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Isotachophoresis of Anthocyanins

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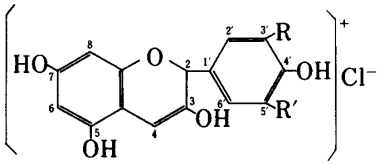
A new system for the analysis of anthocyanins, red or purple plant pigments, has been established using capillary isotachophoresis. Under the conditions employed, authentic anthocyanins were successfully separated into individual zones, and the compounds in plant extracts were also identified on the basis of enhancement of the appropriate zones in runs involving mixed charging of extracts with authentic samples. It was considered that, in comparison with the conventional chromatographic techniques commonly used in the analysis of anthocyanins, this new method has the advantages of simplicity and good resolution with adequate reproducibility.

Keywords——anthocyanin; plant pigment; capillary isotachophoresis; potential unit value; quinoid-pseudobase form; phenopyrillium form

Introduction

Isotachophoresis (ITP) has become a powerful tool for rapid and reproducible microanalysis of electrically charged substances such as metal ions,[1] organic[2] and inorganic[3] acids, amino acids,[4] nucleotides,[5] oligopeptides[6] and other low molecular weight compounds in addition to the proteins in human serum[7] and cerebrospinal fluid,[8] *etc.* However, this new technique has not so far been exploited widely in the field of plant biochemistry.

Anthocyanins are plant pigments which occur widely in fruits, petals, stems, *etc.* and have a red or purple color. They are easily extracted as the stable chlorides (Chart 1) by treating the plant materials with methanolic HCl. Concerning separation and identification of these pigments, earlier workers[9,10] employed paper electrophoresis along with paper chromatography, although the methods commonly used at present are chromatographic ones, including thin layer chromatography (TLC) and high-performance liquid chromatography (HPLC), because the old electrophoresis technique required too long a time to obtain good resolution. The authors therefore tried to apply ITP to the analysis of anthocyanins.



pelargonidin: R=R′=H
cyanidin: R=OH, R′=H
delphinidin: R=R′=OH
malvidin: R=R′=OCH_3

Chart 1. Structures of Anthocyanins

These compounds are present in the plant tissues as various glycosides, and are extracted as stable chlorides with 0.1% (v/v) CH_3OH–HCl.

Experimental

Reagents and Samples——All the reagents were purchased from Wako Pure Chemicals. The authentic samples of anthocyanins (chloride forms) were isolated from plant materials; cyanidin-3-*O*-glucoside and cyanidin-3-*O*-

rhamnoglucoside from the pericarp of *Osmanthus fragnans*,[11] cyanidin-3,5-di-*O*-glucoside, pelargonidin-3,5-di-*O*-glucoside and malvidin-3,5-di-*O*-glucoside from the petals of dahlia, fuchsia and salvia, respectively; delphinidin-3-*O*-(*p*-coumaroyl)glucosylglucoside-5-*O*-glucoside from the petals of *Epimedium grandiflorum*.[12] The plant extracts containing anthocyanins as the chlorides were prepared by the standard method from 1—2 g (fresh weight) of *Rosa* sp. petals (containing cyanidin-3,5-di-*O*-glucoside and pelargonidin-3,5-di-*O*-glucoside) and *Polygonum cuspidatum* SIEB. *et* ZUCC. stems (containing cyanidin-3-*O*-glucoside and cyanidin-3-*O*-rhamnoglucoside). The authentic specimens (about 0.1—0.5 mg each) and the plant extracts were dissolved in 1 ml of the leading electrolytes described later, and 5—25 μl aliquot of the solutions thus obtained were injected into the ITP analysis system.

ITP of Anthocyanins——ITP was carried out with Shimadzu IP-1B and IP-2A isotachophoretic analyzers equipped with a PDG-1 potential detector. The separation was run in a Teflon capillary tube (1 mm × 4 cm + 0.5 mm × 15 cm) maintained at 20 °C. The driving current was stabilized at 200 μA for 10 min, and 75 μA thereafter. Chart speed was 20 or 40 mm/min. The combinations of leading (L) and terminating (T) electrolytes were 0.01 M HCl adjusted to pH 8.3 with trishydroxymethylaminomethane (Tris) (L) and 0.005 M phenol adjusted to pH 10.0 with $Ba(OH)_2$ (T) (alkaline condition) and 0.01 M HCl adjusted to pH 5.8 with L-histidine (L) and 0.01 M 2-(*N*-morpholino)ethanesulfonic acid (MES) adjusted to pH 7.2 with Tris (T) (neutral condition).

Results and Discussion

The six authentic anthocyanins examined in this study (Chart 1) differed from each other in molecular weight, numbers of free phenolic hydroxyls, *etc.* It is known that anthocyanin chlorides maintain the phenopyrillium salt structure in solutions below pH 7, but are immediately transformed into quinoid (B-ring)—pseudobase (interconvertible) form at alkaline pH. The isotachopherograms of the anthocyanins obtained under the alkaline (Fig. 1) and the neutral (Fig. 2) conditions indicated that these compounds were clearly separated into individual zones. Under both conditions, anthocyanins with lower molecular weight and larger numbers of free phenolic hydroxyls generally had higher migration speeds (*e.g.* cyanidin-3-*O*-glucoside > cyanidin-3-*O*-rhamnoglucoside > cyanidin-3,5-di-*O*-glucoside > pelargonidin-3,5-di-*O*-glucoside). However, as summarized in Table I, the potential unit (PU) values of quinoid or pseudobase form compounds under the alkaline condition were larger than those of phenopyrillium compounds under the neutral condition in the cases of pelargonidin-3,5-di-*O*-glucoside and malvidin-3,5-di-*O*-glucoside, but not in the cases of cyanidin and delphinidin glycosides. Therefore, some discrepancies were observed between the orders of migration speed under the two conditions. For instance, the PU value of delphinidin-3-*O*-(*p*-coumaroyl)glucosylglucoside-5-*O*-glucoside was smaller than that of pelargonidin-3,5-di-*O*-glucoside (but larger than that of malvidin-3,5-di-*O*-glucoside) only under the alkaline condition, while pelargonidin-3,5-di-*O*-glucoside migrated faster than malvidin-3,5-di-*O*-glucoside (but slower than cyanidin-3,5-di-*O*-glucoside) only under the neutral condition. It appears that much more work is needed to establish the relation between

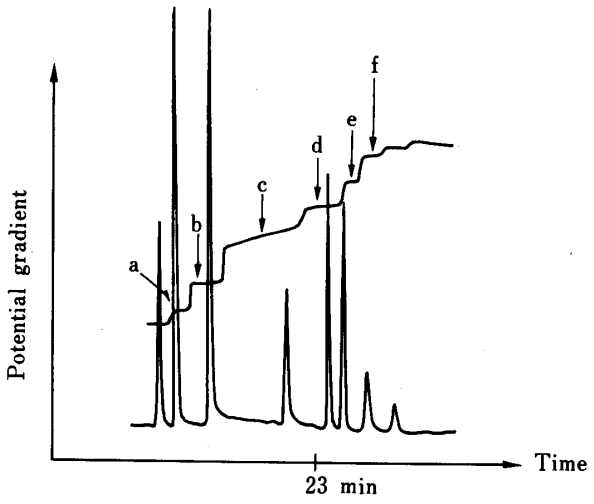


Fig. 1. Isotachophoretic Separation of Anthocyanins (Quinoid or Pseudobase Form) under the Alkaline Condition

Leading electrolyte, 0.01 M HCl–Tris (pH 8.3); terminating electrolyte, 0.005 M phenol–$Ba(OH)_2$ (pH 10.0). Sample: combined leading electrolyte solution (0.5 mg/ml each) of cyanidin-3-*O*-glucoside (5 μl) (a; PU value 0.08), cyanidin-3-*O*-rhamnoglucoside (5 μl) (b; 0.25), cyanidin-3,5-di-*O*-glucoside (25 μl) (c; 0.47), malvidin-3,5-di-*O*-glucoside (10 μl) (d; 0.64), delphinidin-3-*O*-(*p*-coumaroyl)glucosylglucoside-5-*O*-glucoside (5 μl) (e; 0.79) and pelargonidin-3,5-di-*O*-glucoside (5 μl) (f; 0.91). Migration current, 75 μA; chart speed, 40 mm/min.

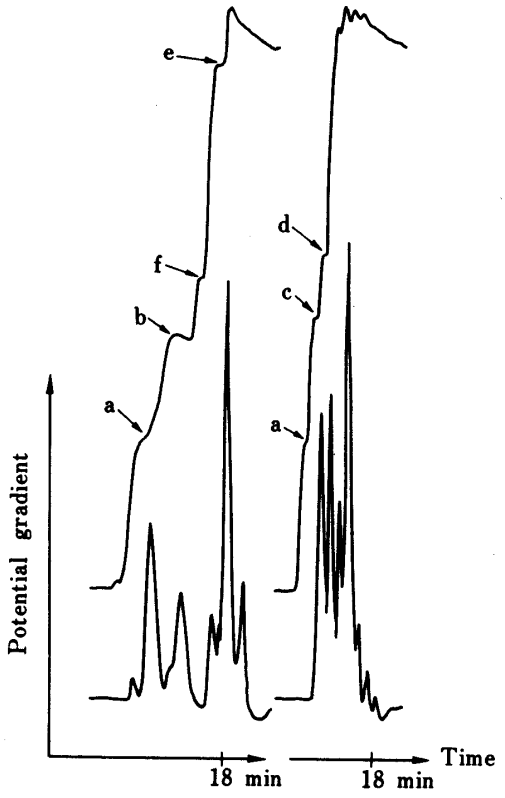


Fig. 2. Isotachophoretic Separation of Anthocyanins (Phenopyrillium Form) under the Neutral Condition

Leading electrolyte, 0.01 M HCl–L-histidine (pH 5.8); terminating electrolyte, 0.01 M MES–Tris (pH 7.2). Samples: combined leading electrolyte solution (0.1—0.2 mg/ml each) of cyanidin-3-*O*-glucoside (10 μl) (a; PU value 0.25), cyanidin-3-*O*-rhamnoglucoside (20 μl) (b; 0.44), pelargonidin-3,5-di-*O*-glucoside (5 μl) (f; 0.54) and delphinidin-3-*O*-(*p*-coumaroyl)glucosylglucoside-5-*O*-glucoside (5 μl) (e; 0.90) (left) and cyanidin-3-*O*-glucoside (5 μl) (a; 0.25), cyanidin-3,5-di-*O*-glucoside (5 μl) (c; 0.50) and malvidin-3,5-di-*O*-glucoside (5 μl) (d; 0.58) (right). Migration current, 75 μA; chart speed, 20 mm/min.

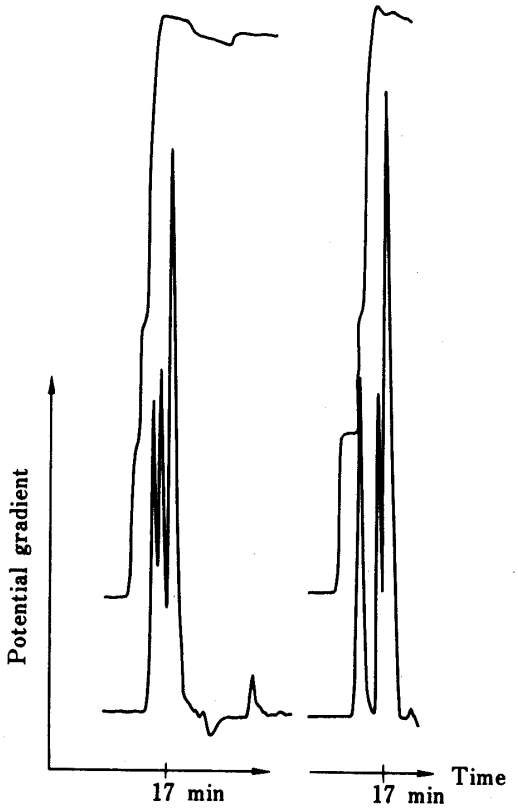


Fig. 3. Isotachopherograms of *Polygonum cuspidatum* Stem Extract (Left) and a Mixture of It and Authentic Cyanidin-3-*O*-glucoside under the Neutral Condition

Leading electrolyte, 0.01 M HCl–L-histidine (pH 5.8); terminating electrolyte, 0.01 M MES–Tris (pH 7.2); migration current, 75 μA; chart speed, 20 mm/min. Samples: 5 μl each of leading electrolyte solution of *P. cuspidatum* stem extract (1 ml of solution of the extract from 1—2 g fresh weight) (A) and authentic cyanidin-3-*O*-glucoside (0.2 mg/ml) (B). The left and right isotachopherograms were obtained by the ITP analysis of A alone (left) and A+B (right), respectively. Zones 1 and 2 correspond to cyanidin-3-*O*-glucoside (PU value 0.25) and cyanidin-3-*O*-rhamnoglucoside (0.44), respectively.

structure and isotachophoretic behavior among anthocyanins.

These ITP analysis systems were then applied to the identification of anthocyanins in plant extracts whose pigment constituents are known. Thus, cyanidin-3-*O*-glucoside (Fig. 3) and cyanidin-3-*O*-rhamnoglucoside in the extract of *Polygonum cuspidatum* stems, as well as cyanidin-3,5-di-*O*-glucoside and pelargonidin-3,5-di-*O*-glucoside in *Rosa* sp. petals, were identified on the basis of the enhancement of the appropriate zones after mixed charging of samples with the authentic specimens under the neutral condition. (The isotachopherograms of crude plant extracts under the alkaline condition exhibited rather linear elevation of the potential gradient, indicating failure to achieve isotachophoretic separation; the reason for this is not yet known).

The separation and identification of anthocyanins have been conventionally carried out by means of TLC and HPLC. However, the former simple technique has the disadvantage that the analysis of crude plant extracts often results in tailing of the pigment spots. On the other hand, the latter offers good resolution of the peaks with adequate reproducibility,

TABLE I. PU Values (× 100) of Anthocyanins under the Alkaline and Neutral Conditions

Compound	Alkaline[a] condition	Neutral[b] condition
Cyanidin-3-*O*-glucoside	8	25
Cyanidin-3-*O*-rhamnoglucoside	25	44
Cyanidin-3,5-di-*O*-glucoside	47	50
Delphinidin-3-*O*-(*p*-coumaroyl)glucosylglucoside-5-glucoside	79	90
Malvidin-3,5-di-*O*-glucoside	64	58
Pelargonidin-3,5-di-*O*-glucoside	91	54

a) L: 0.01 M HCl–Tris (pH 8.3). T: 0.005 M Phenol–$Ba(OH)_2$ (pH 10.0). *b*) L: 0.01 M HCl–L-histidine (pH 5.8). T: 0.01 M MES–Tris (pH 7.2).

although the procedure for accomplishing linear gradient elution is complex.[13,14] The ITP method presented here, especially that employing the neutral condition, allows us to separate and identify anthocyanins in plant extracts with simplicity, good resolution and reproducibility on a microscale. (The time and sample quantity requirements are similar to those of conventional techniques). The zones of anthocyanins appeared clearly even after the injection of 5 μl of the leading buffer solution (0.1 mg/ml), and one analysis required less than 25 min. Attempts to improve this new technique and apply it to surveys on the distribution patterns of anthocyanins and other flavonoids in large numbers of plant specimens are in progress.

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