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学位論文全文に代わる要約
Extended Summary in Lieu of Dissertation

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学位論文題目：
Title of Dissertation

New food technology for the purification and utilization of anthocyanins by forming supramolecular complexes
(超分子複合体法によるアントシアニンの精製法の開発と利用)

学位論文要約：
Dissertation Summary

Anthocyanins, one of the flavonoids, are the most important pigments of the vascular plants, giving a wide range of colors, such as red, blue, purple and black, to many flowers, vegetables, fruits, and other plants. Therefore, many plants containing anthocyanins from red *Perilla* leaves, skin of grapes and berries, red cabbages, and so on, have been used as colorants in processed foods such as pickled plums, red wines, jams, and so on. Moreover, anthocyanins are receiving great attention, not only as safe food colorants, but also as potential therapeutic components. The physiological functions of anthocyanins such as antioxidant activity, anticancer activity, antidiabetes activity, and so on, have been often reported, recently. For all-purpose usage of anthocyanins, separation and purification of various anthocyanins may be in dispensable. When they were used as food colorants, pharmaceutical ingredients and other purposes, plant extractions require the high quality of the colorants to avoid turbidity, off-flavor, low color values, low efficiency of target functions. Foreign substances such as sugars, proteins, organic acids, phenolic compounds, and off-flavor compounds from the original plant materials may be major components to interfere and decrease the quality. Additionally, during concentration process of anthocyanin extracts, some unstable anthocyanins may be decomposed by thermal treatment. The most common method for purifying and isolating individual anthocyanins is preparative high performance liquid chromatography (Preparative HPLC) with ultraviolet-visible or photodiode array detectors. However, pure anthocyanins obtained by Preparative HPLC are expensive for food manufacturing as food additives and are only available in small amounts even though we found valuable biological benefits. Cost performance by Preparative HPLC is limited. Therefore, the development of new purification techniques more easy and cheap is important as a new technology.

In this study, we introduce the unique idea for isolation of anthocyanins with the combination of supramolecules formation and solid phase extraction (SPE). Supramolecule is a stoichiometric self-assembled

complex that consists of anthocyanins that have *ortho*-dihydroxyl groups on the B ring, specific flavonoids and metal ions. Supramolecules recognize the stereostructure of specific anthocyanins and flavonoids that may have great affinity and higher matching each other without covalent bonds. Therefore, the character of supramolecules is strictly to exclude undesired components from the mixtures of target anthocyanins and other molecules and then dissociate target anthocyanins with specific flavonoids by adding weak acid. This is the concept of anthocyanins purification by supramolecular. To perform efficient purification of anthocyanins, we have to consider the separation techniques of individual anthocyanins and specific flavonoids from the supramolecule by means of an efficient, cheap, convenient, and simple method. Solid phase extract (SPE) is, therefore, considered to be a useful method for separation of anthocyanins from other components.

In this dissertation, I would like to emphasize two aspects: 1) the formation of supramolecules that are applicable for common anthocyanins found in nature. 2) separation technique of anthocyanins and flavonoids that were used for the supramolecule formation. Through these studies, we could develop new type of supramolecules consisting of anthocyanidin 3-glycosides, flavocommelin (FC) and aluminum ion (Al^{3+}). Anthocyanidin 3-glycosides that are quite abundant in nature are good target for supramolecular formation. The supramolecular formation is not found in nature but it is useful and applicable for many kinds of anthocyanins' purification.

In the second chapter, appropriate SPE resins that will separate anthocyanin 3,5-diglucoside and their derivatives, and flavonoids were selected by using well-known supramolecules of natural pigments (commelinin from *Commelina communis* and protocyanin from *Centaure cyanus*), hydrolysate of these supramolecules and artificial *Perilla* supramolecule recounstructed from *Perilla* crude pigment, FC and magnesium ion (Mg^{2+}). Two SPE cartridges (Discovery DPA-6S and DSC-SCX) were found to be suitable for separation and purification of anthocyanins from supramolecules. As the results, malonylawobanin from commelinin supramolecular complex yielded 79.5% with 85.7% purity using a DPA-6S (2.5 cm i.d. × 4.0 cm), while a DSC-SCX (2.5 cm i.d. × 3.3 cm) gave a rather lower yield (62.5%) of malonylawobanin with high purity (90.8%). Delphinidin 3,5-diglucoside and awobanin from acidic hydrolysis of commelinin were easily separated using a DPA-6S (2.5 cm i.d. × 4.0 cm). Secondary, succinylcyanin, which is a component of protocyanin, yielded 64.3% with 86.1% purity using a DPA-6S (2.5 cm i.d. × 4.0 cm) and 51.8% with 74.1% purity using a DSC-SCX (2.5 cm i.d. × 3.3 cm). Moreover, cyanidin 3,5-diglucoside yielded 43.4% with 97.0% purity using a DPA-6S (3.5 cm i.d. × 12.0 cm) after partial acidic hydrolysis of protocyanin as shown in Figure 1. Finally, artificial *Perilla* supramolecules (reconstructed from *Perilla* crude pigment, flavocommelin and

magnesium ion (Mg^{2+})) gave a high purity of a malonylshisonin and shisonin mixture (96.1%) with DSC-SCX (2.5 cm i.d. × 3.3 cm).

As far as a cationic exchange resin (DSC-SCX cartridge) for protocyanin, malonylflavone (MF) was eluted quickly with water as the eluent because of the repelling action between the negatively charged glucuronide in MF and DSC-SCX. Conversely, the cationic flavylium ion in SC was attracted to the negatively charged DSC-SCX, and therefore SC was retained in the resin well and eluted by 60% MeOH as described above. Moreover, when many neutral impurities of flavonoids and other chemicals coexist with anthocyanins in *Perilla* leaves, a DSC-SCX cartridge was essential for purification. For example, cationic charged MS from FC and other neutral impurities was purified by means of ion exchange force. However, generally, cation exchange columns such as the Dowex-50 and Amberlite IR120 often show a lower recovery of anthocyanins because of high adsorption of the flavylium type of anthocyanins to the resins. Instead, anthocyanins using DSC-SCX was easy and simple to be desorbed from the resin. Therefore, a DSC-SCX cartridge makes it possible and effective to isolate various flavylium type natural anthocyanins from neutral impurities.

Hydrophobic interaction and hydrogen binding with the amide group (DPA-6S cartridge) are useful for separating anthocyanins from natural supramolecules such as commelinin and protocyanin. Generally the retention force of polyamide type resins for the following aromatic compounds is in descending order, phloroglucinol, resorcinol and pyrocatechol. The retention force of the phenolic compounds against the amide group on the resin is said to be due to the increase in the number of hydroxyl groups and the distance between the hydroxyl groups. In this experiment, MA has many phenolic hydroxyl groups, but FC does not. Therefore, the late elution of MA may be partly explained by the interactions. Moreover, pure Dp3,5diG and Aw which were hydrolyzed from MA in commelinin were successfully isolated by a DPA-6S cartridge. FC which has one phenolic hydroxyl group was eluted faster than Dp3,5diG which bears many phenolic hydroxyl groups, even though elution order of Dp3,5diG and FC in ODS-HPLC system is Dp3,5diG first and then FC later, meaning the polarity of Dp3,5diG is higher than that of FC. Therefore, the number of phenolic hydroxyl groups was thought to be most important for retaining in the resin. In cases that compounds have the same number of phenolic hydroxyl groups, the difference in polarity was essential in separation. Thus, anthocyanidin 3,5-diglucosides and acylated (cinnamoyl derivatives) anthocyanidin 3,5-diglucosides such as Dp3,5diG and Aw were separated with 30% MeOH and 60% MeOH using a DPA-6S cartridge. Moreover, efficient separating of anthocyanidin 3,5-diglucoside from anthocyanidin 3-glucoside might be possible because Dp3,5diG and Dp3G from acidic hydrolysates of commelinin were different in polality.

Consequently, it was found that anthocyanidin 3,5-diglucosides and their related chemicals using supramolecule techniques and Discovery DPA-6S and DSC-SCX cartridges was purified effectively, conveniently and practically even if anthocyanins was purified in a large scale.

In the third chapter, anthocyanidin 3-glycosides in bilberry were tried to form new type of supramolecules with FC and metal ions even though supramolecules of anthocyanidin 3-glycosides have not been found so far in nature. Bilberry pigment contains 15 different anthocyanidin 3-glycosides. The supramolecular complexes of anthocyanidin 3-glycosides with FC and Al^{3+} was successfully established and then applied to isolate specific anthocyanidin 3-glycosides bearing *ortho*-dhydroxyl group on B ring (9 types, 3 delphinidin 3-glycosides, 3 cyanidin 3-glycosides, and 3 petunidin 3-glycosides) from other anthocyanidin 3-glydosides (6 types, 3 peonidin 3-glycosides and 3 malvidin 3-glycosides) as shown in Figure 2. On the other hands, other metal ions (Mg^{2+}, Zn^{2+} and Fe^{3+}) did not form the supramolecule like complexes.

Complex blue pigment (CP, 1.30g) was formed from 1.86 g (1.5 mmol) of bilberry pigment, 1.82 g (3.0 mmol) of FC and 3 mL of 0.5 M aluminum chloride aqueous solution (1.5 mmol), yielding 40.2% recovery of anthocyanidin 3-glycosides in the complex after precipitation by adding ethanol. The purity of anthocyanidin 3-glycosides after purification with a Sephadex G-10 column (1.0 cm i.d. × 15.0 cm) and a Discovery DPA-6S cartridge (2.5 cm i.d. × 4.0 cm) was 96.9%, and the recovery rate of anthocyanidin 3-glycosides from CP was 90.6%. Additionally, the composition of anthocyanidin 3-glycosides bearing *ortho*-dihydroxyl groups on the B ring was occupied 99.1% in the isolated pigment. Moreover, purified anthocyanidin 3-glycosides from CP contained only 0.05 ppm of aluminum against 250 ppm of anthocyanidin 3-glycosides solution. It means contamination of final product anthocyanidin 3-glycosides was trace in amount. Thus, with this technique, the safety of food colorants with aluminum contamination can be guaranteed. Furthermore, purified anthocyanidin 3-glycosides were increased DPPH radical scavenging activity against the original bilberry pigment.

Thus, this separation technique for anthocyanidin 3-glycosides bearing an *ortho*-dihydroxyl group on the B ring by supramolecular-like complex formation and using Discovery DPA-6S cartridge might offer one advanced technology to obtain some functional anthocyanins.

In the fourth chapter, another type of isolation of pure anthocyanin from Delaware grape skin extract was introduced. The main anthocyanins of Delaware grape skin [cyanidin 3-glucoside (Cy3G), cyanidin 3-(6-*O*-*p*-coumaroylglucoside) (Cy3-*p*C·G), peonidin 3-glucoside (Pn3G), and peonidin 3-(6-*O*-*p*-coumaroylglucoside) (Pn3-*p*C·G)] were selectivity isolated from Delaware crude pigment by combination with complex formation with flavocommelin and aluminum ion (Al^{3+}), and a Discovery DPA-6S

cartridge. Complex blue pigment (CP) was formed from Delaware crude pigment, flavocommelin (2 equiv against anthocyanins in Delaware crude pigment) and 0.5 M aluminum chloride aqueous solution (Al^{3+}, 1 equiv against anthocyanins in Delaware crude pigment). CP was obtained 250 ± 12 mg and supernatant (Sn) was obtained 139 ± 19 mg in triplicate. CP was selectivity contained anthocyanins bearing an *ortho*-hydroxyl group on the B ring (Cy3G and Cy3-*p*C·G). The percentages of Cy3G and Cy3-*p*C·G in CP were increased more than 90%, though the percentage of Cy3G and Cy3-*p*C·G in original Delaware crude pigment was 57%. Therefore, *ortho*-dihydroxyl group was essential to form high selective formation of CP, however *p*-coumaric acid group was not affected to selectivity. On the other hand, Sn contained mainly Pn group (around 70%) with a little of Cy group (around 30%).

The results of electrophoresis with cellulose acetate paper were showed that CP was contained both of Cy3G and FC, and it gave a vivid blue spot in color even though Delaware grape skin extract (Cy3G) and FC mixture without Al^{3+} were located in different positions each other on the sheet. This might be the evidence of aluminum complex of Cy3G with FC.

After forming CP, Cy3G and Cy3-*p*C·G were isolated in CP, and the mixture of Pn3G and Cy3G and that of Pn3-*p*C·G and Cy3-*p*C·G remained in Sn as shown in Figure 3. Finally, Cy3G was yielded 48.2% with 95.2% purity and Cy3-*p*C·G was yielded 44.9% with 91.4% purity from Delaware crude pigments (13.0% purity of total anthocyanins). Moreover, Pn3G rich fraction was isolated with 35.8% recovery and 94.5% purity and Pn3-*p*C·G rich fraction was isolated with 33.9% recovery and 88.9% purity from Delaware crude pigment.

The antioxidant activities of isolated anthocyanins were measured by two methods, DPPH radical scavenger activity (DRSA) and lipid peroxidation inhibition activity (thiobarbituric acid reactive substances (TBARS) assay). As the result of DRSA in triplicate, Cy group (Cy3G: 4.5 ± 0.08 μmol TE/mg, Cy3-*p*C·G: 4.5 ± 0.04 μmol TE/mg) showed significantly higher activity ($p < 0.05$) than Pn group (Pn3G: 4.0 ± 0.05 μmol TE/mg, Pn3-*p*C·G: 3.9 ± 0.02 μmol TE/mg). On the other hand, the result of TBARS assay in quadruplicate, Pn group (Pn3G: 22.93 ± 6.45 nmol TBARS/mg linoleic acid, Pn3-*p*C·G: 16.46 ± 6.16 nmol TBARS/mg linoleic acid) showed significantly higher activity ($p < 0.05$) than Cy group (Cy3G: 41.78 ± 3.00 nmol TBARS/mg linoleic acid, Cy3-*p*C·G: 36.06 ± 2.54 nmol TBARS/mg linoleic acid). Anyway, anthocyanins isolated showed high antioxidative activity.

Consequently, the proposed separation technique for anthocyanindin 3-glucosides and acylated anthocyanidin 3-glucosides using supramolecular complex formation and solid phase cartride (Discovery DPA-6S) revealed a potential for advanced technology to obtain functional anthocyanins.

In the fifth chapter, the chemical structure of anthocyanidin 3-glycoside complex with FC and Al^{3+} was demonstrated from several physical analyses (UV-vis and CD spectra, electrophoresis with cellulose acetate paper, ICP emission spectroscopy, and ESI-MS analysis) by means of simple complex of delphinidin 3-glucoside, FC and Al^{3+} (Dp3G complex). Thus, the composition of Dp3G complex was determined to be one molecule each of Dp3G, FC and Al^{3+} by ICP, HPLC quantitative analysis and ESI TOF-MS. The molecular weight for Dp3G complex was determined by ESI TOF-MS to be one molecule each of the components.

Furthermore, we summarized new isolation technique of anthocyanin by forming supramolecules and SPE cartridges from plants as shown Figure 4. SPE cartridges should be one of choices by polarity and charge difference of anthocyanin molecules. Separation and purification techniques for various anthocyanins in terms of the combination of complex formation and SPE separation have successfully offered the development of new technology to obtain some functional anthocyanins.

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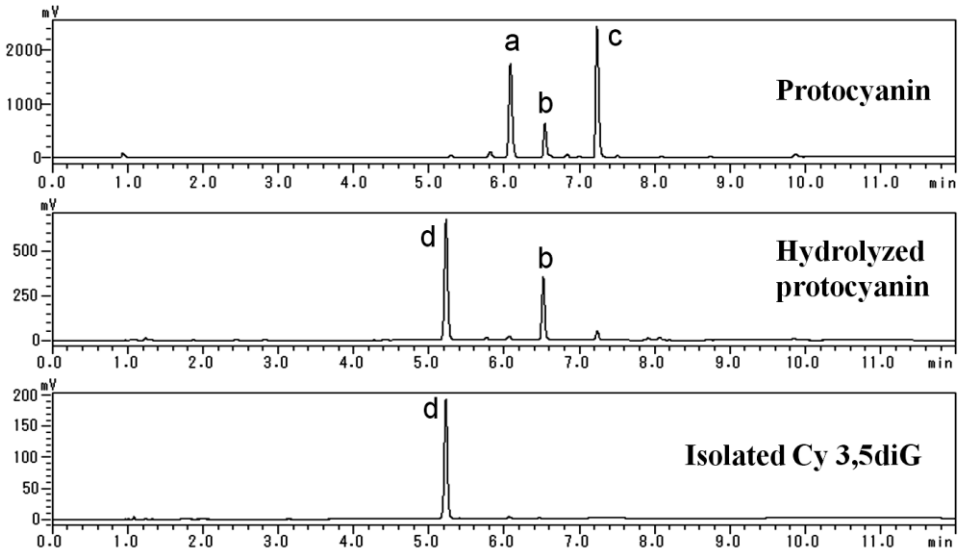
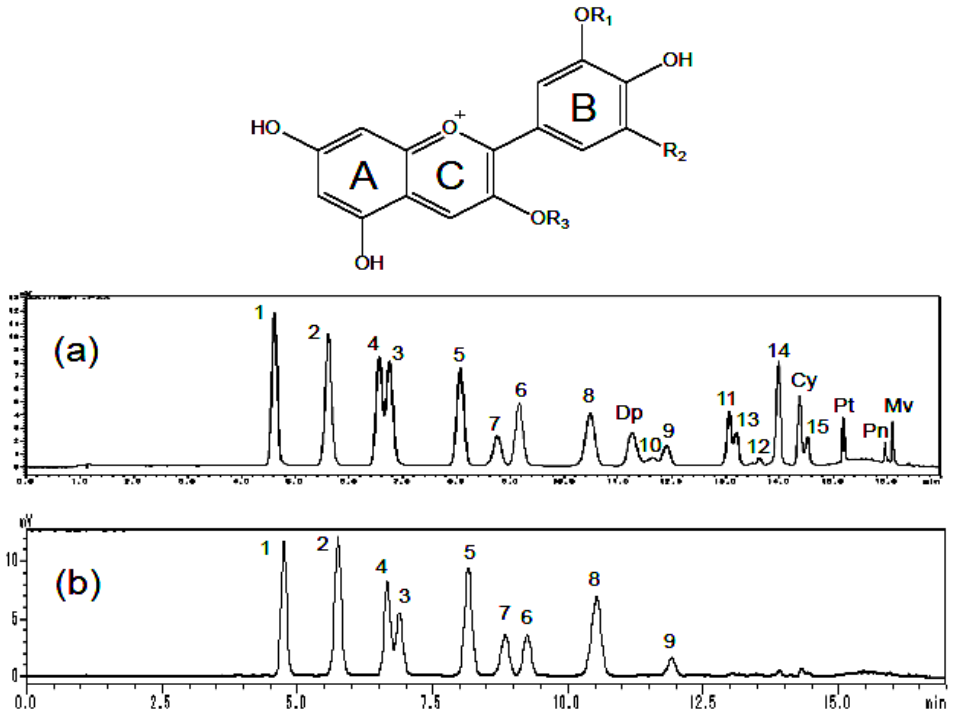


Figure 1. UFLC chromatograms of protocyanin and anthocyanins isolated from the hydolysates using a Discovery DPA-6S detected at 270 nm.

a: succinylcyanin, b: apigenin 7-glucuronide-4'-glucoside, c: malohylflavone,
d: cyanidin 3,5-diglucoside (Cy3,5diG)



Peak	Anthocyanin	R_1	R_2	R_3
1	Delphinidin 3-galactoside	H	OH	Galactose
2	Delphinidin 3-glucoside	H	OH	Glucose
3	Delphinidin 3-arabinoside	H	OH	Arabinose
4	Cyanidin 3-galactoside	H	H	Galactose
5	Cyanidin 3-glucoside	H	H	Glucose
6	Cyanidin 3-arabinoside	H	H	Arabinose
7	Petunidin 3-galactoside	H	OCH_3	Galactose
8	Petunidin 3-glucoside	H	OCH_3	Glucose
9	Petunidin 3-arabinoside	H	OCH_3	Arabinose
10	Peonidin 3-galactoside	CH_3	H	Galactose
11	Peonidin 3-glucoside	CH_3	H	Glucose
12	Peonidin 3-arabinoside	CH_3	H	Arabinose
13	Malvidin 3-galactoside	CH_3	OCH_3	Galactose
14	Malvidin 3-glucoside	CH_3	OCH_3	Glucose
15	Malvidin 3-arabinoside	CH_3	OCH_3	Arabinose

Figure 2. Structure and chromatogram of bilberry anthocyanins (a) and CP (b).

Dp: Delphinidin, Cy: Cyanidin, Pt: Petunidin, Pn: Peonidin, Mv: Malvidin (Aglycones).

Analytical condition of UFLC is described in the materials and methods with Shimadzu LC-20AV equipped with a Shim-pack XR-ODS column (100 × 3.0mm i.d.; 2.2 μm) at 520 nm.

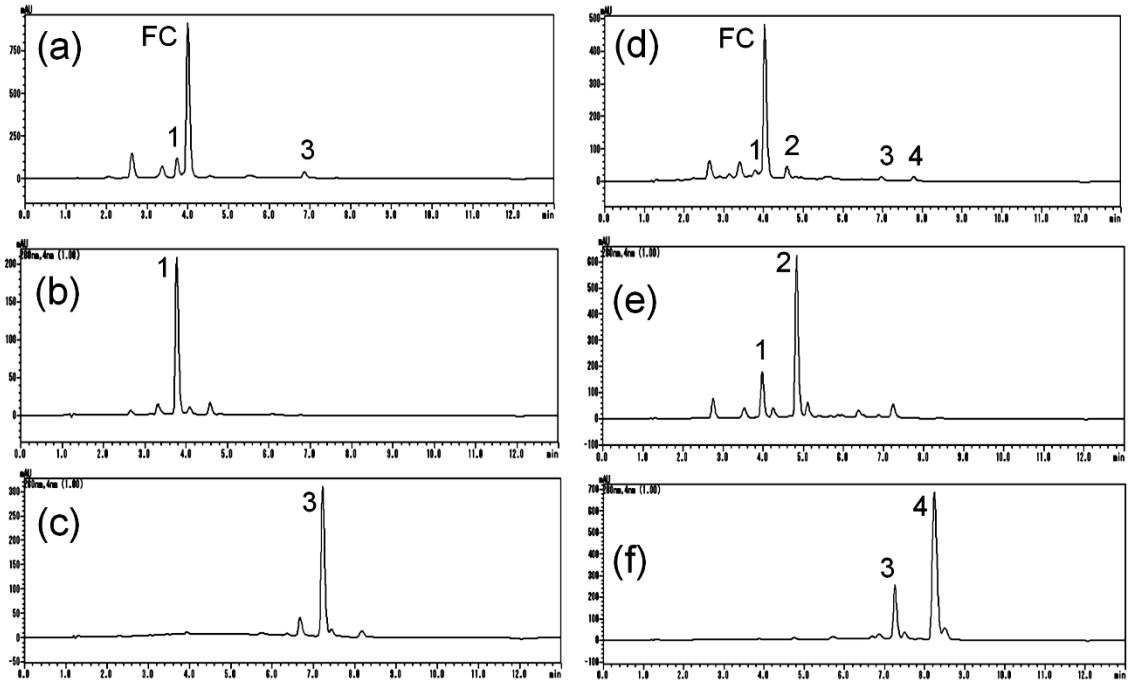


Figure 3. UFLC chromatograms of CP, Sn and isolated anthocyanins using a Discovery DPA-6S.

Analytical condition of UFLC is described in Materials and methods using a SHIMADZU LC-20AD pump equipped with a Shim-pack XR-ODS column (100 × 3.0 mm i.d.; 2.2 μm) at 280 nm.

(a): precipitate, (b): Fr.3, (c): Fr.5, (d): supernatant, (e): Fr.3', (f): Fr.5'.FC: flavocommelin

1: cyanidin 3-glucoside, 2: peonidin 3-glucoside, 3: cyanidin 3-(6-*O*-*p*-coumaroylglucoside),

4: peonidin 3-(6-*O*-*p*-coumaroylglucoside).

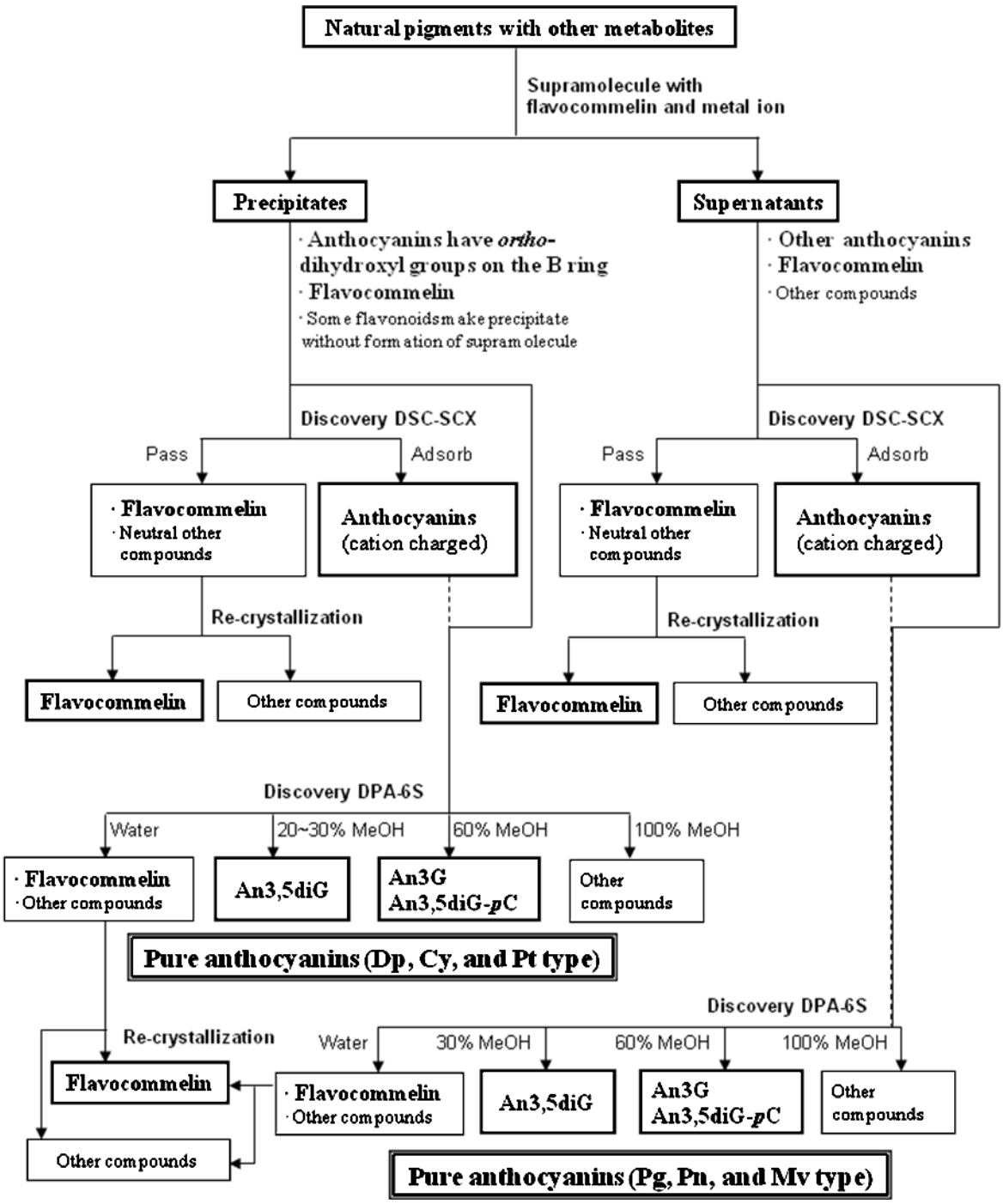


Figure 4. Flowchart of anthocyanins isolation.

An3,5diG: anthocyanindin 3,5-diglucoside, An3G: anthocyanidin 3-glucoside, An3,5diG-*p*C: *p*-coumaloyl anthocyanindin 3,5-diglucoside, Dp: delphinidin, Cy: cyanidin, Pt: petunidin, Pg: pelargonidin, Pn: peonidin, Mv: malvidin