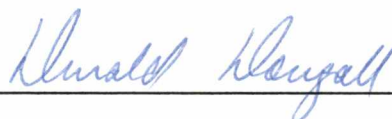


To the Graduate Council:

I am submitting herewith a thesis written by Marc A. Redus entitled "Biosynthesis and Chemical Constants of Mono-Acylated Anthocyanins." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Life Sciences.



Donald K. Dougall, Major Professor

We have read this thesis
and recommend its acceptance.



Accepted for the Council:



Associate Vice Chancellor and
Dean of the Graduate School

**Biosynthesis and Chemical Constants of
Mono-Acylated Anthocyanins**

A Thesis
Presented for the
Master of Science Degree
The University of Tennessee, Knoxville

Marc Redus
December, 1996

Dedication

This thesis is dedicated to my wife, Naomi, who nagged me unmercifully until I got all the necessary work done; and to my son, Aaron, who gave me a reason to graduate so I could get a job; and to my Grandmother, "Mamaw," who always morally supported me throughout school.

Acknowledgments

I would like to thank several people who helped me during my thesis research. First, I am deeply indebted to Dr. Dougall, who helped the most of all, and from whom I learned a great deal during the course of my Master's degree. Next, I would like to give special thanks to the other members of my thesis committee, Dr. Howell, Dr. Baker, and Dr. Mullin, who all offered some excellent help and guidance. Also, I would like to extend my sincere appreciation to Dr. G. Frazier and Dr. T. Bradrick, who also helped in various ways.

Abstract

In this study, a number of non-natural anthocyanins were biosynthesized by the addition of various organic acids to suspension cultures of wild carrot. This resulted in the incorporation of the organic acid residue in a specific place on the anthocyanin molecule, and the resulting novel anthocyanin was purified and characterized. The color of anthocyanins generally decreases with increasing pH due to the conversion of the flavylum ion into a hemiacetal. In addition, the flavylum ion can be converted into blue quinonoid forms at increased pH. Because these reactions result in loss of or changed color of the anthocyanins, their rate constants and equilibrium constants are a measure of color retention of the anthocyanin; if the factors which determine this were understood, this could result in the eventual marketing of anthocyanin preparations with increased color retention. For each anthocyanin, K_h , the equilibrium constant for the (colorless) hemiacetal formation, k_1 and k_2 , the forward and reverse rate constants for this reaction respectively, and K_a , the equilibrium constant for formation of the quinonoid forms, were measured.

Anthocyanins with no acyl group had the largest K_h , smallest k_2 , and a k_1 which was among the largest in the series of 21 compounds measured, and thus retained the least amount of color.

For anthocyanins acylated with benzoic acids, the $\log K_h$, $\log k_1$, and $\log k_2$ were linearly related to the electronegativity of the substituents showing that the effects on these constants were linearly related to the electron density of the aromatic system. $\log K_a$ was not linearly

related to the electron donating capacity of the substituents, so another factor was affecting formation of quinonoid forms.

The K_h and k_1 was larger in the benzoyl compounds than in the cinnamoyl series, and k_2 was smaller, indicating that benzoylated anthocyanins retained less color than did the cinnamoylated ones.

For the anthocyanins acylated with cinnamic acids, a plot of $\log K_h$ and $\log k_1$ vs. electronegativity of the substituents showed that these compounds were scattered about a straight line. This suggests that both the electron density in the acyl aromatic ring and another factor were both important in determining color retention by a cinnamoylated anthocyanin.

The $\log K_a$ and $\log k_2$ in the 4-substituted cinnamoyl series were linearly related to the electronegativity of the substituents on the acyl group, indicating that electron density in the acyl aromatic ring was the primary factor controlling formation of the quinonoid form as well as the reverse reaction from hemiacetal to flavylum ion in these compounds.

A comparison of the compounds acylated with 4-hydroxycinnamic acid, 4-hydroxybenzoic acid, and 3-(4-hydroxyphenyl)propionic acid shows that the 2-carbon chain in acyl groups, as well as the presence of the double bond in cinnamic acids, affected the K_h 's and k_2 's significantly. Removal of the carbon chain also increased k_1 , but removal of the double bond did not have as much affect.

The k_2 's appear to be a major factor that influences stability. Rather than showing an increase in k_1 as stability decreases, many of the least stable compounds instead showed a decrease in k_2 .

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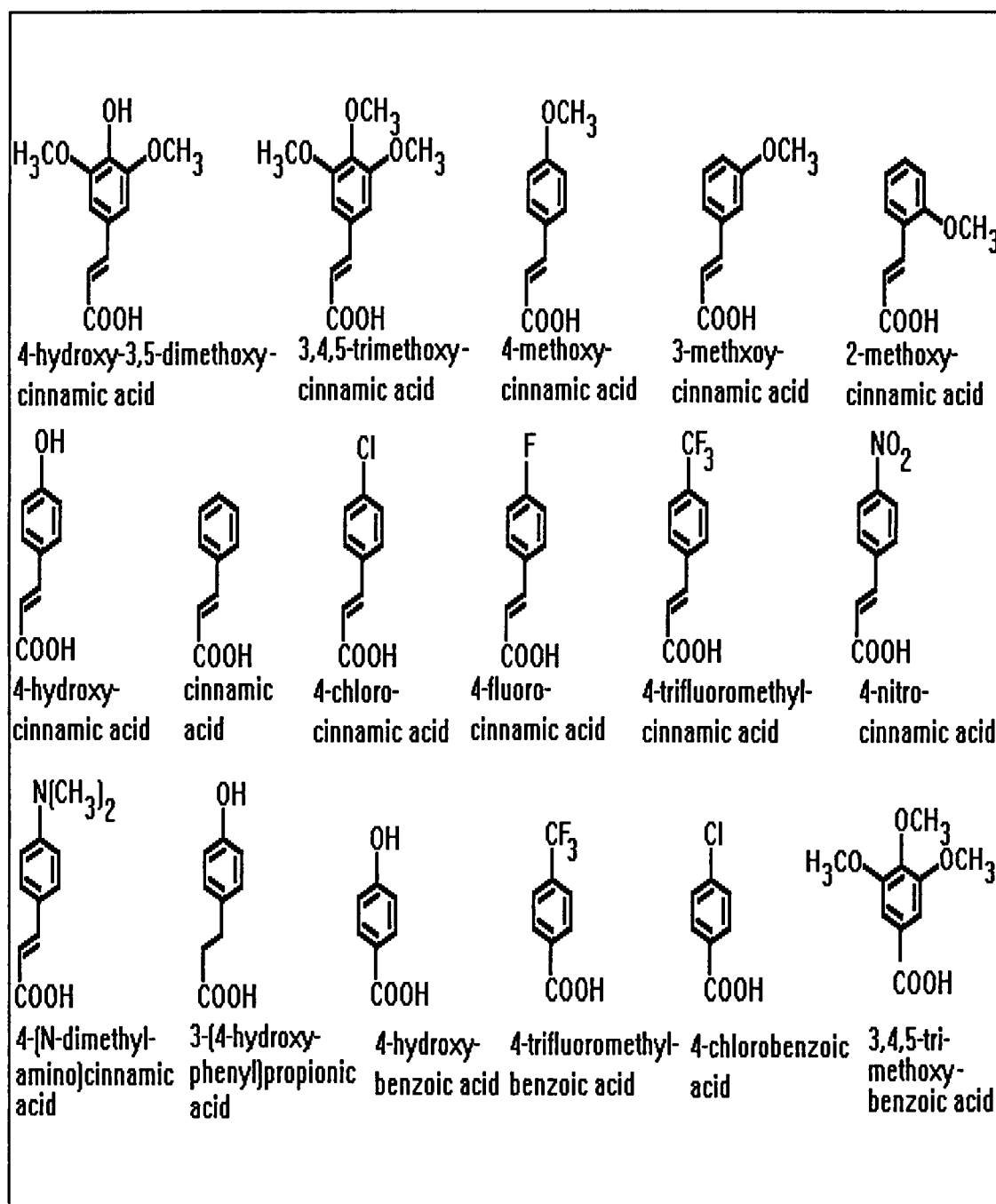
List of Abbreviations

The following abbreviations are for the anthocyanins used in this study. The acyl group of each anthocyanin is listed below, and is attached to the glucose residue of the anthocyanin in the position shown in Fig. 5.

Abbreviation	Acyl Group
DC	4-hydroxy-3,5-dimethoxycinnamic acid
DCX . . .	4-hydroxy-3,5-dimethoxycinnamic acid, minus xylose
TC	3,4,5-trimethoxycinnamic acid
TCX	3,4,5-trimethoxycinnamic acid, minus xylose
2MC	2-methoxycinnamic acid
3MC	3-methoxycinnamic acid
4MC	4-methoxycinnamic acid
4HC	4-hydroxycinnamic acid
C	cinnamic acid
CC	4-chlorocinnamic acid
FC	4-fluorocinnamic acid
TFMC	4-trifluoromethylcinnamic acid
NC	4-nitrocinnamic acid
DMA	4-(N-dimethylamino)cinnamic acid
4HP	3-(4-hydroxyphenyl)propionic acid
4HB	4-hydroxybenzoic acid
TB	3,4,5-trimethoxybenzoic acid
CB	4-chlorobenzoic acid
TFMB	4-trifluoromethylbenzoic acid
CYANGXG	none
CYANGX	none, minus glucose

Structures of Organic Acids

The following are structures for the organic acids which serve as acyl groups for the anthocyanins given in the List of Abbreviations. The acyl groups are incorporated into the anthocyanins in the position shown in fig. 5



I. Introduction

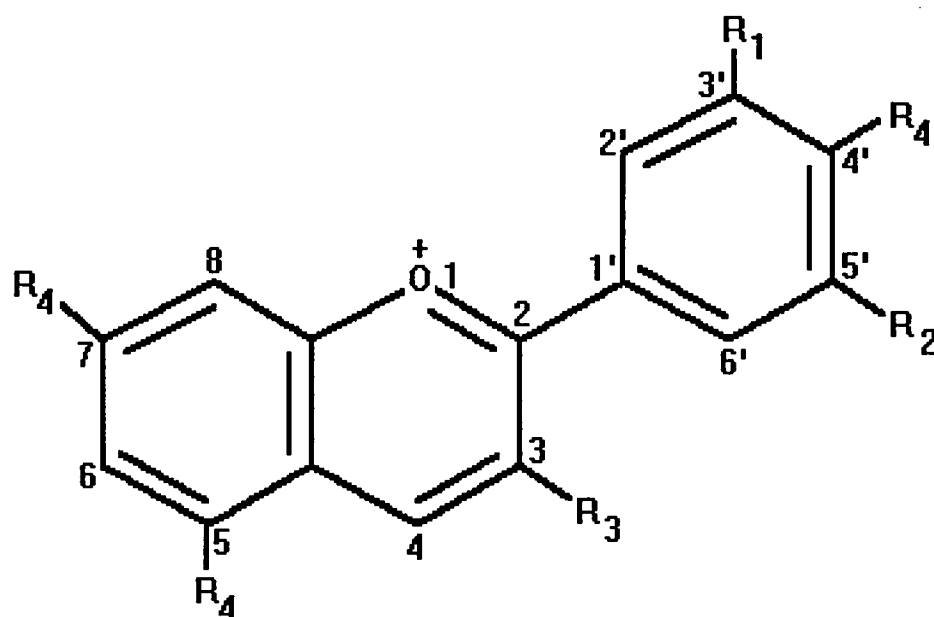
Introduction to Anthocyanins

Anthocyanins are pigments found in plants. They account for most of the color in flowers, fruits, and berries, and are typically red, purple, orange, or blue. The anthocyanin molecule itself consists of a flavylum nucleus with one or more sugar residues attached, and which may be acylated with one or several R groups; see Fig. 1 (Mazza and Miniati, 1993).

There are several beneficial uses for anthocyanins. One is medicinal: anthocyanins are good antioxidants, as well as possessing other medicinal properties (Harborne, 1988). Anthocyanins are also present in natural foods; thus, they could be used as food and drug colorings. The major problem with this, unfortunately, is that most of them are unstable. Some anthocyanins are used as food colorings now--in the US, three anthocyanin preparations are currently commercially available as food colorants, two of which are derived from grapes and one from red cabbage (Hari et al., 1994). These, however, are at the upper end of the stability scale. Some anthocyanins, in fact, are much more stable than others.

Stability of Anthocyanins

Before going any further, it is necessary to define "stability." In anthocyanins, stability is related to color retention; any breakdown of the aromatic structure of an



Flavylium cation.

R_1 and R_2 = H, OH, or OCH_3

R_3 = glycosyl or H

R_4 = glycosyl or OH

Fig. 1. Flavylium nucleus, showing positions for R groups. In cyanidin (the nucleus of the anthocyanins on which this study is based) R_2 is -H, and R_1 , R_3 , and R_4 are -OH (adapted from Mazza and Miniati, 1993).

anthocyanin molecule results in immediate color loss. Moreover, stability can be defined in several different ways; i.e., having to do with retention of color over time at different temperatures, different light intensities, different pH's, or other factors (Markakis, 1992; Elhabiri, 1996). The specific definition used here is stability, i.e., retention of color, as it is influenced by pH. As pH increases, the color of most anthocyanins decreases. The reactions of anthocyanins to increased pH are well-documented from a chemical standpoint; a general summary of these reactions is shown in Fig. 2 (adapted from Dangles et al., 1993b). At low pH, anthocyanins maintain their color relatively well, existing primarily or exclusively in the red flavylium ion form. At higher pH's, they can become deprotonated and hydrated to form the colorless hemiacetal, or alternatively, simply deprotonated to shift to the blue quinonoid form. For any anthocyanin, one or both of these two reactions can occur, and both are reversible. However, further reactions can take place beyond the hemiacetal, and can result in irreversible color loss. Thus the reaction to hemiacetal is the key reaction in anthocyanin color stability, and pH is the primary factor which influences this.

The stability of an anthocyanin (as measured by retention of color at a fixed pH of about 6.0 over time) has been previously shown to be connected to the presence of several cinnamic acid residues in the molecule. In general, the more cinnamic acid residues, the more stable the

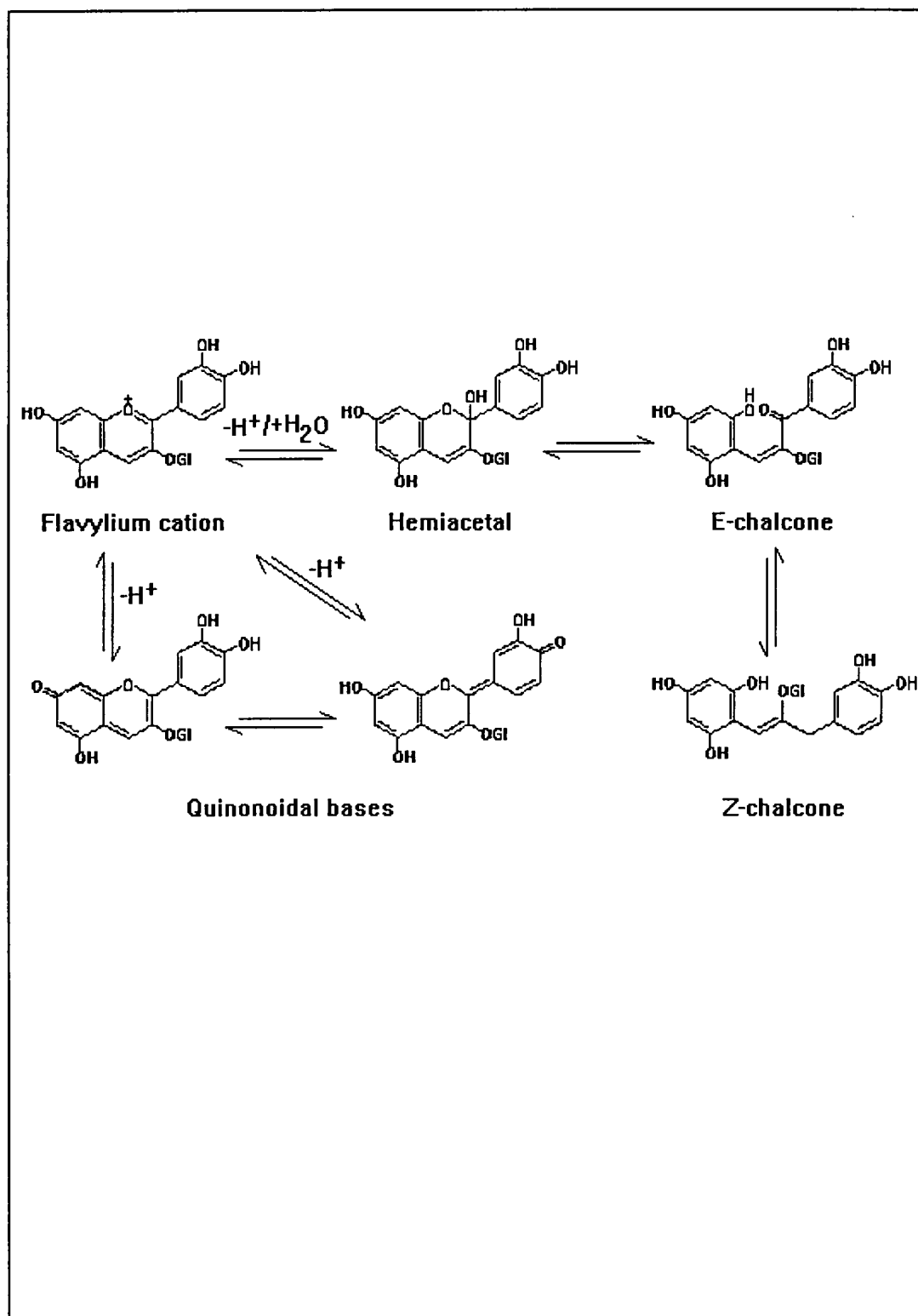


Fig. 2. Flavylium ion, hemiacetal, quinonoid, and chalcone forms of carrot anthocyanins (adapted from Dangles et al., 1993b).

anthocyanin. The ability of cinnamic acids to stabilize the anthocyanins is believed to be the result of a sandwich-type stacking that occurs between these residues and the central ring of the flavylium nucleus (Mazza and Miniati, 1993; see Fig. 3), which results in protection of the central ring from deprotonation. The discovery that altanin-C, a mono-cinnamoylated anthocyanin, had a comparable stability to a di-cinnamoylated one extended the generalization concerning stability of these molecules (Yoshida et al., 1991a and b). If the stability of a mono-acylated anthocyanin is similar to that of a multi-acylated one, then this would greatly simplify a study on the effects of the acyl group on anthocyanin stability. By comparing the effects of different acyl groups on stability of a monoacylated anthocyanin, the mechanism by which this group stabilizes the anthocyanin could be determined.

Biosynthesis of Mono-acylated Anthocyanins

The previous studies of anthocyanin stability have used different anthocyanins from various natural sources; Dangles et al. (1993b) used four different natural anthocyanins for stability testing, each one of which had a different complement of acyl groups (see Fig. 4, from Dangles et al. 1993b). The problem with this is that naturally-produced anthocyanins are limited in the types of acyl groups they contain, and vary widely in the location of these groups on the anthocyanin molecule (refer again to Fig. 1). Other studies on anthocyanin stability were also done using anthocyanins from natural sources or chemically modified

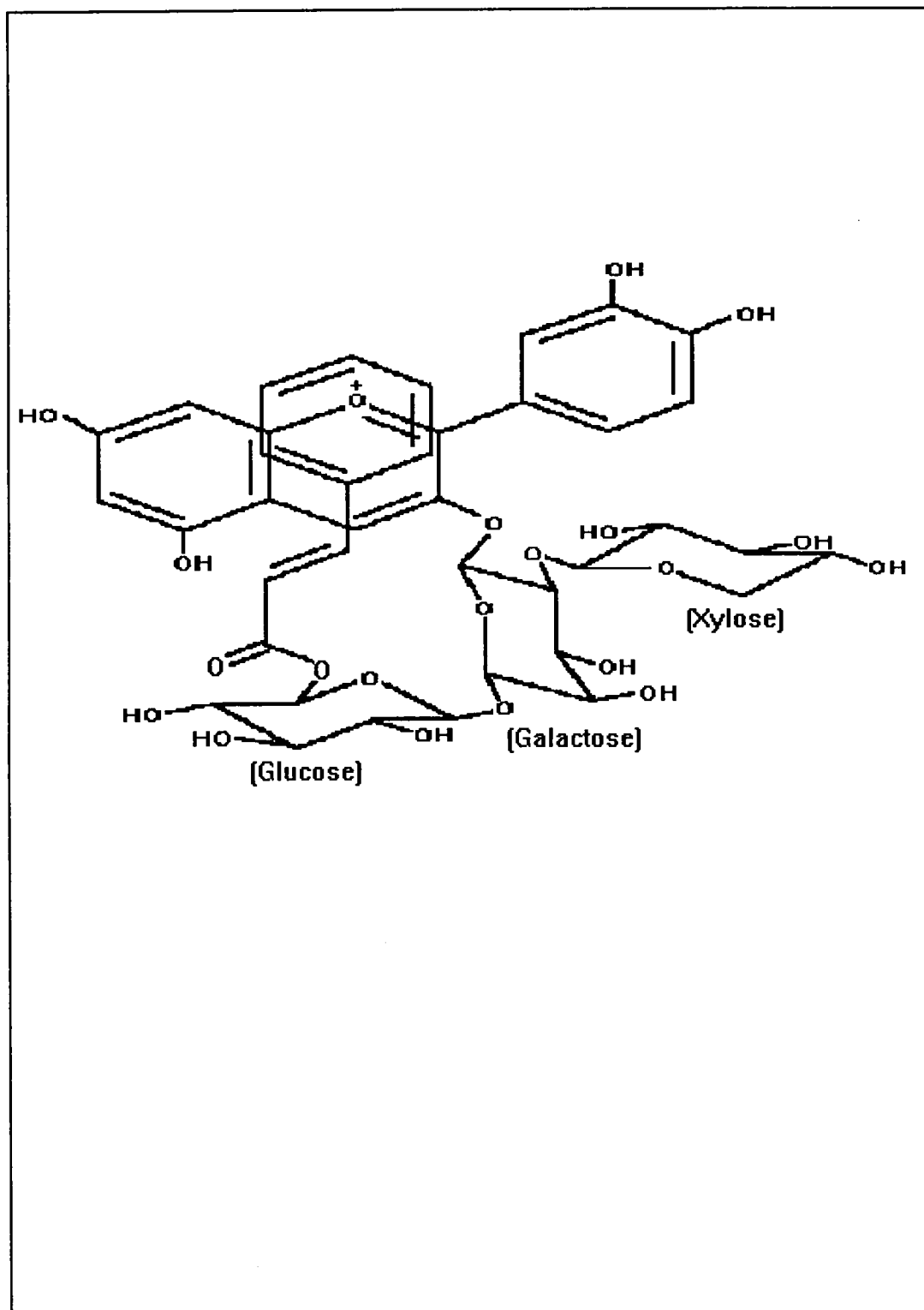


Fig. 3. "Stacking" in a mono-acylated anthocyanin. This drawing represents wild carrot anthocyanin acylated with cinnamic acid. Two or more molecules could associate to protect the central ring from both sides.

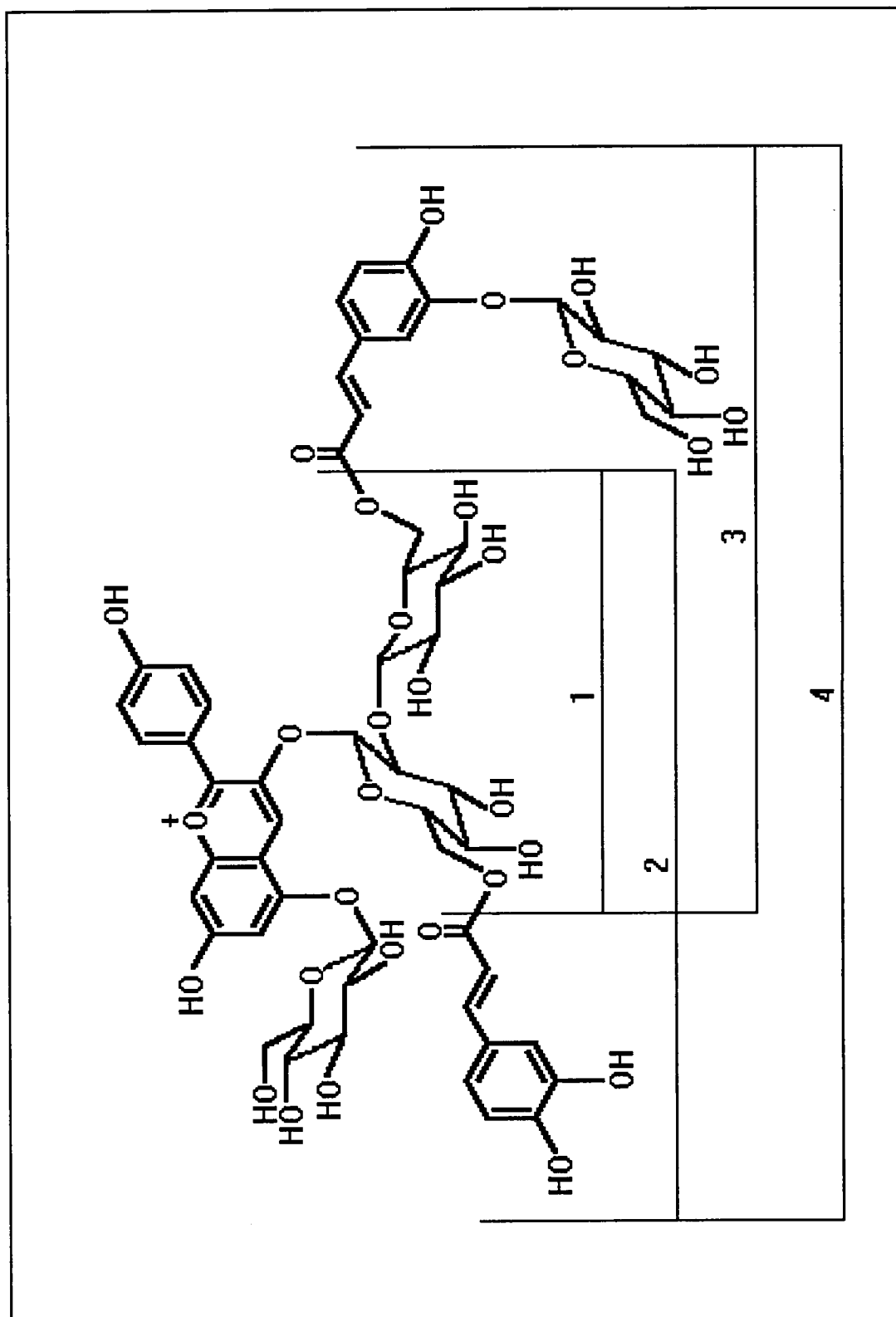


Fig. 4. The anthocyanins used in Dangles et al. (1993b) study: An example of the limitations imposed by nature.

anthocyanins, but there are not a wide variety of modifications possible using present chemical methods (Dangles et al., 1993a, Elhabiri et al., 1996, Figueiredo et al., 1996). Information from these is useful for making general comparisons, but because of the inherent limitations imposed by nature, making more specific comparisons within the acyl group itself is not possible. In order to make specific comparisons of this type, it would be necessary to use mono-acylated anthocyanins that contained a range of acyl groups, but which were identical in all other respects. But because natural anthocyanins contain only a very limited number of different acyl groups, any such comparison would require the use of non-natural acyl groups.

Cultures of wild carrot, *Daucus carota*, produce natural anthocyanins as shown in Fig. 5, which are known to be acylated with ferulic, sinapic, 4-coumaric, and 4-hydroxybenzoic acids (see Figs. 6 and 7). The cultures also produce the non-acylated anthocyanin, as well as one without the glucose residue shown in Fig. 5 (Gläßgen et al., 1992a, 1992b, 1992c; Baker et al., 1994). Baker et al. (1994) have shown that some cinnamic acids, when fed to anthocyanin-producing carrot cell cultures, were incorporated into the specific location on the anthocyanin molecule shown in Fig. 5. The proportion of anthocyanins acylated with these acids was also greatly increased. Two non-natural cinnamic acids--those not naturally biosynthesized in the carrot system--were also incorporated, which resulted in the synthesis of two anthocyanins not normally found in the culture. These

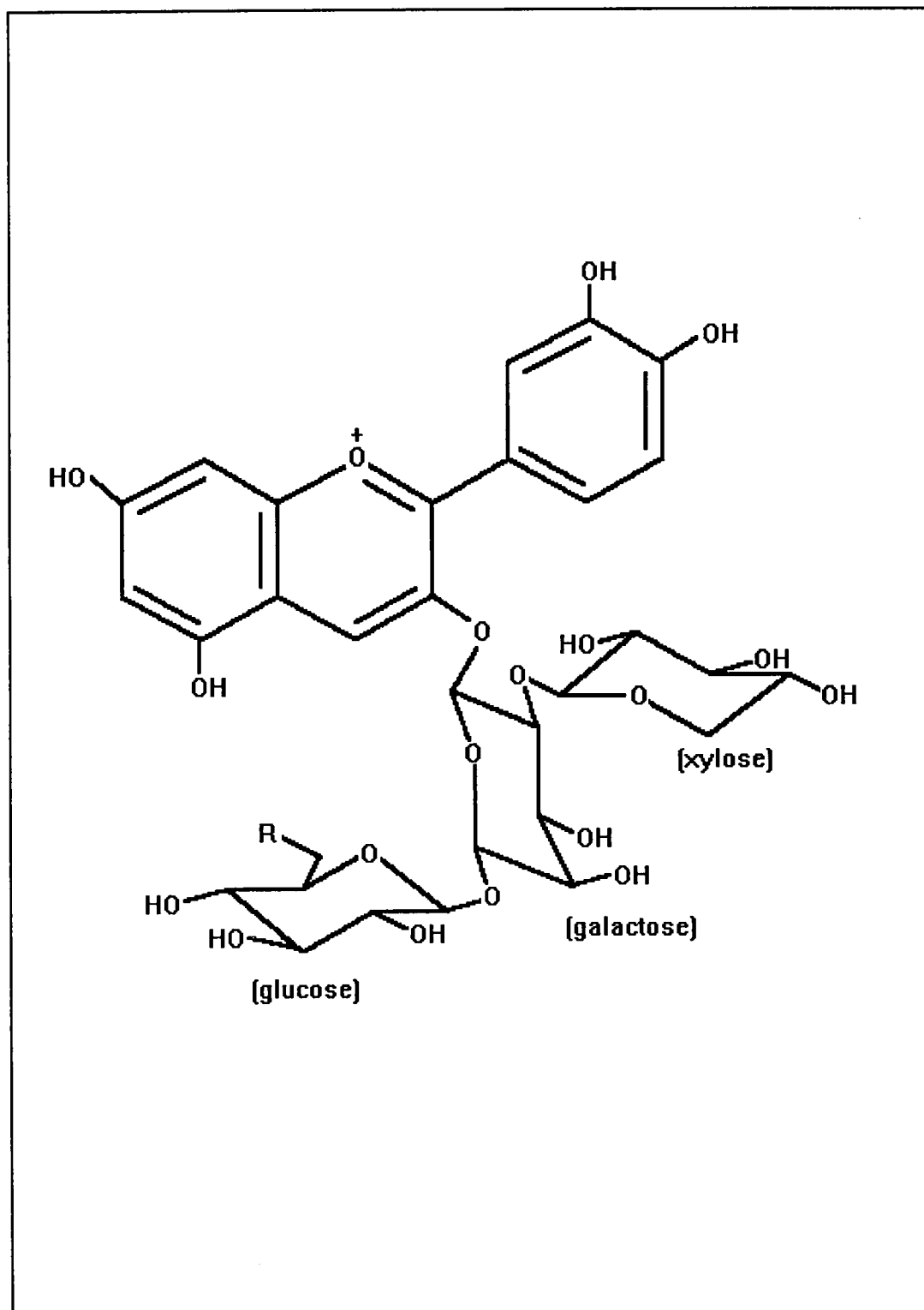


Fig. 5. Anthocyanin produced by carrot cultures . See fig. 6 for R groups; the cultures also produce the deacylated anthocyanin (R = H), as well as the one without the glucose residue (from Gläßgen et al., 1992b).

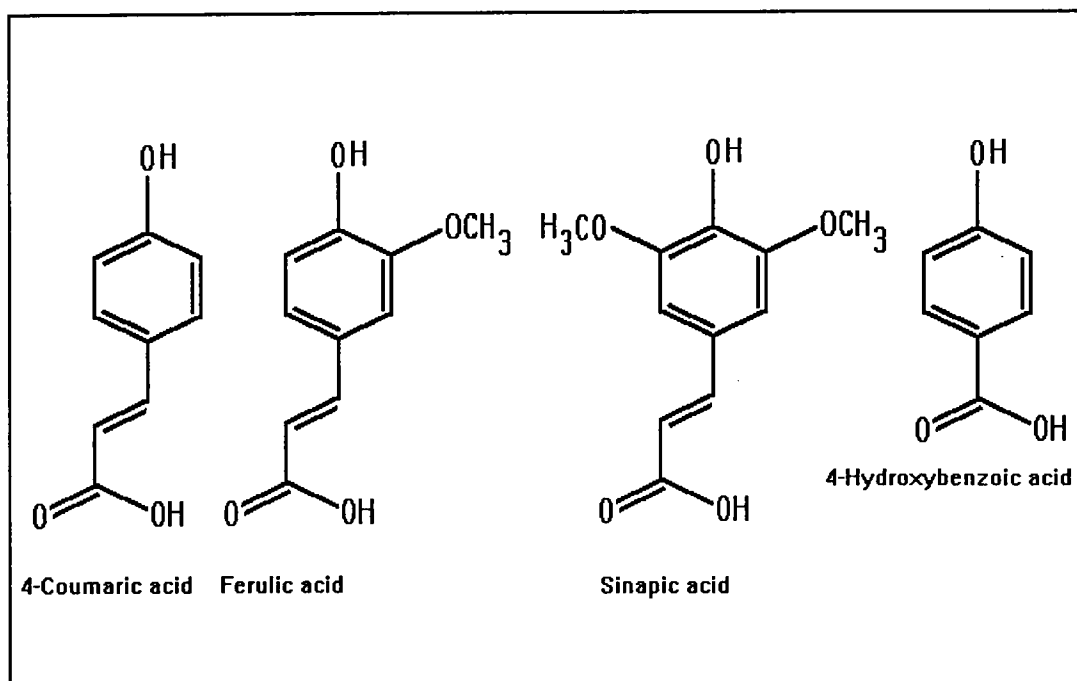


Fig. 6. Known organic acids that are incorporated into carrot anthocyanins in nature.

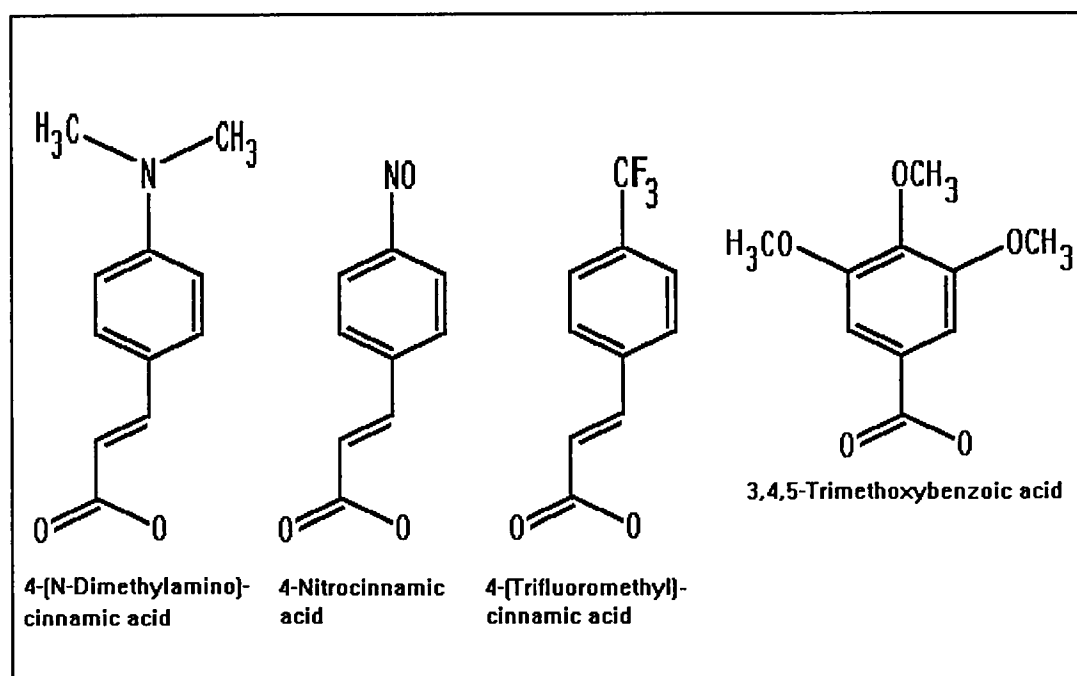


Fig. 7. Examples of non-natural organic acids that can be incorporated into carrot anthocyanins.

observations suggest that the carrot cell cultures are able to incorporate a range of organic acids into the anthocyanins they produce. Feeding a range of acids to carrot cell suspension cultures may, therefore, produce a range of anthocyanins which differ only in the acyl group. For example, an anthocyanin acylated with 4-methoxycinnamic acid could be biosynthesized by feeding this acid to the culture. Likewise, by feeding 4-hydroxycinnamic acid, an anthocyanin which had -OH instead of -OCH₃, but identical in all other respects could be made. By feeding cinnamic acid, a similar anthocyanin but with hydrogen in the 4-position could result. Anthocyanins acylated with the corresponding benzoic acids could also be made (see Fig. 7 for examples).

Measurement of Rate and Equilibrium Constants of the Interconversions of Anthocyanins

By feeding specific organic acids to carrot cell suspension cultures as described above, making a range of anthocyanins with the desired acyl groups should be possible. These could be purified and their stability measured, so that the effects on stability of each differing substituent of the group could be determined.

Using the methods of Dangles et al. (1993b), the chemical constants of each anthocyanin can be measured. These comprise the equilibrium constant between the flavylium ion and the hemiacetal on the one hand, and the equilibrium constant between the flavylium ion and the quinonoid form on the other, as well as the rate constants

of the forward and reverse reaction between flavylum ion and hemiacetal. [Referring to Fig. 2, the flavylum form exists at low pH and one or both of the other two forms predominate as pH increases.]

Because hemiacetal formation is increased at higher pH and the hemiacetal is colorless, this reaction is central to the pH effect on color retention, i.e., color stability. Therefore, quantitative measurements of this reaction for a number of anthocyanins, each with a different acyl group, would allow the effect of each acyl group on anthocyanin color stability to be evaluated and could lead to an understanding of the mechanism(s) involved in the acyl groups' effect on this reaction.

The objectives of this study, therefore, are to 1) biosynthesize several new anthocyanins by feeding non-natural acids to carrot culture; 2) isolate and purify quantities of these; 3) measure the equilibrium constants between the flavylum ion and the quinonoid and hemiacetal forms, as well as the forward and reverse hydration rate constants between the flavylum ion and the hemiacetal of these anthocyanins. Finally, the chemical constants of the modified anthocyanins should be correlated to the structure of the acyl groups they contain, in order to discern structural characteristics of these groups which influence the function--i.e. stability--of the anthocyanin molecule.

II. Materials and Methods

This section will be divided into the following general categories:

- A. Stock Cell Cultures and their Maintenance
- B. Determination of the Concentrations of Acids to be Supplied to the Cultures
- C. Large-Scale Preparations
- D. Purifications
- E. Equilibrium and Rate Constant Measurements
- F. Other Experiments

In the listing above, the first five items are a sequence leading directly from growing inoculum to the measurement of chemical constants of the final product. The last category contains several topics which are indirectly related to the main theme of this thesis.

A. Stock Cell Cultures and their Maintenance

The cell cultures used throughout these experiments were suspension cultures of wild carrot, *Daucus carota*, spp. *carota*. The subclones used were 13.1 SKZB (Dougall and Vogelien, 1990) and 13.1 SKZB M-1 (see "Selection of Cell Line with Increased Anthocyanin Accumulation," under "Other Experiments" here and in the Results section). These were routinely subcultured at a dilution of 1:20 every 14 days in WCM-1 medium (Wetherell, 1969), and were maintained in flasks with cotton plugs at 25° C with shaking at 115 rpm with

occasional light (light was used only for illumination when people were in the incubation room).

B. Determination of the Concentrations of Acids to be Supplied to the Cultures

Inoculum for experiments was obtained by making a 1:5 dilution of the stock culture and growing for 7 days in WCM-1 (pH 5.8), then repeating this for another 7 days. The first dilution was into 25 ml of medium in 125-ml Erlenmyer flasks with cotton plugs; the second was into 100 ml of medium in 500-ml Erlenmyer flasks with silicone closures. All flasks of medium were sterilized by autoclaving at 15 psi and 121° C for 20 min.

Inoculum for the feeding experiments was from the second 1:5 dilution in WCM-1. After 7 days growth, the inoculum was diluted 1:10 into WCM-4 (Dougall and Weyraugh, 1980), which was buffered at pH 4.5 and which stimulates anthocyanin production. Fifty ml of WCM-4 medium in DeLong flasks with silicone closures was used for these experiments.

Half a milliliter of a specific organic acid solution in DMSO (sterilized by autoclaving for 15 min. at 15 psi and 121° C, except for 4-(-dimethylaminocinnamic acid, which was sterilized for 10 minutes at 15 psi and 121° C, because it changed color if autoclaved for 15 minutes) was added to each flask at day 4, and repeated at day 8. For a preliminary experiment, the acid concentrations used were typically from 0 - 100 mM in 20 mM increments, with each concentration used

in duplicate. In some experiments, treatments with no addition were also included. In subsequent experiments, a narrower concentration range of the acid was used to find an optimum concentration for production of the novel anthocyanins.

On day 12 or 13, samples were taken for optical density and dry weight determination in triplicate from each flask. For optical density, a sample of one or two ml was centrifuged for 8 minutes at 1,500 rpm, the medium was removed, and 5 ml of 1% HCl/95% methanol was added. These were refrigerated overnight, vortexed, centrifuged for 8 minutes at 1,500 rpm, and the optical density of the supernatant was measured at 530 nm (around the peak absorbance for most anthocyanins). The mean and standard deviation of the OD (corrected for dilution) was then calculated.

Dry weight was determined by filtering 1 ml samples with S/P™ 291 grade glass fiber filters (Baxter International, Inc., Mc Graw Park, IL) that were previously dried at 60° C overnight and weighed to get tare weight. Then, the samples were washed, dried at 60° C overnight, and weighed. Blanks were included which were washed but contained no sample material to determine the effect of washing the filters (usually this removed about 0.08 mg of the filter material); 1 in 8 filters was a blank. Net weight was determined by subtracting the tare weight and adding the average difference in weight found from the blanks. Thus, the mg/ml dry weight of tissue that was present in the culture was determined. The

mean and standard deviation of this was then calculated.

The anthocyanins produced by the cultures were examined by HPLC analysis, using samples of washed tissue (500 mg. $\pm$ 10 mg.), obtained by filtering the contents of the duplicate flasks for each treatment. The tissue was extracted with a mixture of 5.0 ml of chloroform, 1.5 ml of water, and 2.5 ml of 5% formic acid in methanol ("Folch partition:" Folch et al., 1957), which was vortexed and refrigerated overnight. The next day these were re-vortexed, centrifuged, and a sample of the upper (aqueous) layer containing the anthocyanins was filtered through an ACROTM GLC3S 0.45 μ m filter (Gelman Sciences, Ann Arbor, MI). Injections into the HPLC port were 10 or 15 μ L. HPLC pumps were Waters (Milford, MA) model M-45, controlled by a Waters model 660 solvent programmer, and the spectrophotometer was Waters model 440 absorbance detector, measuring at 546 nm. Column was 0.5 cm X 20 cm MICROSORB-MV with 5 μ m C-18 beads (Rainin Instrumentation Co. Inc., Emeryville, CA). Peak heights were recorded with an OmniScribe chart recorder (Houston Instruments, Austin, TX), which was set to 5 min./inch [Peak heights were used instead of area, because the peaks were narrow enough that height was directly proportional to the quantity of compound present]. Flow rate was 1.0 ml/min and could be run isocratically or using a gradient (all HPLC gradients used in these experiments were linear). Solvents were 5% formic acid in water (solvent "A"), and 55% methanol/10% acetonitrile/5% formic acid (solvent "B").

C. Large-Scale Preparations

Once an optimal concentration of acid was determined, a large-scale preparation of tissue was then made using this concentration. About twenty 500-ml Erlenmyer flasks with silicone closures, each containing 100 ml WCM-4, were autoclaved 20 min. at 15 psi and 121° C. The acid additions were made on days 4 and 8 with 1 ml of the acid in DMSO added each time, and the tissue was harvested on day 12 or 13. The tissue was then combined, filtered on Miracloth™ (Calbiochem, San Diego, CA, with pore size of 22 - 25 μ m), washed with water, and either frozen in liquid nitrogen and stored at -84° C or immediately extracted for anthocyanins.

D. Purifications

The anthocyanins were extracted from the tissue and purified by the following series of steps:

1. Extractions
 - a. Acidic Methanol Extraction
 - b. Methylene Chloride Extraction
 - c. Ethyl Acetate Extraction
2. Chromatography
 - a. XAD-7 Chromatography
 - b. C-18 Chromatography
 - c. LH-20 Chromatography
3. Quantitation

1. Extractions

a. Acidic Methanol Extraction

The anthocyanins were extracted from tissue with 5% formic acid/methanol (6 ml per gram fresh weight), stored overnight at 4° C, and then filtered through Miracloth™. The remaining tissue was washed two or three times with 100 ml of 5% formic acid/85% MeOH to remove the remaining anthocyanin. The combined filtrates were then concentrated under vacuum using a rotary evaporater to remove the methanol.

b. Methylene Chloride Extraction

The evaporated sample was then extracted with methylene chloride. The sample was added to 100 ml of methylene chloride, then mixed and allowed to separate; sometimes water was also added. After separation, the methylene chloride was removed and the extraction was repeated. If necessary, a small quantity of water was added to the spent methylene chloride after extraction to remove any anthocyanin which did not separate into the aqueous phase (as evidenced by a red coloration in the methylene chloride); this was then separated and added back into the aqueous phase.

c. Ethyl Acetate Extraction

The aqueous layer was then extracted with ethyl acetate with the same procedure used in the methylene chloride extraction. The residual aqueous solution was next evaporated in vacuo until all the remaining methylene chloride and ethyl acetate was removed, to a volume of about 100 ml.

2. Chromatographies

a. XAD-7 Chromatography

The solvent-extracted sample was then chromatographed on a 20 cm x 2.5 cm Amberlite™ XAD-7 column using moderately hydrophobic beads with 90 Å pore diameter and 450 m²/g surface area (Sigma Chemical Co., St. Louis, MO). The sample was loaded onto the column, washed in with 0.1% formic acid in water, then a gradient elution was begun. The gradient was provided by two one-liter cylinders connected at the bottom, with 900 ml of 0.1% formic acid in water in the first cylinder (which feeds into the column), and with 1 L of 0.1% formic acid in methanol in the second cylinder. The first cylinder was magnetically stirred. Fractions were collected in about 10-ml portions and were analyzed by HPLC. Those containing the novel anthocyanin were combined and evaporated in vacuo to about 10 ml.

b. C-18 Chromatography

The combined fractions containing the novel anthocyanin from the XAD-7 column were then chromatographed on a 44 cm x 1.5 cm reverse-phase C-18 column, using 28 - 40 µm beads (E. Merck, Gibbstown, NJ). The anthocyanins were eluted from the column with a gradient consisting of 900 ml of 5% formic acid in the first cylinder, and a liter of 5% formic acid/10% acetonitrile/55% methanol in the second cylinder. Fractions of about 10 ml were collected and analyzed by HPLC. The fractions consisting of 95% or more of new anthocyanin were again combined and evaporated in vacuo to 5 ml or less.

c. LH-20 Chromatography

The sample from the C-18 column was then loaded onto a 20 cm x 2.5 cm Sephadex™ LH-20 column, which separates by lipophilic adsorption (Pharmacia, Piscataway, NJ), and eluted isocratically with 0.1% formic acid in water. Fractions of 5 to 10 ml were collected and analyzed by HPLC, and the fractions containing the new anthocyanin (with less than 5% contaminants) were combined. These were then evaporated to dryness, and reconstituted with a known volume (usually from 1 - 5 ml) of 0.1% formic acid in water.

3. Quantitation

The amount of anthocyanin in a purified sample was determined by making a 1:200 dilution into 1% HCl/95% MeOH and measuring the absorbance spectrum in the visible-light region using a Hitachi model U2000 spectrophotometer (Mt. View, CA). The absorbance at the visible maximum (typically at 510 - 540 nm) was divided by the millimolar extinction coefficient for cyanidin (Dougall and Weyrauch, 1980) and multiplied by the dilution to give the molarity of anthocyanin in the original sample. From the molecular weight and sample volume, the mg/ml and total mg of anthocyanin were calculated.

After quantitation, molecular weight was verified for each sample by mass spectroscopy, and structure was verified by NMR spectroscopy. Mass spectroscopy was performed under the direction of Dr. Albert Tuinman of the Chemistry Department at the University of Tennessee. Analysis was

performed using either electrospray-ionization (ESI) or fast-atom bombardment (FAB), with instrumentation in the former case consisting of a Quatro-II mass spectrometer by Micro-Mass, Inc. (Manchester, England). Anthocyanins were analyzed by ESI mass spectroscopy at 10 $\mu\text{g/ml}$ in 1:1 MeOH/H₂O. FAB mass spectroscopy was with a ZAB-EQ mass spectrometer, by UG Analytical (Manchester, England). Samples were analyzed by FAB mass spectroscopy in thioglycerol. [Sample size was 100 μg in 10 μl of 1% trifluoroacetic acid in both cases.]

NMR analysis was by either Dr. Elana Gakh or Neil Whitemore, both of the Chemistry Department at the University of Tennessee, using a Bruker AMX#400 instrument (Billerica, CA) with either a 5 mm QNP (¹H, ¹³C, ³¹P, and ¹¹B nuclei) or 5 mm proton-selective probe. For this analysis, a sample of 1 mg. was dissolved in 0.5 ml of deuterated methanol MeOH-d₄, to which approximately 40 mg of deuterio-trifluoroacetic acid (CF₃CO₂D) was added.

E. Equilibrium and Rate Constant Measurements

The purified anthocyanin samples were used for the equilibrium and rate measurements. The protocols for this were from Dangles et al. (1993b) with a few modifications. The basic procedure for this is as follows:

1. Dilution in Acid/Salt
2. Spectrophotometric Measurements
3. pH Measurements
4. Calculation of Chemical Constants

1. Dilution in Acid/Salt

Initially, a dilution of anthocyanin was made in 0.1 M acetic acid/1.0 M sodium chloride, sufficient to give an OD of about 1.5 at 500 nm. This was used as a stock solution for all rate and equilibrium experiments done on that sample.

2. Spectrophotometric Measurements

For these measurements, one milliliter of the stock solution was mixed with one milliliter of a NaOH solution. The NaOH solutions were 0.0025 M - 0.01 M in 0.0025 M increments and 0.015 M - 0.050 M in 0.005 M increments. A solution of 0.2 N HCl was also used. A series of nine consecutive concentrations of NaOH was used for each anthocyanin (using higher molarity NaOH solutions for the more stable anthocyanins, and lower molarity NaOH solutions for the less stable ones). After addition of the NaOH or HCl, spectrophotometric readings were immediately taken every 0.5 second at 500 nm over two minutes (unless otherwise specified). The reason for measurement at 500 nm was to avoid interference from the quinonoid form, which absorbs at a higher wavelength than the flavylum ion. The spectrophotometer used was a Perkin-Elmer Lambda 3B UV/VIS Spectrometer (Norwalk, CT), to which was attached a HAAKE A80 Heater/Cooler (Berlin, Germany) to maintain the cuvette receptacle at 25° C. This instrument was interfaced with a computer that was capable of storing all spectrophotometric data on 5-1/4" floppy disks, using software called "UVS." The elapsed time between initial mixing and the first reading was about 6 seconds. At least two complete sets of triplicate

readings were taken for each NaOH concentration (unless otherwise specified), with two quadruplicate sets for the 0.2 N HCl, and each set (of triplicate NaOH readings + quadruplicate HCl reading) was stored on a separate disk.

3. pH Measurements

After the spectrophotometric readings for a particular set were complete, the pH of each solution was measured with an Orion model 606-A digital pH meter (Cambridge, MA) which was equipped with an Orion Ross model 8103 electrode.

4. Calculation of Chemical Constants

Once all the measurements were complete, the chemical constants for the anthocyanins were calculated. This was accomplished using the following series of steps:

- a. Calculation of the Apparent Rate Constant k
- b. Calculation of the Rate and Equilibrium Constants

a. Calculation of the Apparent Rate Constant k

Upon completion of the absorbance and pH measurements, the spectrophotometric data were used to calculate k , the apparent rate constant for flavylium ion disappearance. This involves the use of two computer programs which arrive at a value of k for each individual treatment.

The first of these programs was used to remove some extraneous information from the file containing the spectrophotometric data, which the UVS program recorded as part of each file. The extra data were deleted in most data

sets using Word Perfect, version 5.1, and the file was renamed and saved as an ASCII file. With a few sets of data, Q-edit, a Norton Utilities program, was used.

The second program calculated the apparent rate constant k for each treatment. This program, written by Tom Bradrick, is named Tom1c, and uses a nonlinear-least-squares fit of the data to the equation $OD = Ae^{-kt} + B$, where

A = difference in absorbance between time 0 and
at equilibrium

B = absorbance at equilibrium

t = time from the start of the measurement of OD

The filename, time interval, and number of data points were supplied to the program, which calculated the apparent rate constant k for the absorbance readings in that file.

b. Calculation of Rate and Equilibrium Constants

The next set of calculations produce the equilibrium constants and the rate constants for each anthocyanin. ASEASY version 5.7, a spreadsheet program similar to LOTUS, was used to calculate these. The constants which were calculated are:

1). The sum ($K_h + K_a$)

2). K_h, K_a, k_1, k_2

where

K_h is the hydration equilibrium constant

$([\text{hemiacetal}] \times [\text{H}^+]) / [\text{flavylium}]$ at equilibrium)

K_a is the acidity equilibrium constant

($[\text{quinonoid}] \times [\text{H}^+] / [\text{flavylium}]$ at equilibrium)

k_1 is the hydration rate constant

($\text{flavylium} + \text{H}_2\text{O} \rightarrow \text{hemiacetal} + \text{H}^+$)

k_2 is the dehydration rate constant

($\text{hemiacetal} + \text{H}^+ \rightarrow \text{flavylium} + \text{H}_2\text{O}$)

1) Calculation of the Sum $K_h + K_a$

This calculation, which was based on the methods of Dangles et al. (1993b), uses only the pH measurements and the lowest (= equilibrium) absorbance reading from each treatment (which reach equilibrium after 2 minutes, with one exception). The absorbance readings from the HCl treatments were also used: All the points of all four of all the HCl readings were averaged, and this value was given the label " D_0 ." This value represents anthocyanin molecules completely in the flavylium ion (= red) form; i.e., total anthocyanin.

The lowest absorbance readings from each treatment were labeled " D ," and these represent the equilibrium values of the flavylium ion form for each treatment individually. Using the spreadsheet, the quantity $D_0 / (D_0 - D)$ was plotted vs. $10^{-\text{pH}}$ for each value of D , and the line of best fit by method of least squares was calculated. The slope/intercept of this line is equal to the apparent equilibrium constant, the sum $K_h + K_a$ (see appendices A1 - A21 for examples).

2) Calculation of K_a , K_h , k_1 , k_2

These chemical constants were derived from separate calculations, also based on the methods of Dangles et al. (1993b). This involves the linear relationship between

$(K_h + K_a + 10^{-pH})/k$ and 10^{pH} of all the treatments in a set of data.

$K_h + K_a$ was the value obtained from the previous calculation, and is the same for every treatment within a set of data. The k used in this calculation is unique for each treatment, and was obtained by the Tomlc computer program. The pH's were the same or very close (usually $\pm$ within 0.01 pH unit) for triplicates but about 0.1 pH unit different between successive treatments.

From the graph of $(K_h + K_a + 10^{-pH})/k$ vs. 10^{pH} , the line of best fit by method of least squares was calculated. The slope of this line was equal to K_a/k_2 and the intercept was equal to $1/k_2$. Therefore, from this graph,

$$K_a = \text{slope}/\text{intercept}$$

$$K_h = (K_h + K_a) \text{ (from the earlier calculation)} - K_a$$

$$k_2 = 1/\text{intercept}$$

$$k_1 = K_h \times k_2$$

From the two or more separate sets of absorbance measurements for each compound, the average and standard deviation of its chemical constants were calculated.

F. Other Experiments

The experiments described here are those which do not fit neatly into any other category. These are:

1. Selection of a Cell Line with Increased Anthocyanin Production
2. Removal of Xylose from Selected Anthocyanins

1. Selection of a Cell Line with Increased Anthocyanin Production

One experiment that was not related to the others was the selection of a new cell line with increased anthocyanin production to use as stock inoculum. This was because anthocyanin production of the line 13.1 SKZB had declined.

This experiment involved a macroscopic selection technique. Under sterile conditions, about 20 of the most colored clusters of cells were selected from a culture of 13.1 SKZB with pasteur pipets and placed in a beaker. These clusters were about the smallest size that could be seen with the naked eye. Then about 100 ml of fresh WCM-4 was added, and the cells were re-selected and removed to a new beaker. This was repeated 2 more times to wash out any white cells that might still be present.

Each cluster was placed in 50-ml screw-cap tubes, and 2 ml of medium was added to each. This medium consisted of 50% used WCM-4 (which had been taken from a culture after 12 days) and 50% new WCM-4. These tubes were put on a roller drum at 25° C with occasional light.

After 2 weeks, most of the tubes showed growth of both red and white cells. Some of them, however, showed growth of only red cells with no white cells. The entire contents of the best of these (selected visually for brightest color) was added to a flask with 25 ml of WCM-4, and was grown at 25° C with shaking.

After 2 more weeks, the new subline, designated 13.1 SKZB M-1, was subcultured routinely in WCM-1. About 8 weeks after the experiment was started, the new subline was compared to the old. For this, a 1:10 dilution of both 13.1 SKZB and the subline 13.1 SKZB-M1 was made in duplicate into WCM-4 and 1% v/v DMSO was added to all flasks at days 4 and 8. On the 12th day samples were taken from each flask for OD's and Folch partitions. HPLC analysis was using 35% "B" isocratically.

2. Removal of Xylose from Selected Anthocyanins

In order to investigate the effects of the xylose residue on stability, this monosaccharide unit was removed from the anthocyanin acylated with 4-hydroxy-3,5-dimethoxycinnamic acid (DC) and the one acylated with 3,4,5-trimethoxycinnamic acid (TC) so that the chemical constants of these compounds both with and without xylose could be compared.

The starting material had been previously purified through at least XAD-7 and C-18 chromatography as previously described. The xylose was removed by acid hydrolysis in a 1 mM solution of the starting material in 5 N HCl and was incubated for about 72 hours at 25° C. Samples were taken at

approximate 24 hour intervals and diluted 1:50 with water (to prevent further reaction) and were chromatographed by HPLC to determine composition. After 3 days, a solution of NaOH (1 N or 0.5 N) sufficient to neutralize 95% of the HCl was added to stop the reaction. Each product was then purified using XAD-7, C-18, and LH-20 chromatography as previously described.

III. Results

This section will give the results and some conclusions from the experiments which were previously described. The subjects to be covered are:

- A. Determination of the Concentrations of Acids to be Supplied to the Cultures
- B. Large-Scale Preparations
- C. Other Experiments
- D. Rate and Equilibrium Experiments

A. Determination of the Concentrations of Acids to be Supplied to the Cultures

The first steps in the preparation of an anthocyanin acylated with a particular organic acid were the feeding experiments. In these experiments, carrot cell cultures were first fed a range of concentrations of a particular acid, then the results were analyzed, and usually a follow-up experiment was performed to determine the optimal concentration of that acid, i.e., that which produces the highest quantity of the desired acylated anthocyanin. The following three cinnamic acids were used:

1. 4-Trifluoromethylcinnamic Acid
2. 4-Nitrocinnamic Acid
3. 4-(N-Dimethylamino)cinnamic Acid

1. Trifluoromethylcinnamic Acid

In the first experiment with 4-trifluoromethylcinnamic acid, the concentration range used in the stock solutions were from 0 - 100 mM in 20 mM increments. No HPLC analysis was done on this experiment, as the concentration range was obviously too high, with growth and anthocyanin production inhibited at even the lowest concentration of 4-trifluoromethylcinnamic acid. The data for OD and dry weight is given in Table 1.

In the second experiment, the concentration of 4-trifluoromethylcinnamic acid used in the stock solution was from 0 - 10 mM in 2 mM increments, with an additional treatment of 20 mM so that it would overlap with the first experiment. HPLC analysis, using a linear gradient from 25 - 65% B over 20 minutes, showed two new peaks at 16 and 18 minutes; all peaks from the untreated flasks appeared within 10 minutes. When eluted isocratically with 65% B, all peaks appeared within 10 minutes, as shown in Fig. 8. Data for HPLC peak heights, optical density, and dry weights are given in Table 2.

The 4-trifluoromethylcinnamic acid treatment produced not one but two new anthocyanins. The significance of this will be discussed in the section on Large-Scale Preparations. From the product of (novel peak height)/total HPLC peak height) X total OD (which is a measure of the amount of novel anthocyanin in that treatment), the optimal 4-trifluoromethylcinnamic acid concentration in the stock solution was determined to be 6 mM.

Table 1. First experiment showing the effects of 4-trifluoromethylcinnamic acid concentration on growth (dry weight) and on total anthocyanin (given as O.D.) in carrot suspension cultures (clone 13.1 SKZB). Data given is mean $\pm$ standard deviation.

Treatment ¹	Dry Wt. (mg/ml)	O.D. (@ 530 nm)
No addition	4.8 $\pm$ 0.3	0.81 $\pm$ 0.08
DMSO only	4.4 $\pm$ 0.3	1.25 $\pm$ 0.44
20 mM	0.8 $\pm$ 0.1	0.02 $\pm$ 0.01
40 mM	0.9 $\pm$ 0.1	0.01 $\pm$ 0.00
60 mM	0.9 $\pm$ 0.1	----
80 mM	1.0 $\pm$ 0.1	----
100 mM	1.0 $\pm$ 0.2	----

¹ Concentrations refer to stock solutions of 4-trifluoromethylcinnamic acid in DMSO which was added to cultures (1% v/v) on days 4 and 8.

Table 2. Second experiment showing the effects of 4-trifluoromethylcinnamic acid concentration on growth (dry weight), on total anthocyanin (given as O.D.), and on individual anthocyanins present (HPLC) in carrot suspension cultures (clone 13.1 SKZB).

Trt ¹	Dry Wt ² .	OD ²	HPLC Peak Heights (inches)			
	(mg/ml)	@530 nm	Total	Novel Peak 1	Novel Peak 2	1+2/Tot X OD
no add.	5.5 ± 0.2	1.06 ± 0.12	---	---	---	---
DMSO only	4.8 ± 0.2	1.83 ± 0.12	9.7	0.0	0.0	---
2 mM	4.6 ± 0.2	1.42 ± 0.10	10.5	1.0	2.4	0.46
4 mM	4.6 ± 0.2	1.50 ± 0.04	9.3	1.5	3.9	0.86
6 mM	4.6 ± 0.1	1.43 ± 0.33	10.4	1.9	5.5	1.02
8 mM	4.7 ± 0.4	0.89 ± 0.06	6.4	1.1	3.9	0.67
10 mM	3.7 ± 0.5	0.62 ± 0.04	3.8	0.7	2.5	0.53
20 mM	0.8 ± 0.1	0.03 ± 0.01	---	---	---	---

¹ Concentrations refer to stock solutions of 4-trifluorocinnamic acid in DMSO which were added to cultures (1% v/v) on days 4 & 8.

² Mean ± standard deviation.

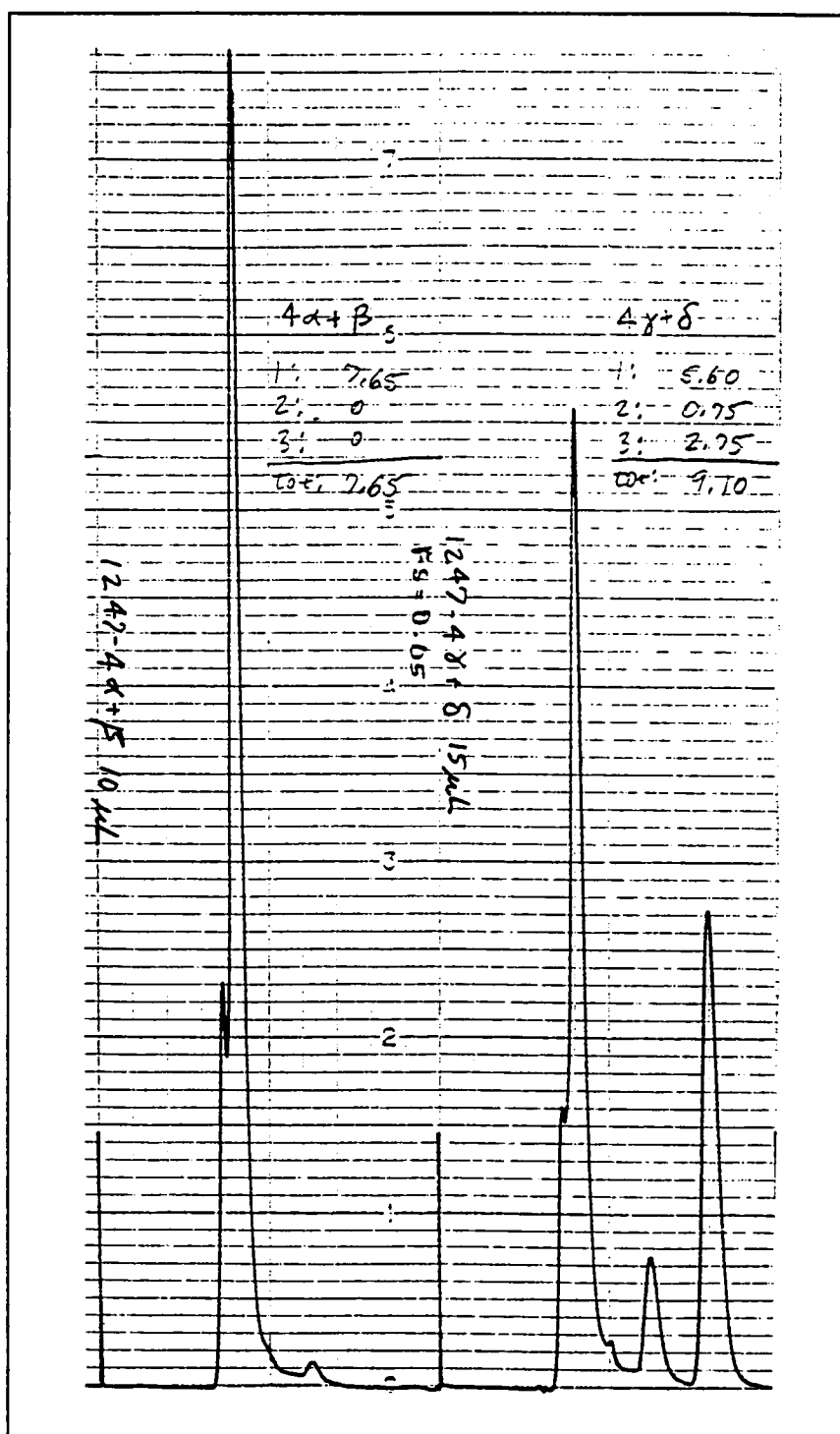


Fig. 8. HPLC peak trace. The peak on the left is anthocyanin from carrot culture treated with DMSO alone, while the peaks on the right side are anthocyanins from culture treated with 6 mM 4-trifluoromethylcinnamic acid in DMSO. Elution was with 65% B.

2. Nitrocinnamic Acid

4-Nitrocinnamic acid was initially tested using concentrations from 0 - 100 mM in 20 mM increments. In this experiment, a total loss of color occurred in all save the 0 mM treatment, and even the flasks which received the 20 mM stock solution appeared to be dead the day after the first addition was made. No measurements were made.

The second experiment was done with 4-nitrocinnamic acid in the concentration range of 0 - 12 mM in 2 mM increments, with an additional treatment of 20 mM. HPLC analysis using 35% solvent B isocratically showed a novel peak at 9.5 minutes; all other peaks were at or before 7.5 minutes. Data for HPLC peak heights, dry weights, and OD's are shown in Table 3. From this experiment, the optimal concentration of 4-nitrocinnamic acid in the stock solution was determined to be 8 mM.

3. 4-(*N*-Dimethylamino)cinnamic Acid

The first experiment with 4-(*N*-dimethylamino)cinnamic acid used stock solutions from 0 - 30 mM in 5 mM increments. HPLC analysis using 40% B isocratically showed the presence of a new peak at 10 minutes; other peaks all appeared in less than 7 minutes. Data for this experiment is shown in Table 4. These data suggested that 20 mM 4-(*N*-dimethylamino)cinnamic acid gave the highest yield of the novel anthocyanin.

In a second experiment, stock solutions of 0 - 45 mM were used in order to confirm the optimum concentration of 4-(*N*-

Table 3. The effects of 4-nitrocinnamic acid concentrations on growth (dry weight), on total anthocyanin accumulation (given as O.D.), and on individual anthocyanins present (HPLC) in carrot suspension cultures (13.1 SKZB).

Treat- ment ¹	Dry Wt ² .	Optical Density ²	HPLC peak heights (inches)		
	(mg/ml)	(@530 nm)	Total	Novel Peak	N.P./Tot X OD
DMSO only	5.4 ± 0.1	1.91 ± 0.26	12.6	0.00	----
2 mM	5.1 ± 0.2	1.51 ± 0.09	10.8	1.25	0.18
4 mM	4.9 ± 0.2	1.18 ± 0.32	7.0	1.75	0.30
6 mM	5.0 ± 0.2	1.34 ± 0.14	7.2	2.35	0.41
8 mM	4.7 ± 0.1	1.14 ± 0.06	6.8	3.10	0.52
10 mM	4.3 ± 0.1	0.94 ± 0.22	4.9	2.50	0.47
12 mM	4.3 ± 0.2	0.80 ± 0.13	5.5	3.25	0.47
20 mM	0.6 ± 0.2	0.05 ± 0.01	0.4	0.20	0.03

¹ Concentrations refer to stock solution of 4-nitrocinnamic acid in DMSO which were added to the cultures (1% v/v) at days 4 and 8.

² Mean ± standard deviation.

Table 4. First experiment showing the effects of 4-(N-dimethylamino)cinnamic acid concentration on growth (dry weight), on total anthocyanin accumulation (given as O.D.), and on individual anthocyanins present (HPLC) in carrot suspension cultures (13.1 SKZB).

Treat- ment ¹	Dry Wt ² .	OD ²	HPLC Peak Heights (inches)		
	(mg/ml)	(@530 nm)	Total	Novel Peak	N.P./Tot X OD
DMSO only	5.1 ± 0.2	2.89 ± 0.10	16.7	0.0	----
5 mM	5.0 ± 0.2	2.33 ± 0.08	10.3	0.6	0.14
10 mM	5.5 ± 1.0	2.32 ± 0.14	9.3	1.2	0.25
15 mM	4.8 ± 0.4	1.96 ± 0.15	7.5	2.2	0.57
20 mM	5.3 ± 0.2	1.88 ± 0.23	6.6	2.1	0.60
25 mM	4.8 ± 0.3	1.46 ± 0.03	5.6	2.1	0.54
30 mM	4.7 ± 0.4	1.38 ± 0.15	4.1	1.7	0.55

¹ Concentrations refer to stock solutions of 4-(N-dimethylamino)cinnamic acid in DMSO which were added to cultures (1% v/v) on days 4 and 8.

² Mean ± standard deviation.

dimethylamino)cinnamic acid that resulted in highest novel anthocyanin yield, and to test the effects of higher 4-(*N*-dimethylamino)cinnamic acid concentrations on the cultures. HPLC analysis was performed isocratically using 40% B. Data is shown in Table 5.

From the second experiment, the data suggests that the optimal concentration of 4-(*N*-dimethylamino)cinnamic acid to produce the desired anthocyanin would be 25 mM instead of 20 mM. However, because of experimental variation, no further experiments were done in order to further refine the concentration of 4-(*N*-dimethylamino)cinnamic acid.

B. Large-Scale Preparations

Once an optimal concentration of an organic acid was found in the feeding experiments, a large quantity of tissue was then grown using that concentration, and from the tissue, the desired anthocyanin was purified. The results for the large-scale preparations using the three acids which were previously described (4-trifluoromethylcinnamic acid, 4-nitrocinnamic acid, and 4-(*N*-dimethylamino)cinnamic acid) are given in Table 6. The purified compounds produced from these experiments were later used in rate and equilibrium experiments, and representative samples of each compound were analyzed by mass spectroscopy and NMR spectroscopy.

Mass spectroscopic analysis of the anthocyanins acylated with 4-(*N*-dimethylamino)cinnamic acid (DMA) and 4-nitrocinnamic acid (NC) gave mass/charge ratios of 918.2 for NC and 916.2 for DMA. The expected molecular weights were 918 and 916, respectively.

Table 5. Second experiment showing the effects of 4-(*N*-dimethylamino)cinnamic acid on growth (dry weight), on total anthocyanin accumulation (given as O.D.), and on individual anthocyanins present (HPLC) in carrot suspension cultures (13.1 SKZB).

Treat- ment ¹	Dry Wt ² .	Optical Density ²	HPLC Peak Heights (inches)		
	(mg/ml)	(@530 nm)	Total	Novel Peak	N.P./Tot X OD
DMSO only	5.2 ± 0.4	1.48 ± 0.06	12.4	0.0	----
20 mM	4.9 ± 0.3	1.16 ± 0.11	7.3	2.1	0.33
25 mM	5.4 ± 0.2	1.10 ± 0.05	6.8	2.4	0.39
30 mM	4.9 ± 0.3	0.91 ± 0.04	5.3	1.9	0.33
35 mM	5.1 ± 0.1	0.77 ± 0.06	4.6	1.8	0.30
40 mM	4.7 ± 0.3	0.77 ± 0.11	4.0	1.6	0.31
45 mM	4.9 ± 0.2	0.79 ± 0.11	4.6	1.8	0.31

¹ Concentrations refer to stock solutions of 4-(*N*-dimethylamino)cinnamic acid in DMSO which were added to cultures (1% v/v) on days 4 and 8.

² Mean ± standard deviation.

Table 6. Fresh weight and anthocyanin yield obtained from large-scale preparations of carrot suspension culture (13.1 SKZB) .

Exp. #	Acid Used	Stock Soln. conc. (mM)	Volume of Medium (L)	Fresh Wt. (g/L)	Antho. Yield (mg/L)
1242	4-Trifluoromethyl-cinnamic Acid	6	1.0	84.4	4.7
1257	4-Trifluoromethyl-cinnamic Acid	6	3.0	97.4	2.2
1242	4-Nitrocinnamic Acid	8	1.0	76.5	5.9
1270	4-Nitrocinnamic Acid	8	1.9	65.1	1.1
1254	4- (N-Dimethyl-amino) - cinnamic Acid	20	1.1	75.0	0.5 ¹
1259	4- (N-Dimethyl-amino) - cinnamic Acid	20	3.0	90.1	3.5

¹ Most of compound was lost during purification, due to a spillage accident during LH-20 chromatography.

In the biosynthesis experiments with 4-trifluoromethylcinnamic acid, as mentioned earlier, two compounds were produced instead of one, which were separated by C-18 chromatography. One of the two compounds (the second-eluting peak) gave a mass/charge ratio of 941.3; expected molecular weight for the anthocyanin acylated with 4-trifluoromethylcinnamic acid (TFMC) was 941. The first-eluting peak gave a mass/charge ratio of 915.2, which corresponds to the expected molecular weight of an anthocyanin acylated with 4-trifluoromethylbenzoic acid.

Later NMR spectral analysis confirmed the expected structures of NC, DMA, and TFMC. Analysis of the first-eluting peak from the 4-trifluoromethylcinnamic acid experiment, however, showed the spectrum expected for a benzoic rather than a cinnamic acid, which was consistent with the mass spectrum. In later experiments, feeding 4-trifluoromethylbenzoic acid to carrot suspension cultures resulted in the production of a compound that was shown to be identical in mass/charge ratio and NMR spectrum to the first-eluting peak from the 4-trifluoromethylcinnamic acid experiments. Experiments with another compound showed another example of an anthocyanin acylated with a benzoic acid produced by the cultures when they were fed the corresponding cinnamic acid. Apparently, there are enzymes in the carrot system which are capable of removing two carbon atoms from certain cinnamic acids and giving the corresponding benzoic acids.

C. Other experiments

As previously mentioned, this category is for experiments that do not fit neatly into other categories. The subjects to be covered here are:

1. Experiments on Feeding Protocols
2. Enzyme Inhibition Experiments
3. Selection of a Cell Line with Increased Anthocyanin Production
4. Removal of Xylose from Selected Anthocyanins

1. Experiments on Feeding Protocols

Because the addition of cinnamic acids to the cultures resulted in lowered total anthocyanin yield, the effects of different schedules for addition of stock solutions to culture was evaluated.

Two experiments were done to test this, one with 8 mM 4-nitrocinnamic acid and the other with 6 mM trifluoromethylcinnamic acid. In both experiments, the effects of the stock solution (in DMSO) and DMSO alone was tested for all treatments. Results are given in Table 7 for 4-nitrocinnamic acid and in Table 8 for 4-trifluoromethylcinnamic acid.

From the data, it is apparent that the addition of the stock solution on days 2, 4, 6, and 8 resulted in the highest production of novel anthocyanin in both experiments. However, because of time considerations and higher risk of contamination with 4 additions, the schedule of addition on days 4 and 8 was followed on all subsequent experiments.

Table 7. Effects of time of addition of 8 mM 4-nitrocinnamic acid in DMSO on growth (expressed as dry weight), on total anthocyanin (given as O.D.), and on individual anthocyanins present (HPLC) in 50 ml of carrot suspension cultures of 13.1 SKZB. Cells were harvested on day 12.

Treatment	Days of Solution Addition	Amount of Solution Added Each Time (ml)	Dry Wt. ¹	O.D. ¹	HPLC Peak Height (inches).			
DMSO ± Acid			mg/ml	@ 530 nm	Total Pk. Height	Novel Pk. Height	N.P./Tot.	N.P./Tot. X OD
1 - Acid	Day 2	1.00	5.5 ±0.2	4.31 ±0.25	24.6	0.00	-----	-----
1 + Acid	Day 2	1.00	0.6 ±0.1	0.01 ±0.00	-----	-----	-----	-----
2 - Acid	Day 4	1.00	6.0 ±0.3	2.48 ±0.24	17.8	0.0	-----	-----
2 + Acid	Day 4	1.00	5.3 ±0.2	1.51 ±0.21	9.9	1.4	0.14	0.21
3 - Acid	Day 6	1.00	6.3 ±0.3	2.26 ±0.24	14.7	0.0	-----	-----
3 + Acid	Day 6	1.00	5.5 ±0.3	1.26 ±0.10	5.1	1.7	0.33	0.42
4 - Acid	Day 8	1.00	6.5 ±0.5	1.75 ±0.05	10.3	0.0	-----	-----
4 + Acid	Day 8	1.00	5.7 ±0.6	1.36 ±0.18	8.5	2.6	0.30	0.41
5 - Acid	Days 2 & 6	0.50	5.6 ±0.3	3.17 ±0.13	34.2	0.0	-----	-----
5 + Acid	Days 2 & 6	0.50	3.5 ±0.6	3.06 ±0.62	19.6	0.8	0.04	0.13
6 - Acid	Days 4 & 8	0.50	6.1 ±0.4	2.53 ±0.17	16.5	0.0	-----	-----
6 + Acid	Days 4 & 8	0.50	5.4 ±0.4	1.22 ±0.16	17.4	7.2	0.41	0.50
7 - Acid	Days 6 & 10	0.50	6.4 ±0.6	1.88 ±0.17	13.5	0.0	-----	-----
7 + Acid	Days 6 & 10	0.50	5.7 ±0.3	1.41 ±0.09	8.4	3.6	0.43	0.60
8 - Acid	Days 2, 4, 6, & 8	0.25	5.5 ±0.2	2.54 ±0.18	18.6	0.0	-----	-----
8 + Acid	Days 2, 4, 6, & 8	0.25	5.4 ±0.4	2.25 ±0.55	16.3	5.3	0.32	0.81

¹ Mean ± standard deviation.

Table 8. Effects of time of addition of 6 mM 4-trifluoromethylcinnamic acid in DMSO on growth (expressed as dry weight), total anthocyanin (given as O.D.), and individual anthocyanins present (HPLC) in carrot suspension cultures of 13.1 SKZB. Cells were harvested on day 12.

Treatment	Days of Solution Addition	Amount of Solution Added Each Time (ml)	Dry Wt. ¹	O.D. ¹	HPLC Peak Height (inches).			
			mg/ml	@ 530 nm	Tot. Pk. Height	Novel Pk. Height	N.P./Tot.	N.P./Tot. X OD
0	no addition	no addition	6.49 +0.33	1.66 +0.09	11.4	0.00	-----	-----
1 Acid -	Day 2	1.00	5.0 +0.3	5.44 +0.67	28.7	0.00	-----	-----
1 Acid +	Day 2	1.00	0.3 +0.2	0.00 +0.00	-----	-----	-----	-----
2 Acid -	Day 4	1.00	5.0 +0.3	0.10 +0.19	11.6	0.0	-----	-----
2 Acid +	Day 4	1.00	1.2 +0.3	0.17 +0.03	4.6	3.0	0.66	0.11
3 Acid -	Day 6	1.00	5.1 +0.2	2.20 +0.08	13.4	0.0	-----	-----
3 Acid +	Day 6	1.00	5.0 +0.2	1.29 +0.06	7.3	3.4	0.46	0.59
4 Acid -	Day 8	1.00	5.4 +0.2	1.62 +0.10	11.5	0.0	-----	-----
4 Acid +	Day 8	1.00	5.1 +0.2	1.58 +0.13	9.1	3.5	0.38	0.61
5 Acid -	Days 2 & 4	0.50	5.3 +0.2	4.40 +0.69	36.8	0.0	-----	-----
5 Acid +	Days 2 & 4	0.50	2.8 +0.1	1.48 +0.04	20.3	14.0	0.69	1.02
6 Acid -	Days 4 & 8	0.50	5.6 +0.3	2.65 +0.21	19.9	0.0	-----	-----
6 Acid +	Days 4 & 8	0.50	5.4 +0.2	1.85 +0.08	12.1	6.0	0.50	0.92
7 Acid -	Days 6 & 10	0.50	5.7 +0.5	2.20 +0.11	14.6	0.0	-----	-----
7 Acid +	Days 6 & 10	0.50	5.2 +0.2	1.29 +0.16	8.8	3.9	0.44	0.57
8 Acid -	Days 2, 4, 6, & 8	0.25	5.4 +0.3	3.64 +0.20	20.3	0.0	-----	-----
8 Acid +	Days 2, 4, 6, & 8	0.25	5.2 +0.3	3.51 +0.07	18.3	9.5	0.52	1.81
9 Acid -	Days 4, 6, 8, & 10	0.25	5.8 +0.2	2.53 +0.16	18.3	0.0	-----	-----
9 Acid +	Days 4, 6, 8, & 10	0.25	5.4 +0.2	2.11 +0.16	12.2	6.3	0.51	1.08

¹ Mean $\pm$ standard deviation.

2. Enzyme Inhibition Experiments

Tables 1 - 3, as well as Tables 7 and 8, show that in general, 4-nitrocinnamic acid and 4-trifluoromethylcinnamic acid inhibited anthocyanin accumulation and also reduced the dry weight of the cultures. The experiments with 4-(*N*-dimethylamino)cinnamic acid, on the other hand (see Tables 4 and 5), show that at all concentrations tested it reduces anthocyanin accumulation, but does not affect dry weight significantly. One possibility is that 4-(*N*-dimethylamino)cinnamic acid may be competing with 4-coumaric acid, and interfering with anthocyanin biosynthesis at an early stage in the pathway. In order to investigate this, a concentration of the acid was used that was high enough to inhibit anthocyanin production significantly but not high enough to inhibit growth substantially. Also added to flasks containing culture plus acid at this concentration were intermediates in the biosynthetic pathway that are believed to be subsequent to the enzymatic step which was inhibited, in order to see if anthocyanin production can be restored (see Fig. 9 [adapted from Hahlbrock, 1981, and Grisebach, 1982]). Theoretically, if the added acid looks enough like an intermediate in the pathway, the enzyme using that intermediate as substrate may be inhibited by addition of the acid. Therefore, adding an intermediate PAST this step should restore anthocyanin production, assuming it enters the cell.

The possibility that 4-(*N*-dimethylamino)cinnamic acid was an inhibitor of one of these enzymes (i.e., 4-coumarate:CoA ligase, referring to Fig. 9) was investigated using

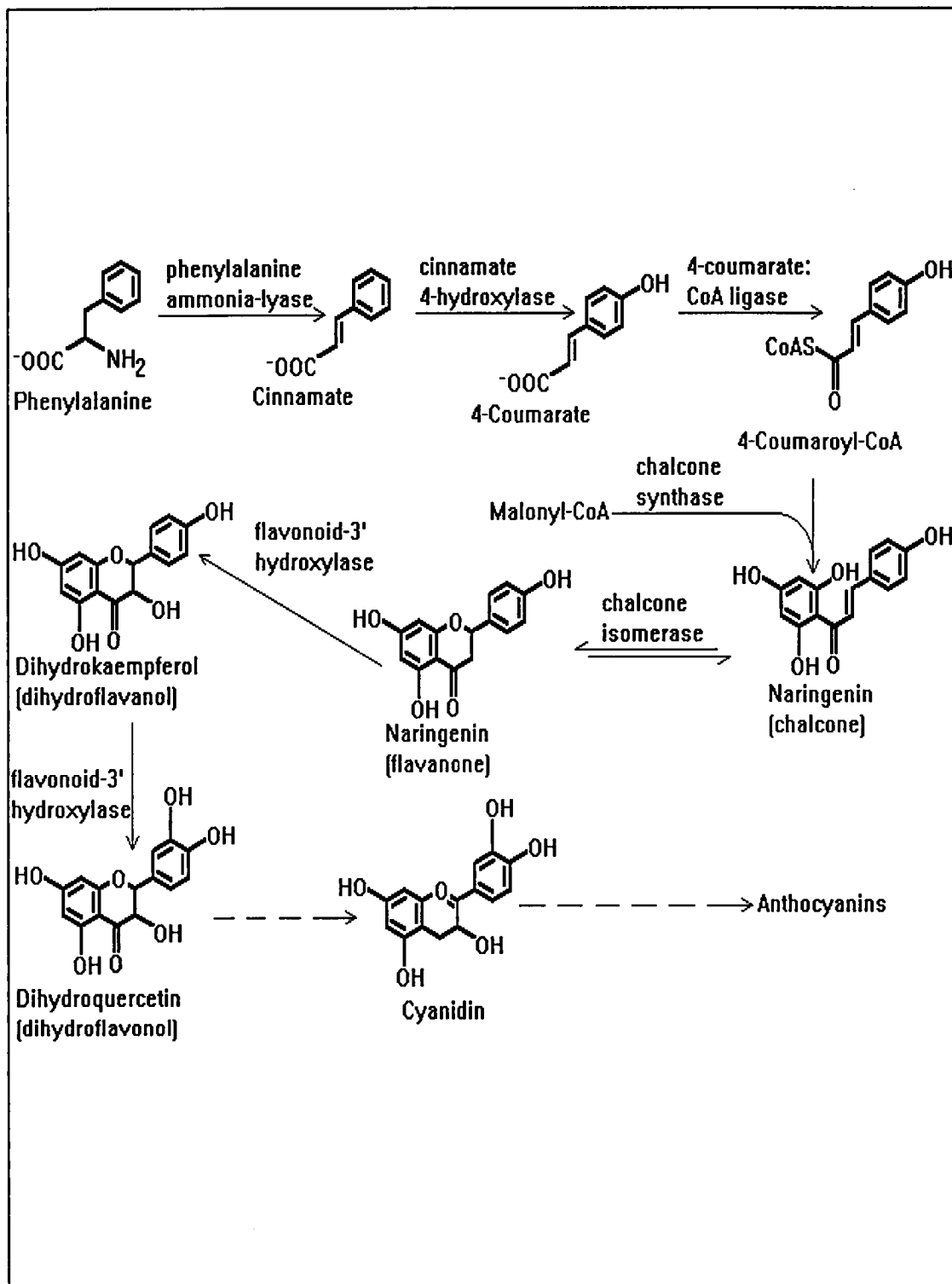


Fig. 9. Proposed sequence of reactions in anthocyanin biosynthesis (abbreviated). Adapted from Hahlbrock, 1981, & Grisebach, 1982.

dihydroquercetin and naringenin as later intermediates in the pathway. Results are given in Table 9.

The data show an increase in total anthocyanin in the treatments with 4-(*N*-dimethylamino)cinnamic acid plus either dihydroquercetin or naringenin compared to those with the acid alone, as well as an increase in all four of the individual anthocyanins. Some peaks, however, increased much more dramatically than others; in fact, the proportion of anthocyanin acylated with 4-(*N*-dimethylamino)cinnamic acid to total anthocyanin decreased substantially in both of the treatments with the acid + intermediates compared to the treatment with acid alone. However, the total HPLC peak heights, as well as total OD of both treatments with the acid plus intermediate was still less than those of the DMSO-only control, showing that anthocyanin production was only partially restored. The conclusion from these data is that even though anthocyanin production was partially restored, thus supporting the enzyme-inhibition hypothesis, there is a second site of inhibition by 4-(*N*-dimethylamino)cinnamic acid later on down the pathway. Another possible explanation for the partial restoration may be that the cells are more permeable to DMA than to NAR or DHQ. Also, from a purely practical standpoint, the use of these intermediates would not be efficient in large-scale preparations of the novel anthocyanin.

3. Selection of a Cell Line with Increased Anthocyanin Production

Total anthocyanin (O.D. at 530 nm) produced by the old vs. new cell line (13.1 SKZB vs. 13.1 SKZB-M1) treated with 1%

Table 9. The effects of dihydroquercetin (DHQ) and naringenin (NAR), late intermediates in anthocyanin biosynthesis, on the inhibition of anthocyanin accumulation in carrot suspension cultures (13.1 SKZB) by 4-(N-dimethylamino)cinnamic acid (DMA).

Treat- ment ¹	Dry Wt ² .	O.D ² .	HPLC Peak Heights (in.). Common peaks are at 3.5, 4, and 5 min., with novel peak at 7 min.						
	mg/ml	@ 530 nm	3.5 min peak	4 min. peak	5 min. peak	7 min. peak	Total Peak Ht.	7min/ Tot.	7min/ Tot. X OD
0) no add	5.6 ±0.3	1.23 ±0.10	2.0	6.3	1.5	0.0	9.7	---	---
1) DMSO only	5.0 ±0.1	1.93 ±0.15	3.6	9.8	2.0	0.0	15.4	---	---
2) 40 mM DMA in DMSO	4.7 ±0.1	1.03 ±0.02	1.5	0.8	0.8	2.1	5.2	0.41	0.42
3) 25 mM DHQ in DMSO	5.1 ±0.1	2.79 ±0.10	4.8	14.5	2.1	0.0	21.4	---	---
4) 10 mM NAR in DMSO	5.3 ±0.2	2.53 ±0.27	5.1	12.4	2.0	0.0	19.5	---	---
5) 40 mM DMA/25 mM DHQ in DMSO	4.5 ±0.2	1.77 ±0.20	3.6	2.8	1.2	2.8	10.4	0.27	0.47
6) 40 mM DMA/10 mM NAR in DMSO	4.6 ±0.3	1.49 ±0.08	3.7	2.9	1.0	2.6	10.2	0.26	0.38

¹ Concentrations are final conc. of stock solution, which were added to the cultures (1% v/v) on days 4 and 8.

² Mean ± standard deviation.

v/v DMSO added on days 4 and 8 are shown below:

Treatment	OD ₅₃₀ *
13.1 SKZB	1.29 ± 0.15
13.1 SKZB M-1	15.67 ± 1.05
new/old	12.1

*Mean ± standard deviation

Total OD measured for the new cell line, 13.1 SKZB M-1, grown in WCM-4 was more than ten times that of the old cell line, 13.1 SKZB, grown under identical conditions. The peak heights for the components separated by HPLC from extracts of the two cell lines are shown in Table 10. The HPLC analysis showed that the new cell line contained the same components as the old line, with peak heights increasing from two-fold to nineteen-fold, with an average increase of over ten-fold. Microscopically, the cells of the new sub-line were predominantly red. This line was used for all subsequent preparations.

4. Removal of Xylose from Selected Anthocyanins

The removal of xylose from anthocyanins was first observed by Dr. E. Gakh in NMR samples acidified with DCl. Subsequently the best conditions for preparative partial hydrolysis to remove xylose was determined to be these used here (Dougall, unpublished). In these experiments, the xylose was removed from the anthocyanin acylated with 4-hydroxy-3,5-dimethoxycinnamic acid (DC) and the anthocyanin acylated with 3,4,5-trimethoxy-cinnamic acid (TC).

Table 10. HPLC peak heights of old and new cell line.

Peaks ¹ (min.)	HPLC Peak Height (inches)		
	13.1 SKZB	13.1 SKZB M-1	new/old
3	1.4	11.0	7.9
3.5	0.2	2.3	11.5
4.5	0.8	15.2	19.0
15.5	0.4	1.4	3.5
21	1.5	24.4	16.3
24	0.2	0.4	2.0
31	0.05	1.0	20.0
35.5	0.3	2.0	6.7
total	4.9	57.7	11.8

¹ Peaks represent anthocyanins found naturally in carrot culture. The six largest are the known compounds shown in Figs. 5 and 6; the remaining two are minor peaks which are not at present identified.

Table 11. HPLC peak heights at different times of treatment of DC with 5 N HCl to remove xylose.

Time (hrs.)	DC Peak Height (in.)	DCX Peak Height (in.)	DCX/Total
0	12.0	0	0
21.5	12.0	3.1	0.21
45.5	5.8	3.8	0.40
67.0	4.1	4.3	0.51

Approximately 14 mg. of DC was used in the first experiment. The HPLC analysis of samples taken at several time periods are shown in Table 11. [DC without xylose is referred to as DCX; TC without xylose is abbreviated to TCX].

After neutralization (at 67 hours) and purification, this experiment yielded 3.3 mg of DCX. A later experiment, starting with approximately 25 mg of DC, yielded 7.7 mg. [Note: previous experiments by Dougall (unpublished) had shown that after approximately 3 days, the compound without xylose (but with the acyl group still intact) begins to decrease.]

Two experiments were also done to remove xylose from TC. The first was discontinued because most of the TCX disappeared; this was probably because solid NaOH was used to neutralize the HCl, and it also apparently removed the acyl group from the anthocyanin as well (HPLC analysis showed that there was a peak in the area expected for deacylated anthocyanin, and very little of the TC peak). NaOH can be used to deacylate anthocyanin in a concentration of 1 N over 7 minutes. The second, using 1 N NaOH to neutralize the HCl, and starting with about 100 mg of TC, produced 47 mg. of purified TCX.

Mass spectroscopy of DCX gave a mass/charge ratio of 817.3; the expected value was 817. Mass spec analysis of TCX gave a mass/charge reading of 831.3. NMR spectroscopy confirmed the structures in both cases.

D. Equilibrium and Rate Constants

The final measurements made on each purified compound were the rate and equilibrium constant measurements. The results are given in Table 12. More detailed data for each compound, including linear regression data and graphs of $D_0/(D_0-D)$ vs. $10^{-\text{pH}}$ and $(K_h + K_a + 10^{-\text{pH}})/k$ vs. 10^{pH} are given in Appendices 1 - 21. Results are discussed in the next section.

One compound that bears mentioning is the one acylated with 4-(*N*-dimethylamino)cinnamic acid (DMA). Data for DMA was not included in Table 12 because the plot of $D_0/(D_0-D)$ vs. $10^{-\text{pH}}$ showed a definite tendency to curve (see Appendix 14). This is probably because the dimethylamino group of this compound was positively charged at a low pH, and as pH increased this group lost its charge. Any attempt to graph this compound at different pH's would therefore be taking more than one variable into account, thus making interpretation impossible.

Data for this compound is not strictly comparable to any of the other data because of this, but it is still useful for some purposes. An interesting feature of this compound is that the pH's measured about 0.1 lower than in any other compounds. This was probably an effect from the positively-charged dimethylamino group.

Table 12. Chemical constants for anthocyanins. K_h and K_a are the equilibrium constants between flavylum ion and hemiacetal, and k_1 and k_2 are the forward and reverse rate constants between flavylum ion and hemiacetal (see Fig. 2). Values given are averages $\pm$ standard deviations.

		DC	DCX	3MC	TC	TCX	2MC	4HC	C	4MC	CC
# Data Sets		3	2	2	2	2	2	2	2	2	2
$K_h + K_a \times 10^5$	Av. S.D.	4.77 ± 0.62	5.47 ± 0.40	6.05 ± 0.26	6.89 ± 0.34	6.98 ± 0.23	6.96 ± 0.17	8.27 ± 0.26	8.84 ± 0.26	9.11 ± 0.20	10.13 ± 0.12
$K_h \times 10^5$	Av. S.D.	3.88 ± 0.49	5.13 ± 0.00	5.87 ± 0.28	5.24 ± 0.26	5.38 ± 0.08	4.95 ± 0.15	6.64 ± 0.78	7.28 ± 0.14	7.93 ± 0.68	8.16 ± 0.02
$K_a \times 10^5$	Av. S.D.	0.89 ± 0.35	0.35 ± 0.01	0.19 ± 0.02	1.65 ± 0.08	1.60 ± 0.30	2.02 ± 0.02	1.63 ± 0.52	1.56 ± 0.12	1.19 ± 0.49	1.97 ± 0.13
$k_1^1 \times 10^2$	Av. S.D.	2.46 ± 0.39	2.01 ± 0.06	3.67 ± 0.10	3.57 ± 0.04	2.78 ± 0.08	3.61 ± 0.02	4.80 ± 0.93	4.64 ± 0.15	4.68 ± 0.54	4.82 ± 0.07
k_2^2	Av. S.D.	633 ± 52	394 ± 20	626 ± 12	684 ± 42	519 ± 20	732 ± 18	716 ± 56	636 ± 8	588 ± 17	590 ± 9

		4FC	TFMC	4HB	TB	CB	NC	TFMB	4HP	CYANGX	CYANGXG
# Data Sets		2	4	2	2	2	2	2	2	2	2
$K_h + K_a \times 10^5$	Av. S.D.	11.81 ± 0.04	13.70 ± 1.24	13.81 ± 0.69	16.22 ± 0.78	17.92 ± 0.27	21.76 ± 0.03	26.11 ± 1.12	31.82 ± 0.94	43.00 ± 3.20	47.27 ± 5.92
$K_h \times 10^5$	Av. S.D.	9.27 ± 0.06	11.45 ± 1.50	10.98 ± 0.39	14.89 ± 0.82	15.75 ± 0.12	19.25 ± 0.02	23.73 ± 1.13	28.24 ± 0.88	38.82 ± 1.34	42.99 ± 5.71
$K_a \times 10^5$	Av. S.D.	2.55 ± 0.10	2.25 ± 0.37	2.83 ± 0.30	1.33 ± 0.03	2.16 ± 0.15	2.52 ± 0.01	2.39 ± 0.09	3.58 ± 0.05	4.18 ± 1.87	4.29 ± 0.21
$k_1^1 \times 10^2$	Av. S.D.	5.34 ± 0.03	6.73 ± 0.28	6.90 ± 0.23	8.73 ± 0.58	8.11 ± 0.13	7.88 ± 0.03	10.79 ± 0.56	6.01 ± 0.15	8.08 ± 0.33	5.74 ± 1.06
k_2^2	Av. S.D.	577 ± 1	601 ± 106	629 ± 1	586 ± 6	515 ± 4	409 ± 1	455 ± 2	213 ± 1	208 ± 1	139 ± 1

¹ Units are sec^{-1} .

² Units are $\text{sec}^{-1}\text{M}^{-1}$.

IV. Discussion

The major topics to be covered in discussion are:

- A. K_h is Greater than K_a for Every Compound
- B. Deacylated vs. Acylated Compounds
- C. Variation in Rate and Equilibrium Constants with Hammett constants
- D. Stability of Compounds with 3,5-Dimethoxy Substituents
- E. *Ortho*-, *meta*-, and *para*- Methoxycinnamic Compounds
- G. Summary and Conclusions

A. K_h is Greater than K_a for every compound

From Table 12, it is immediately apparent that for every compound, the K_h , reflecting conversion of flavylum ion to hemiacetal, is always greater than K_a , which reflects conversion of flavylum ion to the quinonoid form. It can be concluded from this that in all compounds tested, a much greater proportion of the anthocyanin is converted into hemiacetal than into the quinonoid form upon increase of pH. The studies by Dangles et al. (1993a and b) and by Figueiredo et al. (1996) showed similar findings. The K_h is the best indication of a compound's stability because the quinonoid form (reflected by K_a) is colored and the hemiacetal (reflected by K_h) is not. The higher the K_h , the less stable the compound. Therefore, the stabilities of the compounds

examined in this study range over more than an order of magnitude.

B. Deacylated vs. Acylated Compounds

The comparisons of the deacylated compounds to the acylated ones show the most extreme effects on rate and equilibrium constants. The deacylated compounds, CYANGXG and CYANGX, both had similar rate constants, and both had K_h 's much higher than any of the other compounds. The K_a 's for these were also the highest of all, about twice the K_a 's for most of the other compounds.

The k_1 's of the deacylated compounds, interestingly, are not much different than those of the least stable of the acylated compounds, while the k_2 's are much lower than any of the others, except the 3-(4-hydroxyphenyl)propionoyl compound (4HP). This shows that the forward reaction does not occur at a rate any faster than in any of the less stable acylated compounds, but the reverse reaction is much slower. This shifts the equilibrium position towards a much higher K_h , because $K_h = k_1/k_2$.

C. Variation in Rate and Equilibrium Constants with Hammett Constants

There are at present two main theories that try to account for the role of the acyl group in the stability of anthocyanins. One involves purely steric effects--the acyl

group is thought to physically protect the central ring of the flavylum nucleus from attack by water, which would result in the hydration reaction leading to formation of the colorless hemiacetal (see Fig. 2). The other theory involves electronic effects, in which the acyl group lies in a plane directly above and/or below the plane of the flavylum nucleus (see Fig. 3), sharing electrons with this part of the molecule, thus adding strength to the electron density of the molecule as a whole and helping to resist attack of the B-ring by water.

In a diacylated anthocyanin, it is conceivable that the two acyl groups could provide a "sandwich" effect which protect the flavylum nucleus from attack from both above and below. In the case of mono-acylated anthocyanins, however, the acyl group of one anthocyanin molecule could only sterically protect that molecule from one side. The only way to sterically protect both sides of the anthocyanin at once would be if two or more molecules were to associate, with the two flavylum nuclei close together and with one acyl group from each molecule on either side.

It is possible that both the steric and electronic effects could contribute to stability. In order to determine which plays the dominant role, the Hammett constants for the substituents on the different acyl groups were used. Hammett constants are a measure of the electron donating or withdrawing capacity of different substituents, and are based

on empirical measurements of the ionization of substituted benzoic acids in water at 25° C. Benzoic acid, by definition, is given a Hammett constant of 0. The Hammett equation is

$$\log (k/k_0) = \sigma p,$$

where

k_0 = ionization constant of the unsubstituted benzene derivative

k = ionization of the substituted benzene derivative

σ = Hammett constant

p = proportionality constant, dependent on the nature of the reaction and conditions.

Hammett constants are not applicable in the case of *ortho*-substituted compounds, where the substituents are close to the reaction center. Where there is more than one substituent, the Hammett constants are additive (Taft, 1956).

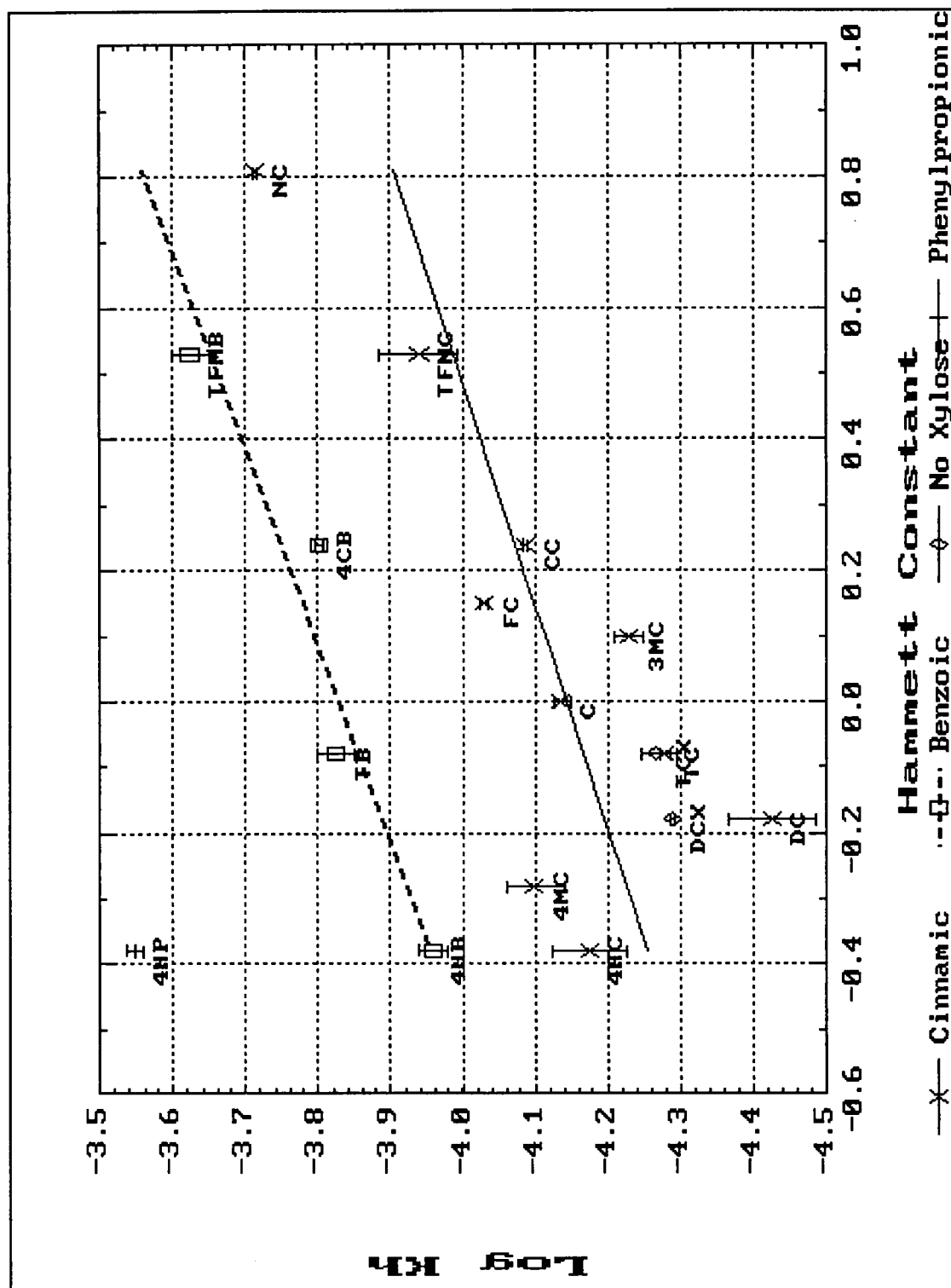
A list of the Hammett constants for the substituents used in this study are given in Table 13.

In order to evaluate the electronic effects of the acyl groups on K_h , K_a , k_1 , and k_2 , the log of each of these constants was plotted against the Hammett constants for the substituents of the acyl group for each compound. In each graph, the compounds acylated with cinnamic acids, with benzoic acids, with cinnamic acids minus xylose, and with 3-(4-hydroxyphenyl)propionic acid will be examined separately so that the only difference within a category is the ring

Table 13. Selected Hammett Constants for substituted benzene derivatives (March, 1992). Electron withdrawing capacity is proportional to Hammett value.

Group	<i>para</i> -	<i>meta</i> -
N(Me) ₂	-0.63	-0.10
OH	-0.38	0.13
OMe	-0.28	0.10
H	0	0
F	0.15	0.34
Cl	0.24	0.37
CF ₃	0.53	0.46

substituents of the acyl group. The reason for using the log plot is that the plot of $\log k$ vs. Hammett constant for a series of compounds that differ only in ring substituents should give a straight line (Taft, 1956). If the points fall in a straight line, this would show that only electronic effects are involved in modifying the characteristics of the reaction; if they do not, then other factors (i.e., steric effects) are involved. The plots of $\log K_h$, K_a , k_1 , and k_2 vs. Hammett constants are given in figs. 10 - 13, and the line of best fit by method of least sum of squares is shown in each graph. The regression data is given in Table 14, including the regression error, which is the sum of the squares of the deviation of the points from the line of best fit to the data. The following discussion is an evaluation of each of these plots.



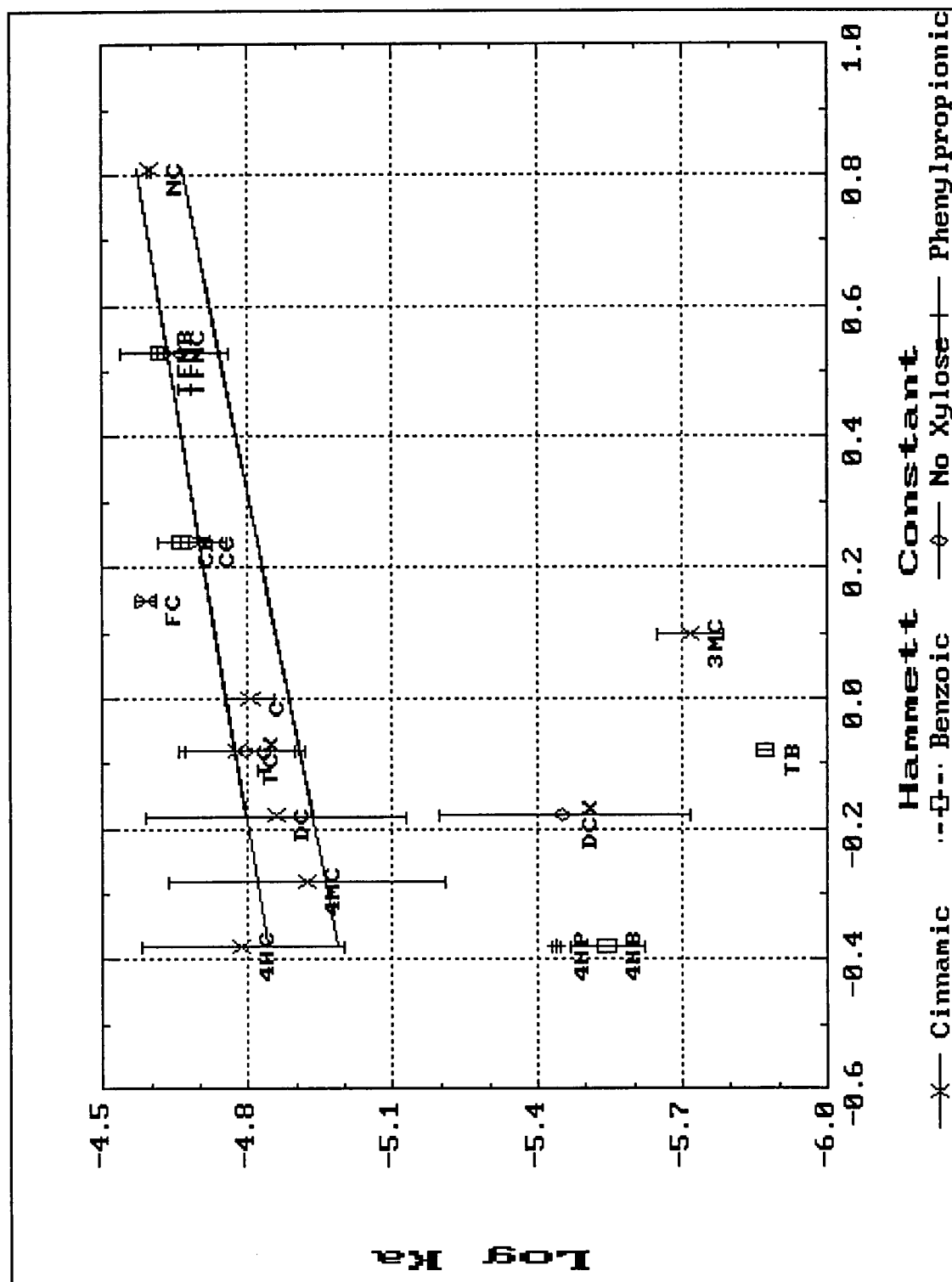


Fig. 11. $\text{Log } K_a$ vs. Hammett constants for anthocyanins acylated with cinnamic, benzoic, and phenylpropionic acids. Regression lines are for cinnamoyl compounds that are mono-substituted and those that are 4-substituted.

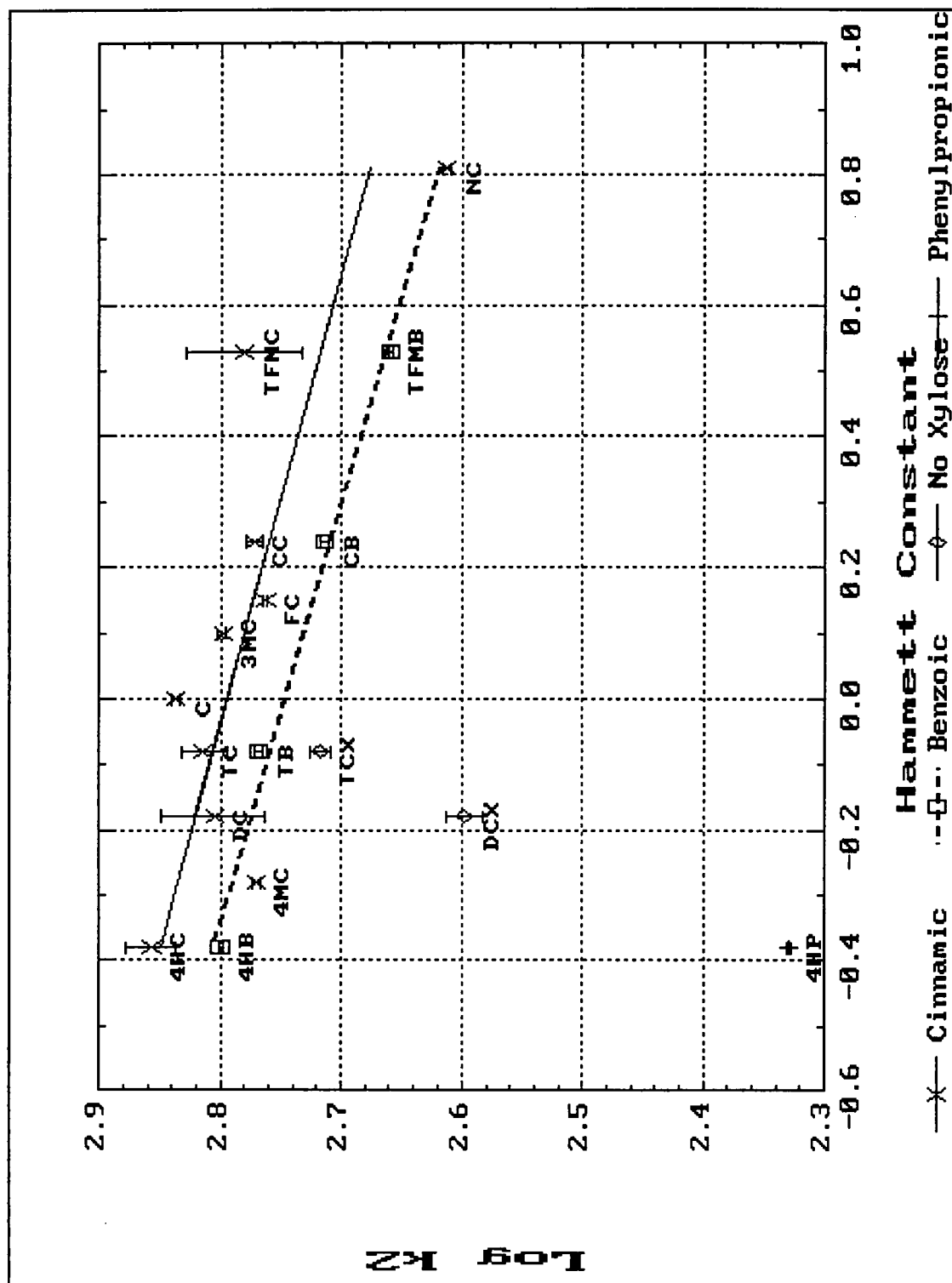


Fig. 13. $\log k_2$ vs. Hammett constants for anthocyanins acylated with cinnamic, benzoic, and phenylpropionic acids. The regression line of the cinnamoyl compounds was calculated using the mono-substituted compounds only.

Table 14. Regression data for log K 's and log k 's vs. Hammett constants

Acyl Group		K_h	K_a	k_1	k_2
Benzoyl (using all points)	Intercept	-3.83	n/a	-1.08	2.75
	Slope	0.34	n/a	0.18	-0.16
	Reg.Err.	0.05	n/a	0.05	0.01
Cinnamoyl (using all points w/ xylose)	Intercept	-4.15		-1.36	
	Slope	0.29		0.27	
	Reg.Err.	0.15		0.11	
Cinnamoyl (mono-substituted only)	Intercept		-4.89		2.79
	Slope		0.27		-0.15
	Reg.Err.		0.38		0.05
Cinnamoyl (4-substituted only)	Intercept		-4.76		
	Slope		0.23		
	Reg.Err.		0.08		

1. Log K_h vs. Hammett constant

The graph of log K_h vs. Hammett constants (Fig. 10) for the benzoyl series is linear, showing that electronic effects due to the substituents cause the changes in K_h . The same pattern holds for the cinnamoyl series, but there is more scatter of the points and the regression error is larger than in the benzoyl series (see Table 14). Thus electronic effects due to the substituents cause part but not all of the changes in K_h of the cinnamoyl compounds, indicating that other factors are also important in these. If all the cinnamoyl compounds were moved as close to the regression line as their standard deviations would allow, however, these would be very close to linear, thus accounting for K_h on the basis of electron density alone. A comparison of the two series with one another shows 1) similarity of relative K_h 's of each compound within the series, with the slopes of the two lines roughly parallel, and 2) that the K_h for the benzoyl compounds is larger than K_h for the corresponding cinnamoyl compounds--i.e. benzoyl compounds are less stable than cinnamoyl compounds. The highly stable 3,4,5-substituted compounds (DC, DCX, TC, and TCX) are all clustered at the lower end of the graph; this indicates the presence of an effect other than electron-donating capacity. However, the standard deviation for DC (represented by the error bars in the graph) allows the possibility that this compound is actually much closer to the regression line.

The points for the compounds without xylose are close to the corresponding compounds with xylose in this graph. This indicates that xylose does not have much effect on K_h . The compound with the 3-(4-hydroxyphenyl)propionyl group (4HP) had

a much higher K_h than the 4-hydroxycinnamoyl compound (4HC), with the 4-hydroxybenzoyl compound (4HB) in-between. This indicates that 1) the length of the carbon chain and 2) the presence of the double bond in the organic acid is important in stability.

2. Log K_a vs. Hammett constant

In the benzoyl compounds, the relationship between log K_a and Hammett constants was not linear (Fig. 11). This indicates that the electronic effects of the substituents on the benzoyl ring are not affecting K_a significantly.

For the 4-substituted cinnamoyl compounds, however, the relationship was linear (Fig. 11). The 3,4,5- substituted compounds, except DCX, also fit in with these (DCX would be much closer if the upper value allowed by its standard deviation were used). The *meta*- compound had a K_a far less than any of the other cinnamoyl compounds. For this compound, electronic effects do not seem to be the factor governing its K_a .

The 3,4,5-trimethoxycinnamoyl compound (TC) was very close to its counterpart without xylose (TCX) but the 4-hydroxy-3,5-dimethoxycinnamoyl compound (DC) was separated by a significant distance from the corresponding compound minus xylose (DCX). DCX was the only compound measured that did not reach equilibrium within 2 minutes. It also has the lowest k_1 and a relatively low k_2 . The reason(s) for this is not immediately apparent; perhaps the xylose residue (Fig. 5) sterically hinders the acyl group's complexation with the flavylum nucleus, so removal of the xylose residue actually improves stability.

3. Log k_1 vs. Hammett constant

This plot (Fig. 12) shows a linear relationship in the benzoyl series. This shows that the electron density of the benzoyl group completely accounts for the changes in the rate constant of the reaction forming the hemiacetal and thus color loss. The k_1 's are also increasing in the same direction as K_h .

The cinnamoyl series (Fig. 12) is also roughly linear, but with more scatter than the benzoyl series. This means there are some electronic effects, but that other factors are also important (there is enough error in the standard deviations of some of these to allow them the possibility of being closer to the regression line, however). The k_1 's are increasing in the same direction as K_h , as with the benzoyl compounds, and the tri-substituted compounds that were clustered at the bottom right corner of the log K_h vs. Hammett constant plot are clustered in the same corner in this graph. The slope of the line of best fit for the cinnamoyl compounds is about one and a half times the slope of the benzoyl line, but the benzoyl compounds can be seen to approximately parallel their cinnamoyl counterparts.

These observations suggest 1) a very strong correlation between electron donating capability and the forward hydration reaction of flavylum ion to hemiacetal in anthocyanins acylated with both benzoic and cinnamic acids, and 2) the absence of a carbon chain in the benzoic acid compounds increases the rate of this reaction. The k_1 for the

phenylpropionoyl compound, however, was between the k_1 's of the corresponding cinnamoyl and benzoyl compounds.

The compounds without xylose were consistently lower than the same compounds with xylose, but not by much. The conclusion that can be drawn from this is that xylose does not greatly affect the forward rate of the reaction.

4. Log k_2 vs. Hammett constant

The last graph (Fig. 13) shows a high linearity among the benzoyl series. The slope is negative, as would be expected (the k_2 's would be expected to vary in the opposite direction as k_1 as K_h increased, because $K_h = k_1/k_2$).

The mono-substituted cinnamoyl series is also linear, and the slope of the graph is close to that of the benzoyl series. The intercept positions of these 2 series are reversed from those in the K_h and k_1 graphs, showing that the rate constant for hemiacetal change is always lower for the benzoyl series than for the 4-substituted cinnamoyl series.

These observations reflect 1) a negative relationship between the Hammett constant and the rate of the reverse reaction from hemiacetal to flavylum within a series, which show that the electronegativity of the substituents is the principal factor controlling k_2 within these series, and 2) cinnamoyl compounds undergo the reverse reaction much more rapidly than the corresponding benzoyl ones. The reason for this could be that the presence of the additional double-bonded carbon atoms in the cinnamoyl acyl group is just the right length so as to permit the most efficient association

between the acyl group and the flavylum nucleus, which would favor the reaction of hemiacetal to flavylum ion in this species much more favorably than in the benzoyl compounds.

The 4-hydroxy-3,5-dimethoxy compound without xylose (DCX) had a lower k_2 than all but the 3-(4-hydroxyphenyl)propionyl compound. This compound (DCX) has proven to be aberrant in several ways, and was the only compound that did not reach equilibrium in 2 minutes. The other compound without xylose (TCX) is also lower than any of the other cinnamoyl compounds. This indicates that xylose does decrease the reverse reaction rate.

The k_2 for the 3-(4-hydroxyphenyl)propionic acid compound was much lower than any of the other compounds, which shows that it has the slowest reverse reaction. This is what would be expected, based on this compounds' high K_h .

E. Ortho-, meta-, and para- methoxycinnamic compounds

There are several factors that influence the comparison between the 2-, 3-, and 4- substituted methoxycinnamic acid compounds. One of these is the electron donating capacity, which is -0.28 for the para- substituted compound and +0.10 in the meta- substituted one, and is not even applicable in ortho- compounds. For the ortho- substituents, another factor is steric effects, with the resulting bond strains and free energy differentials that these entail.

From the data (Table 12), the K_h 's and k_1 's of these compounds are ordered 4MC > 3MC > 2MC, and the k_2 's are in the opposite order. This is consistent with the definition $K_h =$

k_1/k_2 . The K_a 's of the compounds are $2MC > 4MC > 3MC$. Thus, for this reaction the effects of the position of the methoxy group are different from the effects on K_h . It is a notable fact that the K_a for 3MC was the lowest one of all.

F. Stability of compounds with 3,5-Dimethoxy substituents

The last comparison is between those compounds that have acyl groups with 4-hydroxy and 4-methoxy substituents vs. those that have these plus 3,5-dimethoxy groups. These compounds are 4HC vs. DC and 4MC vs. TC. The prediction based on Hammett constants is that the extra *meta*-methoxy groups decrease electron donating potential of the *para*-substituent, because the Hammett constants are additive and the positive value of the *meta*-methoxy groups will decrease the total electron donating capacity of the 4-hydroxy and 4-methoxy substituents.

In both cases, however, the opposite seems to be the case. The K_h 's and k_1 's for these compounds are in the order $4HC > DC$ and $4MC > TC$. The K_a 's of each compound are probably not significantly different. For the k_2 's, $TC > 4MC$ and 4HC is probably not significantly different from DC. The relative sizes of k_1 and k_2 are consistent with the relative sizes of K_h amongst these 4 compounds given that $K_h = k_1/k_2$.

G. Summary and Conclusions

The conclusions from these comparisons are that the length of the carbon chain, as well as the presence of the double bond, are important in stability. Also, the electronegativity of the substituents of the acyl group can be directly correlated to K_h , k_1 , and k_2 in the benzoyl series,

and partially correlated to all the rate and equilibrium constants in the cinnamoyl series. The most probable explanation for the divergence from direct linearity in this series is steric effects. The presence of the xylose residue in the cinnamoyl series has some effect, but does not affect overall stability (measured by $K_h + K_a$) significantly.

Based on these observations, a prediction for the most stable mono-acylated anthocyanin (based on a measure of its K_h) would be one with cinnamoyl acyl group with an -OH substituent in the 4-position, and *tert*-butyl substituents in the 3- and 5-positions; the *meta*-C(Me)₃ group has a Hammett constant of -0.09. Although there are *meta*- groups with higher Hammett constants (the Hammett constants for *meta*-O⁻ and *meta*-N(Me)₂ are -0.47 and -0.10, respectively), these groups are ionizable. The dimethylamino groups would probably behave similarly as the one on the anthocyanin acylated with 4-(N-dimethylamino)cinnamic acid, which should have been the most stable according to the Hammett constant for the *ortho*-N(Me)₂ group, which is -0.81, but it instead appeared to be one of the least stable anthocyanins (no constants were tabulated for this compound; see Appendix 14). This was probably due to de-ionization of the N(Me)₂ group as pH increased. The O⁻ group would have the opposite effect, ionizing at higher pH's, but at a pH high enough to cause ionization of this group (which would be at a pH above 7) the anthocyanin would already have been converted almost completely out of the flavylum ion form.

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Appendix

Index to Appendix

Abbreviation	Acyl Group	Appendix #
DC (4H35DCIN)	4-hydroxy-3,5-dimethoxy-cinnamic acid	1
DCX (4H35DC-X)	4-hydroxy-3,5 dimethoxy-cinnamic acid (minus xylose)	2
TC (345TCIN)	3,4,5-trimethoxycinnamic acid	3
TCX (345TC-X)	3,4,5-trimethoxycinnamic acid (minus xylose)	4
2MC (2MCIN)	2-methoxycinnamic acid	5
3MC (3MCIN)	3-methoxycinnamic acid	6
4MC (4MCIN)	4-methoxycinnamic acid	7
4HC (4HCIN)	4-hydroxycinnamic acid	8
C (CIN)	cinnamic acid	9
CC (4CCIN)	4-chlorocinnamic acid	10
FC (4FCIN)	4-fluorocinnamic acid	11
TFMC (4TFMC)	4-trifluoromethylcinnamic acid	12
NC (4NITRCIN)	4-nitrocinnamic acid	13
DMA (4DMACIN)	4-(N-dimethylamino)cinnamic acid	14
4HP (4HPPROP)	3-(4-hydroxyphenyl)propionic acid	15
4HB (4HBENZ)	4-hydroxybenzoic acid	16
TB (345TBENZ)	3,4,5-trimethoxybenzoic acid	17
CB (4CBENZ)	4-chlorobenzoic acid	18
TFMB (4TFMB)	4-trifluoromethylbenzoic acid	19
CYANGXG	none	20
CYANGX	none, minus glucose	21

Appendix 1. Regression Data for DC
(Anthocyanin acylated with 4-hydroxy-3,5-dimethoxycinnamic acid)

Compound: DC (#5)
 Experiment #: 1249
 Date: 1/26/96
 Dilution: 1:400
 Reaction Time: 2 min.
 Wavelength: 500 nm
 NaOH Conc. Used: 0.02 - 0.07 M
 Comments: Note differences between graphs in
 this compound vs. 1263, CYANGXG.

Calculation #1:		Calculation #2:	
Linear Regression		Linear Regression	
[D ₀ /(D ₀ -D) vs. 10 ^{-pH}]		[(K _b +K _a +10 ^{-pH})/k vs. 10 ^{pH}]	
[w/ 0.02 - 0.06 points]			
Intercept	1.57	Intercept	1.42 x 10 ⁻³
Slope	29307	Slope	1.95 x 10 ⁻⁸
Count	12	Count	10
Kh+Ka (from calc. #1) =	5.35 x 10 ⁻⁵		
Kh = (Kh+Ka) - Ka, =	3.97 x 10 ⁻⁵		
Ka = slope/intercept, =	1.38 x 10 ⁻⁵		
k1 = Kh*k2, =	0.0280		
k2 = 1/intercept, =	706		

=====

Compound: DC (#7)
 Experiment #: 1249
 Date: 4/10/96
 Dilution: 1:400
 Reaction Time: 2 min.
 Wavelength: 500 nm
 NaOH Conc. Used: 0.015 - 0.05 M
 Comments:

Calculation #1:		Calculation #2:	
Linear Regression		Linear Regression	
[D ₀ /(D ₀ -D) vs. 10 ^{-pH}]		[(K _b +K _a +10 ^{-pH})/k vs. 10 ^{pH}]	
[Using 0.02 - 0.05 points]			
Intercept	1.59	Intercept	1.66 x 10 ⁻³
Slope	31559		

Appendix 1. DC (cont.)

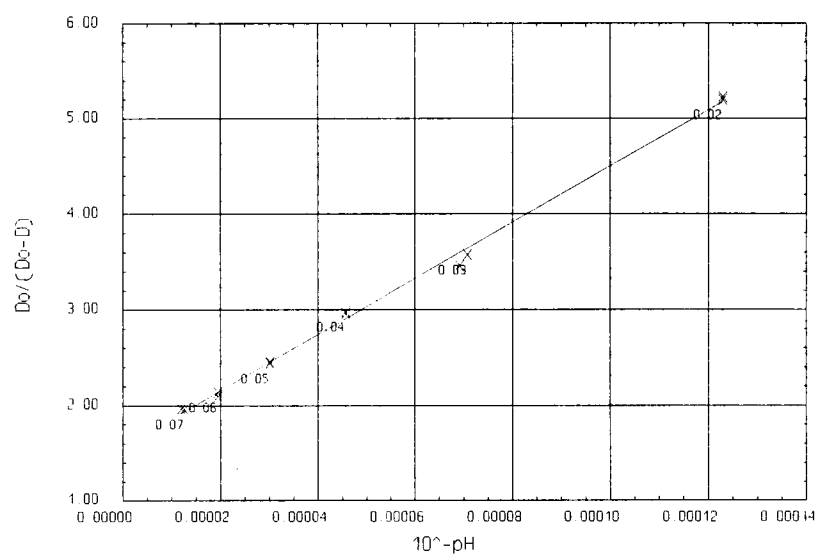
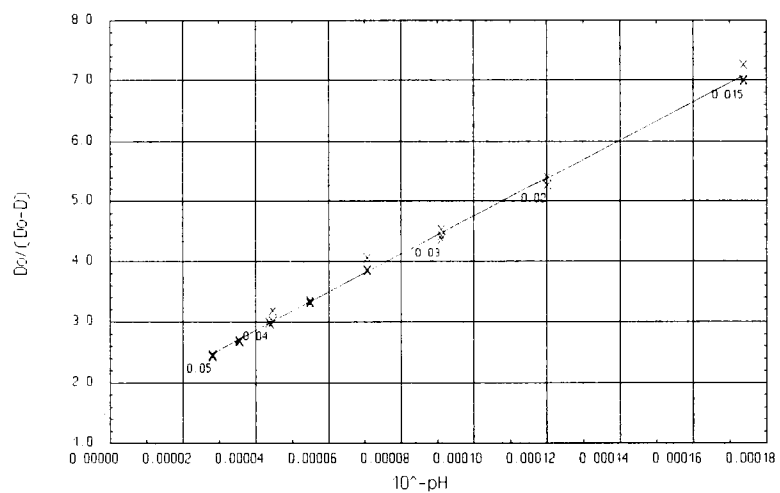
Compound: DC (#8)
 Experiment #: 1249
 Date: 4/12/96
 Dilution: 1:400
 Reaction Time: 2 min.
 NaOH Conc. Used: 0.015 - 0.06 M
 Wavelength: 500 nm
 Comments:

Calculation #1:
 Linear Regression
 $[D_0/(D_0-D) \text{ vs. } 10^{-\text{pH}}]$
 $[w/ 0.025 - 0.06 \text{ pts}]$
 Intercept 1.34
 Slope 34335
 Count 20

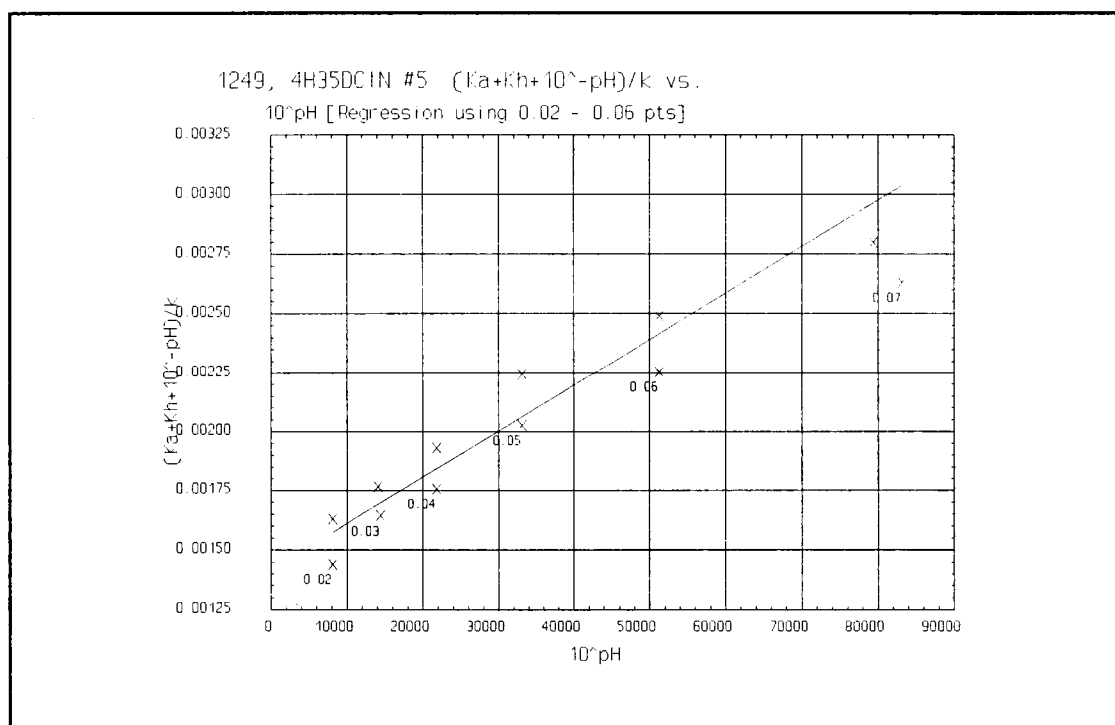
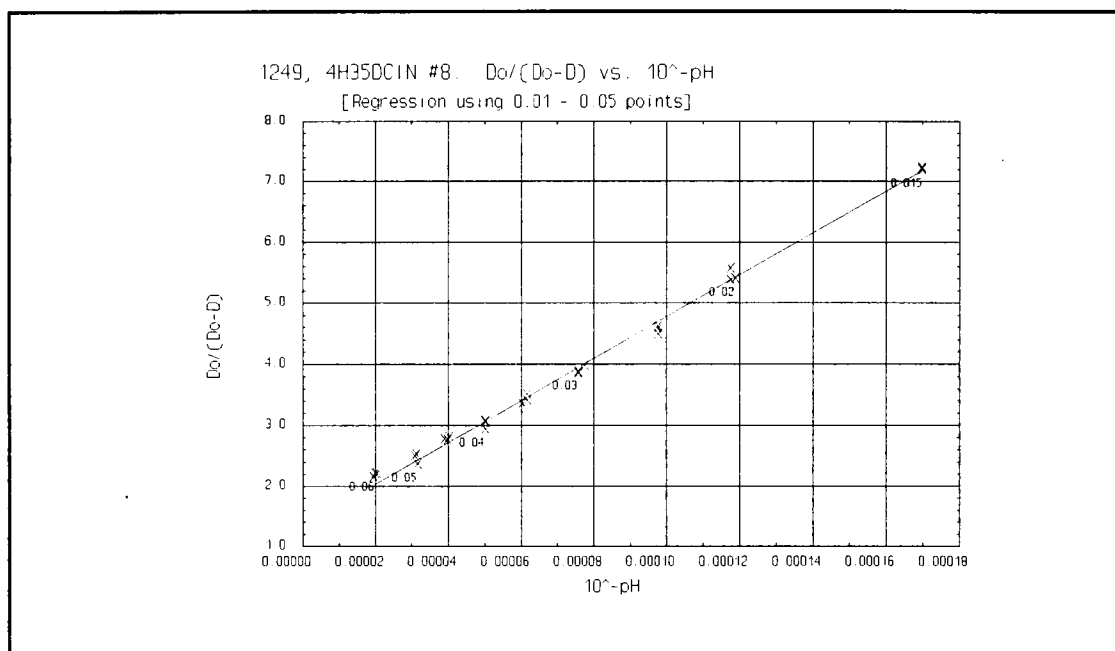
Calculation #2:
 Linear Regression
 $[(K_h+K_a+10^{-\text{pH}})/k \text{ vs. } 10^{\text{pH}}]$
 $[w/ 0.015 - 0.05 \text{ points}]$
 Intercept 1.69×10^{-3}
 Slope 1.12×10^{-8}
 Count 27

K_h+K_a (from calc. #1) = 3.91×10^{-5}
 $K_h = (K_h+K_a) - K_a$, = 3.25×10^{-5}
 $K_a = \text{slope/intercept}$, = 0.66×10^{-5}
 $k_1 = K_h \cdot k_2$, = 0.0192
 $k_2 = 1/\text{intercept}$, = 590

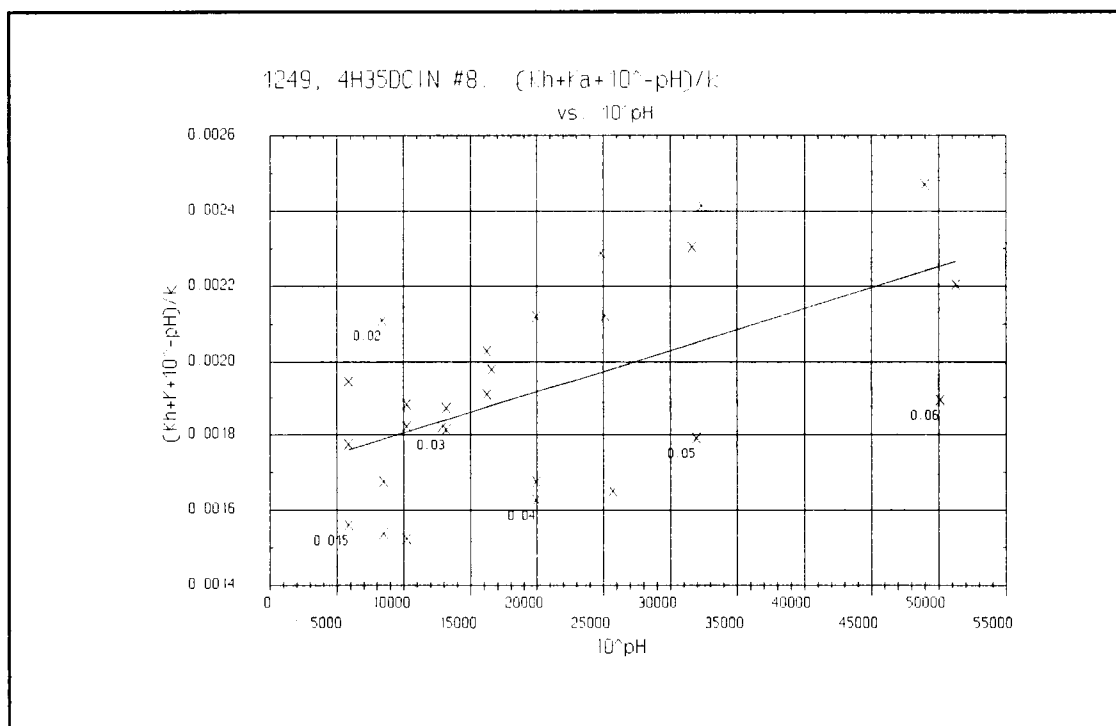
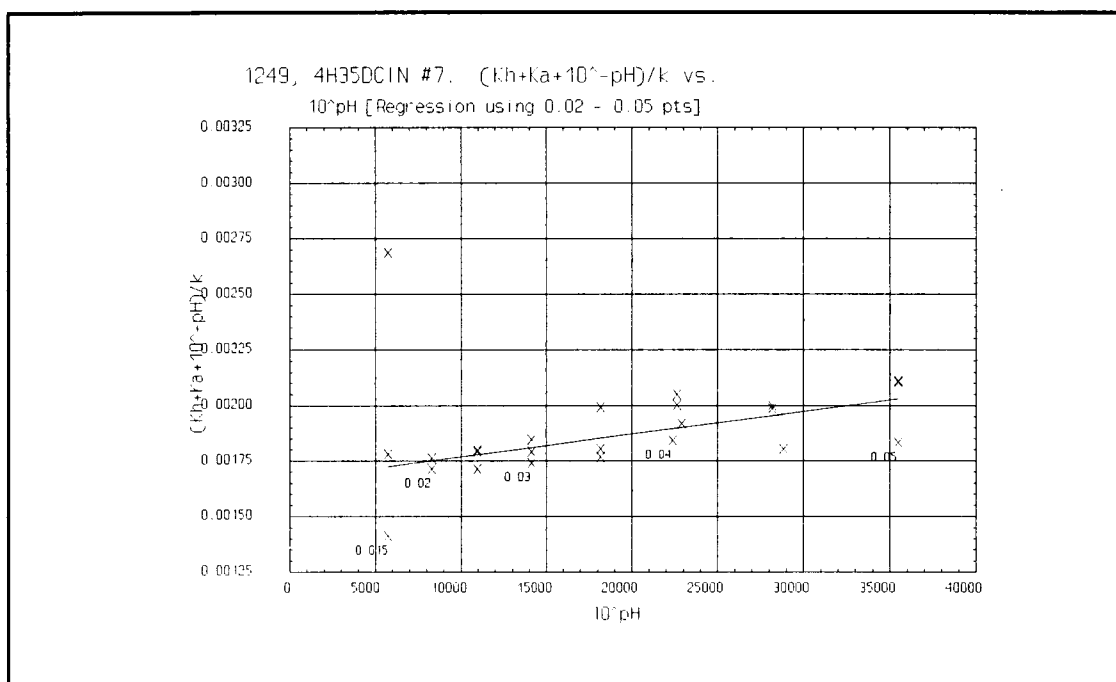
Appendix 1. DC (cont.)

1249, 4H35DCIN #5. $D_o/(D_o-D)$ vs. 10^{-pH} 1249, 4H35DCIN #7. $D_o/(D_o-D)$ vs. 10^{-pH} 

Appendix 1. DC (cont.)



Appendix 1. DC (cont.)



Appendix 2. Regression Data for DCX
(Anthocyanin acylated with 4-hydroxy-3,5-dimethoxycinnamic acid, minus xylose)

Compound: DCX (#3)
 Experiment #: 1349
 Date: 8/13/96
 Dilution: 1:120
 Reaction Time: 5 min.
 Wavelength: 500 nm
 NaOH Conc. Used: 0.015 - 0.05 M
 Comments: Only compound that did not reach equilibrium in 5 mins.

Calculation #1:	Calculation #2:
Linear Regression	Linear Regression
$[D_0/(D_0-D) \text{ vs. } 10^{-\text{pH}}]$	$[(K_h+K_a+10^{-\text{pH}})/k \text{ vs. } 10^{\text{pH}}]$
	[w/o 0.02#1 & 0.05 #1 pts.]
Intercept 1.38	Intercept 2.42×10^{-3}
Slope 27162	Slope 0.87×10^{-8}
Count 24	Count 22

K_h+K_a (from calc. #1) =	5.07×10^{-5}
$K_h = (K_h+K_a) - K_a$, =	4.72×10^{-5}
$K_a = \text{slope/intercept}$, =	0.358×10^{-5}
$k_1 = K_h \cdot k_2$, =	0.0195
$k_2 = 1/\text{intercept}$, =	414

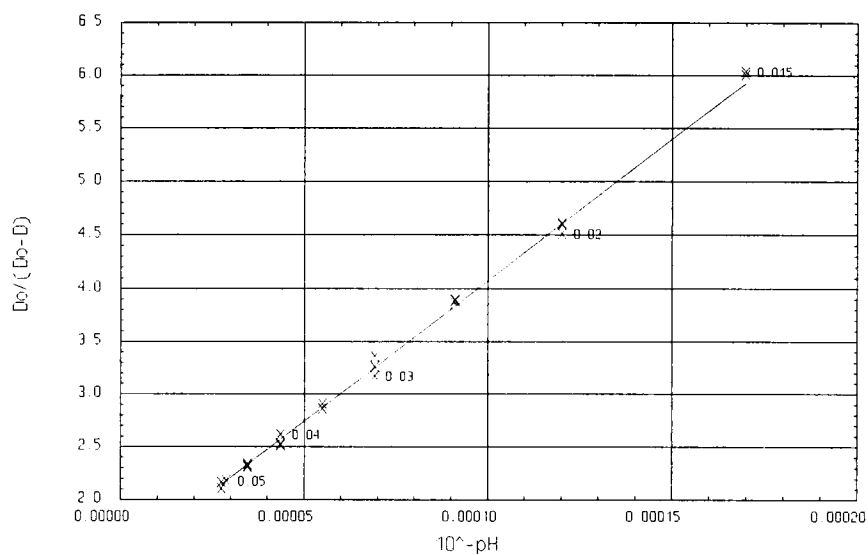
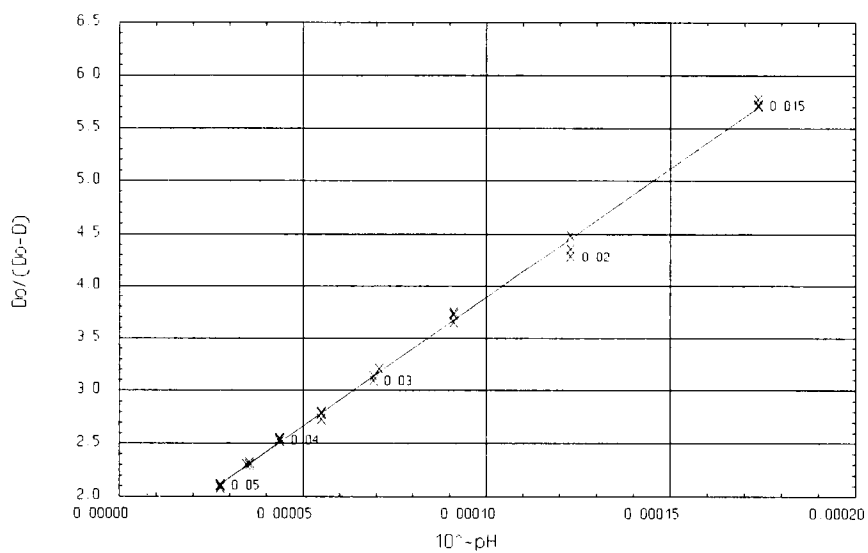
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Compound: DCX (#4)
 Experiment #: 1349
 Date: 8/14/96
 Dilution: 1:120
 Reaction Time: 5 min.
 Wavelength: 500 nm
 NaOH Conc. Used: 0.015 - 0.05 M
 Comments: Only compound that did not reach equilibrium in 2 mins.

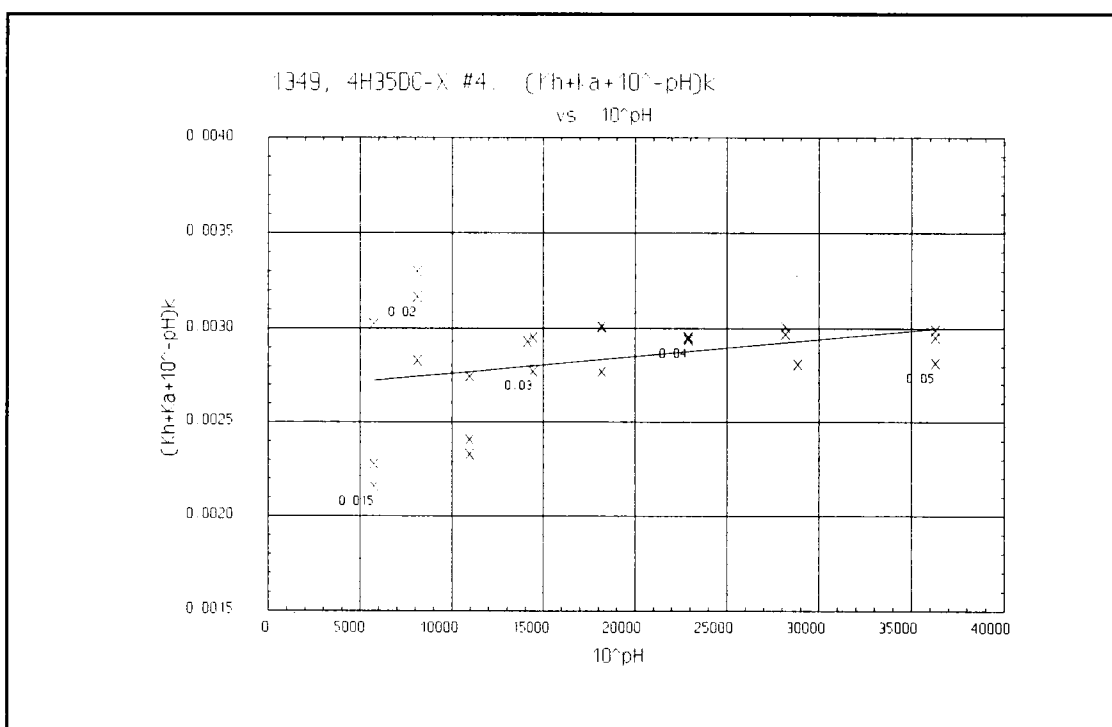
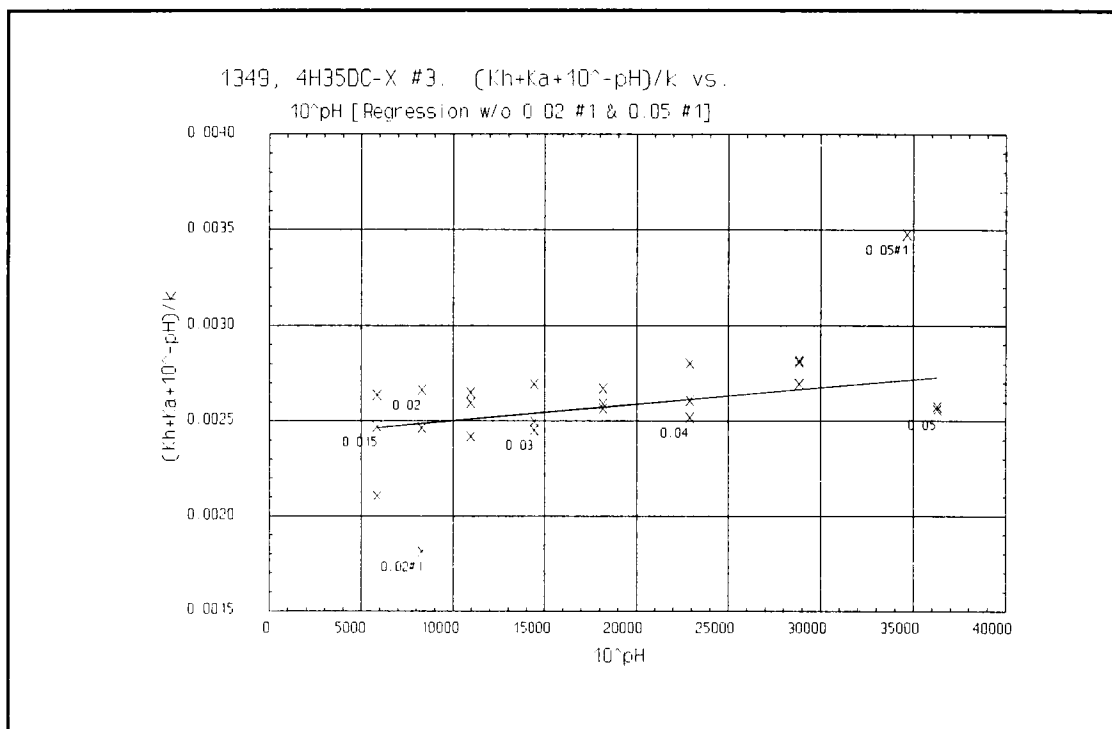
Calculation #1:	Calculation #2:
Linear Regression	Linear Regression
$[D_0/(D_0-D) \text{ vs. } 10^{-\text{pH}}]$	$[(K_h+K_a+10^{-\text{pH}})/k \text{ vs. } 10^{\text{pH}}]$
Intercept 1.44	Intercept 2.67×10^{-3}
Slope 24521	Slope 0.90×10^{-8}
Count 24	Count 24

K_h+K_a (from calc. #1) =	5.86×10^{-5}
$K_h = (K_h+K_a) - K_a$, =	5.53×10^{-5}
$K_a = \text{slope/intercept}$, =	0.34×10^{-5}
$k_1 = K_h \cdot k_2$, =	0.0207
$k_2 = 1/\text{intercept}$, =	374

Appendix 2. DCX (cont.)

1349, 4H35DC-X #3. $D_o/(D_o-D)$ vs. 10^{-pH} 1349, 4H35DC-X #4. $D_o/(D_o-D)$ vs. 10^{-pH} 

Appendix 2. DCX (cont.)



Appendix 3. Regression Data for TC
(Anthocyanin acylated with 3,4,5-trimethoxycinnamic acid)

Compound: TC (#1)
 Experiment #: 1314
 Date: 4/1/96
 Dilution: 1:150
 Reaction Time: 2 min.
 Wavelength: 500 nm
 NaOH Range: 0.01 - 0.05 M
 Comments:

Calculation #1:		Calculation #2:	
Linear Regression		Linear Regression	
[D ₀ /(D ₀ -D) vs. 10 ^{-pH}]		[(K _a +K _a +10 ^{-pH})/k vs. 10 ^{pH}]	
[w/ 0.015 - 0.045 points]		[w/ 0.015 - 0.045 points]	
Intercept	1.51	Intercept	1.38 x 10 ⁻³
Slope	23095	Slope	2.16 x 10 ⁻⁸
Count	28	Count	22

Kh+Ka (from calc. #1) = 6.55 x 10⁻⁵
 Kh = (Kh+Ka) - Ka, = 4.98 x 10⁻⁵
 Ka = slope/intercept, = 1.57 x 10⁻⁵
 k1 = Kh*k2, = 0.0361
 k2 = 1/intercept, = 726

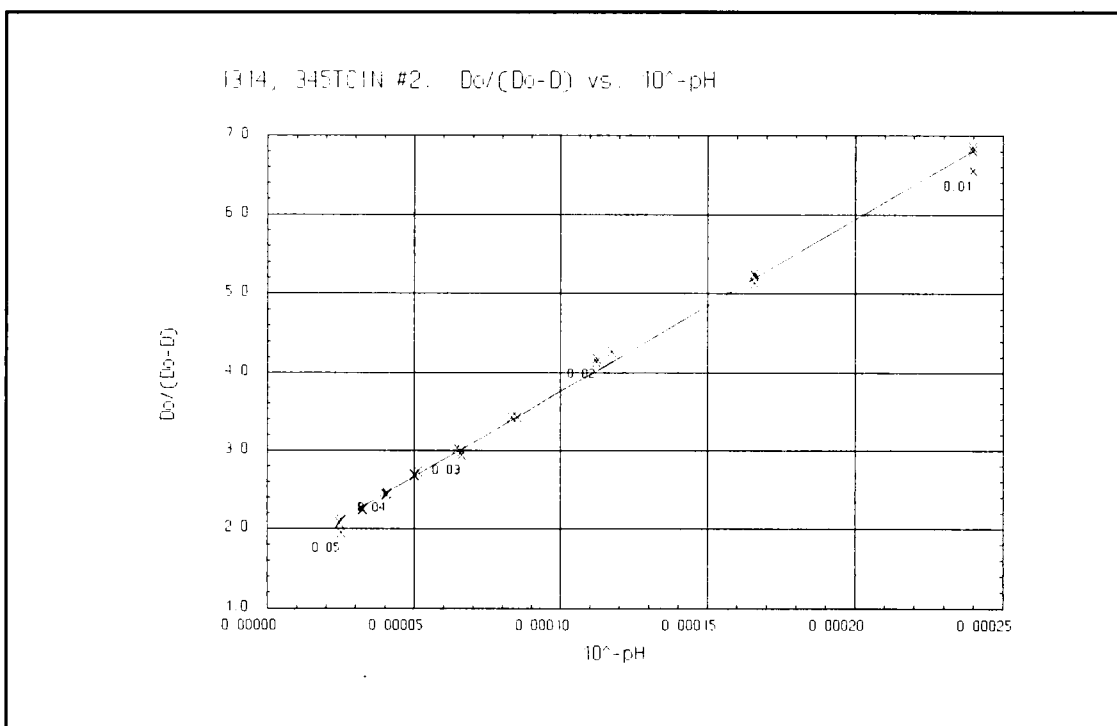
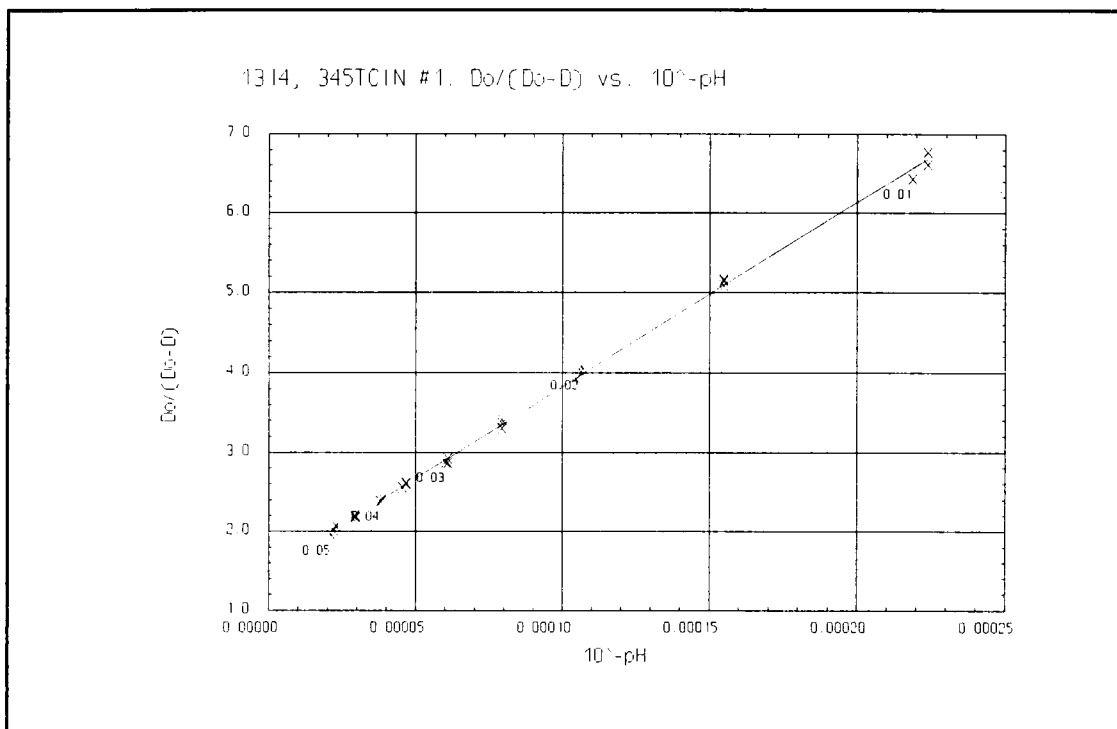
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Compound: TC (#2)
 Experiment #: 1314
 Date: 4/8/96
 Dilution: 1:150
 Reaction Time: 2 min.
 Wavelength: 500 nm
 NaOH Range: 0.01 - 0.05 M
 Comments:

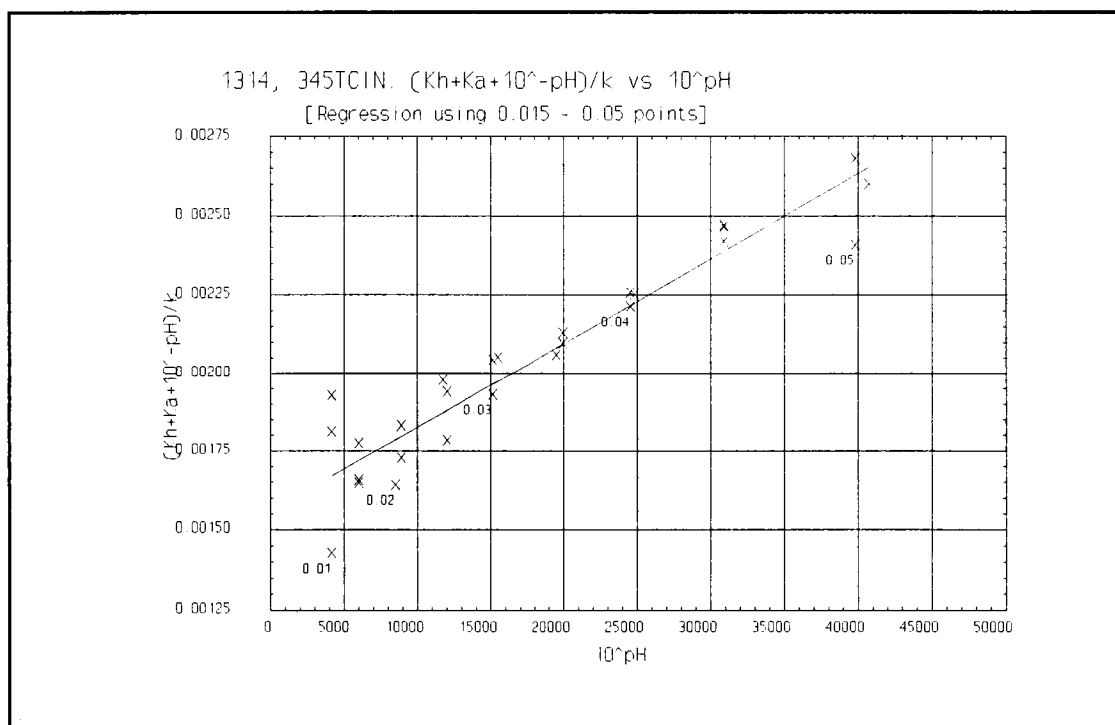
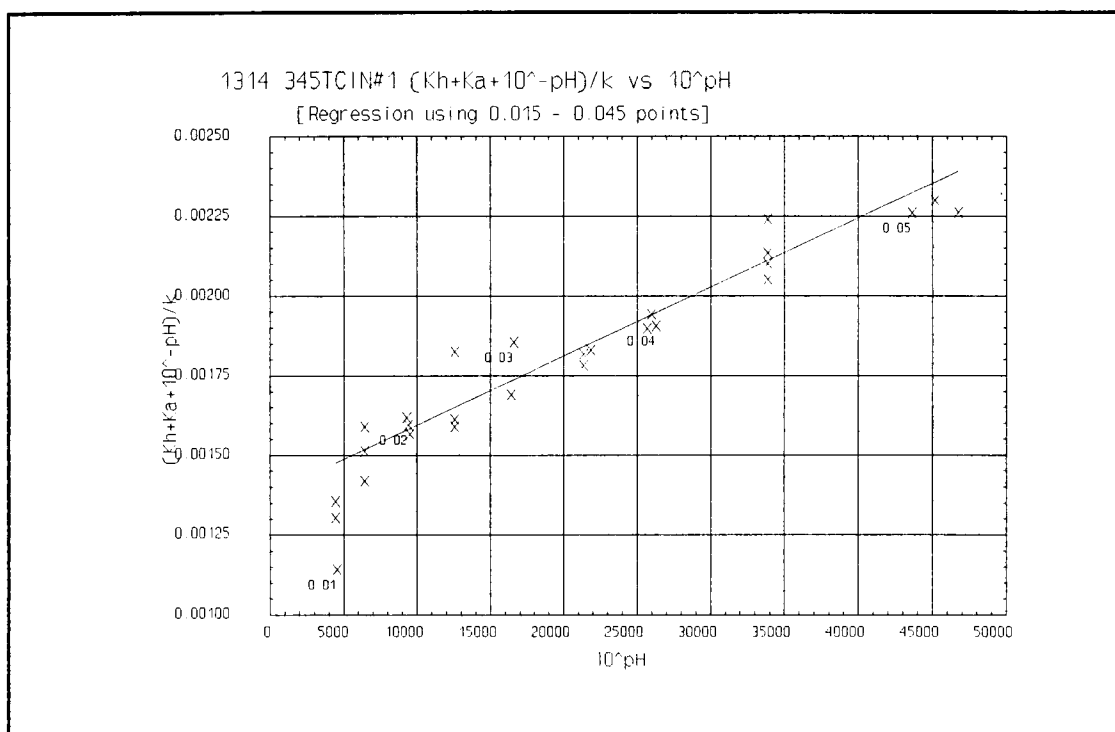
Calculation #1:		Calculation #2:	
Linear Regression		Linear Regression	
[D ₀ /(D ₀ -D) vs. 10 ^{-pH}]		[(K _a +K _a +10 ^{-pH})/k vs. 10 ^{pH}]	
[w/ 0.015 - 0.05 points]		[w/ 0.015 - 0.05 points]	
Intercept	1.57	Intercept	1.56 x 10 ⁻³
Slope	21807	Slope	2.69 x 10 ⁻⁸
Count	27	Count	24

Kh+Ka (from calc. #1) = 7.22 x 10⁻⁵
 Kh = (Kh+Ka) - Ka, = 5.49 x 10⁻⁵
 Ka = slope/intercept, = 1.72 x 10⁻⁵
 k1 = Kh*k2, = 0.0353
 k2 = 1/intercept, = 642

Appendix 3. TC (cont.)



Appendix 3. TC (cont.)



Appendix 4. Regression Data for TCX
(Anthocyanin acylated with 3,4,5-trimethoxycinnamic acid, minus xylose)

Compound: TCX (#4)
 Experiment #: 1354
 Date: 9/20/96
 Dilution: 1:440
 Reaction Time: 2 mins.
 Wavelength: 500 nm
 NaOH Conc. Used: 0.01 - 0.05 M
 Comments:

Calculation #1:
 Linear Regression
 $[D_0/(D_0-D) \text{ vs. } 10^{-pH}]$

Intercept 1.49
 Slope 20746
 Count 27

Calculation #2:
 Linear Regression
 $[(K_h+K_a+10^{-pH})/k \text{ vs. } 10^{pH}]$
 $[w/ 0.02 - .05 \text{ points}]$

Intercept 1.86×10^{-3}
 Slope 3.54×10^{-8}
 Count 21

K_h+K_a (from calc. #1) = 7.20×10^{-5}
 $K_h = (K_h+K_a) - K_a$, = 5.30×10^{-5}
 $K_a = \text{slope/intercept}$, = 1.90×10^{-5}
 $k_1 = K_h \cdot k_2$, = 0.0285
 $k_2 = 1/\text{intercept}$, = 538

=====

Compound: TCX (#5)
 Experiment #: 1354
 Date: 9/20/96
 Dilution: 1:440
 Reaction Time: 2 mins.
 Wavelength: 500 nm
 NaOH Conc. Used: 0.01 - 0.05 M
 Comments:

Calculation #1:
 Linear Regression
 $[D_0/(D_0-D) \text{ vs. } 10^{-pH}]$

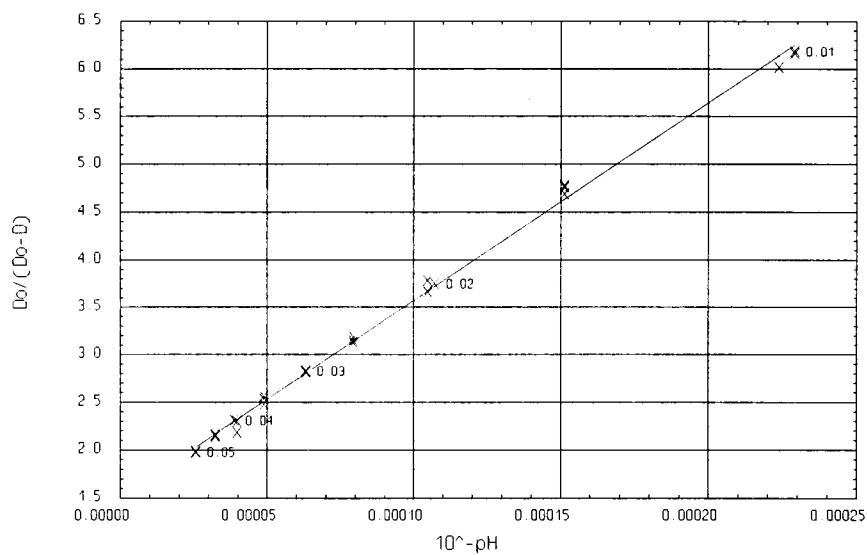
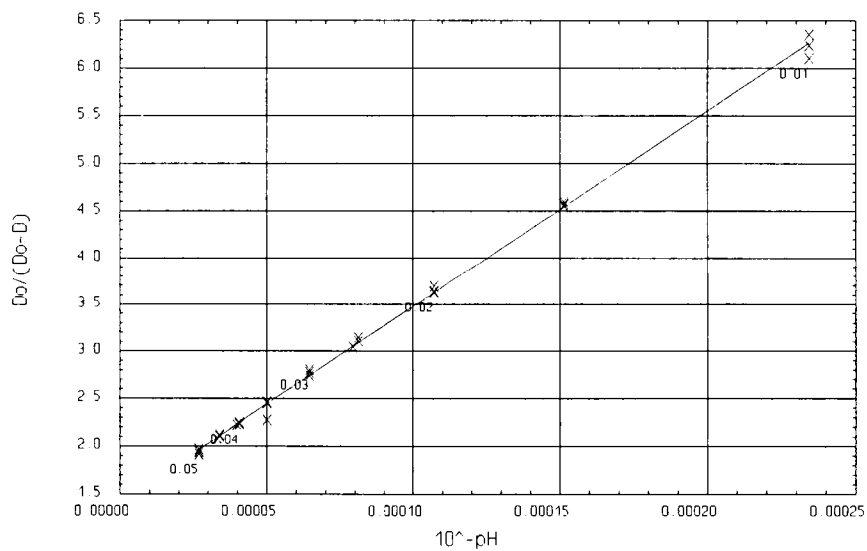
Intercept 1.40
 Slope 20754
 Count 27

Calculation #2:
 Linear Regression
 $[(K_h+K_a+10^{-pH})/k \text{ vs. } 10^{pH}]$
 $[w/ 0.015 - 0.05 \text{ points}]$

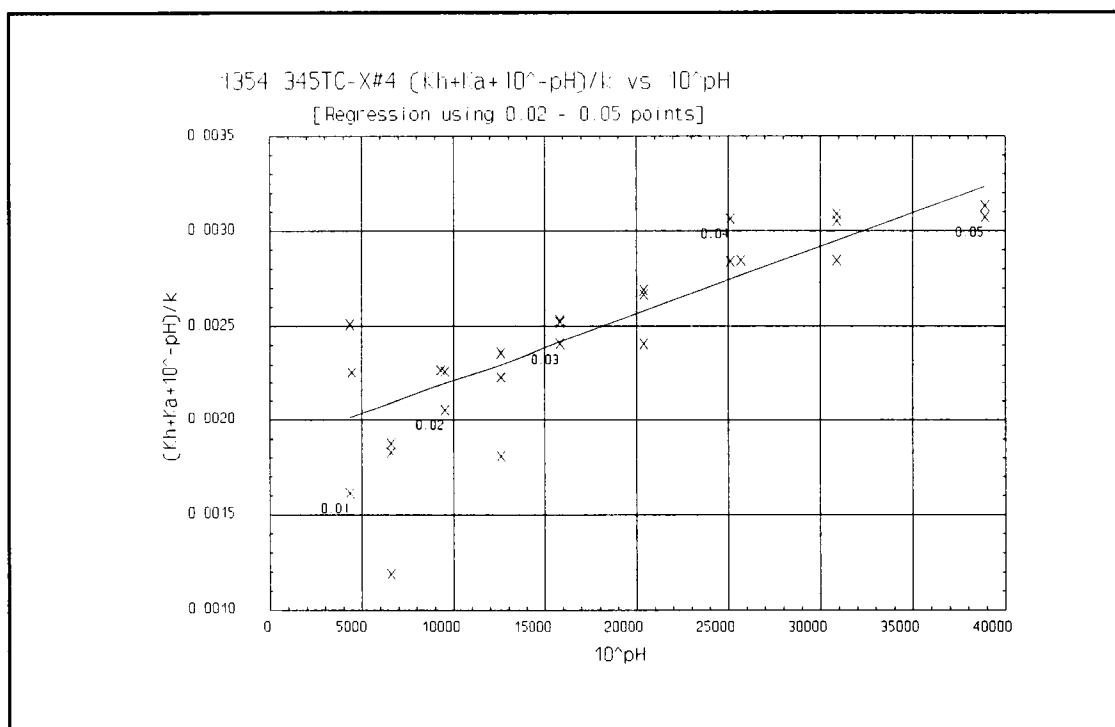
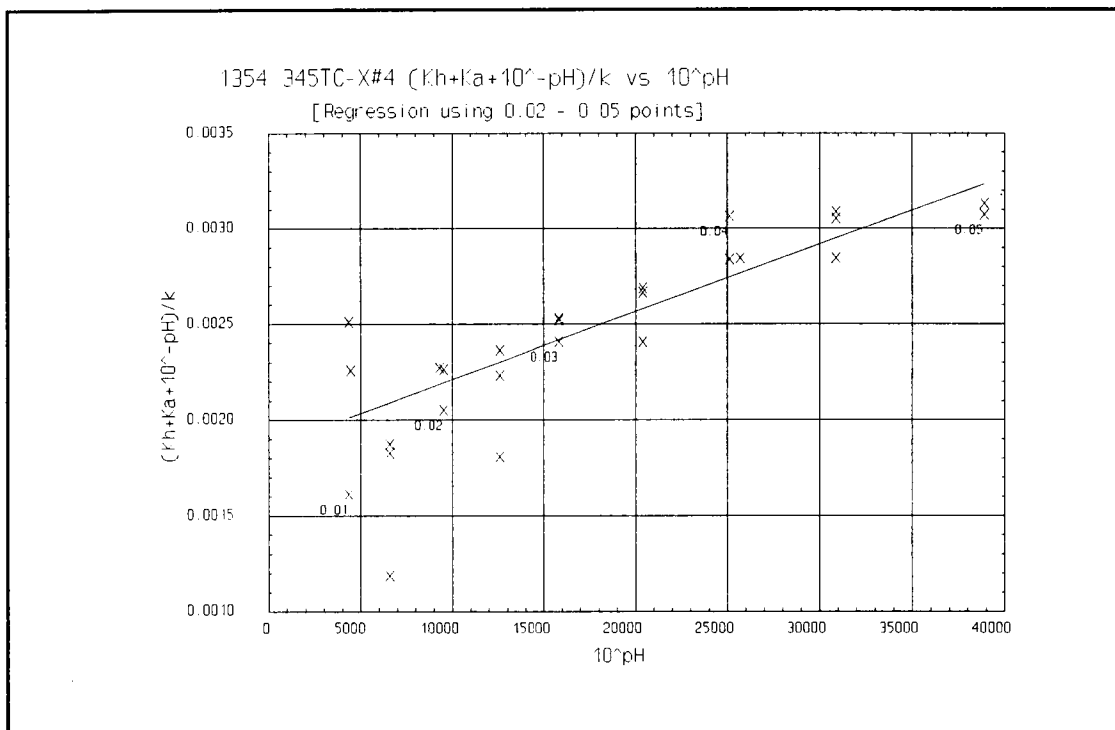
Intercept 2.01×10^{-3}
 Slope 2.70×10^{-8}
 Count 24

K_h+K_a (from calc. #1) = 6.75×10^{-5}
 $K_h = (K_h+K_a) - K_a$, = 5.41×10^{-5}
 $K_a = \text{slope/intercept}$, = 1.30×10^{-5}
 $k_1 = K_h \cdot k_2$, = 0.0272
 $k_2 = 1/\text{intercept}$, = 503

Appendix 4. TCX (cont.)

1354, 345TC-X #4. $D_o/(D_o-D)$ vs. 10^{-pH} 1354, 345TC-X #5. $D_o/(D_o-D)$ vs. 10^{-pH} 

Appendix 4. TCX (cont.)



Appendix 5. Regression Data for 2MC
(Anthocyanin acylated with 2-methoxycinnamic acid)

Compound: 2MC (#1)
Experiment #: 1340
Date: 8/2/96
Dilution: 1:250
Reaction Time: 2 mins.
Wavelength: 500 nm
Comments:

Calculation #1:
Linear Regression
[D₀/(D₀-D) vs. 10^{-pH}]

Intercept 1.46
Slope 20517
Count 27

Calculation #2:
Linear Regression
[(K_h+K_a+10^{-pH})/k vs. 10^{pH}]
[w/o 0.015 #1]

Intercept 1.40 x 10⁻³
Slope 2.86 x 10⁻⁸
Count 26

Kh+Ka (from calc. #1) = 7.13 x 10⁻⁵
Kh = (Kh+Ka) - Ka, = 5.09 x 10⁻⁵
Ka = slope/intercept, = 2.04 x 10⁻⁵
k1 = Kh*k2, = 0.0363
k2 = 1/intercept, = 714

=====

Compound: 2MC (#2)
Experiment #: 1340
Date: 8/2/96
Dilution: 1:250
Reaction Time: 2 mins.
Wavelength: 500 nm
Comments:

Calculation #1:
Linear Regression
[D₀/(D₀-D) vs. 10^{-pH}]

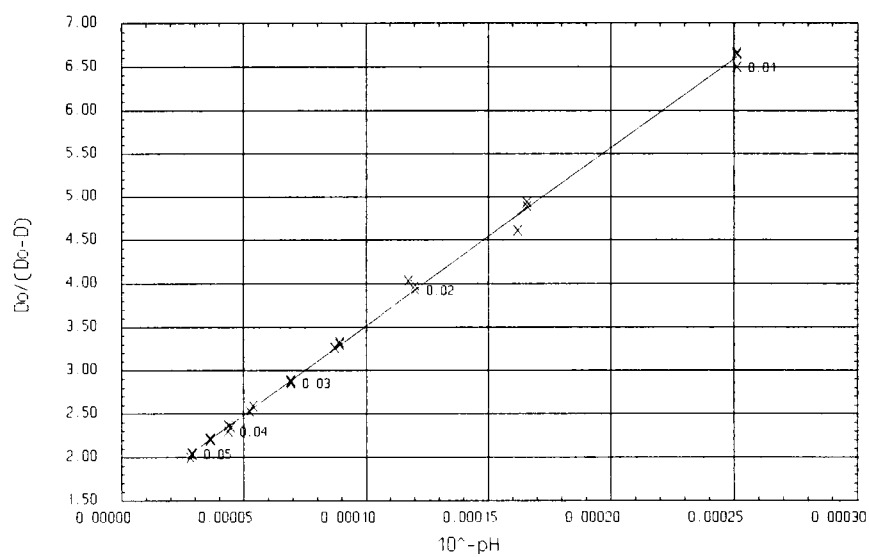
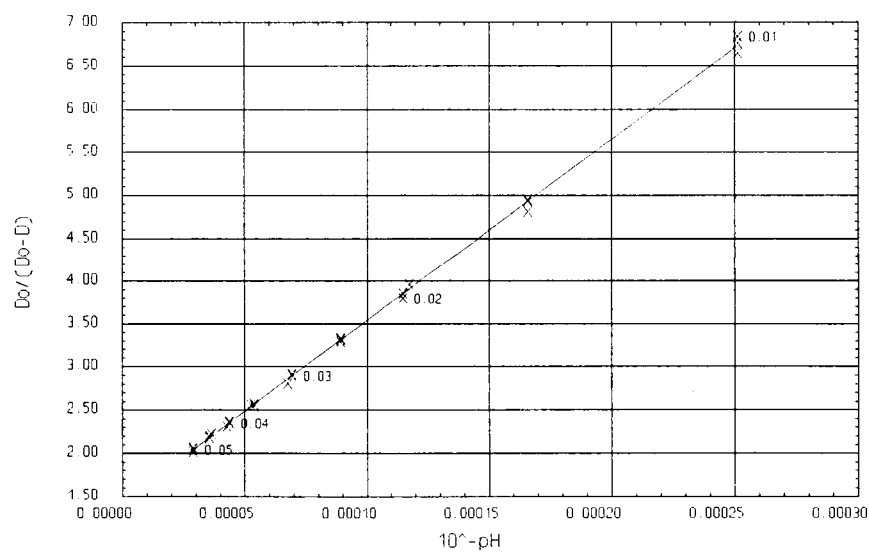
Intercept 1.43
Slope 21076
Count 27

Calculation #2:
Linear Regression
[(K_h+K_a+10^{-pH})/k vs. 10^{pH}]
[w/o 0.01 #2]

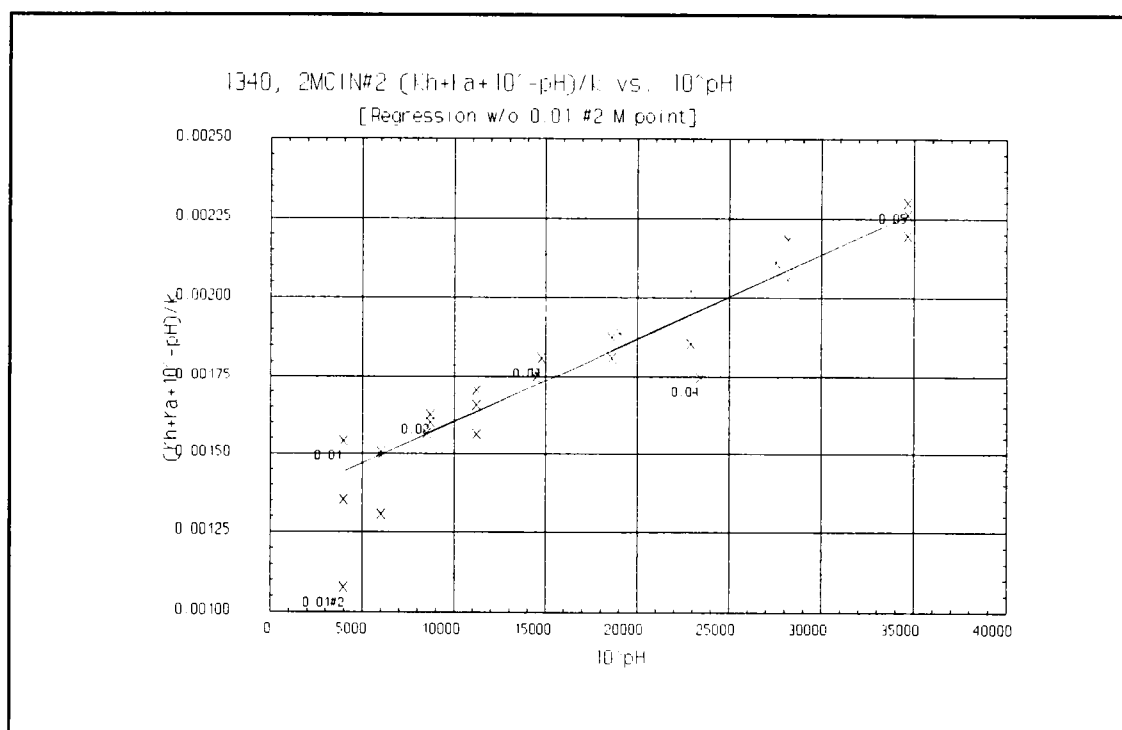
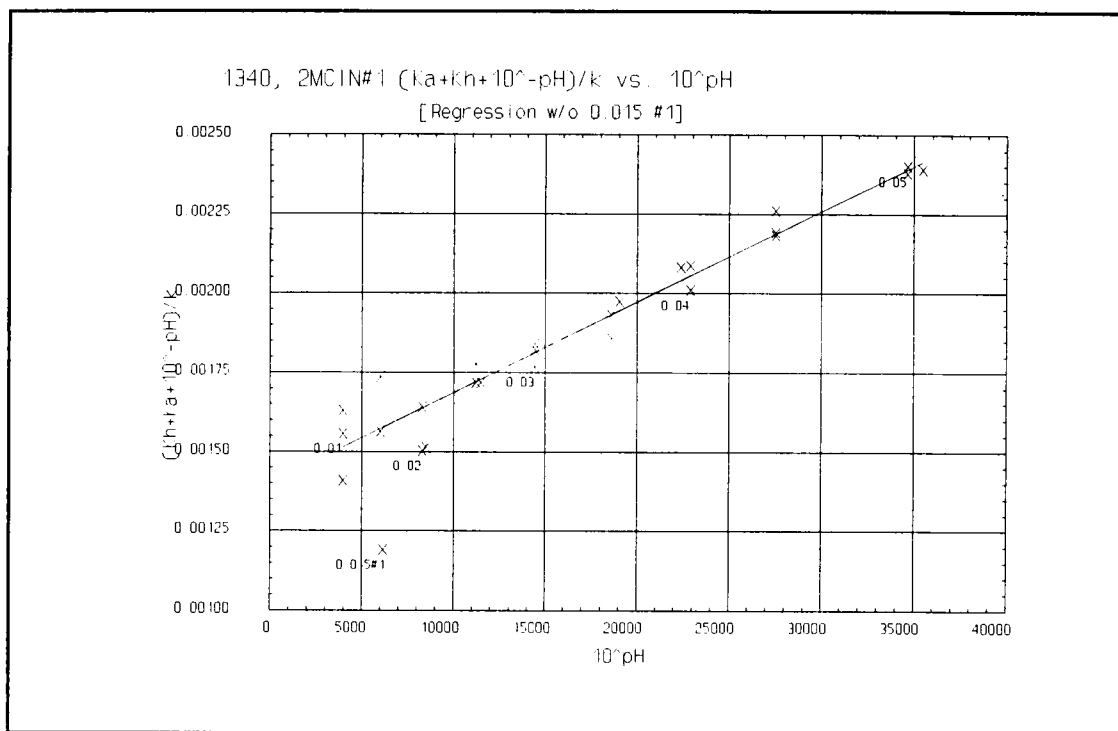
Intercept 1.34 x 10⁻³
Slope 2.67 x 10⁻⁸
Count 26

Kh+Ka (from calc. #1) = 6.79 x 10⁻⁵
Kh = (Kh+Ka) - Ka, = 4.80 x 10⁻⁵
Ka = slope/intercept, = 2.00 x 10⁻⁵
k1 = Kh*k2, = 0.0359
k2 = 1/intercept, = 749

Appendix 5. 2MC (cont.)

1340, 2MCIN #1. $Do/(Do-D)$ vs. 10^{-pH} 1340, 2MCIN #2. $Do/(Do-D)$ vs. 10^{-pH} 

Appendix 5. 2MC (cont.)



Appendix 6. Regression Data for 3MC
(Anthocyanin acylated with 3-methoxycinnamic acid)

Compound: 3MC (#1)
 Experiment #: 1347
 Date: 9/12/96
 Dilution: 1:240
 Reaction Time: 2 mins.
 Wavelength: 500 nm
 NaOH Conc. Used: 0.01 - 0.05 M
 Comments:

Calculation #1:		Calculation #2:	
Linear Regression		Linear Regression	
[D ₀ /(D ₀ -D) vs. 10 ^{-pH}]		[(K _b +K _a +10 ^{-pH})/k vs. 10 ^{pH}]	
		[w/ 0.02 - 0.045 M points]	
Intercept	1.31	Intercept	1.57 x 10 ⁻³
Slope	22684	Slope	0.32 x 10 ⁻⁸
Count	27	Count	18

Kh+Ka (from calc. #1) = 5.79 x 10⁻⁵
 Kh = (Kh+Ka) - Ka, = 5.59 x 10⁻⁵
 Ka = slope/intercept, = 0.21 x 10⁻⁵
 k1 = Kh*k2, = 0.0357
 k2 = 1/intercept, = 638

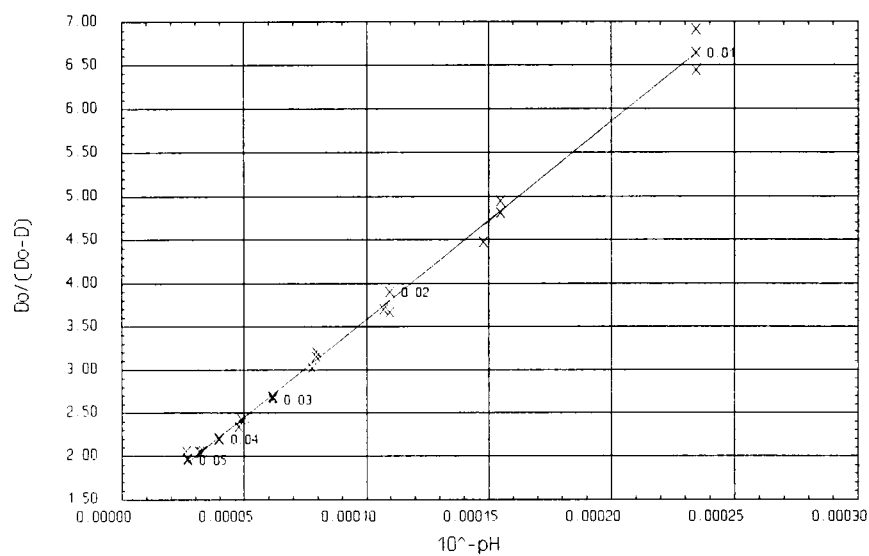
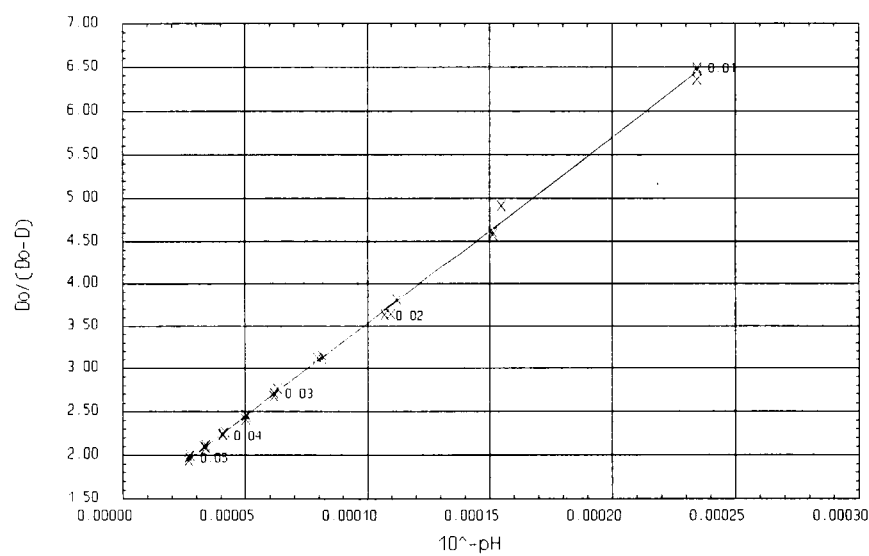
=====

Compound: 3MC (#2)
 Experiment #: 1347
 Date: 9/12/96
 Dilution: 1:240
 Reaction Time: 2 mins.
 Wavelength: 500 nm
 NaOH Conc. Used: 0.01 - 0.05 M
 Comments:

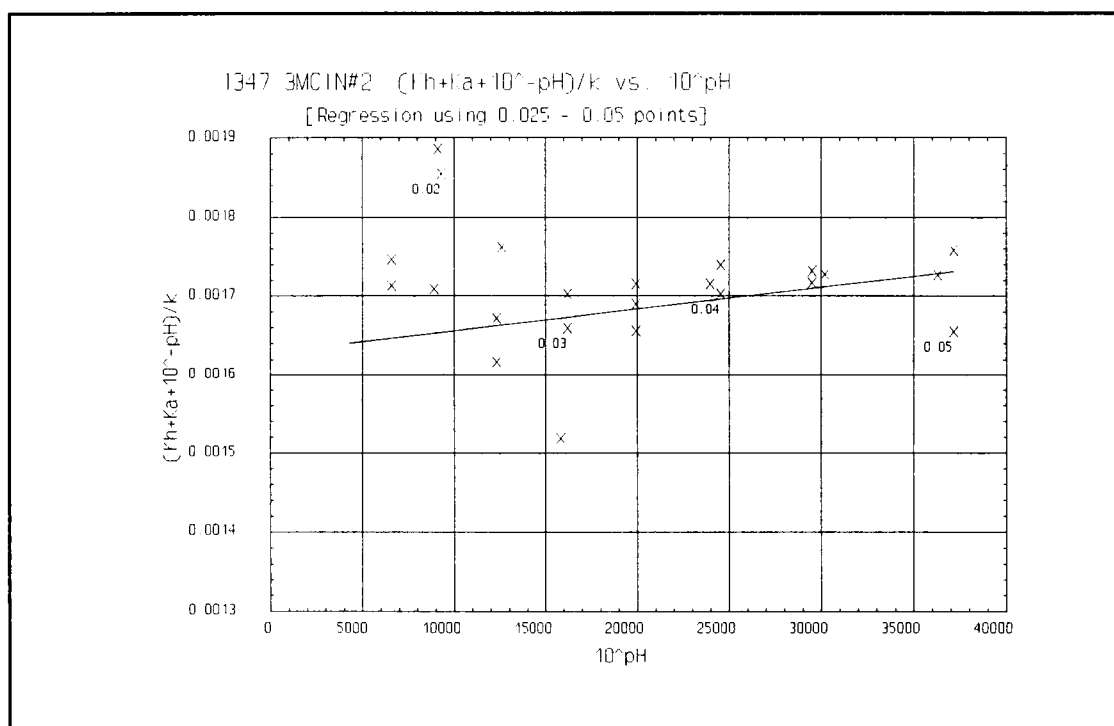
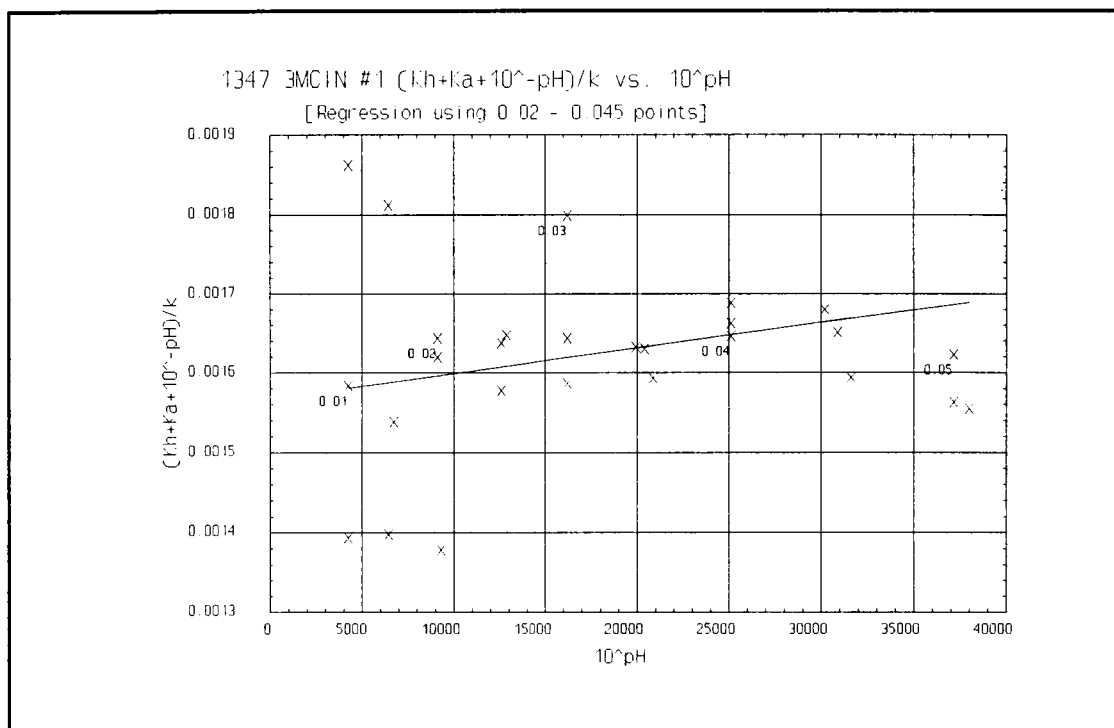
Calculation #1:		Calculation #2:	
Linear Regression		Linear Regression	
[D ₀ /(D ₀ -D) vs. 10 ^{-pH}]		[(K _b +K _a +10 ^{-pH})/k vs. 10 ^{pH}]	
		[w/ 0.025 - 0.05 M points]	
Intercept	1.37	Intercept	1.60 x 10 ⁻³
Slope	21653	Slope	0.28 x 10 ⁻⁸
Count	27	Count	18

Kh+Ka (from calc. #1) = 6.31 x 10⁻⁵
 Kh = (Kh+Ka) - Ka, = 6.14 x 10⁻⁵
 Ka = slope/intercept, = 0.17 x 10⁻⁵
 k1 = Kh*k2, = 0.0377
 k2 = 1/intercept, = 614

Appendix 6. 3MC (cont.)

1347, 3MCIN (#1). $D_o/(D_o-D)$ vs. 10^{-pH} 1347, 3MCIN #2. $D_o/(D_o-D)$ vs. 10^{-pH} 

Appendix 6. 3MC (cont.)



Appendix 7. Regression Data for 4MC
(Anthocyanin acylated with 4-methoxycinnamic acid)

Compound: 4MC (#1)
Experiment #: 1306
Date: 4/25/96
Dilution: 1:50
Reaction Time: 2 min.
Wavelength: 500 nm
NaOH Conc. Used: 0.01 - 0.05 M
Comments:

Calculation #1:
Linear Regression
[D₀/(D₀-D) vs. 10^{-pH}]

Intercept 1.49
Slope 16001
Count 30

Calculation #2:
Linear Regression
[(K_a+K_a+10^{-pH})/k vs. 10^{pH}]
[w/0 0.01 & 0.015 #2]

Intercept 1.65 x 10⁻³
Slope 1.15 x 10⁻⁸
Count 23

Kh+Ka (from calc. #1) = 9.30 x 10⁻⁵
Kh = (Kh+Ka) - Ka, = 8.60 x 10⁻⁵
Ka = slope/intercept, = 0.70 x 10⁻⁵
k1 = Kh*k2, = 0.0521
k2 = 1/intercept, = 606

=====

Compound: 4MC (#2)
Experiment #: 1306
Date: 4/26/96
Dilution: 1:50
Reaction Time: 2 min.
Wavelength: 500 nm
NaOH Conc. Used: 0.02 - 0.05 M
Comments: no 0.01 or 0.015 treatments used

Calculation #1:
Linear Regression
[D₀/(D₀-D) vs. 10^{-pH}]

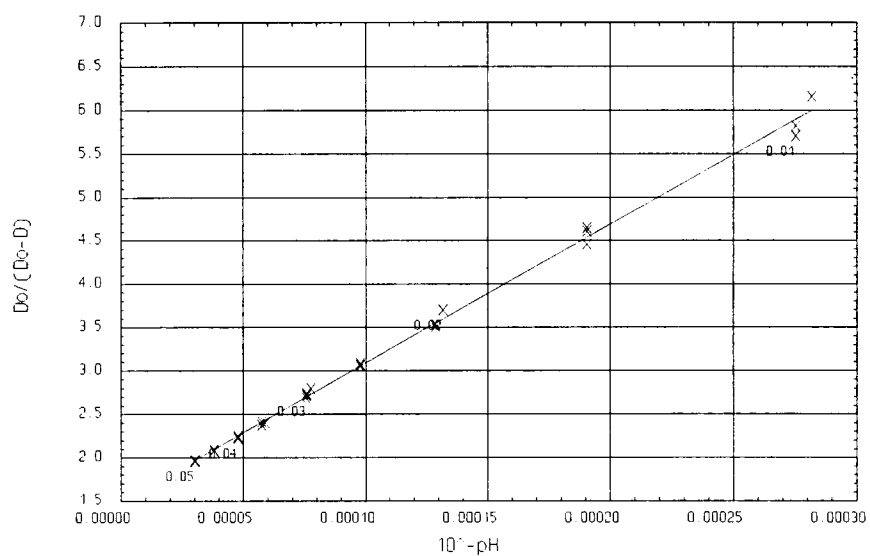
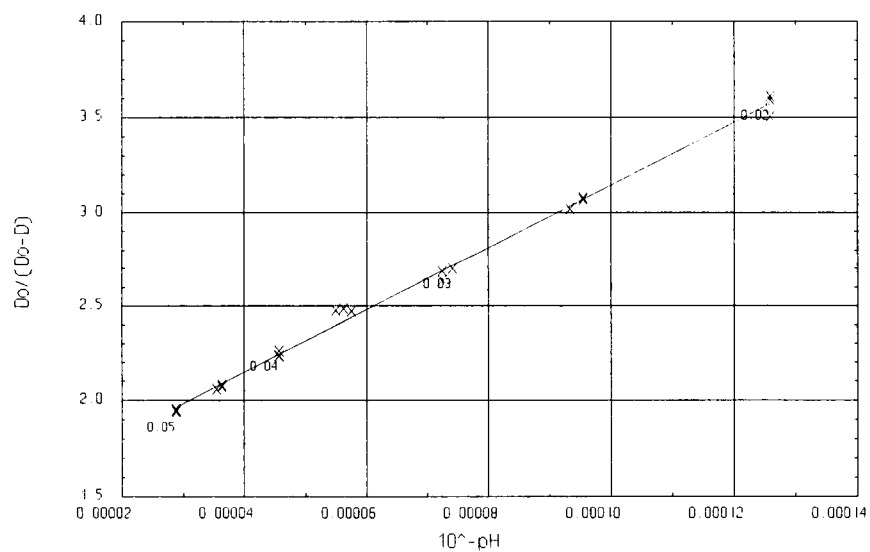
Intercept 1.48
Slope 16619
Count 22

Calculation #2:
Linear Regression
[(K_a+K_a+10^{-pH})/k vs. 10^{pH}]

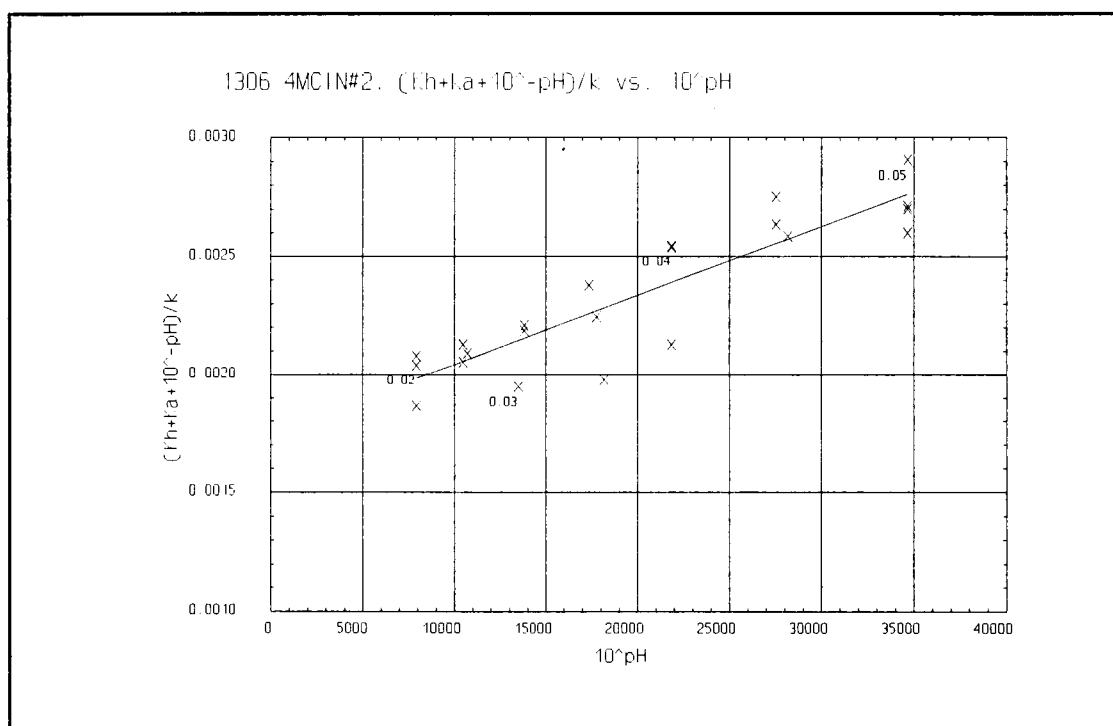
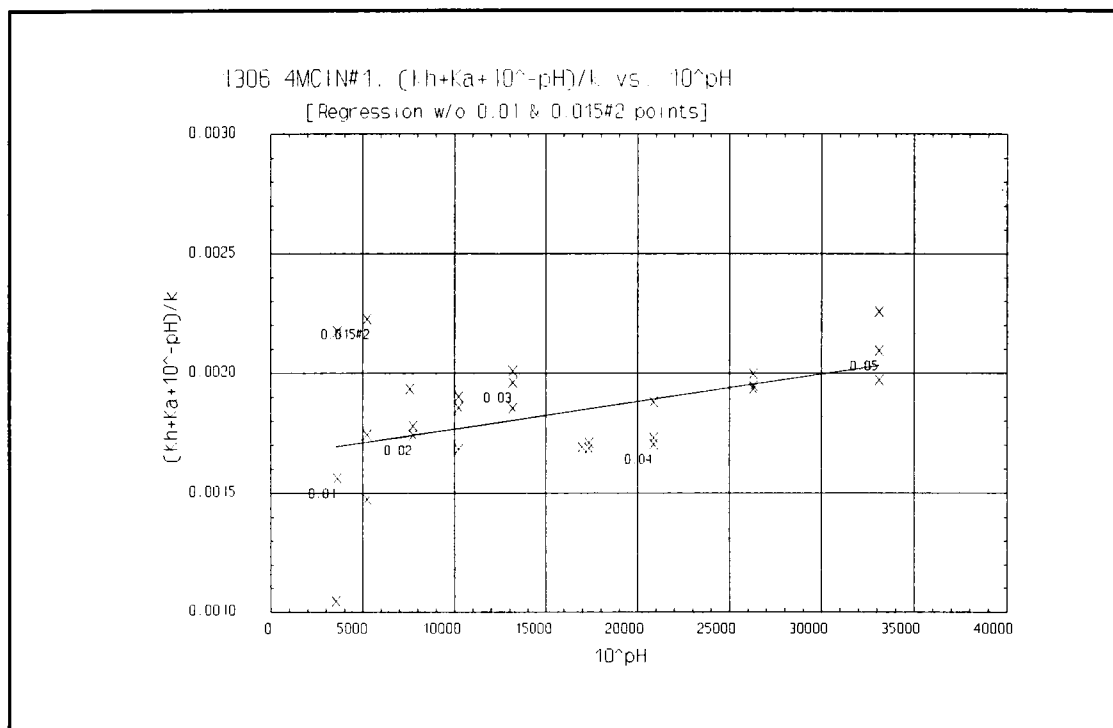
Intercept 1.75 x 10⁻³
Slope 2.92 x 10⁻⁸
Count 22

Kh+Ka (from calc. #1) = 8.91 x 10⁻⁵
Kh = (Kh+Ka) - Ka, = 7.25 x 10⁻⁵
Ka = slope/intercept, = 1.67 x 10⁻⁵
k1 = Kh*k2, = 0.0414
k2 = 1/intercept, = 571

Appendix 7. 4MC (cont.)

1306, 4MCIN #1. $D_o/(D_o-D)$ vs. 10^{-pH} 1306, 4MCIN #2. $D_o/(D_o-D)$ vs. 10^{-pH} 

Appendix 7. 4MC (cont.)



Appendix 8. Regression Data for 4HC
(Anthocyanin acylated with 4-hydroxycinnamic acid)

Compound: 4HC (#1)
 Experiment #: 1321
 Date: 5/20/96
 Dilution: 1:80
 Reaction Time: 2 min.
 Wavelength: 500 nm
 NaOH Range: 0.01 - 0.05 M
 Comments: A lot of scatter in graph
 of $(K_h + K_a + 10^{-pH})/k$ vs. 10^{pH} .

Calculation #1:	Calculation #2:
Linear Regression	Linear Regression
$[D_o / (D_o - D)]$ vs. 10^{-pH}	$[(K_h + K_a + 10^{-pH})/k]$ vs. 10^{pH}
Intercept 1.59	Intercept 1.30×10^3
Slope 18664	Slope 1.44×10^8
Count 27	Count 25

$K_h + K_a$ (from calc. #1) = 8.53×10^{-5}
 $K_h = (K_h + K_a) - K_a$, = 7.42×10^{-5}
 $K_a = \text{slope/intercept}$, = 1.11×10^{-5}
 $k_1 = K_h \cdot k_2$, = 0.0573
 $k_2 = 1/\text{intercept}$, = 772

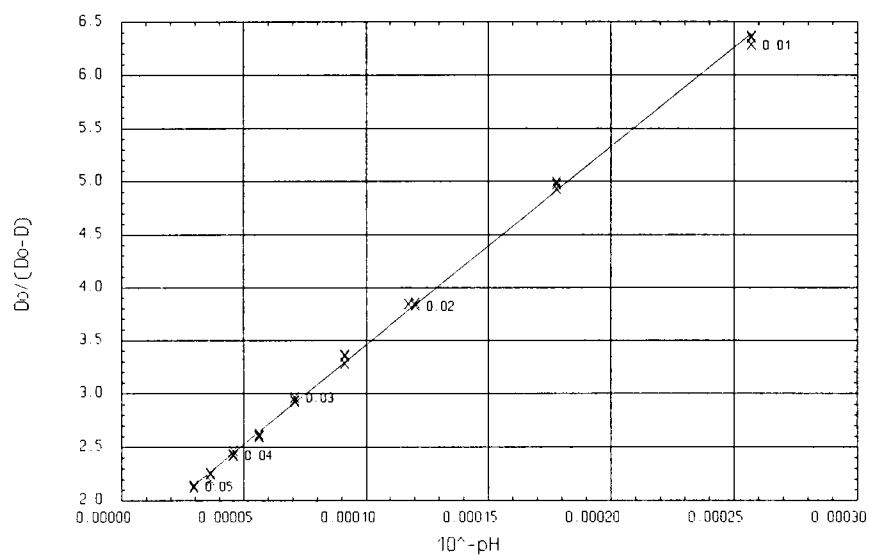
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Compound: 4HC (#2)
 Experiment #: 1321
 Date: 5/28/96
 Dilution: 1:80
 Reaction Time: 2 min.
 Wavelength: 500 nm
 NaOH Range: 0.01 - 0.05 M
 Comments:

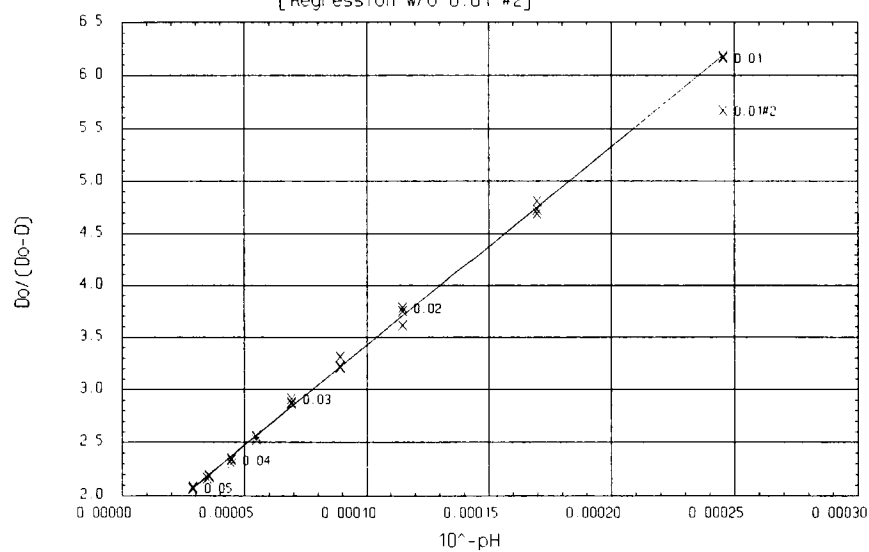
Calculation #1:	Calculation #2:
Linear Regression	Linear Regression
$[D_o / (D_o - D)]$ vs. 10^{-pH}	$[(K_h + K_a + 10^{-pH})/k]$ vs. 10^{pH}
	[w/ 0.01 - 0.045 points]
Intercept 1.52	Intercept 1.52×10^{-3}
Slope 19005	Slope 3.26×10^{-8}
Count 26	Count 24

$K_h + K_a$ (from calc. #1) = 8.01×10^{-5}
 $K_h = (K_h + K_a) - K_a$, = 5.86×10^{-5}
 $K_a = \text{slope/intercept}$, = 2.15×10^{-5}
 $k_1 = K_h \cdot k_2$, = 0.0387
 $k_2 = 1/\text{intercept}$, = 660

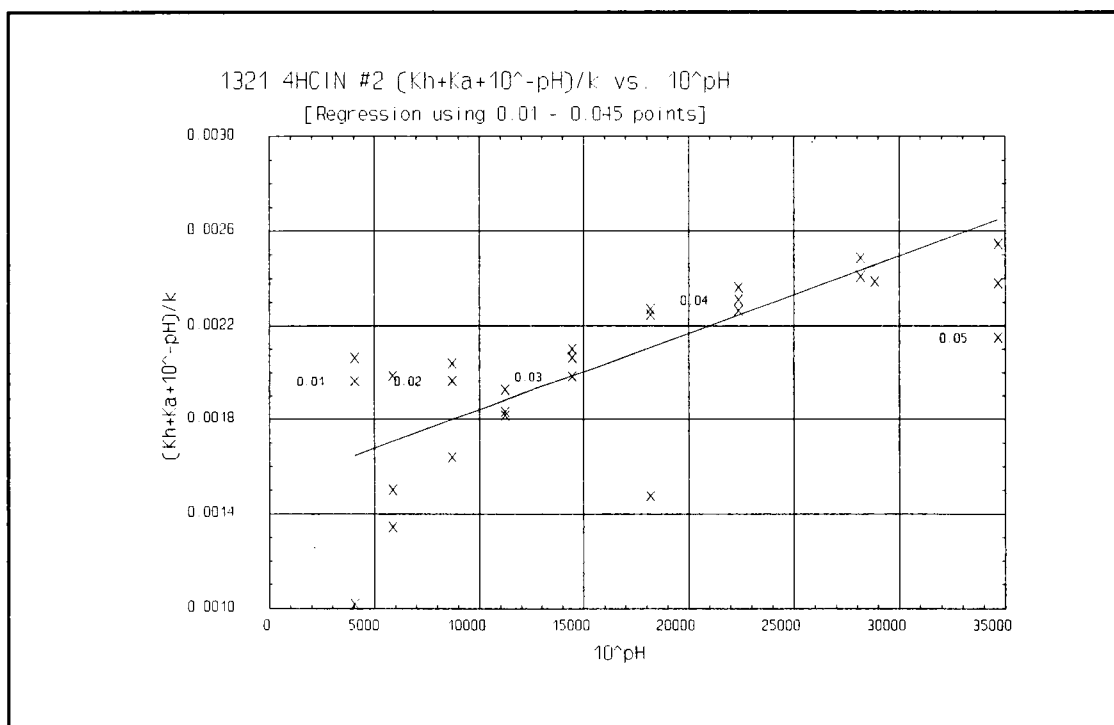
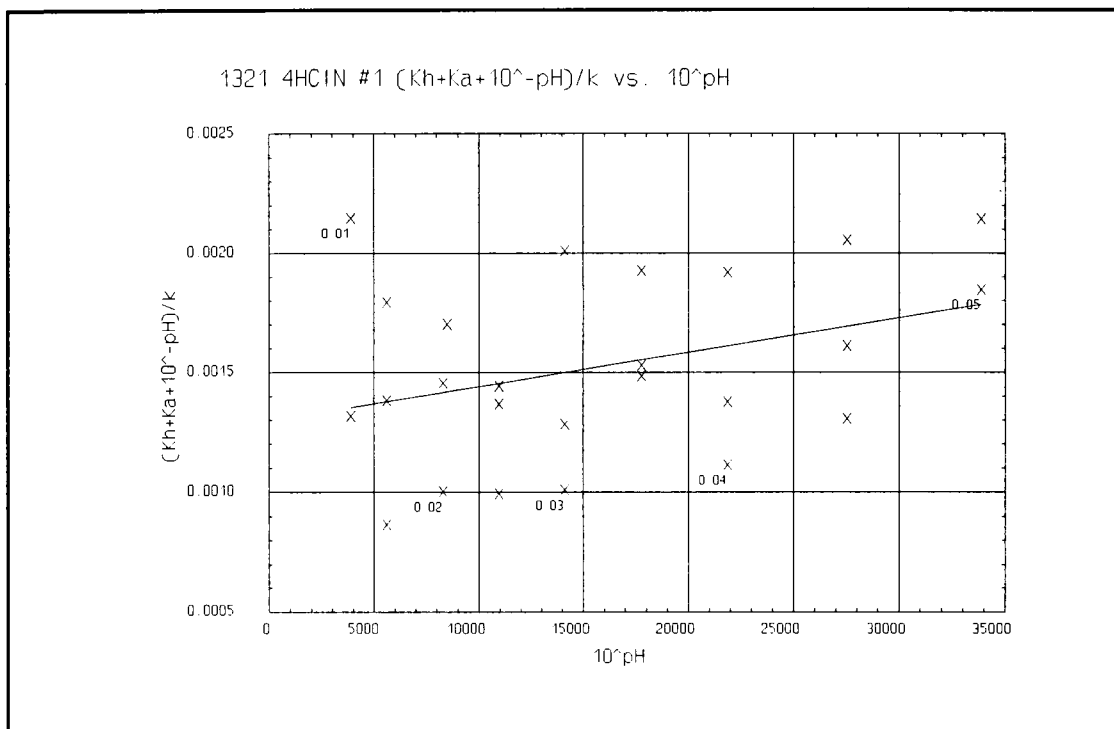
Appendix 8. 4HC (cont.)

1321, 4HCIN #1. $Do/(Do-D)$ vs. 10^{-pH} 1321, 4HCIN #2. $Do/(Do-D)$ vs. 10^{-pH}

[Regression w/o 0.01 #2]



Appendix 8. 4HC (cont.)



Appendix 9. Regression Data for C
(Anthocyanin acylated with cinnamic acid)

Compound: C (#1)
 Experiment #: 1360
 Date: 10/17/96
 Dilution: 1:254
 Reaction Time: 2 mins.
 Wavelength: 500 nm
 NaOH Conc. Used: 0.01 - 0.05 M
 Comments: A combination of 5 bulk preps.

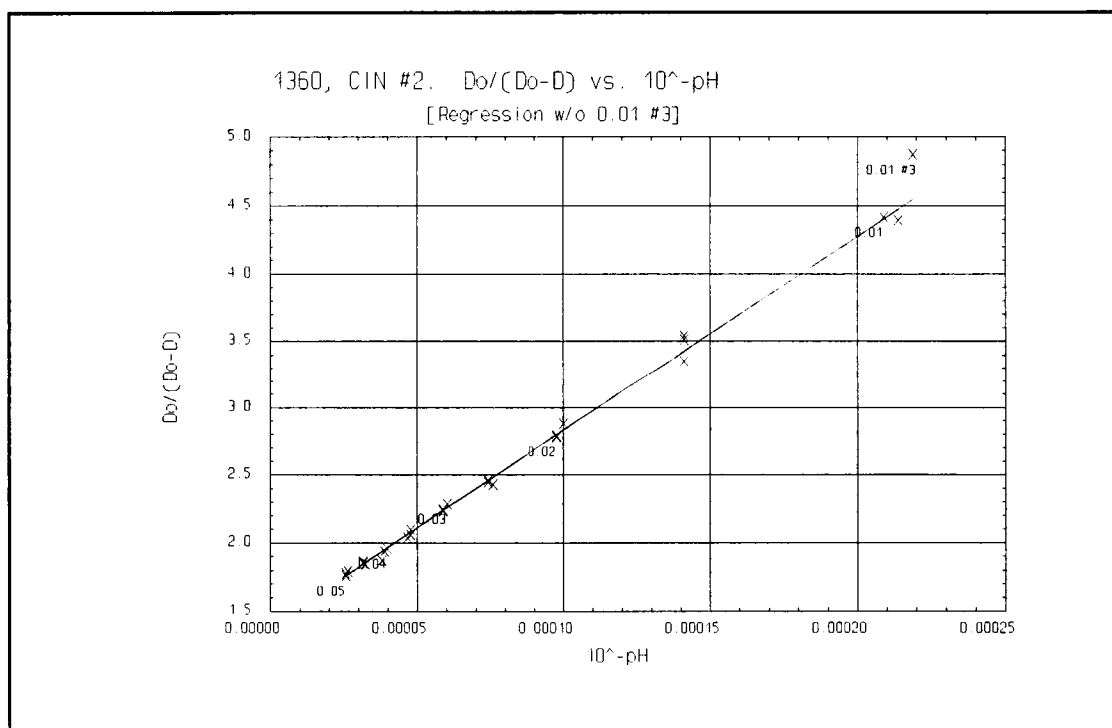
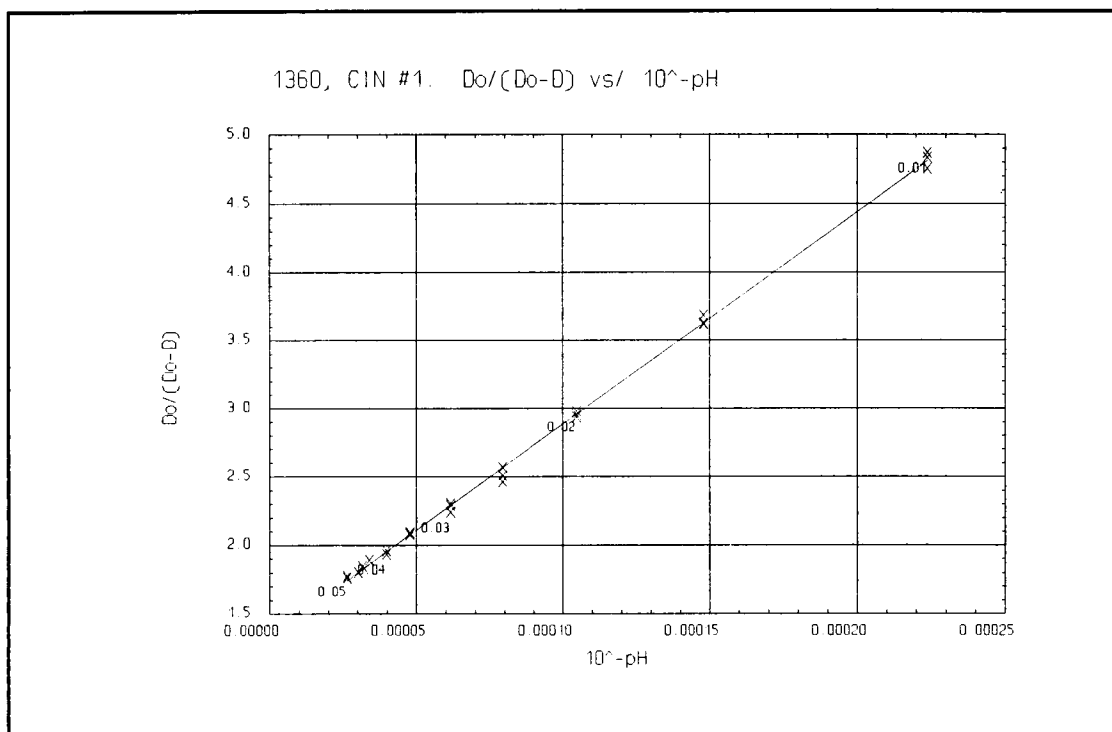
Calculation #1:	Calculation #2:
Linear Regression	Linear Regression
$[D_0/(D_0-D)]$ vs. 10^{-PH}	$[(K_h+K_a+10^{-PH})/k]$ vs. 10^{PH}
	[w/o 0.03 #1]
Intercept 1.33	Intercept 1.59×10^{-3}
Slope 15533	Slope 2.29×10^{-8}
Count 27	Count 27
K_h+K_a (from calc. #1) =	8.58×10^{-5}
$K_h = (K_h+K_a) - K_a,$ =	7.14×10^{-5}
$K_a = \text{slope/intercept},$ =	1.44×10^{-5}
$k_1 = K_h \cdot k_2,$ =	0.0449
$k_2 = 1/\text{intercept},$ =	629

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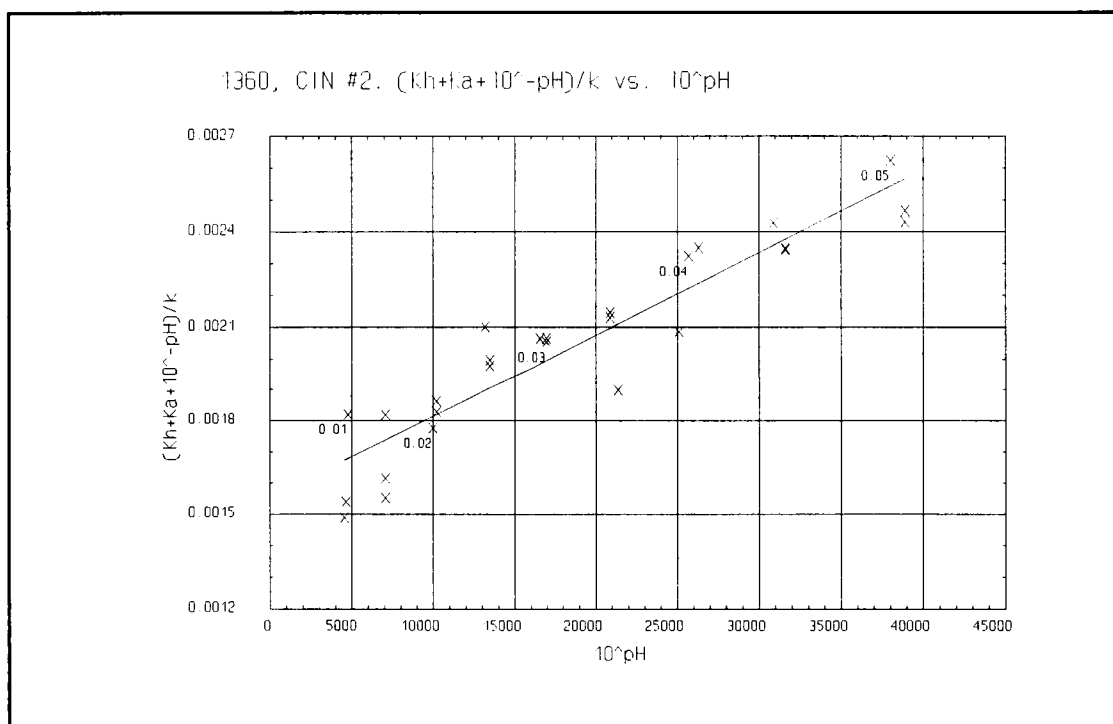
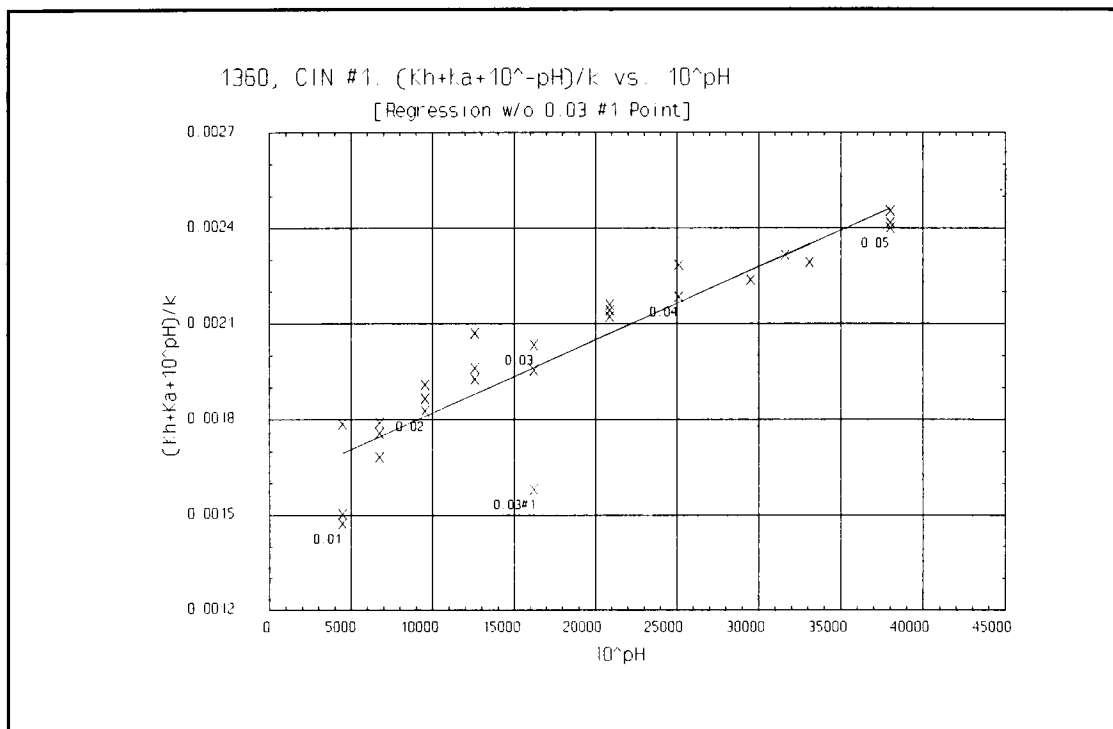
Compound: C (#2)
 Experiment #: 1360
 Date: 10/17/96
 Dilution: 1:254
 Reaction Time: 2 mins.
 Wavelength: 500 nm
 NaOH Conc. Used: 0.01 - 0.05 M
 Comments: A combination of 5 bulk preps.

Calculation #1:	Calculation #2:
Linear Regression	Linear Regression
$[D_0/(D_0-D)]$ vs. 10^{-PH}	$[(K_h+K_a+10^{-PH})/k]$ vs. 10^{PH}
Intercept 1.36	Intercept 1.55×10^{-3}
Slope 14936	Slope 2.60×10^{-8}
Count 26	Count 27
K_h+K_a (from calc. #1) =	9.09×10^{-5}
$K_h = (K_h+K_a) - K_a,$ =	7.42×10^{-5}
$K_a = \text{slope/intercept},$ =	1.67×10^{-5}
$k_1 = K_h \cdot k_2,$ =	0.0478
$k_2 = 1/\text{intercept},$ =	644

Appendix 9. C (cont.)



Appendix 9. C (cont.)



Appendix 10. Regression Data for CC
(Anthocyanin acylated with 4-chlorocinnamic acid)

Compound: CC (#1)
 Experiment #: 1348
 Date: 9/23/96
 Dilution: 1:400
 Reaction Time: 2 mins.
 Wavelength: 500 nm
 NaOH Conc. Used: 0.01 - 0.05 M
 Comments: pk 2 (major peak) from preparative HPLC of culture grown w/ 4-chlorocinnamic acid.

Calculation #1:		Calculation #2:	
Linear Regression		Linear Regression	
[D ₀ /(D ₀ -D) vs. 10 ^{-pH}]		[(K _a +K _s +10 ^{-pH})/k vs. 10 ^{pH}]	
[w/ 0.01 - 0.045 points]		[w/ 0.01 - 0.045 points]	
Intercept	1.33	Intercept	1.72 x 10 ⁻³
Slope	13320	Slope	3.16 x 10 ⁻⁸
Count	25	Count	22

Kh+Ka (from calc. #1) = 10.01 x 10⁻⁵
 Kh = (Kh+Ka) - Ka, = 8.17 x 10⁻⁵
 Ka = slope/intercept, = 1.84 x 10⁻⁵
 k1 = Kh*k2, = 0.0475
 k2 = 1/intercept, = 581

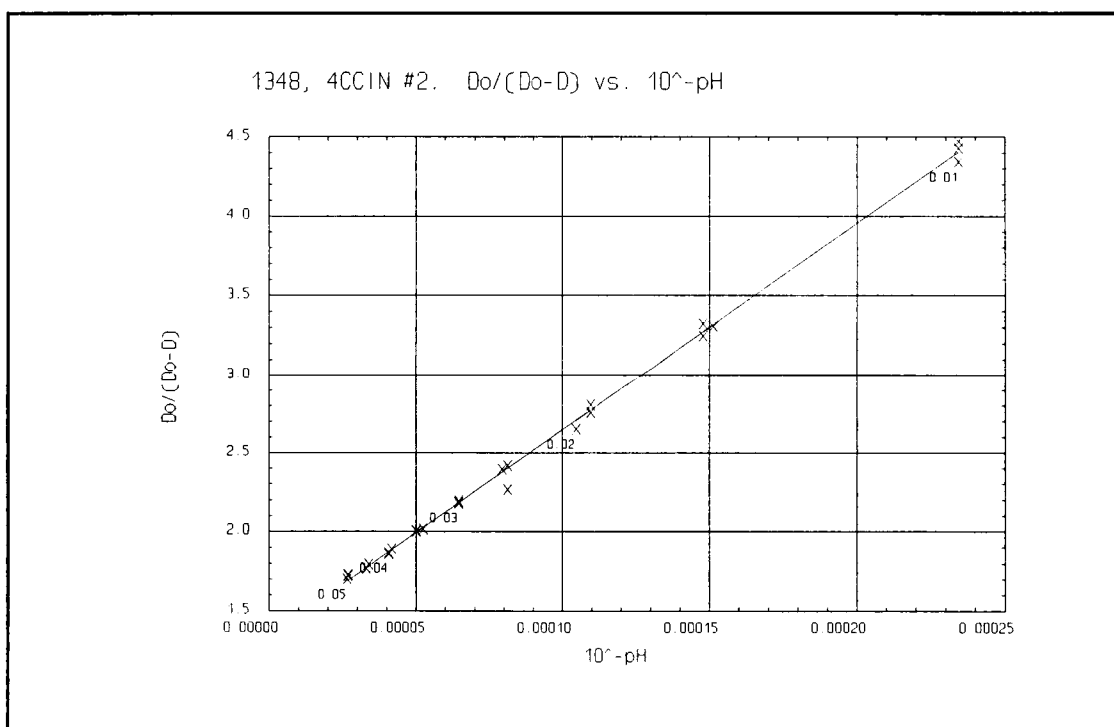
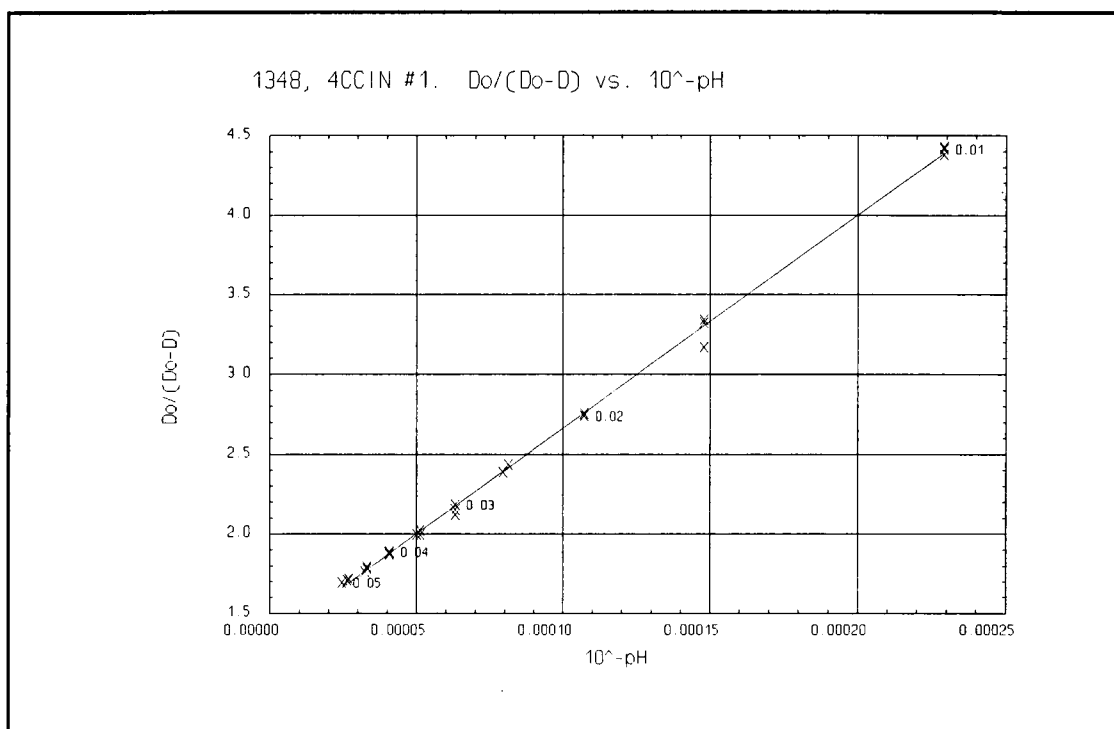
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Compound: CC (#2)
 Experiment #: 1348
 Date: 9/23/96
 Dilution: 1:400
 Reaction Time: 2 mins.
 Wavelength: 500 nm
 NaOH Conc. Used: 0.01 - 0.05 M
 Comments: Peak 2 (major peak) from preparative HPLC of culture grown w/ 4-chlorocinnamic acid.

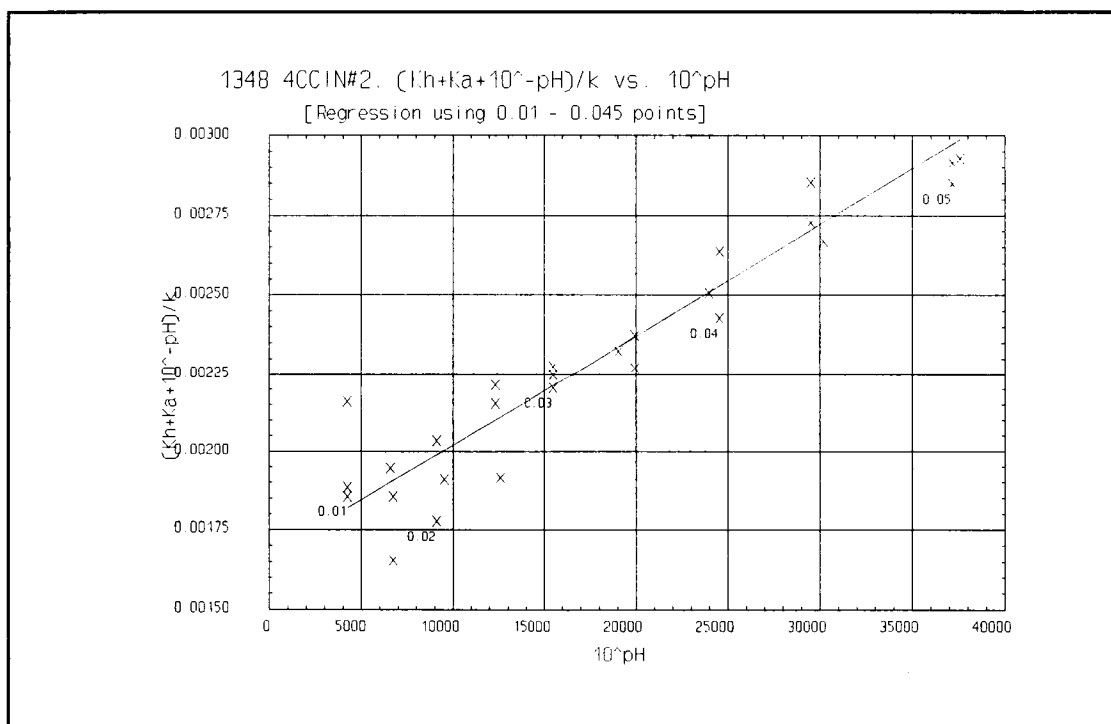
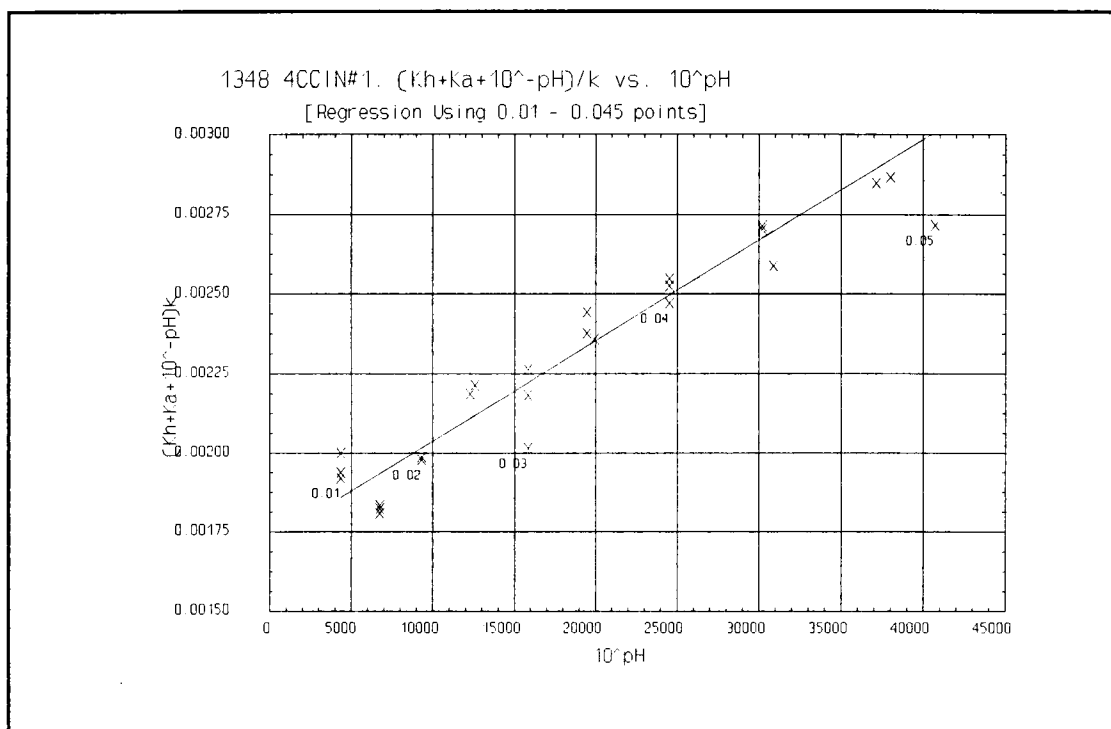
Calculation #1:		Calculation #2:	
Linear Regression		Linear Regression	
[D ₀ /(D ₀ -D) vs. 10 ^{-pH}]		[(K _a +K _s +10 ^{-pH})/k vs. 10 ^{pH}]	
[w/ 0.01 - 0.045 points]		[w/ 0.01 - 0.045 points]	
Intercept	1.34	Intercept	1.67 x 10 ⁻³
Slope	13072	Slope	3.51 x 10 ⁻⁸
Count	27	Count	24

Kh+Ka (from calc. #1) = 10.24 x 10⁻⁵
 Kh = (Kh+Ka) - Ka, = 8.14 x 10⁻⁵
 Ka = slope/intercept, = 2.10 x 10⁻⁵
 k1 = Kh*k2, = 0.0488
 k2 = 1/intercept, = 599

Appendix 10. CC (cont.)



Appendix 10. CC (cont.)



Appendix 11. Regression Data for FC
(Anthocyanin acylated with 4-fluorocinnamic acid)

Compound: FC (#1)
Experiment #: 1350
Date: 9/11/96
Dilution: 1:280
Reaction Time: 2 mins.
Wavelength: 500 nm
NaOH Conc. Used: 0.01 - 0.05 M
Comments:

Calculation #1:		Calculation #2:	
Linear Regression		Linear Regression	
[D ₀ /(D ₀ -D) vs. 10 ^{-pH}]		[(K _b +K _a +10 ^{-pH})/k vs. 10 ^{pH}]	
[w/ 0.01 - 0.045 points]		[w/ 0.01 - 0.045 points]	
Intercept	1.33	Intercept	1.74 x 10 ⁻³
Slope	11261	Slope	4.26 x 10 ⁻⁸
Count	27	Count	24

Kh+Ka (from calc. #1) = 11.77 x 10⁻⁵
 Kh = (Kh+Ka) - Ka, = 9.32 x 10⁻⁵
 Ka = slope/intercept, = 2.45 x 10⁻⁵
 k1 = Kh*k2, = 0.0536
 k2 = 1/intercept, = 576

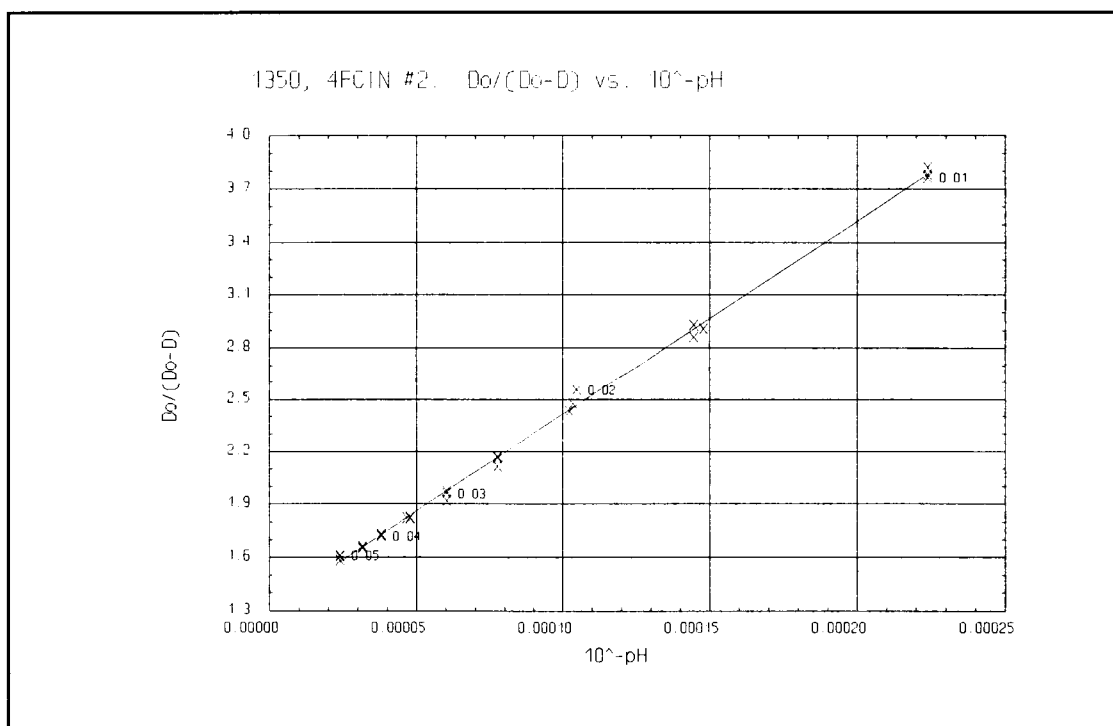
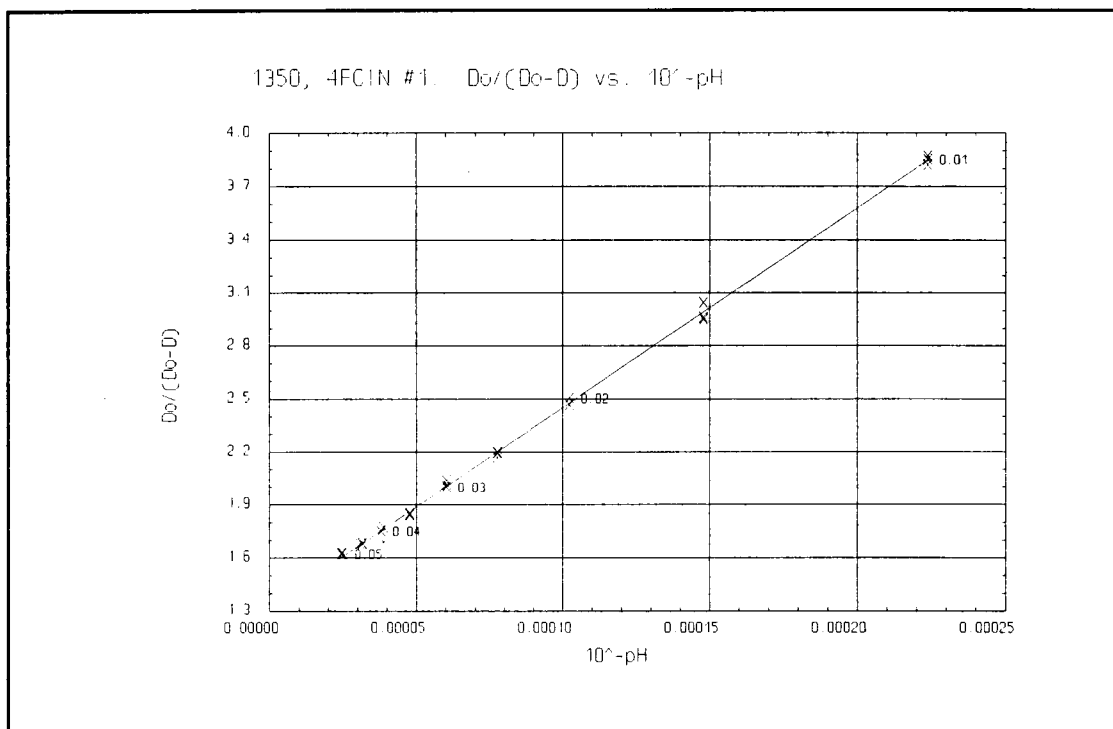
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Compound: FC (#2)
Experiment #: 1350
Date: 9/11/96
Dilution: 1:280
Reaction Time: 2 mins.
Wavelength: 500 nm
NaOH Conc. Used: 0.01 - 0.05 M
Comments:

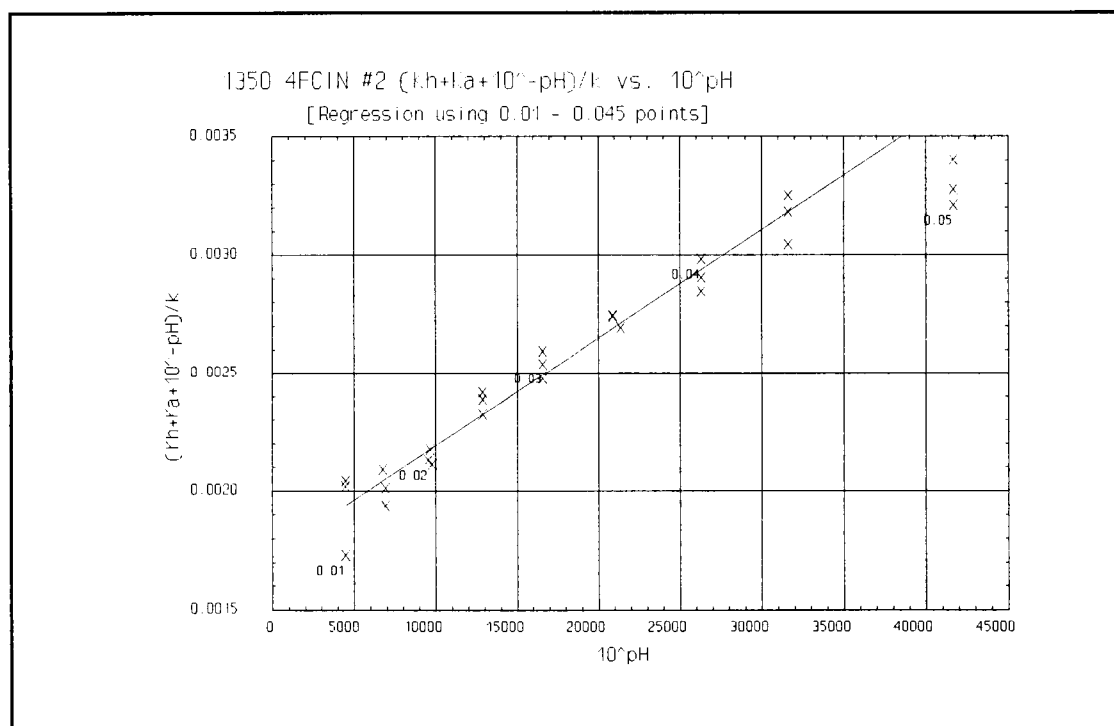
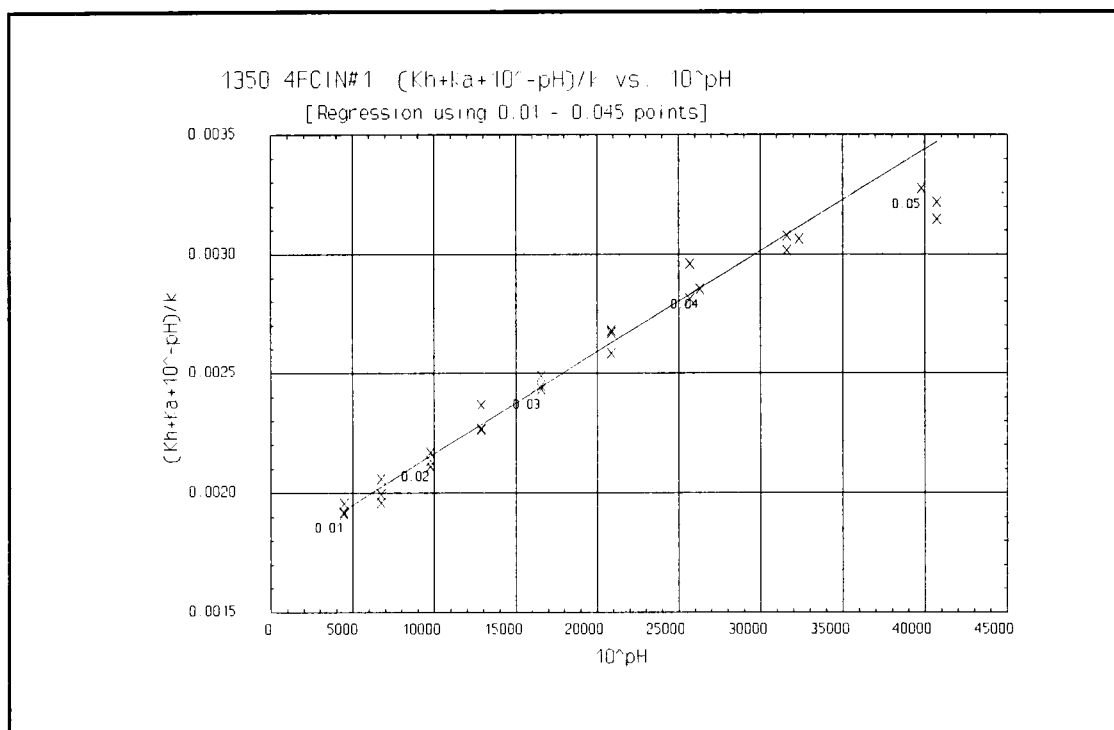
Calculation #1:		Calculation #2:	
Linear Regression		Linear Regression	
[D ₀ /(D ₀ -D) vs. 10 ^{-pH}]		[(K _b +K _a +10 ^{-pH})/k vs. 10 ^{pH}]	
[w/ 0.01 - 0.045 points]		[w/ 0.01 - 0.045 points]	
Intercept	1.31	Intercept	1.73 x 10 ⁻³
Slope	11043	Slope	4.58 x 10 ⁻⁸
Count	27	Count	24

Kh+Ka (from calc. #1) = 11.85 x 10⁻⁵
 Kh = (Kh+Ka) - Ka, = 9.21 x 10⁻⁵
 Ka = slope/intercept, = 2.64 x 10⁻⁵
 k1 = Kh*k2, = 0.0531
 k2 = 1/intercept, = 577

Appendix 11. FC (cont.)



Appendix 11. FC (cont.)



Appendix 12. Regression Data for TFMC
(Anthocyanin acylated with 4-trifluoromethylcinnamic acid)

Compound: TFMC (#1)
 Experiment #: 1323
 Date: 5/10/96
 Dilution: 1:50
 Reaction Time: 2 min.
 Wavelength: 500 nm
 NaOH Range: 0.01 - 0.05 M
 Comments:

Calculation #1:		Calculation #2:	
Linear Regression		Linear Regression	
[D ₀ /(D ₀ -D) vs. 10 ^{-pH}]		[(K _h +K _a +10 ^{-pH})/k vs. 10 ^{pH}]	
		[w/ 0.01 - 0.045 points]	
Intercept	1.34	Intercept	1.31 x 10 ⁻³
Slope	10902	Slope	3.75 x 10 ⁻⁸
Count	27	Count	24

Kh+Ka (from calc. #1) = 12.28 x 10⁻⁵
 Kh = (Kh+Ka) - Ka, = 9.41 x 10⁻⁵
 Ka = slope/intercept, = 2.87 x 10⁻⁵
 k1 = Kh*k2, = 0.0720
 k2 = 1/intercept, = 765

=====

Compound: TFMC (#2)
 Experiment #: 1323 + 1313
 Date: 5/16/96
 Dilution: 1:50 (1323) + 1:12.5 (1313)
 Reaction Time: 2 min.
 Wavelength: 500 nm
 NaOH Range: 0.01 - 0.05 M
 Comments:

Calculation #1:		Calculation #2:	
Linear Regression		Linear Regression	
[D ₀ /(D ₀ -D) vs. 10 ^{-pH}]		[(K _h +K _a +10 ^{-pH})/k vs. 10 ^{pH}]	
		[w/ 0.015 - 0.05 points]	
Intercept	1.36	Intercept	2.04 x 10 ⁻³
Slope	8829	Slope	4.28 x 10 ⁻⁸
Count	27	Count	24

Kh+Ka (from calc. #1) = 15.43 x 10⁻⁵
 Kh = (Kh+Ka) - Ka, = 13.33 x 10⁻⁵
 Ka = slope/intercept, = 2.10 x 10⁻⁵
 k1 = Kh*k2, = 0.0654
 k2 = 1/intercept, = 490

Appendix 12. TFMC (cont.)

Compound: TFMC (#3)
 Experiment #: 1323 + 1313
 Date: 6/14/96
 Dilution: 1:70
 Reaction Time: 2 min.
 Wavelength: 500 nm
 NaOH Range: 0.01 - 0.05 M
 Comments: Compound was recycled from exps. 1 & 2.

Calculation #1:	Calculation #2:
Linear Regression	Linear Regression
$[D_0/(D_0-D) \text{ vs. } 10^{-\text{pH}}]$	$[(K_h+K_a+10^{-\text{pH}})/k \text{ vs. } 10^{-\text{pH}}]$
Intercept 1.35	Intercept 1.89×10^{-3}
Slope 9420	Slope 3.66×10^{-8}
Count 27	Count 26

K_h+K_a (from calc. #1) = 14.28×10^{-5}
 $K_h = (K_h+K_a) - K_a$, = 12.34×10^{-5}
 $K_a = \text{slope/intercept}$, = 1.94×10^{-5}
 $k_1 = K_h \cdot k_2$, = 0.0654
 $k_2 = 1/\text{intercept}$, = 530

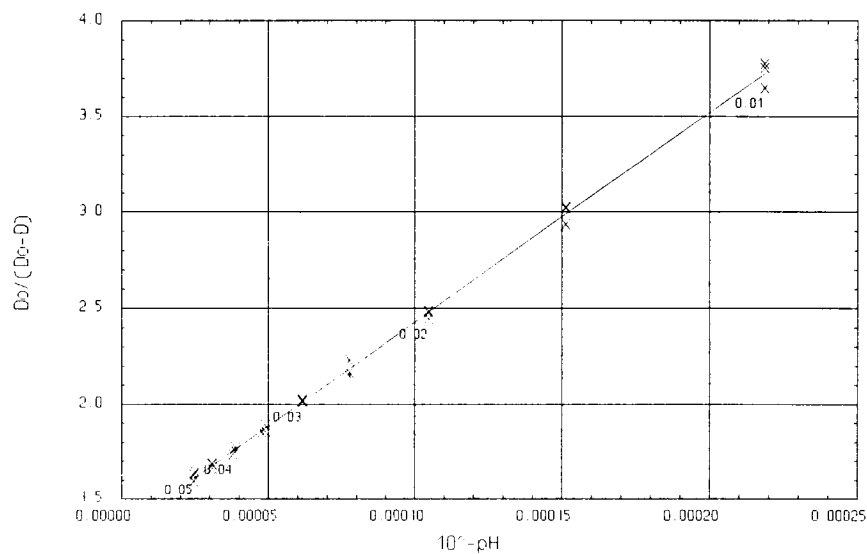
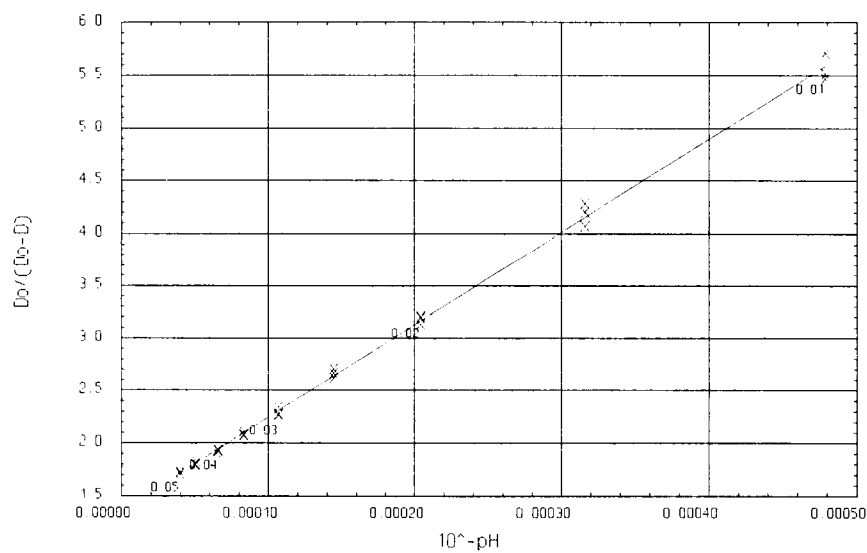
=====

Compound: TFMC (#4)
 Experiment #: 1323 + 1313
 Date: 6/19/96
 Dilution: 1:70
 Reaction Time: 2 min.
 Wavelength: 500 nm
 NaOH Range: 0.01 - 0.05 M
 Comments: Compound was recycled from exps. 1 & 2.

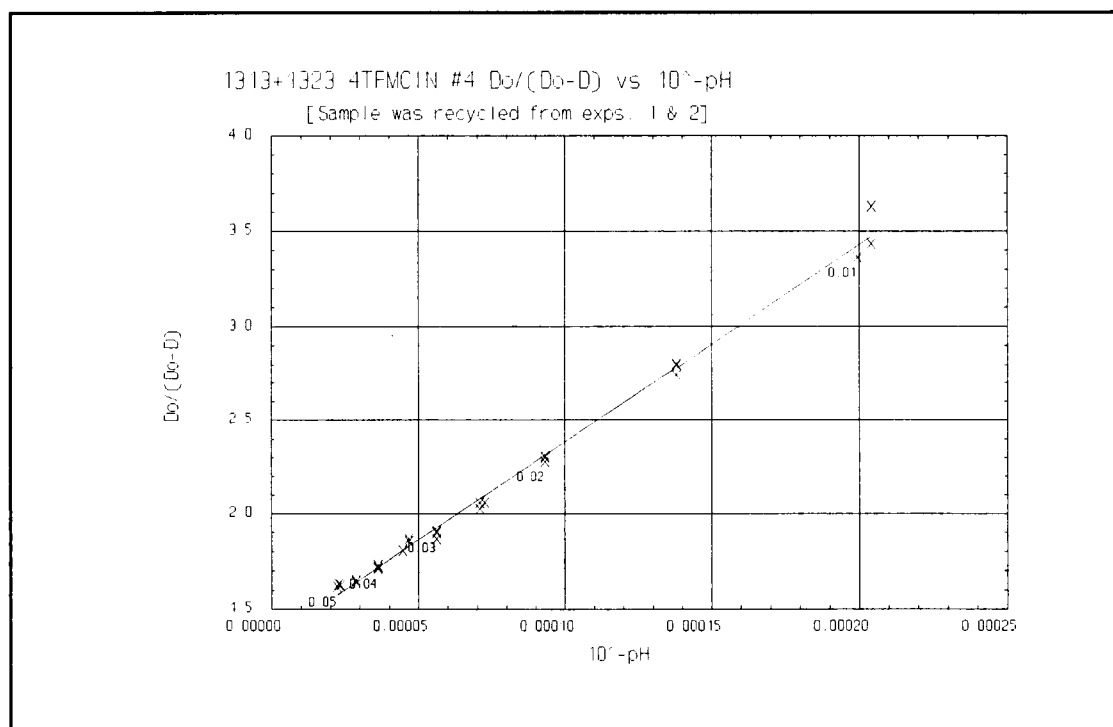
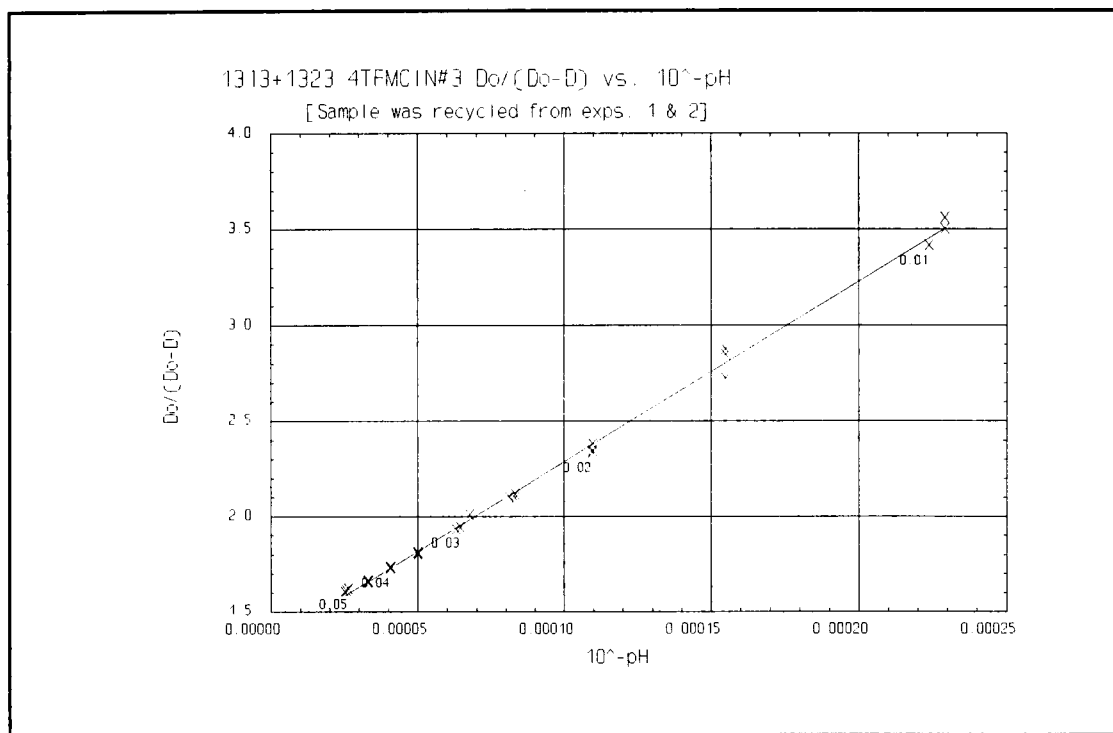
Calculation #1:	Calculation #2:
Linear Regression	Linear Regression
$[D_0/(D_0-D) \text{ vs. } 10^{-\text{pH}}]$	$[(K_h+K_a+10^{-\text{pH}})/k \text{ vs. } 10^{-\text{pH}}]$
	[w/ 0.01 - 0.045 points]
Intercept 1.34	Intercept 1.62×10^{-3}
Slope 10450	Slope 3.36×10^{-8}
Count 27	Count 24

K_h+K_a (from calc. #1) = 12.80×10^{-5}
 $K_h = (K_h+K_a) - K_a$, = 10.73×10^{-5}
 $K_a = \text{slope/intercept}$, = 2.07×10^{-5}
 $k_1 = K_h \cdot k_2$, = 0.0663
 $k_2 = 1/\text{intercept}$, = 618

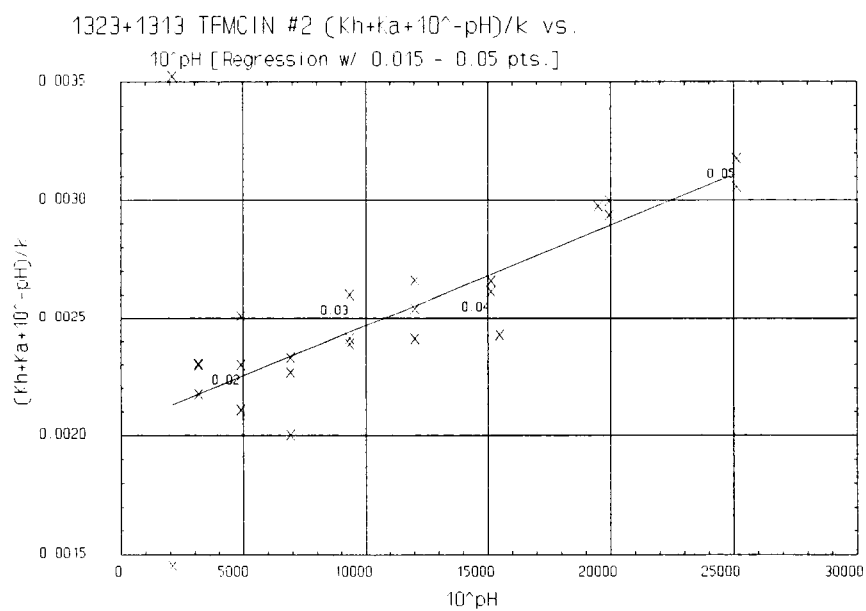
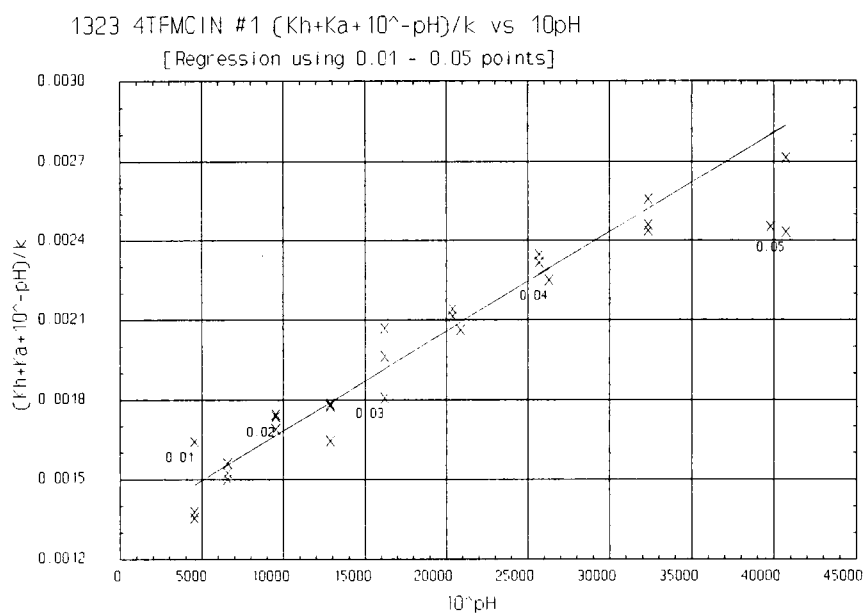
Appendix 12. TFMC (cont.)

1323, 4TFMCIN #1. $D_o/(D_o-D)$ vs. 10^{-pH} 1323+1313 TFMCIN #2 $D_o/(D_o-D)$ vs. 10^{-pH} 

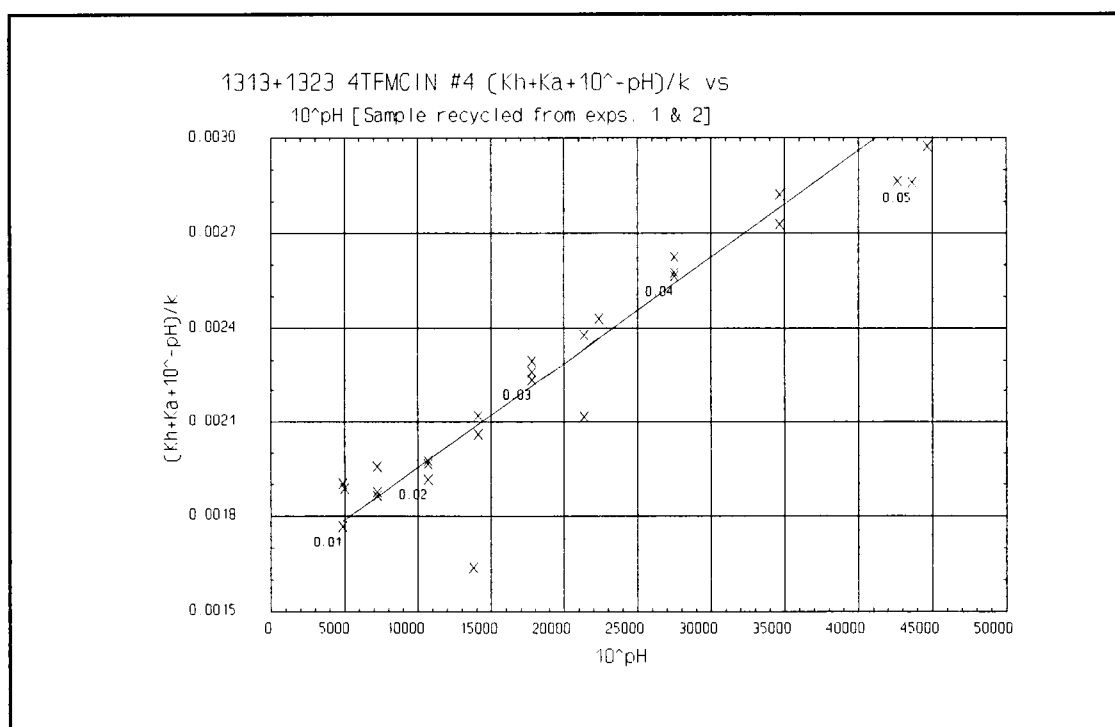
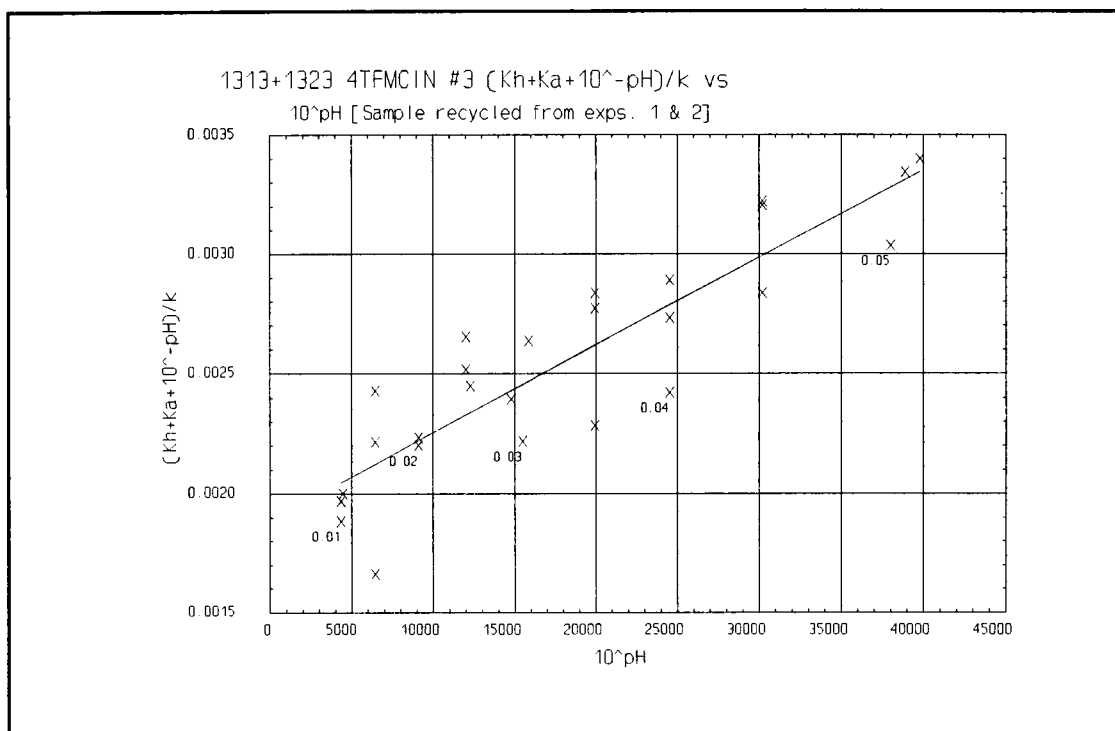
Appendix 12. TFMC (cont.)



Appendix 12. TFMC (cont.)



Appendix 12. TFMC (cont.)



Appendix 13. Regression Data for NC
(Anthocyanin acylated with 4-nitrocinnamic acid)

Compound: NC (#1)
 Experiment: 1332
 Date: 6/28/96
 Dilution: 1:80
 Reaction Time: 2 min.
 Wavelength: 500 nm
 NaOH Conc. Used: 0.01 - 0.05 M
 Comments:

Calculation #1: Linear Regression $[D_0/(D_0-D) \text{ vs. } 10^{-\text{pH}}]$ Intercept 1.22 Slope 5626 Count 26	Calculation #2: Linear Regression $[(K_h+K_a+10^{-\text{pH}})/k \text{ vs. } 10^{-\text{pH}}]$ Intercept 2.44×10^{-3} Slope 6.11×10^{-8} Count 26
--	--

Kh+Ka (from calc. #1) =	21.73×10^{-5}
Kh = (Kh+Ka) - Ka,	= 19.23×10^{-5}
Ka = slope/intercept,	= 2.51×10^{-5}
k1 = Kh*k2,	= 0.0790
k2 = 1/intercept,	= 411

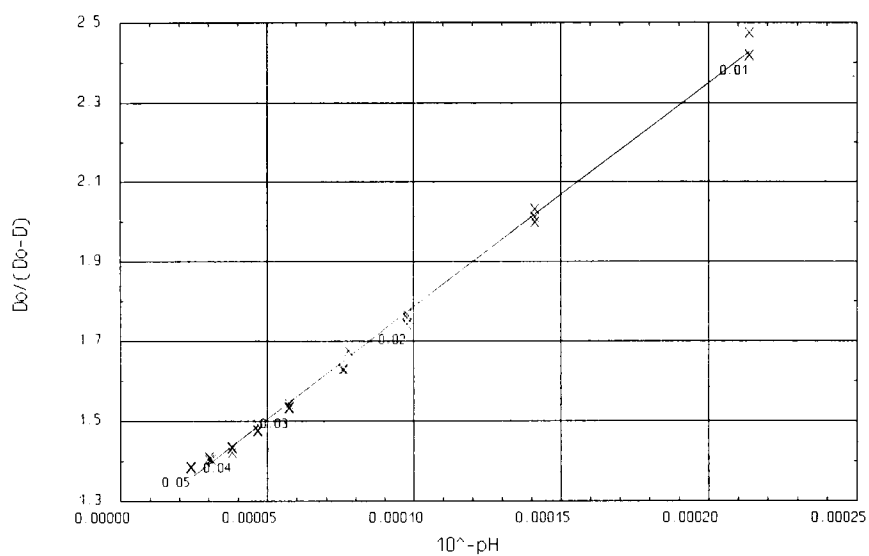
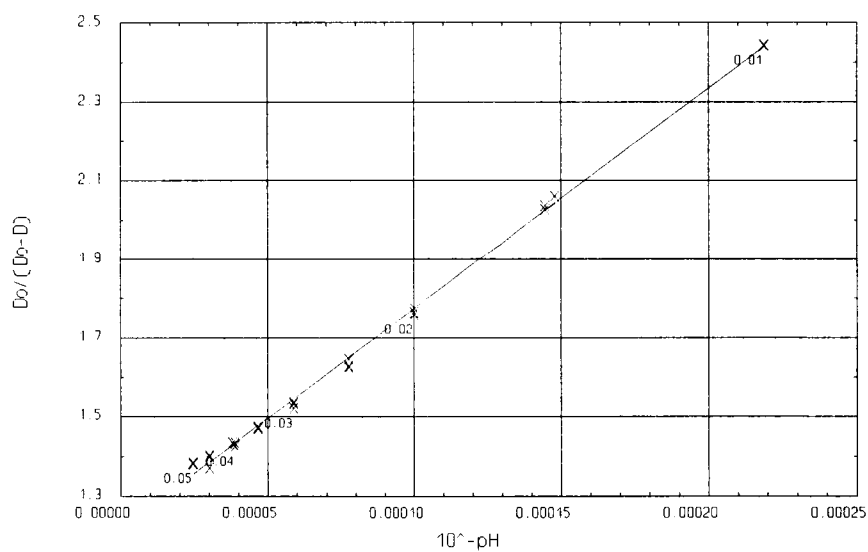
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Compound: NC (#2)
 Experiment: 1332
 Date: 6/28/96
 Dilution: 1:80
 Reaction Time: 2 min.
 Wavelength: 500 nm
 NaOH Conc. Used: 0.01 - 0.05 M
 Comments:

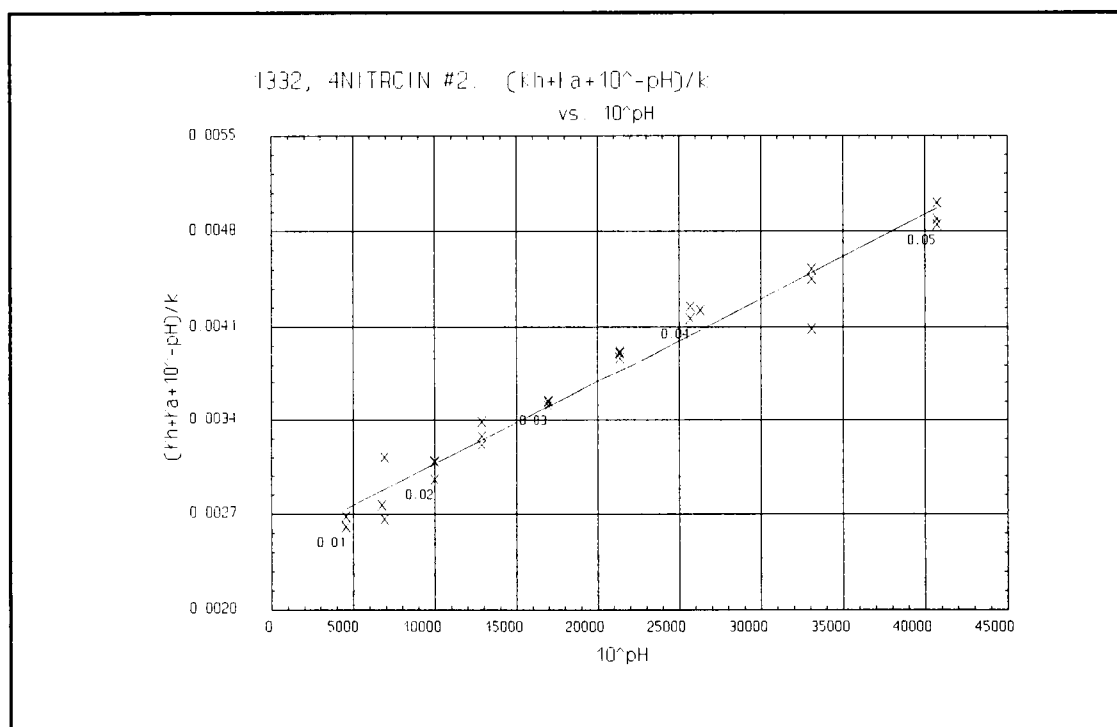
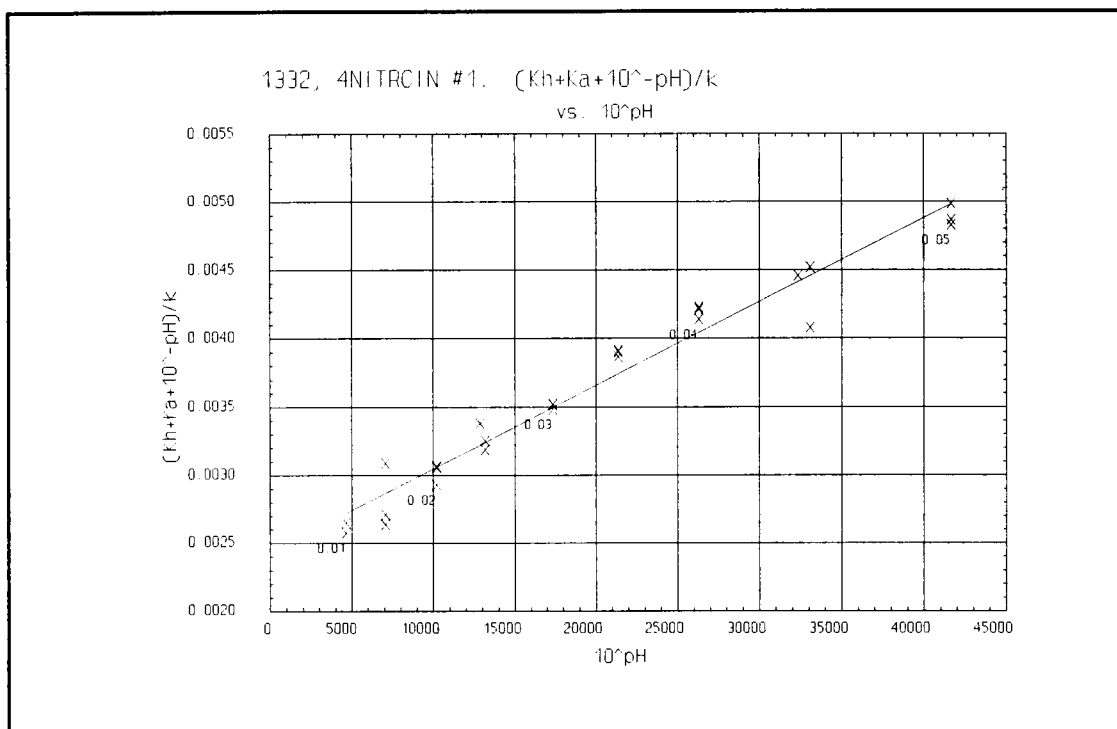
Calculation #1: Linear Regression $[D_0/(D_0-D) \text{ vs. } 10^{-\text{pH}}]$ Intercept 1.22 Slope 5586 Count 27	Calculation #2: Linear Regression $[(K_h+K_a+10^{-\text{pH}})/k \text{ vs. } 10^{-\text{pH}}]$ Intercept 2.45×10^{-3} Slope 6.17×10^{-8} Count 27
--	--

Kh+Ka (from calc. #1) =	21.78×10^{-5}
Kh = (Kh+Ka) - Ka,	= 19.26×10^{-5}
Ka = slope/intercept,	= 2.52×10^{-5}
k1 = Kh*k2,	= 0.0785
k2 = 1/intercept,	= 408

Appendix 13. NC (cont.)

1332, 4NITRCIN #1. $D_o/(D_o-D)$ vs. 10^{-pH} 1332, 4NITRCIN #2. $D_o/(D_o-D)$ vs. 10^{-pH} 

Appendix 13. NC (cont.)



Appendix 14. Regression Data for DMA
(Anthocyanin acylated with 4-(N-dimethylamino)cinnamic acid)

Compound: DMA (#1)
 Experiment #:1335
 Date: 7/15/96
 Dilution: 1:35
 Reaction Time: 2 min.
 Wavelength: 500 nm
 NaOH Conc. Used: 0.01 - 0.05 M
 Comments: Graph of $D_0/(D_0-D)$ curved; this compound may not be measurable using this protocol.
 Also, pH's measured about 0.1 lower than other compounds.

Calculation #1:	Calculation #2:
Linear Regression	Linear Regression
$[D_0/(D_0-D) \text{ vs. } 10^{-\text{pH}}]$	$[(K_h+K_a+10^{-\text{pH}})/k \text{ vs. } 10^{-\text{pH}}]$
	$[w/ 0.025 - 0.05 \text{ points}]$
Intercept 1.76	Intercept 3.02×10^{-3}
Slope 11235	Slope 2.32×10^{-3}
Count 18	Count 27

K_h+K_a (from calc. #1) =	15.69×10^{-5}
$K_h = (K_h+K_a) - K_a,$ =	14.92×10^{-5}
$K_a = \text{slope/intercept},$ =	0.77×10^{-5}
$k_1 = K_h \cdot k_2,$ =	0.0494
$k_2 = 1/\text{intercept},$ =	331

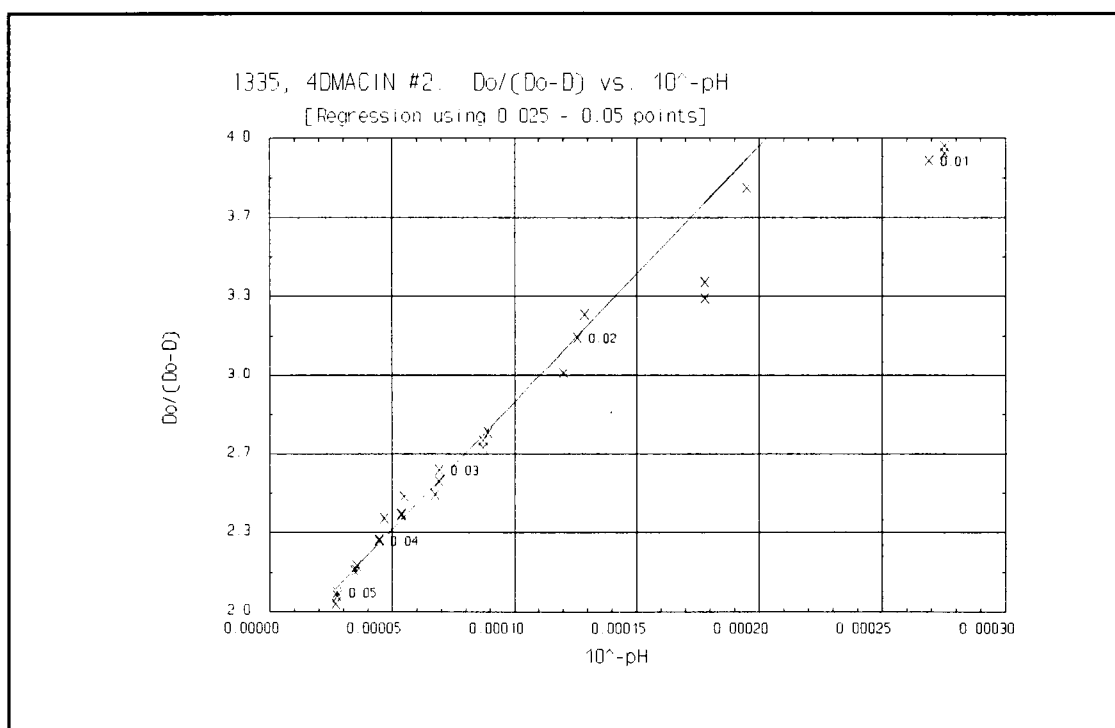
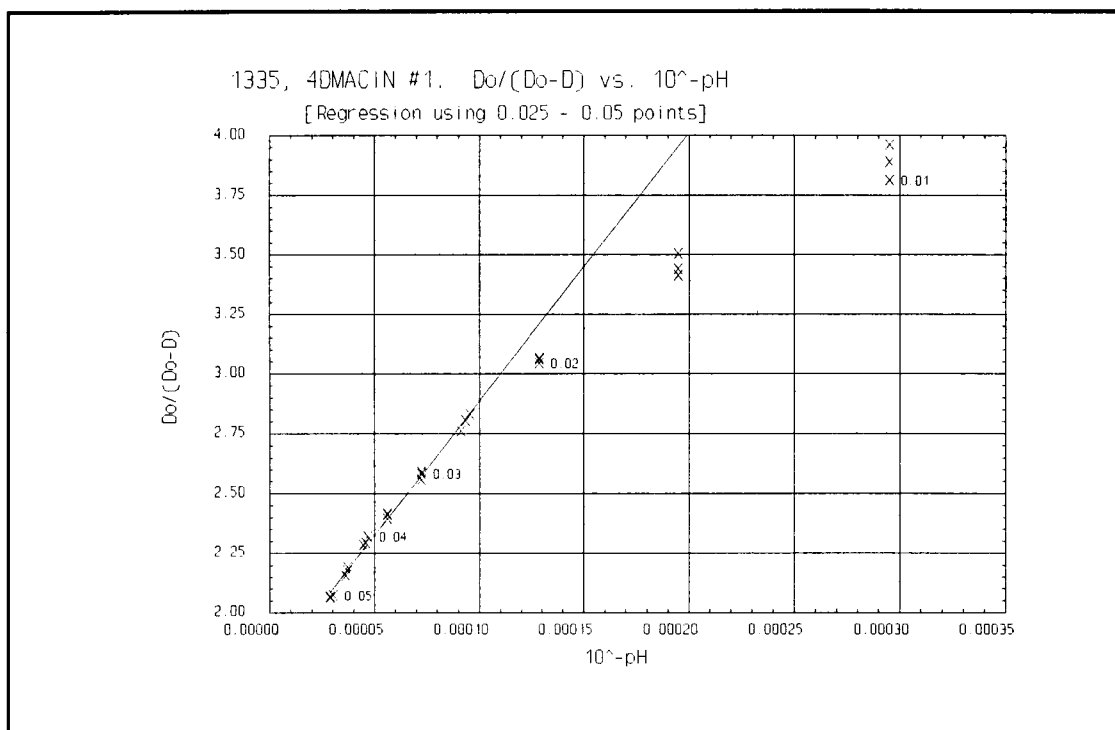
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Compound: 4DMACIN (#2)
 Experiment #: 1335
 Date: 7/17/96
 Dilution: 1:35
 Reaction Time: 2 min.
 Wavelength: 500 nm
 NaOH Conc. Used: 0.01 - 0.05 M
 Comments: Graph of $D_0/(D_0-D)$ curved; this compound may not be measurable using this protocol.
 Also, pH's measured about 0.1 lower than other compounds.

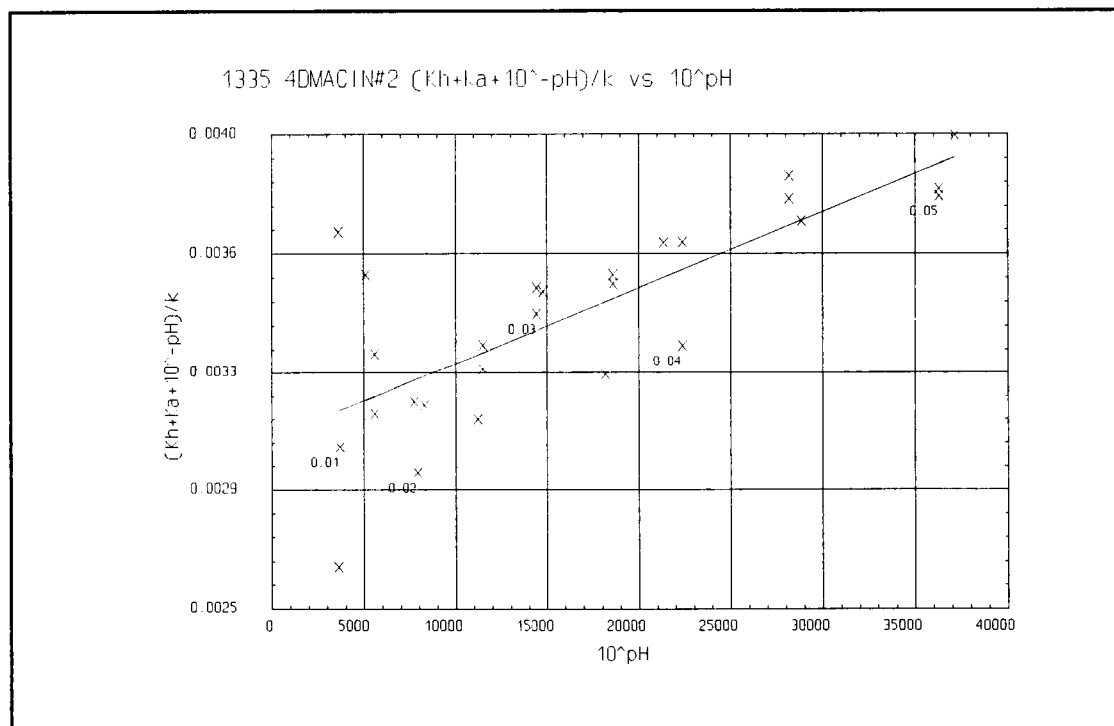
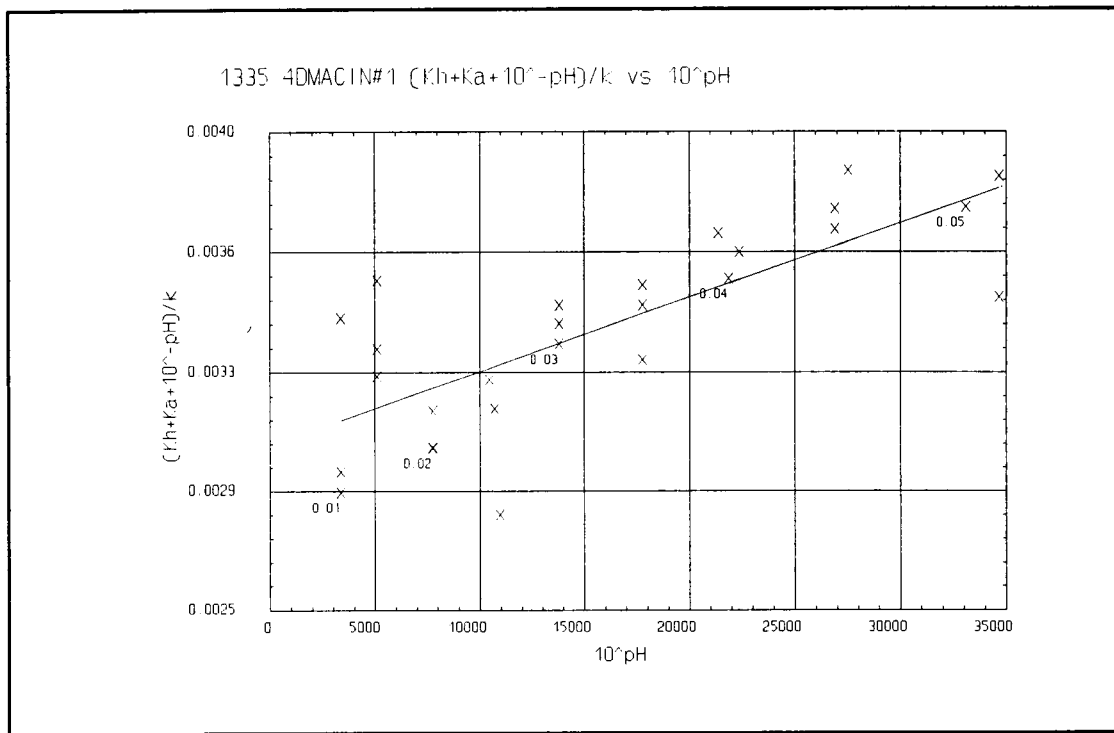
Calculation #1:	Calculation #2:
Linear Regression	Linear Regression
$[D_0/(D_0-D) \text{ vs. } 10^{-\text{pH}}]$	$[(K_h+K_a+10^{-\text{pH}})/k \text{ vs. } 10^{-\text{pH}}]$
	$[w/ 0.025 - 0.05 \text{ points}]$
Intercept 1.81	Intercept 3.04×10^{-3}
Slope 10814	Slope 2.39×10^{-3}
Count 18	Count 27

K_h+K_a (from calc. #1) =	16.69×10^{-5}
$K_h = (K_h+K_a) - K_a,$ =	15.91×10^{-5}
$K_a = \text{slope/intercept},$ =	0.79×10^{-5}
$k_1 = K_h \cdot k_2,$ =	0.0523
$k_2 = 1/\text{intercept},$ =	329

Appendix 14. DMA (cont.)



Appendix 14. DMA (cont.)



Appendix 15. Regression Data for 4HP
(Anthocyanin acylated with 4-hydroxyphenylpropionic acid)

Compound: 4HP (#1)
 Experiment: 1329
 Date: 5/30/96
 Dilution: 1:160
 Reaction Time: 2 min.
 Wavelength: 500 nm
 NaOH Conc. Used: 0.005 - 0.05 M
 Comments:

Calculation #1:	Calculation #2:
Linear Regression	Linear Regression
$[D_0/(D_0-D) \text{ vs. } 10^{-\text{pH}}]$	$[(K_h+K_a+10^{-\text{pH}})/k \text{ vs. } 10^{-\text{pH}}]$
Intercept 1.19	Intercept 4.72×10^{-3}
Slope 3636	Slope 17.18×10^{-8}
Count 30	Count 30

Kh+Ka (from calc. #1) = 32.76×10^{-5}
 Kh = (Kh+Ka) - Ka, = 29.12×10^{-5}
 Ka = slope/intercept, = 3.64×10^{-5}
 k1 = Kh*k2, = 0.0617
 k2 = 1/intercept, = 212

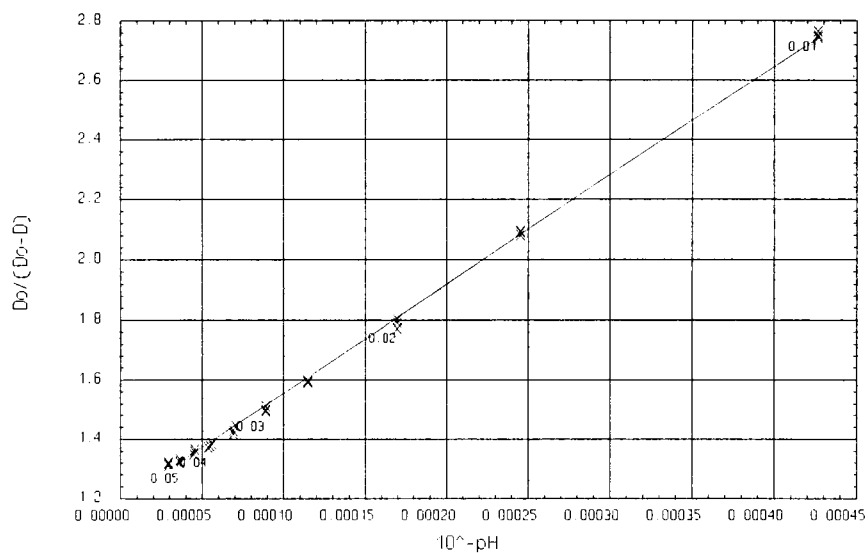
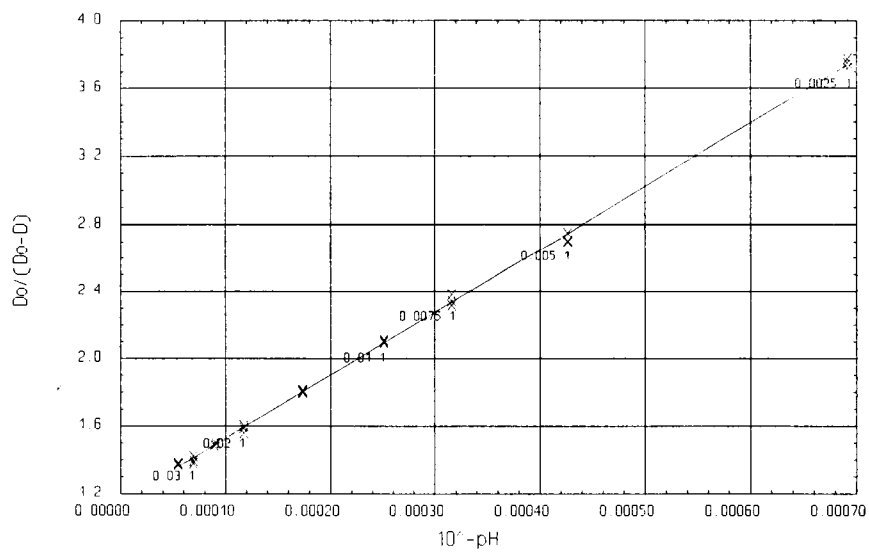
=====

Compound: 4HP (#2)
 Experiment: 1329
 Date: 5/31/96
 Dilution: 1:160
 Reaction Time: 2 min.
 Wavelength: 500 nm
 NaOH Conc. Used: 0.0025 - 0.035 M
 Comments: Used lower NaOH conc. than 1st exp.

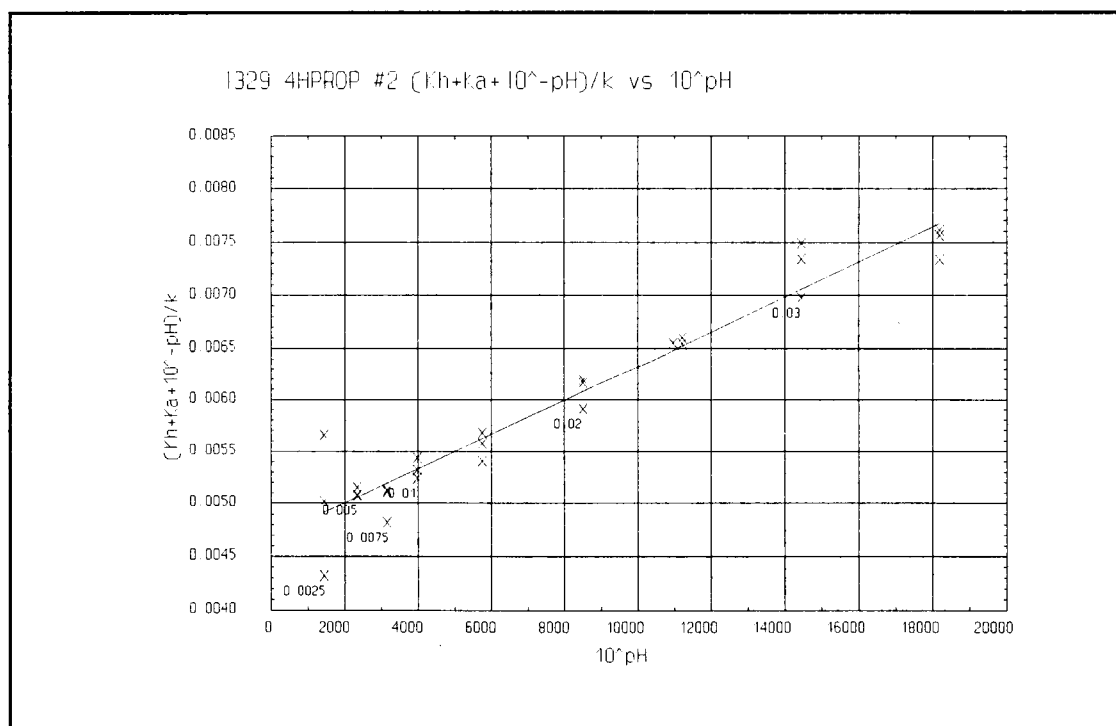
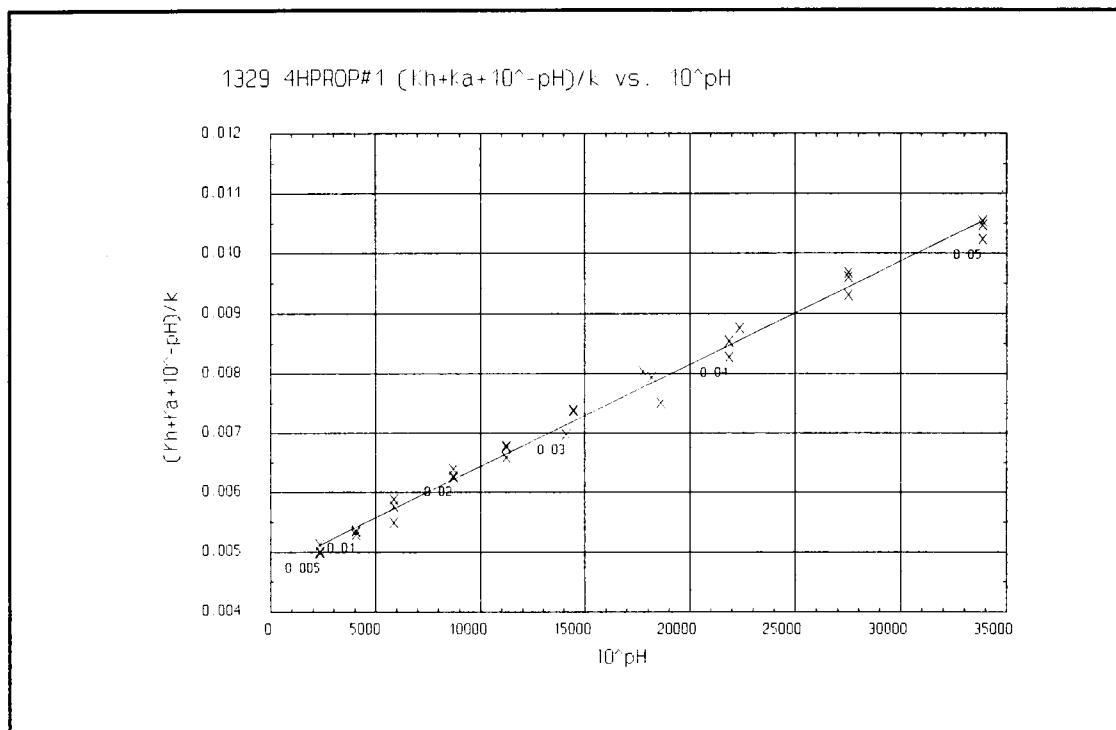
Calculation #1:	Calculation #2:
Linear Regression	Linear Regression
$[D_0/(D_0-D) \text{ vs. } 10^{-\text{pH}}]$	$[(K_h+K_a+10^{-\text{pH}})/k \text{ vs. } 10^{-\text{pH}}]$
Intercept 1.15	Intercept 4.67×10^{-3}
Slope 3735	Slope 16.49×10^{-8}
Count 27	Count 27

Kh+Ka (from calc. #1) = 30.89×10^{-5}
 Kh = (Kh+Ka) - Ka, = 27.36×10^{-5}
 Ka = slope/intercept, = 3.53×10^{-5}
 k1 = Kh*k2, = 0.0586
 k2 = 1/intercept, = 214

Appendix 15. 4HP (cont.)

1329, 4HPROP #1. $D_o/(D_o-D)$ vs. 10^{-pH} 1329, 4HPROP (#2). $D_o/(D_o-D)$ vs. 10^{-pH} 

Appendix 15. 4HP (cont.)



Appendix 16. Regression Data for 4HB
(Anthocyanin acylated with 4-hydroxybenzoic acid)

Compound: 4HB (#2)
 Experiment #: 1339
 Date: 8/22/96
 Dilution: 1:80
 Reaction. Time: 2 min.
 Wavelength: 500 nm
 NaOH Conc. Used: 0.0075 - 0.045 M
 Comments: Peak 2 from semi-prep HPLC of
 culture grown w/ 4-hydroxybenzoic acid.

Calculation #1:	Calculation #2:
Linear Regression	Linear Regression
$[D_0/(D_0-D) \text{ vs. } 10^{-\text{pH}}]$	$[(K_h+K_a+10^{-\text{pH}})/k \text{ vs. } 10^{-\text{pH}}]$
Intercept 1.27	Intercept 1.59×10^{-3}
Slope 9685	Slope 4.02×10^{-8}
Count 27	Count 27

Kh+Ka (from calc. #1) = 13.12×10^{-5}
 Kh = (Kh+Ka) - Ka, = 10.59×10^{-5}
 Ka = slope/intercept, = 2.53×10^{-5}
 k1 = Kh*k2, = 0.0667
 k2 = 1/intercept, = 630

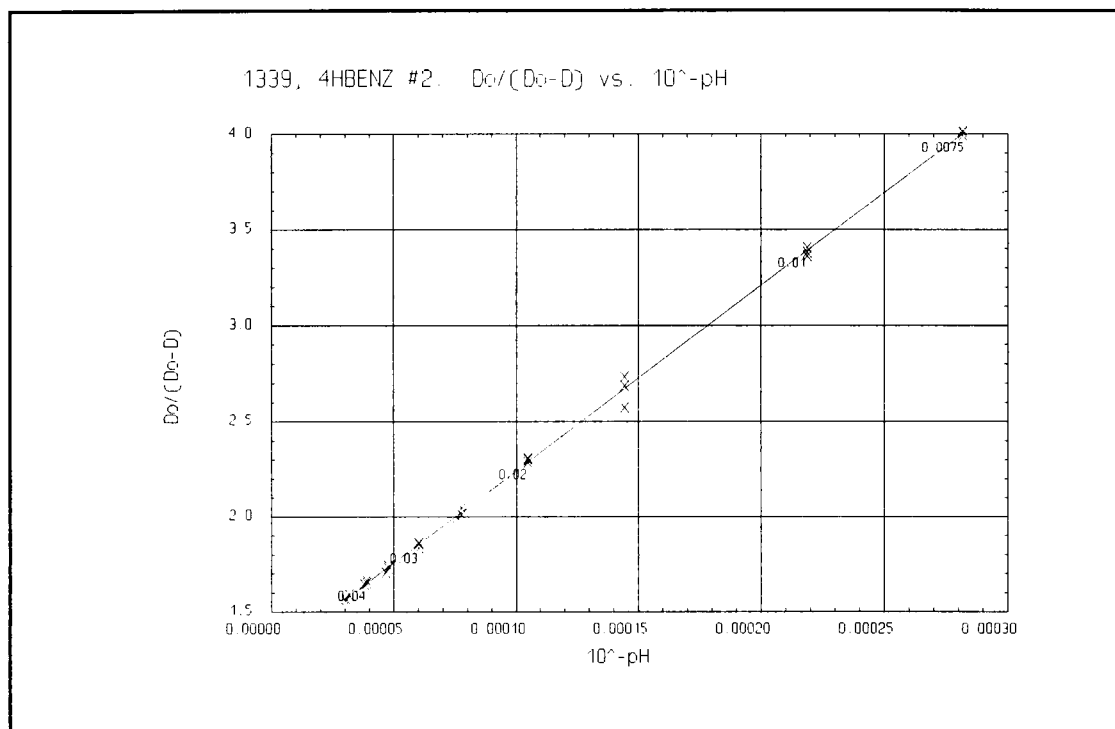
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Compound: 4HB (#3)
 Experiment #: 1339
 Date: 8/22/96
 Dilution: 1:80
 Reaction. Time: 2 min.
 Wavelength: 500 nm
 NaOH Conc. Used: 0.0075 - 0.045 M
 Comments: Peak 2 from semi-prep HPLC of
 culture grown w/ 4-hydroxybenzoic acid.

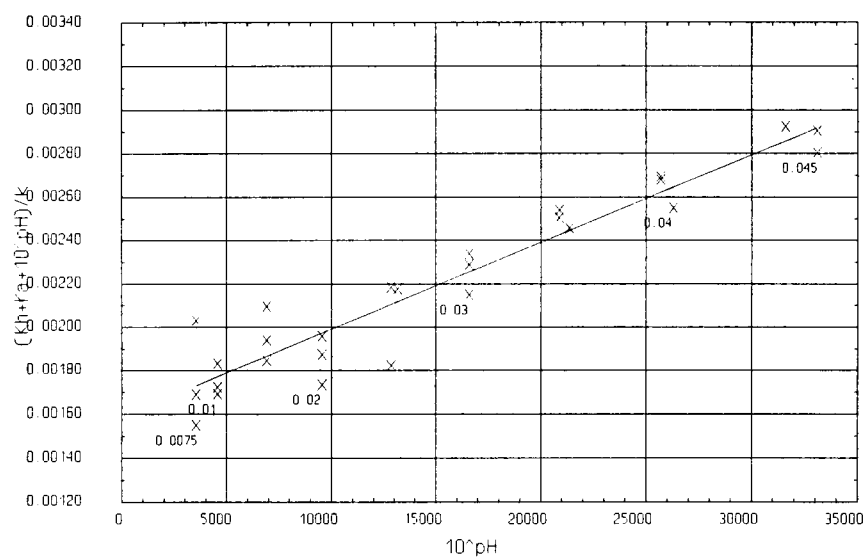
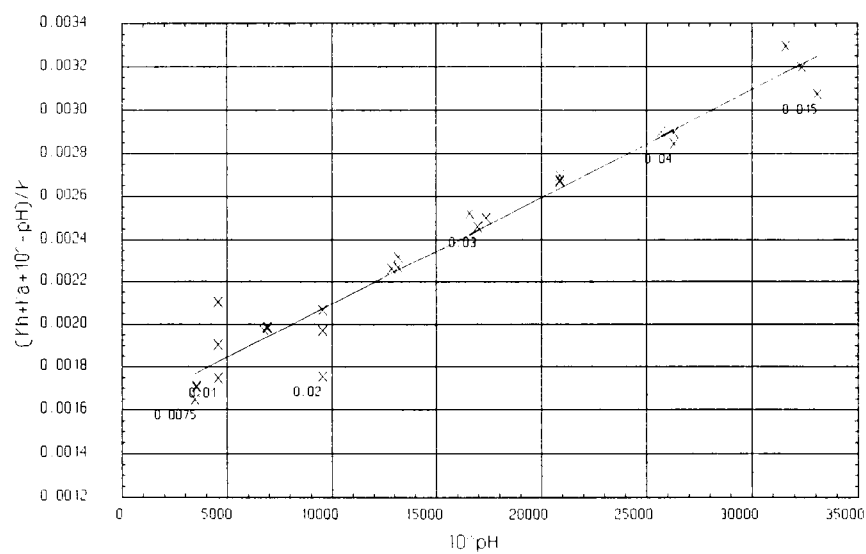
Calculation #1:	Calculation #2:
Linear Regression	Linear Regression
$[D_0/(D_0-D) \text{ vs. } 10^{-\text{pH}}]$	$[(K_h+K_a+10^{-\text{pH}})/k \text{ vs. } 10^{-\text{pH}}]$
Intercept 1.31	Intercept 1.59×10^{-3}
Slope 9018	Slope 4.99×10^{-8}
Count 27	Count 27

Kh+Ka (from calc. #1) = 14.50×10^{-5}
 Kh = (Kh+Ka) - Ka, = 11.36×10^{-5}
 Ka = slope/intercept, = 3.13×10^{-5}
 k1 = Kh*k2, = 0.0713
 k2 = 1/intercept, = 627

Appendix 16. 4HB (cont.)



Appendix 16. 4HB (cont.)

1339 4HBENZ #2 $(K_h + K_a + 10^{-pH})/k$ vs 10^{pH} 1339 4HBENZ #3 $(K_h + K_a + 10^{-pH})/k$ vs 10^{pH} 

Appendix 17. Regression Data for TB
(Anthocyanin acylated with 3,4,5-trimethoxybenzoic acid)

Compound: TB (#1)
 Experiment: 1333
 Date: 7/1/96
 Dilution: 1:260
 Reaction Time: 2 min.
 Wavelength: 500 nm
 NaOH Conc. Used: 0.01 - 0.05 M
 Comments:

Calculation #1:	Calculation #2:
Linear Regression	Linear Regression
$[D_0/(D_0-D)] \text{ vs. } 10^{-pH}$	$[(K_h+K_a+10^{-pH})/k \text{ vs. } 10^{-pH}]$
Intercept 1.24	Intercept 1.73×10^{-3}
Slope 8015	Slope 2.36×10^{-8}
Count 27	Count 27

Kh+Ka (from calc. #1) = 15.44×10^{-5}
 Kh = (Kh+Ka) - Ka, = 14.07×10^{-5}
 Ka = slope/intercept, = 1.36×10^{-5}
 k1 = Kh*k2, = 0.0815
 k2 = 1/intercept, = 579

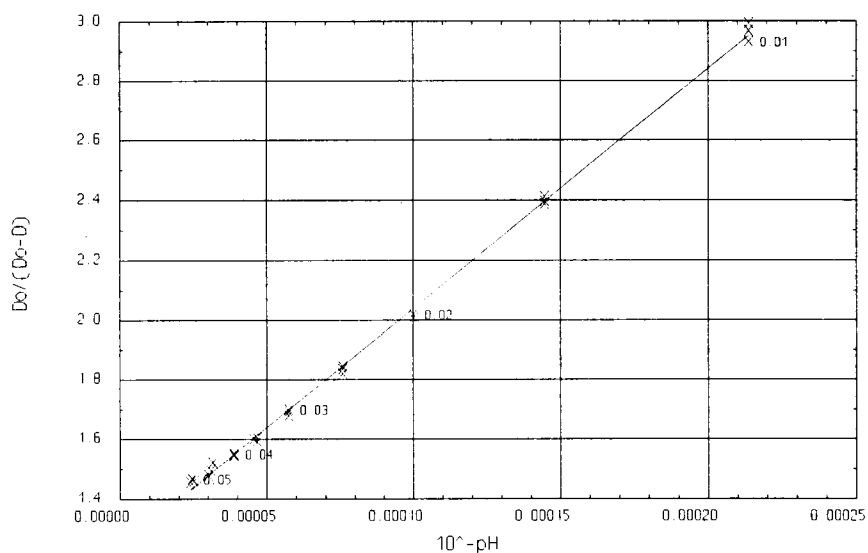
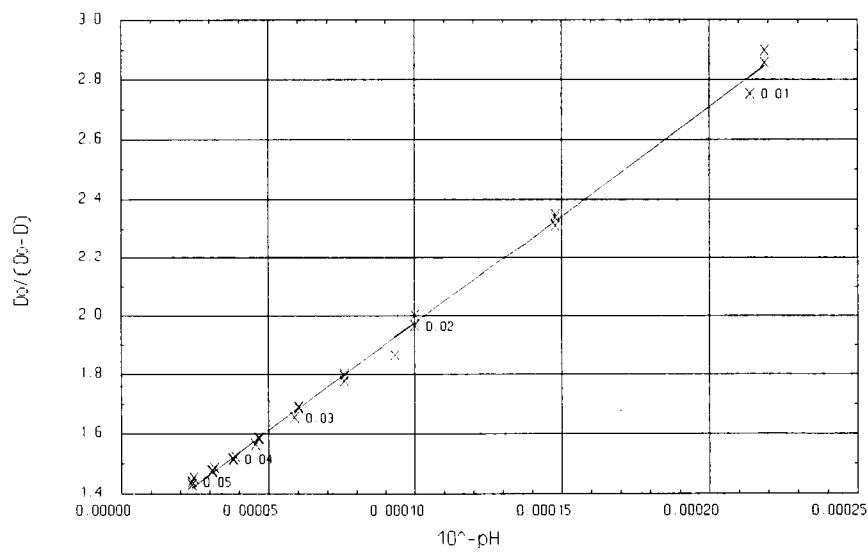
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Compound: TB (#2)
 Experiment: 1333
 Date: 7/2/96
 Dilution: 1:260
 Reaction Time: 2 min.
 Wavelength: 500 nm
 NaOH Conc. Used: 0.01 - 0.05 M
 Comments:

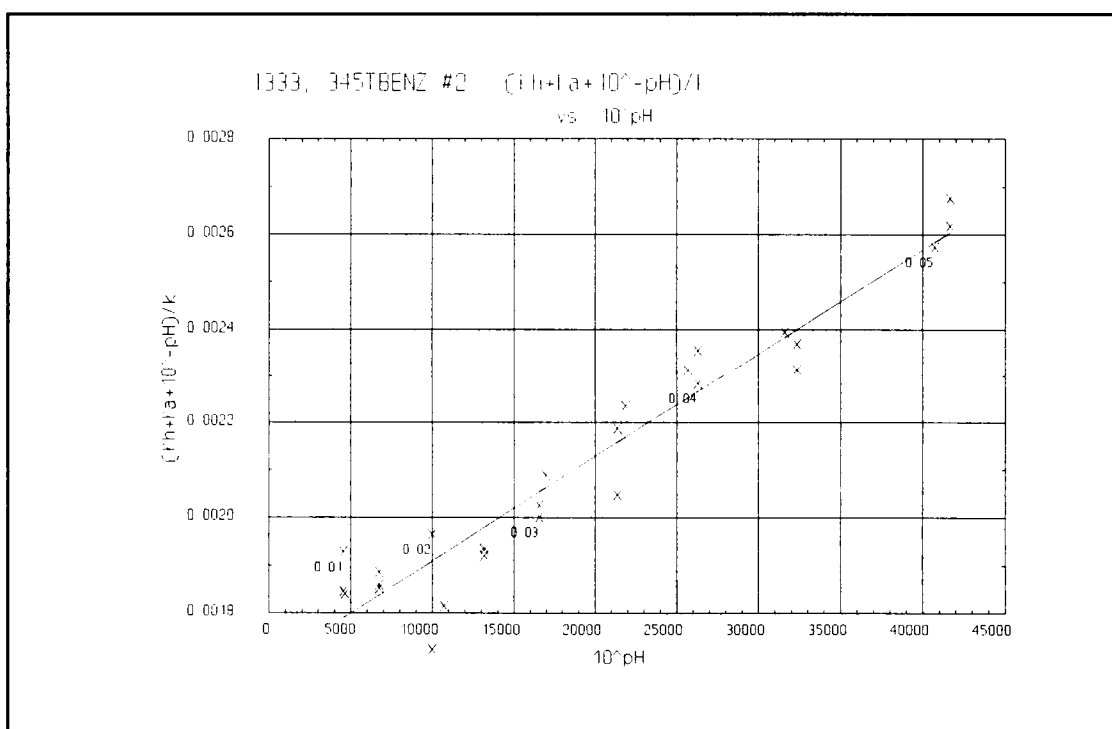
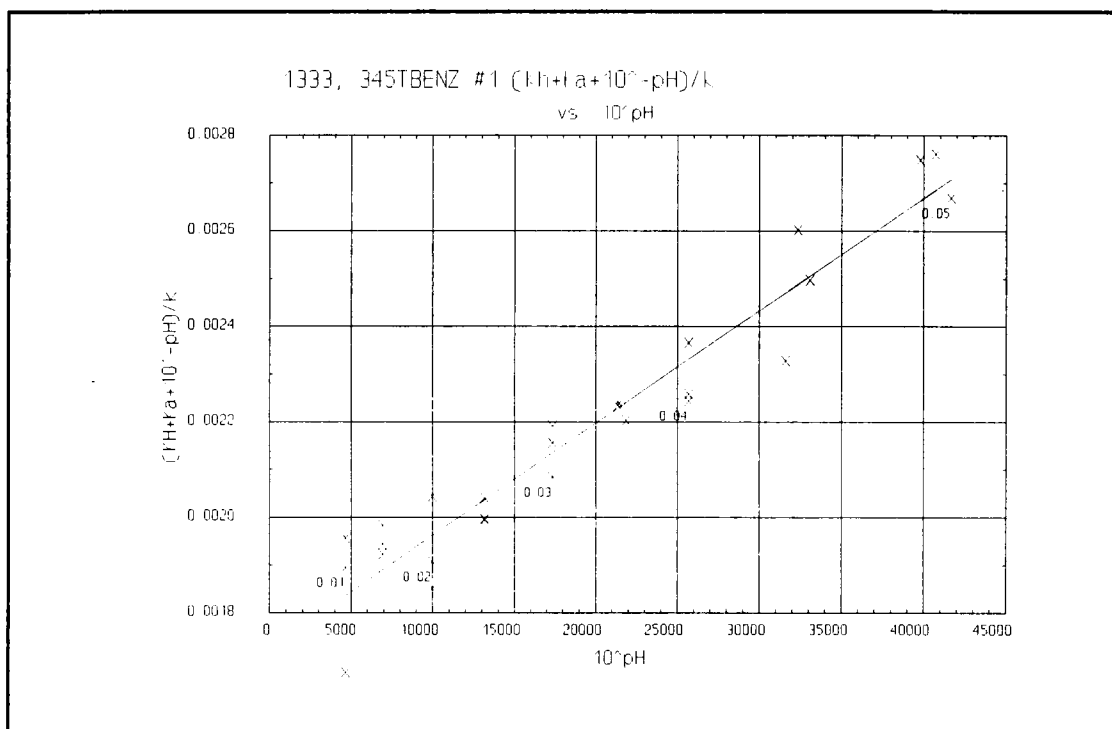
Calculation #1:	Calculation #2:
Linear Regression	Linear Regression
$[D_0/(D_0-D)] \text{ vs. } 10^{-pH}$	$[(K_h+K_a+10^{-pH})/k \text{ vs. } 10^{-pH}]$
Intercept 1.24	Intercept 1.69×10^{-3}
Slope 7317	Slope 2.20×10^{-8}
Count 27	Count 27

Kh+Ka (from calc. #1) = 17.00×10^{-5}
 Kh = (Kh+Ka) - Ka, = 15.70×10^{-5}
 Ka = slope/intercept, = 1.30×10^{-5}
 k1 = Kh*k2, = 0.0930
 k2 = 1/intercept, = 592

Appendix 17. TB (cont.)

1333, 345TBENZ #1. $D_o/(D_o-D)$ vs. 10^{-pH} 1333, 345TBENZ #2. $D_o/(D_o-D)$ vs. 10^{-pH} 

Appendix 17. TB (cont.)



Appendix 18. Regression Data for CB
(Anthocyanin acylated with 4-chlorobenzoic acid)

Compound: CB (#1)
 Experiment #: 1348
 Date: 10/15/96
 Dilution: 1:128
 Reaction Time: 2 mins.
 Wavelength: 500 nm
 NaOH Conc. Used: 0.005 - 0.04 M
 Comments: Peak 1 (minor peak) from preparative HPLC of culture grown w/ 4-chlorocinnamic acid.

Calculation #1:	Calculation #2:
Linear Regression	Linear Regression
$[D_0/(D_0-D)] \text{ vs. } 10^{-\text{pH}}$	$[(K_h+K_a+10^{-\text{pH}})/k] \text{ vs. } 10^{-\text{pH}}$
Intercept 1.11	Intercept 1.96×10^{-3}
Slope 6308	Slope 3.94×10^{-8}
Count 27	Count 27

K_h+K_a (from calc. #1) = 17.65×10^{-5}
 $K_h = (K_h+K_a) - K_a$, = 15.63×10^{-5}
 $K_a = \text{slope/intercept}$, = 2.01×10^{-5}
 $k_1 = K_h \cdot k_2$, = 0.0798
 $k_2 = 1/\text{intercept}$, = 511

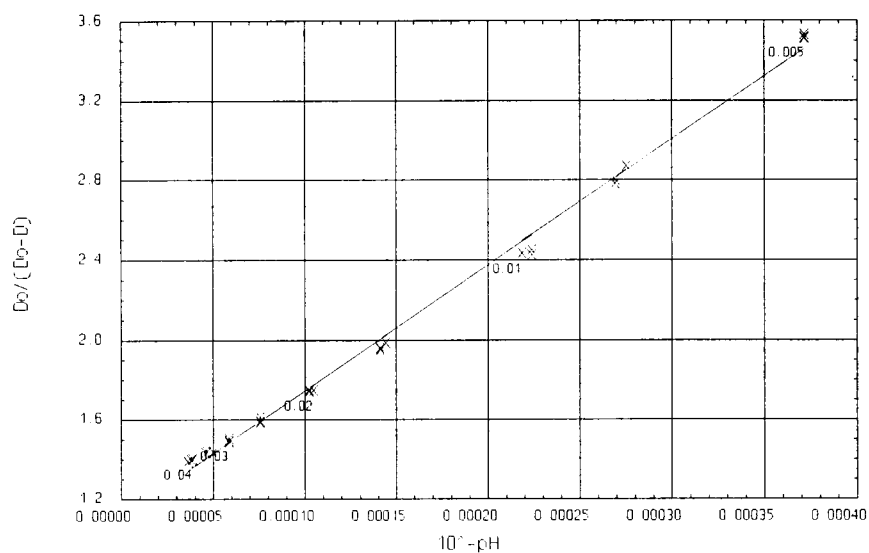
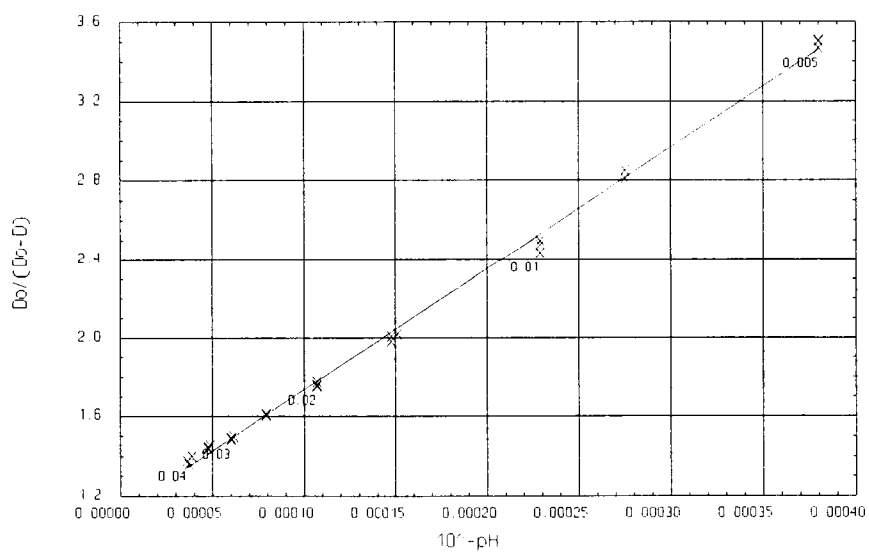
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Compound: CB (#2)
 Experiment #: 1348
 Date: 10/15/96
 Dilution: 1:128
 Reaction Time: 2 mins.
 Wavelength: 500 nm
 NaOH Conc. Used: 0.005 - 0.04 M
 Comments: Peak 1 (minor peak) from preparative HPLC of culture grown w/ 4-chlorocinnamic acid.

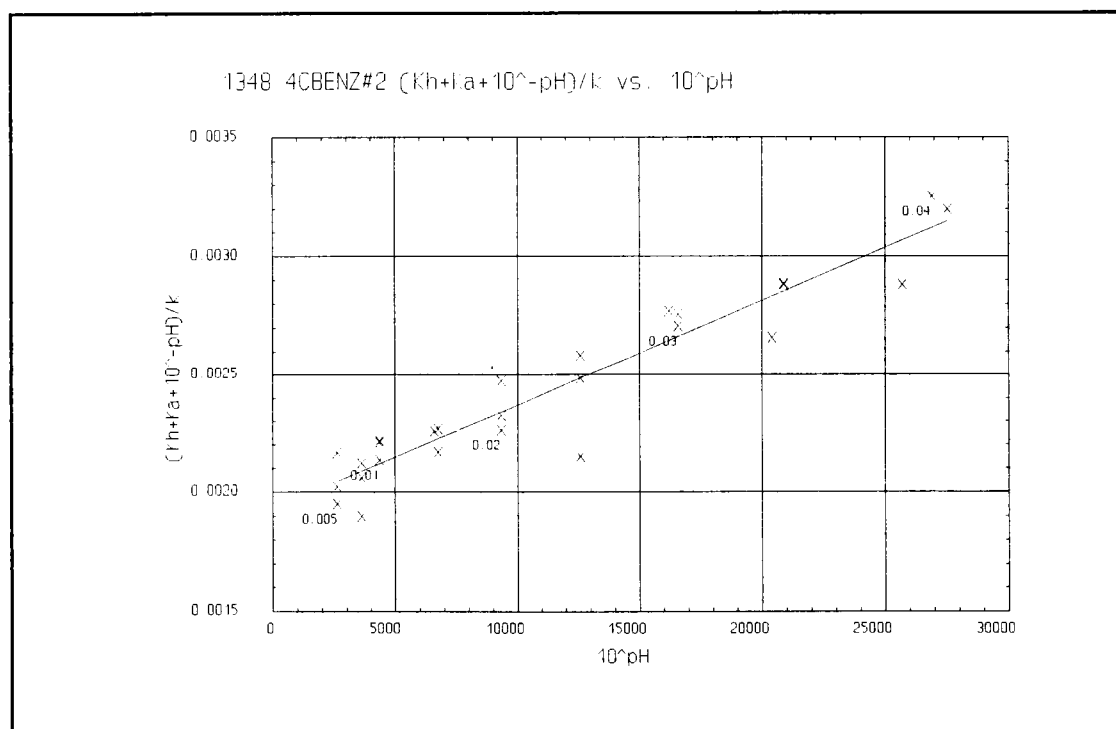
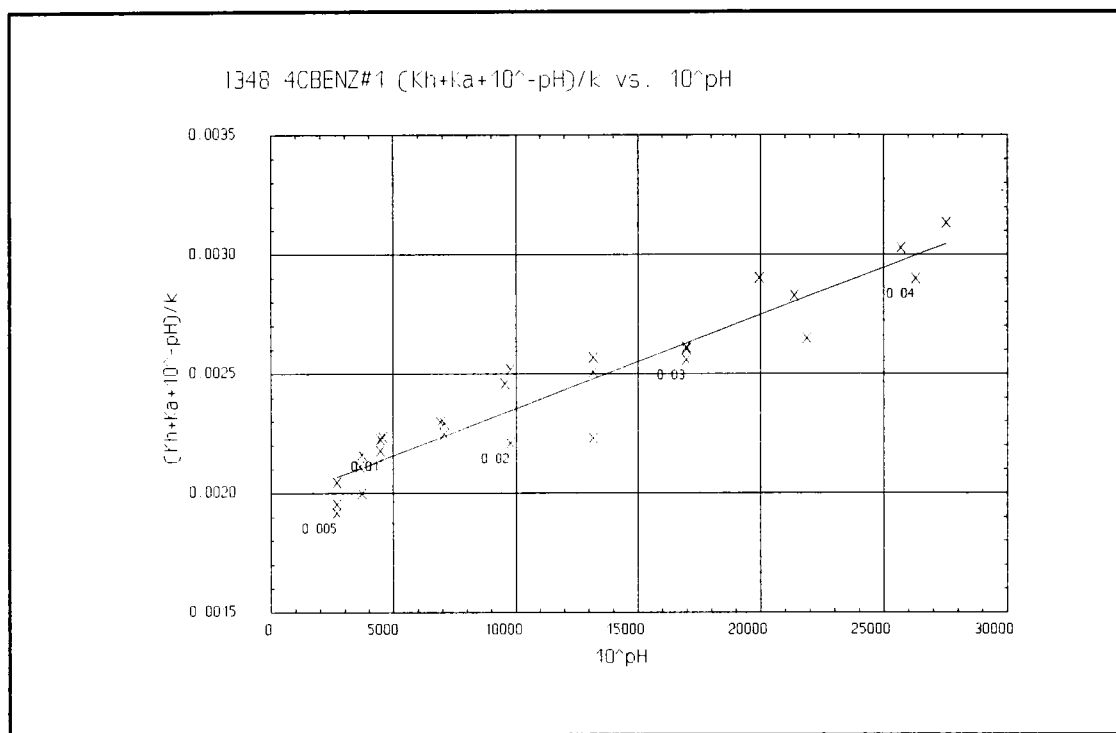
Calculation #1:	Calculation #2:
Linear Regression	Linear Regression
$[D_0/(D_0-D)] \text{ vs. } 10^{-\text{pH}}$	$[(K_h+K_a+10^{-\text{pH}})/k] \text{ vs. } 10^{-\text{pH}}$
Intercept 1.12	Intercept 1.93×10^{-3}
Slope 6155	Slope 4.44×10^{-8}
Count 27	Count 27

K_h+K_a (from calc. #1) = 18.18×10^{-5}
 $K_h = (K_h+K_a) - K_a$, = 15.87×10^{-5}
 $K_a = \text{slope/intercept}$, = 2.31×10^{-5}
 $k_1 = K_h \cdot k_2$, = 0.0824
 $k_2 = 1/\text{intercept}$, = 519

Appendix 18. CB (cont.)

1348, 4CBENZ #1. $D_o/(D_o-D)$ vs. 10^{-pH} 1348, 4CBENZ. $D_o/(D_o-D)$ vs. 10^{-pH} 

Appendix 18. CB (cont.)



Appendix 19. Regression Data for TFMB
(Anthocyanin acylated with 4-trifluoromethylybenzoic acid)

Compound: TFMB (#1)
Experiment #: 1334
Date: 7/10/96
Dilution: 1:142
Reaction Time: 2 min.
Wavelength: 500 nm
NaOH Conc. Used: 0.005 - 0.04 M
Comments:

Calculation #1:	Calculation #2:
Linear Regression	Linear Regression
$[D_0/(D_0-D)] \text{ vs. } 10^{-\text{pH}}$	$[(K_h+K_a+10^{-\text{pH}})/k] \text{ vs. } 10^{-\text{pH}}$
Intercept 1.13	Intercept 2.21×10^{-3}
Slope 4587	Slope 5.09×10^{-8}
Count 27	Count 27

Kh+Ka (from calc. #1) = 24.90×10^{-5}
 Kh = (Kh+Ka) - Ka, = 22.60×10^{-5}
 Ka = slope/intercept, = 2.30×10^{-5}
 k1 = Kh*k2, = 0.102
 k2 = 1/intercept, = 453

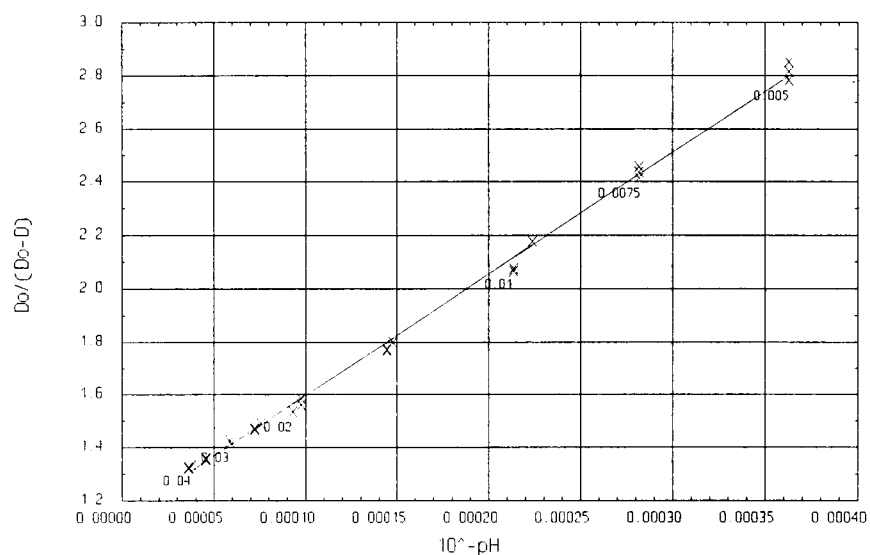
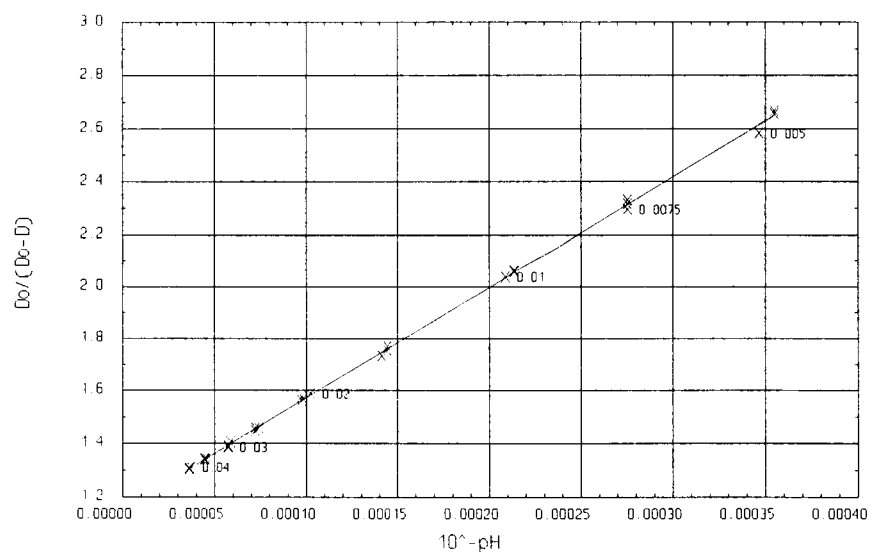
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Compound: TFMB (#2)
Experiment #: 1334
Date: 7/10/96
Dilution: 1:142
Reaction Time: 2 min.
Wavelength: 500 nm
NaOH Conc. Used: 0.005 - 0.04 M
Comments:

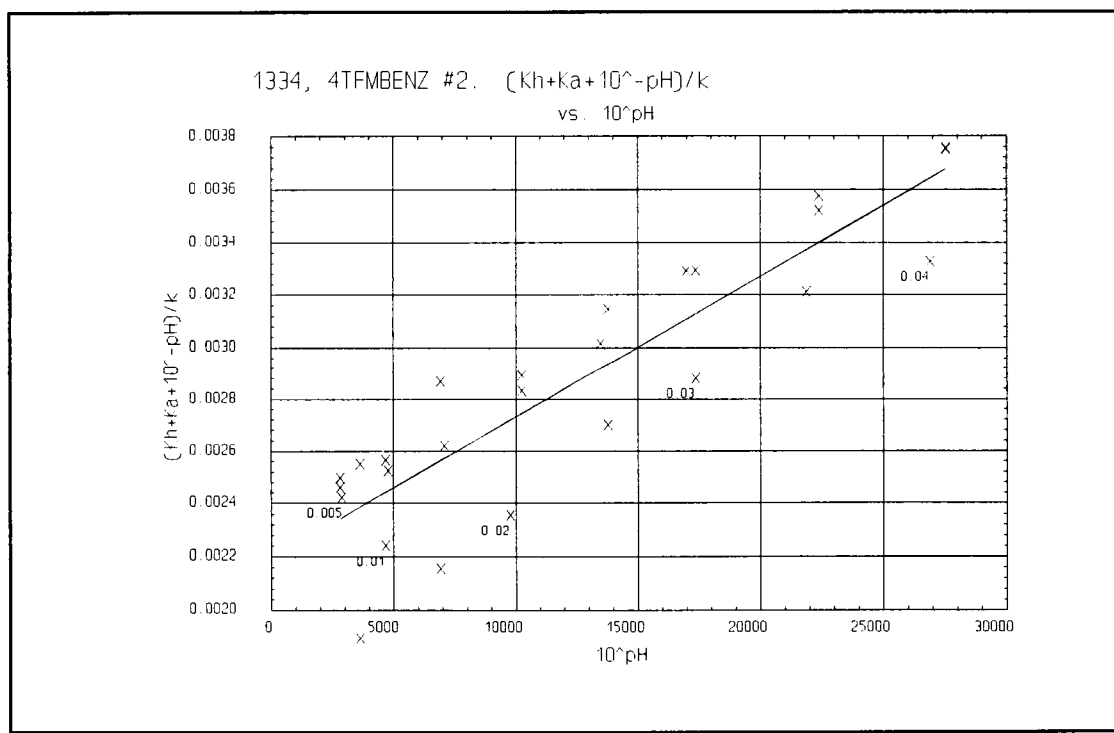
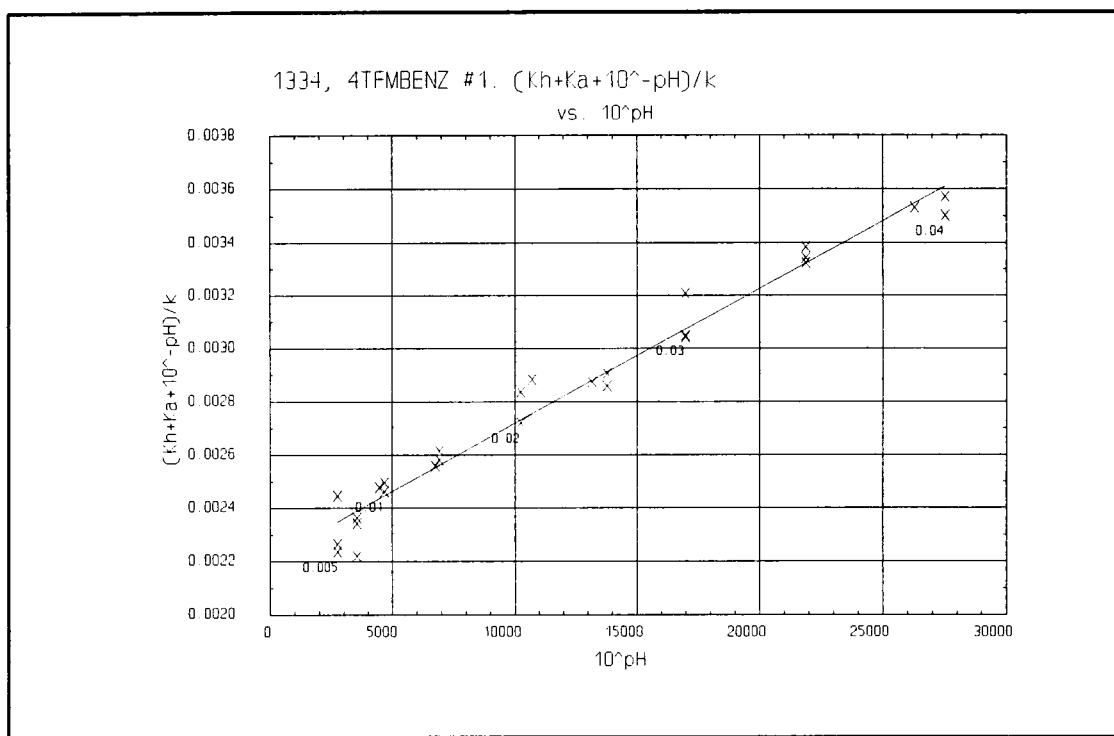
Calculation #1:	Calculation #2:
Linear Regression	Linear Regression
$[D_0/(D_0-D)] \text{ vs. } 10^{-\text{pH}}$	$[(K_h+K_a+10^{-\text{pH}})/k] \text{ vs. } 10^{-\text{pH}}$
Intercept 1.15	Intercept 2.19×10^{-3}
Slope 4217	Slope 5.40×10^{-8}
Count 27	Count 26

Kh+Ka (from calc. #1) = 27.32×10^{-5}
 Kh = (Kh+Ka) - Ka, = 24.85×10^{-5}
 Ka = slope/intercept, = 2.47×10^{-5}
 k1 = Kh*k2, = 0.114
 k2 = 1/intercept, = 457

Appendix 19. TFMB (cont.)

1334, 4TFMBENZ #1. $D_o/(D_o-D)$ vs. 10^{-pH} 1334, 4TFMBENZ #2. $D_o/(D_o-D)$ vs. 10^{-pH} 

Appendix 19. TFMB (cont.)



**Appendix 20. Regression Data for CYANGXG
(Non-acylated anthocyanin)**

Compound: CYANGXG (#1)
 Experiment #: 1263
 Date: 2/19/96
 Dilution: 1:300
 Reaction Time: 2 min.
 Wavelength: 500 nm
 Comments: One of first experiments. Note that concentrations of NaOH above 0.04 M tend to skew graph of $D_0/(D_0-D)$ vs. 10^{-pH} ; this was not the case with most other compounds.

Calculation #1:	Calculation #2:
Linear Regression	Linear Regression
$[D_0/(D_0-D) \text{ vs. } 10^{-pH}]$	$[(K_h+K_a+10^{-pH})/k \text{ vs. } 10^{-pH}]$
[w/ 0.005 - 0.04 points]	[w/ 0.005 - 0.04 points]
Intercept 1.12	Intercept 7.17×10^{-3}
Slope 2097	Slope 32.27×10^{-8}
Count 10	Count 10

K_h+K_a (from calc. #1) =	53.19×10^{-5}
$K_h = (K_h+K_a) - K_a,$ =	48.69×10^{-5}
$K_a = \text{slope/intercept},$ =	4.50×10^{-5}
$k_1 = K_h \cdot k_2,$ =	0.0679
$k_2 = 1/\text{intercept},$ =	139

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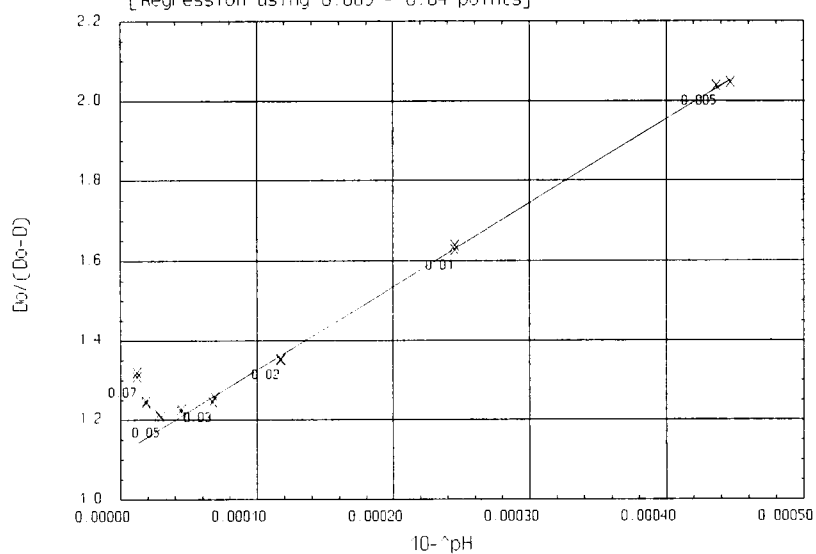
Compound: CYANGXG (#6)
 Experiment #: 1263
 Date: 4/15/96
 Dilution: 1:300
 Reaction Time: 2 min.
 Wavelength: 500 nm
 Comments: One of least stable compounds.

Calculation #1:	Calculation #2:
Linear Regression	Linear Regression
$[D_0/(D_0-D) \text{ vs. } 10^{-pH}]$	$[(K_h+K_a+10^{-pH})/k \text{ vs. } 10^{-pH}]$
[w/ 0.005 - 0.04 points]	[w/o 0.01 #3 point]
Intercept 1.09	Intercept 6.39×10^{-3}
Slope 2624	Slope 24.27×10^{-8}
Count 27	Count 29

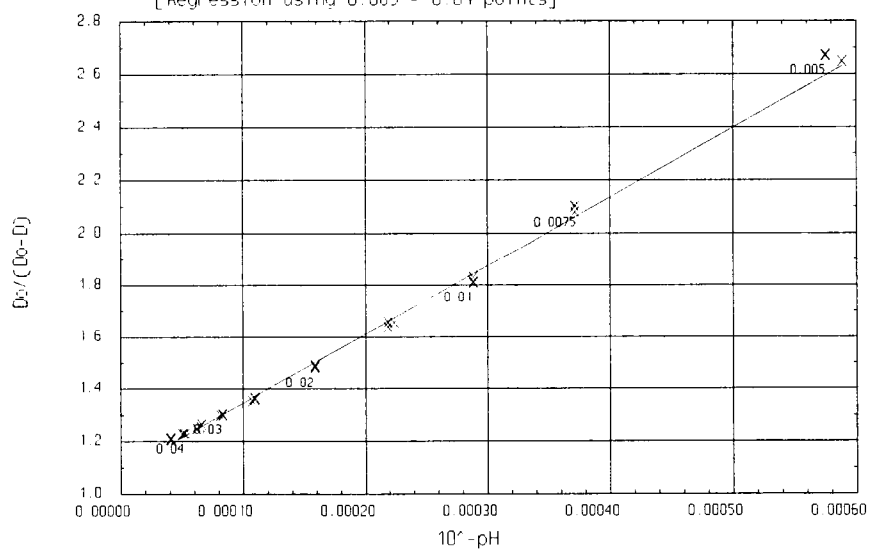
K_h+K_a (from calc. #1) =	41.35×10^{-5}
$K_h = (K_h+K_a) - K_a,$ =	37.55×10^{-5}
$K_a = \text{slope/intercept},$ =	3.80×10^{-5}
$k_1 = K_h \cdot k_2,$ =	0.0588
$k_2 = 1/\text{intercept},$ =	157

Appendix 20. CYANGXG (cont.)

1263, CYANGXG #1. $D_o/(D_o-D)$ vs. 10^{-pH}
 [Regression using 0.005 - 0.04 points]



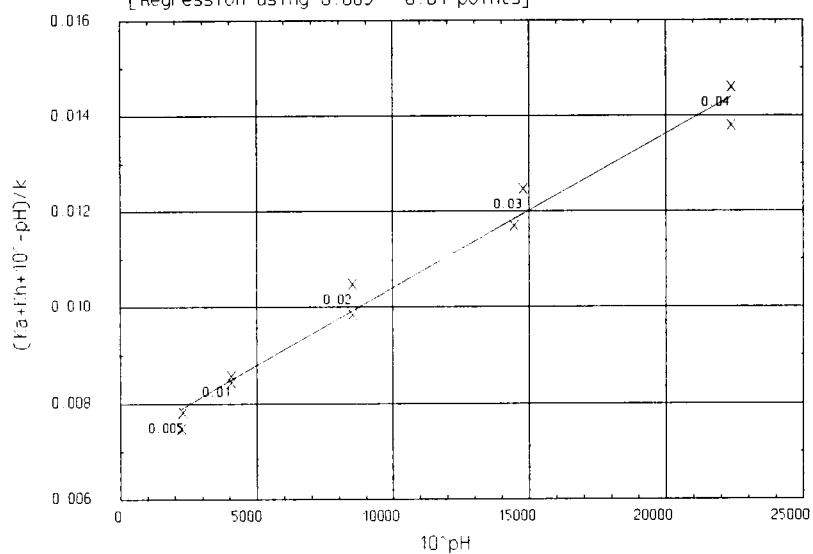
1263, CYANGXG. $D_o/(D_o-D)$ vs. 10^{-pH}
 [Regression using 0.005 - 0.04 points]



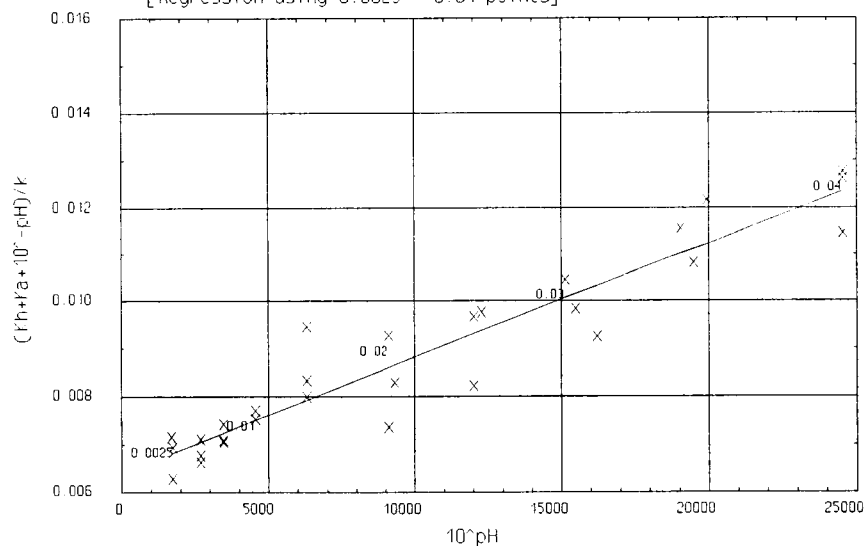
Appendix 20. CYANGXG (cont.)

1263 CYANGXG#1 $(K_a + K_h + 10^{-pH})/k$ vs 10^{pH}

[Regression using 0.005 - 0.04 points]

1263 CYANGXG#6 $(K_h + K_a + 10^{-pH})/k$ vs 10^{pH}

[Regression using 0.0025 - 0.04 points]



**Appendix 21. Regression Data for CYANGX
(Non-acylated anthocyanin without glucose)**

Compound: CYANGX (#1)
 Experiment #: 1339
 Date: 8/16/96
 Dilution: 1:28
 Reaction Time: 2 mins.
 Wavelength: 500 nm
 NaOH Conc. Used: 0.01 - 0.05 M
 Comments: Peak 1 from preparative HPLC of
 culture grown w/ 4-hydroxybenzoic acid.

Calculation #1:	Calculation #2:
Linear Regression	Linear Regression
$[D_0/(D_0-D) \text{ vs. } 10^{-PH}]$	$[(K_h+K_a+10^{-PH})/k \text{ vs. } 10^{-PH}]$
[w/ 0.01 - 0.05 M points]	[w/ 0.025 - 0.05 M points]
Intercept 1.03	Intercept 4.84×10^{-3}
Slope 2595	Slope 11.19×10^{-8}
Count 27	Count 18

Kh+Ka (from calc. #1) =	39.80×10^{-5}
Kh = (Kh+Ka) - Ka, =	37.48×10^{-5}
Ka = slope/intercept, =	2.31×10^{-5}
k1 = Kh*k2, =	0.0775
k2 = 1/intercept, =	207

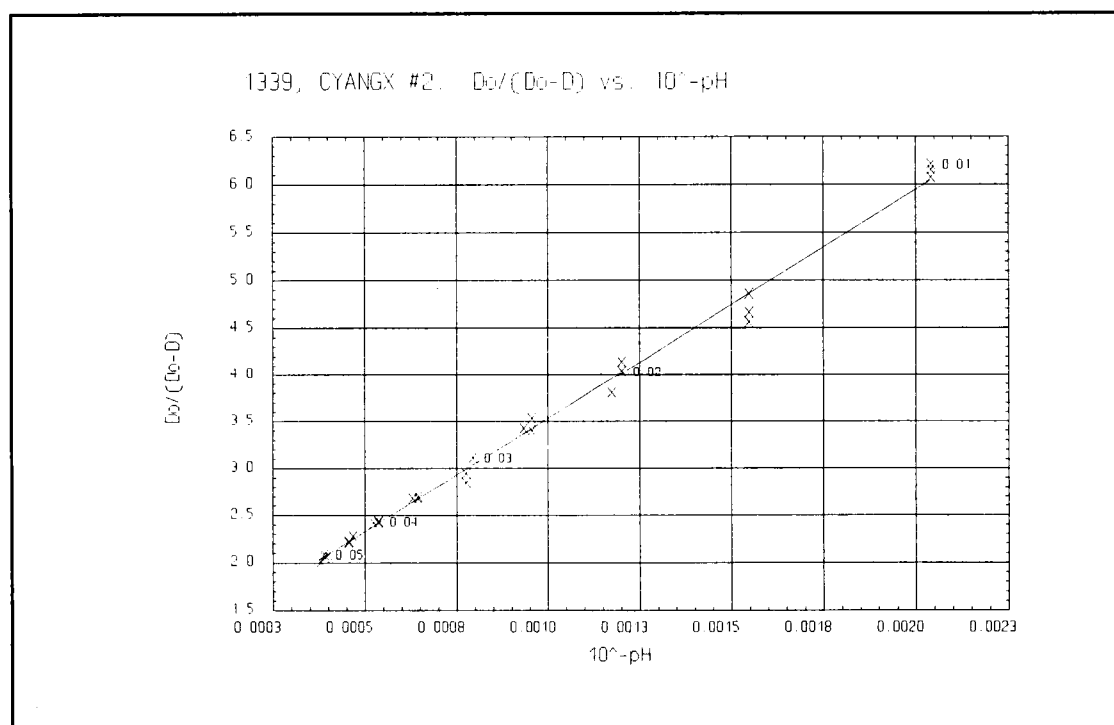
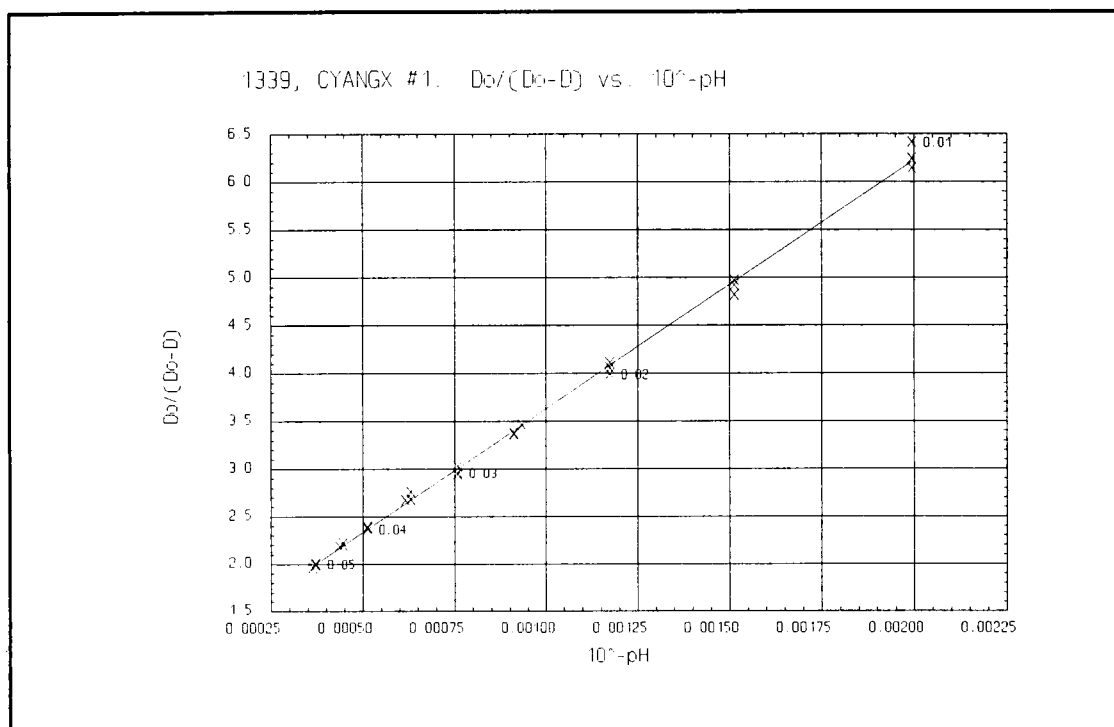
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Compound: CYANGX (#2)
 Experiment #: 1339
 Date: 8/16/96
 Dilution: 1:28
 Reaction Time: 2 mins.
 Wavelength: 500 nm
 NaOH Conc. Used: 0.01 - 0.05 M
 Comments: Peak 1 from preparative HPLC of
 culture grown w/ 4-hydroxybenzoic acid.

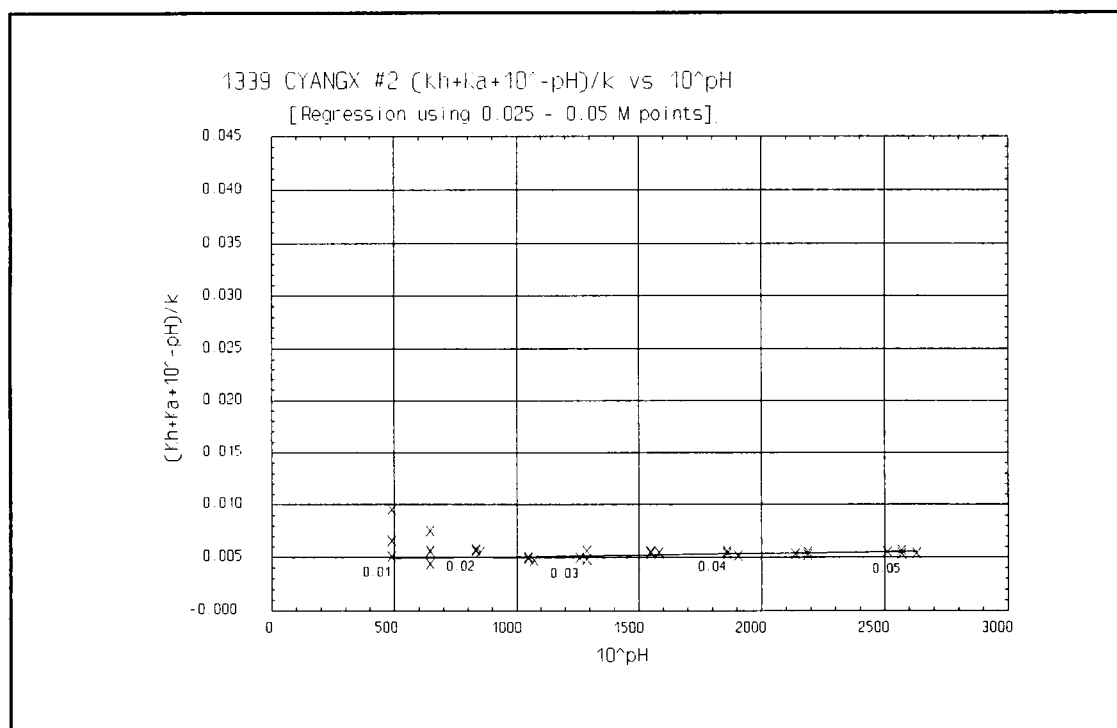
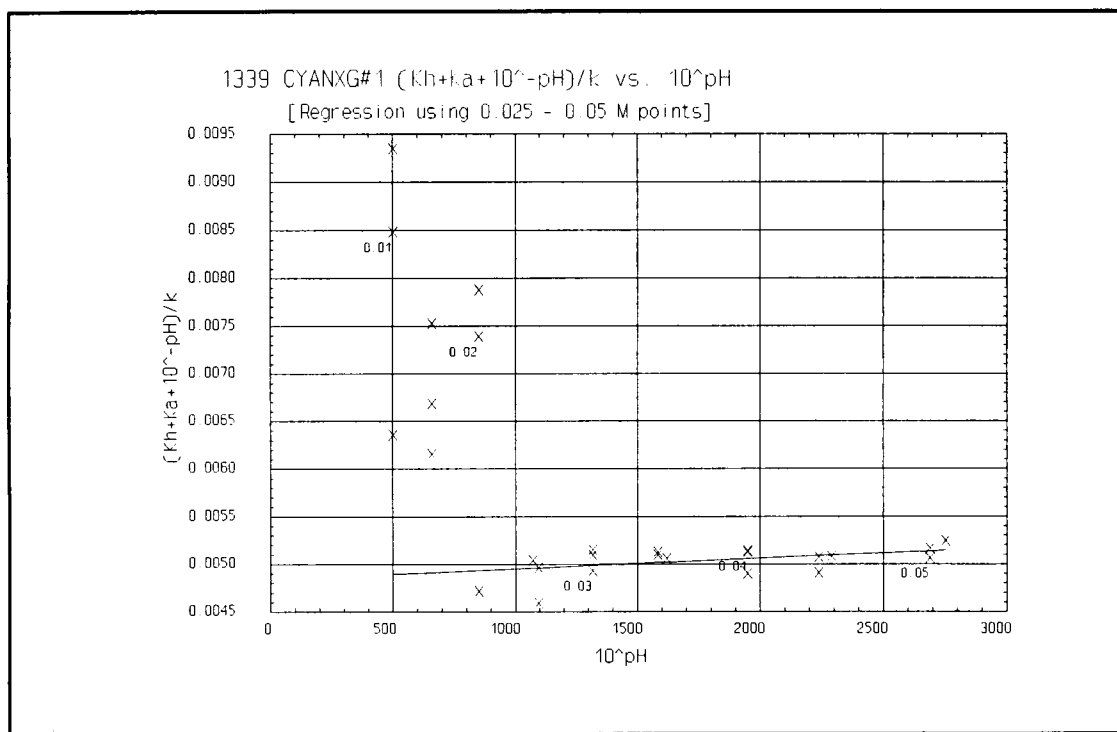
Calculation #1:	Calculation #2:
Linear Regression	Linear Regression
$[D_0/(D_0-D) \text{ vs. } 10^{-PH}]$	$[(K_h+K_a+10^{-PH})/k \text{ vs. } 10^{-PH}]$
[w/ 0.015 - 0.05 M points]	[w/ 0.025 - 0.05 M points]
Intercept 1.19	Intercept 4.77×10^{-3}
Slope 2309	Slope 28.88×10^{-8}
Count 25	Count 19

Kh+Ka (from calc. #1) =	46.20×10^{-5}
Kh = (Kh+Ka) - Ka, =	40.15×10^{-5}
Ka = slope/intercept, =	6.05×10^{-5}
k1 = Kh*k2, =	0.0841
k2 = 1/intercept, =	209

Appendix 21. CYANGX (cont.)



Appendix 21. CYANGX (cont.)



Vita

Marc A. Redus was born on April 26, 1962, in Inglewood, California, to Marc Boswell and Mary Frances Redus. He attended various public schools in Southern California and graduated from Ventura High School, Ventura, California, in June 1980. After high school, he took two years of classes at Ventura College.

Marc then took an 8-year break from school, working at various different jobs, and by the end of that time somehow ended up living in Alabama. During that time he also got married, and his wife, Naomi, eventually convinced him to go back to school. He graduated from Auburn University at Montgomery in December, 1993, with the degree of Bachelor of Science in Biology.

In August 1994 he began studying for his Master's degree in Life Sciences (Biotechnology program) at the University of Tennessee at Knoxville, Tennessee, and began working on a research project for Dr. Donald K. Dougall, which is described in this thesis. He was awarded this degree in December 1996.

Although Marc had originally planned to immediately pursue a Ph.D. degree after he had his M.S., he was forced to reconsider this course of action upon the birth of his son, Marc Aaron, in May of 1996, and as of this writing his current plans are to get a job in the Biotechnology field.