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# Studies on the Flower Colours in *Rhododendron*

## I. Pigment Constitutions of the Elepidote and Some Lepidote Rhododendrons

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### Introduction

Rhododendrons and azaleas, both belonging to the genus *Rhododendron*, have been highly evaluated because of their outstanding ornamental beauties. They have marvelous range of flower colours from white to deepest reds and violets through various shades of yellow, orange, pink, mauve and purple.

The investigations on their flower colours, however, especially those laying the emphasis on the flower colours related to future breeding, have been mainly conducted with those of evergreen azaleas[9-12,24-28], while in rhododendrons the attention of investigators has been rather focused on the chemo-taxonomic problems associated with the classification of this huge and complicated genus[17,22,23,30,31].

In addition, techniques most widely used for the separation and identification of flavonoids have been column, paper or thin-layer chromatographies[6,9,10,27-30]. Although good results have generally been obtained with these techniques, they demand hours to perform and in their resolutions they are not so completely sensitive. Therefore, at the case that pigment composition is highly complex in each plant material, which is frequently encountered in *Rhododendron*[6,9,10,27-30], it has been rather difficult to apply these conventional techniques to the detailed quantitative analysis of the respective constituent pigments.

In recent years, the high performance liquid chromatography (HPLC) was introduced as a new analytical technique and has offered new horizons in the field of the qualification and quantification of the flavonoids[1,4,5,7,15,32,33], because it has many advantages over the conventional chromatographic methods. De Loose[13,14] examined its applicability to the analysis of flavonoids of some evergreen azaleas, and obtained the results that it might quantitate each flavonoid glycoside and might be feasible for the identification of commercial cultivars.

Although in aglycone level, in this experiment the qualification and quantification of the respective constituent anthocyanidins and flavonols were done, by means of HPLC using a total of 165 cultivars and individual plants of rhododendrons, in order to get more accurate pigment composition of each garden form for evaluating their colour-modifying factors and getting some clues for the mode of pigment inheritance and the realization of new flower colours.

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## Materials and Methods

The materials used were 139 cultivars of garden hybrids and 26 individuals (plants) of 13 species, consisting of 157 elepidotes and 8 lepidotes. The colours of these rhododendrons were determined on the basis of Royal Horticultural Society Colour Chart (RHSCC). The petal samples were then lyophilized at −80°C and stored in a desiccator.

### 1. Qualification and quantification of anthocyanidins and flavonol aglycones by HPLC

(1) Standard samples

Standard samples were prepared from the following sources. Each aglycone in the acid hydrolysates was purified by the repeated preparative paper chromatographies and used for the experiments.

| | |
|---|---|
| Delphinidin | Petal of sweet pea "Danny" |
| Petunidin | Ditto |
| Malvidin | Ditto |
| Cyanidin | Petal of rose "Red Devil" |
| Peonidin | Petal of *Paeonia officinalis* |
| Pelargonidin | Petal of gladiolus "Firebrand" |
| Gossypetin | Petal of *Gossypium* sp. |
| Myricetin | Bark of *Myrica rubra* |
| Quercetin | Authentic rutin |
| Kaempferol | Petal of *Robinia pseudoacacia* |
| 5-O-methylmyricetin | Petal of azalea "Oomurasaki" |
| 5-O-methylquercetin | Petal of azalea "Red Wing" |
| 5-O-methylkaempferol | Petal of azalea "Oomurasaki" |

(2) Procedures of analysis by HPLC

Although many investigators used the eluent containing acetic acid for the analysis of flavonoid pigments[1,4,5,7], in our analysing system the mixtures of acetic acid based on Wilkinson *et al.*,[32] did not give so good a result in spite of the various mixtures and flow rates being tested. Replacement of acetic acid with $HClO_4$, however, seemed to give a promising resolution, and the various mixtures of MeOH and $HClO_4$ combined with the various flow rates were examined under the lower concentration of $HClO_4$. The best resolution was obtained in the following conditions, *i.e.*, eluent for anthocyanidin, 50% MeOH containing 0.1% $HClO_4$ and that for flavonol aglycone, 60% MeOH containing 0.1% $HClO_4$; flow rate for anthocyanidin, 0.2 ml/min and that for flavonol aglycone, 0.15 ml/min, respectively[3]. The detailed procedures of analysis in the present experiment were shown in Fig. 1.

The acid hydrolysates were filtered with Millex-SR, Millipore and the filtrates were applied to Sep-Pak cartridge, $C_{18}$, Waters. The aglycones on the cartridge were washed successively with water and 30% MeOH, each in twice, to eliminate the water-soluble, hydrophilic contaminants and were eluted with 80% MeOH. HPLC of the eluates were conducted in the following conditions;

| | |
|---|---|
| Instrument; | BIP–I Micro HPLC System, JASCO with column of 1.5 mm (ID) × 250 mm |
| Adsorbent; | Nucleosil 7–$C_{18}$, Nagel |
| Detector; | UVIDEC–100 IV, JASCO |
| Integrator; | Intelligent Integrator Model 5000E, System Instrument |
| Eluent; | Above mentioned |

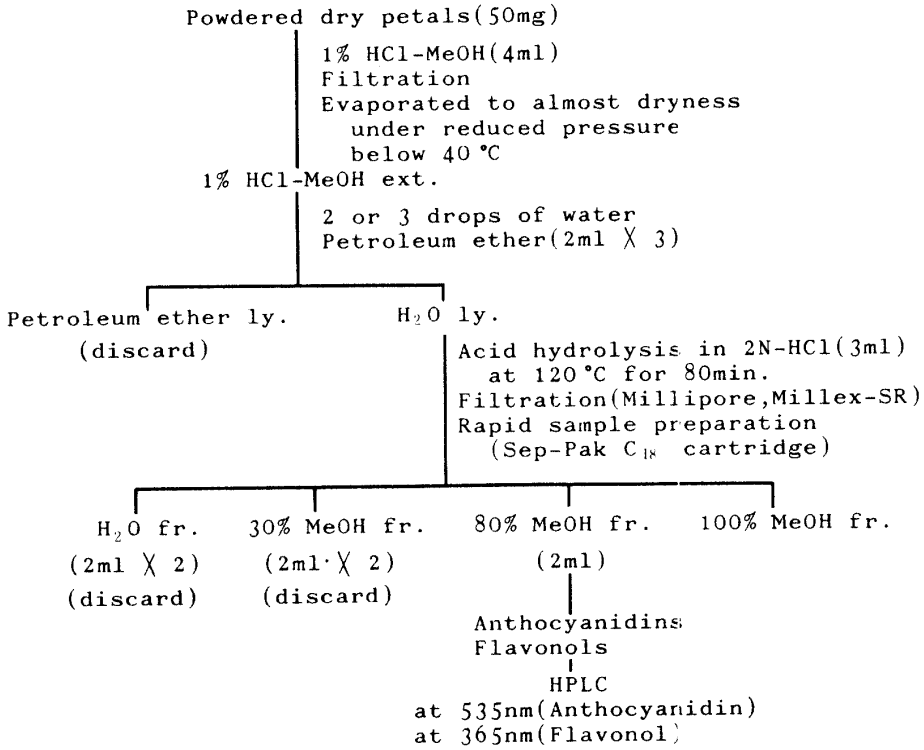



Fig. 1. Procedures of analysis of anthocyanidins and flavonols of *Rhododendron*.

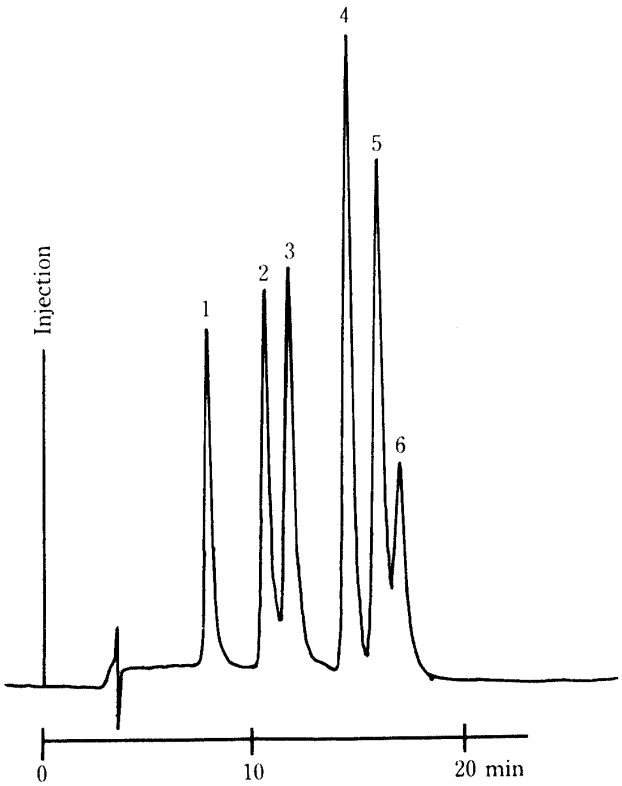



Fig. 2. Separation of the six prevailing anthocyanidins on Nucleosil 7-$C_{18}$ with 50% MeOH as eluent (with 0.1% $HClO_4$, flow rate 0.2 ml/min). For peaks see Table 1.

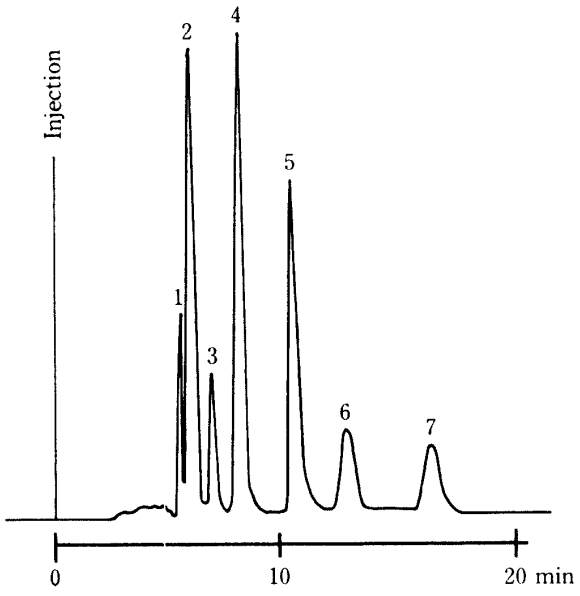


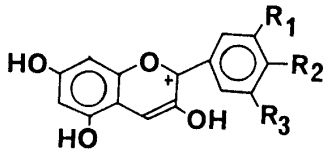

Fig. 3. Separation of seven flavonols occurred in *Rhododendron* on Nucleosil 7-$C_{18}$ with 60% MeOH as eluent (with 0.1% $HClO_4$, flow rate 0.15 ml/min). For peaks see Table 2.

Flow rate; Before mentioned

Wavelength; For anthocyanidin, 535 nm and for flavonol aglycone 365 nm

The HPLC charts obtained were shown in Fig. 2 for anthocyanidins and in Fig. 3 for flavonol aglycones. Tables 1 and 2 show the structures and retention times of these anthocyanidins and flavonols, respectively. As seen in these figures the clear-cut separations were obtained. In addition, the retention times were shorter than 20 min, indicating the capability of rapid analysis as compared with paper or thin-layer chromatographies. The qualification and quantification of each pigment were based on the numerical values indicated by Integrator.

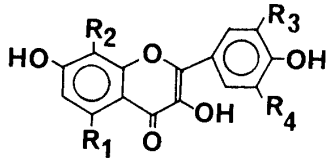
Table 1. Structures and retention times of the six prevailing anthocyanidins




| Peak number | Compound | $R_1$ | $R_2$ | $R_3$ | $t_R$ (min) |
|---|---|---|---|---|---|
| 1 | Delphinidin | OH | OH | OH | 7.66 |
| 2 | Cyanidin | OH | OH | H | 10.52 |
| 3 | Petunidin | $OCH_3$ | OH | OH | 11.96 |
| 4 | Pelargonidin | H | OH | H | 14.72 |
| 5 | Peonidin | $OCH_3$ | OH | H | 16.38 |
| 6 | Malvidin | $OCH_3$ | OH | $OCH_3$ | 17.38 |

For the peak numbers and the running conditions see Fig. 2.

Table 2. Structures and retention times of seven flavonols occurred in *Rhododendron*




| Peak number | Compound | $R_1$ | $R_2$ | $R_3$ | $R_4$ | $t_R$ (min) |
|---|---|---|---|---|---|---|
| 1 | 5-O-methylmyricetin | $OCH_3$ | H | OH | OH | 5.62 |
| 2 | Gossypetin | OH | OH | OH | H | 5.98 |
| 3 | Myricetin | OH | H | OH | OH | 6.96 |
| 4 | 5-O-methylquercetin | $OCH_3$ | H | OH | H | 8.21 |
| 5 | Quercetin | OH | H | OH | H | 10.57 |
| 6 | 5-O-methylkaempferol | $OCH_3$ | H | H | H | 12.80 |
| 7 | Kaempferol | OH | H | H | H | 16.71 |

For the peak numbers and the running conditions see Fig. 3.

## 2. Determination of total amount of anthocyanins and flavonols

In getting the informations about the co-pigmentation, it is prerequisite to evaluate the total amount of anthocyanins and flavonols. This evaluation was conducted by spectrophotometric measurement using a double-beam spectrophotometer, Shimadzu UV-200.

Anthocyanins; 25 mg of powdered dry petal was allowed to stand overnight in 0.5% HCl in

MeOH at room temperature and was filtered. The filtrate was diluted volumetrically to appropriate concentration and the extinction value was measured at 535 nm. The total amount of anthocyanin, mg per 100 mg of dried petal, was calculated from calibration curve prepared from the authentic cyanidin-3: 5-diglucoside.

Flavonol; The extinction value of flavonols is affected with the existence of other contaminants, especially that of anthocyanins, and it is necessary to eliminate these interferences.

25 mg of powdered dry petal was mixed with MeOH. After standing overnight at 50°C, filtration and volumetric dilution, 5–6 drops of 5% $AlCl_3$ in MeOH were added to 4 ml of sample solution and the extinction value was measured at 440 nm 5 min after the addition of $AlCl_3$.

To eliminate the interference of the co-existing anthocyanins to the true extinction value of flavonols at 440 nm, the extinction value of authentic cyanidin-3: 5-diglucoside with $AlCl_3$ was measured at the same wavelength and was subtracted from the above-mentioned, apparent extinction value of flavonols. Based on this corrected value the total amount of flavonol, mg per 100 mg of dried petal, was calculated from calibration curve prepared from authentic quercetin-3-rutinoside.

As the results of the foregoing formalizing procedures, the following expressions for total amount of anthocyanins and flavonols were obtained;

$$\text{Total anthocyanin (mg per 100 mg dry petal)} = \frac{X}{0.65}$$

$$\text{Total flavonol (mg per 100 mg dry petal)} = \frac{[Y-(0.281X-0.01)]-0.0164}{0.3784}$$

where X was the extinction value of anthocyanins at 535 nm and Y was the extinction value of flavonols at 440 nm (not corrected, but apparent value).

## Results and Discussion

### 1. Distribution of anthocyanins and flavonols

The detailed constitutions of each anthocyanidin and flavonol aglycone of 157 elepidotes and 8 lepidotes were presented elsewhere[3] and in this paper only some representative constitutions are shown in Table 3. Table 4 shows the diversity in various attributes concerned with the flower colours.

Although Spathmann[30] suspected the presence of pelargonidin in the species of Section Vireya, the anthocyanins detected were exclusively confined to either delphinidin or cyanidin series and were free from pelargonidin, as far as the present experiment was concerned.

Within cyanic types, 45% of cultivars and species examined contained exclusively the anthocyanins of cyanidin series, whereas the remaining 55% containing delphinidin series were always accompanied by more or less cyanidin series, two extremes of which were seen in cv. "Russautinii" (with 93% delphinidin) and "Grand Slam" (only 1% delphinidin). Moreover, the variation between these two extremes was almost continuous.

According to Heursel and Horn[26] the hydroxylation of anthocyanins, delphinidin *vs.* cyanidin, in the evergreen azaleas was postulated to be controlled by a single gene, O. However, the situation encountered in rhododendrons was excessively complicated, because the range of variation covered almost continuously from the pure cyanidin type to the type highly pigmented with delphinidin. Thus, it seemed to be impossible that the hydroxylation of anthocyanins in the elepidote rhododendron should be ascribed to the action of a single gene as in the evergreen azaleas, although

Table 3. Some representative constitutions of anthocyanins and flavonols in *Rhododendron*

| Cultivars | RHSCC | Y | Percentages of constituent Anthocyanidins | | | | | Flavonols | | | | | | | Total amount of | | Indices*2 of | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Dp | Pt | Mv | Cy | Pn | 5M-My | 5M-Qu | 5M-Km | My | Qu | Km | Gp | An(mg) | Fl(mg) | Dp | M-An | M-Fl | Fl/An |
| Passionate Purple*1 | 90-D | 20.9 | 4 | 5 | 57 | 30 | 4 | 13 | 81 | 1 | 1 | 5 | — | — | 0.26 | 9.54 | 6.6 | 6.6 | 9.4 | 36.7 |
| Russautinii*1 | 88-C | 25.6 | 5 | 5 | 83 | 5 | 3 | 2 | 93 | — | — | 5 | — | — | 0.47 | 2.81 | 9.3 | 9.1 | 9.5 | 5.98 |
| Blue Jay | 84-A | 20.9 | 20 | 6 | 40 | 23 | 12 | 34 | 6 | — | 45 | 15 | — | — | 0.44 | 3.02 | 6.6 | 5.7 | 4.0 | 6.86 |
| Blue Frost | 81-C | 28.5 | 5 | — | 60 | 4 | 32 | 26 | 44 | 5 | 9 | 13 | 3 | — | 0.46 | 3.48 | 6.4 | 9.2 | 7.5 | 7.57 |
| Red Eye | 80-A | 9.8 | 11 | 4 | 65 | 5 | 16 | 50 | 25 | — | 16 | 10 | — | — | 1.24 | 2.53 | 7.9 | 8.5 | 7.4 | 2.04 |
| Smokey #9 | 78-A | 12.8 | 20 | 14 | 19 | 40 | 7 | 37 | 18 | — | 30 | 15 | — | — | 4.40 | 8.00 | 5.3 | 4.0 | 5.5 | 1.82 |
| Mahmoud | 75-C | 54.6 | 61 | — | — | 39 | — | — | — | — | — | 100 | — | — | 0.13 | 2.73 | 6.1 | — | — | 13.0 |
| Roseann | 73-A | 28.0 | 2 | — | — | 99 | — | — | — | — | — | 77 | 23 | — | 0.77 | 0.74 | 0.2 | — | — | 0.96 |
| Purple Lace | 72-B | 13.3 | 41 | 7 | 18 | 29 | 6 | — | — | — | 35 | 65 | — | — | 1.23 | 5.72 | 6.5 | 3.1 | — | 4.65 |
| Grand Slam | 67-A | 15.4 | 1 | — | — | 76 | 22 | — | 88 | — | — | 12 | — | — | 1.18 | 4.70 | 0.1 | 2.2 | 8.8 | 3.98 |
| Hallelujah | 66-C | 26.7 | 3 | — | — | 97 | — | — | 56 | — | — | 44 | — | — | 0.57 | 2.04 | 0.3 | — | 5.6 | 3.58 |
| Anna Rose Whitney | 64-C | 22.1 | 2 | — | — | 75 | 22 | — | 69 | — | — | 31 | — | — | 0.90 | 1.61 | 0.2 | 2.2 | 6.9 | 1.79 |
| Bruce Brechtbill | 62-A | 39.8 | 13 | — | — | 87 | — | — | 10 | — | — | 66 | 1 | 22 | 0.18 | 6.91 | 1.3 | — | 1.0 | 38.4 |
| William Austin | 61-B | 11.8 | 9 | 8 | 1 | 63 | 20 | — | — | — | 9 | 91 | — | — | 1.85 | 3.37 | 1.8 | 2.8 | — | 1.82 |
| Kubla Khan | 58-C | 23.5 | 6 | — | — | 94 | — | — | — | — | — | 100 | — | — | 0.89 | 0.83 | 0.6 | — | — | 0.34 |
| Mrs. C. B. van Nes | 57-C | 15.8 | — | — | — | 82 | 18 | — | — | — | — | 51 | 49 | — | 0.93 | 2.65 | — | 1.8 | — | 2.85 |
| Golden Gate | 57-D | 21.7 | — | — | — | 100 | — | — | 30 | — | 2 | 7 | — | 61 | 0.55 | 4.60 | — | — | 3.0 | 8.36 |
| Twilight Pink | 57-D | 21.7 | — | — | — | 100 | — | — | — | — | 8 | 82 | 10 | — | 0.21 | 4.44 | — | — | — | 21.14 |
| Ruby Hart | 53-A | 8.3 | — | — | — | 100 | — | — | — | — | — | — | — | — | 3.34 | — | — | — | — | — |
| Halfdan Lem | 53-C | 15.5 | — | — | — | 100 | — | — | 57 | 3 | — | 30 | 10 | — | 0.67 | 1.18 | — | — | 6.0 | 1.76 |
| Bambi | 52-A | 18.8 | — | — | — | 100 | — | — | — | — | — | 37 | — | 63 | 0.35 | 0.96 | — | — | — | 2.74 |
| RM-11 | 50-B | 24.7 | — | — | — | 100 | — | — | — | — | — | 56 | 44 | — | 0.36 | 0.31 | — | — | — | 0.86 |
| Polynesian Sunset | 47-D | 30.3 | — | — | — | 100 | — | — | — | — | — | 33 | 2 | 65 | 0.14 | 1.35 | — | — | — | 9.64 |
| Elizabeth | 46-A | 9.4 | — | — | — | 100 | — | — | — | — | — | 97 | 3 | — | 0.76 | 0.79 | — | — | — | 1.04 |
| Vulcan's Flame | 46-C | 16.1 | — | — | — | 94 | 6 | — | — | — | — | 100 | — | — | 1.03 | 0.51 | — | 0.6 | — | 0.50 |
| Whitney Dwarf Red | 45-A | 11.9 | — | — | — | 96 | 4 | — | — | — | — | 97 | 3 | — | 0.74 | 0.07 | — | 0.4 | — | 0.09 |
| Oh-Too | 43-D | 43.1 | — | — | — | 100 | — | — | — | — | — | 44 | — | 56 | 0.22 | 2.54 | — | — | — | 11.5 |
| Grumpy | 38-A | 47.4 | — | — | — | 100 | — | — | — | — | — | 55 | — | 45 | 0.15 | 0.77 | — | — | — | 5.13 |
| Ostbo's Low Yellow | 27-A | 73.8 | — | — | — | 100 | — | — | 24 | — | 4 | 44 | 3 | 25 | 0.37 | 4.33 | — | — | 2.4 | 11.70 |
| Golden Pheasant | 16-C | 69.6 | — | — | — | 100 | — | — | — | — | 8 | 14 | — | 79 | 0.03 | 1.95 | — | — | — | 65.0 |
| Hotei | 5-A | 72.9 | — | — | — | — | — | — | — | — | 4 | 31 | — | 65 | — | 1.83 | — | — | — | — |
| Unique | 2-D | 83.2 | — | — | — | — | — | — | 20 | — | — | 50 | 2 | 28 | — | 5.39 | — | — | 2.0 | — |
| Crest | 1-B | 76.0 | — | — | — | — | — | — | 3 | — | — | 40 | — | 57 | — | 8.50 | — | — | 0.3 | — |
| Mrs. A. T. de la Mare | white | 88.9 | — | — | — | — | — | 3 | 46 | 11 | 3 | 33 | 5 | — | — | 5.10 | — | — | 5.9 | — |
| White Pearl | white | 88.9 | — | — | — | — | — | — | 19 | 5 | 12 | 38 | 26 | — | — | 2.14 | — | — | 2.4 | — |

*1 Lepidote (Cultivars without asterisk are elepidote)

*2 Index of delphinidin (Dp); (Amount of anthocyanidin of delphinidin series/Total amount of anthocyanidin) × 10
Index of methylated anthocyanidin (M-An); (Amount of methylated anthocyanidin/Total amount of anthocyanidin) × 10
Index of methylated flavonol (M-Fl); (Amount of 5-methylated flavonol/Total amount of flavonol) × 10
Index of flavonol/anthocyanidin (Fl/An); Total amount of flavonol/Total amount of anthocyanidin

Table 4. Diversity in various attributes of flower colours in *Rhododendron*[*1]

| Item[*2] | Number of cv. present/absent | Variation in various attributes[*3] Frequency distribution | Max. | Mean |
|---|---|---|---|---|
| | | 0 1 2 3 4 5 6 7 8 9 10 (Index) | | |
| Dp | 80/85 | 29 12 12 4 4 3 8 2 4 1 | 9.3 | 2.77 |
| M-An | 63/102 | 17 11 12 5 2 3 7 — 4 2 | 9.2 | 3.13 |
| M-Fl | 74/91 | 12 3 4 1 9 8 9 17 5 6 | 9.6 | 5.34 |
| | | 0 2 6 10 15 20 25 35 45 55 (Index) | | |
| Fl/An | 138/27[*4] | 42 38 16 15 12 4 6 3 1 1 | 65.0 | 8.60 |
| | | 0 5 10 15 20 25 30 35 40 45 50 (%) | | |
| Km | 60/105 | 40 7 5 2 2 1 1 — 1 1 | 49.0 | 7.12 |
| | | 0 10 20 30 40 50 60 70 (%) | | |
| Gp | 26/139 | 1 2 4 5 3 6 4 1 | 79.0 | 43.35 |

*1 Prepared from the data of 165 cultivars and individuals presented in detail in the report of Arisumi *et al.*[3]

*2 For detail for index and percentage see Table 3.

*3 Numerical values are based on the case in which the pigment of each item is present.

*4 In these 27 cultivars either anthocyanin or flavonol is absent.

Heursel and Horn[26] stated that in O-delphinidin genotypes the presence of traces of the oo-controlled cyanidin could be ascertained.

The similar situation was also encountered in the methylation of anthocyanins, in that the variation ranged almost continuously from cvs. "Russautinii" and "Blue Frost" (both with 91–92% methylated anthocyanin), through such as "Anna Rose Whitney" (22%), "Vulcan's Flame" (6%) to the various cultivars whose anthocyanins were exclusively confined to the non-methylated one. In the evergreen azaleas, Heursel and Horn[26] attributed the methylation of anthocyanins to the action of a single gene, P, although this gene permits such situation as peonidin containing flowers invariably hold a certain amount of cyanidin, and flowers containing cyanidin as their principal pigment (=no peonidin) often show traces of peonidin. In rhododendrons, however, the mode of inheritance of methylation seemed to be a little but definitely different from that of azaleas on account of the continuance found in their variation.

As for the distribution of flavonols the following three items might be worthy of mention, namely, the presences of i) methylated flavonol, ii) kaempferol and iii) gossypetin. Of these items, the last two, especially their significance for the future breeding, will be discussed later.

As in the cases of the hydroxylation and methylation of anthocyanins, the methylation of flavonols of rhododendrons was again very variable and continuous, from *R. ponticum* and cv. "Russautinii" (with 96 and 95% methylated flavonol) through "Hallelujah" (56%) to the non-methylated forms. Methylation of flavonols in the evergreen azaleas was ascribed to be controlled by a single gene, M.[26] In rhododendrons, however, their methylation of flavonol was highly complicated and did not seem to be due to a single gene.

In any way, the great diversity in pigment constitutions in rhododendrons might be the reflex

of their genetic complexity due probably to the involvement of diverse species and to their complicated interspecific hybridization in the course of the formation of garden forms.

## 2. Variation and modifying factors of flower colours

(1) Variation

The variation in the flower colours based on the RHSCC was presented in Fig. 4. As seen in the figure, in which white forms were omitted, the variation found in rhododendrons was very wide, ranging from green yellow through orange, red, purple, violet to violet blue. However, the frequency distribution was converged in the region from red to violet and was rather low from yellow to orange red. It also was devoid of true blue shade.

Except the pigments in the blotch of upper lobe or in the throat, no carotenoid and chlorophyll was found to be concerned with the pigmentation of the elepidotes[30]. From microscopic examination, it was revealed that the only exception in the elepidotes was *R. sanguineum* and some garden hybrids[30,31]. In other words, in the elepidotes the flower colours of various yellow tones are almost exclusively based on the presence of one of the flavonols, gossypetin[19,21].

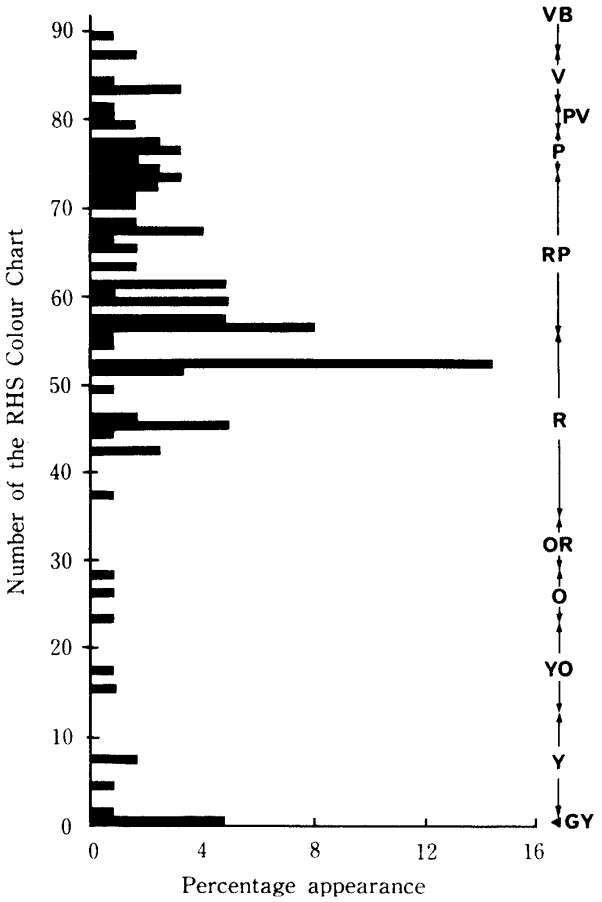



Fig. 4. Frequency distributions of various colour types on the basis of RHS Colour Chart.

VB; violet blue, V; violet, PV; purple violet, P; purple, RP; red purple, R; red, OR; orange red, O; orange, YO; yellow orange, Y; yellow, GY; green yellow.

As seen in Table 3, gossypetin can coexist with anthocyanins and other flavonols in the same epidermal cells, which indicates the competition in the course of their biosynthetic pathway. This might be one of the reasons why the garden forms with intense yellow or orange shade have been rather rare. As will

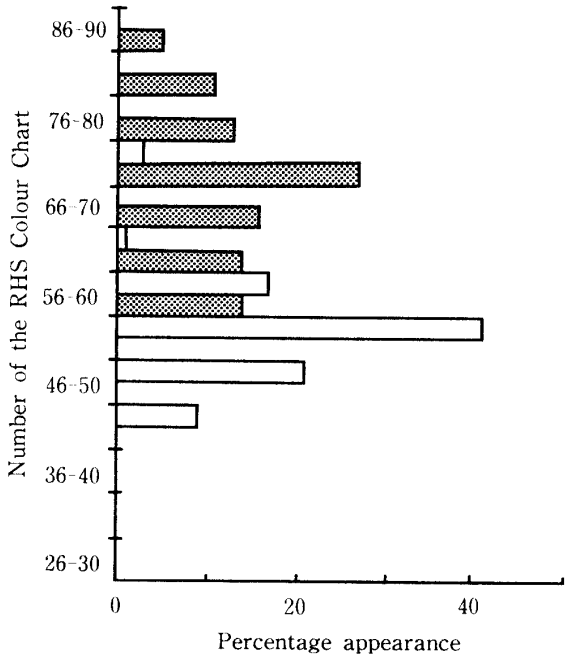



Fig. 5. Difference in colour shades in accordance with the hydroxylation of anthocyanins.

Open bars; the forms exclusively containing anthocyanin of cyanidin series, cross-hatched bars; those containing anthocyanin of delphinidin series in addition to cyanidin series.

be seen later, however, the improvement towards intenser yellow shades might be possible with the increase in the level of total amount of flavonoids and in the relative amount of gossypetin within flavonols, together with the decrease in the amount of anthocyanins.

(2) Modifying factors of flower colours

It has been well known that the flower colours based on the anthocyanins are modified by various factors[18]. One of the most important is the hydroxylation of anthocyanin. As mentioned before, the constitutional pattern of anthocyanins and flavonols in rhododendrons was very variable and continuous from one extreme to another. Therefore, when we evaluate the effectiveness of various modifying factors, it was not so easy to set a clear-cut assortment, in other words, to decide where is the most pertinent point in dividing the continuous variation.

Fig. 5 shows the difference in colour shades due to the hydroxylation of anthocyanins, based on the assortment according to whether anthocyanins of delphinidin series were present or not. It is clear from this figure that the co-occurrence of delphinidin series gives much bluer tone, as compared with the forms with pure cyanidin series.

As shown in Figs. 6–8, the similar blueing effects were observed in the other cases, although the degrees of effectiveness in these cases were consistently lower than that of hydroxylation.

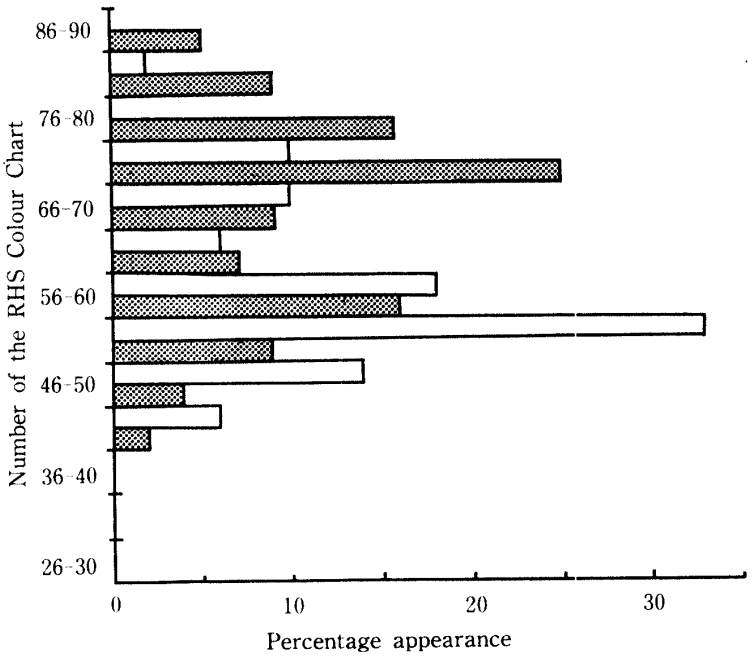



Fig. 6. Difference in colour shades in accordance with the methylation of anthocyanins.
Open bars; the forms exclusively containing non-methylated anthocyanins, cross-hatched bars; those containing both methylated and non-methylated anthocyanins.

Fig. 6 shows the difference in colour shades due to the methylation of anthocyanins. As mentioned previously, the methylation pattern in the anthocyanins of rhododendrons was highly variable, and in this figure it was conveniently assorted in accordance with the presence or the absence of methylated anthocyanins.

The methylation of anthocyanins is said to have some reddening effect; in other words the freedom from methylation, on the contrary, slightly intensifies blue tones.[18] As seen in Fig. 6, however, the methylation of anthocyanins in rhododendrons had a weak but definite blueing effect, as against the generalization of methylation in the other plants.

The methylation of flavonols also displayed a blueing effect as presented in Fig. 7, which was summarized on the basis of the presence or the absence of methylated flavonol, similar to the other foregoing cases. This blueing effect of methylated flavonols agreed with the report of Spathmann[30].

Fig. 8 shows the effect of the well-known co-pigmentation. It was assorted for convenience by

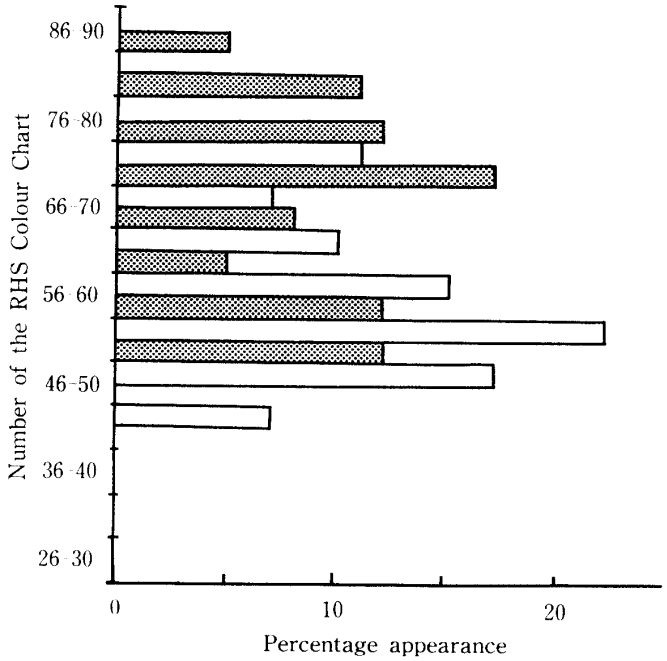



Fig. 7. Difference in colour shades in accordance with the 5-methylation of flavonols.
Open bars; the forms exclusively containing non-methylated flavonols, cross-hatched bars; those containing both methylated and non-methylated flavonols.

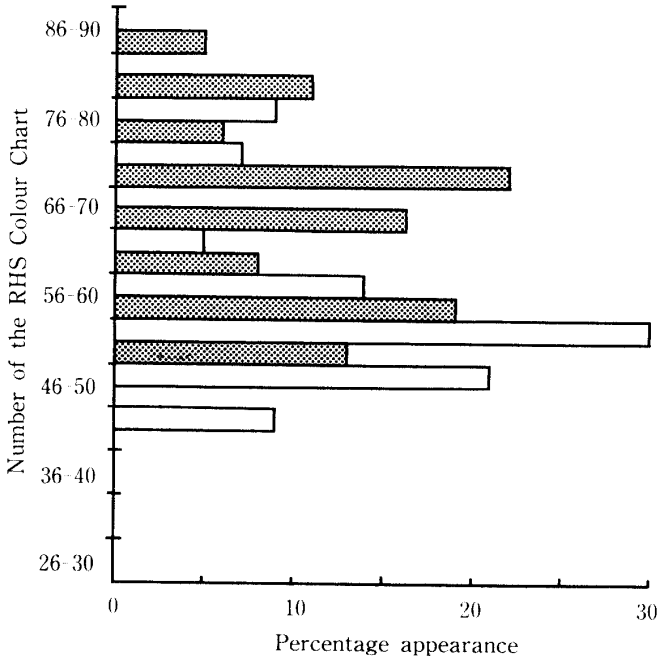



Fig. 8. Difference in colour shades in accordance with the relative amount of flavonols to anthocyanins.
Open bars; the forms whose ratio of flavonols to anthocyanins is below 2, cross-hatched bars; 2 and over.

index number 2 of the relative amount of flavonol to anthocyanin. The blueing effect of co-pigmentation in the genus *Rhododendron* was clearly demonstrated by Asen *et al.*[6] in their novel experiments on an orange mutant of "Red Wing" azalea, and it has been regarded as one of the most important modifying factors of flower colours in the evergreen azaleas[11,25]. As seen in Fig. 8, it apparently gave the bluer shades to rhododendrons, but its effectiveness was not so great as in the case of the hydroxylation of anthocyanins.

### 3. Yellow flower colours

Although the carotenoids play an important role in various intense yellow shades in some rhododendrons and azaleas[30], especially in the species and hybrids of Sections Pentanthera and Vireya, the yellow colours in the species and hybrids examined in this experiment were chiefly based on a flavonol, gossypetin. Fig. 9 shows the distribution of this pigment in various colour types. Its distribution clearly showed two peaks, one being in yellow region and the other being from red to lower half of red purple region, and no gossypetin was found in the region from upper half of red purple to violet blue. In other words, gossypetin could co-exist with anthocyanin, but the co-existance was mainly with cyanidin and not with delphinidin. The reason why gossypetin could not co-exist with delphinidin was not clear, but some explanations would be that the difficulty might be the genetic difficulty due to the difference in the hydroxylation level of B ring of delphinidin and

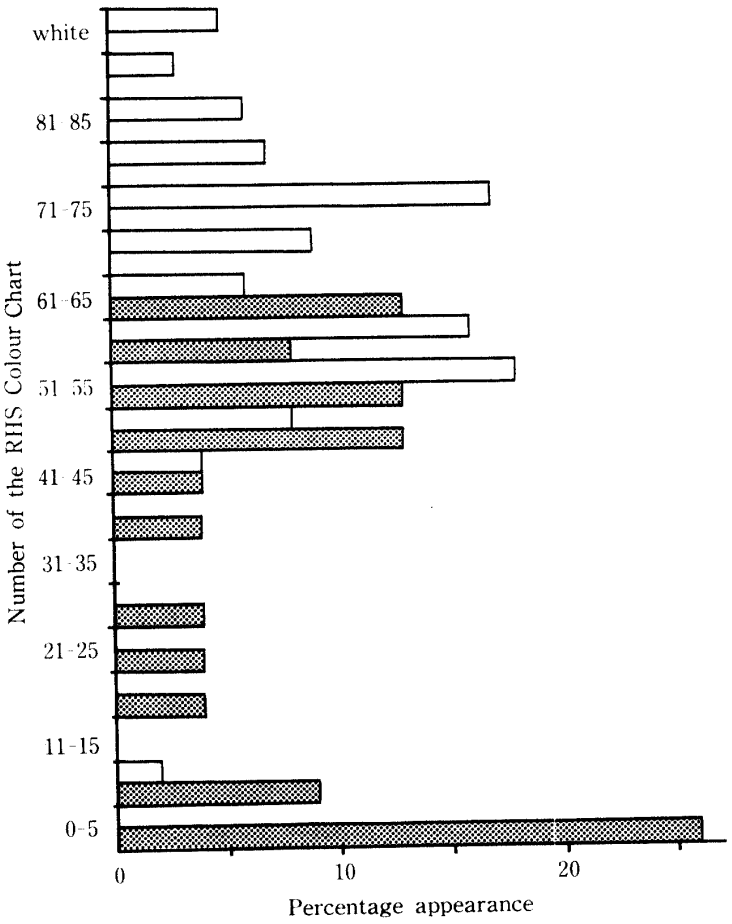



Fig. 9. Distribution of gossypetin in various colour type.

Open bars; the forms without gossypetin, cross-hatched bars; those with gossypetin and other flavonol.

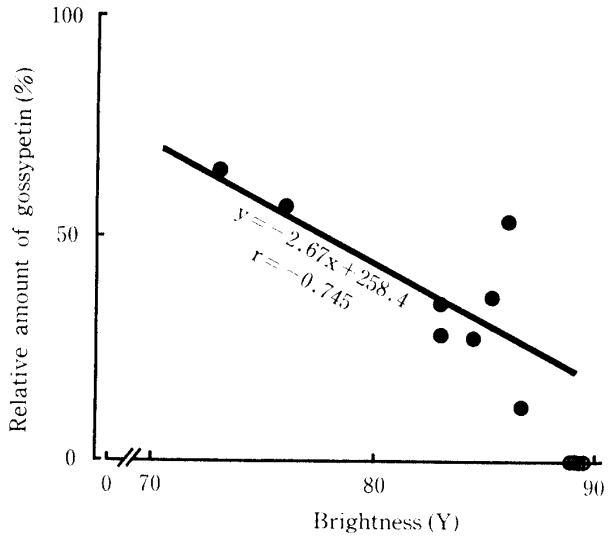



Fig. 10. The effect of gossypetin on the depth of yellow flower colour.
Open circles on the horizontal line; white forms, closed circle; yellow ones.

gossypetin (although, in the detailed inspection of our data[3], the hydroxylation pattern between anthocyanin and flavonol in the elepidote rhododendrons was considerably distorted, as compared with that of *Antirrhinum*, in which the perfect correlation was noticeable[16]), and/or that the co-existance of delphinidin and gossypetin might afford unpleasant flower colours and the relevant seedlings might be selected out in the practical breeding.

When it occurred, gossypetin always accompanied the other flavonols. Fig. 10 shows the interrelationship between the brightness (Y) and the relative amount of gossypetin to the other flavonols, which was prepared basing solely on the data of pure yellow forms, *i.e.*, the anthocyanin-containing forms being omitted. As seen in the figure, there was the apparent negative correlation between the relative amount of gossypetin and the brightness, indicating clearly that the increasing in the relative amount of gossypetin can give deeper yellow colours.

## 4. Some aspects for future breeding

(1) Blue shades

As seen in the foregoing discussion, the primary problem concerning in realizing true blue rhododendrons would be how to eliminate the anthocyanins of cyanidin series, tenaciously co-occurred with blue delphinidin. In addition, it might intensify the blue shade to fill simultaneously the following items; i) 100% malvidin and methylated flavonols and ii) the high amount of the latter pigments sufficient to form full co-pigments.

Although in this investigation the detailed analysis of the glycosylation of anthocyanins has not been conducted yet, it is well known that the 3: 5-diglycoside may afford bluer shade than the corresponding 3-monoglycoside[11]. Furthermore, in their preliminary research the authors observed the phenomenon looking like acylation in some garden forms. De Loose[10] also observed the acylation in the evergreen azaleas. Therefore, it would be quite reasonable to presume that the systematic and composite planning of future breeding might realize much bluer shades than those which have been attained until nowadays, although De Loose[12] concluded in *Azalea indica* that the prospects for obtaining a blue flower colour are quite small, because of its low epidermal pH which interferes the formation and stabilization of co-pigmentation between delphinidin and flavonol.

(2) Yellow shades

In the elepidote rhododendrons, it would be one of the most important future subjects to intensify the yellow colours. In this context, the following item must be emphasized, *i.e.*, how to increase the total amount of flavonol together with the increase of relative amount of gossypetin within various flavonols.

In the evergreen azaleas, Heursel[25] speculated on the realization of yellow colours and stated that if the flavonol content were 3.5 times higher than the highest content found in *R. simsii*, the lemon-coloured flowers might be the result. As seen in Table 3 and elsewhere[3], the flavonoid pigment content including anthocyanins was very variable in rhododendrons, from 0 to 4.40 mg per 100 mg of dry petal in anthocyanin and from 0 to 9.54 mg in flavonol. Furthermore, the elepidote rhododendrons can fortunately produce a yellow flavonol, gossypetin, the highest relative amount of which was 79% in cv. "Golden Pheasant" in anthocyanin-containing cultivars and 65% in "Hotei" in pure yellow cultivars.

Thus, there might be a possibility for further improvement towards intenser yellow colours by the simultaneous increasing in the total amount of flavonol and the relative amount of gossypetin within various flavonols, together with decreasing in the amount of anthocyanins.

(3) Introduction of pelargonidin production

In the present investigation, it was revealed that *ca.* 36% of cultivars contained kaempferol and/or its 5-methylether as the constituent of flavonols. The most excellent cases were "Mrs. C. B. van Nes" and "RM 11", the percentage of kaempferol within total flavonols in these cultivars being 49% and 44%, respectively.

The production of kaempferol is closely related to the production of pelargonidin, and *vice versa*[2,16]. For example, the garden hybrids of *Streptocarpus* were derived essentially from the cross of the delphinidin-containing *S. rexii* (genotype Or) and the cyanidin-containing *S. dunnii* (genotype oR). In the $F_2$, the production of a new anthocyanin occurred, *i.e.*, the pelargonidin-containing forms (genotype or) were segregated out, as the result of the accumulation of two recessive genes, both of which control the hydroxylation of anthocyanin in dominant allele[20]. In addition, on this particular colour types the entirely new production of kaempferol occurred, which was not known otherwise in the garden hybrids and did not occur elsewhere in the genus or even in the same family, Gesneriaceae[20].

Furthermore, Cornu *et al.*[8] succeeded in *Petunia* in having first induced the production of pelargonidin in the following procedures; namely, they applied the mutagenic treatments to cyanidin and quercetin lines, obtained the carriers of a recessive mutation (k) which promoted the synthesis of kaempferol at the expense of quercetin, and added the selection to those kaempferol lines.

As mentioned previously, Spathmann[30] suspected the presence of pelargonidin in the species of Section Vireya. In this investigation, however, any trace of this pigment could not be detected in the elepidotes. Pelargonidin gives various, brilliant orange shades, such as scarlet, vermilion, orange, coral or salmon. Therefore, it would be worth trying to pursue the accumulation of kaempferol by the cross and selection, if needed, combined with mutagenic treatment of kaempferol lines, to realize the production of pelargonidin and, thus, to introduce the entirely new colours into the elepidote rhododendrons.

## Summary

The qualification and quantification of the constituent anthocyanins and flavonols of rhodo-

dendrons were done, by means of high performance liquid chromatography using a total of 165 cultivars and individual plants, in order to get more accurate pigment composition of each garden form for evaluating their colour-modifying factors and getting some clues for the mode of pigment inheritance and the realization of new flower colours.

In connection with the hydroxylation and methylation of anthocyanins and the methylation of flavonols, the distribution pattern of constituent pigments was various and continuous from one extreme to another, which was interpreted to indicate the perticipation of plural genes in the respective modifications of anthocyanins and flavonols.

The great diversity in pigment constitutions encountered was also interpreted to be the reflex of their genetic complexity, due probably to the involvement of diverse species and to their complicated interspecific hybridization in the course of the formation of garden forms.

Except white forms, the variation of flower colours based on the RHSCC was very wide ranging from 1D (green yellow) to 90D (violet blue), although the frequency distribution was converged in the region from red to violet and was rather rare from yellow to orange red.

The various yellow shades in the elepidote rhododendrons was based on the presence of a flavonol, gossypetin. The relative amount of it within total flavonols was revealed to be directly correlated to the intensity of yellow shades. However, gossypetin always co-existed with the other flavonoids. The competition between gossypetin and the other flavonols in their biosynthetic pathway, was deduced to cause the situation why the garden forms with intense yellow or orange shades were rather rare.

As in the other plants, the hydroxylation of anthocyanins and the co-pigmentation between anthocyanins and flavonols exhibited the blueing effect. The methylation of flavonols also modified the flower colours towards bluer side. In rhododendrons, however, the methylation of anthocyanins displayed a little but definite blueing effect, as against the reddening effect which was generalized in the other plants.

On the basis of these findings, several aspects for future breeding were discussed.

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