 The effects of higher molecular weight diols

4.10.1 General

It was decided to try to reduce the oiliness of the petals by investigating the effects of other higher molecular weight diols such as pentan-1,5 -diol and hexan-1,6-diol. Being solid at room temperature (M.P., 41-43 °C) it was hoped that hexan-1,6-diol

would not only reduce oiliness but would also improve firmness. Higher molecular weight diols were not considered at this stage, partly due to cost.

4.10.2 Experimental

The following solutions were prepared:

i. 25% v/v pentan-1,5-diol, 75 % v/v propan-1,2-diol, 25 g/L *p*-toluenesulphonic acid

ii. 50% v/v pentan-1,5-diol, 50% v/v propan-1,2-diol, 25 g/L *p*-toluenesulphonic acid

iii. 75 % v/v pentan-1,5-diol, 25% v/v propan-1,2-diol, 25 g/L *p*- toluenesulphonic acid

a second set of solutions in which hexan-1,6-diol was used in place of the pentan-1,5-diol was also prepared.

Red rose petals (variety: Sacha) were processed in the above solutions at 80 °C for periods of 10-30 minutes.

4.10.3 Results and discussion

Increasing the proportion of the higher molecular weight diol resulted in firmer petals, however, in the case of pentan-1,5-diol the petals also became more oily. Pentan-1,5-diol is more expensive (Appendix 4) than the other diols used to date therefore there appeared to be no advantage in using pentan-1,5-diol as an ingredient.

Hexan-1,6-diol, on the other hand, had the effect of improving the body and firmness of the petals whilst reducing oiliness. At levels of 50-75% w/v hexan-1,6-diol, however, the petals became too rigid with crystals. Processing petals in the 75% w/v propan-1,2-diol and 25% w/v hexan-1,6-diol solution initially produced firm, non-oily petals, however after two weeks crystals began to appear and the petals took on a slightly papery feel.

4.11 Processing of whole roses using hexan-1,6-diol as an ingredient

4.11.1 General

As hexan-1,6-diol is relatively inexpensive (Appendix 4) and appeared to have the desired effects (i.e. it improved firmness and reduced oiliness), it was decided to continue trials with this ingredient. To counteract the dryness it was decided that butan-1,4-diol should also be used in the mixture and that optimisation trials using various proportions of propan-1,2-diol, butan-1,4-diol and hexan-1,6-diol should be carried out.

4.11.2 Experimental

The following solutions each containing 25 g/L *p*-toluenesulphonic acid, 10 g/L copper sulphate and a chlorophyll boost (nettles, 100g /L) were prepared (Table 4.1). Red roses (unknown varieties) were processed in these solutions at 80 °C for periods of between 30-60 minutes. The roses were rinsed with propan-2-ol and then oven dried at 50 °C.

Solution	% Propan-1,2-diol	% Butan-1,4-diol	% hexan-1,6-diol
1	65	10	25
2	70	5	25
3	50	10	40
4	75	-	25
5	50	50	-
6	55	20	25
7	60	15	25
8	-	50	50
9	65	25	10
10	45	45	10
11	65	17.5	17.5

Table 4.1 Solutions of diol mixtures.

4.11.3 Results and discussion

From these trials it emerged that the optimum processing time for both heads and leaves was around 30 minutes and that the stage of development and the condition of the plant were important contributory factors to successful processing (poor results were obtained for damaged plants or those processed at a stage too early or late in their development).

In general it appeared that the hexan-1,6-diol gave body and firmness to the heads and leaves (although too much of this diol, as in the case of solution (3), resulted in the appearance of crystals on drying) and that butan-1,4-diol helped to keep the petals and leaves supple. The role of the propan-1,2-diol was uncertain (since using the pure diol caused the petals to become limp), but in the absence of this diol the petals and leaves became extremely oily as was the case for solution (8) (Table 4.1).

The processing of foliage emerged to be more problematic than the processing of flower heads. This was possibly due, in part, to the fact that temperature variations at around 80 °C appear to be more critical for foliage and also to the need to maintain sufficient levels of copper ions and chlorophyll in the processing solution.

Overall, however, the most consistently superior results for both flower heads and foliage were for those processed in solution (1). Solution (9) also gave promising results.

4.12 Prevention of growth of hexan-1,6-diol crystals

4.12.1 General

Although solution (1) had initially given excellent results, after about one month of normal laboratory conditions, crystals of hexan-1,6-diol (identified by melting point and ^{1}H NMR), were observed on the surface of the leaves and petals processed in this solution. It had been noticed that under conditions of high temperature (>45 °C) or high humidity that the crystals did not develop (or, in the case of previously processed flowers, disappeared). The former of these observations could be attributed to the melting point of crystals and the latter to the high solubility of the crystals in water. The development of crystals appeared to be due to evaporation of

the process fluid, possibly due to evaporation of the lowest molecular weight diol, but more likely to the evaporation of any water remaining in the processed plant. A means of preventing this evaporation, for instance by a adding a hygroscopic substance such as glycerol or some other suitable high molecular weight ingredient such as polyethylene glycols could also be considered. Another possibility was to physically seal the water and process fluid inside the leaves and petals by applying a suitable coating material, for example, the coating material mentioned in the foliage process (Polidene 33-004), which is claimed to produce a soft water resistant film.
It was decided to continue trials with a mixture based on solution (1) and to attempt the above ideas as a means of improvement.

4.12.2 Experimental

4.12.2.1 The use of glycerol in the process solution

Solutions were prepared in which 1-5% v/v of glycerol was used as a replacement for an equivalent proportion of propan-1,2-diol, butan-1,4-diol and hexan-1,6-diol in solution (1), for example:

i. 65% v/v propan-1,2-diol, 20% v/v hexan-1,6-diol, 5% v/v glycerol, 10% v/v butan-1,4-diol, 10 g/L copper sulphate, 20 g/L *p*-toluenesulphonic acid.

ii. 65% v/v propan-1,2-diol, 25% v/v hexan-1,6-diol, 5% v/v butan-1,4-diol, 5% v/v glycerol, 10 g/L copper sulphate, 20 g/L *p*-toluenesulphonic acid.

iii. 60% v/v propan-1,2-diol, 5% v/v glycerol, 25% v/v hexan-1,6-diol, 10% v/v butan-1,4-diol, 10 g/L copper sulphate, 20 g/L *p*-toluenesulphonic acid.

(the chlorophyll boost was not considered necessary for this investigation)

Red roses (variety: Sacha), blood red carnations, red carnations and purple irises were processed in these solutions at 80 °C for periods of 30 minutes. The flowers were rinsed with propan-2-ol and dried at 50 °C for two days.

4.12.2.2 The use of polyethylene glycols in the process solution

Solutions were prepared in which 5% of the hexan-1,6-diol from solution (1) was replaced by PEG 400 (hygroscopic) and PEG 600 respectively. Red roses (variety: Sacha), blood red carnations, red carnations and purple irises were processed in these solutions at 80 °C for 30 minutes. The flowers were then rinsed with propan-2-ol and dried at 50 °C for two days.

4.12.2.3 The use of Polidene coatings

Whole roses (variety:Sacha) previously processed in solution (1), rinsed with propan-2-ol and then dried at 50 °C were dipped in 10% v/v, 15% w/v and 20% v/v aqueous solutions of the Polidene preparation and then left to dry under normal laboratory conditions.

4.12.3 Results and discussion

4.12.3.1 The use of glycerol in the process solution

In all cases the formation of crystals was reduced by the addition of glycerol (compared to the control-solution (1)) with the slowest rate of crystal growth for solutions containing 5% glycerol (no crystals for up to 2 months), however the heads tended to become limp and slightly oily. The best overall results were for flowers processed in solutions in which the glycerol replaced 5% of the propan-1,2-diol.
It appeared that increasing the glycerol content slowed down the formation of crystals. This could have been due to the ability of glycerol to retain the water needed to keep the hexan-1,6-diol in solution and/or to the fact that it does not evaporate readily and was therefore also available as a solvent for hexan-1,6-diol.

4.12.3.2 The use of polyethylene glycols in the process solution

The results for both PEG solutions were similar. In general it was observed that although the PEG solutions slowed down and reduced the severity of crystal growth (compared to the control-solution (1)) the results were worse than for glycerol in this respect. The PEG solutions, however, produced less oily petals than the glycerol solutions.

4.12.3.3 The use of Polidene coatings

For both the rose heads and leaves the 10% Polidene coating appeared to slow down the formation of crystals (up to 2 months) and reduce their quantities considerably

(compared to the control). The worst area of crystal growth on the petals was around the peripheries.

The 15% and 20% Polidene coatings gave very similar results in that no crystals appeared on the rose heads or leaves. The higher concentrations of coating improved the suppleness of the leaves but were unsuitable for the flower heads in that the petals acquired an unnatural rubbery appearance.

From the above results it seemed that a 10% Polidene coating was not providing sufficient cover to prevent evaporation and that the 15% and 20% coatings were too heavy.

Coating was, however, still considered to be an option but it was felt that more research into suitable coating materials was required and that the product should be as refined as possible before coating.

4.13 The effects of adding alternative ingredients to hexan-1,6-diol

4.13.1 General

Since the problem of the hexan-1,6-diol crystallisation had not been resolved it was decided to replace this ingredient with alternative substances which it was hoped would provide the firmness and body contributed by the hexan-1,6-diol but without the associated crystallisation problems. Substances considered were polyethylene glycols (PEGs), sugars (as these have been used as bulking and impregnating agents in the conservation of wood[4]) synthetic polymers and polysaccharides (in particular setting agents such as pectin and carrageenan which it was hoped would produce the same petal firmness and body as hexan-1,6-diol).

4.13.2 Experimental

4.13.2.1 Polyethylene glycols as replacements for hexan-1,6-diol

Solutions based on solution (1) in which the 25% w/v hexan-1,6-diol was replaced by PEG 400, PEG 600, PEG 900, PEG 1000, PEG 1500 and PEG 2000 respectively, were prepared. Red roses (variety:Sacha) and red carnations were processed in the above solutions at 80 °C for 30 minutes, rinsed with propan-2-ol and dried at 50 °C.

4.13.2.2 Sugars as replacements for hexan-1,6-diol.

Solutions based on solution (1) in which the hexan-1,6-diol was replaced by glucose, fructose, lactose, inositol, trehalose and sorbitol (the alcohol corresponding to glucose) were prepared. Red petals (variety: Sacha) were processed in these solutions for 20 minutes at 80 °C, then rinsed in propan-2-ol and oven dried at 50 °C.

4.13.2.3 Polymeric materials as replacements for hexan-1,6-diol

Attempts were made to prepare solutions based on solution (1) in which the 25% hexan-1,6-diol was replaced by polyvinyl alcohol and carboxymethylcellulose repectively. Both these materials, however, appeared to be insoluble in the diol mixtures and further attempts to prepare 1% solutions or to dissolve the polymers in water prior to adding the diols were also unsuccessful. 25% w/v aqueous solutions were prepared and red rose petals (variety: Sacha) were processed in these solutions for 20 minutes at 80 °C.

4.13.2.4 Polysaccharides as replacements for hexan-1,6-diol

1. Starch

Attempts were made to prepare a solution based on solution (1) in which the hexan-1,6-diol was replaced by starch, however the starch appeared to be insoluble in the diol mixture.

A 20% w/v aqueous solution was prepared and red rose petals (variety: Sacha) were processed in this solution for 20 minutes at 80 °C.

2. Carrageenan, Pectin, polygalacturonic acid

1% w/v and 5% w/v aqueous and non-aqueous solutions (based on 90% v/v propan-1,2-diol, 10% v/v butan-1,4-diol, 20 g/L *p*-toluenesulphonic acid, 10 g/L copper sulphate) of carrageenan (a carbohydrate product extracted from seaweed), pectin (a carbohydrate product containing methyl pectate) and polygalacturonic acid (pectic acid) were prepared.

1% w/v and 5% w/v pectin solutions based on the sugar solutions of (4.13.2.2) were also prepared (in a attempt to improve setting). Red rose petals (variety: Sacha) were processed in these solutions at 80 °C for 20 minutes.

4.13.3 Results and discussion

4.13.3.1 Polyethylene glycols as replacements for hexan-1,6-diol

It was observed that for solutions containing PEGs of molecular weight higher than 600, the flower heads appeared to collapse and become dry and brittle with time

(after approximately one month). This effect was seen to progressively worsen with increasing molecular weight. Flowers processed in PEG 400 and PEG 600 suffered considerable colour loss, particularly at the edges of petals.

It seemed that the higher molecular weight PEGs had not penetrated the cell walls and that some form of osmotic collapse had taken place. PEG 400 and PEG 600 did not appear to have distributed the acid evenly throughout the petals (as adding acid to the colourless areas regenerated the red colouration indicating that the pigment was still present in these areas).

Based on these observations, polyethylene glycols were considered unsuitable replacements for hexan-1,6-diol at levels of 25% or greater.

4.13.3.2 Sugars as replacements for hexan-1,6-diol

On processing red petals in these solutions the body and firmness of the petals was greatly reduced compared to solution (1) petals and with the exception of inositol, all the petals processed in the sugar solutions had slightly darkened in colour. It was noted that lactose and inositol were only sparingly soluble in the diol mixture and that sucrose and fructose appeared to caramelise (possibly forming furfural and 5-hydroxymethyl-furfural which are formed when sugars are heated with acids[3]).

Although the sugars did not fulfil their role as "bulking agents" the darkening in colour of the processed petals indicated that the sugars were passing through the cell walls and possibly interacting with the anthocyanin pigments.

4.13.3.3 Polymeric materials as replacements for hexan-1,6-diol

Processing in aqueous solutions of these polymers caused the petals to loose all body and eventually to disintegrate. These polymeric materials were therefore unsuitable "bulking agents" but could possibly be used as coating materials (Chapter 6).

4.13.3.4 Polysaccharides as replacements for hexan-1,6-diol

The petals processed in the aqueous starch solution lost all body and disintegrated as did the petals processed in the aqueous carrageenan, pectin and polygalacturonic acid solutions.

The petals processed in the 1% and 5% carrageenan gave promising results in that the body and firmness of the petals appeared to be slightly better than the blank (no added setting agents). As for the non-aqueous pectin solutions, the results were similar to those of the blank but there was a problem with residue on the petals (that could have been due to non-dissolved pectin). The polygalacturonic acid solutions gave similar results to the blank with regards to body and firmness but some colour loss was observed. The petals processed in the pectin and sugar solutions generally gave darker petals but showed no obvious improvements on the blank with regards body and firmness.

From the above observations it appeared that non-aqueous solutions of carrageenan gave the best overall results with regards to body and firmness. Whole red roses and carnations processed in this solution also gave good results although it may have been the case that the carrageenan had not actually penetrated the cell walls but had formed a coating on the surfaces of the leaves and petals. After a period of about one month, however, there appeared to be some fading of colour in the petals.

4.14 Reduction of hexan-1,6-diol content

4.14.1 General

It was decided, therefore, to return to the original mixture containing the 3 diols (propan1,2-diol, butan-1,4-diol and hexan-1,6-diol) (Table 4.1) and to consider reducing the proportion of hexan-1,6-diol and increasing the proportion of butan-1,4-diol in an attempt to keep the hexan-1,6-diol in solution. Solution (9), which had also been shown during the trials to produce good results, was chosen.

4.14.2 Experimental

Solution (9), containing 65% v/v propan-1,4-diol, 25% v/v butan-1,4-diol, 10% v/v hexan-1,6-diol, 20 g/L *p*-toluenesulphonic acid and 10 g/L copper sulphate was prepared.

Red roses (variety: Sacha), blood red carnations, red carnations and purple irises were processed in this solution at 80 °C for periods of 20-30 minutes. Solution (1) was used as a control.

4.14.3 Results and discussion

All flowers processed well and initially there were no significant differences between flowers processed in solution (9) and those processed in solution (1). After a period of 3 weeks in dry conditions crystals had appeared on the flowers processed in solution (1), whereas 3 months later no crystals had developed on the solution (9) flowers.

In as far as it provided body, firmness and texture to the flowers, solution (9) was considered to be a suitable alternative to solution (1) for the processing of whole flowers. Furthermore, with regards to colour, both solutions gave visually identical results for a given flower variety.

4.15 The effects of various combinations of acid, copper salt and chlorophyll on the processing of dark and pale coloured flowers

4.15.1 General

It was noted that the colour of the processed flower was seldom the same as the colour of the unprocessed flower and that solutions containing a chlorophyll boost always produced flower heads that were darker in colour than those without chlorophyll. In the case of red and other dark coloured flowers the chlorophyll solutions still produced attractive colours. However, since commercially there appeared to be a demand for a full range of preserved flower colours, it was decided that an investigation into the processing of paler coloured flowers was required before any scaling up of the process commenced.

4.15.2 Experimental

Solutions, as described in Table 4.2, with the following base mix and concentrations of acid, copper salt and chlorophyll boost were prepared:

Base mix: 65% v/v propan-1,2-diol, 25% v/v butan-1,4-diol, 10% v/v hexan-1,6-diol (solution (9)), acid: 20 g/L w/v *p*-toluenesulphonic acid, copper salt: 10 g/L copper sulphate, chlorophyll boost: 100 g/L grass.

Solution	Acid	Chlorophyll	Copper salt
A	+	+	+
B	+	-	-
C	-	-	-
D	-	+	-
E	-	+	+
F	+	-	+
G	-	-	+
H	+	+	-

Table 4.2. The various combinations of acid, copper sulphate and chlorophyll added to the process fluid.

Petals from red, pale yellow and pale peach roses (unknown varieties) were processed in the above solutions at 80 °C for 20 minutes and then rinsed with propan-2-ol. The petals were then subjected to 7 days of light box treatment (at 20,000 lux).

4.15.3 Results and discussion

The colours of the three varieties of roses after processing and light box testing were as described in Table 4.3. In general, the best results for all colours of flower with regards to light fastness and attractiveness were for those processed in solution F (containing acid and copper). Solution A (containing acid, copper and chlorophyll) also gave good results for light fastness but the resulting colours for pale flowers were less pleasing as a result of the apparent discolouration by chlorophyll.

Solution	After Processing	After Light Box Test
Red Petals		
A	Dark red	Dark red
B	Red	Deep pink
C	Pale lilac	V.pale lilac/white centre
D	Green	Cream
E	Grey	Pink/green
F	Red	Red
G	Lilac	Pale lilac
H	Red	Peach
Yellow petals		
A	Yellow/green	"dirty" yellow/green
B	Pale yellow	Faint yellow
C	Yellow	Cream
D	Bright green	Cream
E	Blue/green	Dull green
F	Yellow/blue tinge	Yellow
G	Gold/yellow	Cream/yellow
H	Dull green	Pale yellow
Pale Peach Petals		
A	Pink/green tinge	Peach/green tinge
B	Pink	Pale peach
C	Yellow	Cream/yellow
D	Bright green	Cream
E	Pink/grey/green	"dirty" grey green
F	Pink	Pink/peach
G	Yellow	Mushroom
H	Pink/green tinge	"dirty"peach

Table 4.3 The effects of various combinations of copper sulphate, chlorophyll and acid on the processing of petals.

The red pigments (anthocyanins) had behaved as expected in that they had stayed red in acidic solution (Fig. 3.9) and light stability had been improved by the addition of metal ions (in this case Cu (II)). The lilac colourations observed for the red petals after processing in solutions C and G could have been due to formation of the purple anhydrobase at higher pH (Fig. 3.9), which was stabilised slightly by the presence of copper ions in solution G.

The yellow pigments (which could also have combined with the red pigments to produce peach) were also stabilised by acid and copper. The identities of the yellow pigments, from the particular flowers studied, were unknown but it was most likely that they were flavonoids, (section 3.4) rather than the more strongly coloured carotenoids (section 3.3).

The problem of chlorophyll discolouration meant that a one-stage process for the processing of pale coloured flowers using acid, copper salt and chlorophyll was unsuitable. It therefore appeared that a return to a two-stage process, in which the foliage was processed separately from the flower heads, was going to be necessary if pale flowers were required.

4.16 The effect of copper sulphate concentration on the processing of red roses

4.16.1 General

The foliage process had required a copper sulphate concentration of 10 g/L, which also appeared to be suitable for the stabilisation of the floral pigments (section 4.15). Changing to a two-stage process, although practically problematic, meant that lower concentrations of copper sulphate could be considered. From a commercial

viewpoint any reduction in reagent costs would be beneficial and could possibly make the two-stage process a viable option.

4.16.2 Experimental

Petals from the varieties of red rose, First Red and Danse de Feu were processed for 30 minutes at 80 °C in solutions containing 65% v/v propan-1,2-diol, 25% v/v butan-1,4-diol, 10% w/v hexan-1,6-diol, 20 g/L *p*-toluenesulphonic acid and copper sulphate at levels of 0.0 g/L (1), 0.5 g/L (2), 1 g/L (3) and 10 g/L (4). The petals were then subjected to 7 days of light box treatment (operating at 20,000 Lux)

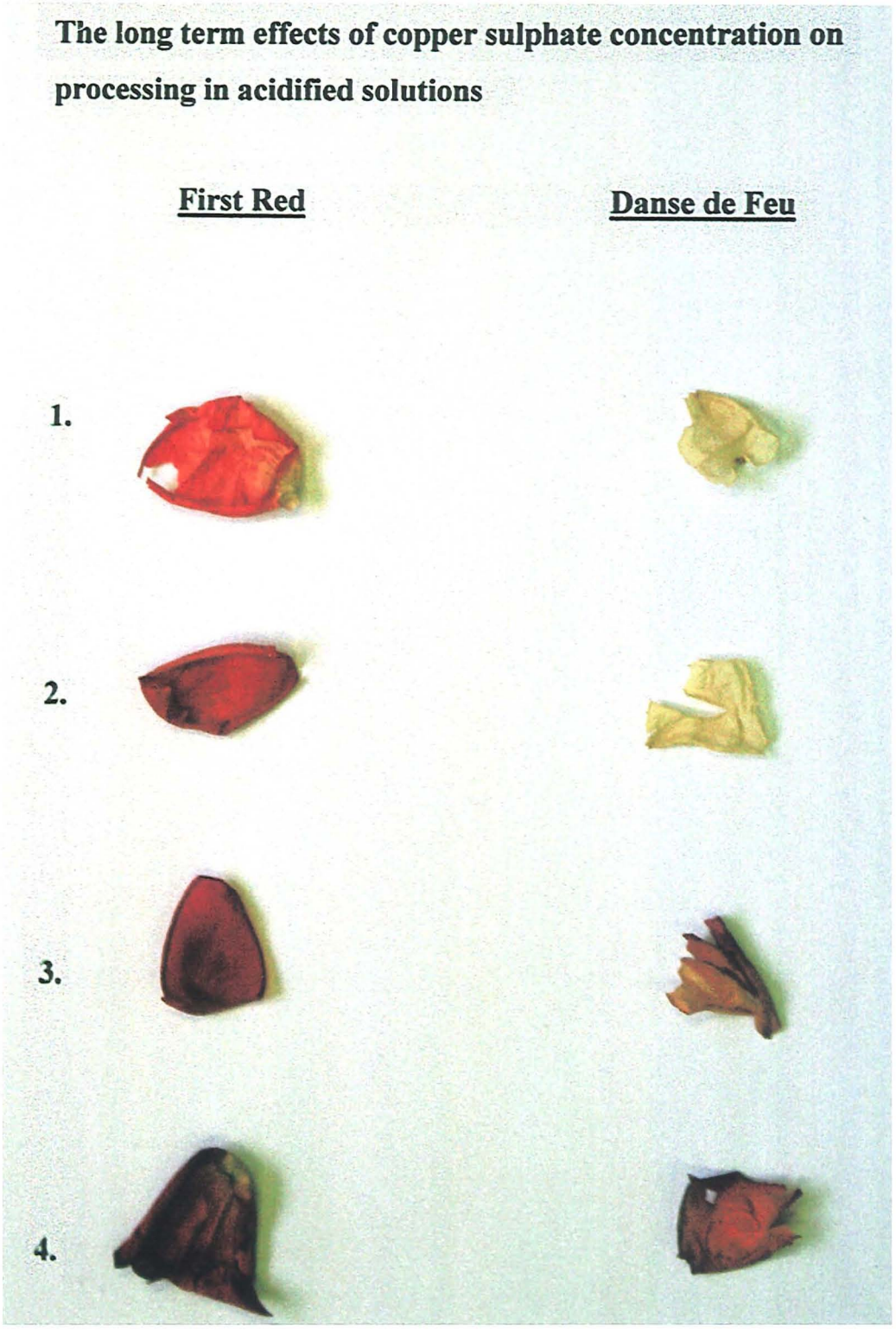
4.16.3 Results and discussion

The colours of the two varieties of roses on processing in solutions (1)- (4) and then

Solution	After processing	After light box test
First Red (dark red in colour)		
1	Red	Pale red
2	Dark red	Light red
3	Dark red	Red
4	Dark red	Red
Danse de Feu (light red in colour)		
1	Pink	Cream
2	Pink	Cream/yellow
3	Orange/red	Orange
4	Orange/red	Orange

Table 4.4 The effects of copper sulphate concentration on the processing of red roses.

1. 0.0 g/L $CuSO_4$ 2. 0.5 g/L $CuSO_4$ 3. 1.0 g/L $CuSO_4$ 4. 10 g/L $CuSO_4$



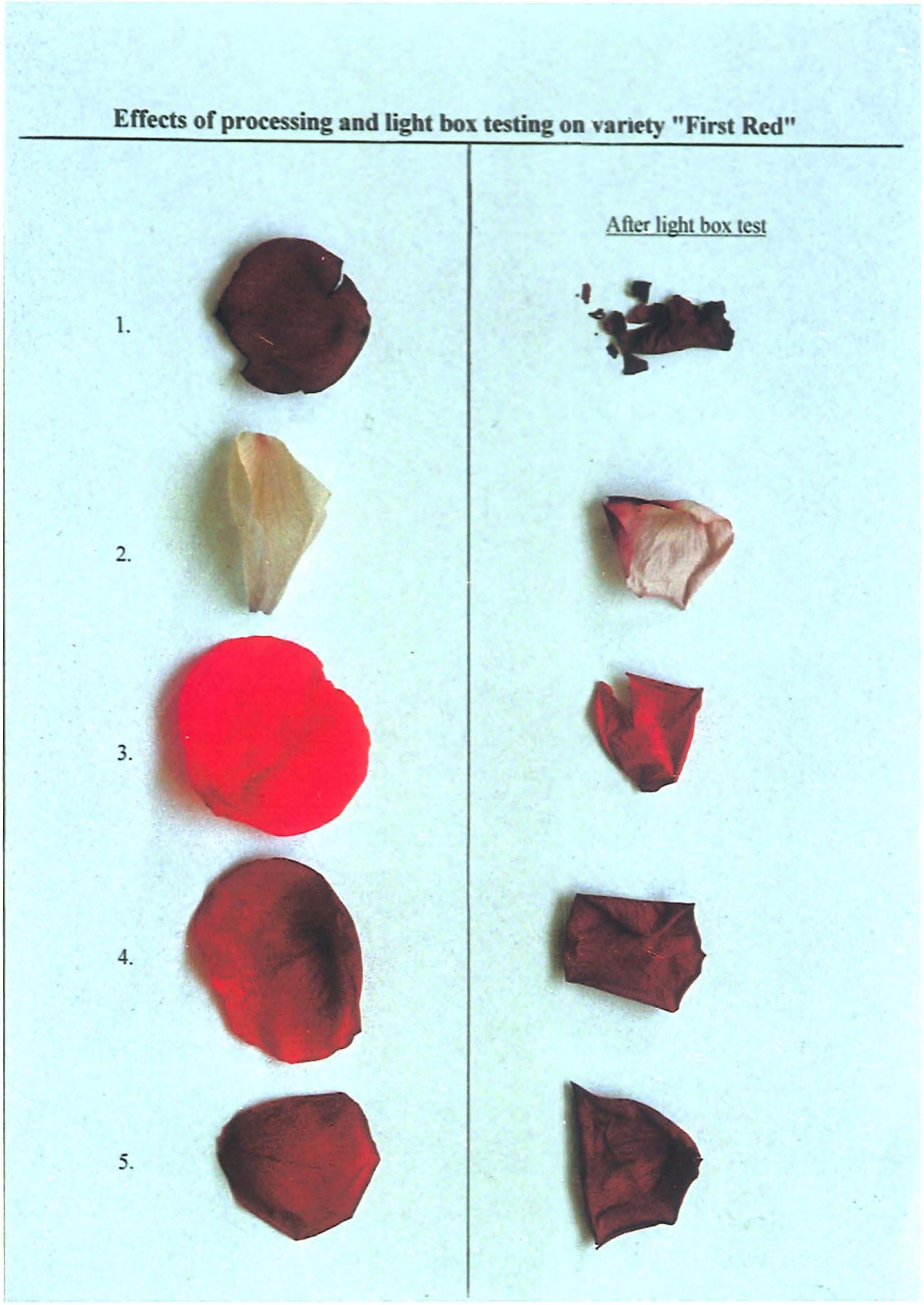
Photograph 4.2 The long term effects of copper sulphate concentration on the processing of red roses in acidified solutions (approximately 12 months after light box testing).

1. 0.0g/L$CuSO_4$ 2. 0.5g/L$CuSO_4$ 3. 1.0g/L $CuSO_4$ 4. 10g/L $CuSO_4$

subjecting to light box treatment were as described in Table 4.4. On leaving the petals to stand for approximately 1 year under normal laboratory conditions, the colours were as depicted in Photograph 4.2.

These results indicated that although the addition of copper sulphate down to levels of 0.5 g/L still gave reasonably good light stabilisation for the rose variety First Red, this level did not appear to be sufficient to maintain the red colouration in the second variety Danse de Feu. One possibility for this result was that Danse de Feu contained a higher proportion of flavonol pigments to anthocyanins than First Red and that the copper ions complexed preferentially with the flavonols[5] due to the presence of the hydroxyketo grouping (Fig. 3.4). This might also have explained the yellow/orange tinge to the Danse de Feu petals processed in the copper containing solutions. However, another explanation could have been that the main anthocyanin pigment was the orange/ red anthocyanin, pelargonin (Fig. 3.5), which may have needed a higher concentation of copper ions for stabilisation (although this stabilisation was unlikely to have been as a result of metal chelation due to the absence of a catechol group). In any case, it appeared that a concentration of above 0.5 g/L was generally required.

It was also noted that in the case of First Red, that the colour of the petals processed with concentrations of copper sulphate of 1 g/L and above were very close in colour to the unprocessed petals (Photograph 4.3).



Photograph 4.3 The effects of processing and 24 hour light box testing of variety First Red

1. fresh petal. 2. Petal processed without acid or copper sulphate. 3. Petal processed with acid but without copper sulphate.
4. Petal processed with acid and 1g/L copper sulphate. 5. Petal processed with acid and 10g/L Copper sulphate.

4.17 The effects of adding alternative metal salts to the process solution

4.17.1 General

As it is known that anthocyanins form stable coloured complexes with metal salts (section 3.6.6) it was decided to investigate the use of a range of metal salts in both acidified and non-acidified process solutions.

4.17.2 Experimental

4.17.2.1 The effects of alternative metal salts in the acidified process solutions

Solutions containing 65% v/v propan-1,2-diol, 25% w/v hexan-1,6-diol, 10% v/v butan-1,4-diol and 20 g/L *p*-toluenesulphonic acid to which 10 g/L each of the following metal salts had been added, were prepared:

1. Calcium sulphate, Ca (II)
2. Chromic sulphate, Cr (III)
3. Aluminium sulphate, Al (III)
4. Ferric chloride, Fe (III)
5. Ferrous sulphate, Fe (II)
6. Manganese sulphate, Mn (II)
7. Stannous sulphate, Sn (II)
8. Zinc sulphate, Zn (II)
9. Copper sulphate, Cu (II)
10. Cobalt sulphate, Co (II)
11. Nickel sulphate, Ni (II)
12. Magnesium sulphate, Mg (II)
13. Sodium molybdate Mo (VI))
14. Sodium tungstate, W(VI)

Solutions containing 1 g/L of these metal salts were also prepared and a blank (15) containing acid but no metal was run for comparison.

Petals from rose variety First Red were processed in the above solutions for 30 minutes at 80 °C. The petals were then rinsed with water and subjected to light box treatment for 7 days (operating at 20,000 lux). Petals from two (unknown) varieties of yellow rose and two other (unknown) varieties of red rose were processed (under the same conditions) in solutions (1)-(12) at metal concentrations of 10 g/L and then subjected to light box treatment.

4.17.2.2 The effects of alternative metal salts in the non-acidified process solutions

Solutions containing 10 g/L metal salts as in section (4.17.2.1) were prepared without acid. All varieties of rose were processed in solutions (1)-(9) for 30 minutes at 80 °C and then subjected to the 7 day light box test (continuous exposure to 20,000 lux for 7 days).

4.17.3 Results and discussion

4.17.3.1 The effects of alternative metal salts in the acidified process solutions

The effects on petals of rose variety First Red of processing in acidified solutions (1)-(15), and then light box testing and leaving to stand under normal laboratory conditions for approximately 6 months are shown in Photograph 4.4, for the 10 g/L metal solutions and Photograph 4.5, for the 1 g/L metal solutions.

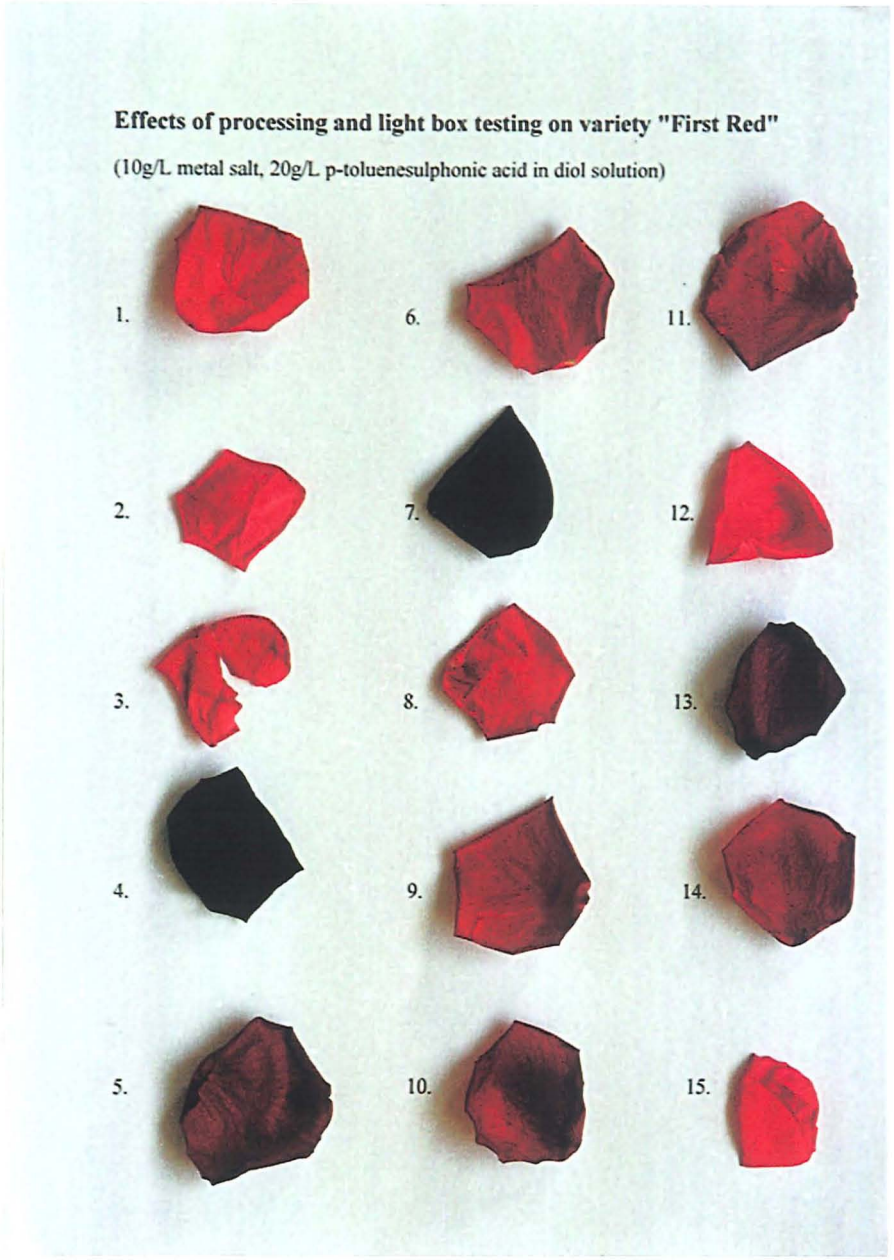
The additions of copper sulphate, cobalt sulphate, ferrous sulphate, sodium molybdate, sodium tungstate, manganese sulphate and nickel sulphate to the process solution at levels of 10 g/L appeared to give light fast colours very close to the original colours. The ferric chloride and stannous chloride salts gave reasonably light

fast red/brown and deep purple petals, respectively, for the 10 g/L solutions, but these colours were not obtained at the lower concentration of 1 g/L. The 1 g/L metal solutions generally gave slightly lighter petals than the corresponding 10 g/L metal solutions, with the exception of the copper, chromium, aluminium and magnesium metal salts which appeared to give the same colours for both concentrations (with only copper giving light fast colours).

In the case of the other flower varieties it also appeared that copper gave superior results for the two red varieties and showed some colour stabilisation of the yellow varieties. One variety red of rose was seen to have generally poorer light stability than the other, but the colour was seen to be stabilised with chromic sulphate as well as copper. Again ferric salts and tin salts changed the colours of the red petals to red/purple and deep purple respectively. The pale yellow petals gave a blue/black with ferric chloride (indicative of phenolic compounds) and stable deep yellows for stannous chloride.

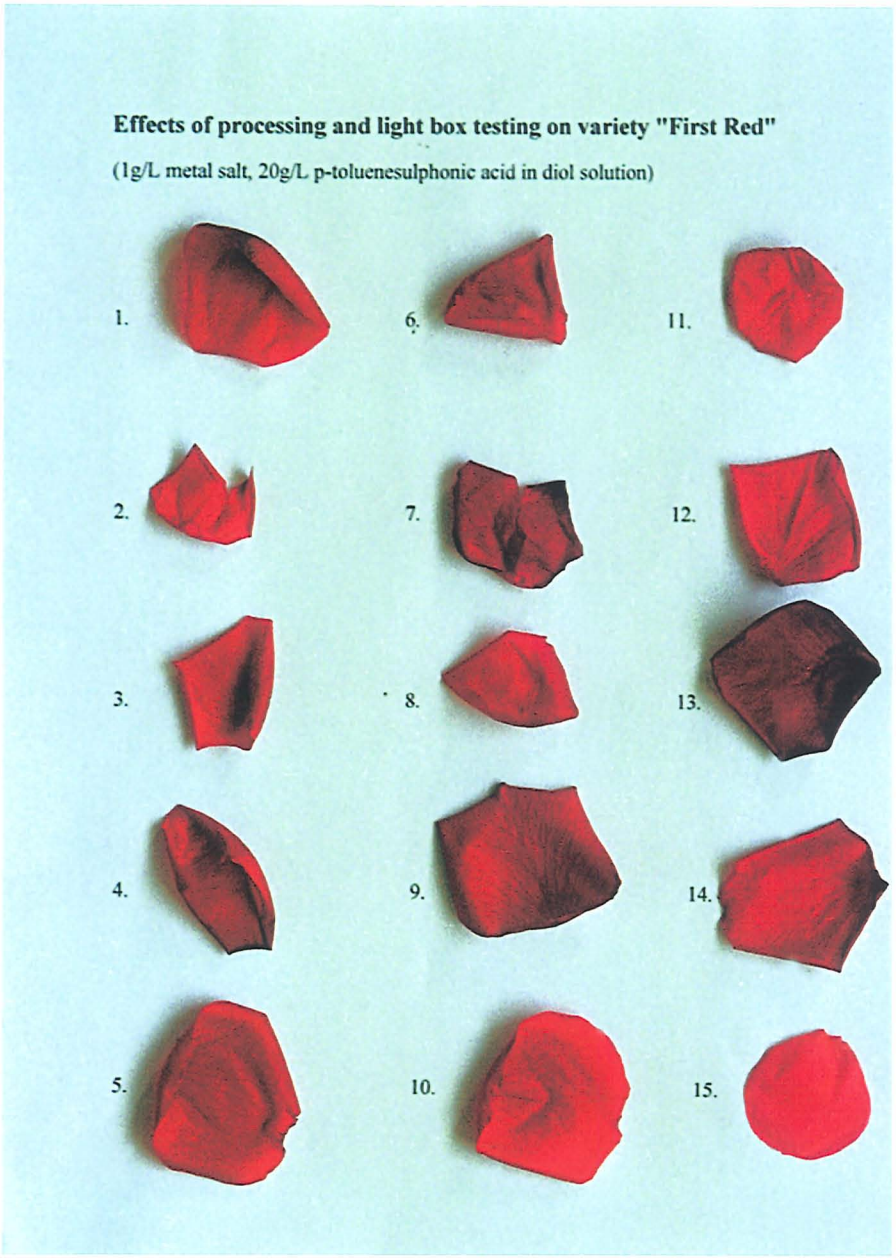
From the above observations it could be concluded that the resulting colour and light-fastness of processed petals was, amongst other factors, a result of the metal ions present and also the pigments and possibly copigments present in that particular variety. In general, however, it appeared that acidified diol solutions containing certain metal ions (i.e. Cu (II), Co (II), Fe (II), Fe (III), Sn (II), Mo(VI), W (VI) and Mn(II)) were capable of producing preserved petals with light stable colours at metal concentrations of 1 g/L and above.

In Chapter 5 the effects of the different metal ions are treated in a more quantitative manner, in attempt to find the most suitable metal and metal concentration.



Photograph 4.4 The long term effects of 10g/L metal salts on the processing of rose variety First Red in acidified solutions (approximately 6 months after light box testing).

1. Ca (II) 2. Cr (III) 3. Al (III) 4. F e(III) 5. Fe (II) 6. Mn (II) 7. Sn (II) 8. Zn (II) 9. Cu (II) 10. Co (II) 11. Ni (II) 12. Mg (II) 13. Mo (VI) 14. W (VI) 15. Acidified blank



Photograph 4.5 The long term effects of 1g/L metal salts on the processing of rose variety First Red in acidified solutions (approximately 6 months after light box testing).

1. Ca (II) 2. Cr (III) 3. Al (III) 4. F e(III) 5. Fe (II) 6. Mn (II) 7. Sn (II) 8. Zn (II) 9. Cu (II) 10. Co (II) 11. Ni (II) 12. Mg (II) 13. Mo (VI) 14. W (VI). 15. Acidified blank.

4.17.3.2 The effects of alternative metal salts in the non-acidified process solutions

Only red rose petals processed in solutions in which the metal salt itself acidified the process fluid gave rise to light-fast coloured petals. For example, for the red petals, the ferric chloride solution (pH 0.8) produced light stable red/brown petals, the stannous chloride solution (pH 1.05) produced crimson or purple petals. All other metals gave pale lilac colours from the red petals. The yellow petals although initially bright after processing (with the exception of blue/black streaks for the ferric chloride chloride solutions) were all seen to fade on light box treatment.

In general it appeared that solutions were required to be acidic in order to produce light stable petal colours with metal salts.

4.18 The effects of adding copigments to the process solution

4.18.1 General

One of the most efficient copigments (Appendix 2) and widely distributed and plant chemicals is the flavonol rutin (quercetin 3-rutinoside). Rutin (Fig. 3.4) occurs in up to 25% of any given local flora (e.g. magnolia, pansy, viola, horse-chestnut, tobacco leaf, plane, rhubarb and tea) and is extracted commercially, at relatively low cost from buckwheat.[6] It was decided to add this copigment to the process fluid in various combinations with acid and copper sulphate in an attempt to improve colour stability.

Another naturally abundant and commercially available copigment is ferulic acid (Fig. 3.6), which although not one of the most efficient copigments (Appendix 2) has other additional properties, which made it promising as an ingredient for the process

fluid. Ferulic acid can be extracted at low cost from cereals and agricultural by-products such as wheat bran, maize bran, barley spent grain and sugar beet pulp.[7] A component of the plant cell wall, ferulic acid maintains plant rigidity by forming chemical crosslinks that act as 'spot welds' to reinforce the cell wall (Fig 2.5), thereby providing a protective barrier against invading pathogens and also preventing oxidative damage by free radicals. It is this antioxidant activity, combined with antimicrobial properties and ability to strengthen the cell wall that has drawn the attention of the food industry to the potential uses of ferulic acid.[7] For example, as an antioxidant it can be used to stabilise fats and oils, as an antimicrobial reagent to protect fruit from spoilage and as a cell wall strengthener to prevent textural changes during cooking. Since all of these properties were also relevant to the flower preservation process, it was decided to add ferulic acid to the process fluid in an attempt to stabilise colour, improve firmness and to prevent oxidative and microbial damage.

4.18.2 Experimental

4.18.2.1 The effects of adding rutin to the process solution

Eight solutions were prepared from the base process diol mixture (65%v/v propan-1,2-diol, 25% v/v butan-1,4-diol, 10% w/v hexan-1,6-diol) by adding the following:

1. 20 g/L *p*-toluenesulphonic acid
2. 20 g/L *p*-toluenesulphonic acid + 10 g/L $CuSO_4$
3. 20 g/L *p*-toluenesulphonic acid + 10 g/L $CuSO_4$ + 0.1 g/L rutin
4. 20 g/L *p*-toluenesulphonic acid + 0.1 g/L rutin

5. 10 g/L $CuSO_4$ + 0.1 g/L rutin

6. 0.1 g/L rutin

7. 10 g/L $CuSO_4$

8. blank

Petals from rose variety First Red were processed in these solutions for 30 minutes at 80 °C and then subjected to light box treatment (7 days at 20,000 lux).

4.18.2.2 The effects of adding ferulic acid to the process solution

The base process diol mixture was prepared as in (4.18.2.1) and the following ingredients added:

1. 20 g/L *p*-toluenesulphonic acid + 10 g/L $CuSO_4$

2. 20 g/L *p*-toluenesulphonic acid + 10 g/L $CuSO_4$ + 5 g/L ferulic acid

3. 5 g/L ferulic acid

4. 10 g/L $CuSO_4$ + 5 g/L ferulic acid

5. 20 g/L *p*-toluenesulphonic acid

6. 20 g/L *p*-toluenesulphonic acid + 0.5 g/L $CuSO_4$

7. 20 g/L *p*-toluenesulphonic acid + 0.5 g/L $CuSO_4$ + 5 g/L ferulic acid

8. 0.5 g/L $CuSO_4$ + 5 g/L ferulic acid

Petals (previously frozen) from rose varieties First Red and Danse de Feu were processed in the above solutions for 30 minutes at 80 °C and then subjected to light box treatment (7 days at 20,000 lux)

4.18.3 Results and discussion

4.18.3.1 The effects of adding rutin to the process solution

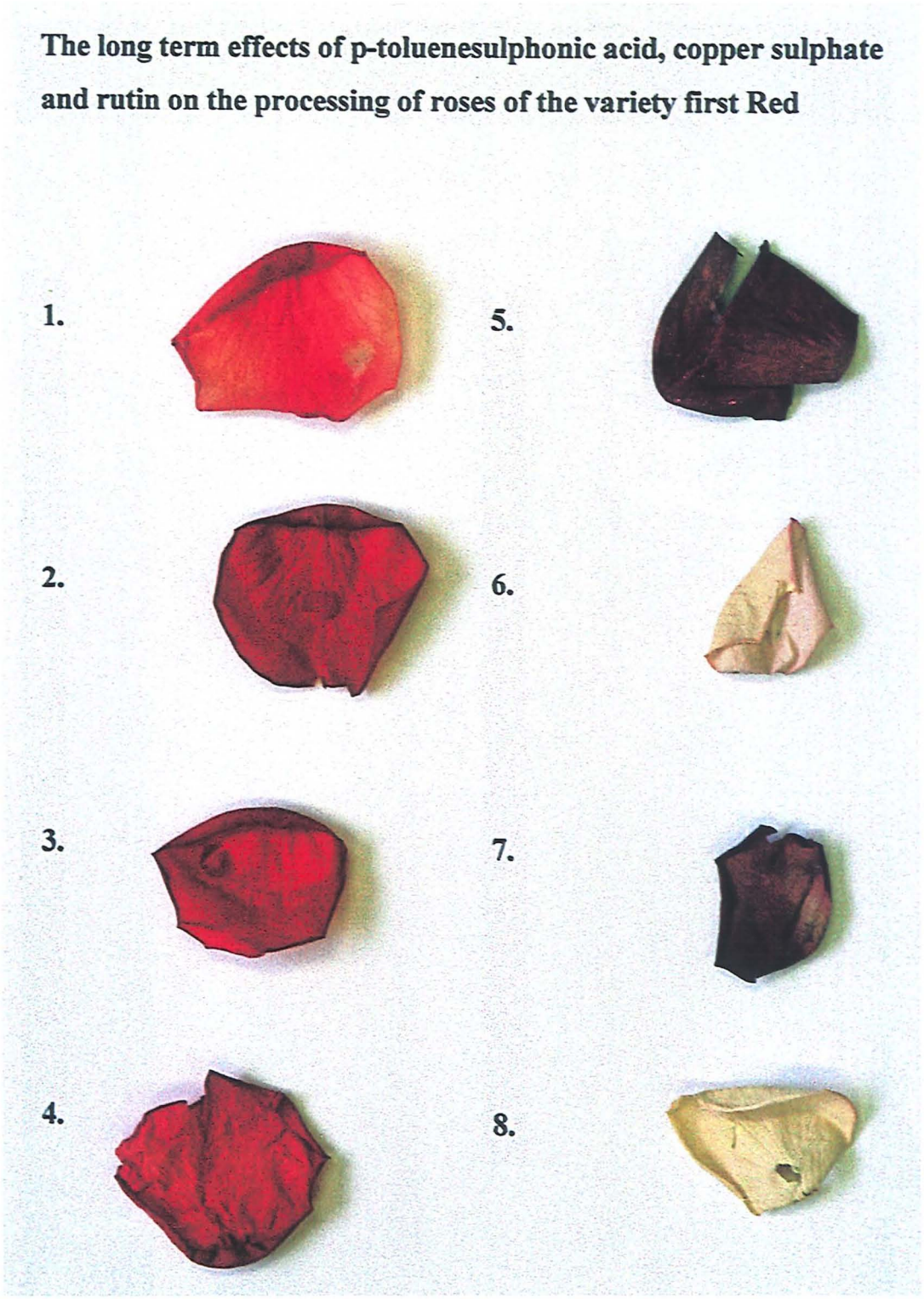
Processing in solutions (1)-(8) gave the petal colours described in Table 4.5.

Solution	After processing	After light box test
1	Red	Pale red
2	Dark red	Red
3	Dark red	Red
4	Red	Orange/red
5	Light purple	Light purple
6	Cream/purple tinge	Cream
7	Pale purple	Pale purple
8	Cream/purple tinge	Cream

Table 4.5 The effects of adding *p*-toluenesulphonic acid, copper sulphate and rutin to the process solution

1. Acid. 2. Acid + copper sulphate. 3. Acid + copper sulphate + rutin 4. Acid + rutin 5. Copper sulphate + rutin 6. Rutin 7. Copper sulphate 8. blank

After further exposure to normal laboratory conditions (for approximately 4 months) the results were as shown in Photograph 4.6. The effects, if any, of adding rutin to the acidified solutions containing 10 g/L copper sulphate were not detectable by visual examination (2) & (3). However, there did appear to be a slight difference in light stability between those petals processed in acidified solution without $CuSO_4$ (1) and those processed in the acidified solution without $CuSO_4$ but containing rutin (4). These effects are looked at more quantitatively in Chapter 5.



Photograph 4.6 The long term effects of p-toluenesulphonic acid, copper sulphate and rutin on the processing of rose variety First Red (approximately 4 months after light box testing).

1. Acid. 2. Acid + copper sulphate. 3. Acid +copper sulphate + rutin 4. Acid + rutin 5. Copper sulphate + rutin 6. Rutin 7. Copper sulphate 8. blank

4.18.3.2 The effects of adding ferulic acid to the process solution

Processing in solutions (1)-(8) initially produced petal colours as described in Table 4.6.

Solution	After processing	After light box test
First Red		
1	Dark red	Red
2	Dark red	Red
3	Pale lilac	Cream/pink tinge
4	Pink/purple	Pink purple
5	Red	Pale red
6	Red	Pale Red
7	Red	Red
8	Light pink/purple	Light pink/purple
Danse de Feu		
1	Dark orange/red	Orange/red
2	Dark orange/red	Dark orange/red
3	Pale lilac	Cream/lilac tinge
4	Dark pink	Brown/pink tinge
5	Bright pink	Cream
6	Bright pink	Cream
7	Bright pink	Pale orange
8	Lilac	Beige/pink tinge

Table 4.6 The effects of adding *p*-toluenesulphonic acid (PTSA), copper sulphate and ferulic acid to the process solution

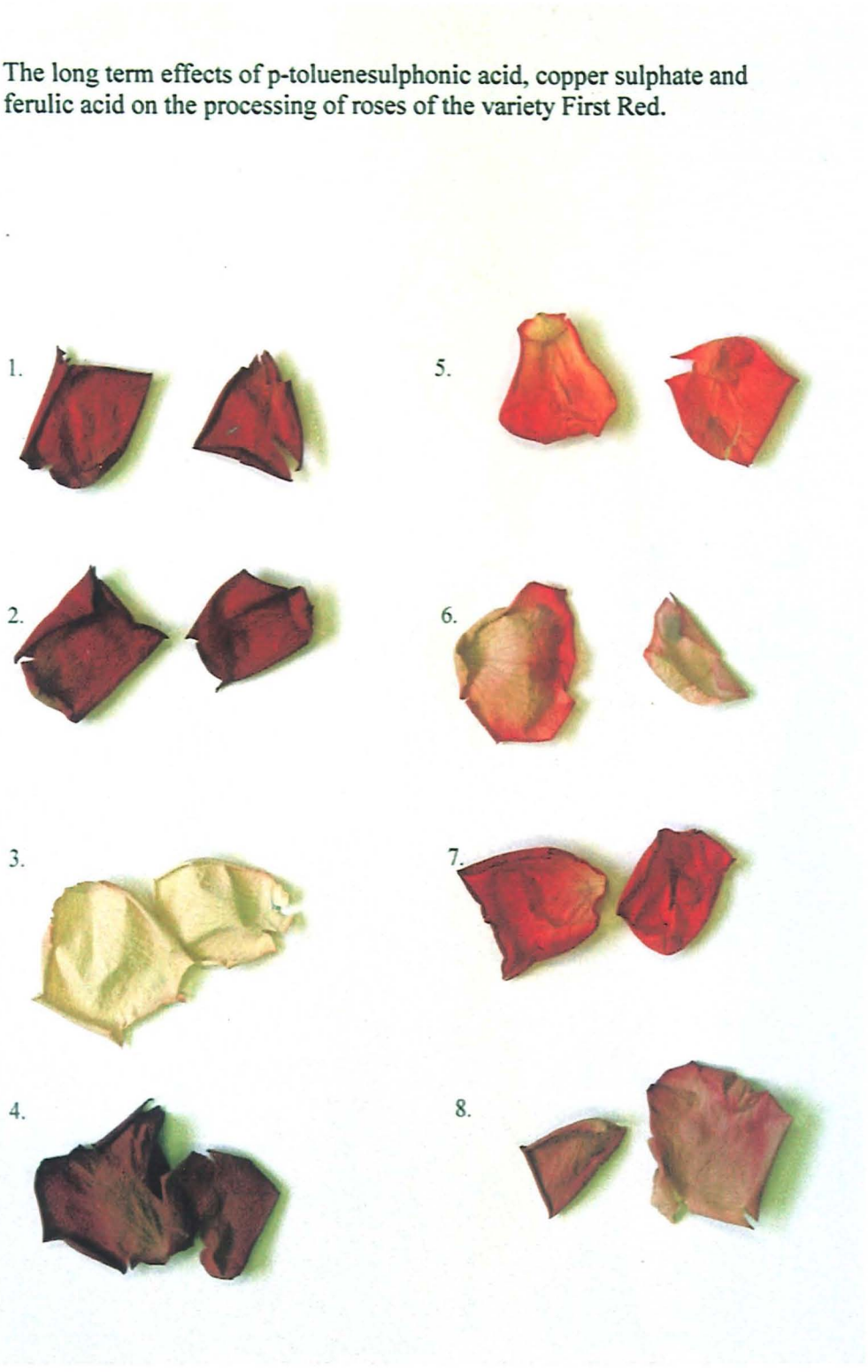
1. PTSA + 10 g/L copper sulphate. 2. PTSA + 10 g/L copper sulphate + ferulic acid. 3. Ferulic acid. 4. 10 g/L copper sulphate + ferulic acid. 5. PTSA. 6. PTSA + 0.5 g/L copper sulphate. 7. PTSA + 0.5 g/L copper sulphate + ferulic acid. 8. 0.5 g/L copper sulphate + ferulic acid.

After a further 12 months of exposure to normal laboratory conditions the results for First Red and Danse de Feu were as shown in Photograph 4.7 and Photograph 4.8 respectively.

Initially, for First Red, it appeared that for solutions containing 10 g/L $CuSO_4$ and 20 g/L *p*-toluenesulphonic acid, (1) & (2), that ferulic acid had no obvious colour stabilisation effect. However, for the solutions containing 0.5 g/L $CuSO_4$ and *p*-toluenesulphonic acid, there did appear to be some stabilisation to light on adding ferulic acid (6) & (7). On leaving the petals to stand for a further 12 months, however, the stabilising effects of ferulic acid in solutions containing *p*-toluenesulphonic acid and $CuSO_4$ were much more obvious (Photograph 4.7). For the solutions without *p*-toluenesulphonic acid but containing $CuSO_4$, the addition of ferulic acid gave rise to light- fast pink/purple petals, which were darker for the higher $CuSO_4$ content (Table 4.6 (4) & (8)) and more pink than for 10 g/L $CuSO_4$ alone (Table 4.5 (7)).

In the case of Danse de Feu the effects were similar to those for First Red, but more pronounced in that there was a visual difference between (1) & (2) and (6) & (7) after light box testing, whereas for First Red the differences were only obvious after a further 6-12 months. This could have been due to the condition of the plants in each case or to the nature of the pigments present, their concentrations.

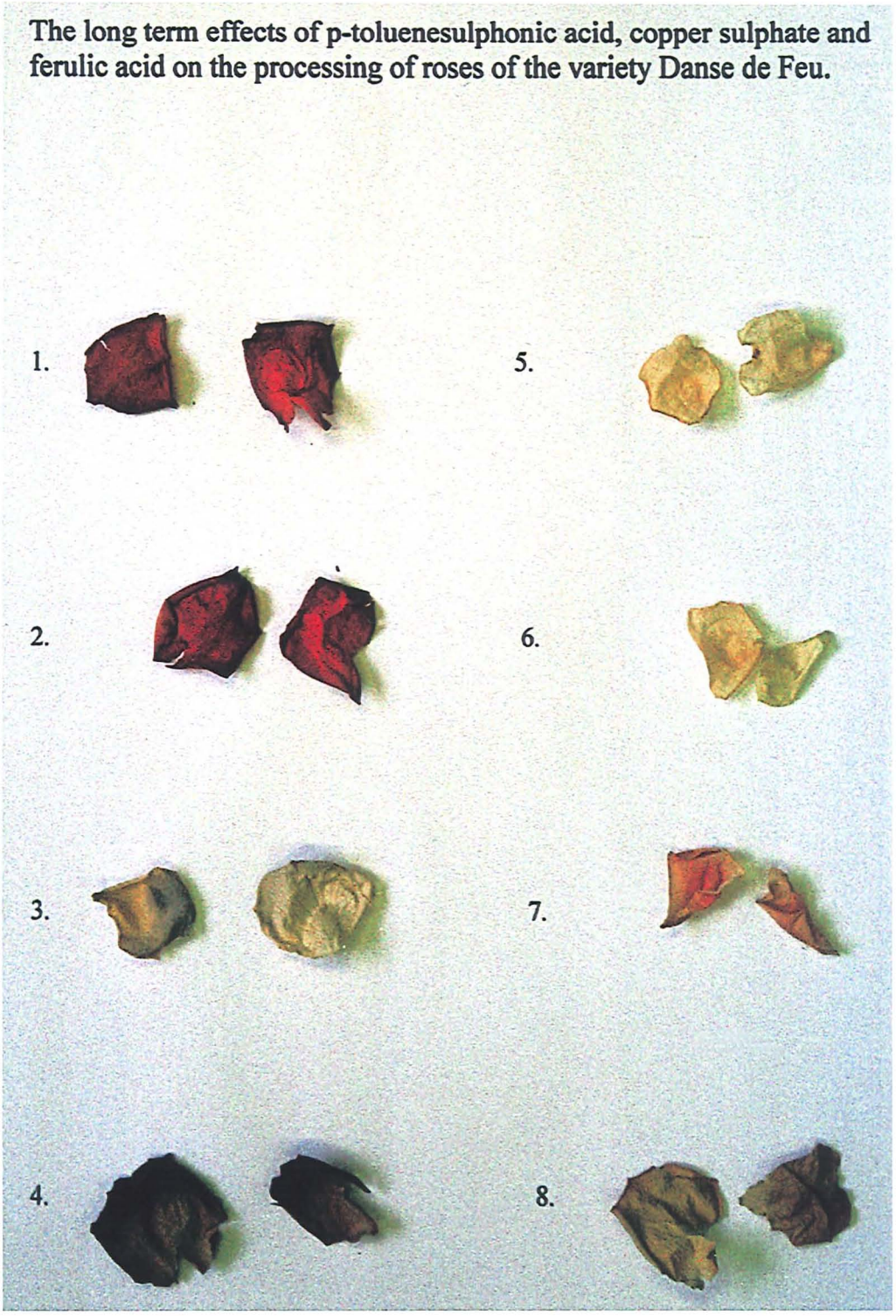
Overall, however it appeared that ferulic acid was having a long term stabilising effect on petal colour, stabilising both the flavylium cations, as in solutions (2) & (7) and also the anhydrobases, as in (4) & (8). Furthermore, the copper ions appeared to enhance stabilisation, since the higher the copper ion concentration, the deeper the colours of the petals (2) & (7) and (4) & (8).



Photograph 4.7 The long term effects of PTSA, copper sulphate and ferulic acid on the processing of rose variety First Red (approximately 12 months after light box testing).

1. PTSA + 10g/L copper sulphate. 2. PTSA + 10g/L copper sulphate + ferulic acid. 3. Ferulic acid.

4. 10g/L copper sulphate + ferulic acid. 5. PTSA. 6. PTSA + 0.5g/L copper sulphate. 7. PTSA + 0.5g/L copper sulphate + ferulic acid. 8. 0.5g/L copper sulphate + ferulic acid.



Photograph 4.8 The long term effects of PTSA, copper sulphate and ferulic acid on the processing of rose variety Danse de Feu (approximately 12 months after light box testing).

1. PTSA + 10g/L copper sulphate. 2. PTSA + 10g/L copper sulphate + ferulic acid. 3. Ferulic acid.

4. 10g/L copper sulphate + ferulic acid. 5. PTSA. 6. PTSA + 0.5g/L copper sulphate. 7. PTSA + 0.5g/L copper sulphate + ferulic acid. 8. 0.5g/L copper sulphate + ferulic acid.

These effects are dealt with again in Chapter 5 when dealing with solutions of pigment extracts.

There was possibly some improvement in tissue strength, however this effect was not obvious enough to be conclusive.

4.19 Implications

The aim of this section of work was to adapt the existing SEPAL technology in order to preserve whole flowers (in the first instance red roses) and in particular to solve the problem of colour loss in flower heads. To this end the work had been fairly successful in that satisfactory colour retention and light-fastness for several varieties of flowers containing anthocyanin pigments had been achieved by the addition of acid and metal salts to the process solution.[8] Other problems concerning the physical appearance of the flower heads were also addressed with the resulting situation being that, at laboratory scale, it was possible to produce a selection of preserved flowers of natural appearance.

At this stage, the most promising process mixture was considered to be one containing 65% v/v propan-1,2-diol, 25% v/v butan-1,4-diol, 10% w/v hexan-1,6-diol, 20-25 g/L *p*-toluenesulphonic acid and 1-10 g/L of $CuSO_4$. The addition of other metal salts such as $CoSO_4$ and $FeSO_4$ also improved light stability and the work carried out with copigments such as rutin and ferulic acid indicated that they were also potential pigment stabilisers.

To some extent the process times appeared to be related to the size of the petals and their accessibility, but in general process times of 15-30 minutes were required. With

regards process temperature, it was observed that temperatures of greater than 60 °C were necessary and that a temperature of 80 °C appeared to give satisfactory results. The following two chapters deal with the attempts to increase the life span of the preserved flowers, firstly by refining the existing process in order to improve pigment stability and secondly by providing a suitable barrier coating as a means of protecting the preserved plant material from potentially deleterious environmental conditions.

4.20 References

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CHAPTER 5

Chapter 5

Process refinement

(a semi-quantitative approach to pigment stabilization)

5.1 Introduction

Having developed the basic process for preserving flowering plants, through studying the effects of various treatments on intact petals and flowers, this chapter deals with the attempts to gain a more in depth understanding of the effects of the preservation process on the stability of the floral pigments themselves. By doing so it was hoped to be able to refine the existing process by highlighting areas for possible improvement.

Initially, this was to involve extracting and identifying the main pigments from several commercial varieties of red of rose in order to confirm the information from the literature on the nature of red rose pigments. It was then decided to concentrate on one of the commonest commercial varieties, the rose variety First Red, in an attempt to investigate the behaviour of its pigments towards various aspects of the existing preservation process.

This was approached in several ways. Firstly the identity and proportions of pigments extracted from fresh First Red petals were compared with those extracted from processed petals and secondly the effects of acidification, process temperature, process time and copper concentration on the stability of solutions of pigment extracts were studied. These solutions of pigment extracts were not only aqueous but also included the process fluid 'diol ' mixture and a 'diol/water' mixture (once it had been established that the processed petals contained such a mixture). The non-

aqueous media were of particular interest because although there has been some research into the stabilisation of anthocyanins in aqueous solutions (as a consequence of water being their natural medium), very little is known about their behaviour in non-aqueous solutions.

Finally, the effects of a range of alternative metal salts on the stability of the First red pigments in diol and diol/water mixtures was reinvestigated, as was the addition of other possible stabilising plant constituents.

5.2 Equipment and Materials

5.2.1 Ultraviolet and visible (UV/VIS) Spectroscopy

Absorption spectra were recorded on a Cintra 20 UV-Visible Spectrometer (GBC Scientific Equipment) in double beam mode using 10mm quartz cells. The scan range was 200-900 nm. The appropriate media were used as blanks.

5.2.2 High performance liquid chromatography (HPLC)

The HPLC equipment for the analysis of anthocyanidins and anthocyanins consisted of a BioCad Sprint System with a dual wavelength UV/VIS detector. Analysis was carried out on a reverse-phase C18 column, Luna (5μ, 250 × 4.6 mm), preceded by a guard cartridge (4 × 3 mm), both from Phenomenex. Separation was by gradient elution with: Solvent A, formic acid water (5:95 v/v) and solvent B, methanol. The solvent flow rate was 1.0 ml/min (in the first instance), the column pressure was 30-40 bar and the injection volume 30 μL. Detection was performed simultaneously at 260 nm and 450 nm.

The HPLC equipment for the analysis of flavonoid aglycones (non-anthocyanin) consisted of a Waters 660 gradient pump system fitted with a Waters 996 photodiode array (PDA) detector and Millennium version 3.2 software. Analysis was performed on a reverse-phase Spherisorb ODS column (5μ, 150 × 4.6 mm) with guard column (30 × 4.6 mm) from Phenomenex. Separation was by gradient elution using the following solvents: Solvent A, water, solvent B, methanol, solvent C, orthophosphoric acid (5:95 v/v). The flow rate was 1.2 ml/min, the injection volume was 20 μL and detection at 350 nm was recorded.

All HPLC separations were carried out at room temperature and all solutions and solvents were of HPLC grade and filtered through a 0.5 μm Millipore filter (Millex-SR, France) before use.

5.2.3 Matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS)

Molecular ion identification was carried out using MALDI-TOF MS on a Micromass Tof Spec 2E mass spectrometer (Manchester, UK).

For analysis of the anthocyanin aglycones, the matrix used was 2,6-dihydroxyaceto-phenone (DHAP) doped with sodium and/or potassium ions (NaI / KI). A two point external calibration was performed using dimethylnaphthdicarboxylate and warfarin. Analysis of the glycosides was carried out using DHAP and also 3-aminoquinoline (3AQ) as matrixes, with external calibration using dibenzylmatairesinol and sucrose octaacetate. In all cases the ratio of matrix solution to anthocyanin extracts was 1:1, with 1.0 μL being applied to the probe and allowed to air dry.

5.2.4 pH

As described in section 4.2

5.2.5 Paper chromatography (PC)

Paper chromatography was carried out Whatman No.1 and No.3 paper (for large-scale separations).

5.2.6 Thin layer chromatography (TLC)

Thin layer chromatography was performed on sheets of TLC pre-coated silica gel 60, sheets of HPTLC cellulose and PLC cellulose plates, all were from Merk and all were without fluorescent indicator.

5.2.7 Light box tests

As described in section 4.2.

5.2.8 Heating

As described in section 4.2

5.2.9 Humidity and temperature

Relative humidity and ambient temperature measurements were made on a digital thermo-hygrometer from The LabSales Company.

5.2.10 Standards and reagents

The anthocyanidin standards cyanidin, pelargonidin and peonidin were purchased from Polyphenols AS (Norway) and were of HPLC grade.

Rutin, quercetin, catechin, coumarin, *p*-coumaric acid and ferulic acid were purchased from Sigma. Gallic acid was from Fisons and protocatechuic acid was donated by E Ferreria at The Royal Scottish Museum (Edinburgh).

Matrix and calibrants and doping materials for MALDI-TOF MS were supplies by Sigma.

5.3 Extraction and Identification of the Flavonoid Pigments

5.3.1 General

In this section attempts were made to extract and identify the main anthocyanin pigments in the rose variety First Red, since this was the most likely rose variety to be used commercially. As it is known that the anthocyanins are almost invariably accompanied by and indeed stabilised by, other flavonoid compounds such as the flavonols and flavones (the most universal of the flavonoids), it was decided that these species should also be looked for.

As stated previously, (section 3.4) flavonoids are generally present in plants bound to sugar as glycosides, with any one flavonoid aglycone possibly occurring in several glycosidic combinations. For this reason, it was decided to simplify the identification procedure by examining the aglycones present in the hydrolysed extracts before considering the complexity of the glycosides present.

The ionic character of the anthocyanins and their much greater stability at low pH, required the use of acidic solvents, particularly in the case of the aglycones.

Extraction of the glycosides, however, involved the use of particularly mild conditions in order to prevent hydrolysis. Furthermore, due to the possible presence of labile, highly acylated anthocyanins, it was necessary to perform extractions using a weak acid such as acetic acid rather than HCl (as used for the aglycones).[1] Although aqueous solvents have been shown to give the best results for anthocyanin extractions,[2] an alcoholic mixture was chosen for ease of drying.

Once extracted, separation and identification of the flavonoid aglycones and glycosides was carried out using PC, TLC, HPLC, UV/VIS spectroscopy and MALDI-TOF MS. A short summary of these techniques and their applicability to the examination of floral pigments will now be given.

5.3.1.1 PC [2,3]

PC is particularly applicable to water soluble plant constituents such as the flavonoids and has the advantage over other methods in that it is quick, relatively inexpensive and the R_f values (mobility relative to solvent front) obtained are of considerable reproducibility.[3] This was useful in the case of the anthocyanins where careful comparison with the literature data avoided the need to purchase a range of expensive standard materials. In fact, only the anthocyanidin pigments commonly found in red roses (i.e. cyanidin, pelargonidin and peonidin) were purchased.

5.3.1.2 TLC [2,3]

The special advantages of TLC compared to PC include versatility (due to range of adsorbents available), speed (due to the compact nature of the adsorbent) and sensitivity (in that separations can be achieved with μg amounts). The main

disadvantages, however, are that are pre-coated plates are expensive and R_f values are considerably less reproducible than on paper, making it essential to include one or more reference compounds as markers due to lack of consistent literature data. For these reasons PC was the method of choice for identification purposes, although preparative TLC plates were chosen in preference to paper for their superior large-scale separations.

5.3.1.3 HPLC [3,4,5]

HPLC is a useful complementary technique to PC and TLC and has become popular for the identification, quantification and separation (using preparative columns) of non-volatile plant constituents. It is a technique that has basically arisen from the application to liquid chromatography (LC) of the theories and instrumentation that were originally developed for gas liquid chromatography (GLC). It differs from GLC in that the stationary phase is held in a narrow- bore stainless steel column and the liquid mobile phase is forced through under considerable pressure. The apparatus for HPLC is more expensive than for GLC, mainly because a suitable pumping system is required and all connections have to be screw jointed to withstand the pressures involved. The mobile phase is a miscible solvent mixture, which either remains constant (isocratic separation) or, may be changed continuously in its proportions by including a mixing chamber in the set up (gradient elution). The latter situation is used when the range of retention times of solutes on the column is so large that they cannot be eluted in a reasonable time using a single solvent or solvent mixture.

The stationary phases are microparticulate column packings and are commonly uniform, porous silica particles of nominal diameters of 10, 5 or 3 μm to which different chemical groups have been bonded (bonded phases). Chromatography

suppliers list a variety of these but most separations are carried out using either a silica microporous particle column (for non polar compounds) or a reverse-phase C-18 bonded phase column (for polar compounds) in which C-18 alkyl groups are attached to the surface of the silica particles. The compounds are monitored as they elute from the column by means of a detector, usually measuring in the ultraviolet. In recent years, photodiode-array detection has been developed. This is a system in which the sample is scanned every few milliseconds, generating UV/VIS spectral data and calculating the absorbance maxima making it a particularly useful technique for the identification of unknown compounds.

For our purposes HPLC with photodiode array detection was the main technique used for the separation and identification of the non-anthocyanin flavonoid aglycones and it was also useful in the detection of other plant phenolics which were present. HPLC with the dual wavelength detection was used to identify the anthocyanidins present and to check the purity of the coloured extracts.

5.3.1.4 UV/VIS spectroscopy [2, 3, 5, 6]

UV/VIS spectroscopy is, on its own, a particularly useful tool for the examination of plant extracts. This is mainly because the majority of the plant constituents of interest are conjugated aromatic systems showing intense characteristic absorption bands in the UV and visible regions of the spectrum. The value of UV and visible spectra in identifying unknown constituents is related to the relative complexity of the spectrum and to the general position of the wavelength maxima. The characteristic spectral properties of some of the main flavonoid classes are shown in Table 5.1.[3]

With regards to the anthocyanins, it has been shown that if the 5-position is free, the spectrum has an inflection at 440 nm. Thus, the ratios of the absorbances at 440 nm to those at the visible maxima for 3-glycosides are twice those for 3,5-diglycosides.[2]

Principal maxima (nm)	**Subsidiary maxima (nm) (with relative intensities)**	**Indication**
475-650	Ca. 275 (55%)	Anthocyanins
390-430	240-270 (32%)	Aurones
365-390	240-260 (30%)	Chalcones
350-390, 250-270	Ca. 300 (40%)	Flavonols
330-350, 250-270	Absent	Flavones

Note: all values are approximate, the actual values varying according to the solvent used and the pH.

Table 5.1 Spectral characteristics of some of the main flavonoid classes.

For the acylated anthocyanins the spectra can be considered as the superimposition of the spectra of the acyl residues present in the pigment onto the simple anthocyanin spectrum. Therefore, the presence of aromatic acyl compounds e.g. coumaric, ferulic and caffeic acids shows up as an extra absorbance peak in the 310-330 nm region. Moreover, the ratio of the absorbance at the maximum of the acid (310-330 nm) to the visible maximum of the pigment (500-530 nm) is proportional to the number of aromatic acid residues present per molecule of pigment i.e. for one acyl substituent the ratio is 50 to 60% and for two acyl groups about 90%.[2]

5.3.1.5 MALDI-TOF MS [7,8]

Finally one other technique which proved to be particularly useful for the identification of the anthocyanin aglycones and glycosides was MALDI-TOF MS.

This is a relatively new (first introduced in 1987), widely applicable technique, with high sensitivity but with good tolerance towards contaminants, making it particularly suitable for the analysis of complex mixtures.

MALDI-TOF MS is a soft ionisation method in which the sample is mixed with a UV absorbing matrix and applied to a target plate. The co-crystals are then irradiated with a UV laser which causes ionisation. The time taken for the ions to travel to the detector is measured and converted to a mass measurement, larger ions travelling more slowly than smaller ones.

The matrix plays a key role in this technique by absorbing the laser light energy and causing the matrix and the sample embedded in it to vaporise. The matrix also serves to minimise sample damage from the laser by absorbing most of the incident energy and is further believed to facilitate the ionization of the analyte molecules. Ionization can occur by several mechanisms including protonation and cationization, both of which involve the non-covalent addition of a positively charged ion (e.g. H^+, Na^+ or K^+) to a neutral molecule thus giving rise to a charged complex. In such cases the major ion observed in the positive ion mode is the MH^+ ion, with MNa^+ and MK^+ ions also seen (particularly if assisted by doping). In the case of compounds which are already positively charged, the charged molecules are simply transferred from the condensed phase into the gas phase giving rise to M^+ ions.

Fragmentation does not normally occur in MALDI-TOF MS, but spectra usually contain a number of peaks as well as the species of interest. Some of these peaks occur regularly at the same mass and can therefore be attributed to matrix related peaks, however, other peaks are of less predictable mass, making it impossible to obtain information on sample purity. Furthermore no two species will ionise identically therefore relative concentration data can not normally be obtained.

In order to maintain high accuracy it is necessary to calibrate the mass spectrometer on a regular basis. In general spectra are routinely externally calibrated using two calibrants which bracket the molecular weight of the species of interest.

The fact that this technique allows for the analysis of relatively small quantities of material (due to the efficient and directed energy transfer during a matrix-laser-induced desorption) and that it requires minimal sample preparation and analysis time meant that MALDI-TOF MS was a powerful tool for the analysis of unstable compounds such as the anthocyanins in complex biological samples such as plant extracts.

5.3.2 Experimental

5.3.2.1 Anthocyanidins

Approximately 1 g of fresh petals (First Red) was heated in 10 ml of 2M HCl in a test tube for 40 minutes at 100 °C (30-40 minutes being sufficient time to complete the hydrolysis of the anthocyanin glycosides[2]). The coloured extract was then cooled and decanted from the plant tissue. This extract was washed twice with ethyl acetate (2 × 5 ml) to remove the other (non anthocyanin) flavonoid aglycones and these washings were kept for further analysis (section 5.3.2.3). The remaining aqueous layer was heated at 80 °C for 3-4 minutes to remove the last traces of ethyl acetate and then re-extracted with approximately 3 ml of amyl alcohol (just sufficient to form an upper layer above the aqueous hydrolysate). The pigmented extract was pipetted off and concentrated to dryness on a watch glass on a boiling water bath (taking care not to prolong heating as overheating destroys the pigment). The residue was then taken up in a few drops of 1% methanolic HCl and chromatographed one

dimensionally on paper alongside the anthocyanidin standards. The following solvents were used: Forestal = conc.HCl-HOAc-H_2O(3:30:10); Formic = conc.HCl-HCO_2H-H_2O (2:5:3); BAW = n-BuOH-HOAc-H_2O (4:1:5). TLC on silica was also carried out using EtOAc-HCO_2H-2M HCl (85:6:9).

The extraction procedure was repeated to the evaporation stage with a further 3 lots of First Red petals and was taken up in 1 ml of MAW = MeOH-HOAc-H_2O (10:1:9) for HPLC analysis (with further 1-2 dilution with the same solvent), 0.1% methanolic HCl for MALDI TOF Mass Spectrometry and approximately 10 ml of 0.01% methanolic HCl for UV/VIS spectroscopy of the anthocyanidins separated on paper in the Formic solvent (with further 1-10 dilution for the magenta band).

At this stage all standards were run for identification purposes only (quantitative analysis being discussed in section 5.5), however, care was taken to ensure that, wherever possible, concentrations and experimental conditions were comparable for samples and standards.

Out of interest PC was repeated on every new batch of First Red and was also carried out on two other varieties of rose, namely Sacha and Danse de Feu.

5.3.2.2 Anthocyanins

Approximately 2 g of fresh petals (First Red) were crushed in a test tube with approximately 2 ml of MAW = MeOH-HOAc-H_2O (10:1:9) The extracts were then directly chromatographed on paper (No1) in three solvents; BAW = n-BuOH-HOAc-H_2O (4:1:5), BuHCl = n-BuOH-2M HCl (1:1, top layer) and 1% HCl = conc. HCl-H_2O (3:97).

The extraction procedure was repeated and the extracts were decanted off and filtered for HPLC examination before being streaked on cellulose PLC plates. The

plates were run in BAW and after drying the coloured bands were scraped off and re-extracted into 1 ml of 0.1% methanolic HCl for a PC check and for analysis by MALDI-TOF MS. This was followed by a 1-10 dilution with methanol to give solutions in 0.01% methanolic HCl suitable for analysis by UV/VIS spectroscopy (with further 1-10 dilution with 0.01% methanolic HCl for the magenta band).

5.3.2.3 Flavonoid Aglycones (Hydrolysed Extracts from Ethyl Acetate Layer)

The ethyl acetate layer from the hydrolysis of approximately 1 g of fresh First Red petals with 2M HCl, followed by extraction into ethyl acetate (section 5.3.2.1), was evaporated to dryness under vacuum at 50 °C. The evaporate was then taken up in 1 ml of 95% and ethanol and chromatographed on paper in Forestal, BAW, 50% aqueous acetic acid and in water.

HPLC analysis was carried out after a 1-20 dilution of the alcoholic extracts with MeOH: H_2O (1:1).

5.3.3 Results and discussion

5.3.3.1 Anthocyanidins

Results from the initial PC, TLC and UV/VIS data were as in Table 5.2.

PC carried out on the hydrolysed extracts of successive batches of First Red gave identical results and the varieties Sacha and Danse de Feu were also found to contain cyanidin and pelargonidin although from visual comparison both varieties contained appreciably more pelargonidin than did First Red.

HPLC analysis of the hydrolysed extracts of First Red petals with detection at 450 nm gave two peaks of retention times 26.9 and 27.6 minutes and approximate peak area ratio of 12:1 respectively (Fig 5.1 a). The retention time of the cyanidin

		R_f x 100				
Sample	**Visible colour**	**Forestal**	**Formic**	**BAW**	**EtOAc/ Formic acid/HCl**	**Visible max. (nm)** 0.01% HCl/MeOH
Cyanidin	Magenta	50	31	78	50	537
Pelargonidin	Red	71	45	88	62	522
Peonidin	Magenta	65	43	82	56	539
First Red (1)	Magenta	50	31	78	50	537
First Red (2)	Red	71	45	88	62	522

Table 5.2 Results from PC, TLC and UV/VIS analysis of anthocyanidins.

standard was 27.0 minutes (Fig. 5.1 b). Problems with the pump pressure, however, caused difficulties in running further solutions. The problem was resolved by reducing the flow rate to 0.5 ml/min (adjusting the solvent gradient accordingly) and then running the petal extract solution, followed by solutions of the petal extracts to which a small amount of each of the pelargonidin and cyanidin standards had been added. The corresponding increase in peak heights when cyanidin and pelargonidin were added to the extract solutions verified that the larger of the two peaks was cyanidin and that the smaller was pelargonidin.

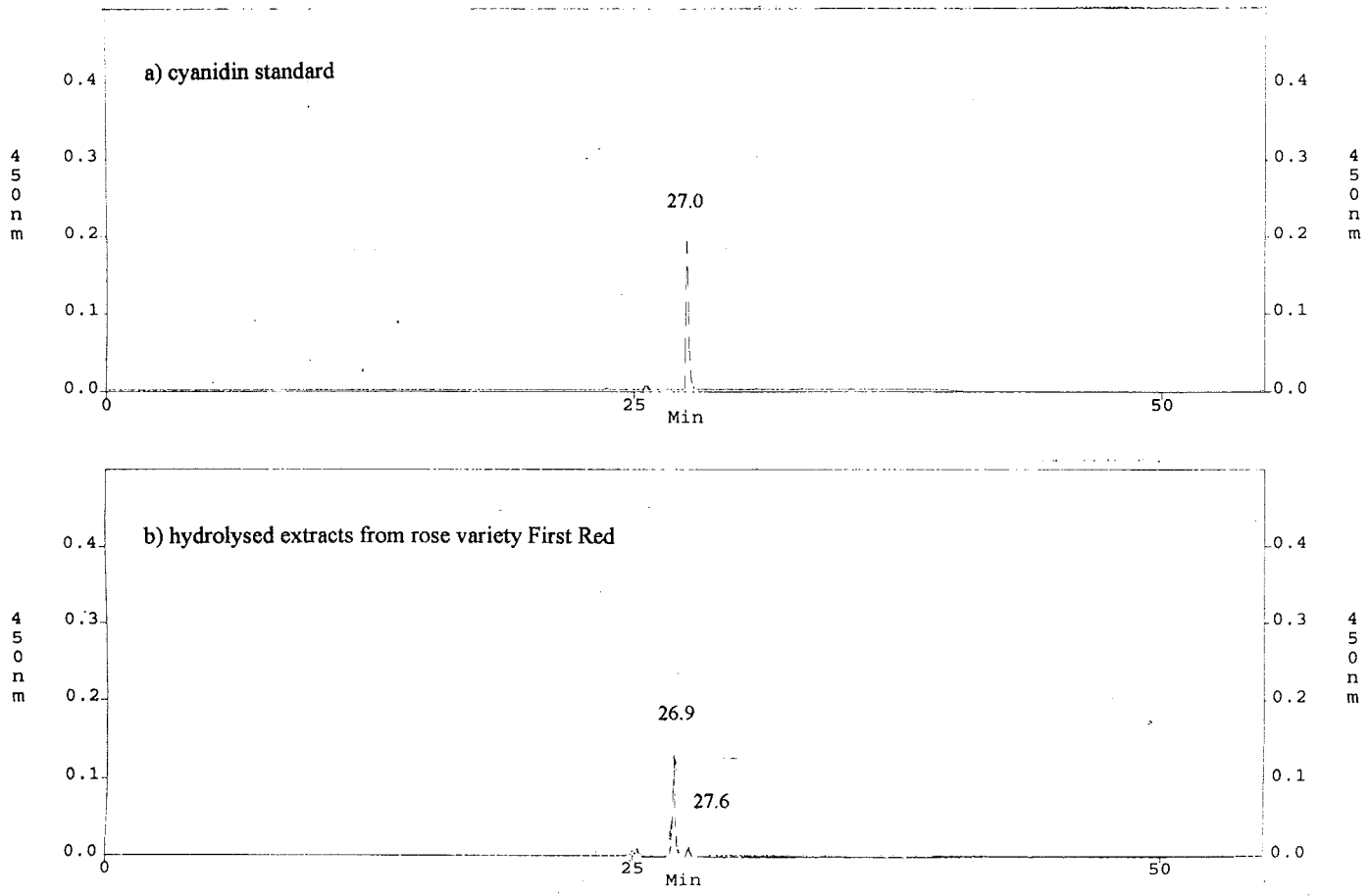


Fig. 5.1 HPLC chromatograms of anthocyanidins

It was also noted that a solution of the pelargonidin standard in MAW had become almost colourless overnight (with a corresponding drop in peak height of 95%) and that the colour was not regenerated on further acidification. On the other hand there was no obvious loss of colour for the cyanidin standard after 24 hours and the peak height remained constant.

Analysis by MALDI-TOF mass spectrometry (matrix DHAP) of the cyanidin, pelargonidin and peonidin standards gave good signals for the molecular ions; M^+ (or considered as MH^+ if in the quinoidal form), MNa^+, MK^+ (Table 5.3) indicating that the flavylium ions were suitable for analysis by this method. Analysis of the hydrolysed petal extracts was then carried out and the presence of cyanidin and pelargonidin in the extracts was confirmed (Fig. 5.2 a, Table 5.3).

	M^+/MH^+		MNa^+		MK^+	
Sample	**Expected**	**Present**	**Expected**	**Present**	**Expected**	**Present**
Cyanidin	287.05	287.04	309.04	309.05	325.01	325.00
Pelargonidin	271.06	271.09	293.04	293.04	309.02	-
Peonidin	301.07	301.09	323.05	323.06*	339.03	339.09
First Red		287.15		309.13	-	-
		271.13		293.12	-	-

*matrix signal at 322, therefore not conclusive, - not doped

Table 5.3 Anthocyanidin masses obtained using MALDI-TOF MS

On consideration of the collected results it was concluded that the aglycones present in rose variety First Red were cyanidin and pelargonidin in the ratio of approximately 12:1.

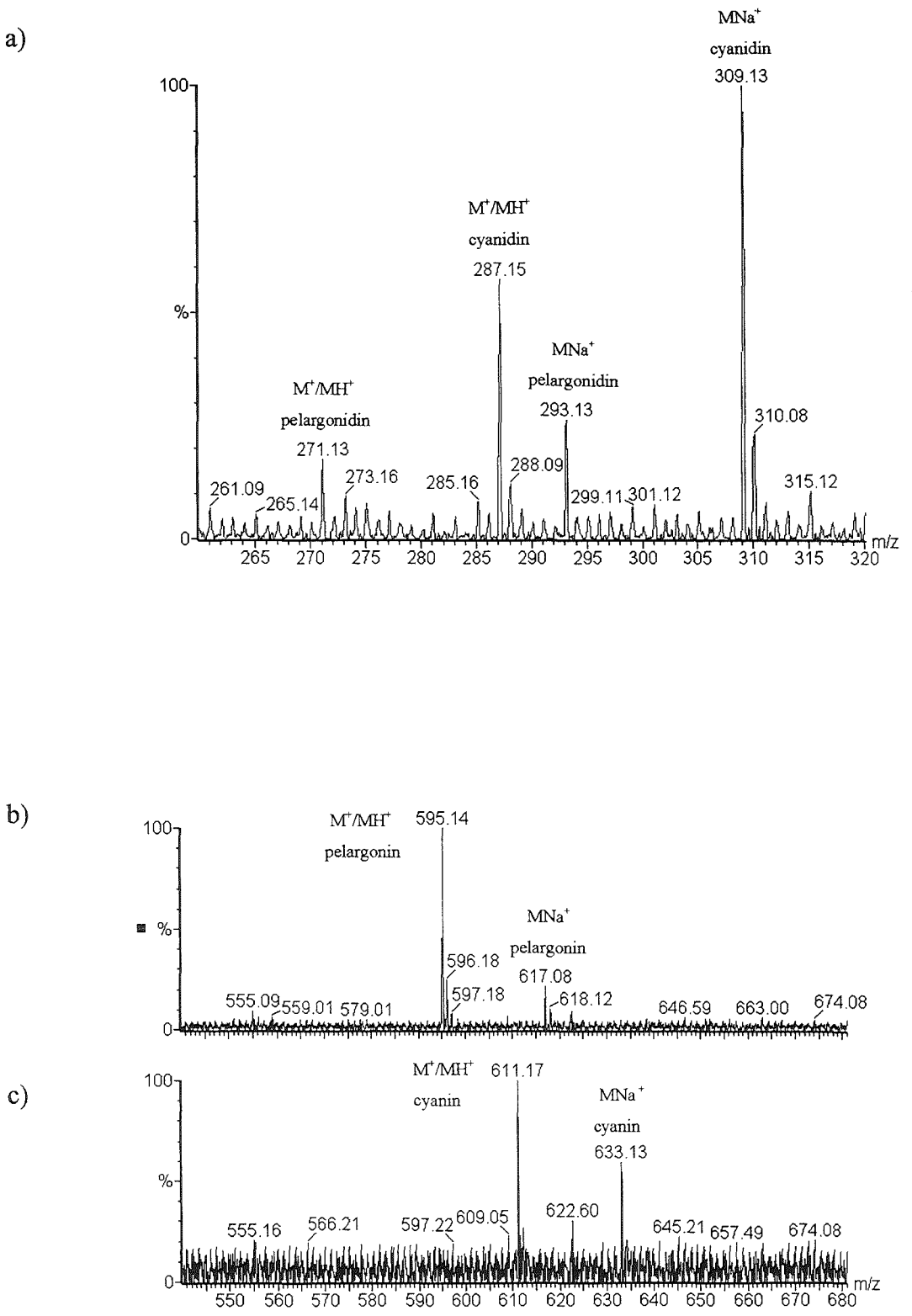


Fig. 5.2. MALDI-TOF MS positive ion spectra (matrix DHAP) of anthocyanidins and anthocyanins extracted from rose variety First Red

a) anthocyanidins b) anthocyanins (orange/pink band) c) anthocyanins (magenta band)

5.3.3.2 Anthocyanins

HPLC analysis of the MAW extracts with detection at 450 nm gave two peaks at 23.0 and 23.7 minutes when operating at a flow rate of 1.0 ml/min. No other peaks were detected indicating that no hydrolysed anthocyanins were present. As standards were not available no further information was obtained from the chromatograms.

PC analysis of the material extracted by MAW gave rise to two spots in each solvent system, one being magenta in colour and the other orange/pink and florescent under UV light. PC analysis of the material extracted from the two bands separated on cellulose each gave single spots, which matched those from the unseparated extracts. The results of PC analysis and UV/VIS spectroscopy of the separated materials are given in Table 5.4.

		R_f x 100				
Sample	**Visible colour**	**BAW**	**BuHCl**	**1% HCl**	**Visible max. (nm)** 0.01% HCl/MeOH	**Abs.**
First Red (1)	Magenta	27 (28)[9]	6 (6)[9]	16 (16)[2]	525 (524)[9] (522)[16]	0.939 x 10
First Red (2)	Orange/pink	33 (33)[9]	14 (14)[9]	24 (23)[3]	506 (504)[9]	0.782
Cyanidin					537 (535)[3]	
Pelargonidin					522 (520)[3]	

Table 5.4 Results from PC and UV/VIS analysis of anthocyanin extracts.

The figures in brackets alongside the experimental values for the magenta compound refer to the literature values for cyanin (cyanidin 3,5-diglucoside) and the figures next to the results for the orange/pink compound refer to pelargonin (pelargonidin 3,5-diglucoside).[2, 3, 9] The visible absorption maxima for each of the two spots was

found to be 2 nm more than their corresponding literature values but since this difference between experimental and literature values was also observed for the anthocyanidin standards (when measured under identical conditions) then the anthocyanin values were considered accordingly. The differences in the literature values for the positions of the visible absorption maxima of the 3- and 3,5-diglycosides were too close to permit unequivocal assignment but the absence of a distinct shoulder between 410 and 450 nm in the anthocyanin spectra indicated that the anthocyanins were substituted in the 5-position (section 5.3.1.4) impling a 3,5-glycoside rather than the 3-glycoside, as anthocyanins with only one sugar are rarely substituted in the 5-position. [2, 9] Indeed, this deduction was supported in the case of the pelargonidin glycoside by the fact that the orange spots were observed to fluoresce under U.V. light, a distinguishing property of the 3,5-diglycosides of pelargonidin, peonidin and malvidin.[3] Furthermore, the absence of peaks in the 310-330 nm region in either of the anthocyanin spectra ruled out acylation of the sugar molecules with aromatic acids e.g. caffeic, coumaric and ferulic acids (section 5.3.1.4).

Using the DHAP matrix, MALDI-TOF MS analysis of the materials extracted from the magenta and from the orange/pink bands gave molecular ions corresponding to those expected for cyanin (cyanidin 3,5-diglucoside) and pelargonin (pelargonidin 3,5-diglucoside) (Table 5.5, Fig 5.2 b & c). It was noted, however, that when 3AQ was used as a matrix, that additional molecular ions corresponding to the loss of a glucose fragment were observed for the spectra of both bands. In the case of the magenta band, molecular ions appeared at m/z = 449.14 and 471.17 corresponding to the expected M^+/MH^+ and MNa^+ ions for cyanidin monoglucoside. For the

orange/pink band the molecular ions appeared at 433.18 and 455.25 corresponding to the expected M^+/MH^+ and MNa^+ ions for pelargonidin monoglucoside. The phenomenon of fragmentation by loss of a glycoside residue is known to occur in the MALDI-TOF MS analysis of glycosylated natural products,[8] therefore it was most likely that the molecular ions corresponding to the monoglucosides were present as a result of fragmentation by the laser treatment, rather than being present in the original plant extracts, particularly since previous analysis had not detected their presence.

	M^+/MH^+		MNa^+	
Sample	**Expected**	**Present**	**Expected**	**Present**
Cyanin	611.16		633.15	
Pelargonin	595.17		617.15	
Magenta band		611.17		633.13
Orange/pink band		595.14		617.08

Table 5.5 Anthocyanin masses obtained using MALDI-TOF MS

Analysis of the extracts from the magenta and orange/pink bands by MALDI-TOF MS was therefore further confirmation that it was the cyanidin and pelargonidin 3,5-diglycosides that were present in the petals of First Red and in particular that it was glucose that was used for glycosidation. This conclusion was in keeping with previous studies on the identity of red rose pigments, which found that on the analysis of 160 varieties of red rose that only glucose was used for glycosidation and that the 3,5-diglucosides were the dominant pigments.[10]

From the UV/VIS spectroscopic measurements, the ratio of cyanin to pelargonin was found to be approximately 12:1. It was noted, however, that the colour of the cyanin solution in 0.01% methanolic HCl began to fade after 2 days (with corresponding drop in visible absorbance of approximately 50%) whereas the colour of the pelargonin solution remained strong (with no significant drop in absorbance). This was the reverse of the situation with the aglycones, where cyanidin was observed to be more stable than pelargonidin.

5.3.3.3 Flavonoid aglycones (non-anthocyanin)

HPLC analysis of the alcoholic extracts using the PDA detection system, identified peaks at 19.8 and 22.2 minutes as the flavonol aglycones quercetin and kaempferol respectively. A peak at 17.1 minutes was attributed to ellagic acid (Fig 5.3). Confirmation of these assignations was by PC in solvents Forestal and BAW for which reference values were available (Table 5.6). The lack of mobility of the spots in water indicated that glycoflavones were absent.[3]

Other HPLC peaks, which were not identified, could possibly have been due to organic acids or to tannins and their breakdown products. The detection of ellagic acid after acid hydrolysis was in itself indicative of the presence of ellagitannins [2,3]

	Colour	R_f x 100	
Sample	**U.V.→ U.V + NH_3** [3,11]	**Forestal**	**BAW**
Spot (1)	Bright yellow → bright yellow	71 (55)[3]	82 (83)[3]
Spot (2)	Bright yellow →bright yellow	53 (41)[3]	62 (64)[3]
Spot (3)	Violet fluorescence → pale yellow green	40 (33)[11]	21 (18-11)[11]

Table 5.6 Results from PC analysis of Flavonoid aglycones (non-anthocyanin)

The figures in brackets next to the experimental R_f values for spots 1, 2 and 3 refer to the literature values (under the same conditions) for kaempferol, quercetin and ellagic acid repectively. The spots were quite diffuse making accurate measurement difficult but the colour changes were as predicted. [3, 11]

To conclude, it would appear that the non-anthocyanin phenolic compounds expected to be present in red roses i.e. the flavonol glycosides of quercetin and kaempferol and the ellagitannins [10] were in fact present in First Red.

It was also noted that when the ethyl acetate extractable materials from Sacha and Danse de Feu were chromatographed in the same PC systems that they appeared to contain slightly more quercetin than First Red, especially the pinker variety Danse de Feu.

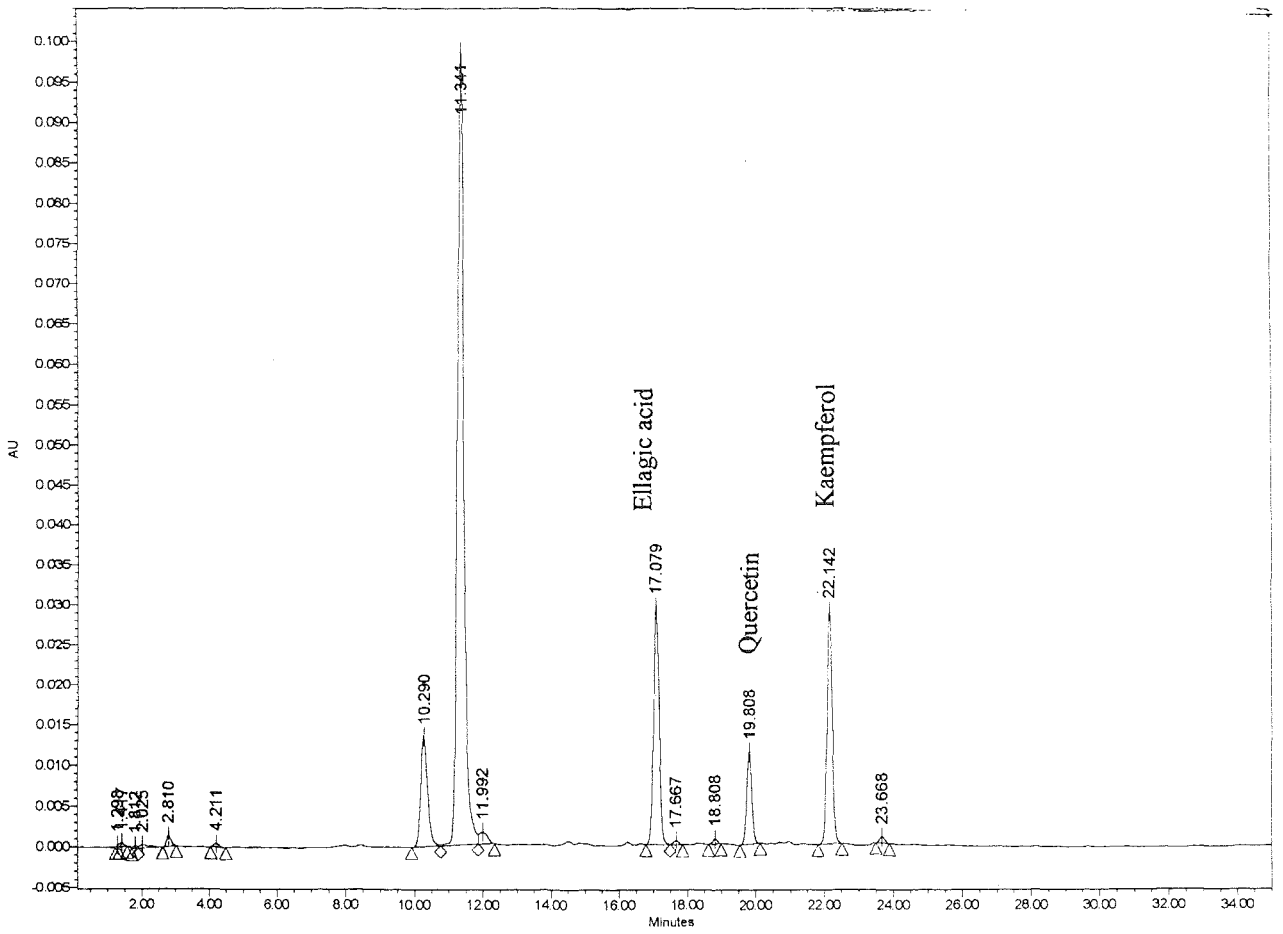


Fig. 5.3 HPLC chromatogram of flavonoid aglycones (non-anthocyanin) extracted from the fresh petals of rose variety First Red (recorded at 350nm).

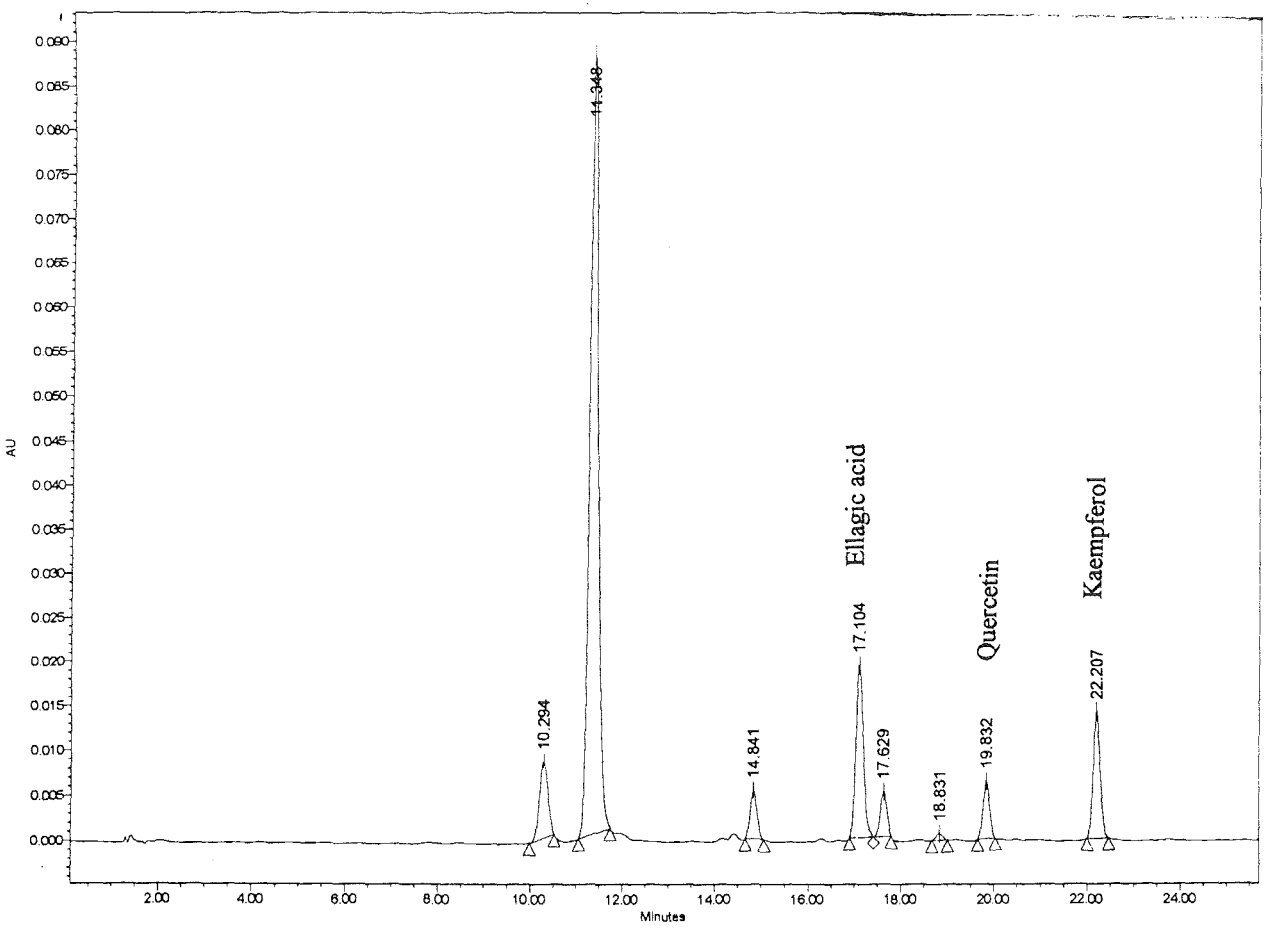


Fig. 5.4 HPLC chromatogram of flavonoid aglycones (non-anthocyanin) extracted from the processed petals of rose variety First Red (recorded at 350nm)

5.4 Comparison of extractable materials from processed roses with those from fresh roses

5.4.1 General

The petals from prosessed roses of the variety First Red (processed at 80 °C for 30 minutes in a solution containing 65% propan-1,2-diol, 25% butan-1,4-diol, 10% hexan-1,6-diol, 20 g/L *p*-toluenesulphonic acid and 10 g/L $CuSO_4$) were extracted and identified by identical methods to those for the fresh roses and the results compared.

5.4.2 Experimental

Extraction and identification of the anthocyanidins, anthocyanins and flavonoid aglycones was carried out as in 5.3.2. (including TLC on cellulose with BAW) and run in parallel with the analysis of the fresh petals.

5.4.3 Results and discussion

PC and TLC (as carried out in 5.3.2) of the processed petal extracts gave identical spots and R_f values to those of the fresh petals.

Separation of the anthocyanins on cellulose gave rise to a magenta band and an orange/pink band, as for the fresh petals. MALDI TOF MS analysis of the material from each of these bands gave molecular ions corresponding to those for cyanin and pelargonin. The UV/VIS absorption spectra for these bands gave an equivalent visible maximum wavelength for the magenta bands to the fresh and petals but the maximum wavelength for the orange/pink band was 1-2 nm higher than that for the

fresh petals. This difference could possibly have been due to traces of diols being present.

HPLC analysis with detection at 450 nm for the anthocyanidins gave two peaks of ratio 12:1 corresponding to the retention times for cyanidin and pelargonidin. HPLC for the anthocyanins gave two peaks of the same retention times and peak area ratio as those of the fresh petals and could therefore be attributed to cyanin and pelargonin.

Furthermore, on analysis of the processed petal extracts, there was no evidence of any hydrolysed material present nor did their appear to be any significant drop in concentration of the anthocyanins when compared with the fresh petals.

HPLC comparison of the flavonoid aglycones from the fresh and processed petals gave similar profiles (Figs. 5.3 and 5.4), with the exception of two extra small peaks for the processed petals at retention times of 14.8 and 17.6 minutes, which could possibly have been attributed to breakdown products.

It would appear therefore, that processing had very little effect on the pigment concentrations and relative and the relative proportions of the plant constituents detected.

5.5 Determination of total Anthocyanin content

5.5.1 General

In this section an attempt was made to measure the total anthocyanin content of the rose variety First Red using UV/VIS spectroscopy.

According to Beer's Law, the absorbance at any wavelength λ (in a given solvent) is proportional to the concentration of the species in solution (denoted c) which is responsible for the absorbing radiation, and proportional to the distance (denoted d) that the radiation passes through the solution.

Thus for a given wavelength:

$$A_\lambda = E\,c\,d$$

Where:

A_λ = absorbance at wavelength λ

$E = E^{1\%}_{1cm,\lambda}$ = absorption in a 1 cm cell at a concentration of 1% w/v at wavelength λ (absorption or extinction coefficient)

c = concentration in g /100ml

d = path length in cm

E values can be obtained from the literature (or derived if standard material is available), therefore when d =1 the concentration can be readily determined.

(note: the absorbance of pigments is more often expressed as the molar absorptivity, which is defined as the absorbance of one molecular weight in one litre, with c in moles/L, however the two systems are interconvertible).[6, 9, 12]

For this work, three batches of roses were examined (Appendix 8), each using a different method of extraction; one being the hydrolysis method for the extraction of the aglycones as described in section 5.3.2.1 and the other two being milder extraction methods using EtOH:HOAc:H_2O (EAW,10:1:9)[13] and 95% EtOH:1.5M HCl (85:15).[12] For the milder methods, in order to achieve complete extraction of the pigments, it was necessary to disrupt the plant tissues by macerating the samples in a

blender and then leaving the slurries to stand overnight to allow the anthocyanins to diffuse through the cell membranes.

Since no other substances absorbing energy in the range of the anthocyanins (500-550 nm) were expected to be present, no further purification procedures were considered necessary and the amount of pigment present was measured simply by measuring the absorbance at the given wavelength.

Final absorbance measurements were made in three different media. In the case of the aglycones the solvent used was 0.01% HCl in MeOH and a standard solution was prepared using the same solvent. In the case of the anthocyanins, the two solvents were 0.1% HCl in EtOH and 95% EtOH:1.5M HCl (85:15). Literature extinction (absorption) coefficients (E) were available for cyanin in 0.1% HCl in EtOH and were used to calculate the anthocyanin content of the extracts in this solvent. However, literature values for the extinction coefficent of cyanin in 95% EtOH:1.5M HCl (85:15) were unavailable and since standard material was also unavailable, it was necessary to derive this value by calculating the ratio of absorbances of the pigment extracts (extracted by the same EAW solvent) in 0.1% HCl in EtOH to the absorbances in 95% EtOH:1.5M HCl (85:15). This derived (E) value was then used to calculate the concentration of the anthocyanins extracted with 95% EtOH:1.5M HCl.

The total anthocyanin content was calculated and expressed in terms of the major pigment cyanin. It was considered that any error introduced would not be significant since the proportion of pelargonin in the sample was relatively small (12:1) and it is known that the anthocyanins do not vary appreciably in absorbance with minor changes in structure.[12]

5.5.2 Experimental

5.5.2.1 Extraction following hydrolysis

20.59 g of shredded fresh petals were heated in a beaker in 200 ml of 2M HCl for 45 minutes at a temperature of 100 °C. The coloured extract was cooled and suction filtered through a Whatman No 44 filter paper and the residue washed with a further 20 ml of water. The aqueous extract was then washed twice with ethyl acetate (2 × 30 ml) and the ethyl acetate layer discarded. The aqueous layer was heated on a water bath at 80 °C to remove any traces of ethyl acetate before the pigment was extracted into 30 ml of amyl alcohol. The amyl alcohol solution was evaporated to dryness under vacuum at approximately 40 °C and the residue weighed. 0.0054 g of the residue was then dissolved in 5 ml of 0.01 % HCl in MeOH and diluted (1-10) for measurement of absorbance at 537 nm (the maximum wavelength of cyanidin absorption in 0.01% HCl in MeOH).

A standard solution of cyanidin in 0.01% HCl in MeOH containing 0.05 mg/ml of cyanidin was also prepared and the absorbance measured at 537 nm.

5.5.2.2 Extraction with EtOH:HOAc:H_2O (EAW,10:1:9 v/v)

10.01 g of fresh petals were blended at for 2 minutes with 100 ml of EtOH:HOAc: H_2O (10:1:9) in a blender. The macerate was quantitatively transferred to a beaker with approximately 50 ml of the extracting solvent, covered with cling film and stored refrigerator overnight. The mixture was filtered with suction through a Whatman No 44 filter paper and the residue was washed repeatedly with the extracting solvent until approximately 300 ml of the extract solution had been

collected. This solution was then evaporated on the rotary evaporator at approximately 30 °C and the dried extracts weighed.

0.0055 g of these extracts were then dissolved in 2 ml of 0.1% HCl in EtOH and diluted (1-10) for absorbance measurement at 535 nm (the literature maximum wavelength of cyanin 0.1% HCl in EtOH [12]).

A further two lots of the extracted material, 0.0208 g and 0.0207 g, were then each dissolved in 5 ml of a 95% EtOH:1.5M HCl (85:15 v/v) solution, diluted (1-10) and the absorbances measured at 535 nm.

*Note: On a previous occasion a different batch of roses was also extracted with the EAW solvent and the absorbances measured in the same solvents as above.

(i.e. 20.58 g petals in 200 ml EAW, 0.0059 g extract dissolved in 2 ml of 0.1% HCl in EtOH and diluted (1-10) and 0.0110 g and 0.0100 g extracts each dissolved in 5 ml of 95% EtOH:1.5M HCl (85:15) and diluted (1-10) and (1-5) respectively, all absorbances being measured at 535 nm). Since the petals were not macerated with the solvent before leaving to stand overnight the extraction of anthocyanins may not have been complete and the resulting concentrations would possibly have been too low. The ratio of the absorbances of the extracted material in the two different solvents was, however, still useful in determining E for cyanin in 95% EtOH:1.5M HCl, section 5.5.3.3).

5.5.2.3 Extraction with 95% EtOH:1.5M HCl (85:15 v/v)

10.09 g of fresh petals were macerated in a blender with 100 ml of 95% EtOH:1.5M HCl (85:15) for 2 minutes and transferred quantitatively to a beaker with a further 50 ml of the extracting solvent. The covered mixture was allowed to stand overnight in

the refrigerator and was then filtered under suction through a Whatman No 44 filter paper. The beaker and the residue on the filter paper were washed repeatedly with the acidic ethanol solvent and then made up to 250 ml in a volumetric flask. Absorbance was measured on a 1-10 dilution of the extract solution at 535 nm.

5.5.3 Results and discussion

5.5.3.1 Extraction following hydrolysis

Petal solution

Wt of petals = 20.59 g

Total wt of material extracted = 0.2243 g

Wt of extracted material for absorbance measurement = 0.0054 g

Medium = 0.01% HCl in MeOH

Volume made up = 5 ml

Dilution = 1-10

Absorbance at 537 nm = 0.802

Standard solution

Standard solution = 0.05 mg/ml cyanidin

Medium = 0.01% HCl in MeOH

Absorbance at 537 nm = 0.372

Therefore $E^{1\%}_{1cm,}537 = 74.4$

($E^{1\%}_{1cm,}$ 537 = absorption measured in a 1 cm cell at 537 nm at a concentration of 1%)

Calculation of cyanin content

Asorbance of 1 g of cyanidin in 100 ml of 0.01% HCl in MeOH = 74.4

Therefore absorbance of 1mg in 1 ml = 7.44

Sample absorbance = 10 × 0.802

Therefore concentration of cyanidin in sample solution = (10 × 0.802)/ 7.44 = 1.08 mg/ml

Therefore (1.08 × 5) mg = 5.4 mg cyanidin in 5 ml extracting solution

Therefore 5.4 mg cyanidin in 0.0054 g of extracts

or 5.4 mg cyanidin in 0.4957 g petals (since 0.2243 g extracts is produced from 20.59 g petals) or 1089 mg cyanidin in 100 g petals

or 2125 mg cyanin in 100 g petals (since ratio of cyanin to cyanidin = 1.95:1)

5.5.3.2 Extraction with EtOH:HOAc:H_2O (EAW, 10:1:9)

(followed by absorbance measurements in two different media)

Petal solutions

Wt of petals = 10.01 g

Total wt of material extracted = 1.2727 g

Wt of extracted material taken for measurement of absorbance = 0.0055 g

Medium = 0.1% HCl in EtOH

$E^{1\%}_{1cm,}$ 535 cyanin in 0.1% HCl in EtOH = 188 (literature value[12])

Volume made up = 2 ml

Dilution = 1-10

Absorbance at 535 nm = 0.948

Calculation of cyanin content

As in section 5.5.3.1,

gives 2335 mg cyanin in 100 g petals

Determination of $E^{1\%}_{1cm}$, 535 for cyanin in 95% EtOH:1.5M HCl

The absorbance values for the pigments extracted (as above) with EAW but measured in 95% EtOH:1.5M HCl (85:15) were as follows:

Petal solutions

Wt of petals = 10.01 g

Total wt of material extracted = 1.2727 g

wts of extracted materials taken for measurement of absorbance = 0.0207 g, 0.0208 g

Medium = 95% EtOH:1.5M HCl (85:15)

Volumes made up = 5 ml

Dilutions = 1-10

Absorbances at 535 nm = 1.2494, 1.2928

The ratio of absorbances for the pigment extracts in the two solvents, when measured at the same concentration, were used to calculate the $E^{1\%}_{1cm}$, 535 of cyanin in the second solvent (95% EtOH:1.5M HCl).

Ratio of absorbances at 535 nm of:

i) 1 g of extracts in 100 ml of 0.1% HCl in EtOH to

ii) 1g of extracts in 95% EtOH:1.5M HCl

i) absorbance of 0.0055 g extracts in 2 ml 0.1% HCl in EtOH , dilution 1-10 = 0.948

therefore absorbance of 0.0055 g in 2 ml = 9.48

therefore absorbance of 0.275 g in 100 ml = 9.48

therefore absorbance of 1 g extracts in 100 ml 0.1% HCl in EtOH = 34.5

ii) absorbance of 0.0207 g & 0.0208 g extracts in 5 ml each of 95% EtOH:1.5M HCl, dilution 1-10 = 1.2494 & 1.2928 respectively. As calculated as above, this gives an average value of absorbance for 1 g of extracts in 100 ml of 95% EtOH:1.5M HCl (85:15) = 30.6

The ratio absorbances of i) to ii) = 0.89

$E^{1\%}_{1cm,}$ 535 for cyanin in 0.1% HCl in EtOH = 188, therefore

$E^{1\%}_{1cm,}$ 535 for cyanin in 95% EtOH:1.5M HCl (85:15) = 188 × 0.89 = 167

*Note: this ratio of absorbances of the extracts in the two media was also obtained for the batch of roses in which extraction was thought to be incomplete and therefore, as such, unsuitable for direct calculation of anthocyanin content

i.e. 0.0059 g extracts made up to 2 ml in 0.1% HCl in EtOH, diluted 1-10, gave absorbance 0.8116 and 0.0110 g & 0.0100 g extracts each made up to 5 ml with 95% EtOH:1.5M HCl and diluted 1-10 and 1-5, gave absorbances 0.5412 and 0.9823 respectively.

The values, however, still served the purpose of determining the ratios of absorbances in the two media i.e. 1 g of extracts in 100 ml of 0.1% HCl in EtOH at 535 nm = absorbance of 27.5 and 1 g of extracts in 95% EtOH:1.5M HCl (85:15) at 535 nm = 24.6 (average of two sets of absorbances) giving a ratio 0.89.

5.5.3.3 Extraction with 95% EtOH:1.5M HCl (85:15 v/v)

Petal solution

Wt of petals = 10.09 g

Medium = 95% EtOH:1.5M HCl (85:15)

Volume made up = 250 ml

Dilution = 1-10

$E^{1\%}_{1cm}$, 535 cyanin in 95% EtOH:1.5M HCl (85:15),

(calculated in section 5.5.3.2) = 167

Absorbance = 1.483

Calculation of cyanin content

Absorbance of 1 g of cyanin in 100 ml of 95% EtOH:1.5M HCl (85:15) = 167

Therefore absorbance of 1 mg in 1 ml = 16.7

Sample absorbance = 1.4833 × 10

Therefore concentration of cyanin in sample = 1.4833 ×10 /16.7 mg/ml = 0.89 mg/ml

Therefore (0.89 × 250) mg = 222.5 mg in 250 ml extracting solution

Therefore 222.5 mg in 10.09 g petals

Therefore 2205 mg in 100 g petals.

Using three different extraction methods and three different media for measuring absorbances, the values for the anthocyanin content of the petals from three different batches of roses were 2125 mg, 2335 mg and 2205 mg in 100 g of petals, giving an average value of 2220 mg of anthocyanins per 100 g of petals for the variety First Red. This would indicate that, as expected, the anthocyanin content of a given variety of flowers is fairly constant.[1, 3]

5.6 Determination of diol/ moisture content of processed petals

5.6.1 General

To re-cap on the theory behind this method of preservation; the process solution is believed to exchange with the cell sap of the plant material when submerged in the process fluid for a period of time at temperatures of approximately 80 °C. In this section an attempt was made to measure the extent of this exchange by measuring the diol (65% propan-1,2-diol, 25% butan-1,4-diol, 10% hexan-1,6-diol) and water content of the processed petals using simple oven drying methods. This involved two approaches, one of which was based on the assumption that the weight of processed petals was made up from the weights of diols (process fluid), water (cell sap), extractable material (plant pigments and other solutes) and fibrous material. The other approach involved comparison of the oven drying of solutions of diols and water with the oven drying of processed petals.

In either case, however, the measurement of the moisture content of the processed petals was complicated by the fact that this value was not constant and was considerably affected by the external conditions. Bearing this in mind the environmental conditions were kept constant (as far as possible) and weighing of the

pre-dried processed petals was made when the processed weight was equal to the fresh weight.

5.6.2 Experimental

5.6.2.1 Determination of moisture and solids content of fresh petals

2.205 g, 8.470 g and 8.285 g of fresh First Red petals from three different batches of roses were oven dried at 100 °C for 24 hours and allowed to cool in a desiccator before re-weighing.

5.6.2.2 Determination of extractable material content of fresh petals

10.29 g, 10.01 g and 11.90 g of fresh petals from three different batches of First Red were each macerated in a blender with 100 ml of EAW (as in section 5.5.2.2) and quantitatively transferred to a beaker with approximately 50 ml of solvent and left to stand overnight. The extracts were then suction filtered through a No 44 Whatman filter paper and washed repeatedly with solvent. The solvent was evaporated off on a rotary evaporator at 30-40 °C and the residue weighed.

5.6.2.3 Determination of diol + extractable material content of processed petals.

11.69 g, 8.47 g and 5.95 g of processed petals from three different batches of First Red were extracted with EAW as for the fresh petals (section 5.5.2.2) and the filtered material repeatedly washed with EAW. The filtrate was transferred to a distillation apparatus and the ethanol (bp 78 °C), acetic acid (bp 118 °C) and water (bp 100 °C)

distilled over. The remaining solution of diols (bp > 186 °C) and petal extracts was then weighed.

5.6.2.4 Oven drying of processed petals and water/diol solutions

10ml of distilled water, 2 × 10 ml of diols, 2 × 10 ml of 60:20 diol: water solution and two lots of processed petals were weighed out and then dried in the oven at 100 °C for 24 hours. The dried solutions and petals were allowed to cool in a desiccator and were then re-weighed.

5.6.2.5 Investigation into the moisture absorption properties of processed petals

Two weighed flower heads (of unknown varieties), one red and one white were left to stand in a humid environment (relative humidity 70-80 %) for one week and were then re-weighed.

5.6.3 Results and discussion

5.6.3.1 Determination of moisture and solids content of fresh petals

The results of oven drying the fresh petals (Table 5.7) were used to determine the average moisture content (pre-processing) of the fresh petals and the weight of the remaining solids were used to determine the average (fibre + extractable material) content.

Wt of petals before drying (g)	Wt of petals after drying (g)	% Moisture content	% Extractable/ fibrous material content
2.205	0.731	83.2	16.8
8.470	1.517	82.1	17.9
8.285	1.376	83.4	16.6

Table 5.7 Moisture and extractable/ fibrous material content of fresh petals.

Average moisture content = 82.9%.....(1).

Therefore since:

wt of fresh petals = wt of moisture + wt of extractable material + wt of fibrous material, then:

100% - (1) = 100% - 82.9%

= Average (extractable + fibrous) material content = 17.1%.....(2).

5.6.3.2 Determination of extractable material content of fresh petals

The results of extractions with EAW (Table 5.8) were used to determine the average extractable material content of fresh petals (i.e. pigments and other solutes).

Wt of petals (g)	Wt of extracted material (g)	% extractable material
10.29	1.2900	12.5
10.01	1.2727	12.7
11.90	1.3565	11.4

Table 5.8 Extractable material content of fresh petals.

Average extractable material content = 12.2%.....(3)

5.6.3.3 Determination of diol + extractable material content of processed petals

The weight of substances remaining, following the extraction of the processed petals with EAW and then removal of the solvent (Table 5.9), was used to determine the average (diols + extractable materials) content of the processed petals.

Wt of processed petals (g)	Wt of diols/extractable materials (g)	% diols/extractable materials
11.69	8.86	75.8
8.47	6.49	76.6
5.95	4.26	71.6

Table 5.9 Diols and extractable material content of processed petals.

Average (diols + extractable materials) content = 74.7%.....(4).

Therefore, assuming that the weight of extractable materials is the some for both processed and fresh petals (since any extractable material lost on processing would possibly be cancelled by a gain in copper sulphate and *p*-toluenesulphonic acid content), then:

wt of diols = wt of (diols + extractable materials) - wt of extractable materials

then:

(4)-(3) = 74.7%- 12.2%

= average diols content = 62.5%.....(5).

From 5.6.1:

Wt of processed petals = wt of diols + wt of water + wt of extractable materials + wt of fibrous materials.

therefore

100% - % diol content - % (extractable + fibrous material) content (or residue on drying fresh petals)

= 100% - (5) - (2)

= 100% - 62.5% - 17.1%

= average water content = 20.4%.....(6).

Therefore the approximate ratio of diols to water in processed petals is 60: 20.

5.6.3.4 Oven drying of processed petals and water/diol solutions

The percentage loss on drying of solutions of diols and water was compared with the percentage loss on drying (under identical conditions) of processed petals (Table 5.10).

Sample	Wt before drying (g)	Wt after drying (g)	% wt loss
Water	10.0250	0.0001	100
Diols	10.0361	5.6438	43.8
Diols	10.0307	5.7534	42.6
60:20 water/diols	10.0964	3.7862	62.5
60:20 water/diols	10.1212	3.8144	62.3
Processed petals	4.9477	1.7376	64.9
Processed petals	4.0901	1.4188	65.3

Table 5.10 Losses on drying for processed petals and diol/water solutions.

From these results it could be seen that the percentage losses on drying the processed petals were relatively close to the losses on drying the 60:20 diol/ water solutions.

These results would therefore support the previous estimate of a 60:20 diol/water content for processed petals (section 5.6.3.3) for petals and flowers stored under normal laboratory conditions (i.e. Temperature of approximately 20° C, relative humidity of approximately 45%).

5.6.3.5 Investigation into the moisture absorption properties of processed petals.

From the results of this experiment (Table 5.11) it appeared that although the diol content of the processed petals presumably remained constant at room temperature, the water content of processed petals was very much dependent on environmental conditions (i.e. in a humid environment they were found to absorb moisture and become limp and in dry conditions they were observed to become papery and brittle).

Wt of flower head before storage (g)	Wt of flower head after storage (g)	% Wt gain
9.135	10.728	17.4
4.925	5.673	15.2

Table 5.11 Moisture absorption properties of processed flower heads

5.7 Acidification of pigment extracts

5.7.1 General

The pH has been shown to be the most important factor affecting the colour of anthocyanins (section 3.5) and previous work on maintaining the colour of fresh petals by acidification concluded that *p*-toluenesulphonic acid (PTSA) was the most suitable commercial ingredient for this purpose (section 4.5).

In this section an attempt was made to find the optimum concentration of PTSA required in order to produce sufficient colour in solutions of the extracts of the rose variety First Red.

The extracts used were the total petal extracts from extraction with EAW (section 5.5.2.2) as it seemed pertinent to study the anthocyanin pigments in association with other cellular constituents with which they could be interacting.

Plant extracts dissolved in the commercial diol mixture were studied, as were those in a 60:20 diol/water mixture and those in an aqueous solution.

5.7.2 Experimental

10.01 g of fresh First Red petals were extracted with EAW (as in 5.5.2.2) to give 1.2727 g of extracts. 0.0355 g of these extracts were dissolved in 5 ml of diol solution and 1 ml of this solution was diluted to 10ml to give an anthocyanin concentration of 1.8×10^{-4} M.

PTSA was added in increments, starting at 5 mg and increasing to 1000 mg (to give 0.05%-10 % w/v PTSA in solution). The pH and UV/VIS absorption spectra were recorded after allowing the solutions to stand for approximately 20 minutes after each addition.

This procedure was then repeated with an aqueous solution of the pigment extracts and also with a 60:20 diol/water solution (1.7×10^{-4} M and 1.6×10^{-4} M solutions of anthocyanins respectively).

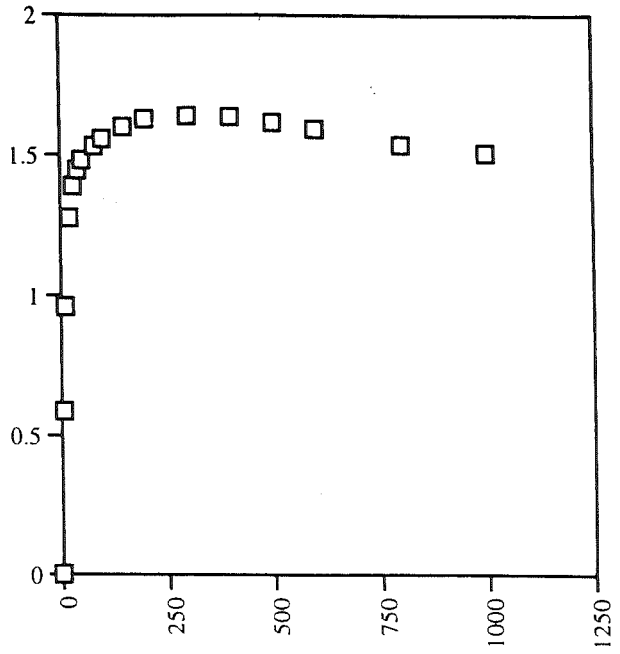
5.7.3 Results and discussion

The results of the addition of PTSA to 10 ml of the diol solution of pigment extracts were as presented in Fig. 5.5. From a plot of absorbance against milligrams of PTSA added (Fig 5.5 a) it could be seen that the maximum absorbance occurred after the addition of approximately 200-300 mg of acid (2.0-3.0% PTSA solutions). From a plot of absorbance against pH (Fig. 5.5 b) it could be seen that this maximum took place at a pH of less than 1.7. The wavelength of maximum absorbance was approximately 530 nm and no spectral shifts were observed with increasing the concentration of acid.

For the 60:20 diol/ water solution and the aqueous solution of pigments extracts, the maximum absorbance was also reached at PTSA concentrations of approximately 2.0-3.0%, and at a pH of less than 1.7. The maximum absorbance for the 60:20 diol/ water solution occurred at a wavelength of approximately 527 nm and again there was no spectral shift observed on addition of acid. Addition of acid to the aqueous solution of extracts, however, resulted in a shift in maximum absorbance to longer wavelength with each addition of acid, giving an overall shift of 110-119 nm. The fact that this shift in the maximum wavelength only appeared to occur in the aqueous solution was possibly a manifestation of the hydrophobic effect (section 3.6.2.).

Bearing in mind that in the processed petals the pigments would be in a diol/water solution and that costs were to be kept to a minimum, an acid concentration of approximately 2% w/v and pH 1.6-1.7 was considered sufficient. This was in keeping with previous work on the processing of whole flowers which indicated that satisfactory colour was achieved with process solutions containing 2-2.5% w/v acid at a pH of 2 and below (section 4.5).

a)



b)

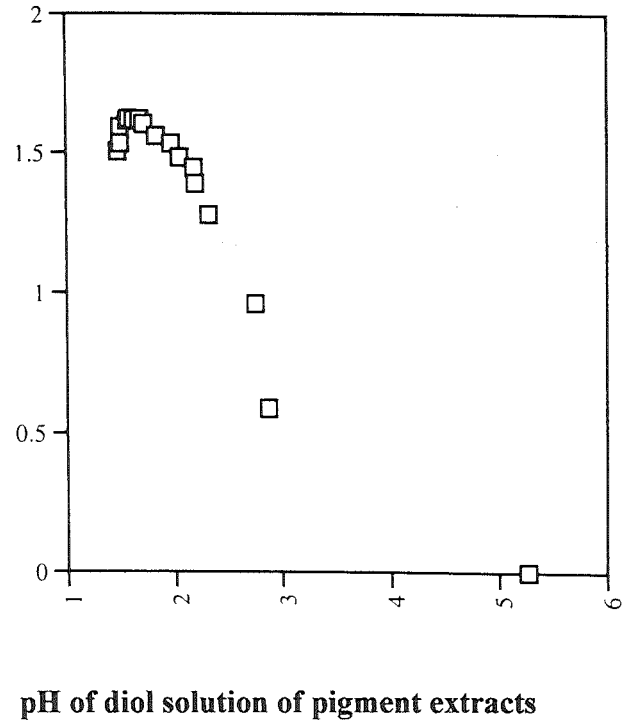


Fig. 5.5 The addition of *p*-toluenesulphonic acid (PTSA) to a diol solution of pigment extracts (rose variety First Red)

a) Plot of maximum visible absorbance against mg of PTSA added to pigment extracts in diol solution

b) Plot of maximum visible absorbance against pH of diol solution of pigment extracts

5.8 The study of acidified $CuSO_4$ solutions of pigment extracts

5.8.1 General

Petals processed in the diol solutions containing 2% w/v PTSA initially gave strong colours, however, the processed colour was generally a lighter shade than the fresh petals and on exposure to light, the colours were observed to fade slightly (Table 4.4) Previous work on whole petals had indicated light stability could be improved by the addition of metal salts (section 4.17) and $CuSO_4$ was of particular interest as it was also used to stabilise chlorophyll in the foliage process.

In this section the effects of Cu (II) ions on acidified solutions of pigment extracts of the rose variety First Red were studied. Initially this involved studying the effects of various concentrations of copper ions on the stability of pigment extracts in three different media (diol, 60:20 diol/water and water), followed by studies of the stabilising effects of copper after processing at different processing temperatures and times. Process temperatures below 70 °C were not considered since, in general, the fluid exchange process between process solution and cell sap did not take place below 60-70 °C and process times of 15 minutes and above were considered necessary in order to ensure that the exchange was completed (section 4.4).

This *in vitro* work involved mimicking the process by heating solutions of the pigment extracts in a water bath for specified times and temperatures. The processed solutions were cooled and then exposed to the same light box treatment as was used for the whole petals. UV/VIS absorption spectra of the solutions were obtained before and after heating (processing) and after continuous light box exposure for periods of 24 hours and for a further 6 days (giving 7 days in total). The period of 24 hours was considered necessary in case of rapid deterioration of the pigments and it

had also been used commercially as a rapid test for the foliage process (section 4.2.3).

5.8.2 Experimental

5.8.2.1 Investigation into the effects of $CuSO_4$ concentration and solvent composition

10.01 g of fresh First Red petals were extracted with EAW (as in 5.5.2.2) to give 1.2727 g of extracts.

0.0355 g extracts were dissolved in 5 ml diol solution and diluted (1-10) × 4 in stoppered test tubes to give four 1.8×10^{-4} M anthocyanin solutions.

200 mg of PTSA was added to each solution to give 20 g/L (2% w/v) PTSA solutions and the absorption spectra were recorded.

5 mg, 10 mg and 100 mg $CuSO_4$ was added to each of the three solutions and one solution was left without $CuSO_4$. This gave acidified solutions of 0.0 g/L, 0.5 g/L, 1.0 g/L and 10 g/L $CuSO_4$. These solutions were heated in a water bath at 80 °C for 30 minutes and cooled under running water. UV/VIS absorption spectra of the processed solutions were recorded and the solutions were then subjected to light box treatment (operating at 20,000 lux) for a period of 24 hours and then for a further 6 days, after each of which UV/VIS absorption spectra were recorded.

This entire process was repeated for the same concentration of pigment extracts in 60:20 diol /water solutions and also for the extracts in aqueous solutions.

5.8.2.2 Investigation into the effects of $CuSO_4$ concentration, process temperature and process time

Solutions of pigment extracts (1.9×10^{-4} M anthocyanins) and $CuSO_4$ (0.0 g/L, 0.5 g/L, 1.0 g/L and 10 g/L) in a 60:20 diol/water mixture were prepared as in section 5.8.2.1. The solutions were heated at 70 °C for periods of 15, 30 and 60 minutes, 80 °C for periods of 15, 30 and 60 minutes and 90 °C for 30 minutes. UV/VIS absorption spectra were recorded before heating (processing), after heating, after 24 hours light box treatment and after at total of 7 days light box treatment.

5.8.3 Results and discussion.

5.8.3.1 Investigation into the effects of $CuSO_4$ concentration and solvent composition

Before heating, the UV/VIS absorption spectra of the acidified 1.8×10^{-4} M anthocyanin solutions in diols gave an average maximum absorbance of 1.747 at a wavelength of 530 nm.

The changes in absorbance on heating (processing) and on further light box testing were as recorded in (Table 5.12) and as illustrated in (Fig.5.6). There was no change in the wavelength of maximum absorbance on the addition of $CuSO_4$.

From these results it could be seen that the anthocyanins in acidic diol solution were unstable to both heat and light, but that addition of $CuSO_4$ went some way to stabilising the pigments. This breakdown of anthocyanins and the subsequent drop in concentration could have been one of the reasons that the colour of the petals processed in solutions without $CuSO_4$ often appeared slightly lighter in shade than those processed with the copper salt (Table 4.4).

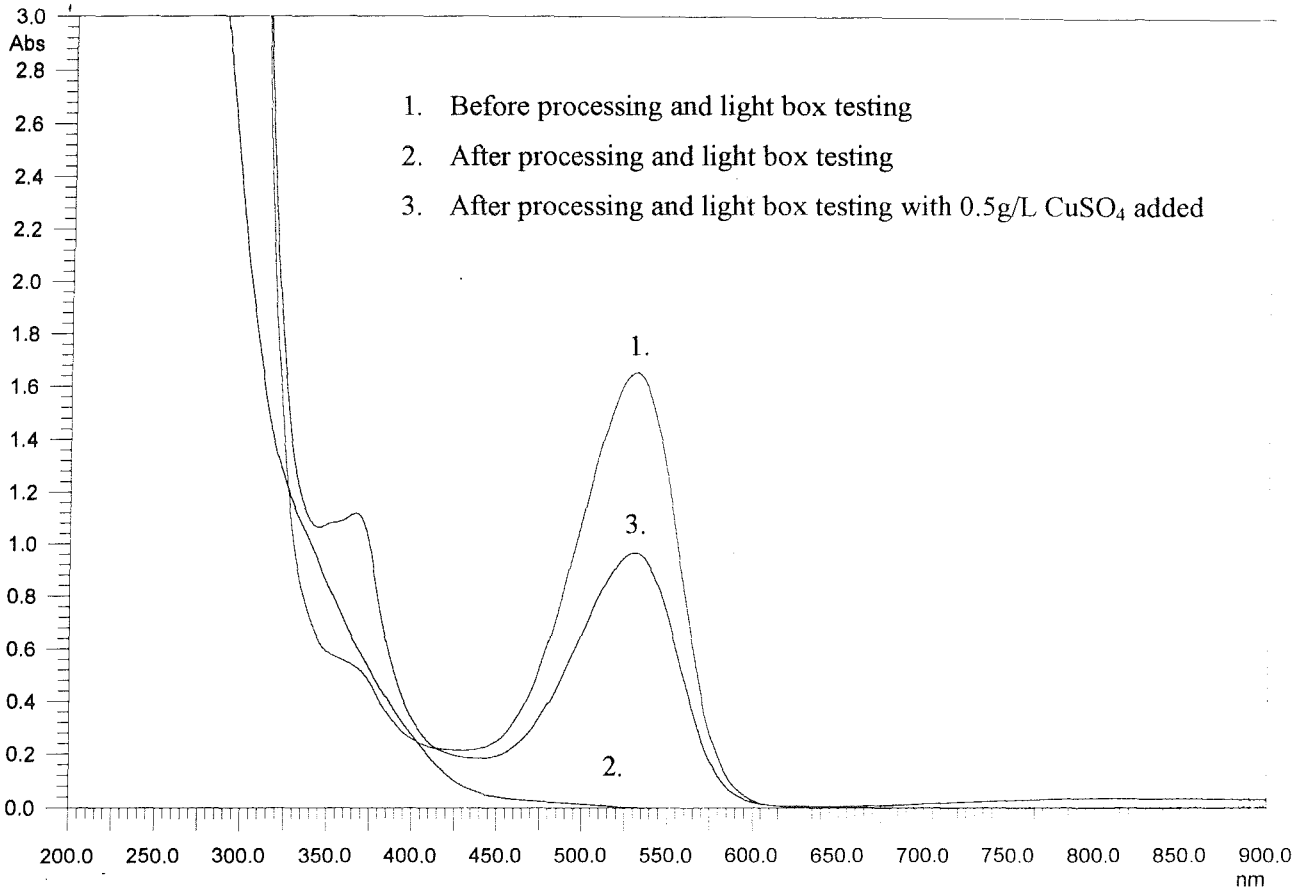


Fig. 5.6 UV/VIS absorption spectra of pigment extracts (rose variety First Red) in acidified diol solutions.

$CuSO_4$ concentration (g/L)	Change in absorbance on heating (processing)	Change in absorbance after 24 Hour light box test (from processing)	Change in absorbance after 7 day light box test (from processing)
0.0	- 0.694	-0.862	No absorbance
0.5	-0.131	-0.175	-0.613
1.0	-0.164	-0.199	-0.751
10.0	-0.147	-0.211	-0.703

Table 5.12 changes in the visible absorbance of diol solutions containing pigment extracts, *p*-toluenesulphonic acid and copper sulphate.

For the same concentration of extracts in 60:20 diol/water solutions the average value of maximum absorbance before processing was 1.667, occurring at 527 nm. The changes in absorbance on heating and light box testing were as in Table 5.13 and as illustrated in (Fig. 5.7) by the comparison of spectra of solutions containing 0.5 g/L $CuSO_4$ and those without. Once again there was no shift in the maximum absorbance observed.

$CuSO_4$ Concentration (g/L)	Change in absorbance on heating (processing)	Change in absorbance after 24 hour light box test (from processing)	Change in absorbance after 7 day light box test (from processing)
0.0	- 0.190	-0.166	-0.321
0.5	-0.063	-0.044	-0.240
1.0	-0.046	-0.046	-0.270
10.0	-0.088	-0.038	-0.211

Table 5.13 changes in the visible absorbance of 60:20 diol/water solutions containing pigment extracts, *p*-toluenesulphonic acid and copper sulphate.

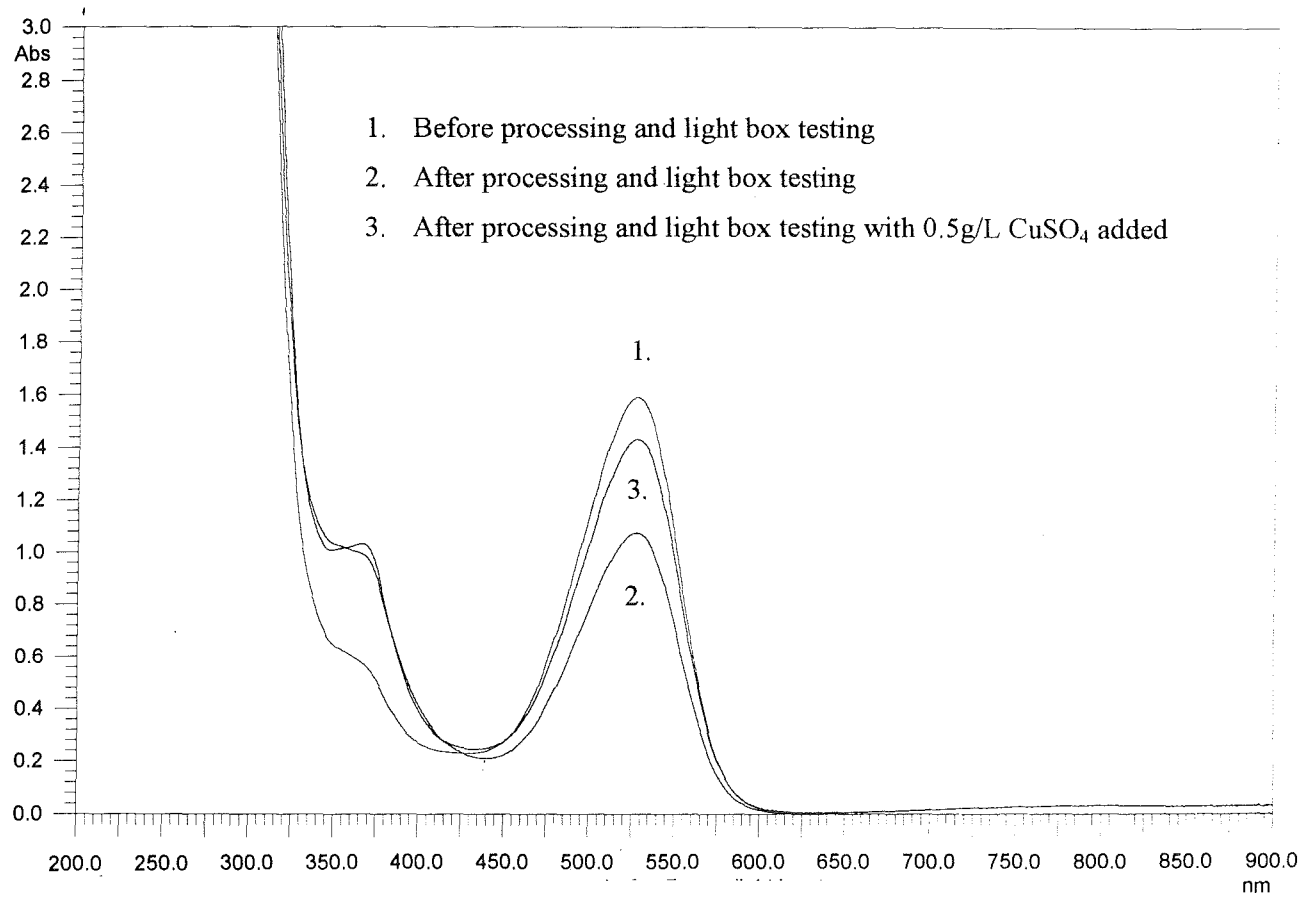


Fig. 5.7 UV/VIS absorption spectra of pigment extracts (rose variety First Red) in acidified 60: 20 diol/water solutions.

From these results the most significant observation was that, even without the addition of $CuSO_4$, the pigments were more stable to heat and light in the acidified 60:20 diol/water solution than they were in the non-aqueous diol solution. At the end of the 7 day light box test the solution without $CuSO_4$ was still coloured whereas in the previous set of results, the diol solution without $CuSO_4$ had become completely colourless. Again, $CuSO_4$ was seen to increase the anthocyanin stability, resulting in relatively strong colour even after light exposure.

In the case of the 1.8×10^{-4} M aqueous anthocyanin solutions, the maximum absorbance before heating was 1.471 at a wavelength of 515 nm. The results of processing and light box treatment were as reported in Table 5.14 and as illustrated in Fig. 5.8.

$CuSO_4$ concentration (g/L)	**Change in absorbance on heating (processing)**	**Change in absorbance after 24 hour light box test (from processing)**	**Change in absorbance after 7 day light box test (from processing)**
0.0	+ 0.008	+ 0.014	+ 0.056
0.5	- 0.008	+ 0.014	0.000
1.0	+ 0.002	+ 0.011	+ 0.023
10.0	- 0.043	+ 0.025	+ 0.067

Table 5.14 Changes in visible the absorbance of aqueous solutions containing pigment extracts, *p*-toluenesulphonic acid and copper sulphate.

Here it could be seen that in aqueous solutions, the anthocyanins were completely stable to processing and light testing and that $CuSO_4$ had little or no effect on this stability.

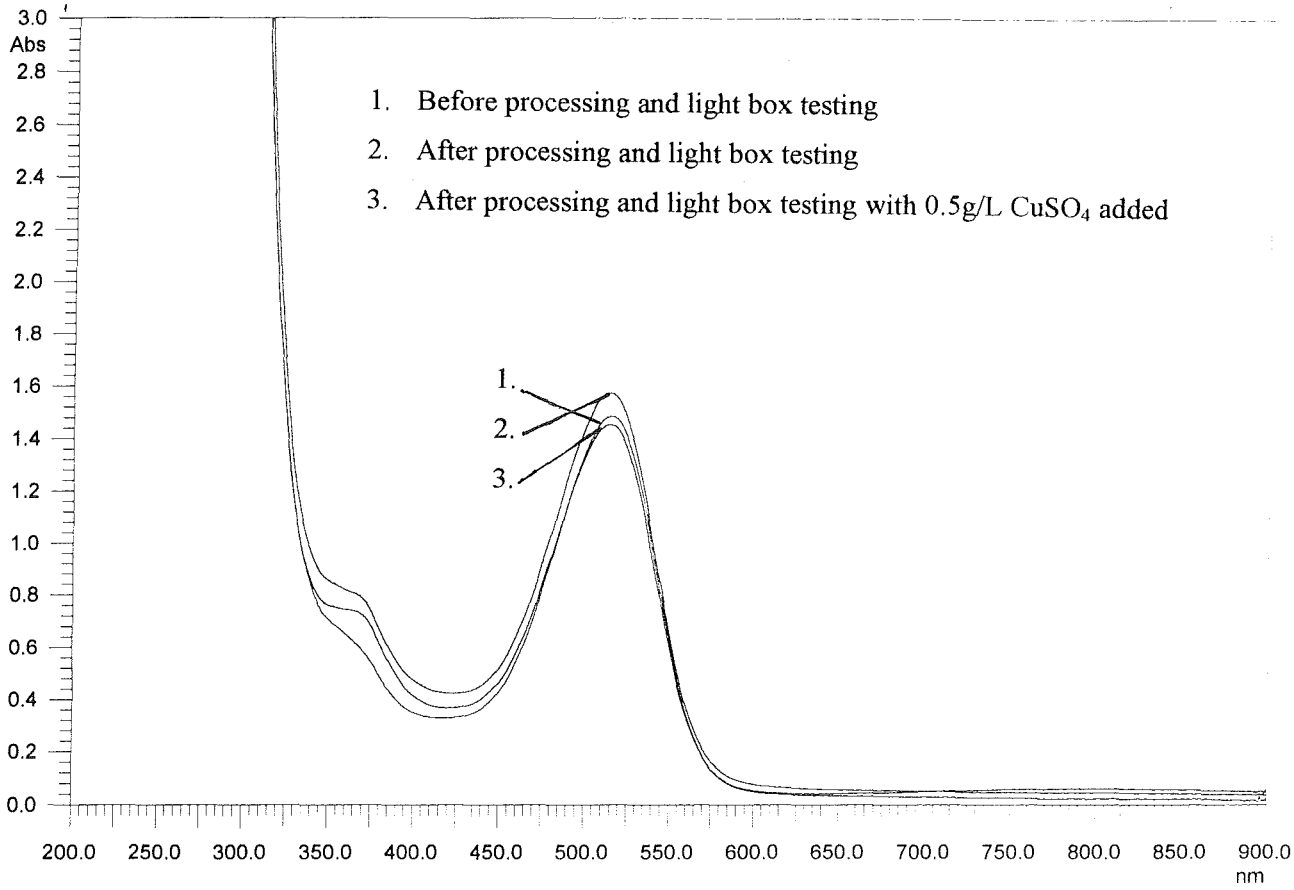


Fig. 5.8 UV/VIS absorption spectra of pigment extracts (rose variety First Red) in acidified aqueous solutions.

This effect; the much greater stability of the anthocyanins in aqueous solution as opposed to their stability in diol solutions could be explained by the phenomenon of copigmentation. Copigmentation, along with pH, is probably the most important single factor influencing flower colour and pigment stability (section 3.6). To recapitualte, copigmenation consists of a loose association between the flavylium cation or quinoidal base and a flavonol or related compound (or in the case of self-association, with another molecule of anthocyanin (section 3.6.3)). Copigments stabilise and enhance the colour of anthocyanins by intermolecular, hydrophobically reinforced π-π stacking of their aromatic nucleii with those of the pigment. This intermolecular stacking shields the pyrilium ring from nucleophilic attack at C2 thereby preventing water nucleophilic addition and subsequent colour loss (section 3.6.2). The structural and electronic features of the of the coloured forms of the anthocyanins i.e. that they are almost planar chromophores with extended delocalization, make them particularly suitable to form molecular, non-covalent complexes. The most effective copigments are also planar, their effectiveness being related to the potential surface available for π-π stacking and amongst the most efficient so far are flavonols such as rutin and quercetin (section 3.6.5). The stabilising effect of copigments is considerably weaker in solvent mixtures other than pure water and it has been found that copigmentation occurs only when sufficient water is present (section 3.6.2), a finding consistent with the disrupting effect of other solvent molecules on the lattice of liquid water. In other words, on the effect of the solvent on the copigmentation phenomenon, no solvent is better than water, indicating that the strength of the copigment effect parallels the cohesion of the hydrogen bonded tetrahedral network of water molecules.

From the above results it was observed that the pigment extracts were extremely stable in aqueous solution, partially stable in the 60:20 diol water solution and unstable in the non-aqueous diol solution. It would appear therefore, that the stability of the anthocyanins in the aqueous solution of extracts was due to a copigmentation effect, most likely by association with one of the quercetin or kaempferol glycosides (which had been detected in the First Red petals (section 5.3)), or possibly by self-association (since the concentration of anthocyanins was of a suitable order i.e.10^{-2} M (section 3.6.5)). This stabilising effect was then disrupted by the introduction of diols to the medium and was destroyed by the total replacement of water by diols. Conveniently, however, the drop in stabilisation by copigmentation was minimised on processing since there still appeared to be an appreciable amount of water present in the processed petals (section 5.6). Furthermore, the ability of diols to mimic water in their ability to hydrogen bond to their neighbours meant that the decrease in the copigment effect was less than it might have been with any other cosolvent.[14]

Addition of copper ions to the diol containing solutions was seen to improve the stability of these pigmented solutions, possibly by strengthening the pigment-copigment interaction and /or by allowing the association of the pigment-copigment units (section 3.6.6). A small shoulder (occurring at approximately 355-368 nm) was observed after heating and light box testing the copper containing diol solutions (Fig. 5.6) and to a lesser extent after heating and light box testing the 60:20 diol/water copper containing solutions (Fig. 5.7). The shoulder for the aqueous solutions was, however, almost negligible as was the stabilising effect of copper (Fig. 5.8). The fact that copper had no apparent stabilising effect on the aqueous solutions of extracts

may have been as a result of the pigment-copigment interaction in water already being in its most stable state.

From the results of this experiment and referring back to the work carried out on petals (section 4.16), it could be deduced that the difference in the observed shade of First Red petals processed with and without copper ions was due the difference in concentration of the anthocyanin pigments (since the maximum wavelength did not alter). The shade of the petals processed with copper ions was very close to that of the fresh petals, whereas the petals processed without copper had suffered a drop in anthocyanin concentration, which continued to fall on light exposure, resulting in paler petals. The very slight difference between the shade of the fresh petals and the shade of the petals processed with copper ions was most likely due to the change in the medium (from water as in Fig. 5.8 to diol/water as in Fig. 5.7) and possibly to an alteration of the physical nature of the epidermal tissues. Changes in the structure and composition of the tissues meant that changes in optical effects such as reflection and scattering were possibly occurring.

The overall effect for the variety First Red was, however, one of very little observable colour change between processed and unprocessed petals. With other varieties this would not necessarily be the case, since the pigments and their stabilising environment could be very different, as could their physical environment. For example, if there happened to be a high proportion of a pelargonidin glycoside present, it might not stabilise as well with copper (e.g. Sacha or Danse de Feu) as a cyanin glycoside due to the absence of a catechol group in the former (section 3.7.7). Or, if the concentration of pigments was too low to permit copigmentation (as was observed when in cyanin extracts were diluted (section 5.3.3.2)) then stabilisation on

processing would not take place. Both of these situations would result in a drop in concentration of pigments in the processed flower and therefore a change in colour. Furthermore, physical changes as a result of storage conditions e.g. freezing, or condition of the plant on processing could also have some effect on the observed colour of processed petals.

5.8.3.2 Investigation into the effects of $CuSO_4$ concentration, process temperature and process time

The percentage changes in absorbance (from the original value) at 527 nm on processing and light box testing a series of acidified (20 g/L PTSA) 60:20 diol/water solutions at different temperatures and process times were as reported in Tables 5.15, 5.16 and 5.17.

From these results it was apparent that both temperature of processing and processing time had a considerable effect on the final absorbance of the solutions processed without copper salt. As expected, due to the thermal degradation of the pigments, the higher the temperature, the greater the decrease in absorbance for a given process time. Likewise, the longer the processing time for a given temperature the lower the absorbance on processing. On light box treatment of the processed solutions the absorbances then were seen to drop further.

It was noted, however, that at low temperatures and short processing times i.e. at 70 °C for 15 or 30 minutes (Table 5.15) and 80 °C for 15 minutes (Table 5.16), that a slight increase in absorbance occurred and that this increase was greatest for the solutions containing copper sulphate. This increase pattern was an indication that some form of stabilisation process was taking place.

$CuSO_4$ concentration (g/L)	% change in absorbance on processing	% change in absorbance after 7 day light box test (from processing)	% total change in absorbance on heating and light box testing.
Process time 15 minutes			
0.0	+ 6	- 21	- 15
0.5	+ 8	- 10	- 2
1.0	+ 7	-11	- 4
10.0	+ 5	-11	- 6
Process time 30 minutes			
0.0	+ 2	-18	-16
0.5	+ 6	-10	- 4
1.0	+ 5	-10	- 5
10	+ 3	- 12	- 9
Process time 60 minutes			
0.0	- 1	- 17	- 18
0.5	+ 3	- 17	- 14
1.0	+ 3	- 22	- 19
10	0	- 17	- 17

Table 5.15 Changes in the visible absorbances of solutions of pigment extracts processed at 70 °C for periods of 15, 30 and 60 minutes.

Lengthening the process time to 60 minutes for a given temperature resulted in little or no overall improvement in stability by the addition of copper ions, implying that any stabilisation effect, possibly by the formation of a loose association, was destroyed by prolonged heating.

$CuSO_4$ concentration (g/L)	% change in absorbance on processing	% change in absorbance after 7 day light box test (from processing)	% total change in absorbance on heating and light box testing.
Process time 15 minutes			
0.0	+ 2	- 22	- 20
0.5	+ 6	- 17	- 11
1.0	+ 6	- 16	- 10
10	+ 4	- 17	-13
Process time 30 minutes			
0.0	- 5	- 20	- 25
0.5	- 1	- 14	- 15
1.0	- 1	- 16	- 17
10.0	- 4	- 13	- 17
Process time 60 minutes			
0.0	- 13	- 15	- 28
0.5	- 11	- 13	- 24
1.0	- 11	- 16	- 27
10.0	- 15	- 15	- 30

Table 5.16 Changes in the visible absorbances of solutions of pigment extracts processed at 80 °C for periods of 15, 30 and 60 minutes.

$CuSO_4$ concentration (g/L)	% change in absorbance on processing	% change in absorbance after 7 day light box test (from processing)	% total change in absorbance on heating and light box testing.
Process time 30 minutes			
0.0	- 44	-16	- 60
0.5	- 12	-11	- 23
1.0	- 18	-12	- 30
10	- 16	-16	- 32

Table 5.17 Changes in the visible absorbances of solutions of pigment extracts processed at 90 °C for 30 minutes.

In general, however, the addition of copper sulphate at concentrations of 0.5 g/L and 1.0 g/L was observed to increase the stability of the pigments to both heat and light, however, at concentrations of 10 g/L copper sulphate the stabilising effect tended to be reduced. Correspondingly, a shoulder/peak at approximately 365 nm was observed for the 0.5 g/L and 1.0 g/L copper sulphate solutions after light box exposure, but this shoulder/peak was not as prominent for the 10 g/L copper sulphate solutions. It appeared, therefore, that the introduction of copper ions at low concentrations strengthened the pigment-copigment (or self-association) interaction and improved stability, whereas this stabilising effect was diminished by high copper sulphate concentrations. As mentioned previously, copigmentation is strictly dependent on any factor acting on the highly structured aqueous medium, therefore, increasing the ionic strength by the addition of large amounts of copper sulphate possibly weakened the pigment-copigment molecular association thereby reducing stability.

On the basis of the above *in vitro* studies the implications for processing would be to keep processing temperatures and process times as low as possible (i.e. 70 °C for 15 minutes) and to maintain copper sulphate concentration below 10 g/L, all of which would be of financial benefit. Moreover, on consideration of the work carried out on petals (section 4.16) a minimum concentration of 0.5 g/L copper sulphate was generally required for overall colour stabilisation and up to 10 g/L did not appear to have a detrimental effect. This was convenient from a practical viewpoint as it meant that the copper sulphate concentration did not have to be monitored too closely providing that it was not allowed to fall below 0.5 g/L.

5.9 The effects of alternative metal salts on acidified solutions of pigment extracts

5.9.1 General

From previous work on whole petals it was observed that metal ions other than copper were capable of stabilising or altering the colour of petals processed in acidified solutions (section 4.17). In this section the effects of various metal ions on the UV/VIS absorbance of solutions of acidified pigment extracts were studied in an attempt to obtain a more quantitative comparison of stabilisation between metals.

Initially pigment extracts of First Red in acidified 60:20 diol/water solutions with metal salt contents of 0.5 g/L, 1.0 g/L and 10 g/L were studied. This was followed by examination of the metal salts in acidified diol solutions at concentrations of 10 g/L, since the stabilisation effect was more pronounced in diol solutions and from the results of the initial work, the higher metal concentrations generally gave better stabilisation for metals other than copper.

5.9.2 Experimental

5.9.2.1 Investigation into the effects of metal salts in acidified 60:20 diol/water solutions

Acidified 60:20 diol/water solutions of First Red extracts containing concentrations of 1.8×10^{-4} M anthocyanins were prepared as in section 5.8.2.1 and the UV/VIS absorbances measured (200-900 nm). 0 mg, 5 mg, 10 mg and 100 mg of each of the following metal salts:

Calcium sulphate	Ferrous sulphate
Chromic sulphate	Magnesium sulphate

Auminium sulphate	Stannous chloride
Ferric chloride	Zinc sulphate

were added to 10 ml of each solution to give metal salt concentrations of 0.0 g/L, 0.5 g/L, 1.0 g/L and 10 g/L respectively. These solutions were heated at 80 °C on a water bath for 30 minutes and cooled under running water. UV/VIS absorption spectra of the processed solutions were recorded and the solutions were then subjected to light box treatment for periods of up to 7 days and again the UV/VIS absorption spectra were recorded.

5.9.2.2 Investigation into the effects of metal salts in acidified diol solutions

Acidified diol solutions of First Red extracts containing 1.8×10^{-4} M anthocyanins were prepared and the UV/VIS absorbances measured.
100 mg of each of the following metal salts were added:

Chromic sulphate	Copper sulphate
Aluminium sulphate	Cobalt sulphate
Ferric chloride	Nickel sulphate
Ferrous sulphate	Manganese sulphate
Magnesium sulphate	Sodium molybdate
Stannous chloride	Sodium tungstate
Zinc sulphate	

to give diol solutions containing 10 g/L of each salt. An acidified diol solution of equivalent anthocyanin content but without metal salt was also prepared and all

solutions were heated on a water bath at 80 °C for 30 minutes. UV/VIS absorption spectra of the cooled solutions were obtained and the solutions were then subjected to light box treatment and the UV/VIS spectra were recorded.

5.9.3 Results and discussion

5.9.3.1 Investigation into the effects of metal salts in acidified 60:20 diol/water solutions

The UV/VIS absorption spectra, after processing and light box testing, of the various metal salt solutions at concentrations of 0.5 g/L, 1.0 g/L and 10 g/L are shown in Figs. 5.9, 5.10 and 5.11.

In general the maximum absorbance in the visible region was found to be at 527 nm and all absorbances were compared with those of the non-metal solution which also displayed a maximum value at this wavelength.

Calcium sulphate was found to be only barely soluble in the diol/water solution but the solutions did show a slight stabilisation to heat and light, increasing with increasing calcium sulphate concentration.

Chromic sulphate also gave slight stability to heat and light with the best results for the 10 g/L solution, which also gave a slight bathochromic shift of 3 nm.

In general, aluminium sulphate did not appear to provide stabilisation to heat or light, although at a concentration of 10 g/L there was a very slight improvement to light stability when compared to the lower concentrations of salt.

Adding ferric chloride to the cold acidified solutions of anthocyanins initially gave deepening shades of purple on increasing the salt concentration from 0.5 g/L to 10 g/L. On heating these solutions the colours changed to red for the 0.5 g/L solution and to orange and green/brown for the 1.0 g/L and 10 g/L solutions respectively. At

0.5 g/L, ferric chloride was found to provide better stability to heat and light than the higher concentrations and a slight shift in maximum absorbance to 523 nm was observed. At 1.0 g/L the absorbance dropped considerably and was accompanied by a hypsochromic shift of 20 nm. The 10 g/L solution showed no stabilisation of absorbance at around 500-550 nm but absorbed strongly below 450 nm. The indication here was that the ferric ions were forming chelates with the phenolic compounds (e.g. at the *ortho* hydroxy grouping of the B ring in cyanin), a characteristic reaction of ferric ions,[15] rather than merely aiding the loose copimentation/self-association interactions. The observed changes in colour were most likely due to competition between the different phenolic compounds present (e.g. anthocyanins -purples, flavonols- red/brown/green, tannins -green).[15]

Ferrous ions, on the other hand, were seen to stabilise the absorbance at 527 nm for all three concentrations of salt with 10 g/L ferrous sulphate giving the best results.

Magnesium sulphate was found to give no stabilisation at any concentration and appeared to cause destabilisation to heat and light.

In the case of the stannous chloride solutions there appeared to be no stabilisation to heat at any concentration of metal salt. At concentrations above 0.5 g/L, the maximum wavelength was seen to shift to 530 nm for 1.0 g/L stannous chloride solution and 545 nm for the 10 g/L solution, giving rise to a purple colouration. As with ferric chloride this was an indication that new coloured a metal complex had been formed.

Zinc chloride appeared to have little effect on the stability of the pigment solutions to heat or light, although there was a slight stabilisation to heat and light for the 10 g/L solutions.

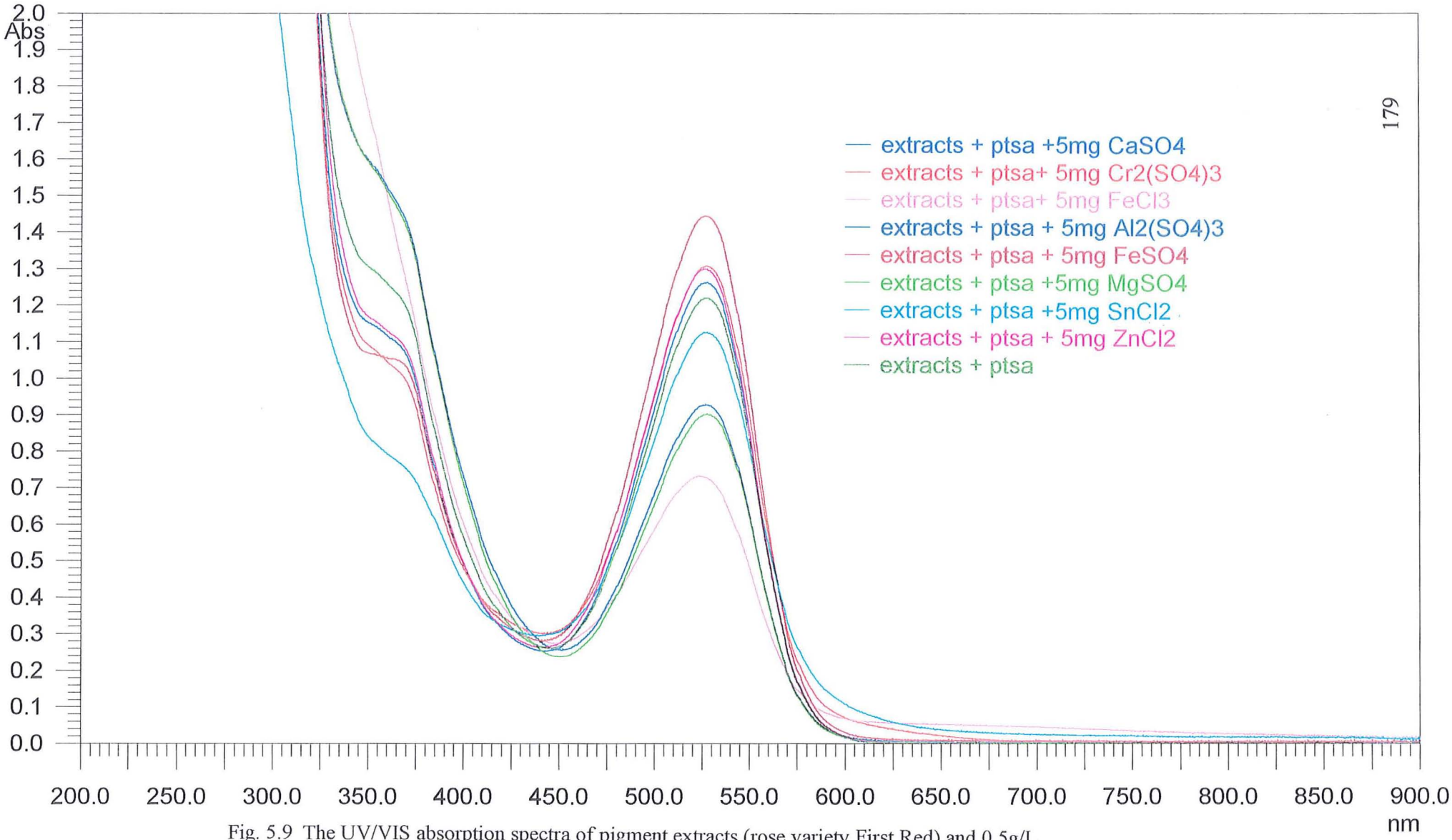


Fig. 5.9 The UV/VIS absorption spectra of pigment extracts (rose variety First Red) and 0.5g/L metal salts in acidified 60:20 diol/water solutions (after processing and light box testing).

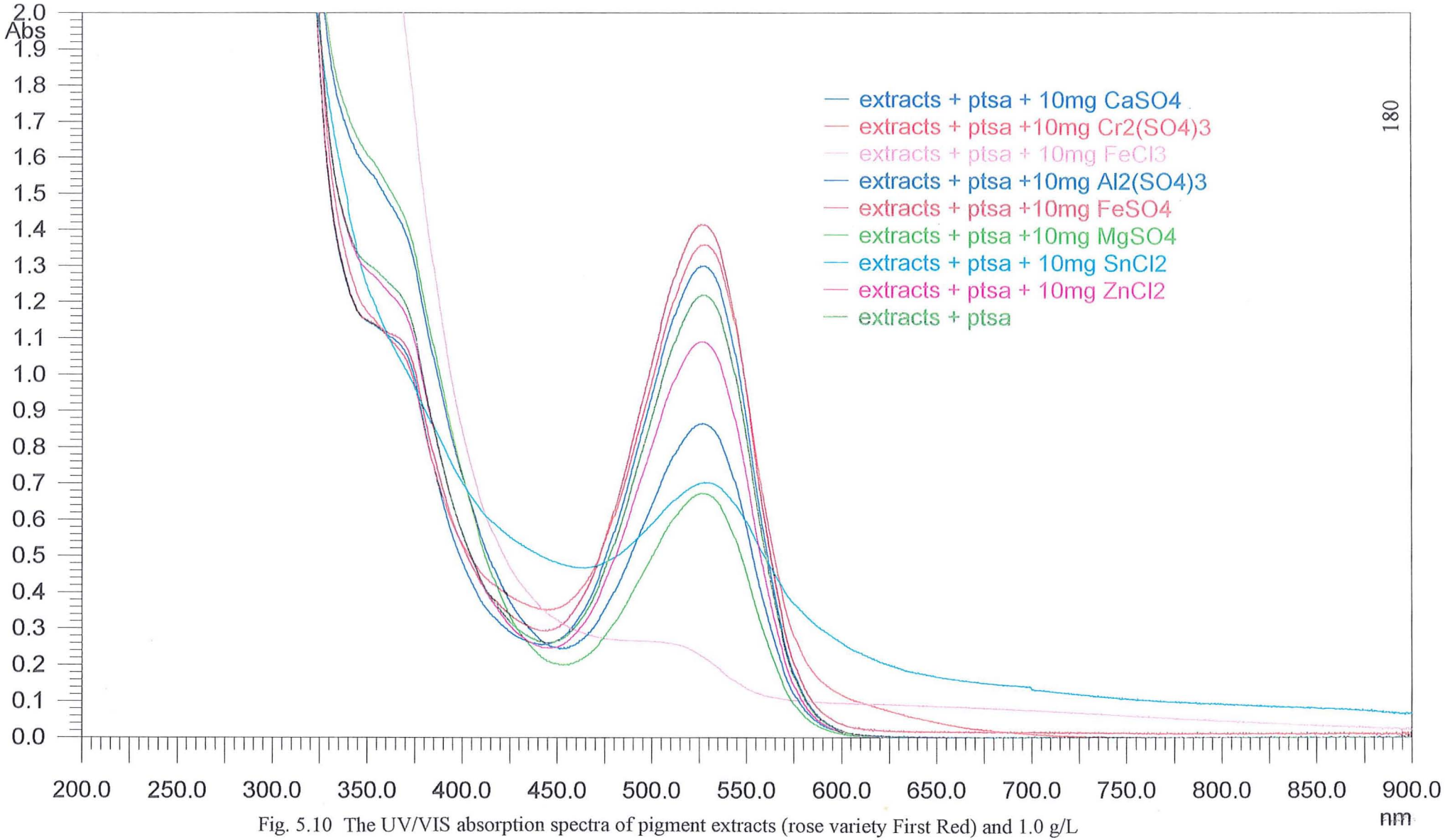


Fig. 5.10 The UV/VIS absorption spectra of pigment extracts (rose variety First Red) and 1.0 g/L metal salts in acidified 60:20 diol/water solutions (after processing and light box testing).

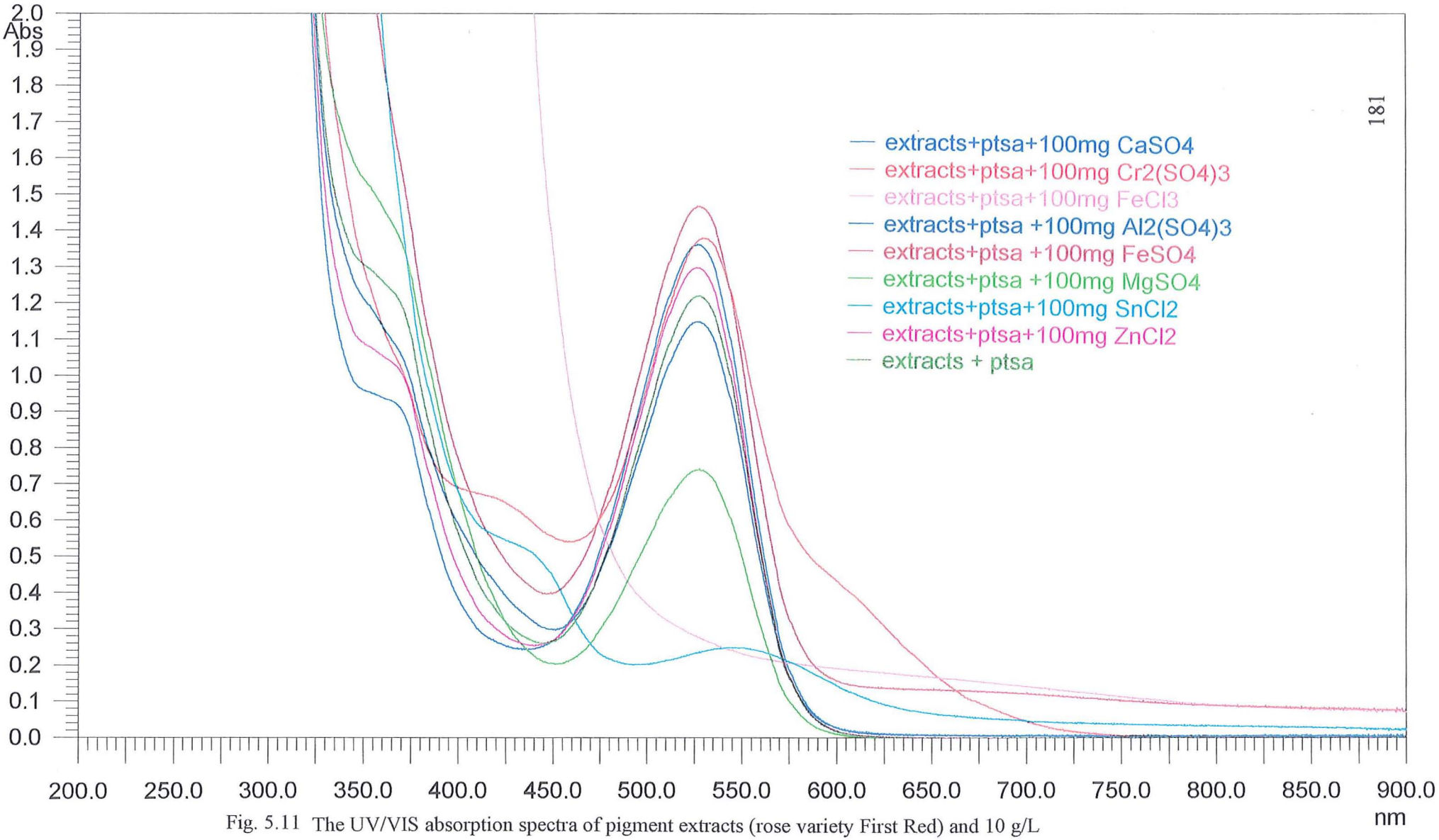


Fig. 5.11 The UV/VIS absorption spectra of pigment extracts (rose variety First Red) and 10 g/L metal salts in acidified 60:20 diol/water solutions (after processing and light box testing).

5.9.3.2 Investigation into the effects of metal salts in acidified diol solutions

The percentage changes in absorbance (from the original value) on heating and light box testing of the metal containing diol solutions are given in Table 5.18.

Metal salt (10 g/L)	% change in absorbance on processing	% change in absorbance on 7 day light box test (from processing)	% total change in absorbance (from original value)
No metal	-33	-66	-99
$Cr_2(SO_4)_3$	-27	-71	-98
$Al_2(SO_4)_3$	-27	-71	-98
$FeCl_3$	*	*	*
$FeSO_4$	- 7	- 30	- 37
$MgSO_4$	- 34	- 64	- 98
$SnCl_2$	*	*	*
$ZnSO_4$	- 40	- 58	- 98
$CuSO_4$	- 8	- 26	- 34
$CoSO_4$	- 5	- 24	- 29
$NiSO_4$	- 34	- 56	- 89
$MnSO_4$	- 14	- 23	- 37
Na_2MoO_4	- 15	-18	- 33
Na_2WO_4	- 7	- 30	- 37

Fig. 5.18 Changes in the visible absorbance of acidified diol solutions containing pigment extracts and 10 g/L of metal salts.

With the exception of $FeCl_3$ (an orange/brown solution absorbing strongly below 450 nm) and $SnCl_2$ (a purple solution absorbing at 558 nm and 490 nm) the maximum wavelength of absorbance was 530 nm. $CoSO_4$ itself was found to absorb

at 530 nm and this was corrected for in the above results. $CaSO_4$ was not considered, as like $BaSO_4$, it appeared to be insoluble in the diol solutions.

Cobalt (II), copper (II), iron (II) and tungsten (VI) salts all provided protection to heat, with the salts of manganese (II) and molybdenum (VI) also offering some thermal stability. Light stability was most improved by the addition of sodium molybdate, with the cobalt (II), copper (II), maganese (II), iron (II) and tungsten (VI) salts also providing increased light stability, as did nickel sulphate but considerably less so.

It was also noted that in the case of cobalt and copper salts, a small shoulder/peak (previously observed for copper solutions (section 5.8.3.1)) was detected after heating. This shoulder/peak, which was not present when any of the other metal ions were added, could possibly have been due to the interaction of the copper or cobalt ions with one of the anthocyanin degradation products (e.g. a chalcone), or to the interaction of the metal ions with one of the other plant constituents or their degradation products (e.g. a flavonol glycoside). Any of these possibilities could subsequently have produced a new species, or stabilised an existing one, that was also capable of improving the stability of the anthocyanins. However, it is also possible that the small shoulder/peak was merely an indication of the strengthened copigmentation/self-association interaction as a result of adding the highly effective copper or cobalt ions.

In any case, it appeared that cobalt and copper salts had the greatest stabilising effect on the pigment extracts, molybdenum, tungsten, manganese and ferric salts were observed to provide moderate stability and the ferric and stannous salts completely changed the colour of the solutions. The poor performance of the remaining metal ions may have been due to their inability to form chelates with the anthocyanins or to

the reducing properties of these metals in acidic solution, possibly by reduction of the double bond in the central pyran ring and the subsequent destruction of the conjugation between the two benzene rings.[2]

The results for the addition of metal ions to the solutions of acidified pigment extracts (with or without water present) were found to correlate well with the results for whole petals (section 4.17), with the exception of nickel sulphate which faired slightly better in petals at concentrations of 10 g/L than in solution. The additions of cobalt sulphate or copper sulphate to the process solution at concentrations of 10 g/L were seen to give petals with the most light-fast colours (Photograph 4.4), followed by molybdenum, tungsten and ferrous salts. The petals processed in the 1.0 g/L metal salt solutions (Photograph 4.5) did not give the red/brown and the purple colours for ferric chloride and tin chloride solutions as had been observed for the petals processed in the 10 g/L solutions. This was an indication that the Fe (III) and Sn (II) ions had complexed with the other flavonoids (i.e. the glycosides of quercetin or kaempferol) present before complexing with the anthocyanins. This stronger tendency of the flavonols towards complex formation is due to the presence of the hydroxyketo grouping as opposed to the *ortho* hydoxy grouping of the B ring in anthocyanins such as cyanin.

This would also explain the slight yellow tinge of the petals processed in the 1.0 g/L tin solution, since it had previously been noted that tin stabilised the yellow flavonoid pigments in pale flowers (section 4.17). Similarly the red/ brown of the petals processed in the 10 g/L ferric chloride solution and the red of the petals processed in the 1.0 g/L solution reflected the change in maximum absorbance of the corresponding 60:20 diol/ water extract solutions (section 5.9.3.1). For petals processed in the 1.0 g/L solutions of ferrous sulphate, zinc sulphate, cobalt sulphate

nickel sulphate, sodium tungstate and sodium molybdate, all gave slightly paler petals than for the corresponding 10 g/L solutions as was reflected in the absorbance values (where available) for the extract solutions (section 5.9.3.1). Petals processed using the remaining metal salts were found to have very similar shades for processing in both 1.0 g/L and 10 g/L solutions and again this was reflected in the work carried out on the extract solutions (section 5.9.3.1).

On assessing the performance of the various metal salts on their abilities to stabilise the pigments (both *in vivo* and *in vitro*) it appeared that overall, copper sulphate was the most effective metal salt. This was a convenient conclusion commercially, since copper sulphate was already an ingredient in the foliage process, it appeared to be most effective at low concentrations and it is relatively inexpensive and safe (Appendix 4).

5.10 The effects of copigments on (diol) solutions of pigment extracts

5.10.1 General

Previous investigations into the effects of adding the copigments rutin and ferulic acid to the process fluid indicated that ferulic acid did have some stabilising effect on the pigments of processed petals under a variety of conditions, however, the results for rutin were less conclusive (section 4.18).

In this section a more quantitative approach was taken in order to confirm the findings for ferulic acid and also to determine whether any improvements in stabilisation were occurring with rutin.

5.10.2 Experimental

5.10.2.1 Investigation into the effects of adding rutin to the process solution

5 × 10 ml diol solutions of First Red extracts each containing 1.8×10^{-4} M anthocyanins were prepared and the following added:

1. 20 g/L *p*-toluenesulphonic acid (PTSA)
2. 20 g/L PTSA + 10 g/L $CuSO_4$
3. 20 g/L PTSA + 10 g/L CuSO4 + 0.1 g/L (1.6×10^{-4}M) rutin
4. 20 g/L PTSA + 0.1 g/L rutin
5. 10 g/L $CuSO_4$ + 0.1 g/L rutin

The UV/VIS absorbance spectra of the solutions were measured before heating at 80 °C for 30 minutes (processing), after processing and cooling and then again after the 7 day light box test.

5.10.2.2 Investigation into the effects of adding ferulic acid to the process solution

9 × 10ml diol solutions of First Red extracts containing 1.7×10^{-4} M anthocyanins were prepared and the following added:

1. 20 g/L PTSA + 10 g/L $CuSO_4$
2. 20 g/L PTSA + 10 g/L $CuSO_4$ + 5 g/L (2.6×10^{-2}M) ferulic acid
3. 5 g/L ferulic acid
4. 10 g/L CuSO4 + 5 g/L ferulic acid
5. 20 g/L PTSA

6. 20 g/L PTSA + 0.5 g/L CuSO4
7. 20 g/L PTSA + 0.5 g/L CuSO4 + 5 g/L ferulic acid
8. 0.5 g/L CuSO4 + 5 g/L ferulic acid
9. 10 g/L CuSO4

UV/VIS absorbances of the solutions were measured before heating at 80 °C for 30 minutes (processing), after processing and cooling and then again after the 7 day light box test.

5.10.3 Results and discussion

5.10.3.1 Investigation into the effects of adding rutin to the process solution

Maximum absorbance in the visible occurred at 530 nm for all solutions acidified with *p*-toluenesulphonic acid, with an average absorbance value of 1.757 before heating.

The percentage changes in maximum absorbance (from the original value) on heating and light box testing of the pigment extract solutions were as recorded in Table 5.19.

Solution (5) immediately turned pale lilac and then colourless after heating, with no absorbance in the visible. This was an indication that rutin had no stabilising effect on colour in non-acidified diol solutions at the anthocyanin and rutin concentrations given. However, rutin did appear to provide some stabilisation to light in the acidified diol solutions (2, 3 & 4) and the heat and light stabilisation in the acidified solution containing both copper ions and rutin (3) was just marginally better than for the corresponding solution without rutin (2).

Solution	% change in absorbance on processing	% change in absorbance on 7 day light box test (from processing)	% total change in absorbance (from original value)
1	- 33	- 66	- 99
2	- 8	- 26	- 34
3	- 1	- 25	- 26
4	- 41	- 22	- 63

Table 5.19 Changes in the visible absorbance of diol solutions of pigment extracts containing PTSA, copper sulphate and rutin

1.Acid 2. Acid + copper sulphate 3. Acid + copper sulphate + rutin 4. Acid + rutin

These results were an indication that rutin did have some stabilising effect on colour even at low pigment and copigment concentrations (of the order 10^{-4} M), where the copigmentation effect is generally at its minimum in aqueous solutions (section 3.6). Rutin was therefore to be considered as a potential ingredient for the process fluid, once suitable concentrations and pigment to copigment ratios were established.

5.10.3.2 Investigation into the effects of adding ferulic acid to the process solution

Maximum absorbance in the visible, before heating, for all solutions containing *p*-toulenesulphonic acid occurred at 530 nm, with an average maximum absorbance value of 1.580.

The percentage changes in maximum absorbance on heating and light box testing the solutions of pigment extracts were as recorded in Table 5.20.

Solution	% change in absorbance on processing	% change in absorbance on 7 day light box test (from processing)	% total change in absorbance (from original value)
1	- 15	- 33	- 48
2	- 14	- 34	- 48
5	- 31	- 69	- 100
6	- 11	- 34	- 45
7	- 10	- 35	- 45

Table 5.20 Changes in the visible absorbance of diol solutions containing PTSA, copper sulphate and ferulic acid

1. PTSA + 10 g/L copper sulphate 2. PTSA + 10 g/L copper sulphate + ferulic acid 5. PTSA 6. PTSA + 0.5 g/L copper sulphate 7. PTSA + 0.5 g/L copper sulphate + ferulic acid

As was the case for First Red petals processed in the corresponding acidified solutions (section 4.18), the addition of ferulic acid was not appear to increase the stabilising effect of the copper sulphate solutions immediately after heating and light box testing. For the petals, it was only after at least a further 6-12 months (of normal laboratory conditions) that slight improvements in the colour stability of petals processed with ferulic acid became evident. Unfortunately, the variety Danse de Feu, for which the stabilising effects had been more immediate, was unavailable.

In the case of the solutions without *p*-toluenesulphonic acid; solution (3), which contained only extracts and ferulic acid turned colourless, with no absorbance at 530 nm; solution (4) which contained extracts, 10 g/L $CuSO_4$ and 5 g/L ferulic acid was pale purple in colour with a maximum absorbance of 0.855 at 530 nm; solution (9) containing only 10 g/L $CuSO_4$ had an absorbance of only 0.372 at 530 nm and Solution (8), which contained extracts, 0.5 g/L $CuSO_4$ and 5 g/L ferulic acid was a slightly paler purple than (4) with an absorbance at 530 nm of 0.373. Therefore,

although there appeared to be no immediate improvement in the stabilisation of the acidified solutions of pigment extracts containing ferulic acid, there did appear to be some improvement in stabilisation of the pigments in the non-acidified solutions containing copper sulphate and ferulic acid.

As with the previous copigment rutin, there were some positive effects on adding ferulic acid to the process solutions and no apparent negative effects.

5.11 Implications

Having made a study of the effects of the preservation process on the stability of pigment extracts and having verified the previous work involving petals and flower heads, the following refinements to the preservation process could be considered:

i) the establishment of a suitable process temperature range i.e. 60-80 °C

ii) the establishment of an adequate process time for flower heads i.e. 15-30 minutes

iii) the establishment of a suitable copper sulphate concentration i.e. 1-10 g/L

iv) the addition of ferulic acid (concentration to be established)

v) the addition of rutin (concentration to be established)

A pH of less than 2 was still considered satisfactory and could be provided by a 2% w/v *p*-toluenesulphonic acid diol solution. Although it appeared that the anthocyanins were more stable in solutions containing some water rather than in diol only solutions, the re-introduction of water into the process solution was not recommended since too much water caused the petals to loose body and become sticky. The stability problem in diol solutions appeared to be solved by the addition

of metal ions such as copper and could potentially be further improved by addition of suitable copigments.

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CHAPTER 6

Chapter 6

The provision of an effective coating system for processed plant material

6.1 Introduction

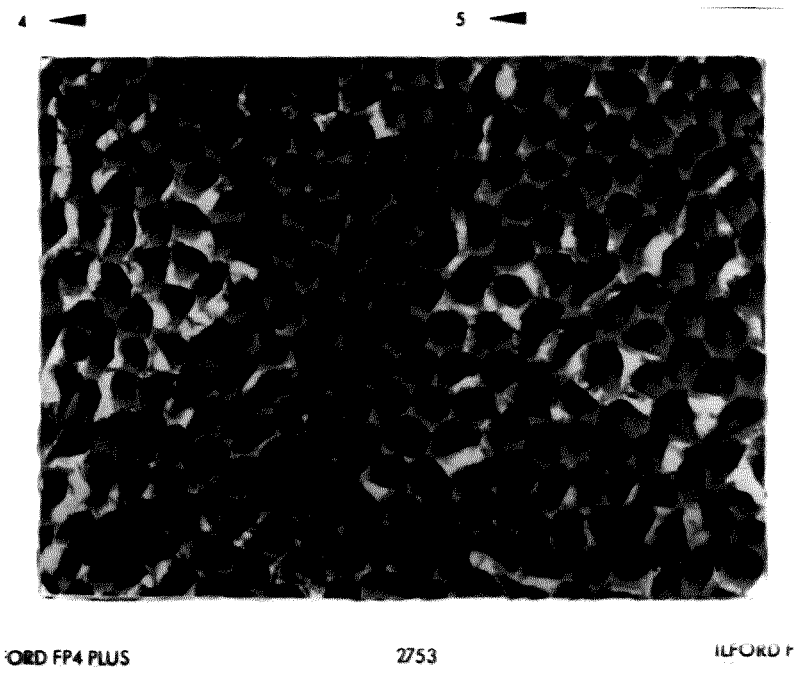
The aim of this project was to adapt existing (SEPAL) technology in order to preserve whole flowers, with the ultimate aim being to develop a commercially viable preservation process. This meant that not only did the process have to be inexpensive and safe but it also had to produce a marketable product i.e. a product that was visually attractive and long lasting under a range of environmental conditions.

From the previous developmental work, it appeared that the product, as processed according to the basic preservation process (4.19), remained in good condition for approximately 4 months under normal laboratory conditions. However, it was observed that under conditions of high humidity (70-80%) the flowers absorbed moisture (section 5.6) and became limp and translucent (possibly due to the filling of the intercellular spaces with water (section 2.5)) and that in hot dry conditions they took on a slightly papery feel and began to dry out at the edges. These effects had been previously noticeable when the process fluid contained a high proportion of hexan-1,6-diol, with crystals of hexan-1,6-diol appearing on the surface of petals as they dried out and then disappearing as they re-absorbed moisture in humid environments (4.12). It appeared, therefore, that some form of physical moisture/air barrier was required in order to seal the plant in its processed condition. In nature, such a barrier is provided by the cuticle (section 2.4), which is presumably destroyed

on processing. The cuticle consists of a layer of fatty substances (cutin), which are mainly high molecular weight lipid polyesters whose structures stem mostly from intermolecular esterification processes between carboxylic and hydroxyl groups of C16 and C18 polyhydroxylated fatty acids. A further layer, the epicuticle, which consists of a layer of waxes, including long chain (greater than C20) alkanes, alcohols acids and esters, normally covers the cuticle thereby providing an additional barrier to water and air.[1]

It was decided that attempts should be made to provide the processed plant material with a coating equivalent to the cuticle; namely that it had to be flexible, invisible, stable and provide an efficient barrier to water and air. Previously, a commercially available synthetic polymer, Polidene 33-004 (a vinylidene chloride/acrylate copolymer emulsion) had been used as a coating for foliage (section 4.3) and attempts had also been made to use it for the coating of flowers (section 4.12). However, although described as a " flexible, water-resistant polymer for coatings, exhibiting fire retardancy and moisture vapour barrier properties" the product did not appear to be entirely satisfactory for foliage in that in the dry air-conditioned environments, where it was generally being displayed, the foliage still dried out too quickly, giving rise to customer dissatisfaction. Furthermore, scanning electron microscope (SEM) images of the surface of a Polidene 33-004 (Polidene) coated processed petal, immediately after coating and then again after approximately two months, indicated that some degree of coating degradation was taking place (Fig 6.1).

(a)



(b)

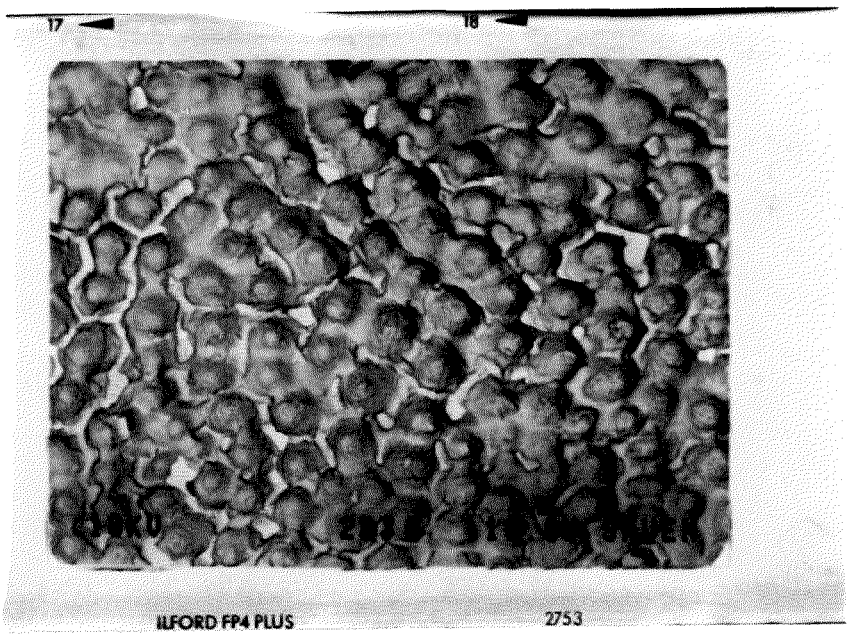


Fig. 6.1 SEM images of processed petals (magnification × 400) coated with Polidene 33-004

(a) Processed petal freshly coated with Polidene 33-004

(b) Processed petal coated with Polidene 33-004 after approximately 2 months

The performance of a coating or film is however, the result of many factors,[2, 3, 4] of which are listed a few:

(i) the type of the coating material (e.g. synthetic polymer, polysaccharide, protein, lipid)

(ii) the nature of the solvent (aqueous, non-aqueous)

(iii) the concentration of the coating material

(iv) the means of application (e.g. spraying, dipping, painting)

(v) the wettability (force of adhesion between the solid and liquid phases)

(vi) the thickness of the coating

(vii) the conditions of drying (temperature, time, humidity, air flow)

(viii) the compatiblitity of the coating with the substrate

(ix) the stability of the coating to environmental stresses

The failure of the Polidene coating to provide a long lasting barrier to moisture could have been attributed to any combination of these factors, but it is quite likely that it was due to the incompatiblity of the coating with the substrate, since the manufacturers advice was to avoid contact with metals such as copper (an ingredient of the process solution). Bearing the manufacturer's warning in mind and also the fact that it had been difficult to prepare a Polidene coating of suitable thickness for flowers (section 4.12), it was decided to carry out a brief investigation into alternative coating systems.

This chapter therefore commences with a look at the range of coating materials on offer (Appendix 5), selecting a few potential coatings from each category in order to assess their general suitability as coatings for processed flowers. Initially this

involved performing preliminary tests on petals in an attempt to find suitable coating materials, solvents and concentrations. The investigations then moved on to assess the means of application of the coating solutions using whole flowers. The assessments were generally visual, examining the effects of possible storage or display conditions on the ability of the coating to protect the flowers, while maintaining their aesthetic qualities. Examination by scanning electron microscopy (SEM) was also used as supporting evidence of the effectiveness of some of the most promising coating materials.

It was apparent, however, that the techniques of film coating called for a high degree of skill, experience and judgement and that the work described here could only be considered as a starting point in the search for a coating system that might compare favourably with nature's coating material, cutin.

6.2 Equipment and materials

6.2.1 Heating

As described in section 4.2

6.2.2 Light exposure

As described in section 4.2

6.2.3 Humidity and temperature

As described in section 5.2

6.2.4 Scanning electron microscopy (SEM)

Scanning electron microscope model JCXA733 from JEOL Ltd (USA).

6.2.5 Spraying

Aerosol spray gun from Fisher Scientific.

6.2.6 Chemicals

General chemicals were of laboratory grade and were supplied by Sigma-Aldrich Company Ltd., Lancaster Synthesis Ltd., or Avocado Research Chemicals Ltd.

6.2.7 Commercial coating preparations

Polidene 33-004, Texicryl 13-802, Texicryl 13-055 all from Scott Bader Company Ltd (Northants)

Febclear Super from Feb Ltd (Manchester)

Agral from ICI Performance Chemicals.

Edible food coatings from Agricoat Industries Ltd (Berkshire)

6.3 Selection of suitable coating materials

6.3.1 General

The applications of coating materials today are extensive, as are the types of materials currently available. Coatings and protective films are employed in almost every industrial sector ranging from agriculture and horticulture (e.g. as pest control

agents), building and construction (e.g. as surface sealers), textiles and paper manufacture (e.g. as flame retardant finishes) through to pharmaceuticals and foods (e.g. as edible coatings).[2, 4, 5] In every case the properties of the coating material are chosen to match the requirements of the product. For example, surface sealers in the building industry are usually required to be mechanically tough and impervious to commonly used chemicals and these requirements are often met by using an acrylic polymer.[2] In the case of the preserved flowers the requirements were for a stable, invisible, flexible, moisture and air resistant barrier that was generally safe and preferably odourless. As these requirements appeared to be very similar to those for coating food products, it seemed that an investigation into commonly used edible food coatings would be worthwhile.

Edible coatings and film materials comprise three general categories: polysaccharides, proteins and lipids (Appendix 5). The first two categories are hydrophilic and are effective at minimizing the transport of gases, but are poor moisture barriers. Lipids, on the other hand, are very effective moisture barriers but somewhat ineffective gas barriers.

Features necessary for a lipid film to have a low water permeability include the absence of highly polar groups and a regular chemical structure with a high degree of saturation such that crystallisation or close packing is encouraged. Gas permeability tends to be proportional to the fraction of the amorphous phase of a film.[3, 4] Many types of lipids have been examined for use as coating or film-forming materials, including waxes, fatty acids and their glyceride and sucrose esters and non-ionic surfactants (Appendix 5).

Waxes, the most superior of the lipid moisture barriers, can be classified on the basis of their source into four general groups: mineral, vegetable, animal or synthetic. The

mineral wax category is dominated by paraffin wax (a purified mixture of hydrocarbons obtained from petroleum with a crystalline structure and a range of melting points), but also includes microcrystalline, petroleum and peat waxes.

The vegetable waxes include, candelilla, carnauba, ricebran and cotton wax. Candelilla wax is produced by reed like plants that grow wild in Mexico and Texas and is considered to have the best moisture barrier properties of all the naturally occurring lipids because of its hydrophobic nature and highly crystalline character. In fact, the moisture barrier properties have been deemed better than low-density polyethylene, the industry standard for efficient, inexpensive moisture barrier polymers.[4]

The animal waxes comprise beeswax, insect wax, spermaceti wax and wool wax. Beeswax is produced by honeybees for building honeycomb cells and is a mixture of wax esters (C26/C28 acids and C30/C32 alcohols) wax acids and hydrocarbons. Beeswax was found to have water permeability indices 25 times weaker than for common oils and a 100 or 200 times weaker than for casein or pectin.[3] Shellac, although not considered a lipid material, is often used as a protective barrier for foods because of its physical toughness and excellent moisture and gas barrier properties. "Shellac" refers to lac, the hardened resinous secretion of the scale insect, *Laccifer lacca,* and is the only commercial resin of animal origin. It is composed of more than 90% resin and less than 5% wax and forms films because of extensive hydrogen bonding.[4]

Synthetic waxes are waxes in the sense that they possess physical properties similar to those of natural waxes. They are composed of long-chain polymers of ethylene or ethylene oxide, halogenated hydrocarbons, hydrogenated waxes and many reaction products involving fatty acids. Oxidised polyethylene (or polyethylene wax) is the

basic resin produced by the mild air oxidation of polyethylene and is the most widely used food coating material of the synthetic waxes.[4]

The other major group of lipid food coatings consists of fatty acids and their glyceride and sucrose esters. The acetylated monoglycerides (or acetylated glyceride monoesters of long chain fatty acids) have been extensively studied for their ability to form flexible and stretchable films, which are relatively impermeable to moisture and oxygen.[3] The sucrose fatty acid (e.g. palmitic, stearic and lauric acid) ester coatings have been found to have similar barrier properties and are currently used to control moisture and gas transport in bananas.[6, 7] The triglycerides, or generally termed "fats" and "oils" are also used as coating materials, the difference between the two groups being their melting point; fats are solid at room temperature, whereas oils are not. Full hydrogenation of vegetable oils or animal oils yields a wax like substance with increased melting point, greater chemical stability and increased moisture barrier properties.

Since the transfer of moisture was causing the most obvious problems for the processed flowers, the majority of the coating materials tested tended to be of a lipid nature. However, several other food coatings from the polysaccharide category as well as corn zein (the alcohol soluble fraction of corn) from the protein category were also tested since they are known to produce relatively water-resistant films.[3] In addition a few synthetic polymeric materials with both efficient moisture and gas barrier properties were considered, as were several multicomponent systems, which were attempting to overcome the negative qualities of the individual components when used separately as coating materials. Also included were several non-ionic surfactants for their wetting and emulsification abilities.[8]

In general, guidance on solvents and concentrations of coating materials was taken from standard food coating guidelines [3] or manufacturers instructions.

6.3.2 Experimental

Coating solutions from each category of material (Appendix 5) were prepared as described in Tables 6.1, 6.2 and 6.3 (in some cases with the aid of heat) and dried processed rose petals were dipped into each solution for approximately 20 seconds and then allowed to air dry.

Material	Concentration	Solvent
Vinylidene chloride/acrylate copolymer emulsion (Polidene 33-004)	10%, 15%, 20% v/v	Water
	10% v/v	70% propan-2-ol
Styrene acrylate copolymer emulsion (Texicryl 13-055, Texicryl 13-802)	10% v/v	Water
	10% v/v	70% propan-2-ol
Acrylic polymer (Febclear Super)	100% v/v	-
Polyvinyl alcohol	1%, 5% w/v	Water

Table 6.1 Plastic and synthetic polymer solutions used to coat processed flowers

Material	Concentration	Solvent
Polysaccharides		
Carboxymethyl cellulose	1%, 2%, 5% w/v	Water
Hydroxypropyl methyl cellulose	2% w/v	Water
Pectin	1% w/v	Water
Gum ghatti	2% w/v	Water
Proteins		
Zein	2% w/v	Water
Lipids		
Beeswax	1%, 5% w/v	Dichloromethane
Candelilla wax	1% w/v	Propan-2-ol (50 °C)
Synthetic wax	1% w/v	Water (80 °C)
(oxidised polyethylene)	1% w/v	Propan-2-ol (50 °C)
Tripalmitin	1% w/v	Propan-2-ol (50 °C)
Tung oil	10% v/v	Propan-2-ol
Sucrose esters of fatty acids	2% v/v	Water
(Agricoat: SPCR, BAN 6)	2% v/v	Propan-2-ol
Shellac (Agricoat: R49, EWW)	2% w/v	Propan-2-ol
Shellac (Agricoat: Applewax)	50% v/v	Water
	100%	-
Alkylphenol ethylene oxide condensate	5%, 10% v/v	Water
(Agral)	100%	-
t-octylphenoxypolyethoxyethanol	10% w/v	Water
(Triton-X-405)		
Stearyl ether (Brij 78)	10% w/v	Water

Table 6.2 Polysaccharide, protein and lipid solutions used to coat processed flowers

Material	Concentration	Solvent
Polidene-Agral mixtures	5%-5%, 10%-10%, 10%-5%, 10%-1%.	Water
Sucrose esters of fatty acids-sodium carboxymethyl cellulose-acetylated monoglycerides (Agricoat-Semperfresh)	0.5%, 1%, 2% v/v	Water
Unspecified preparation Containing carboxymethyl cellulose (Agricoat-Natureseal)	10% v/v	Water

Fig. 6.3 Multicomponent solutions used to coat processed flowers

6.3.3 Results and discussion

Of the plastics and synthetic polymers, the aqueous solutions gave less glossy coatings than the corresponding alcohol based solutions, with the best visual results for the 10% Polidene and the 10% Texicryl solutions. The polyvinyl alcohol solutions gave reasonable results, however the petals became slightly sticky. The polysaccharide materials and the corn zein also tended to give glossy, sticky coatings, with the best results for the hydroxypropyl methyl cellulose coating. The natural wax coatings (i.e. beeswax and candelilla) wax gave a dull appearance and tripalmitin was unsuitable as it left a white deposit, whatever solvent was used. Tung oil and the acrylic polymers (Febclear) were too viscous and proved difficult to thin. The non-ionic surfactants were less firm than the other coatings with Brij 78 and Triton-X-405) appearing to absorb moisture slightly.

The multicomponent systems generally gave visually satisfactory results, as did all the Agricoat food coating formulations.

6.4 The application of coating solutions (spraying versus dipping)

6.4.1 General

The most suitable coating materials (from 6.3) were used to coat whole flowers and visual comparisons were made between flowers coated by spraying and those coated by dipping.

6.4.2 Experimental

The following solutions were prepared:

10% Polidene 33-004 (aq), 10% Texicryl 13-802 (aq), 10% Texicryl 13-055 (aq), 10% synthetic wax (aq), 2% Semperfresh(aq), 2% SPCR (aq), 2 % BAN 6 (aq), 10% Natureseal (aq), 2% hydroxypropyl methyl cellulose (aq), 2% SPCR (propan-2-ol), 2% EWW (propan-2-ol), 2% R49 (propan-2-ol).

Two sets of processed roses were coated; one by spraying with the above solutions and the other by dipping for 20 seconds in these solutions. The coated roses were allowed to dry under normal laboratory conditions.

6.4.3 Results and discussion

In general, the best visual results were for the roses coated by spraying as the petals coated by dipping tended to stick together, possibly due to the weight of the aqueous coating material and the length of time required for drying. For the coating materials that used propan-2-ol as a solvent, the problems of petals sticking together did not occur and there was no obvious difference between the dipped and sprayed roses.

6.5 The barrier properties of applied coatings (air and moisture)

6.5.1 General

The initial coating materials were selected from a vast range of possible materials on account of their alleged properties, which appeared to match the requirements of the processed flowers (6.3). The preliminary investigations based on visual inspection of the newly coated petals and flower heads narrowed the selection further, leaving the effectiveness of these potential coating materials to be assessed in terms of their barrier properties. This involved studying the long-term effects of exposing the coated flowers to different environmental conditions, concentrating on the ability of the coatings to prevent the flowers from drying out or absorbing moisture. Visually this was relatively easy to assess since dry conditions caused the petals to become papery and moist conditions made them limp. The barrier properties with respect to air were considerably less obvious since there had been no immediate visual evidence of any deleterious effects that could have been attributed solely to the ingress of air.

However, on studying the images of the processed petals (coated and un-coated) produced by scanning electron microscopy (SEM)[9, 10] an interesting observation was made (Fig 6.2). It appeared that the epidermal cells of un-coated processed petals collapsed under the vacuum pressure of the microscope column, whereas processed petals coated with Polidene- 33-04, which was alleged to provide an excellent barrier to air, appeared to be resistant to such vacuum damage. On the other hand, petals coated with Agral, a material not renowned for its air barrier properties, appeared to provide only slight protection to vacuum damage and a 10:1 mixture of Polidene and Agral, to provide protection somewhere between the two.

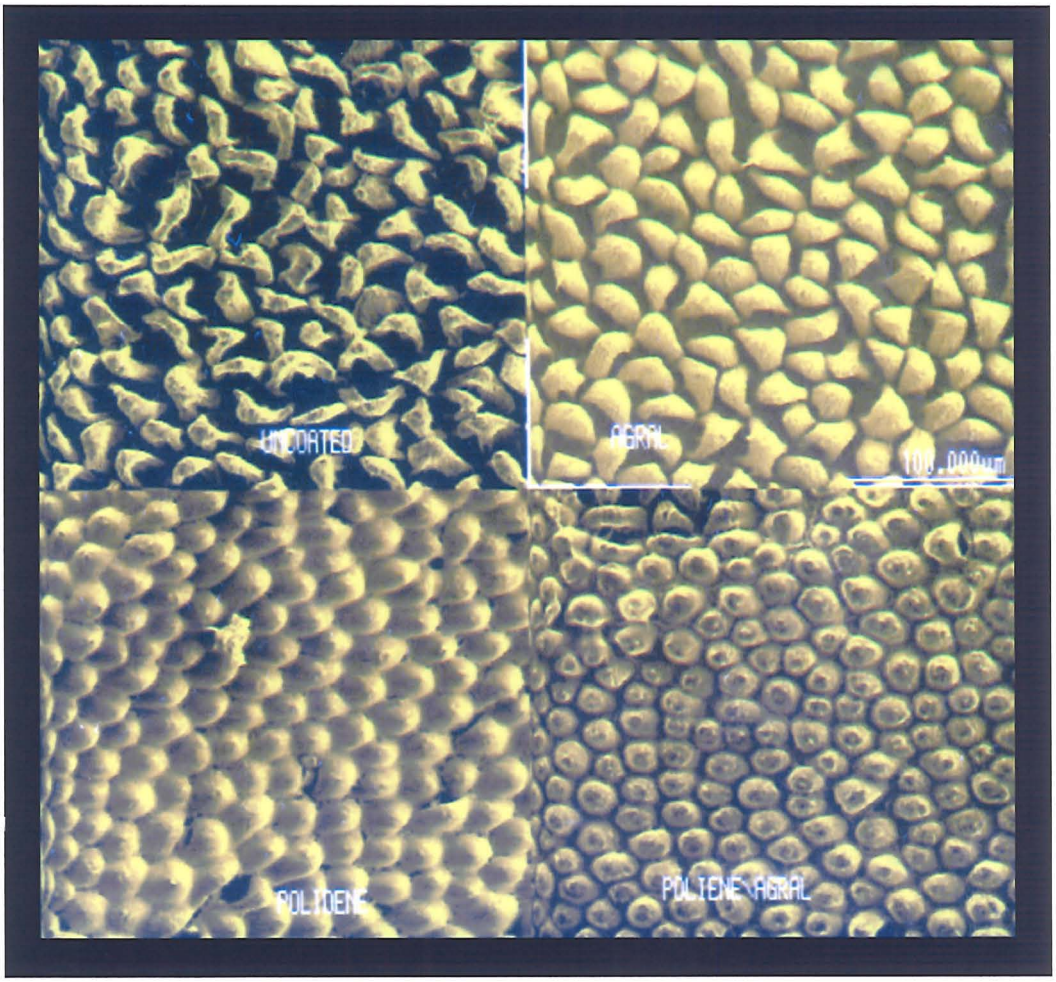


Fig. 6.2 SEM images (magnification × 300) of coated and un-coated processed petals

top LH- un-coated, top RH- Agral coating, bottom LH- Polidene coating, bottom RL-Polidene/Agral coating

Although slightly unorthodox, this means of assessment did appear to provide an indication of coating effectiveness by relating the observed "degree of collapse" of the epidermal cells to the air barrier properties of the coating.

6.5.2 Experimental

Two sets of processed roses were sprayed with the selection of coatings listed in section 6.4. Both sets of roses were stored for 4 months, one set at an average temperature of 30 °C and a relative humidity of 45% (possible display conditions); the other set at an average temperature of 15 °C and a relative humidity of 80% (possible storage conditions). An un-coated rose was used in each case as a control.
A sample from each of the different coated roses was taken for examination by SEM one week after coating (accelerating voltage 10 kV, magnification × 400)

6.5.3 Results and discussion

With regards to the visual examination of the roses, all of the coated roses faired better than the control in either environment, with the best results for the commercial food coatings Semperfresh, EWW, R49 and Applewax.
The SEM images (Appendix 6) also indicated that all of the coatings were providing some barrier to air, since none of the coated petals suffered complete collapse as in the case of the un-coated petal. However, the petals using propan-2-ol as a solvent (i.e. EWW, R49 and SPCR), all appeared to have a slightly more shrivelled surface than for the aqueous based coating, although the cells did not collapse. This may have been an effect unconnected to the vacuum (i.e. that had occurred before examination by SEM) since when processed petals were dipped into propan-2-ol

they appeared to stiffen (section 4.9), indicating contraction of the cell walls, possibly in some form of solvent shock. However, this effect did not detract from the visual appearance of the petals and therefore these coating materials would still be considered suitable.

6.6 Implications

In general, it appeared that the application of any of the coating materials tested in section 6.5 prolonged the life of the preserved flowers to some extent by providing a barrier to either air or moisture or both. However, the most impressive results with regards visual appearance and overall barrier properties were for the shellac coatings from Agricoat i.e. R49, EWW and Applewax. The advantage of the R49 and EWW coating materials over the Applewax coating was that they could be dissolved in propan-2-ol and applied by dipping (without the petals sticking together) thereby reducing the equipment costs associated with spraying.

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CHAPTER 7

Chapter 7

Restaurant Trial

7.1 Introduction

In an attempt to an gain external opinion of the durability, aesthetic appeal and other marketable qualities of the preserved flowers, a local hotel (The Russell Hotel, St. Andrews) agreed to take a supply of the preserved flowers for a trial for a period. The hotel currently spends approximately £10 per week on fresh flowers, often having to replace them in less than 7 days. They therefore welcomed the idea of longer lasting blooms.

7.2 Trial

15 red carnation heads were processed in the acidified process solution (65% propan-1,2-diol, 25% butan-1,4-diol, 10% hexan-1,6-diol, 10 g/L $CuSO_4$, 20 g/L *p*-toluenesulphonic acid) for 30 minutes at 80 °C. 15 white carnation heads were processed in the basic diol mixture without the addition of copper sulphate or acid. The stems were not processed but were allowed to dry out naturally, as the woody nature of the carnation stems meant that when dry they were still capable of supporting the flower heads. The stems and sepals were coated with 2% w/v solutions of the Agricoat coating materials R49 and EWW in propan-1,2-ol (2 lots of 15). The flowers were arranged by the hotel staff into table displays for the restaurant (Photograph 7.1).

Photograph 7.1. Table display at The Russell Hotel (St. Andrews)

7.3 Comments and implications (Appendix 7)

The hotel staff were pleased with the general appearance and durability of the carnations, which were still in good condition (with either coating material) after being displayed for two and a half months in the busy hotel restaurant. However, a decision to remove the carnations after this period was taken because one of the carnations caught alight after coming into contact with an oil burner. This problem could have been anticipated with dried flowers due to the cellulose component of the plant tissues, but since the fire risk was not obvious with the processed flowers, due to their moist appearance, safety warnings would therefore be necessary. However, if safety warnings were not considered sufficient for the product to comply with the current consumer safety legislation, then a fire retardant (as in the Polidene coatings) would have to be incorporated into either the coating or the process mixture.

The initial odour of the processed flowers was also commented on, which although not considered to be unpleasant, was not of a floral nature. This could possibly be rectified by adding floral fragrances to the process fluid or by incorporating such perfumes into the coating material.

Despite these problems, however, the hotel manager was still keen to use the processed flowers and wished to be supplied with a new batch when available (particularly if preserved foliage was also supplied).

At a cost of 5p extra per bloom for preservation chemicals, this could result in a saving of over £28 per month for an establishment of this size, making the product an economical option.

CHAPTER 8

Chapter 8

Conclusion

8.1 Summary

Armed with a basic knowledge of plant anatomy and an insight into the chemistry of floral pigments, the existing foliage process was adapted to enable it to preserve whole flowers. In the case of dark coloured flowers, such as red roses, this required only minimal changes to the composition of the process fluid, namely, the addition of acid to maintain the bright flavylium ion colours and the removal of water and addition of hexan-1,6-diol to improve firmness.

Since the foliage fluid already contained copper ions, which were found to be the one of most cost effective light stabilisers of the anthocyanins, and since the chlorophyll did not cause unpleasant discolouration of the petals, it appeared to be possible to use the same process fluid and equipment for whole flowers as for foliage. In the large-scale operation in America, this consisted of 400-gallon stainless steel heated tanks, to which the foliage was added in layered mesh baskets. The addition of acid would of course be a safety issue, but the precautions already in place for dealing with the hot process fluid would presumably be sufficient for the acidified solutions. It would, however, be necessary to take extra care when preparing solutions and cleaning tanks.

Fortunately, the temperature of processing and the process times for flowers were similar to those for foliage, making a one-stage process for dark coloured flowers an option. In the case of pale coloured flowers, however, solutions containing

chlorophyll were found to be unsuitable, therefore necessitating a two-stage process, with some type of reversible clamping device and twin tanks envisaged. Although more expensive in terms of equipment, time and training, the advantages of treating the flower heads separately would be that the conditions of processing could be fine-tuned to suit the delicate flower heads and pigments. For example, after studying the effects of the process chemicals and conditions on the floral pigment extracts it was discovered that slightly lower temperatures and shorter process times improved the long term stability of the pigments. Furthermore, a two-stage process meant that other anthocyanin stabilisers could be added (i.e. other metals and copigments) without the possibility of any adverse effects to the foliage process.

Studies of the pigment extracts were also useful in determining the optimum pH and copper sulphate concentration for petals. This appeared to be pH<2 (i.e. *p*-toluenesulphonic acid concentration of 2-2.5% w/v) and a copper sulphate concentration of approximately 1 g/L (although up to 10 g/L did not appear to cause problems *in vivo*).

It was also discovered that the anthocyanins were unstable in diol solutions but that their stability increased with increasing water content, possibly as a result of the copigmentation effect. Stabilisation of anthocyanins by copigmentation with other plant constituents (e.g. flavonols) appeared to be at its most effective in aqueous solutions but the addition of complexing metal ions (e.g. Co(II), Cu(II), Sn(II)) improved stability in the solutions containing diols.

The application of a barrier coating to prevent the movement of moisture and air not only improved the physical appearance of the petals (by preventing them from drying out or becoming limp with excess moisture) but may also have prevented the

accelerated breakdown of the pigments by maintaining a small percentage of water in the petals.

8.2 Future work

The preservation process as it stood was successful in that a wide range of anthocyanin colours could be stabilised by the addition of acid and copper ions to the process solution. The safety implications could possibly be improved, however, by reducing the acid content of the process fluid and attempting to stabilise the anthocyanins with copigments effective in diol solutions even at a higher pH. Rutin and ferulic acid, for example, which have been demonstrated to have a stabilising effect in aqueous solutions at pH 3 (Appendix 2) and up to pH 7 for rutin,[1] also showed some potential as stabilisers in the diol solutions at low pH (<2). This work could, therefore be expanded to by attempting to establish optimum concentrations for these and other known copigments over the full range of pH.

With regards to the physical condition of the processed flowers, roses, carnations and irises all retained their firmness and were able to stand unaided in a suitable recepticle. Unfortunately, the stems of delicate flowers such as freesias and tulips became limp and unable to support their heads. A means of strengthening the stems was therefore required. This could possibly be achieved by introducing strengthening cell wall constituents such as ferulic acid or pectin to the process fluid or by coating, or injecting the stems with a thermosetting polymer.

As a result of the hotel trial (Chapter 7), other areas requiring attention were flammability and odour, both of which could be tackled by the incorporation of materials into the coating or by the addition of such materials to the process fluid.

With such improvements in place, supplying the market demand for a full range of attractive preserved flowers could be met by means of a relatively safe, cost-efficient process. Furthermore, the fundamental ability of the plant material to exchange cell sap for process fluid, facilitates the introduction of many ameliorative additives and opens routes in countless directions with regards the future of flower preservation.

8.3 References

1. M. Williams and G. Hrazdina, *Journal of Food Science*, 1979, **44**, 66.

Appendix 1

Pelargonidin Glycosides

Pelargonidin Glycosides

Compound	*Substitution Pattern** 3	5	7	4′	*Source(s)*
Pelargonidin	OH	OH	OH	OH	
Callistephin[67b,74c,76,161]	β-Gl	OH	OH	OH	*Callistephus chinensis* Ness.; ***Primula sinensis;*** ***Fragaria*** species; *Dianthus caryophyllus; Lathyrus odoratus* cv. 'Air Warden'
Fragarin[76,86a,161]	β-Ga	OH	OH	OH	*Fagus sylvatica*
Pelargonidin-3-rhamnoside[67c]	Rh	OH	OH	OH	*Lathyrus odoratus* cv. 'Air Warden'
Pelargonidin-3-rhamnoglucoside[84,95]	RhGl	OH	OH	OH	*Antirrhinum majus; Solanum phureja; Gloxinia* species; *Colombia thyracanthus* var. *rutilans*
Pelargonin[48b,117b]	β-Gl	β-Gl	OH	OH	*Centaurea cyanus* L.; *Impatiens balsamina; Paeonia suffruticosa; Pelargonium peltatum, P. zonale; Pharbitis nil Chois.; Punica granatum*
Pelargonidin-3-rhamnoside-5-glucoside[67b]	Rh	Gl	OH	OH	*Lathyrus odoratus* cv. 'Air Warden'
Pelargonidin-3-cellobioside[90]	Cell	OH	OH	OH	*Campanula medium; Gloxinia* species; *Papaver nudicaule, P. orientale; Tropaeolum majus*
Pelargonidin triglycosides[85]	Cell	Gl	OH	OH	*Raphanus sativus*
	GlGlGl	OH	OH	OH	*Primula sinensis*
	RhGl	Gl	OH	OH	*Solanum phureja*

* Ga galactose, Gen gentiobiose, Gl glucose, Cell cellobiose, Arb arabinose, Rh rhamnose, Xy xylose

Reference: F.M. Dean, *Naturally Occurring Oxygen Ring Compounds*, Butterworths, 1963 London

Cyanidin Glycosides

Compound	*Substitution Pattern* 3	5	7	3′	4′	*Source(s)*
Chrysanthemin[102b,162–5]	β-Gl	OH	OH	OH	OH	*Callistephus chinensis* Ness.; *Calycanthus fertilis; Chrysanthemum indicum* L.; *Fritillaria camschatensis* Ker-Gawl; *Lycoris radiata; Oenothera odorata; Prunus avium, P. cerasus, P. persica; Rubus fructicosus; Sambucus nigra* L.; *Acer* spp.; *Glycine hispida*; maize; soya-bean; blood oranges
Idaein[74d,80,166]	Gal	OH	OH	OH	OH	*Fatsia japonica; Vaccinium vitis-idaea* L; *Fagus sylvatica; Polygonum hydropiper; Pirus malus; Theobroma cacao*
Cyanidin-3-rhamnoside[67c]	Rh	OH	OH	OH	OH	*Lathyrus odoratus* cv. 'Harrow'
Capensinidin-3-rhamnoside[67b]	Rh	OMe	OH	OMe	OH	*Plumbago capensis*
Cyanin[48b,167–9]	β-Gl	β-Gl	OH	OH	OH	*Centaurea cyanus* L.; *Dahlia variabilis; Helenium autumnale* L.; *Pelargonium* species; *Perilla ocimoides* L. var. *crispa* Benth.; *Rosa gallica* var. *rubra; Tulipa* species and many other plants
Cyanidin-5-glucoside-3-rhamnoside[67c]	Rh	Gl	OH	OH	OH	*Lathyrus odoratus* 'Harrow'
Mecocyanin[82,86c]	Cell	OH	OH	OH	OH	*Papaver rhoeas* L.; *Prunus cerasus; Rubia akane; Hibiscus* spp.
Antirrhinin (keracyanin)[170–1]	RhGl	OH	OH	OH	OH	*Antirrhinum majus; Canna generalis; Prunus avium, P. cerasus; Ribes sanguineum; Sambucus nigra* L.; *Solanum phureja; Viola wittrockiana;* olives, etc.
Illicyanin[67b,85,117b] and lycoricyanin	XyGl	OH	OH	OH	OH	*Begonia* species; *Ilex crenata; Lycoris radiata; Sambucus nigra; Lathyrus odoratus* cv. 'Harrow'; *Streptocarpus* species
Cyanidin-3-rhamno-gluco-side-5-glucoside[171]	RhGl	Gl	OH	OH	OH	*Viola wittrockiana*
Fritillaricyanin[160]	XyRh	OH	OH	OH	OH	*Fritillaria camschatensis* Ker-Gawl
Oxycoccicyanin[78,172]	β-Gl	OH	OH	OMe	OH	*Oxycoccus macrocarpus* Pers.; *Viola wittrockiana*
Paeonin[48b,117b]	β-Gl	β-Gl	OH	OMe	OH	*Nectandra eleophora; Paeonia arborea, P. suffruticosa; Pharbitis nil* Chois.; *Lathyrus odoratus* cv. 'Harrow'
Paeonidin-3-diglucoside[92]	GlGl	OH	OH	OMe	OH	*Fuchsia magellanica* var. *corallina*
Rosinin[67a,85]	Gl	Gl	OMe	OMe	OH	*Primula rosea*
Cyanidin-3-arabino-side[67b,122]	Arb	OH	OH	OH	OH	*Theobroma cacao; Rhododendron* and *Hordeum* species, etc.

* Ga galactose, Gen gentiobiose, Gl glucose, Cell cellobiose, Arb arabinose, Rh rhamnose, Xy xylose

Reference: F.M. Dean, *Naturally Occurring Oxygen Ring Compounds*, Butterworths, 1963, London

Glycosides of Delphinidin and its Ethers

Compound	*Substitution Pattern* 3	5	7	3′	4′	5′	*Source(s)*
Delphinidin	OH	OH	OH	OH	OH	OH	
Myrtillin-a[163,171–3]	Gl	OH	OH	OH	OH	OH	*Viola wittrockiana; Vaccinium myrtillus; Verbena* spp; blood oranges
Myrtillin-b[173–4] (empetrin?)	Ga	OH	OH	OH	OH	OH	*Empetrum nigrum; Vaccinium uliginosum*
Delphinidin-x-arabinoside[175]	Arb	OH	OH	OH	OH	OH	*Anemone coronaria* (pollen)
Delphin[84]	Gl	Gl	OH	OH	OH	OH	*Commelina communis; Salvia patens; Verbena hybrida*
Delphinidin-5-glucoside-3-rhamnoside[67b]	Rl	Gl	OH	OH	OH	OH	*Lathyrus odoratus* cv. 'Jupiter'
Hibiscin[176] (delphinidin-x-glucoside-y-pentoside)							*Hibiscus sabdariffa* L.
Delphinidin-3-diglucoside[91,167–8]	GlGl	OH	OH	OH	OH	OH	*Clematis* species; *Linum grandiflorum* var. *rubrum*
Delphinidin-3-rhamnoglucoside[168]	RhGl	OH	OH	OH	OH	OH	*Solanum melongena* L. var. *esculentum* Ness
Petunidin	OH	OH	OH	OMe	OH	OH	
Petunin[81b] (muscadinin?[177])	Gl	Gl	OH	OMe	OH	OH	*Primula obconica*; *Vitis rotundifolia*?
Petunidin-5-glucoside-3-rhamnoside[67b]	Rh	Gl	OH	OMe	OH	OH	*Lathyrus odoratus* 'Jupiter'
Petunidin-3-rhamnoglucoside[85]	RhGl	OH	OH	OMe	OH	OH	*Solanum phureja*
Malvidin[47,171] (syringidin)	OH	OH	OH	OMe	OH	OMe	
Oenin[79,171,178] (cyclamin)	Gl	OH	OH	OMe	OH	OMe	*Cyclamen persicum* Mill.; *Primula sinensis;* black grapes
Primulin[81,117b,171,174] (uliginosin)	Ga	OH	OH	OMe	OH	OMe	*Empetrum nigrum; Primula* species; *Vaccinium uliginosum*
Bladhianin[180]	OH	OH	Gal	OMe	OH	OMe	*Bladhia sieboldii*
Malvin[48,170a]	Gl	Gl	OH	OMe	OH	OMe	*Lespedeza thunbergii; Malva silvestris* L.; *Primula integrifolia, P. viscosa,* and many other sources
Malvidin-5-glucoside-3-rhamnoside[67b]	Rh	Gl	OH	OMe	OH	OMe	*Lathyrus odoratus* cv. 'Jupiter'
Hirsutidin	OH	OH	OMe	OMe	OH	OMe	
Hirsutin[48]	Gl	Gl	OMe	OMe	OH	OMe	*Primula auricala, P. denticulata, P. hirsuta, P. polyanthus, P. purpurea*

* Ga galactose, Gen gentiobiose, Gl glucose, Cell cellobiose, Arb arabinose, Rh rhamnose, Xy xylose

Reference: F.M. Dean, *Naturally Occurring Oxygen Ring Compounds*, Butterworths, 1963, London

Complex Anthocyanins

Compound	*Parent glycoside*	*Esterifying acid(s)*	*Source(s)*
Gesnerin trigallate[180]	gesnerin	gallic acid (3 residues)	'Maxpalxochitl'
Monardein[85a,121]	pelargonin	*p*-coumaric acid (1 residue) + malonic acid (2 residues)	*Monarda didyma*
Salvianin[85a,121]	pelargonin	caffeic acid	*Salvia coccinea, S. splendens*
Pelanin[85]	pelargonidin-3-rhamnoglucoside-5-glucose	*p*-coumaric acid	*Solanum phureja*
Raphanin A[85a–6]	pelargonidin-3-diglucoside-5-glucoside	*p*-coumaric acid	*Raphanus sativa*
Raphanin B[85a–6]	pelargonidin-3-diglucoside-5-glucoside	ferulic acid	*Raphanus sativa*
Sishonin B[167]	cyanin	*p*-coumaric acid	*Perilla ocimoides* L. var. *crispa* Benth.
Cyananin[85]	cyanidin-3-rhamnoglucoside-5-glucoside	*p*-coumaric acid	*Solanum phureja*
Paeonanin[85]	paeonidin-3-rhamnoglucoside-5-glucoside	*p*-coumaric acid	*Solanum phureja*
Perillanin[177]	delphinidin-x-glucoside	protocatechuic acid	*Perilla ocimoides* L. var. *crispa* Benth.
Gentianin[65]	delphinidin-x-glucoside	*p*-coumaric acid	*Gentiana acaulis*
Awobanin[151]	delphin	*p*-coumaric acid	*Commelina communis* var. *hortensis* Makino
Delphinin	delphinidin diglucoside	*p*-hydroxybenzoic acid (2 residues)	*Delphinium consolida* L.
Violanin[68b]	delphinidin-3-rhamnoglucoside	*p*-coumaric acid	*Viola tricolor*
Delphanin (nasunin?)[85,167b–8]	delphinidin-3-rhamnoglucoside-5-glucoside	*p*-coumaric acid	*Solanum melongena, S. phureja, S. seaforthianum*
Petanin (tuberin)[85, 181]	petunin	*p*-coumaric acid	*Solanaceae*, e.g. *S. phureja* and *S. melongera; Atropa belladonna; Lycopersicon esculentum; Petunia* garden hybrids; red cabbage
Ensatin[182]	malvin	*p*-coumaric acid	*Iris ensata* Thunbg.
Negretein[85]	malvidin-3-rhamnoglucoside	*p*-coumaric acid	*Solanum tuberosum* cv. 'Congo'

* Ga galactose, Gen gentiobiose, Gl glucose, Cell cellobiose, Arb arabinose, Rh rhamnose, Xy xylose

Reference: F.M. Dean, *Naturally Occurring Oxygen Ring Compounds*, Butterworths, 1963, London

Appendix 2

Copigmentation of Cyanidin 3,5-diglucoside

Copigmentation of Cyanidin 3,5-diglucoside (2×10^{-3} *M*) at pH 3.32[a]

Copigment (6×10^{-3} *M*)	λ_{max} (nm)	$\Delta\lambda_{max}$ (nm)	*A*/mm at λ_{max}	% *A* increase at λ_{max}
None	508	—	0.500	—
Aurone				
Aureusidin[b]	540	32	2.135	327
Alkaloids				
Caffeine	513	5	0.590	18
Brucine	512	4	1.110	122
Amino acids				
Alanine	508	0	0.525	5
Arginine	508	0	0.600	20
Aspartic acid	508	0	0.515	3
Glutamic acid	508	0	0.530	6
Glycine	508	0	0.545	9
Histidine	508	0	0.595	19
Proline	508	0	0.625	25
Benzoic acids				
Benzoic acid	509	1	0.590	18
o-Hydroxybenzoic acid	509	1	0.545	9
p-Hydroxybenzoic acid	510	2	0.595	19
Protocatechuic acid	510	2	0.615	23
Coumarin				
Esculin	514	6	0.830	66
Cinnamic acids				
m-Hydroxycinnamic acid	513	5	0.720	44
p-Hydroxycinnamic acid	513	5	0.660	32
Caffeic acid	515	7	0.780	56
Ferulic acid	517	9	0.800	60
Sinapic acid	519	11	1.085	117
Chlorogenic acid	513	5	0.875	75
Dihydrochalcone				
Phloridzin	517	9	1.005	101

[a] Reference: S. Ansen, R.N. Stewart and K.H. Norris, *Phytochemistry*, 1972, **11**, 1139

[b] formed a slight precipitate

Copigmentation of Cyanidin 3,5-diglucoside

Copigmentation of Cyanidin 3, 5-diglucoside (2×10^{-3} M) at pH 3.32[a]

Copigment (6×10^{-3} *M*)	λ_{max} (nm)	$\Delta\lambda_{max}$ (nm)	*A*/mm at λ_{max}	% *A* increase at λ_{max}
Flavan-3-ols				
(+)-Catechin	514	6	0.890	78
Flavone				
Apigenin 7-glucoside[b]	517	9	0.840	68
C-glycosyl Flavone				
8-C-Glucosylapigenin (vitexin)	517	9	1.690	238
6-C-Glucosylapigenin (isovitexin)	537	29	1.705	241
6-C-Glucosylgenkwanin (swertisin)	541	33	2.835	467
Flavonones				
Hesperidin	521	13	1.095	119
Naringin	518	10	0.985	97
Flavonols				
Kaempferol 3-glucoside	530	22	1.693	239
Kaempferol 3-robinobioside-7-rhamnoside (robinin)	524	16	1.423	185
Quercetin 3-glucoside (isoquercitrin)	527	19	1.440	188
Quercetin 3-rhamnoside (quercitrin)	527	19	1.588	217
Quercetin 3-galactoside (hyperin)	531	23	1.910	282
Quercetin 3-rutinoside (rutin)	528	20	1.643	228
Quercetin 7-glucoside (quercimeritrin)	518	10	1.363	173
7-*O*-Methylquercetin-3-rhamnoside (xanthorhamnin)	530	22	1.576	215

[a] Reference: S. Ansen, R.N. Stewart and K.H. Norris, *Phytochemistry*, 1972, **11**, 1139

[b] formed a slight precipitate

Appendix 3

Occurrence of Anthocyanidin types

Occurrence of Anthocyanidin Types in Flower Colour Mutants of Garden Plants[a]

Plant	*Colour forms*	*No. of varieties examined*	*Percentage occurrence of*			*Accompanying flavonols*[c]
			Pg	*Cy*	*Dp*[b]	
Lathyrus odoratus (sweet pea)	cerise, pink[d] and salmon	7	100	0	0	Km
	crimson and carmine	5	0	100	0	Km, Qu
	mauve and blue	9	0	0	100	Km, Qu, My
Verbena hybrida (verbena)	pale pink	2	100	0	0	Km, Qu, Ap
	scarlet-magenta	1	95	5	0	
	pink	1	80	20	0	
	scarlet	2	85	10	5	
	maroon	1	40	30	30	
	purple-blue	2	0	15	85	My, Qu, Km, Lu, Ap
	purple	2	0	10	90	
	white	1	0	0	0	Lu, Ap
Hyacinthus orientalis (hyacinth)	deep red "Scarlet O'Hara"	—	90	10	0	Ap, Km
	pink "Pink Perfection"	—	60	40	0	
	mauve "Lord Balfour"	—	20	80	0	
	mauve "Mauve Queen"	—	0	100	0	
	blue "Delft Blue"	—	0	10	90	
	pale blue "Springtime"	—	0	0	100	
Streptocarpus hybrida[e] (cape primrose)	pink	—	100	0	0	Ap, Km
	salmon	—	80	20	0	Ap
	rose and magenta	—	0	100	0	Ap, Lu
	mauve and blue	—	0	0	100	Ap, Lu
Primula sinensis[e] (Chinese primrose)	orange and coral	—	90	10	0	Km
	maroon, mauve and blue	—	0	0	100	Km, Qu, My
Lupinus polyphyllus (lupin)	pink and red	3	40	60	0	Qu, Km, Lu, Ap
	purple, mauve and blue	3	0	20	80	Lu, Ap
Tulipa (tulip)	red and orange	48	46	48	6	Km, Qu
	pink, crimson and deep red	38	36	56	7	
	black, purple and violet	21	6	32	61	Km, Qu, My

[a] T.W. Goodwin (Ed), Chemistry and Biochemistry of Plant Pigments, Academic Press, London, 1965.
[b] Dp = delphinidin; Cy = cyanidin; Pg = pelargonidin. Pigments present in *Lathyrus, Primula* and *Streptocarpus* are mainly methylated (i.e. peonidin, petunidin and malvidin are present).
[c] Km = kaempferol; Qu = quercetin; My = myricetin; Lu = luteolin; Ap = apigenin.
[d] Two pink shades also contain traces of cyanidin
[e] Forms of known genotype were examined in these cases

Appendix 4

Safety codes and prices for proposed chemicals

Chemical	Quantity	Price	Supplier	Hazard code
acetic acid	2.5L	14.10	Lancaster	3A
butan-1,4-diol	2.5L	18.20	Aldrich	1
citric acid	2.5kg	53.70	Aldrich	1
Copper sulphate	2.5kg	26.0	Lancaster	3T
Ferulic acid	100g	93.10	Sigma	2A
Formic acid	2.5L	16.90	Aldrich	3A
Glycerol	2.5L	22.30	Lancaster	1
Heptan-1,7-diol	25ml	57.10	Fluka	1
Hexan-1,6-diol	5kg	48.70	Lancaster	1
Hydrochloric acid (conc.)	2.5L	18.20	Lancaster	3A
1-naphthalenesulphonic acid	100g	75.90	Aldrich	3A, T
2-naphthalenesulphonic acid	500g	42.40	Aldrich	3A,T
Pentan-1,5-diol	1L	27.20	Fluka	1
PEG 400	1kg	18.40	Lancaster	1
PEG 600	1kg	18.40	Lancaster	1
Phosphoric acid	2.5L	37.00	Aldrich	3A
Propan-2-ol	2.5L	7.60	Aldrich	2F
Propan-1, 2-diol	2.5L	19.30	Aldrich	1
rutin	100g	27.30	Sigma	2T
Sulphuric acid (conc.)	2.5L	11.10	Aldrich	4A
p-toluenesulphonic acid	2.5Kg	19.80	Lancaster	2A, T
Triflic acid				4A, T

Hazard Assessment Codes (*University of St. Andrews Safety Handbook*)

5 = highly hazardous
4 = hazardous
3 = moderate hazard
2 = low hazard
1 = no significant hazard

A = corrosive
B = biological hazard
C = carcinogenic
F = flammable
O = oxidising

R = radiation hazard
T = toxic
X = explosive

Appendix 5

Suitable Coatings / Protective films

1. Plastics/synthetic polymers

(Generally effective gas and moisture barriers)

Polyolefins e.g. low density polyethylene

Vinyls e.g. polyvinyl acetate, polyvinylidene chloride,

Styrene polymers e.g. styrene/acrylate copolymers

Miscellaneous- acrylonitrile/methyl acrylate copolymer, polychlorotrifluoroethylene, polyester, nylon, poly (p-xylene)

2. Polysaccharides

(generally effective gas barriers but poor moisture barriers)

Celluloses e.g.hydroxypropyl methyl cellulose

Starches e.g. hydroxypropylated amylose starch

Dextrins e.g. maltodextrin

Gums e.g. agar, carrageenan, pectin, gum ghatti

3. Proteins

(Generally effective gas barriers but poor moisture barriers)

Gelatin

Collagen

Casein

Zein

Soya isolate

4. Lipids

(Effective moisture barriers but generally less effective gas barriers)

Waxes e.g. Paraffin wax (mineral wax), candelilla wax (vegetable wax), shellac wax (insect wax), oxidised polyethylene (synthetic wax).
Fatty acids and their derivatives e.g. triglycerides (fats and oils), acetylated monoglycerides, sucrose esters of fatty acids.
Non-ionic sufactants e.g. alkyl phenol ethothoxides, alkyl polyglucosides

5. Multicomponent Systems

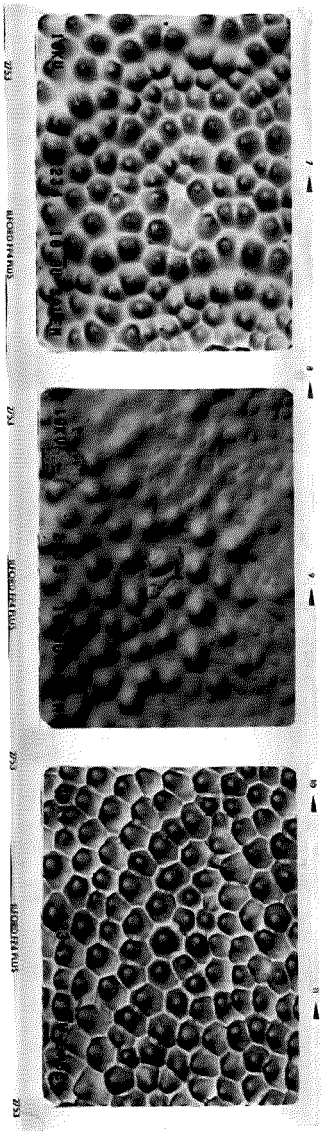
Monolayer emulsions e.g. stearic acid/hydroxypropyl methyl cellulose, palmitic acid/corn zein, fatty acid sucrose esters/ sodium carboxymethyl cellulose/ mono-diglycerides of fatty acids.

Bilayers e.g. stearic/ palmitic acid on hydroxypropyl methyl cellulose

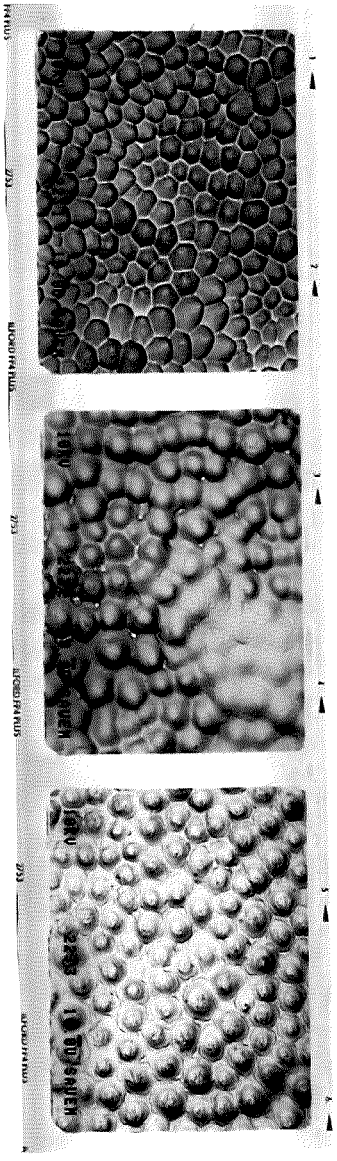
Appendix 6

SEM Images of Coated Processed Petals

(magnification × 400)



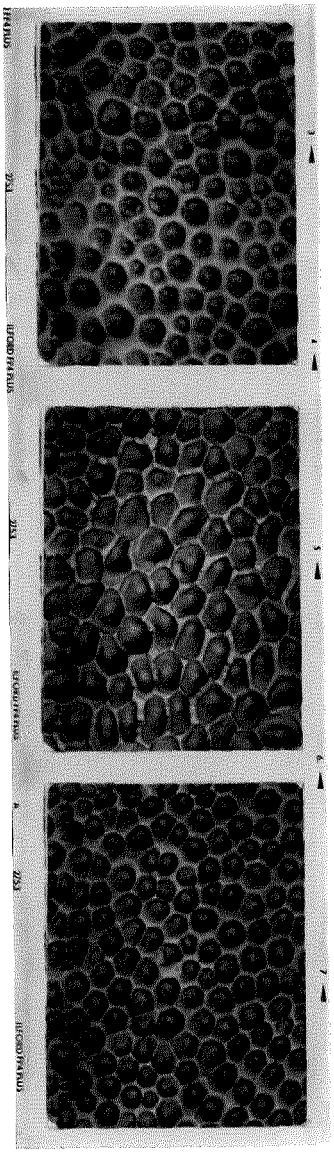
Top LH corner - 10% Applewax (aq)
Middle LH side - 10% Natureseal (aq)
Bottom LH side - 10% synthetic wax (aq)



Top RH corner - 2% Semperfresh (aq)
Middle RH side - 2% SPCR (aq)
Bottom RH side - 2% BAN6 (aq)

SEM Images of Coated Processed Petals

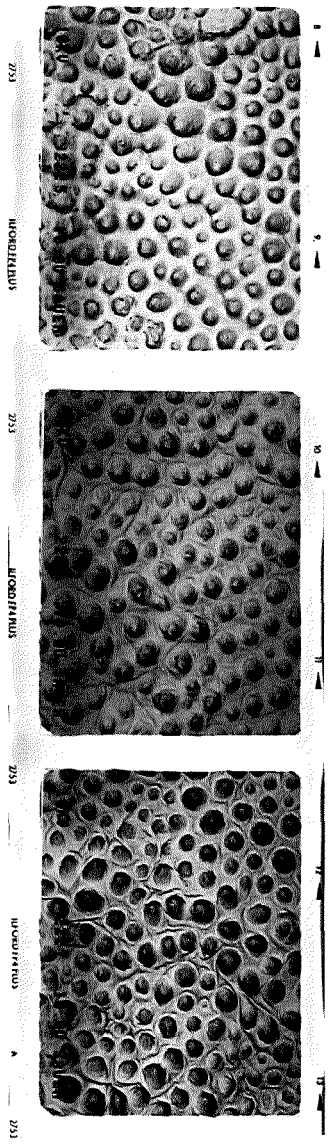
(magnification × 400)



Top LH corner - 10% Texicryl 13-055 (aq)

Middle LH side - 10% Texicryl 13-802 (aq)

Bottom LH corner - 2% HPMC (aq)



Top RH corner - 2% SPCR (propan-2-ol)

Middle RH side - 2% EWW (propan-2-ol)

Bottom RH corner - 2% R49 (propan-2-ol)

Appendix 7

Questionnaire - Preserved Flowers

1. Approximately how much do you spend per month on flowers?

Answer: £30-40.

2. On average how long do your current floral displays last?

Answer: approximatey one week, sometimes less.

3. How long have the preserved flowers lasted?

Answer: approximately 2 and a half months.

4. Where were the flowers displayed?

Answer: tables in dining room.

5. Have you any comments with regards the appearance of the preserved flowers?

Answer: The flower heads looked good, however, the overall appearance of the table displays would be improved if preserved foliage was added, since the dried stems looked slightly withered.

6. Have you any comments with regards to their suitability for hotel/catering use?

Answer: It was necessary to keep them away from flames as one of the flowers went on fire after coming into contact with an oil burner. They also had a slight odour initially.

7. Would you consider using the preserved flowers again?

Answer: Yes, definitely.

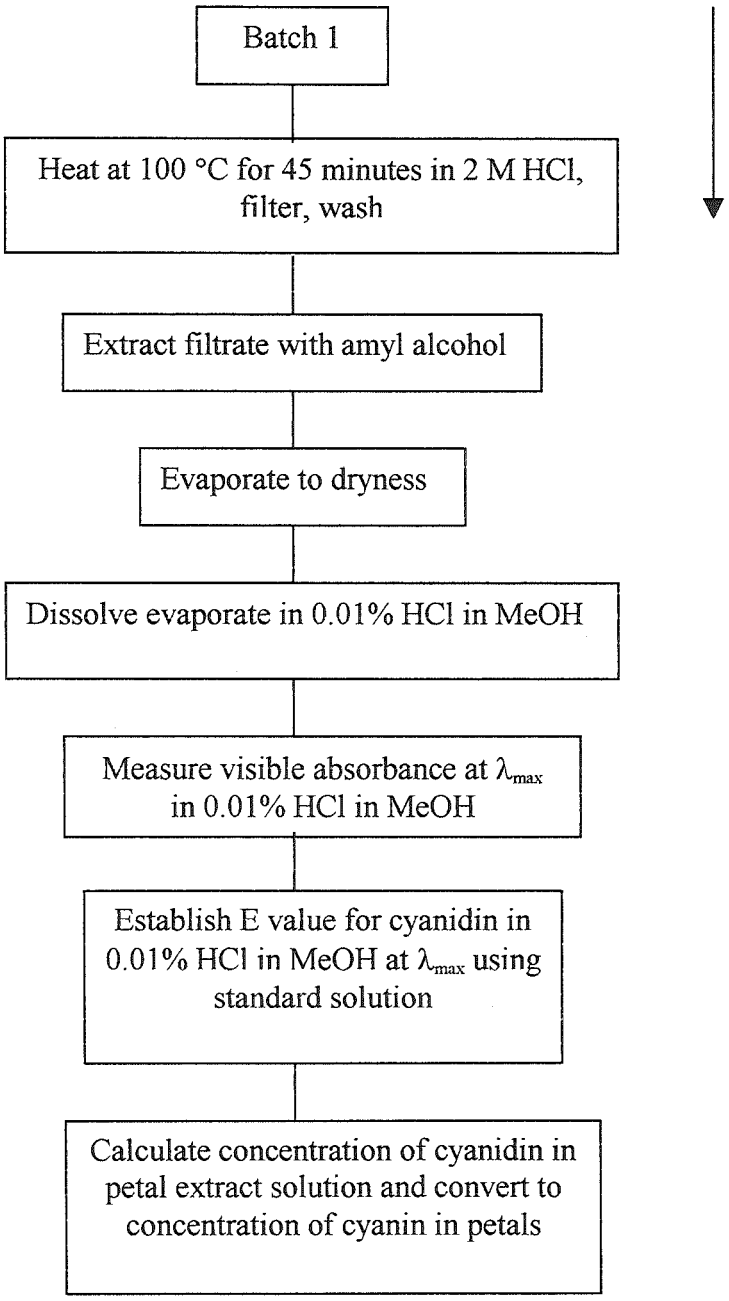
8. Any other comments?

Answer: Carnations with foliage would be good and perhaps a range of colours.

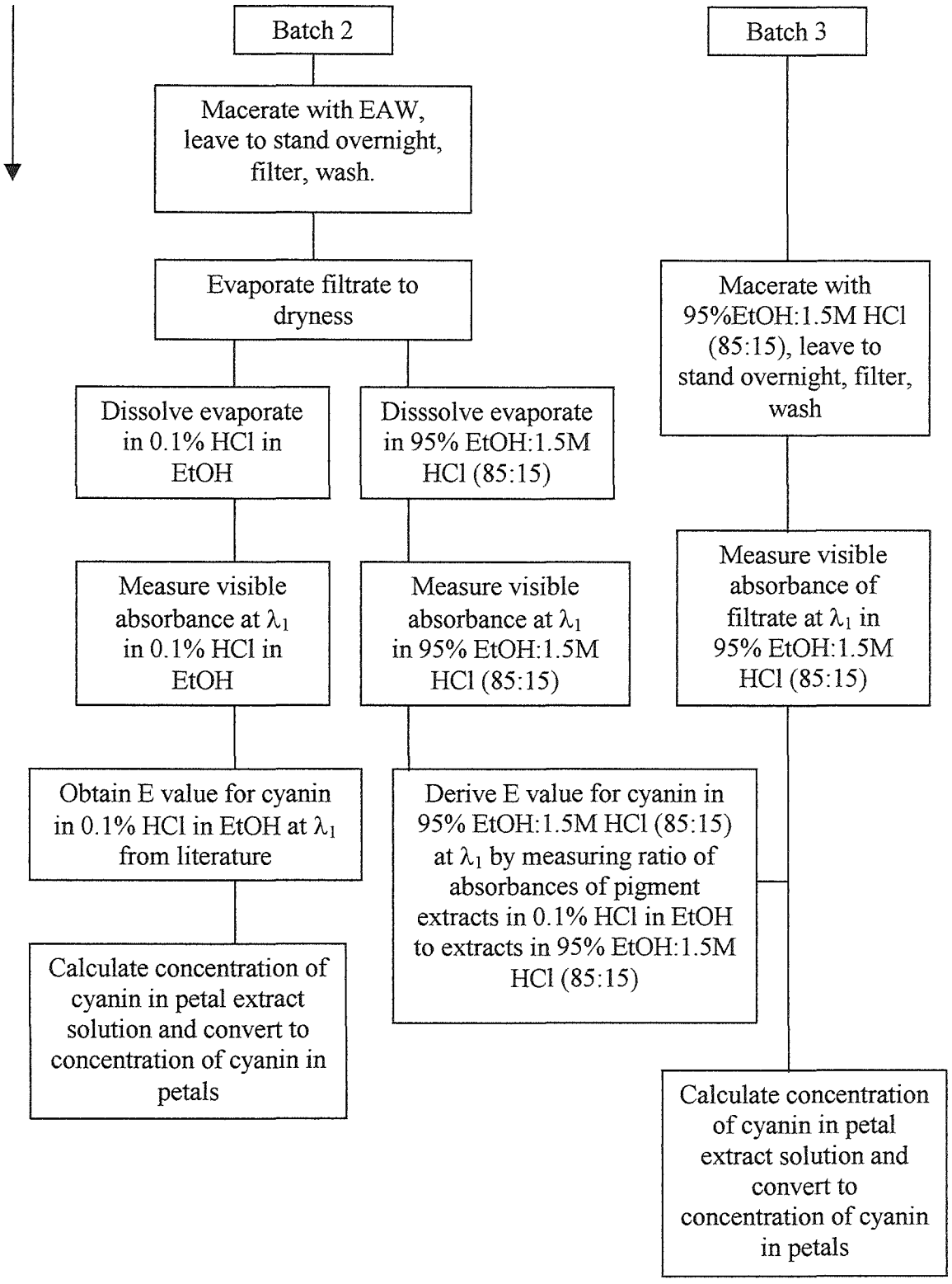
Thank you for participating.

Appendix 8

Procedures for the Determination of the Cyanin content of Petals



Procedures for the Determination of the Cyanin Content of Petals



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