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STABILITY OF THE ANTHOCYANINS FROM *ACALIPHA HISPIDA* AND COPIGMENTATION EFFECT

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The stability and stabilization of the anthocyanins to light and air in the crude and partially purified extracts of the leaves of *Acalipha hispida* were studied at pH 3.0. Addition of non anthocyanic flavonoid fractions from *A. hispida* to the partially purified extracts did not significantly improve the time of half life of the extracts, whereas addition of tannic acid resulted in an increase of $t_{1/2}$ of 60–67%.

Keywords: anthocyanins, copigmentation, *A. hispida*, stability

Consumer's concern (MARKAKIS, 1982) about the use of artifical colours in food and pharmaceutical products has been an important factor in promoting the search for natural non toxic substitutes of some of the artifical colours, mainly red ones.

Anthocyanins are among the natural colours with red to bluish shades that are non toxic, water soluble and could eventually become the most important substitutes for the artificial red colours. Two main problems make this substitution difficult presently: the lack of reliable and cheap sources and the poor stability of anthocyanins to light and high pH. The search for reliable sources of stable anthocyanins has been the subject of many publications i.e.: MOK and HETTIARHCACHY (1991), SHI and co-workers (1992), DOUGALL and co-workers (1997), DUHARD and co-workers (1997). A potential source of anthocyanins for use in food and pharmaceutical products are the leaves of *Acalipha hispida* for which long term toxicity studies are yet to be made.

According to INAMI and co-workers (1996), acylation improves both heat and light stability, whereas glycosidation only stabilized anthocyanins in the presence of light. The use of tannic acid to stabilize the colour of orange juice from Italian red oranges was proposed in a paper by MACCARONE and co-workers (1987).

In a previous paper (BAILONI et al., 1998) the preparation of a methanolic extract from the leaves of *A. hispida* as well as its concentration and partial purification by reverse-osmosis were reported. The main anthocyanin in the extract was cyanidin-3-arabinosyl-glucoside.

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The present paper reports on the stability of the extract and of purified fractions from it as well as on the stabilizing effect of copigmentation.

1. Materials and methods

1.1. Stability of the extract E_1 to light at pH 3.0 under N_2 or air

Five hundred mg of the crude extract, free of methanol, prepared as described in a previous paper (BAILONI et al., 1998), was dissolved in sufficient amount of citrate-phosphate buffer at pH 3.0. The pH of the solution was adjusted to 3.0 by the addition of a few drops of 0.1 N HCl and the volume of the solution made up to 100.00 ml with the buffer solution. The slightly turbid solution was filtered and the clear liquid distributed into 10 ml screw-cap tubes. The head space was thoroughly flushed with N_2 when needed. The screw-cap tubes were divided in 2 lots. One was kept in the dark at 21±1.0 °C, while the other was irradiated at the same temperature between two 40 W lamps, day-light type, with nominal intensity of 2500 lm. Absorptions at 530 nm were measured from time to time until the loss of absorption reached aproximately 50%.

1.2. Fractionation of extract E_1

Extract E_1 was chromatographed on Whatman No. 3 and the chromatogram was developed with 1% HCl (FRANCIS, 1982).

A strong red zone, E_2, near the top was eluted and dried at 36 °C under vacuum.

A strip of paper from the chromatogram, when exposed to ammonia vapor turned yellow indicating the presence of a large zone of non anthocyanic flavonoids (NAF) preceding the E_2 zone. The NAF fraction was eluted and evaporated under vacuum at 36 °C to a pale yellow syrup which turned brown on exposure to air and light. Both E_2 and NAF were stored under N_2 at –18 °C.

Part of fraction E_2 was rechromatographed and the chromatogram was developed with BAW (n-BuOH-HAc-H_2O 6:1:2) (FRANCIS, 1982). A strong red zone, E_3, near the middle part of paper was eluted, dried at 36 °C and stored at –18 °C under N_2.

Part of the E_3 fraction was rechromatographed using Bu-HCl (n-BuOH-2NHCl 1:1 upper phase) as a developer (FRANCIS, 1982). A narrow red zone E_4, in the lower portion of the chromatogram was eluted and dried at 36 °C under vacuum. Fraction E_4 was stored under N_2 at –18 °C.

1.3. Stability of the purified fractions (E_2, E_3, E_4) to light and air

Solutions of each fraction were prepared as in 1.1 and their stability to light and air was estimated as described for fraction E_1.

1.4. Effect of copigmentation on the stability of the anthocyanic fractions

Solutions of each fraction (E_{1-4}) were prepared as described in 1.1. To each fraction sufficient tannic acid was added in order to attain a 3:1 proportion of tannic acid:anthocyanin (w:w) (BOBBIO et al., 1990; 1992).

Approximately the same weight of the fractions E_2 and NAF were used to estimate the copigmentation effects of NAF on the anthocyanins.

2. Results and discussion

Results presented in Table 1, $t_{½}$, were obtained from data of Figs 1–9 which represent the average of the absorbance readings from 2 samples. All values of $t_{½}$ were calculated from absorbance readings up to a maximum of 50% loss of the initial absorbance.

It has been reported as early as 1931 by ROBINSON and ROBINSON, that NAF can act as a stabilizing agent for anthocyanins by copigmentation, and the NAF-anthocyanin association has been considered responsible for the stability of the anthocyanins in plants tissues (ASEM et al., 1972).

Purification of E_1 with separation of colourless flavonoids did not significantly affect the $t_{½}$ values of fraction (E_2) (Table 1), but a considerable decrease of the $t_{½}$ occurred when purifying E_1, which besides removing flavonoids (NAF) also removed other minor anthocyanins as in fractions E_3–E_4.

Table 1

Time of half life (h) for E_1 and purified extracts E_2, E_3, E_4 with copigment at pH 3.0, 20 °C

Reaction conditions		$t_{½}$ (h)
E_1+N_2+D	(Fig. 1)	2772
E_1+N_2+L	(Fig. 2)	721
E_1+A+L	(Fig. 3)	445
E_1+A+L+TA(1:3)	(Fig. 4)	1103
E_2+A+L	(Fig. 5)	459
E_2+A+L+TA (1:2)	(Fig. 6)	1388
E_3+A+L	(Fig. 7)	202
E_4+A+L	(Fig. 8)	103
E_2+A+L+NAF	(Fig. 9)	476

A: air; D: dark; E_1: crude extract; E_2: ext. purified by p.c. 1% HCl; E_3: purified ext. by p.c. BAW; E_4: purified ext. by p.c. Bu-HCl ; L: light; NAF: non anthocyanins flavonoids; TA: tannic acid

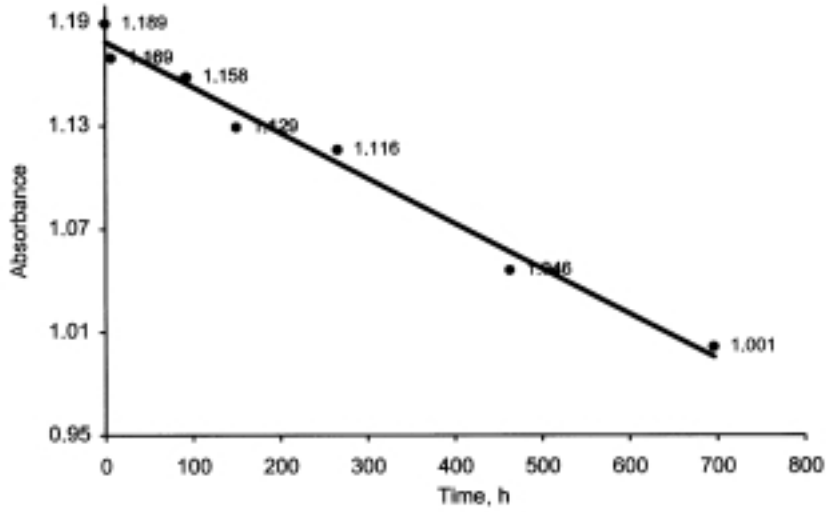

Fig. 1. Loss of absorbance for solutions of *A. hispida* E_1 under N_2 in the dark. Values are the average of two readings from two samples. $y = -0.0003\ x + 1.1784$; $R^2 = 0.9827$

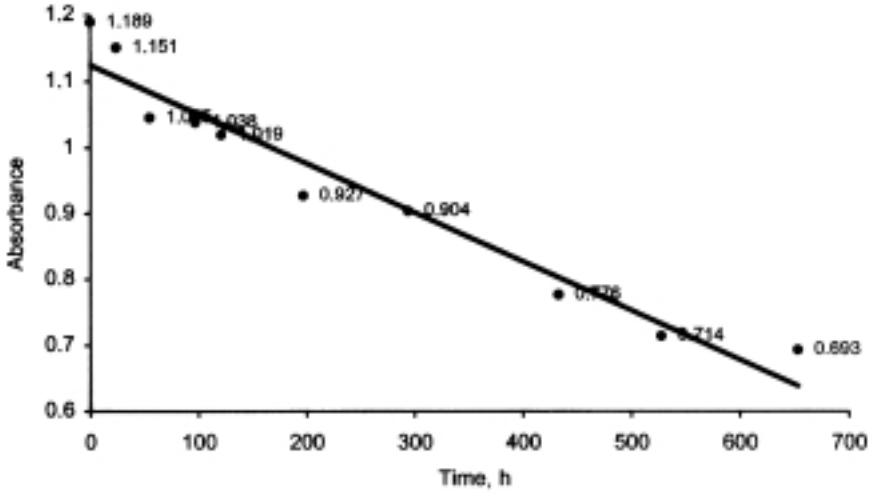

Fig. 2. Loss of absorbance for solutions of *A. hispida* E_1 under N_2 and light. Values are the average of two readings from four samples. $y = -0.0007\ x + 1.1241$; $R^2 = 0.9466$

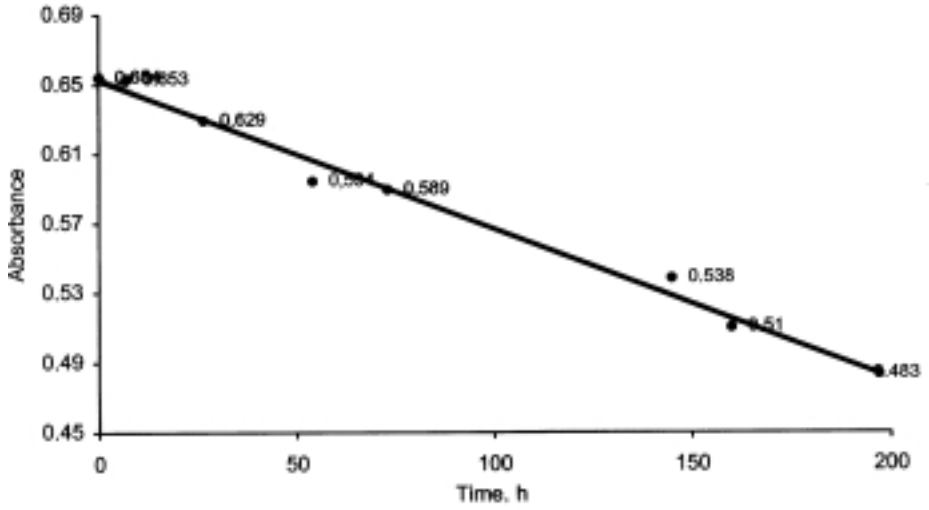

Fig. 3. Loss of absorbance for solutions of *A. hispida* E_1 under air and light. Values are the average of two readings from two samples. $y = -0.0009\ x + 0.6524$; $R^2 = 0.9892$

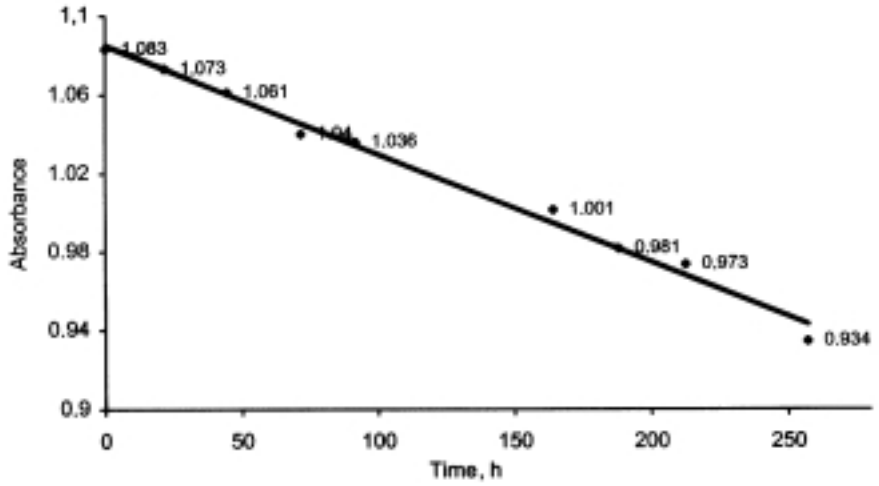

Fig. 4. Loss of absorbance for solutions of *A. hispida* E_1 under light and air with addition of tannic acid (1:3). $y = -0.0006\ x + 1.0848$; $R^2 = 0.9908$

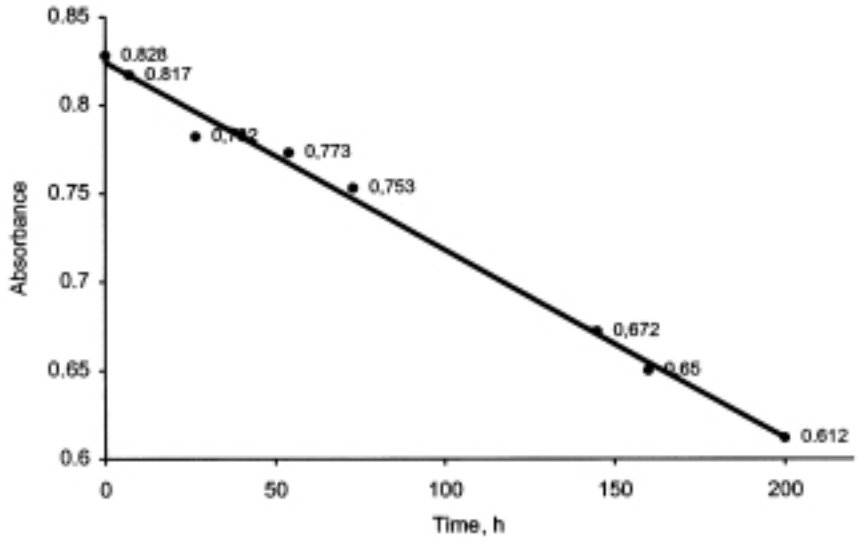

Fig. 5. Loss of absorbance for solutions of *A. hispida* E_2 under air and light. Values are the average of two readings from two samples. $y = -0.0011\ x + 0.8242$; $R^2 = 0.9932$

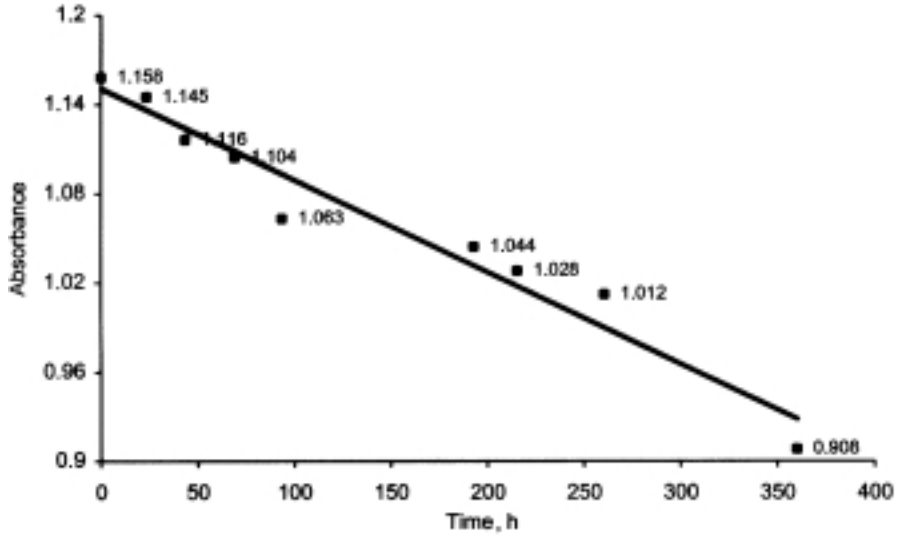

Fig. 6. Loss of absorbance for solutions of *A. hispida* E_2 under light and air with addition of tannic acid (1:2). $y = -0.0006\ x + 1.1505$; $R^2 = 0.953$

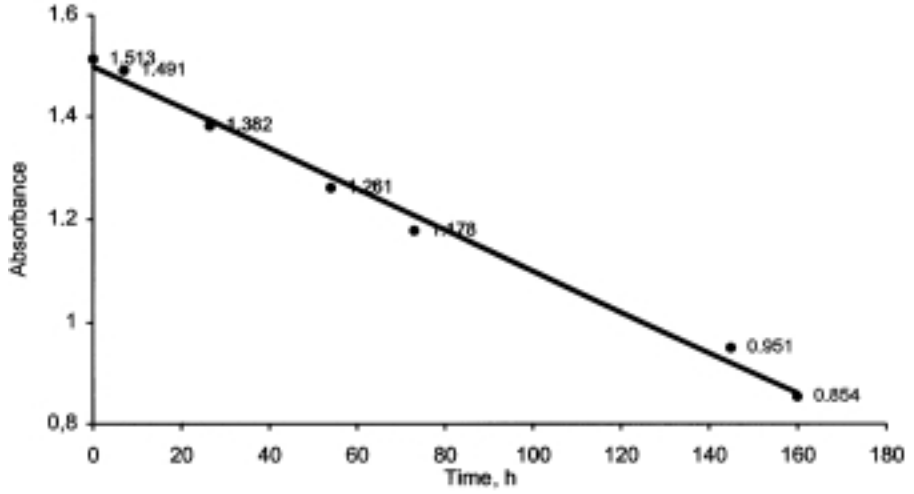

Fig. 7. Loss of absorbance for solutions of *A. hispida* E_3 under air and light. Values are the average of two readings from two samples. $y = -0.004\ x + 1.4976$; $R^2 = 0.9922$

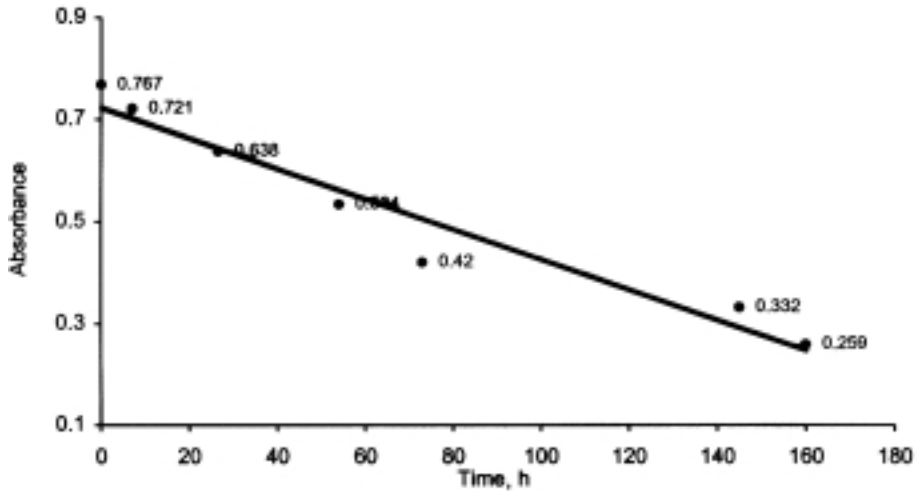

Fig. 8. Loss of absorbance for solutions of *A. hispida* E_4 under air and light. Values are the average of two readings from two samples. $y = -0.003\ x + 0.7215$; $R^2 = 0.9464$

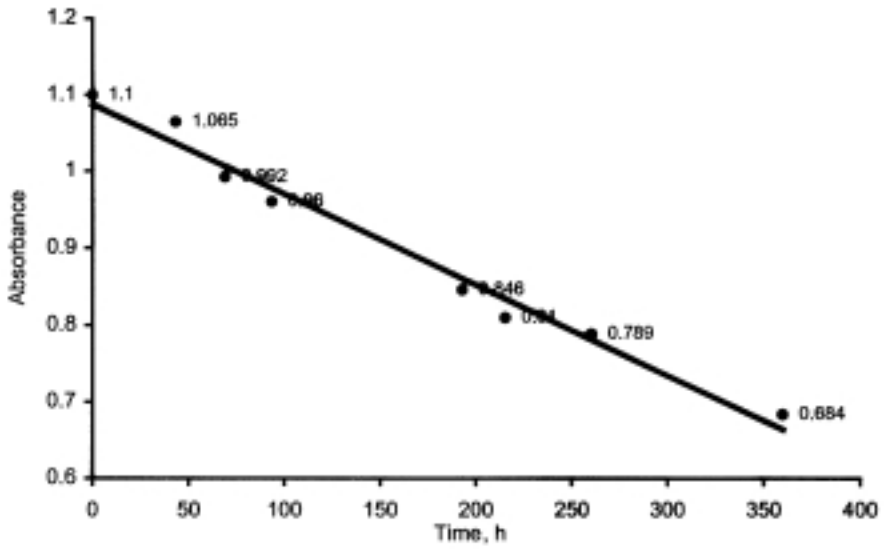

Fig. 9. Loss of absorbance for solutions of *A. hispida* E_2 under air and light, with addition of NAF. $y = -0.0012\ x + 1.0872$; $R^2 = 0.9816$

The addition of the recovered NAF to fraction E_2 did not significantly increase its stability (Table 1). The rapid darkening of the NAF fraction by air and light could be in part responsible for its lack of protective effects. Addition of ascorbic acid to the NAF fraction effectively prevented its darkening but the possibility of using ascorbic acid was precluded by the reported destructive effect of ascorbic acid on anthocyanins (MARKAKIS, 1982).

Intermolecular association of anthocyanins has been considered responsible for the stability of the anthocyanins in plants (SCHEFFELDT & HRAZDINA, 1978). This association as well as copigmentation is easily destroyed by methanol or ethanol (MINIATI et al., 1992). The significant decrease of $t_{½}$ from fraction E_1–E_4 parallels the increase in purity of the main anthocyanin by the progressive elimination of minor anthocyanins, therefore decreasing the possibility of hetero intermolecular associations and stabilization.

Tannic acid, on the other hand, was quite effective in stabilizing the anthocyanin, particularly fraction E_2 (Table 1). The presence of tannic acid apparently retarded the hydration reaction, which becomes important at pH 3.0 and retarded the formation of the hemiacetalic structure, but did not increase the concentration of the flavilium ion since no significant increase in $\lambda_{max.}$ value was observed.

By far the most destructive effect on the anthocyanins (Table 1) resulted from the combination of purification, have the light and oxygen. Tannic acid was an effective stabilizing agent, and for fraction E_2 under light and oxygen, increased the $t_{1/2}$ from 459 h to 1380 h (Table 1).

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